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Antiseptics and Disinfectants: Activity, Action, and Resistance

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INTRODUCTION

Antiseptics and disinfectants are used extensively in hospitals and other health care settings for a variety of topical and hard-surface applications. In particular, they are an essential part of infection control practices and aid in the prevention of nosocomial infections (277, 454). Mounting concerns over the potential for microbial contamination and infection risks in the food and general consumer markets have also led to increased use of antiseptics and disinfectants by the general public. A wide variety of active chemical agents (or "biocides") are found in these products, many of which have been used for hundreds of years for antisepsis, disinfection, and preservation (39). Despite this, less is known about the mode of action of these active agents than about antibiotics. In general, biocides have a broader spectrum of activity than antibiotics, and, while antibiotics tend to have specific intracellular targets, biocides may have multiple targets. The widespread use of antiseptic and disinfectant products has prompted some speculation on the development of microbial resistance, in particular cross-resistance to antibiotics. This review considers what is known about the mode of action of, and mechanisms of microbial resistance to, antiseptics and disinfectants and attempts, wherever possible, to relate current knowledge to the clinical environment.

A summary of the various types of biocides used in antiseptics and disinfectants, their chemical structures, and their clinical uses is shown in Table 1. It is important to note that many of these biocides may be used singly or in combination in a variety of products which vary considerably in activity against microorganisms. Antimicrobial activity can be influenced by many factors such as formulation effects, presence of an organic load, synergy, temperature, dilution, and test method. These issues are beyond the scope of this review and are discussed elsewhere (123, 425, 444, 446, 451).

DEFINITIONS

"Biocide" is a general term describing a chemical agent, usually broad spectrum, that inactivates microorganisms. Because biocides range in antimicrobial activity, other terms may be more specific, including "-static," referring to agents which inhibit growth (e.g., bacteriostatic, fungistatic, and sporistatic) and "-cidal," referring to agents which kill the target organism (e.g., sporicidal, virucidal, and bactericidal). For the purpose of this review, antibiotics are defined as naturally occurring or synthetic organic substances which inhibit or destroy selective bacteria or other microorganisms, generally at low concentrations; antiseptics are biocides or products that destroy or inhibit the growth of microorganisms in or on living tissue (e.g. health care personnel handwashes and surgical scrubs); and disinfectants are similar but generally are products or biocides that are used on inanimate objects or surfaces. Disinfectants can be sporostatic but are not necessarily sporicidal.

Sterilization refers to a physical or chemical process that completely destroys or removes all microbial life, including spores. Preservation is the prevention of multiplication of microorganisms in formulated products, including pharmaceuticals and foods. A number of biocides are also used for cleaning purposes; cleaning in these cases refers to the physical removal of foreign material from a surface (40).

MECHANISMS OF ACTION

Introduction

Considerable progress has been made in understanding the mechanisms of the antibacterial action of antiseptics and disinfectants (215, 428, 437). By contrast, studies on their modes of action against fungi (426, 436), viruses (298, 307), and protozoa (163) have been rather sparse. Furthermore, little is known about the means whereby these agents inactivate prions (503).

Whatever the type of microbial cell (or entity), it is probable that there is a common sequence of events. This can be envisaged as interaction of the antiseptic or disinfectant with the cell surface followed by penetration into the cell and action at the target site(s). The nature and composition of the surface vary from one cell type (or entity) to another but can also alter as a result of changes in the environment (57, 59). Interaction at the cell surface can produce a significant effect on viability (e.g. with glutaraldehyde) (374, 421), but most antimicrobial agents appear to be active intracellularly (428, 451). The outermost layers of microbial cells can thus have a significant effect on their susceptibility (or insusceptibility) to antiseptics and disinfectants; it is disappointing how little is known about the passage of these antimicrobial agents into different types of microorganisms. Potentiation of activity of most biocides may be achieved by the use of various additives, as shown in later parts of this review.

In this section, the mechanisms of antimicrobial action of a range of chemical agents that are used as antiseptics or disinfectants or both are discussed. Different types of microorganisms are considered, and similarities or differences in the nature of the effect are emphasized. The mechanisms of action are summarized in Table 2.

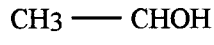
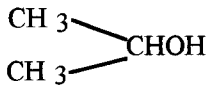
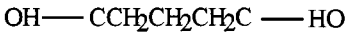
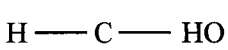
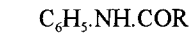
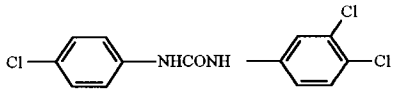
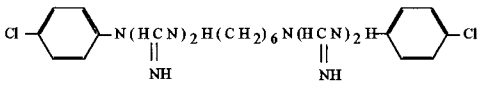
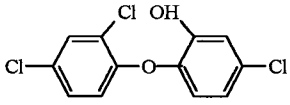
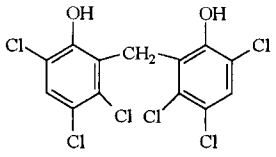
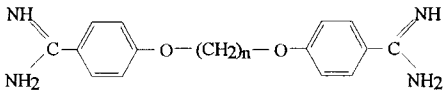
General Methodology

A battery of techniques are available for studying the mechanisms of action of antiseptics and disinfectants on microorganisms, especially bacteria (448). These include examination of uptake (215, 428, 459), lysis and leakage of intracellular constituents (122), perturbation of cell homeostasis (266, 445), effects on model membranes (170), inhibition of enzymes, electron transport, and oxidative phosphorylation (162, 272), interaction with macromolecules (448, 523), effects on macromolecular biosynthetic processes (133), and microscopic examination of biocide-exposed cells (35). Additional and useful information can be obtained by calculating concentration exponents (*n* values [219, 489]) and relating these to membrane activity (219). Many of these procedures are valuable for detecting and evaluating antiseptics or disinfectants used in combination (146, 147, 202, 210).

Similar techniques have been used to study the activity of antiseptics and disinfectants against fungi, in particular yeasts. Additionally, studies on cell wall porosity (117–119) may provide useful information about intracellular entry of disinfectants and antiseptics (204–208).

Mechanisms of antiprotozoal action have not been widely investigated. One reason for this is the difficulty in culturing some protozoa (e.g., *Cryptosporidium*) under laboratory conditions. However, the different life stages (trophozoites and cysts) do provide a fascinating example of the problem

TABLE 1. Chemical structures and uses of biocides in antiseptics and disinfectants

Alcohols	Ethanol		Antisepsis
	Isopropanol		Disinfection
			Preservation
Aldehydes	Glutaraldehyde		Disinfection
	Formaldehyde		Sterilization
			Preservation
Anilides	General structure		Antisepsis
	Triclocarban		
Biguanides	Chlorhexidine		Antisepsis
			Antiplaque agents
	Alexidine, polymeric biguanides		Preservation
			Disinfection
Bisphenols	Triclosan		Antisepsis
			Antiplaque agents
	Hexachlorophene		Deodorants
			Preservation
Diamidines	Propamidine		Antisepsis
			Preservation
	Dibromopropamidine		

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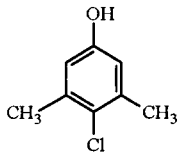
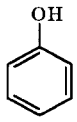
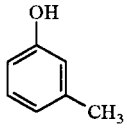
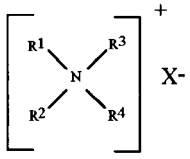
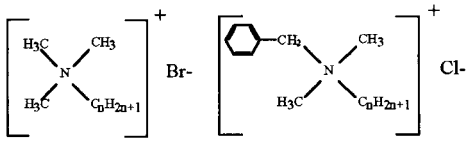
of how changes in cytology and physiology can modify responses to antiseptics and disinfectants. Khunkitti et al. (251–255) have explored this aspect by using indices of viability, leakage, uptake, and electron microscopy as experimental tools.

Some of these procedures can also be modified for studying effects on viruses and phages (e.g., uptake to whole cells and viral or phage components, effects on nucleic acids and proteins, and electron microscopy) (401). Viral targets are

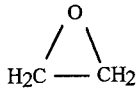
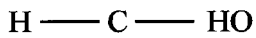
TABLE 1—*Continued*

Halogen-releasing agents	Chlorine compounds	⇳OCl-, HOCl, Cl_2	Disinfection Antisepsis
	Iodine compounds	⇳I_2	Cleaning
Halophenols	Chloroxylenol (PCMX)		Antisepsis Preservation
Heavy metal derivatives	Silver compounds	Ag	Preservation Antisepsis
	Mercury compounds	Hg	Disinfection
Peroxygens	Hydrogen peroxide	H_2O_2	Disinfection
	Ozone	O_3	Sterilization
	Peracetic acid	$CH_3.COOOH$	
Phenols and cresols	Phenol		Disinfection Preservation
	Cresol		
Quaternary ammonium compounds	General structure		Disinfection Antisepsis Preservation
	Cetrimide, benzalkonium chloride		Cleaning

Continued on following page

predominantly the viral envelope (if present), derived from the host cell cytoplasmic or nuclear membrane; the capsid, which is responsible for the shape of virus particles and for the protection of viral nucleic acid; and the viral genome. Release of an intact viral nucleic acid into the environment following capsid destruction is of potential concern since some nucleic acids are infective when liberated from the capsid (317), an aspect that must be considered in viral disinfection. Important considerations in viral inactivation are dealt with by Klein and Deforest (259) and Prince et al.

TABLE 1—*Continued*

Vapor phase	Ethylene oxide		Sterilization Disinfection
	Formaldehyde		
	Hydrogen peroxide	H_2O_2	

(384), while an earlier paper by Grossgebauer is highly recommended (189).

Alcohols

Although several alcohols have been shown to be effective antimicrobials, ethyl alcohol (ethanol, alcohol), isopropyl alcohol (isopropanol, propan-2-ol) and *n*-propanol (in particular in Europe) are the most widely used (337). Alcohols exhibit rapid broad-spectrum antimicrobial activity against vegetative bacteria (including mycobacteria), viruses, and fungi but are not sporicidal. They are, however, known to inhibit sporulation and spore germination (545), but this effect is reversible (513). Because of the lack of sporicidal activity, alcohols are not recommended for sterilization but are widely used for both hard-surface disinfection and skin antisepsis. Lower concentrations may also be used as preservatives and to potentiate the activity of other biocides. Many alcohol products include low levels of other biocides (in particular chlorhexidine), which remain on the skin following evaporation of the alcohol, or excipients (including emollients), which decrease the evaporation time of the alcohol and can significantly increase product efficacy (68). In general, isopropyl alcohol is considered slightly more efficacious against bacteria (95) and ethyl alcohol is more potent against viruses (259); however, this is dependent on the concentrations of both the active agent and the test microorganism. For example, isopropyl alcohol has greater lipophilic properties than ethyl alcohol and is less active against hydrophilic viruses (e.g., poliovirus) (259). Generally, the antimicrobial activity of alcohols is significantly lower at concentrations below 50% and is optimal in the 60 to 90% range.

Little is known about the specific mode of action of alcohols, but based on the increased efficacy in the presence of water, it is generally believed that they cause membrane damage and rapid denaturation of proteins, with subsequent interference with metabolism and cell lysis (278, 337). This is supported by specific reports of denaturation of *Escherichia coli* dehydrogenases (499) and an increased lag phase in *Enterobacter aerogenes*, speculated to be due to inhibition of metabolism required for rapid cell division (101).

Aldehydes

Glutaraldehyde. Glutaraldehyde is an important dialdehyde that has found usage as a disinfectant and sterilant, in particular for low-temperature disinfection and sterilization of endoscopes and surgical equipment and as a fixative in electron

TABLE 2. Summary of mechanisms of antibacterial action of antiseptics and disinfectants

Target	Antiseptic or disinfectant	Mechanism of action
Cell envelope (cell wall, outer membrane)	Glutaraldehyde	Cross-linking of proteins
	EDTA, other permeabilizers	Gram-negative bacteria: removal of Mg^{2+}, release of some LPS
Cytoplasmic (inner) membrane	QACs	Generalized membrane damage involving phospholipid bilayers
	Chlorhexidine	Low concentrations affect membrane integrity, high concentrations cause congealing of cytoplasm
	Diamines	Induction of leakage of amino acids
	PHMB, alexidine	Phase separation and domain formation of membrane lipids
	Phenols	Leakage; some cause uncoupling
Cross-linking of macromolecules	Formaldehyde	Cross-linking of proteins, RNA, and DNA
	Glutaraldehyde	Cross-linking of proteins in cell envelope and elsewhere in the cell
DNA intercalation	Acridines	Intercalation of an acridine molecule between two layers of base pairs in DNA
Interaction with thiol groups	Silver compounds	Membrane-bound enzymes (interaction with thiol groups)
Effects on DNA	Halogens	Inhibition of DNA synthesis
	Hydrogen peroxide, silver ions	DNA strand breakage
Oxidizing agents	Halogens	Oxidation of thiol groups to disulfides, sulfoxides, or disulfoxides
	Peroxygens	Hydrogen peroxide: activity due to from formation of free hydroxy radicals (·OH), which oxidize thiol groups in enzymes and proteins; PAA: disruption of thiol groups in proteins and enzymes

TABLE 3. Mechanism of antimicrobial action of glutaraldehyde

Target microorganism	Glutaraldehyde action
Bacterial spores	Low concentrations inhibit germination; high concentrations are sporicidal, probably as a consequence of strong interaction with outer cell layers
Mycobacteria	Action unknown, but probably involves mycobacterial cell wall
Other nonsporulating bacteria	Strong association with outer layers of gram-positive and gram-negative bacteria; cross-linking of amino groups in protein; inhibition of transport processes into cell
Fungi	Fungal cell wall appears to be a primary target site, with postulated interaction with chitin
Viruses	Actual mechanisms unknown, but involve protein-DNA cross-links and capsid changes
Protozoa	Mechanism of action not known

icroscopy. Glutaraldehyde has a broad spectrum of activity against bacteria and their spores, fungi, and viruses, and a considerable amount of information is now available about the ways whereby these different organisms are inactivated (Tables 2 and 3). Earlier reviews of its mechanisms of action have been published (179, 182, 374, 482).

The first reports in 1964 and 1965 (182) demonstrated that glutaraldehyde possessed high antimicrobial activity. Subsequently, research was undertaken to evaluate the nature of its bactericidal (339–344, 450) and sporicidal (180, 181, 507, 508) action. These bactericidal studies demonstrated (374) a strong binding of glutaraldehyde to outer layers of organisms such as *E. coli* and *Staphylococcus aureus* (179, 212, 339–341, 343, 344), inhibition of transport in gram-negative bacteria (179), inhibition of dehydrogenase activity (343, 344) and of periplasmic enzymes (179), prevention of lysostaphin-induced lysis in *S. aureus* (453) and of sodium lauryl sulfate-induced lysis in *E. coli* (340, 344), inhibition of spheroplast and protoplast lysis in hypotonic media (340, 344), and inhibition of RNA, DNA, and protein synthesis (320). Strong interaction of glutaraldehyde with lysine and other amino acids has been demonstrated (450).

Clearly, the mechanism of action of glutaraldehyde involves a strong association with the outer layers of bacterial cells, specifically with unprotonated amines on the cell surface, possibly representing the reactive sites (65). Such an effect could explain its inhibitory action on transport and on enzyme systems, where access of substrate to enzyme is prohibited. Partial or entire removal of the cell wall in hypertonic medium, leading to the production of spheroplasts or protoplasts and the subsequent prevention of lysis by glutaraldehyde when these forms are diluted in a hypotonic environment, suggests an additional effect on the inner membrane, a finding substantiated by the fact that the dialdehyde prevents the selective release of some membrane-bound enzymes of *Micrococcus lysodeikticus* (138). Glutaraldehyde is more active at alkaline than at acidic pHs. As the external pH is altered from acidic to alkaline, more reactive sites will be formed at the cell surface, leading to a more rapid bactericidal effect. The cross-links thus obtained mean that the cell is then unable to undertake most, if not all, of its essential functions. Glutaraldehyde is also mycobactericidal. Unfortunately, no critical studies have as yet been undertaken to evaluate the nature of this action (419).

The bacterial spore presents several sites at which interaction with glutaraldehyde is possible, although interaction with a particular site does not necessarily mean that this is associated with spore inactivation. *E. coli*, *S. aureus*, and vegetative cells of *Bacillus subtilis* bind more glutaraldehyde than do resting spores of *B. subtilis* (377, 378); uptake of glutaraldehyde is greater during germination and outgrowth than with mature spores but still lower than with vegetative cells. Low concentrations of the dialdehyde (0.1%) inhibit germination, whereas much higher concentrations (2%) are sporicidal. The aldehyde, at both acidic and alkaline pHs, interacts strongly with the outer spore layers (508, 509); this interaction reduces the release of dipicolinic acid (DPA) from heated spores and the lysis induced by mercaptoethanol (or thioglycolate)-peroxide combinations. Low concentrations of both acidic and alkaline glutaraldehyde increase the surface hydrophobicity of spores, again indicating an effect at the outermost regions of the cell. It has been observed by various authors (182, 374, 376, 380) that the greater sporicidal activity of glutaraldehyde at alkaline pH is not reflected by differences in uptake; however, uptake per se reflects binding and not necessarily penetration into the spore. It is conceivable that acidic glutaraldehyde interacts with and remains at the cell surface whereas alkaline glutaraldehyde penetrates more deeply into the spore. This contention is at odds with the hypothesis of Bruch (65), who envisaged the acidic form penetrating the coat and reacting with the cortex while the alkaline form attacked the coat, thereby destroying the ability of the spore to function solely as a result of this surface phenomenon. There is, as yet, no evidence to support this theory. Novel glutaraldehyde formulations based on acidic rather than alkaline glutaraldehyde, which benefit from the greater inherent stability of the aldehyde at lower pH, have been produced. The improved sporicidal activity claimed for these products may be obtained by agents that potentiate the activity of the dialdehyde (414, 421).

During sporulation, the cell eventually becomes less susceptible to glutaraldehyde (see "Intrinsic resistance of bacterial spores"). By contrast, germinating and outgrowing cells reacquire sensitivity. Germination may be defined as an irreversible process in which there is a change of an activated spore from a dormant to a metabolically active state within a short period. Glutaraldehyde exerts an early effect on the germination process. L-Alanine is considered to act by binding to a specific receptor on the spore coat, and once spores are triggered to germinate, they are committed irreversibly to losing their dormant properties (491). Glutaraldehyde at high concentrations inhibits the uptake of L-[^{14}C]alanine by *B. subtilis* spores, albeit by an unknown mechanism (379, 414). Glutaraldehyde-treated spores retain their refractivity, having the same appearance under the phase-contrast microscope as normal, untreated spores even when the spores are subsequently incubated in germination medium. Glutaraldehyde is normally used as a 2% solution to achieve a sporicidal effect (16, 316); low concentrations (<0.1%) prevent phase darkening of spores and also prevent the decrease in optical density associated with a late event in germination. By contrast, higher concentrations (0.1 to 1%) significantly reduce the uptake of L-alanine, possibly as a result of a sealing effect of the aldehyde on the cell surface. Mechanisms involved in the revival of glutaraldehyde-treated spores are discussed below (see "Intrinsic resistance of bacterial spores").

There are no recent studies of the mechanisms of fungicidal action of glutaraldehyde. Earlier work had suggested that the fungal cell wall was a major target site (179, 182, 352), especially the major wall component, chitin, which is analogous to the peptidoglycan found in bacterial cell walls.

Glutaraldehyde is a potent virucidal agent (143, 260). It reduces the activity of hepatitis B surface antigen (HBsAg) and especially hepatitis B core antigen ([HBcAg] in hepatitis B virus [HBV]) (3) and interacts with lysine residues on the surface of hepatitis A virus (HAV) (362). Low concentrations

(<0.1%) of alkaline glutaraldehyde are effective against purified poliovirus, whereas poliovirus RNA is highly resistant to aldehyde concentrations up to 1% at pH 7.2 and is only slowly inactivated at pH 8.3 (21). In other words, the complete poliovirus particle is much more sensitive than poliovirus RNA. In light of this, it has been inferred that glutaraldehyde-induced loss of infectivity is associated with capsid changes (21). Glutaraldehyde at the low concentrations of 0.05 and 0.005% interacts with the capsid proteins of poliovirus and echovirus, respectively; the differences in sensitivity probably reflect major structural variations in the two viruses (75).

Bacteriophages were recently studied to obtain information about mechanisms of virucidal action (298–304, 306, 307). Many glutaraldehyde-treated *P. aeruginosa* F116 phage particles had empty heads, implying that the phage genome had been ejected. The aldehyde was possibly bound to F116 double-stranded DNA but without affecting the molecule; glutaraldehyde also interacted with phage F116 proteins, which were postulated to be involved in the ejection of the nucleic acid. Concentrations of glutaraldehyde greater than 0.1 to 0.25% significantly affected the transduction of this phage; the transduction process was more sensitive to the aldehyde than was the phage itself. Glutaraldehyde and other aldehydes were tested for their ability to form protein-DNA cross-links in simian virus 40 (SV40); aldehydes (i.e., glyoxal, furfural, prionaldehyde, acetaldehyde, and benzylaldehyde) without detectable cross-linking ability had no effect on SV40 DNA synthesis, whereas acrolein, glutaraldehyde, and formaldehyde, which formed such cross-links (144, 271, 297), inhibited DNA synthesis (369).

Formaldehyde. Formaldehyde (methanal, CH_2O) is a monoaldehyde that exists as a freely water-soluble gas. Formaldehyde solution (formalin) is an aqueous solution containing *ca.* 34 to 38% (wt/wt) CH_2O with methanol to delay polymerization. Its clinical use is generally as a disinfectant and sterilant in liquid or in combination with low-temperature steam. Formaldehyde is bactericidal, sporicidal, and virucidal, but it works more slowly than glutaraldehyde (374, 482).

Formaldehyde is an extremely reactive chemical (374, 442) that interacts with protein (156, 157), DNA (155), and RNA (155) in vitro. It has long been considered to be sporicidal by virtue of its ability to penetrate into the interior of bacterial spores (500). The interaction with protein results from a combination with the primary amide as well as with the amino groups, although phenol groups bind little formaldehyde (155). It has been proposed that formaldehyde acts as a mutagenic agent (291) and as an alkylating agent by reaction with carboxyl, sulfhydryl, and hydroxyl groups (371). Formaldehyde also reacts extensively with nucleic acid (489) (e.g., the DNA of bacteriophage T2) (190). As pointed out above, it forms protein-DNA cross-links in SV40, thereby inhibiting DNA synthesis (369). Low concentrations of formaldehyde are sporostatic and inhibit germination (512). Formaldehyde alters HBsAg and HBcAg of HBV (3).

It is difficult to pinpoint accurately the mechanism(s) responsible for formaldehyde-induced microbial inactivation. Clearly, its interactive, and cross-linking properties must play a considerable role in this activity. Most of the other aldehydes (glutaraldehyde, glyoxyl, succinaldehyde, and *o*-phthalaldehyde [OPA]) that have sporicidal activity are dialdehydes (and of these, glyoxyl and succinaldehyde are weakly active). The distance between the two aldehyde groups in glutaraldehyde (and possibly in OPA) may be optimal for interaction of these-CHO groups in nucleic acids and especially in proteins and enzymes (428).

Formaldehyde-releasing agents. Several formaldehyde-releasing agents have been used in the treatment of peritonitis (226, 273). They include noxythiolin (oxymethylenethiourea), tauroline (a condensate of two molecules of the aminosulponic acid taurine with three molecules of formaldehyde), hexamine (hexamethylenetetramine, methenamine), the resins melamine and urea formaldehydes, and imidazolone derivatives such as dantoin. All of these agents are claimed to be microbicidal on account of the release of formaldehyde. However, because the antibacterial activity of taurolin is greater than that of free formaldehyde, the activity of taurolin is not entirely the result of formaldehyde action (247).

TABLE 4. Mechanisms of antimicrobial action of chlorhexidine

Type of microorganism	Chlorhexidine action
Bacterial spores	Not sporicidal but prevents development of spores; inhibits spore outgrowth but not germination
Mycobacteria	Mycobacteristatic (mechanism unknown) but not mycobactericidal
Other nonsporulating bacteria	Membrane-active agent, causing protoplast and spheroplast lysis; high concentrations cause precipitation of proteins and nucleic acids
Yeasts	Membrane-active agent, causing protoplast lysis and intracellular leakage; high concentrations cause intracellular coagulation
Viruses	Low activity against many viruses; lipid-enveloped viruses more sensitive than nonenveloped viruses; effect possibly on viral envelope, perhaps the lipid moieties
Protozoa	Recent studies against *A. castellanii* demonstrate membrane activity (leakage) toward trophozoites, less toward cysts

***o*-Phthalaldehyde.** OPA is a new type of disinfectant that is claimed to have potent bactericidal and sporicidal activity and has been suggested as a replacement for glutaraldehyde in endoscope disinfection (7). OPA is an aromatic compound with two aldehyde groups. To date, the mechanism of its antimicrobial action has been little studied, but preliminary evidence (526) suggests an action similar to that of glutaraldehyde. Further investigations are needed to corroborate this opinion.

Anilides

The anilides have been investigated primarily for use as antiseptics, but they are rarely used in the clinic. Triclocarban (TCC; 3,4,4′-triclorocarbanilide) is the most extensively studied in this series and is used mostly in consumer soaps and deodorants. TCC is particularly active against gram-positive bacteria but significantly less active against gram-negative bacteria and fungi (30) and lacks appreciable substantivity (persistency) for the skin (37). The anilides are thought to act by adsorbing to and destroying the semipermeable character of the cytoplasmic membrane, leading to cell death (194).

Biguanides

Chlorhexidine. Chlorhexidine is probably the most widely used biocide in antiseptic products, in particular in handwashing and oral products but also as a disinfectant and preservative. This is due in particular to its broad-spectrum efficacy, substantivity for the skin, and low irritation. Of note, irritability has been described and in many cases may be product specific (167, 403). Despite the advantages of chlorhexidine, its activity is pH dependent and is greatly reduced in the presence of organic matter (430). A considerable amount of research has been undertaken on the mechanism of the antimicrobial action of this important bisbiguanide (389) (Tables 2 and 4), although most of the attention has been devoted to the way in which it

inactivates nonsporulating bacteria (215, 428, 430, 431, 451). Nevertheless, sufficient data are now available to examine its sporostatic and mycobacteriostatic action, its effects on yeasts and protozoa, and its antiviral activity.

Chlorhexidine is a bactericidal agent (120, 215). Its interaction and uptake by bacteria were studied initially by Hugo et al. (222–224), who found that the uptake of chlorhexidine by *E. coli* and *S. aureus* was very rapid and depended on the chlorhexidine concentration and pH. More recently, by using [^{14}C]chlorhexidine gluconate, the uptake by bacteria (145) and yeasts (204) was shown to be extremely rapid, with a maximum effect occurring within 20 s. Damage to the outer cell layers takes place (139) but is insufficient to induce lysis or cell death. The agent then crosses the cell wall or outer membrane, presumably by passive diffusion, and subsequently attacks the bacterial cytoplasmic or inner membrane or the yeast plasma membrane. In yeasts, chlorhexidine "partitions" into the cell wall, plasma membrane, and cytoplasm of cells (205). Damage to the delicate semipermeable membrane is followed by leakage of intracellular constituents, which can be measured by appropriate techniques. Leakage is not per se responsible for cellular inactivation but is a consequence of cell death (445). High concentrations of chlorhexidine cause coagulation of intracellular constituents. As a result, the cytoplasm becomes congealed, with a consequent reduction in leakage (222–224, 290), so that there is a biphasic effect on membrane permeability. An initial high rate of leakage rises as the concentration of chlorhexidine increases, but leakage is reduced at higher biocide concentrations because of the coagulation of the cytosol.

Chlorhexidine was claimed by Harold et al. (199) to be an inhibitor of both membrane-bound and soluble ATPase as well as of net K^+ uptake in *Enterococcus faecalis*. However, only high biguanide concentrations inhibit membrane-bound ATPase (83), which suggests that the enzyme is not a primary target for chlorhexidine action. Although chlorhexidine collapses the membrane potential, it is membrane disruption rather than ATPase inactivation that is associated with its lethal effects (24, 272).

The effects of chlorhexidine on yeast cells are probably similar to those previously described for bacteria (204–207). Chlorhexidine has a biphasic effect on protoplast lysis, with reduced lysis at higher biguanide concentrations. Furthermore, in whole cells, the yeast cell wall may have some effect in limiting the uptake of the biguanide (208). The findings presented here and elsewhere (47, 136, 137, 527) demonstrate an effect on the fungal plasma membrane but with significant actions elsewhere in the cell (47). Increasing concentrations of chlorhexidine (up to 25 μg/ml) induce progressive lysis of *Saccharomyces cerevisiae* protoplasts, but higher biguanide concentrations result in reduced lysis (205).

Work to date suggests that chlorhexidine has a similar effect on the trophozoites of *Acanthameoba castellanii*, with the cysts being less sensitive (251–255). Furr (163) reviewed the effects of chlorhexidine and other biocides on *Acanthameoba* and showed that membrane damage in these protozoa is a significant factor in their inactivation.

Mycobacteria are generally highly resistant to chlorhexidine (419). Little is known about the uptake of chlorhexidine (and other antiseptics and disinfectants) by mycobacteria and on the biochemical changes that occur in the treated cells. Since the MICs for some mycobacteria are on the order of those for chlorhexidine-sensitive, gram-positive cocci (48), the inhibitory effects of chlorhexidine on mycobacteria may not be dissimilar to those on susceptible bacteria. *Mycobacterium avium-intracellulare* is considerably more resistant than other mycobacteria (48).

Chlorhexidine is not sporicidal (discussed in "Mechanisms of resistance"). Even high concentrations of the bisbiguanide do not affect the viability of *Bacillus* spores at ambient temperatures (473, 474), although a marked sporicidal effect is achieved at elevated temperatures (475). Presumably, sufficient changes occur in the spore structure to permit an increased uptake of the biguanide, although this has yet to be shown experimentally. Little is known about the uptake of chlorhexidine by bacterial spores, although coatless forms take up more of the compound than do "normal" spores (474).

Chlorhexidine has little effect on the germination of bacterial spores (414, 422, 432, 447) but inhibits outgrowth (447). The reason for its lack of effect on the former process but its significant activity against the latter is unclear. It could, however, be reflected in the relative uptake of chlorhexidine, since germinating cells take up much less of the bisbiguanide than do outgrowing forms (474). Binding sites could thus be reduced in number or masked in germinating cells.

The antiviral activity of chlorhexidine is variable. Studies with different types of bacteriophages have shown that chlorhexidine has no effect on MS2 or K coliphages (300). High concentrations also failed to inactivate *Pseudomonas aeruginosa* phage F116 and had no effect on phage DNA within the capsid or on phage proteins (301); the transduction process was more sensitive to chlorhexidine and other biocides than was the phage itself. This substantiated an earlier finding (306) that chlorhexidine bound poorly to F116 particles. Chlorhexidine is not always considered a particularly effective antiviral agent, and its activity is restricted to the lipid-enveloped viruses (361). Chlorhexidine does not inactivate nonenveloped viruses such as rotavirus (485), HAV (315), or poliovirus (34). Its activity was found by Ranganathan (389) to be restricted to the nucleic acid core or the outer coat, although it is likely that the latter would be a more important target site.

Alexidine. Alexidine differs chemically from chlorhexidine in possessing ethylhexyl end groups. Alexidine is more rapidly bactericidal and produces a significantly faster alteration in bactericidal permeability (79, 80). Studies with mixed-lipid and pure phospholipid vesicles demonstrate that, unlike chlorhexidine, alexidine produces lipid phase separation and domain formation (Table 2). It has been proposed (80) that the nature of the ethylhexyl end group in alexidine, as opposed to the chlorophenol one in chlorhexidine, might influence the ability of a biguanide to produce lipid domains in the cytoplasmic membrane.

Polymeric biguanides. Vantocil is a heterodisperse mixture of polyhexamethylene biguanides (PHMB) with a molecular weight of approximately 3,000. Polymeric biguanides have found use as general disinfecting agents in the food industry and, very successfully, for the disinfection of swimming pools. Vantocil is active against gram-positive and gram-negative bacteria, although *P. aeruginosa* and *Proteus vulgaris* are less sensitive. Vantocil is not sporicidal. PHMB is a membrane-active agent that also impairs the integrity of the outer membrane of gram-negative bacteria, although the membrane may also act as a permeability barrier (64, 172). Activity of PHMB increases on a weight basis with increasing levels of polymerization, which has been linked to enhanced inner membrane perturbation (173, 174).

Unlike chlorhexidine but similar to alexidine (Table 2), PHMB causes domain formation of the acidic phospholipids of the cytoplasmic membrane (61–64, 172, 173, 227). Permeability changes ensue, and there is believed to be an altered function of some membrane-associated enzymes. The proposed sequence of events during its interaction with the cell envelope of *E. coli* is as follows: (i) there is rapid attraction of

PHMB toward the negatively charged bacterial cell surface, with strong and specific adsorption to phosphate-containing compounds; (ii) the integrity of the outer membrane is impaired, and PHMB is attracted to the inner membrane; (iii) binding of PHMB to phospholipids occurs, with an increase in inner membrane permeability (K^+ loss) accompanied by bacteriostasis; and (iv) complete loss of membrane function follows, with precipitation of intracellular constituents and a bactericidal effect.

Diamidines

The diamidines are characterized chemically as described in Table 1. The isethionate salts of two compounds, propamidine (4,4-diaminodiphenoxypropane) and dibromopropamidine (2,2-dibromo-4,4-diamidinodiphenoxypropane), have been used as antibacterial agents. Their antibacterial properties and uses were reviewed by Hugo (213) and Hugo and Russell (226). Clinically, diamidines are used for the topical treatment of wounds.

The exact mechanism of action of diamidines is unknown, but they have been shown to inhibit oxygen uptake and induce leakage of amino acids (Table 2), as would be expected if they are considered as cationic surface-active agents. Damage to the cell surface of *P. aeruginosa* and *Enterobacter cloacae* has been described (400).

Halogen-Releasing Agents

Chlorine- and iodine-based compounds are the most significant microbicidal halogens used in the clinic and have been traditionally used for both antiseptic and disinfectant purposes.

Chlorine-releasing agents. Excellent reviews that deal with the chemical, physical, and microbiological properties of chlorine-releasing agents (CRAs) are available (42, 130). The most important types of CRAs are sodium hypochlorite, chlorine dioxide, and the *N*-chloro compounds such as sodium dichloroisocyanurate (NaDCC), with chloramine-T being used to some extent. Sodium hypochlorite solutions are widely used for hard-surface disinfection (household bleach) and can be used for disinfecting spillages of blood containing human immunodeficiency virus or HBV. NaDCC can also be used for this purpose and has the advantages of providing a higher concentration of available chlorine and being less susceptible to inactivation by organic matter. In water, sodium hypochlorite ionizes to produce Na^+ and the hypochlorite ion, OCl^-, which establishes an equilibrium with hypochlorous acid, HOCl (42). Between pH 4 and 7, chlorine exists predominantly as HClO, the active moiety, whereas above pH9, OCl^- predominates. Although CRAs have been predominantly used as hard-surface disinfectants, novel acidified sodium chlorite (a two-component system of sodium chlorite and mandelic acid) has been described as an effective antiseptic (248).

Surprisingly, despite being widely studied, the actual mechanism of action of CRAs is not fully known (Table 2). CRAs are highly active oxidizing agents and thereby destroy the cellular activity of proteins (42); potentiation of oxidation may occur at low pH, where the activity of CRAs is maximal, although increased penetration of outer cell layers may be achieved with CRAs in the unionized state. Hypochlorous acid has long been considered the active moiety responsible for bacterial inactivation by CRAs, the OCl^- ion having a minute effect compared to undissolved HOCl (130). This correlates with the observation that CRA activity is greatest when the percentage of undissolved HOCl is highest. This concept applies to hypochlorites, NaDCC, and chloramine-T.

Deleterious effects of CRAs on bacterial DNA that involve the formation of chlorinated derivatives of nucleotide bases have been described (115, 128, 477). Hypochlorous acid has also been found to disrupt oxidative phosphorylation (26) and other membrane-associated activity (70). In a particularly interesting paper, McKenna and Davies (321) described the inhibition of bacterial growth by hypochlorous acid. At 50 μM (2.6 ppm), HOCl completely inhibited the growth of *E. coli* within 5 min, and DNA synthesis was inhibited by 96% but protein synthesis was inhibited by only 10 to 30%. Because concentrations below 5 mM (260 ppm) did not induce bacterial membrane disruption or extensive protein degradation, it was inferred that DNA synthesis was the sensitive target. In contrast, chlorine dioxide inhibited bacterial protein synthesis (33).

CRAs at higher concentrations are sporicidal (44, 421, 431); this depends on the pH and concentration of available chlorine (408, 412). During treatment, the spores lose refractivity, the spore coat separates from the cortex, and lysis occurs (268). In addition, a number of studies have concluded that CRA-treated spores exhibit increased permeability of the spore coat (131, 268, 412).

CRAs also possess virucidal activity (34, 46, 116, 315, 394, 407, 467, 485, 486). Olivieri et al. (359) showed that chlorine inactivated naked f2 RNA at the same rate as RNA in intact phage, whereas f2 capsid proteins could still adsorb to the host. Taylor and Butler (504) found that the RNA of poliovirus type 1 was degraded into fragments by chlorine but that poliovirus inactivation preceded any severe morphological changes. By contrast, Floyd et al. (149) and O'Brien and Newman (357) demonstrated that the capsid of poliovirus type 1 was broken down. Clearly, further studies are needed to explain the antiviral action of CRAs.

Iodine and iodophors. Although less reactive than chlorine, iodine is rapidly bactericidal, fungicidal, tuberculocidal, virucidal, and sporicidal (184). Although aqueous or alcoholic (tincture) solutions of iodine have been used for 150 years as antiseptics, they are associated with irritation and excessive staining. In addition, aqueous solutions are generally unstable; in solution, at least seven iodine species are present in a complex equilibrium, with molecular iodine (I_2) being primarily responsible for antimictrobial efficacy (184). These problems were overcome by the development of iodophors ("iodine carriers" or "iodine-releasing agents"); the most widely used are povidone-iodine and poloxamer-iodine in both antiseptics and disinfectants. Iodophors are complexes of iodine and a solubilizing agent or carrier, which acts as a reservoir of the active "free" iodine (184). Although germicidal activity is maintained, iodophors are considered less active against certain fungi and spores than are tinctures (454).

Similar to chlorine, the antimicrobial action of iodine is rapid, even at low concentrations, but the exact mode of action is unknown. Iodine rapidly penetrates into microorganisms (76) and attacks key groups of proteins (in particular the free-sulfur amino acids cysteine and methionine [184, 267]), nucleotides, and fatty acids (15, 184), which culminates in cell death (184). Less is known about the antiviral action of iodine, but nonlipid viruses and parvoviruses are less sensitive than lipid enveloped viruses (384). Similarly to bacteria, it is likely that iodine attacks the surface proteins of enveloped viruses, but they may also destabilize membrane fatty acids by reacting with unsaturated carbon bonds (486).

Silver Compounds

In one form or another, silver and its compounds have long been used as antimicrobial agents (55, 443). The most important silver compound currently in use is silver sulfadiazine

(AgSD), although silver metal, silver acetate, silver nitrate, and silver protein, all of which have antimicrobial properties, are listed in *Martindale*, *The Extra Pharmacopoeia* (312). In recent years, silver compounds have been used to prevent the infection of burns and some eye infections and to destroy warts.

Silver nitrate. The mechanism of the antimicrobial action of silver ions is closely related to their interaction with thiol (sulfydryl, –SH) groups (32, 49, 161, 164), although other target sites remain a possibility (397, 509). Liau et al (287) demonstrated that amino acids such as cysteine and other compounds such as sodium thioglycolate containing thiol groups neutralized the activity of silver nitrate against *P. aeruginosa*. By contrast, amino acids containing disulfide (SS) bonds, non-sulfur-containing amino acids, and sulfur-containing compounds such as cystathione, cysteic acid, L-methionine, taurine, sodium bisulfite, and sodium thiosulfate were all unable to neutralize Ag^+ activity. These and other findings imply that interaction of Ag^+ with thiol groups in enzymes and proteins plays an essential role in bacterial inactivation, although other cellular components may be involved. Hydrogen bonding, the effects of hydrogen bond-breaking agents, and the specificity of Ag^+ for thiol groups were discussed in greater detail by Russell and Hugo (443) (Table 2). Virucidal properties might also be explained by binding to –SH groups (510).

Lukens (292) proposed that silver salts and other heavy metals such as copper act by binding to key functional groups of fungal enzymes. Ag^+ causes the release of K^+ ions from microorganisms; the microbial plasma or cytoplasmic membrane, with which is associated many important enzymes, is an important target site for Ag^+ activity (161, 329, 392, 470).

In addition to its effects on enzymes, Ag^+ produces other changes in microorganisms. Silver nitrate causes marked inhibition of growth of *Cryptococcus neoformans* and is deposited in the vacuole and cell wall as granules (60). Ag^+ inhibits cell division and damages the cell envelope and contents of *P. aeruginosa* (398). Bacterial cells increase in size, and the cytoplasmic membrane, cytoplasmic contents, and outer cell layers all exhibit structural abnormalities, although without any blebs (protuberances) (398). Finally, the Ag^+ ion interacts with nucleic acids (543); it interacts preferentially with the bases in DNA rather than with the phosphate groups, although the significance of this in terms of its lethal action is unclear (231, 387, 510, 547).

Silver sulfadiazine. AgSD is essentially a combination of two antibacterial agents, Ag^+ and sulfadiazine (SD). The question whether the antibacterial effect of AgSD arises predominantly from only one of the compounds or via a synergistic interaction has been posed repeatedly. AgSD has a broad spectrum of activity and, unlike silver nitrate, produces surface and membrane blebs in susceptible (but not resistant) bacteria (96). AgSD binds to cell components, including DNA (332, 404). Based on a chemical analysis, Fox (153) proposed a polymeric structure of AgSD composed of six silver atoms bonding to six SD molecules by linkage of the silver atoms to the nitrogens of the SD pyrimidine ring. Bacterial inhibition would then presumably be achieved when silver binds to sufficient base pairs in the DNA helix, thereby inhibiting transcription. Similarly, its antiphage properties have been ascribed to the fact that AgSD binds to phage DNA (154, 388). Clearly, the precise mechanism of action of AgSD has yet to be solved.

Peroxygens

Hydrogen peroxide. Hydrogen peroxide (H_2O_2) is a widely used biocide for disinfection, sterilization, and antisepsis. It is a clear, colorless liquid that is commercially available in a variety of concentrations ranging from 3 to 90%. H_2O_2 is considered environmentally friendly, because it can rapidly degrade into the innocuous products water and oxygen. Although pure solutions are generally stable, most contain stabilizers to prevent decomposition. H_2O_2 demonstrates broad-spectrum efficacy against viruses, bacteria, yeasts, and bacterial spores (38). In general, greater activity is seen against gram-positive than gram-negative bacteria; however, the presence of catalase or other peroxidases in these organisms can increase tolerance in the presence of lower concentrations. Higher concentrations of H_2O_2 (10 to 30%) and longer contact times are required for sporicidal activity (416), although this activity is significantly increased in the gaseous phase. H_2O_2 acts as an oxidant by producing hydroxyl free radicals ($^{\bullet}OH$) which attack essential cell components, including lipids, proteins, and DNA. It has been proposed that exposed sulfhydryl groups and double bonds are particularly targeted (38).

Peracetic acid. Peracetic acid (PAA) (CH_3COOOH) is considered a more potent biocide than hydrogen peroxide, being sporicidal, bactericidal, virucidal, and fungicidal at low concentrations (<0.3%) (38). PAA also decomposes to safe by-products (acetic acid and oxygen) but has the added advantages of being free from decomposition by peroxidases, unlike H_2O_2, and remaining active in the presence of organic loads (283, 308). Its main application is as a low-temperature liquid sterilant for medical devices, flexible scopes, and hemodialyzers, but it is also used as an environmental surface sterilant (100, 308).

Similar to H_2O_2, PAA probably denatures proteins and enzymes and increases cell wall permeability by disrupting sulfhydryl (–SH) and sulfur (S–S) bonds (22, 38).

Phenols

Phenolic-type antimicrobial agents have long been used for their antiseptic, disinfectant, or preservative properties, depending on the compound. It has been known for many years (215) that, although they have often been referred to as "general protoplasmic poisons," they have membrane-active properties which also contribute to their overall activity (120) (Table 2).

Phenol induces progressive leakage of intracellular constituents, including the release of K^+, the first index of membrane damage (273), and of radioactivity from ^{14}C-labeled *E. coli* (242, 265). Pulvertaft and Lumb (386) demonstrated that low concentrations of phenols (0.032%, 320 μg/ml) and other (nonphenolic) agents lysed rapidly growing cultures of *E. coli*, staphylococci, and streptococci and concluded that autolytic enzymes were not involved. Srivastava and Thompson (487, 488) proposed that phenol acts only at the point of separation of pairs of daughter cells, with young bacterial cells being more sensitive than older cells to phenol.

Hugo and Bloomfield (216, 217) showed with the chlorinated bis-phenol fenticlor that there was a close relationship between bactericidal activity and leakage of 260-nm-absorbing material (leakage being induced only by bactericidal concentrations). Fentichlor affected the metabolic activities of *S. aureus* and *E. coli* (217) and produced a selective increase in permeability to protons with a consequent dissipation of the proton motive force (PMF) and an uncoupling of oxidative phosphorylation (41). Chlorocresol has a similar action (124). Coagulation of cytoplasmic constituents at higher phenol concentrations, which causes irreversible cellular damage, has been described by Hugo (215).

The phenolics possess antifungal and antiviral properties. Their antifungal action probably involves damage to the plas-

ma membrane (436), resulting in leakage of intracellular constituents. Phenol does not affect the transduction of *P. aeruginosa* PAO by bacteriophage F116 (301), has no effect on phage DNA within the capsid, and has little effect on several of the phage band proteins unless treatments of 20 min or longer are used (303, 304).

Bis-Phenols

The bis-phenols are hydroxy-halogenated derivatives of two phenolic groups connected by various bridges (191, 446). In general, they exhibit broad-spectrum efficacy but have little activity against *P. aeruginosa* and molds and are sporostatic toward bacterial spores. Triclosan and hexachlorophane are the most widely used biocides in this group, especially in antiseptic soaps and hand rinses. Both compounds have been shown to have cumulative and persistent effects on the skin (313).

Triclosan. Triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether; Irgasan DP 300) exhibits particular activity against gram-positive bacteria (469, 521). Its efficacy against gram-negative bacteria and yeasts can be significantly enhanced by formulation effects. For example, triclosan in combination with EDTA caused increased permeability of the outer membrane (282). Reports have also suggested that in addition to its antibacterial properties, triclosan may have anti-inflammatory activity (25, 522). The specific mode of action of triclosan is unknown, but it has been suggested that the primary effects are on the cytoplasmic membrane. In studies with *E. coli*, triclosan at subinhibitory concentrations inhibited the uptake of essential nutrients, while higher, bactericidal concentrations resulted in the rapid release of cellular components and cell death (393). Studies with a divalent-ion-dependent *E. coli* triclosan mutant for which the triclosan MIC was 10-fold greater than that for a wild-type strain showed no significant differences in total envelope protein profiles but did show significant differences in envelope fatty acids (370). Specifically, a prominent 14:1 fatty acid was absent in the resistant strain, and there were minor differences in other fatty acid species. It was proposed that divalent ions and fatty acids may adsorb and limit the permeability of triclosan to its site of action (370). Minor changes in fatty acid profiles were recently found in both *E. coli* and *S. aureus* strains for which the triclosan MICs were elevated; however, the MBCs were not affected, suggesting, as for other phenols, that the cumulative effects on multiple targets contribute to the bactericidal activity (318, 319).

Hexachlorophene. Hexachlorophene (hexachlorophane; 2,2′-dihydroxy-3,5,6,3′,5′,6′-hexachlorodiphenylmethane) is another bis-phenol whose mode of action has been extensively studied. The primary action of hexachlorophene, based on studies with *Bacillus megatherium*, is to inhibit the membrane-bound part of the electron transport chain, and the other effects noted above are secondary ones that occur only at high concentrations (92, 158, 241, 481). It induces leakage, causes protoplast lysis, and inhibits respiration. The threshold concentration for the bactericidal activity of hexachlorphene is 10 μg/ml (dry weight), but peak leakage occurs at concentrations higher than 50 μg/ml and cytological changes occur above 30 μg/ml. Furthermore, hexachlorophene is bactericidal at 0°C despite causing little leakage at this temperature. Despite the broad-spectrum efficacy of hexachlorophene, concerns about toxicity (256), in particular in neonates, have meant that its use in antiseptic products has been limited.

Halophenols

Chloroxylenol (4-chloro-3,5-dimethylphenol; *p*-chloro-*m*-xylenol) is the key halophenol used in antiseptic or disinfectant formulations (66). Chloroxylenol is bactericidal, but *P. aeruginosa* and many molds are highly resistant (66, 432). Surprisingly, its mechanism of action has been little studied despite its widespread use over many years. Because of its phenolic nature, it would be expected to have an effect on microbial membranes.

Quaternary Ammonium Compounds

Surface-active agents (surfactants) have two regions in their molecular structures, one a hydrocarbon, water-repellent (hydrophobic) group and the other a water-attracting (hydrophilic or polar) group. Depending on the basis of the charge or absence of ionization of the hydrophilic group, surfactants are classified into cationic, anionic, nonionic, and ampholytic (amphoteric) compounds. Of these, the cationic agents, as exemplified by quaternary ammonium compounds (QACs), are the most useful antiseptics and disinfectants (160). They are sometimes known as cationic detergents. QACs have been used for a variety of clinical purposes (e.g., preoperative disinfection of unbroken skin, application to mucous membranes, and disinfection of noncritical surfaces). In addition to having antimicrobial properties, QACs are also excellent for hard-surface cleaning and deodorization.

It has been known for many years that QACs are membrane-active agents (221) (Table 2) (i.e., with a target site predominantly at the cytoplasmic (inner) membrane in bacteria or the plasma membrane in yeasts) (215). Salton (460) proposed the following sequence of events with microorganisms exposed to cationic agents: (i) adsorption and penetration of the agent into the cell wall; (ii) reaction with the cytoplasmic membrane (lipid or protein) followed by membrane disorganization; (iii) leakage of intracellular low-molecular-weight material; (iv) degradation of proteins and nucleic acids; and (v) wall lysis caused by autolytic enzymes. There is thus a loss of structural organization and integrity of the cytoplasmic membrane in bacteria, together with other damaging effects to the bacterial cell (120).

Useful information about the selectivity of membrane action can be obtained by studying the effects of biocides on protoplasts and spheroplasts suspended in various solutes. QACs cause lysis of spheroplasts and protoplasts suspended in sucrose (107, 215, 243, 428). The cationic agents react with phospholipid components in the cytoplasmic membrane (69), thereby producing membrane distortion and protoplast lysis under osmotic stress. Isolated membranes do not undergo disaggregation on exposure to QACs, because the membrane distortion is not sufficiently drastic. The non-QAC agents TCC and trichlorosalicylanide have specific effects: TCC induces protoplast lysis in ammonium chloride by increasing Cl^- permeability, whereas trichlorosalicylanide induces lysis in ammonium nitrate by increasing NO_3^- permeability (428). In contrast, QACs (and chlorhexidine) induce lysis of protoplasts or spheroplasts suspended in various solutes because they effect generalized, rather than specific, membrane damage.

The bacterial cytoplasmic membrane provides the mechanism whereby metabolism is linked to solute transport, flagellar movement, and the generation of ATP. Protons are extruded to the exterior of the bacterial cell during metabolism. The combined potential (concentration or osmotic effect of the proton and its electropositivity) is the PMF, which drives these ancillary activities (428). The QAC cetrimide was found (121) to have an effect on the PMF in *S. aureus*. At its bacteriostatic concentration, cetrimide caused the discharge of the pH component of the PMF and also produced the maximum amount of 260-nm-absorbing material.

QACs are also believed to damage the outer membrane of gram-negative bacteria, thereby promoting their own uptake. This aspect of QACs is considered below (see "Intrinsic resistance of gram-negative bacteria").

The QAC cetylpyridium chloride (CPC) induces the leakage of K^+ and pentose material from the yeast *S. cerevisiae* and induces protoplast lysis as well as interacting with crude cell sap (205). Unlike chlorhexidine, however, no biphasic effect on protoplast lysis was observed. The initial toxic effect of QACs on yeast cells is a disorganization of the plasma membranes, with organized lipid structures in the membranes (and in lipid bilayers) being disrupted.

QACs are sporostatic; they inhibit the outgrowth of spores (the development of a vegetative cell from a germinated spore) but not the actual germination processes (development from dormancy to a metabolically active state), albeit by an unknown mechanism (414). Likewise, the QACs are not mycobactericidal but have a mycobacteriostatic action, although the actual effects on mycobacteria have been little studied (419).

The QACs have an effect on lipid, enveloped (including human immunodeficiency virus and HBV) but not nonenveloped viruses (394, 485, 486). QAC-based products induced disintegration and morphological changes of human HBV, resulting in loss of infectivity (382). In studies with different phages (298–301, 303–305, 307), CPC significantly inhibited transduction by bacteriophage F116 and inactivated the phage particles. Furthermore, CPC altered the protein bands of F116 but did not affect the phage DNA within the capsid.

Vapor-Phase Sterilants

Many heat-sensitive medical devices and surgical supplies can be effectively sterilized by liquid sterilants (in particular glutaraldehyde, PAA, and hydrogen peroxide) or by vapor-phase sterilization systems (Table 1). The most widely used active agents in these "cold" systems are ethylene oxide, formaldehyde and, more recently developed, hydrogen peroxide and PAA. Ethylene oxide and formaldehyde are both broad-spectrum alkylating agents. However, their activity is dependent on active concentration, temperature, duration of exposure, and relative humidity (87). As alkylating agents, they attack proteins, nucleic acids, and other organic compounds; both are particularly reactive with sulfhydryl and other enzyme-reactive groups. Ethylene oxide gas has the disadvantages of being mutagenic and explosive but is not generally harsh on sensitive equipment, and toxic residuals from the sterilization procedure can be routinely eliminated by correct aeration. Formaldehyde gas is similar and has the added advantage of being nonexplosive but is not widely used in health care. Vapor-phase hydrogen peroxide and PAA are considered more active (as oxidants) at lower concentrations than in the liquid form (334). Both active agents are used in combination with gas plasma in low-temperature sterilization systems (314). Their main advantages over other vapor-phase systems include low toxicity, rapid action, and activity at lower temperature; the disadvantages include limited penetrability and applications.

MECHANISMS OF RESISTANCE

Introduction

As stated above, different types of microorganisms vary in their response to antiseptics and disinfectants. This is hardly surprising in view of their different cellular structure, composition, and physiology. Traditionally, microbial susceptibility to antiseptics and disinfectants has been classified based on these differences; with recent work, this classification can be further extended (Fig. 1). Because different types of organisms react differently, it is convenient to consider bacteria, fungi, viruses, protozoa, and prions separately.

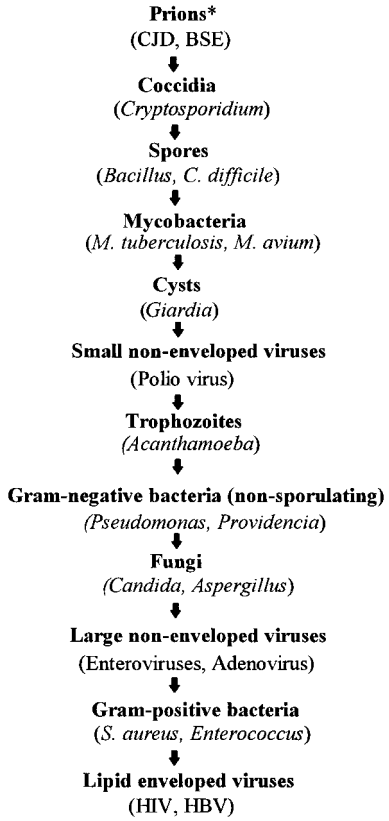


FIG. 1. Descending order of resistance to antiseptics and disinfectants. The asterisk indicates that the conclusions are not yet universally agreed upon.

Bacterial Resistance to Antiseptics and Disinfectants

In recent years, considerable progress has been made in understanding more fully the responses of different types of bacteria (mycobacteria, nonsporulating bacteria, and bacterial spores) to antibacterial agents (43, 84, 414, 415, 419, 422, 496). As a result, resistance can be either a natural property of an organism (intrinsic) or acquired by mutation or acquisition of plasmids (self-replicating, extrachromosomal DNA) or transposons (chromosomal or plasmid integrating, transmissible DNA cassettes). Intrinsic resistance is demonstrated by gram-negative bacteria, bacterial spores, mycobacteria, and, under certain conditions, staphylococci (Table 5). Acquired, plasmid-mediated resistance is most widely associated with mercury compounds and other metallic salts. In recent years, acquired resistance to certain other types of biocides has been observed, notably in staphylococci.

Intrinsic Bacterial Resistance Mechanisms

For an antiseptic or disinfectant molecule to reach its target site, the outer layers of a cell must be crossed. The nature and composition of these layers depend on the organism type and may act as a permeability barrier, in which there may be a reduced uptake (422, 428). Alternatively but less commonly, constitutively synthesized enzymes may bring about degradation of a compound (43, 214, 358). Intrinsic (innate) resistance

TABLE 5. Intrinsic resistance mechanisms in bacteria to antiseptics and disinfectants

Type of resistance	Example(s)	Mechanism of resistance
Impermeability		
Gram-negative bacteria	QACs, triclosan, diamines	Barrier presented by outer membrane may prevent uptake of antiseptic or disinfectant; glycocalyx may also be involved
Mycobacteria	Chlorhexidine, QACs Glutaraldehyde	Waxy cell wall prevents adequate biocide entry Reason for high resistance of some strains of *M. chelonae*(?)
Bacterial spores	Chlorhexidine, QACs, phenolics	Spore coat(s) and cortex present a barrier to entry of antiseptics and disinfectants
Gram-positive bacteria	Chlorhexidine	Glycocalyx/mucoexopolysaccaride may be associated with reduced diffusion of antiseptic
Inactivation (chromosomally mediated)	Chlorohexidine	Breakdown of chlorhexidine molecule may be responsible for resistance

is thus a natural, chromosomally controlled property of a bacterial cell that enables it to circumvent the action of an antiseptic or disinfectant. Gram-negative bacteria tend to be more resistant than gram-positive organisms, such as staphylococci (Table 6).

Intrinsic resistance of bacterial spores. Bacterial spores of the genera *Bacillus* and *Clostridium* have been widely studied and are invariably the most resistant of all types of bacteria to antiseptics and disinfectants (43, 46, 150, 414, 418, 420, 422, 423, 457). Although *Bacillus* species are generally not pathogenic, their spores are widely used as indicators of efficient sterilization. *Clostridium* species are significant pathogens; for example, *C. difficile* is the most common cause of hospital-acquired diarrhea (478). Many biocides are bactericidal or bacteristatic at low concentrations for nonsporulating bacteria, including the vegetative cells of *Bacillus* and *Clostridium* species, but high concentrations may be necessary to achieve a sporicidal effect (e.g., for glutaraldehyde and CRAs). By contrast, even high concentrations of alcohol, phenolics, QACs, and chlorhexidine lack a sporicidal effect, although this may be achieved when these compounds are used at elevated temperatures (475).

A typical spore has a complex structure (29, 151). In brief, the germ cell (protoplast or core) and germ cell wall are surrounded by the cortex, outside which are the inner and outer spore coats. A thin exosporium may be present in the spores of some species but may surround just one spore coat. RNA, DNA, and DPA, as well as most of the calcium, potassium, manganese, and phosphorus, are present in the spore protoplast. Also present are large amounts of low-molecular-weight basic proteins (small acid-soluble spore proteins [SASPs]), which are rapidly degraded during germination. The cortex consists largely of peptidoglycan, including a spore-specific muramic lactam. The spore coats comprise a major portion of the spore. These structures consist largely of protein, with an alkali-soluble fraction made up of acidic polypeptides being found in the inner coat and an alkali-resistant fraction associated with the presence of disulfide-rich bonds being found in the outer coat. These aspects, especially the roles of the coat(s) and cortex, are all relevant to the mechanism(s) of resistance presented by bacterial spores to antiseptics and disinfectants.

Several techniques are available for studying mechanisms of spore resistance (428). They include removing the spore coat and cortex by using a "step-down" technique to achieve a highly synchronous sporulation (so that cellular changes can be accurately monitored), employing spore mutants that do not sporulate beyond genetically determined stages in sporulation, adding an antiseptic or disinfectant at the commencement of sporulation and determining how far the process can proceed, and examining the role of SASPs. Such procedures have helped provide a considerable amount of useful information. Sporulation itself is a process in which a vegetative cell develops into a spore and involves seven stages (designated 0 to VII). During this process, the vegetative cell (stage 0) undergoes a series of morphological changes that culminate in the release of a mature spore (stage VII). Stages IV (cortex development) to VII are the most important in the development of resistance to biocides.

Resistance to antiseptics and disinfectants develops during sporulation and may be an early, intermediate, or (very) late event (103, 375, 378, 429, 474). Useful markers for monitoring the development of resistance are toluene (resistance to which is an early event), heat (intermediate), and lysozyme (late) (236, 237). Studies with a wild-type *B. subtilis* strain, 168, and its Spo$^-$ mutants have helped determine the stages at which resistance develops (262, 375, 474). From these studies (Fig. 2), the order of development of resistance was toluene (marker), formaldehyde, sodium lauryl sulfate, phenol, and phenylmercuric nitrate; *m*-cresol, chlorocresol, chlorhexidine gluconate, cetylpyridinium chloride, and mercuric chloride; and moist heat (marker), sodium dichloroisocyanurate, sodium hypochlorite, lysozyme (marker), and glutaraldehyde. The association of the onset of resistance to a particular antiseptic or disinfectant with a particular stage(s) in spore development is thereby demonstrated.

Spore coat-less forms, produced by treatment of spores un-

TABLE 6. MIC of some antiseptics and disinfectants against gram-positive and gram-negative bacteria[a]

Chemical agent	MIC (μg/ml) for:		
	S. aureus[b]	*E. coli*	*P. aeruginosa*
Benzalkonium chloride	0.5	50	250
Benzethonium chloride	0.5	32	250
Cetrimide	4	16	64–128
Chlorhexidine	0.5–1	1	5–60
Hexachlorophene	0.5	12.5	250
Phenol	2,000	2,000	2,000
o-Phenylphenol	100	500	1,000
Propamine isethionate	2	64	256
Dibromopropamidine isethionate	1	4	32
Triclosan	0.1	5	>300

[a] Based on references 226 and 440.
[b] MICs of cationic agents for some MRSA strains may be higher (see Table 10).

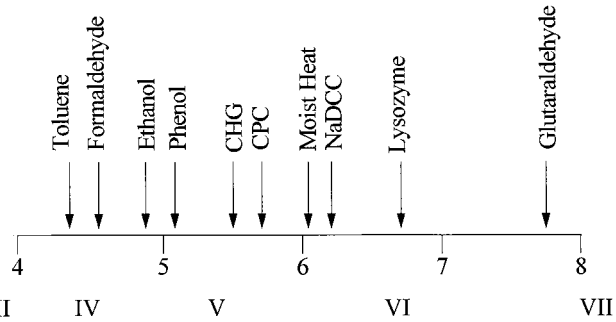


FIG. 2. Development of resistance of *Bacillus subtilis* during sporulation. Roman numerals indicate the sporulation stage from III (engulfment of the forespore) to VII (release of the mature spore). Arabic numbers indicate the time (hours) following the onset of sporulation and the approximate times at which resistance develops against biocides (262). CHG, chlorhexidine; CPC, cetylpyridinium chloride; NaDCC, sodium dichloroisocyanurate.

der alkaline conditions with urea plus dithiothreitol plus sodium lauryl sulfate (UDS), have also been of value in estimating the role of the coats in limiting the access of antiseptics and disinfectants to their target sites. However, Bloomfield and Arthur (44, 45) and Bloomfield (43) showed that this treatment also removes a certain amount of cortex and that the amount of cortex remaining can be further reduced by the subsequent use of lysozyme. These findings demonstrate that the spore coats have an undoubted role in conferring resistance but that the cortex also is an important barrier since (UDS plus lysozyme)-treated spores are much more sensitive to chlorine- and iodine-releasing agents than are UDS-exposed spores.

The initial development and maturity of the cortex are implicated in the development of resistance to phenolics. Likewise, it is now clear that cortex development is at least partially responsible for resistance to chlorhexidine and QACs; this resistance is enhanced in developing spores by the initiation of spore coat synthesis (262). The effect of various concentrations of chlorhexidine, sublethal to vegetative bacteria, on the development of spores of *B. subtilis* 168 MB_2 were investigated by Knott and Russell (261). They found that chlorhexidine affected spore development; as concentrations of the biguanide increased, spore index values (the percentage of cells forming spores) decreased and sensitivity to both heat and toluene increased. By contrast, the control (untreated) culture was highly resistant to both of these agents and had a high spore index value, indicative of high levels of mature spores. The slightly increased resistance to toluene compared to resistance to heat was not surprising, since cells must reach stages V to VI (synthesis of spore coats and maturation) to attain heat resistance but only stage III (forespore engulfment) to attain toluene resistance (Fig. 2); in other words, if sporulation is inhibited by chlorhexidine, more cells are likely to reach stage III than the later stages. While less definitive than the earlier approaches, these procedures provide further evidence of the involvement of the cortex and coats in chlorhexidine resistance.

Development of resistance during sporulation to formaldehyde was an early event but depended to some extent on the concentration (1 to 5% [vol/vol]) of formaldehyde used. This appears to be at odds with the extremely late development of resistance to the dialdehyde, glutaraldehyde. Since glutaraldehyde and the monoaldehyde, formaldehyde, contain an aldehyde group(s) and are alkylating agents, it would be plausible to assume that they would have a similar mode of sporicidal action, even though the dialdehyde is a more powerful alkylating agent. If this were true, it could also be assumed that spores would exhibit the same resistance mechanisms for these disinfectants. In aqueous solution, formaldehyde forms a glycol in equilibrium (512, 524); thus, formaldehyde could well be acting poorly as an alcohol-type disinfectant rather than as an aldehyde (327). Alkaline glutaraldehyde does not readily form glycols in aqueous solution (178). Resistance to formaldehyde may be linked to cortex maturation, and resistance to glutaraldehyde may be linked to coat formation (262).

Setlow and his coworkers (472) demonstrated that α/β-type SASPs coat the DNA in wild-type spores of *B. subtilis*, thereby protecting it from attack by enzymes and antimicrobial agents. Spores ($\alpha^-\beta^-$) lacking these α/β-type SASPs are significantly more sensitive to hydrogen peroxide (471) and hypochlorite (456). Thus, SASPs contribute to spore resistance to peroxide and hypochlorite but may not be the only factors involved, since the coats and cortex also play a role (428).

Two other aspects of spores should be considered: the revival of injured spores and the effects of antiseptics and disinfectants on germinating and outgrowing spores. Although neither aspect is truly a resistance mechanism, each can provide useful information about the site and mechanism of action of sporicidal agents and about the associated spore resistance mechanisms and might be of clinical importance.

The revival of disinfectant-treated spores has not been extensively studied. Spicher and Peters (483, 484) demonstrated that formaldehyde-exposed spores of *B. subtilis* could be revived after a subsequent heat shock process. A more recent finding with *B. stearothermophilus* casts further doubt on the efficacy of low-temperature steam with formaldehyde as a sterilizing procedure (541). The revival of spores exposed to glutaraldehyde, formaldehyde, chlorine, and iodine was examined by Russell and his colleagues (103, 376, 377, 424, 532–537). A small proportion of glutaraldehyde-treated spores of various *Bacillus* species were revived when the spores were treated with alkali after neutralization of glutaraldehyde with glycine (103, 379, 380). Experiments designed to distinguish between germination and outgrowth in the revival process have demonstrated that sodium hydroxide-induced revival increases the potential for germination. Based on other findings, the germination process is also implicated in the revival of spores exposed to other disinfectants.

Intrinsic resistance of mycobacteria. Mycobacteria are well known to possess a resistance to antiseptics and disinfectants that is roughly intermediate between those of other nonsporulating bacteria and bacterial spores (Fig. 1) (177, 345, 419). There is no evidence that enzymatic degradation of harmful molecules takes place. The most likely mechanism for the high resistance of mycobacteria is associated with their complex cell walls that provide an effective barrier to the entry of these agents. To date, plasmid- or transposon-mediated resistance to biocides has not been demonstrated in mycobacteria.

The mycobacterial cell wall is a highly hydrophobic structure with a mycoylarabinogalactan-peptidoglycan skeleton (27, 105, 106, 322, 389, 390, 461, 530). The peptidoglycan is covalently linked to the polysaccharide copolymer (arabinogalactan) made up of arabinose and galactose esterified to mycolic acids. Also present are complex lipids, lipopolysaccharides (LPSs), and proteins, including porin channels through which hydrophilic molecules can diffuse into the cell (232, 356). Similar cell wall structures exist in all the mycobacterial species examined to date (228). The cell wall composition of a particular species may be influenced by its environmental niche (27). Pathogenic bacteria such as *Mycobacterium tuberculosis* exist in a relatively nutrient-rich environment, whereas saprophytic mycobacteria living in soil or water are exposed to natural antibiotics and tend to be more intrinsically resistant to these drugs.

Antiseptics or disinfectants that exhibit mycobacterial activity are phenol, PAA, hydrogen peroxide, alcohol, and glutaraldehyde (16, 17, 99, 419, 425, 455). By contrast, other well-known bactericidal agents, such as chlorhexidine and QACs, are mycobacteristatic even when used at high concentrations (51, 52, 419, 425, 455). However, the activity of these can be substantially increased by formulation effects. Thus, a number of QAC-based products claim to have mycobacterial activity. For example, a newer formulation (Sactimed-I-Sinald) containing a mixture of alkyl polyguanides and alkyl QACs is claimed to be mycobactericidal (211, 353). However, there is some doubt whether the antibacterial agents had been properly quenched or neutralized to prevent carryover of inhibitory concentrations into recovery media.

Many years ago, it was proposed (T. H. Shen, cited in reference 99) that the resistance of mycobacteria to QACs was related to the lipid content of the cell wall. In support of this contention, *Mycobacterium phlei*, which has a low total cell lipid content, was more sensitive than *M. tuberculosis*, which has a higher lipid content. It was also noted that the resistance of various species of mycobacteria was related to the content of waxy material in the wall. It is now known that because of the highly hydrophobic nature of the cell wall, hydrophilic biocides are generally unable to penetrate the mycobacterial cell wall in sufficiently high concentrations to produce a lethal effect. However, low concentrations of antiseptics and disinfectants such as chlorhexidine must presumably traverse this permeability barrier, because the MICs are of the same order as those concentrations inhibiting the growth of nonmycobacterial strains such as *S. aureus*, although *M. avium-intracellulare* may be particularly resistant (51, 52). The component(s) of the mycobacterial cell wall responsible for the high biocide resistance are currently unknown, although some information is available. Inhibitors of cell wall synthesis increase the susceptibility of *M. avium* to drugs (391); inhibition of myocide C, arabinogalactan, and mycolic acid biosynthesis enhances drug susceptibility. Treatment of this organism with *m*-fluoro-DL-phenylalanine (*m*-FL-phe), which inhibits mycocide C synthesis, produces significant alterations in the outer cell wall layers (106). Ethambutol, an inhibitor of arabinogalactan (391, 501) and phospholipid (461, 462) synthesis, also disorganizes these layers. In addition, ethambutol induces the formation of ghosts without the dissolution of peptidoglycan (391). Methyl-4-(2-octadecylcyclopropen-1-yl) butanoate (MOCB) is a structural analogue of a key precursor in mycolic acid synthesis. Thus, the effects of MOCB on mycolic acid synthesis and *m*-FL-phe and ethambutol on outer wall biosynthetic processes leading to changes in cell wall architecture appear to be responsible for increasing the intracellular concentration of chemotherapeutic drugs. These findings support the concept of the cell wall acting as a permeability barrier to these drugs (425). Fewer studies have been made of the mechanisms involved in the resistance of mycobacteria to antiseptics and disinfectants. However, the activity of chlorhexidine and of a QAC, cetylpyridinium chloride, against *M. avium* and *M. tuberculosis* can be potentiated in the presence of ethambutol (52). From these data, it may be inferred that arabinogalactan is one cell wall component that acts as a permeability barrier to chlorhexidine and QACs. It is not possible, at present, to comment on other components, since these have yet to be investigated. It would be useful to have information about the uptake into the cells of these antiseptic agents in the presence and absence of different cell wall synthesis inhibitors.

One species of mycobacteria currently causing concern is *M. chelonae*, since these organisms are sometimes isolated from endoscope washes and dialysis water. One such strain was not killed even after a 60-min exposure to alkaline glutaraldehyde; in contrast, a reference strain showed a 5-log-unit reduction after a contact time of 10 min (519). This glutaraldehyde-resistant *M. chelonae* strain demonstrated an increased tolerance to PAA but not to NaDCC or to a phenolic. Other workers have also observed an above-average resistance of *M. chelonae* to glutaraldehyde and formaldehyde (72) but not to PAA (187, 294). The reasons for this high glutaraldehyde resistance are unknown. However, *M. chelonae* is known to adhere strongly to smooth surfaces, which may render cells within a biofilm less susceptible to disinfectants. There is no evidence to date that uptake of glutaraldehyde by *M. chelonae* is reduced.

Intrinsic resistance of other gram-positive bacteria. The cell wall of staphylococci is composed essentially of peptidoglycan and teichoic acid. Neither of these appears to act as an effective barrier to the entry of antiseptics and disinfectants. Since high-molecular-weight substances can readily traverse the cell wall of staphylococci and vegetative *Bacillus* spp., this may explain the sensitivity of these organisms to many antibacterial agents including QACs and chlorhexidine (411, 417, 422, 428, 451).

However, the plasticity of the bacterial cell envelope is a well-known phenomenon (381). Growth rate and any growth-limiting nutrient will affect the physiological state of the cells. Under such circumstances, the thickness and degree of cross-linking of peptidoglycan are likely to be modified and hence the cellular sensitivity to antiseptics and disinfectants will be altered. For example, Gilbert and Brown (171) demonstrated that the sensitivity of *Bacillus megaterium* cells to chlorhexidine and 2-phenoxyethanol is altered when changes in growth rate and nutrient limitation are made with chemostat-grown cells. However, lysozyme-induced protoplasts of these cells remained sensitive to, and were lysed by, these membrane-active agents. Therefore, the cell wall in whole cells is responsible for their modified response.

In nature, *S. aureus* may exist as mucoid strains, with the cells surrounded by a slime layer. Nonmucoid strains are killed more rapidly than mucoid strains by chloroxylenol, cetrimide, and chlorhexidine, but there is little difference in killing by phenols or chlorinated phenols (263); removal of slime by washing rendered the cells sensitive. Therefore, the slime plays a protective role, either as a physical barrier to disinfectant penetration or as a loose layer interacting with or absorbing the biocide molecules.

There is no evidence to date that vancomycin-resistant enterococci or enterococci with high-level resistance to aminoglycoside antibiotics are more resistant to disinfectants than are antibiotic-sensitive enterococcal strains (9, 11, 48, 319). However, enterococci are generally less sensitive to biocides than are staphylococci, and differences in inhibitory and bactericidal concentrations have also been found among enterococcal species (257).

Intrinsic resistance of gram-negative bacteria. Gram-negative bacteria are generally more resistant to antiseptics and disinfectants than are nonsporulating, nonmycobacterial gram-positive bacteria (Fig. 2) (428, 440, 441). Examples of MICs against gram-positive and -negative organisms are provided in Table 6. Based on these data, there is a marked difference in the sensitivity of *S. aureus* and *E. coli* to QACs (benzalkonium, benzethonium, and cetrimide), hexachlorophene, diamidines, and triclosan but little difference in chlorhexidine susceptibility. *P. aeruginosa* is considerably more resistant to most of these agents, including chlorhexidine, and (not shown) *Proteus* spp. possess an above-average resistance to cationic agents such as chlorhexidine and QACs (311, 440).

The outer membrane of gram-negative bacteria acts as a barrier that limits the entry of many chemically unrelated types

TABLE 7. Possible transport of some antiseptics and disinfectants into gram-negative bacteria[a]

Antiseptic/disinfectant	Passage across OM[b]	Passage across IM[b]
Chlorhexidine	Self-promoted uptake(?)	IM is a major target site; damage to IM enables biocide to enter cytosol, where further interaction occurs
QACs	Self-promoted uptake(?); also, OM might present a barrier	IM is a major target site; damage to IM enables biocide to enter cytosol, where further interaction occurs
Phenolics	Hydrophobic pathway (activity increases as hydrophobicity of phenolic increases)	IM is a major target site, but high phenolic concentrations coagulate cytoplasmic constituents

[a] Data from references 197, 433 to 435, 438, and 439.
[b] OM, outer membrane; IM, inner membrane.

of antibacterial agents (18, 169, 196, 197, 355, 366, 440, 516, 517). This conclusion is based on the relative sensitivities of staphylococci and gram-negative bacteria and also on studies with outer membrane mutants of *E. coli*, *S. typhimurium*, and *P. aeruginosa* (134, 135, 433–435, 438). Smooth, wild-type bacteria have a hydrophobic cell surface; by contrast, because of the phospholipid patches on the cell surface, deep rough (heptose-less) mutants are hydrophobic. These mutants tend to be hypersensitive to hydrophobic antibiotics and disinfectants. Low-molecular-weight (M_r <ca. 600) hydrophilic molecules readily pass via the porins into gram-negative cells, but hydrophobic molecules diffuse across the outer membrane bilayer (Table 7). In wild-type gram-negative bacteria, intact LPS molecules prevent ready access of hydrophobic molecules to phospholipid and thence to the cell interior. In deep rough strains, which lack the O-specific side chain and most of the core polysaccharide, the phospholipid patches at the cell surface have their head groups oriented toward the exterior.

In addition to these hydrophilic and hydrophobic entry pathways, a third pathway has been proposed for cationic agents such as QACs, biguanidies, and diamidines. It is claimed that these damage the outer membrane, thereby promoting their own uptake (197). Polycations disorganize the outer membrane of *E. coli* (520). It must be added, however, that the QACs and diamidines are considerably less active against wild-type strains than against deep rough strains whereas chlorhexidine has the same order of activity (MIC increase about 2 to 3 fold) against both types of *E. coli* strains (434, 435, 439). However, *S. typhimurium* mutants are more sensitive to chlorhexidine than are wild-type strains (433).

Gram-negative bacteria that show a high level of resistance to many antiseptics and disinfectants include *P. aeruginosa*, *Burkholderia cepacia*, *Proteus* spp., and *Providencia stuartii* (428, 440). The outer membrane of *P. aeruginosa* is responsible for its high resistance; in comparison with other organisms, there are differences in LPS composition and in the cation content of the outer membrane (54). The high Mg^{2+} content aids in producing strong LPS-LPS links; furthermore, because of their small size, the porins may not permit general diffusion through them. *B. cepacia* is often considerably more resistant in the hospital environment than in artificial culture media (360); the high content of phosphate-linked arabinose in its LPS decreases the affinity of the outer membrane for polymyxin antibiotics and other cationic and polycationic molecules (97, 516). *Pseudomonas stutzeri*, by contrast, is highly sensitive to many antibiotics and disinfectants (449), which implies that such agents have little difficulty in crossing the outer layers of the cells of this organism.

Members of the genus *Proteus* are invariably insensitive to chlorhexidine (311). Some strains that are highly resistant to chlorhexidine, QACs, EDTA, and diamidines have been isolated from clinical sources. The presence of a less acidic type of outer membrane LPS could be a contributing factor to this intrinsic resistance (97, 516).

A particularly troublesome member of the genus *Providencia* is *P. stuartii*. Like *Proteus* spp., *P. stuartii* strains have been isolated from urinary tract infections in paraplegic patients and are resistant to different types of antiseptics and disinfectants including chlorhexidine and QACs (492, 496). Strains of *P. stuartii* that showed low-, intermediate-, and high-level resistance to chlorhexidine formed the basis of a series of studies of the resistance mechanism(s) (86, 422, 428). Gross differences in the composition of the outer layers of these strains were not detected, and it was concluded that (i) subtle changes in the structural arrangement of the cell envelopes of these strains was associated with this resistance and (ii) the inner membrane was not implicated (230).

Few authors have considered peptidoglycan in gram-negative bacteria as being a potential barrier to the entry of inhibitory substances. The peptidoglycan content of these organisms is much lower than in staphylococci, which are inherently more sensitive to many antiseptics and disinfectants. Nevertheless, there have been instances (discussed in reference 422) where gram-negative organisms grown in subinhibitory concentrations of a penicillin have deficient permeability barriers. Furthermore, it has been known for many years (215, 409, 411) that penicillin-induced spheroplasts and lysozyme-EDTA-Tris "protoplasts" of gram-negative bacteria are rapidly lysed by membrane-active agents such as chlorhexidine. It is conceivable that the stretched nature of both the outer and inner membranes in β-lactam-treated organisms could contribute to this increased susceptibility.

The possibility exists that the cytoplasmic (inner) membrane provides one mechanism of intrinsic resistance. This membrane is composed of lipoprotein and would be expected to prevent passive diffusion of hydrophilic molecules. It is also known that changes in membrane composition affect sensitivity to ethanol (159). Lannigan and Bryan (275) proposed that decreased susceptibility of *Serratia marcescens* to chlorhexidine was linked to the inner membrane, but Ismaeel et al. (230) could find no such role with chlorhexidine-resistant *P. stuartii*. At present, there is little evidence to implicate the inner membrane in biocide resistance. In addition, chlorhexidine degradation was reported for *S. marcescens*, *P. aeruginosa*, and *Achromobacter/Alcaligenes xylosoxidans* (358).

Physiological (phenotypic) adaption as an intrinsic mechanism. The association of microorganisms with solid surfaces leads to the generation of a biofilm, defined as a consortium of organisms organized within an extensive exopolysaccharide exopolymer (93, 94). Biofilms can consist of monocultures, of several diverse species, or of mixed phenotypes of a given species (57, 73, 381). Some excellent publications that deal with the nature, formation, and content of biofilms are available (125, 178, 276, 538). Biofilms are important for several reasons,

TABLE 8. Biofilms and microbial response to antimicrobial agents

Mechanism of resistance associated with biofilms	Comment
Exclusion or reduced access of antiseptic or disinfectant to underlying cell	Depends on (i) nature of antiseptic/disinfectant, (ii) binding capacity of glycocalyx toward antiseptic or disinfectant, and (iii) rate of growth of microcolony relative to diffusion rate of chemical inhibitor
Modulation of microenvironment	Associated with (i) nutrient limitation and (ii) growth rate
Increased production of degradative enzymes by attached cells	Mechanism unclear at present
Plasmid transfer between cells in biofilm?	Associated with enhanced tolerance to antiseptics and disinfectants?

notably biocorrosion, reduced water quality, and foci for contamination of hygienic products (10, 12–14). Colonization also occurs on implanted biomaterials and medical devices, resulting in increased infection rates and possible recurrence of infection (125).

Bacteria in different parts of a biofilm experience different nutrient environments, and their physiological properties are affected (57). Within the depths of a biofilm, for example, nutrient limitation is likely to reduce growth rates, which can affect susceptibility to antimicrobial agents (98, 142, 171, 172). Thus, the phenotypes of sessile organisms within biofilms differ considerably from the planktonic cells found in laboratory cultures (73). Slow-growing bacteria are particularly insusceptible, a point reiterated recently in another context (126).

Several reasons can account for the reduced sensitivity of bacteria within a biofilm (Table 8). There may be (i) reduced access of a disinfectant (or antibiotic) to the cells within the biofilm, (ii) chemical interaction between the disinfectant and the biofilm itself, (iii) modulation of the microenvironment, (iv) production of degradative enzymes (and neutralizing chemicals), or (v) genetic exchange between cells in a biofilm. However, bacteria removed from a biofilm and recultured in culture media are generally no more resistant than the "ordinary" planktonic cells of that species (57).

Several instances are known of the contamination of antiseptic or disinfectant solutions by bacteria. For example, Marrie and Costerton (310) described the prolonged survival of *S. marcescens* in 2% chlorhexidine solutions, which was attributed to the embedding of these organisms in a thick matrix that adhered to the walls of a storage containers. Similar conclusions were reached by Hugo et al. (225) concerning the survival of *B. cepacia* in chlorhexidine and by Anderson et al. (10, 12–14) concerning the contamination of iodophor antiseptics with *Pseudomonas*. In the studies by Anderson et al., *Pseudomonas* biofilms were found on the interior surfaces of polyvinyl chloride pipes used during the manufacture of providone-iodine antiseptics. It is to be wondered whether a similar reason could be put forward for the contamination by *S. marcescens* of a benzalkonium chloride solution implicated in meningitis (468). Recently, a novel strategy was described (540) for controlling biofilms through generation of hydrogen peroxide at the biofilm-surface interface rather than simply applying a disinfectant extrinsically. In this procedure, the colonized surface incorporated a catalyst that generated the active compound from a treatment agent.

Gram-negative pathogens can grow as biofilms in the catheterized bladder and are able to survive concentrations of chlorhexidine that are effective against organisms in noncatheterized individuals (493, 494). Interestingly, the permeability agent EDTA has only a temporary potentiating effect in the catheterized bladder, with bacterial growth subsequently recurring (495). *B. cepacia* freshly isolated from the hospital environment is often considerably more resistant to chlorhexidine than when grown in artificial culture media, and a glycocalyx may be associated with intrinsic resistance to the bisbiguanide (360). *Legionella pneumophila* is often found in hospital water distribution systems and cooling towers. Chlorination in combination with continuous heating (60°C) of incoming water is usually the most important disinfection measure; however, because of biofilm production, contaminating organisms may be less susceptible to this treatment (140). Increased resistance to chlorine has been reported for *Vibrio cholerae*, which expresses an amorphous exopolysaccharide causing cell aggregation ("rugose" morphology [336]) without any loss in pathogenicity.

One can reach certain conclusions about biofilms. The interaction of bacteria with surfaces is usually reversible and eventually irreversible. Irreversible adhesion is initiated by the binding of bacteria to the surface through exopolysaccharide glycocalyx polymers. Sister cells then arise by cell division and are bound within the glycocalyx matrix. The development of adherent microcolonies is thereby initiated, so that eventually a continuous biofilm is produced on the colonized surface. Bacteria within these biofilms reside in specific microenvironments that differ from those of cells grown under normal laboratory conditions and thus show variations in their response to antiseptics and disinfectants.

Recent nosocomial outbreaks due to *M. chelonae* (discussed under "Intrinsic resistance of mycobacteria"), *M. tuberculosis* (4, 323) and HCV (53) underscore the importance of pseudobiofilm formation in flexible fiberoptic scope contamination. These outbreaks were associated with inadequate cleaning of scopes, which compromised subsequent sterilization with glutaraldehyde. While these organisms do not form a true biofilm, the cross-linking action of glutaraldehyde can cause a buildup of insoluble residues and associated microorganisms on scopes and in automated reprocessors.

Biofilms provide the most important example of how physiological (phenotypic) adaptation can play a role in conferring intrinsic resistance (57). Other examples are also known. For example, fattened cells of *S. aureus* produced by repeated subculturing in glycerol-containing media are more resistant to alkyl phenols and benzylpenicillin than are wild-type strains (220). Subculture of these cells in routine culture media resulted in reversion to sensitivity (218). Planktonic cultures grown under conditions of nutrient limitation or reduced growth rates have cells with altered sensitivity to disinfectants, probably as a consequence of modifications in their outer membranes (56, 59, 98). In addition, many aerobic microorganisms have developed intrinsic defense systems that confer tolerance to peroxide stress (in particular H_2O_2) in vivo. The so-called oxidative-stress or SOS response has been well studied in *E. coli* and *Salmonella* and includes the production of neutralizing enzymes to prevent cellular damage (including peroxidases, catalases, glutathione reductase) and to repair DNA lesions (e.g., exonuclease III) (112, 114, 497). In both organisms, increased tolerance can be obtained by pretreatment with a subinhibitory dose of hydrogen peroxide (113, 539). Pretreatment induces a series of proteins, many of which are under the positive control of a sensor/regulator protein (OxyR), including catalase and glutathione reductase (497)

TABLE 9. Possible mechanisms of plasmid-encoded resistance to antiseptics and disinfectants

Chemical agent	Examples	Mechanisms
Antiseptics or disinfectants	Chlorhexidine salts	(i) Inactivation: not yet found to be plasmid mediated; chromosomally mediated inactivation; (ii) efflux: some *S. aureus*, some *S. epidermidis*; (iii) Decreased uptake(?)
	QACs	(i) Efflux: some *S. aureus*, some *S. epidermidis*; (ii) Decreased uptake(?)
	Silver compounds	Decreased uptake; no inactivation (cf. mercury compounds)
	Formaldehyde	(i) Inactivation by formaldehyde dehydrogenase; (ii) Cell surface alterations (outer membrane proteins)
	Acridines[a]	Efflux: some *S. aureus*, some *S. epidermidis*
	Diamidines	Efflux: some *S. aureus*, some *S. epidermidis*
	Crystal violet[a]	Efflux: some *S. aureus*, some *S. epidermidis*
Other biocides	Mercurials[b]	Inactivation (reductases, lyases)
	Ethidium bromide	Efflux: some *S. aureus*, some *S. epidermidis*

[a] Now rarely used for antiseptic or disinfectant purposes.
[b] Organomercurials are still used as preservatives.

and further nonessential proteins that accumulate to protect the cell (338). Cross-resistance to heat, ethanol, and hypochlorous acid has also been reported (81, 128, 335). The oxidative stress response in gram-positive bacteria is less well studied, but *Bacillus* tolerance to H_2O_2 has been described to vary during the growth phase (127) and in mutant strains (67, 200). Similar inducible defense mechanisms were described for *Campylobacter jejuni* (185), *Deinococcus* (528), and *Haemophilus influenzae* (36). However, the level of increased tolerance to H_2O_2 during the oxidative stress response may not afford significant protection to concentrations used in antiseptics and disinfectants (generally >3%). For example, *B. subtilis* mutants have been described to be more resistant at ~0.5% H_2O_2 than are wild-type strains at ~0.34% H_2O_2 (200).

Acquired Bacterial Resistance Mechanisms

As with antibiotics and other chemotherapeutic drugs, acquired resistance to antiseptics and disinfectants can arise by either mutation or the acquisition of genetic material in the form of plasmids or transposons. It is important to note that "resistance" as a term can often be used loosely and in many cases must be interpreted with some prudence. This is particularly true with MIC analysis. Unlike antibiotics, "resistance," or an increase in the MIC of a biocide, does not necessarily correlate with therapeutic failure. An increase in an antibiotic MIC can may have significant consequences, often indicating that the target organism is unaffected by its antimicrobial action. Increased biocide MICs due to acquired mechanisms have also been reported and in some case misinterpreted as indicating resistance. It is important that issues including the pleiotropic action of most biocides, bactericidal activity, concentrations used in products, direct product application, formulation effects, etc., be considered in evaluating the clinical implications of these reports.

Plasmids and bacterial resistance to antiseptics and disinfectants. Chopra (82, 83) examined the role of plasmids in encoding resistance (or increased tolerance) to antiseptics and disinfectants; this topic was considered further by Russell (413). It was concluded that apart from certain specific examples such as silver, other metals, and organomercurials, plasmids were not normally responsible for the elevated levels of antiseptic or disinfectant resistance associated with certain species or strains. Since then, however, there have been numerous reports linking the presence of plasmids in bacteria with increased tolerance to chlorhexidine, QACs, and triclosan, as well as to diamidines, acridines and ethidium bromide, and the topic was reconsidered (83, 423, 427) (Table 9).

Plasmid-encoded resistance to antiseptics and disinfectants had at one time been most extensively investigated with mercurials (both inorganic and organic), silver compounds, and other cations and anions. Mercurials are no longer used as disinfectants, but phenylmercuric salts and thiomersal are still used as preservatives in some types of pharmaceutical products (226). Resistance to mercury is plasmid borne, inducible, and may be transferred by conjugation or transduction. Inorganic mercury (Hg^{2+}) and organomercury resistance is a common property of clinical isolates of *S. aureus* containing penicillinase plasmids (110). Plasmids conferring resistance to mercurials are either narrow spectrum, specifying resistance to Hg^{2+} and to some organomercurials, or broad-spectrum, with resistance to the above compounds and to additional organomercurials (331). Silver salts are still used as topical antimicrobial agents (50, 443). Plasmid-encoded resistance to silver has been found in *Pseudomonas stutzeri* (192), members of the *Enterobacteriaceae* (479, 480, 511), and *Citrobacter* spp. (511). The mechanism of resistance has yet to be elucidated fully but may be associated with silver accumulation (152, 511).

(i) Plasmid-mediated antiseptic and disinfectant resistance in gram-negative bacteria. Occasional reports have examined the possible role of plasmids in the resistance of gram-negative bacteria to antiseptics and disinfectants. Sutton and Jacoby (498) observed that plasmid RP1 did not significantly alter the resistance of *P. aeruginosa* to QACs, chlorhexidine, iodine, or chlorinated phenols, although increased resistance to hexachlorophene was observed. This compound has a much greater effect on gram-positive than gram-negative bacteria, so that it is difficult to assess the significance of this finding. Transformation of this plasmid (which encodes resistance to carbenicillin, tetracycline, and neomycin and kanamycin) into *E. coli* or *P. aeruginosa* did not increase the sensitivity of these organisms to a range of antiseptics (5).

Strains of *Providencia stuartii* may be highly tolerant to Hg^{2+}, cationic disinfectants (such as chlorhexidine and QACs), and antibiotics (496). No evidence has been presented to show that there is a plasmid-linked association between antibiotic resistance and disinfectant resistance in these organisms, pseudomonads, or *Proteus* spp. (492). High levels of disinfectant resistance have been reported in other hospital isolates (195), although no clear-cut role for plasmid-specified resistance has emerged (102, 250, 348, 373, 518). High levels of tolerance to chlorhexidine and QACs (311) may be intrinsic or may have resulted from mutation. It has been proposed (492, 496) that the extensive usage of these cationic agents could be responsible for the selection of antiseptic-disinfectant-, and antibiotic-resistant strains; however, there is little evidence to support this conclusion. All of these studies demonstrated that it was difficult to transfer chlorhexidine or QAC resistance under nor-

TABLE 10. *qac* genes and susceptibility of *S. aureus* strains to some antiseptics and disinfectants

qac gene[a]	MIC ratios[b] of[c]:							
	Proflavine	CHG	Pt	Pi	CTAB	BZK	CPC	DC
qacA	>16	2.5	>16	>16	4	>3	>4	2
qacB	8	1	>4	2	2	>3	>2	2
qacC	1	1	ca. 1	1	6	>3	>4	1
qacD	1	1	ca. 1	1	6	>3	>4	1
MIC (μg/ml) for sensitive strain	40	0.8	<50	50[d]	1	<2	<1	4

[a] *qac* genes are otherwise known as nucleic acid binding (NAB) compound resistance genes (88).

[b] Calculated from the data in reference 289. Ratios are MICs for strains of *S. aureus* carrying various *qac* genes divided by the MIC for a strain carrying no gene (the actual MIC for the test strain is shown in the bottom row).

[c] CHG, chlorhexidine diacetate; Pt, pentamidine isethionate; Pi, propamidime isethionate; CTAB, cetyltrimethylammonium bromide; BZK, benzalkonium chloride; CPC, cetylpyridinium chloride; DC, dequalinium chloride.

[d] The MIC of propamidine isethionate for the sensitive *S. aureus* is considerably higher than the normal quoted value (ca. 2 μg/ml [Table 6]).

mal conditions and that plasmid-mediated resistance to these chemicals in gram-negative bacteria was an unlikely event. By contrast, plasmid R124 alters the OmpF outer membrane protein in *E. coli*, and cells containing this plasmid are more resistant to a QAC (cetrimide) and to other agents (406).

Bacterial resistance mechanisms to formaldehyde and industrial biocides may be plasmid encoded (71, 193). Alterations in the cell surface (outer membrane proteins [19, 246]) and formaldehyde dehydrogenase (247, 269) are considered to be responsible (202). In addition, the so-called TOM plasmid encodes enzymes for toluene and phenol degradation in *B. cepacia* (476).

(ii) Plasmid-mediated antiseptic and disinfectant resistance in staphylococci. Methicillin-resistant *S. aureus* (MRSA) strains are a major cause of sepsis in hospitals throughout the world, although not all strains have increased virulence. Many can be referred to as "epidemic" MRSA because of the ease with which they can spread (91, 295, 317). Patients at particularly high risk are those who are debilitated or immunocompromised or who have open sores.

It has been known for several years that some antiseptics and disinfectants are, on the basis of MICs, somewhat less inhibitory to *S. aureus* strains that contain a plasmid carrying genes encoding resistance to the aminoglycoside antibiotic gentamicin (Table 10). These biocidal agents include chlorhexidine, diamidines, and QACs, together with ethidium bromide and acridines (8, 238, 289, 368, 423, 427, 463). According to Mycock (346), MRSA strains showed a remarkable increase in tolerance (at least 5,000-fold) to povidone-iodine. However, there was no decrease in susceptibility of antibiotic-resistant strains to phenolics (phenol, cresol, and chlorocresol) or to the preservatives known as parabens (8).

Tennent et al. (505) proposed that increased resistances to cetyltrimethylammonium bromide (CTAB) and to propamidine isethionate were linked and that these cationic agents may be acting as a selective pressure for the retention of plasmids encoding resistance to them. The potential clinical significance of this finding remains to be determined.

Staphylococci are the only bacteria in which the genetic aspects of plasmid-mediated antiseptic and disinfectant resistant mechanisms have been described (466). In *S. aureus*, these mechanisms are encoded by at least three separate multidrug resistance determinants (Tables 10 and 11). Increased antiseptic MICs have been reported to be widespread among MRSA strains and to be specified by two gene families (*qacAB* and *qacCD*) of determinants (188, 280, 281, 288, 289, 363–365, 367, 506). The *qacAB* family of genes (Table 11) encodes proton-dependant export proteins that develop significant homology to other energy-dependent transporters such as the tetracycline transporters found in various strains of tetracycline-resistant bacteria (405). The *qacA* gene is present predominantly on the pSK1 family of multiresistance plasmids but is also likely to be present on the chromosome of clinical *S. aureus* strains as an integrated family plasmid or part thereof. The *qacB* gene is detected on large heavy-metal resistance plasmids. The *qacC* and *qacD* genes encode identical phenotypes and show restriction site homology; the *qacC* gene may have evolved from *qacD* (288).

Interesting studies by Reverdy et al. (395, 396), Dussau et al. (129) and Behr et al. (31) demonstrated a relationship between increased *S. aureus* MICs to the β-lactam oxacillin and four antiseptics (chlorhexidine, benzalkonium chloride, hexamine, and acriflavine). A gene encoding multidrug resistance was not found in susceptible strains but was present in 70% of *S. aureus* strains for which the MICs of all four of these antiseptics were increased and in 45% of the remaining strains resistant to at least one of these antiseptics (31). Genes encoding increased QAC tolerance may be widespread in food-associated staphylococcal species (203). Some 40% of isolates of coagulase-negative staphylococci (CNS) contain both *qacA* and *qacC* genes, with a possible selective advantage in possessing both as opposed to *qacA* only (281). Furthermore, there is growing evidence that *S. aureus* and CNS have a common pool of resistance determinants.

Triclosan is used in surgical scrubs, soaps, and deodorants. It is active against staphylococci and less active against most gram-negative organisms, especially *P. aeruginosa*, probably by virtue of a permeability barrier (428). Low-level transferable resistance to triclosan was reported in MRSA strains (88, 90); however, no further work on these organisms has been described. According to Sasatsu et al. (465), the MICs of triclosan against sensitive and resistant *S. aureus* strains were 100 and

TABLE 11. *qac* genes and resistance to quaternary ammonium compounds and other antiseptics and disinfectants

Multidrug resistance determinant[a]	Gene location	Resistance encoded to
qacA	pSK1 family of multiresistant plasmids, also β-lactamase and heavy-metal resistance families	QACs, chlorhexidine salts, diamidines, acridines, ethidium bromide
qacB	β-Lactamase and heavy-metal resistance plasmids	QACs, acridines, ethidium bromide
qacC[b]	Small plasmids (<3 kb) or large conjugative plasmids	Some QACs, ethidium bromide
qacD[b]	Large (50-kb) conjugative, multiresistance plasmids	Some QACs, ethidium bromide

[a] The *qacK* gene has also been described, but it is likely to be less significant than *qacAB* in terms of antiseptic or disinfectant tolerance.

[b] These genes have identical target sites and show restriction site homology.

>6,400 μg/ml, respectively; these results were disputed because these concentrations are well in excess of the solubility of triclosan (515), which is practically insoluble in water. Sasatsu et al. (464) described a high-level resistant strain of *S. aureus* for which the MICs of chlorhexidine, CTAB, and butylparaben were the same as for a low-level resistant strain. Furthermore, the MIC quoted for methylparaben comfortably exceeds its aqueous solubility. Most of these studies with sensitive and "resistant" strains involved the use of MIC evaluations (for example, Table 6). A few investigations examined the bactericidal effects of antiseptics. Cookson et al. (89) pointed out that curing of resistance plasmids produced a fall in MICs but not a consistent decrease in the lethal activity of chlorhexidine. There is a poor correlation between MIC and the rate of bactericidal action of chlorhexidine (88, 89, 319) and triclosan (90, 319). McDonnell et al. (318, 319) have described methicillin-susceptible *S. aureus* (MSSA) and MRSA strains with increased triclosan MICs (up to 1.6 μg/ml) but showed that the MBCs for these strains were identical; these results were not surprising, considering that biocides (unlike antibiotics) have multiple cellular targets. Irizarry et al. (229) compared the susceptibility of MRSA and MSSA strains to CPC and chlorhexidine by both MIC and bactericidal testing methods. However, the conclusion of this study that MRSA strains were more resistant warrants additional comments. On the basis of rather high actual MICs, MRSA strains were some four times more resistant to chlorhexidine and five times more resistant to a QAC (CPC) than were MSSA strains. At a concentration in broth of 2 μg of CPC/ml, two MRSA strains grew normally with a threefold increase in viable numbers over a 4-h test period whereas an MSSA strain showed a 97% decrease in viability. From this, the authors concluded that it was reasonable to speculate that the residual amounts of antiseptics and disinfectants found in the hospital environment could contribute to the selection and maintenance of multiresistant MRSA strains. Irizarry et al. (229) also concluded that MRSA strains are less susceptible than MSSA strains to both chronic and acute exposures to antiseptics and disinfectants. However, their results with 4 μg of CPC/ml show no such pattern: at this higher concentration, the turbidities (and viability) of the two MRSA and one MSSA strains decreased at very similar rates (if anything, one MRSA strain appeared to be affected to a slightly greater extent that the MSSA strain). Furthermore, the authors stated that chlorhexidine gave similar results to CPC. It is therefore difficult to see how Irizarry et al. arrived at their highly selective conclusions.

Plasmid-mediated efflux pumps are particularly important mechanisms of resistance to many antibiotics (85), metals (349), and cationic disinfectants and antiseptics such as QACs, chlorhexidine, diamidines, and acridines, as well as to ethidium bromide (239, 289, 324–336, 363–368). Recombinant *S. aureus* plasmids transferred into *E. coli* are responsible for conferring increased MICs of cationic agents to the gram-negative organism (505, 544). Midgley (324, 325) demonstrated that a plasmid-borne, ethidium resistance determinant from *S. aureus* cloned in *E. coli* encodes resistance to ethidium bromide and to QACs, which are expelled from the cells. A similar efflux system is present in *Enterococcus hirae* (326).

Sasatsu et al. (463) showed that duplication of *ebr* is responsible for resistance to ethidium bromide and to some antiseptics. Later, Sasatsu et al. (466) examined the origin of *ebr* (now known to be identical to *qacCD*) in *S. aureus*; *ebr* was found in antibiotic-resistant and -sensitive strains of *S. aureus*, CNS, and enterococcal strains. The nucleotide sequences of the amplified DNA fragment from sensitive and resistant strains were identical, and it was proposed that in antiseptic-resistant cells there was an increase in the copy number of the *ebr* (*qacCD*) gene whose normal function was to remove toxic substances from normal cells of staphylococci and enterococci.

Based on DNA homology, it was proposed that *qacA* and related genes carrying resistance determinants evolved from preexisting genes responsible for normal cellular transport systems (405) and that the antiseptic resistance genes evolved before the introduction and use of topical antimicrobial products and other antiseptics and disinfectants (288, 289, 365, 367, 368, 405).

Baquero et al. (23) have pointed out that for antibiotics, the presence of a specific resistance mechanism frequently contributes to the long-term selection of resistant variants under in vivo conditions. Whether low-level resistance to cationic antiseptics, e.g., chlorhexidine, QACs, can likewise provide a selective advantage on staphylococci carrying *qac* genes remains to be elucidated. The evidence is currently contentious and inconclusive.

(iii) Plasmid-mediated antiseptic and disinfectant resistance in other gram-positive bacteria. Antibiotic-resistant corynebacteria may be implicated in human infections, especially in the immunocompromised. 'Group JK' coryneforms (*Corynebacterium jeikeium*) were found to be more tolerant than other coryneforms to the cationic disinfectants ethidium bromide and hexachlorophene, but studies with plasmid-containing and plasmid-cured derivatives produced no evidence of plasmid-associated resistance (285). *Enterococcus faecium* strains showing high level resistance to vancomycin, gentamicin, or both are not more resistant to chlorhexidine or other nonantibiotic agents (9, 11, 20, 319). Furthermore, despite the extensive dental use of chlorhexidine, strains of *Streptococcus mutans* remain sensitive to it (235). To date, therefore, there is little or no evidence of plasmid-associated resistance of nonstaphylococcal gram-positive bacteria to antiseptics and disinfectants.

Mutational resistance to antiseptics and disinfectants. Chromosomal mutation to antibiotics has been recognized for decades. By contrast, fewer studies have been performed to determine whether mutation confers resistance to antiseptics and disinfectants. It was, however, demonstrated over 40 years ago (77, 78) that *S. marcescens*, normally inhibited by QACs at <100 μg/ml, could adapt to grow in 100,000 μg of a QAC per ml. The resistant and sensitive cells had different surface characteristics (electrophoretic mobilities), but resistance could be lost when the cells were grown on QAC-free media. One problem associated with QACs and chlorhexidine is the turbidity produced in liquid culture media above a certain concentration (interaction with agar also occurs), which could undoubtedly interfere with the determination of growth. This observation is reinforced by the findings presented by Nicoletti et al. (354).

Prince et al. (383) reported that resistance to chlorhexidine could be induced in some organisms but not in others. For example, *P. mirabilis* and *S. marcescens* displayed 128- and 258-fold increases, respectively, in resistance to chlorhexidine, whereas it was not possible to develop resistance to chlorhexidine in *Salmonella enteritidis*. The resistant strains did not show altered biochemical properties of changed virulence for mice, and some strains were resistant to the QAC benzalkonium chloride. Moreover, resistance to chlorhexidine was stable in *S. marcescens* but not in *P. mirabilis*. Despite extensive experimentation with a variety of procedures, Fitzgerald et al. (148) were unable to develop stable chlorhexidine resistance in *E. coli* or *S. aureus*. Similar observations were made by Cookson et al. (89), who worked with MRSA and other strains of *S. aureus*, and by McDonnell et al. (319), who worked with MRSA and enterococci. Recently, stable chlorhexidine resistance was developed in *P. stutzeri* (502); these strains showed

various levels of increased tolerance to QACs, triclosan, and some antibiotics, probably as a result of a nonspecific alteration of the cell envelope (452). The adaptation and growth of *S. marcescens* in contact lens disinfectants containing chlorhexidine, with cross-resistance to a QAC, have been described previously (166).

Chloroxylenol-resistant strains of *P. aeruginosa* were isolated by repeated exposure in media containing gradually increasing concentrations of the phenolic, but the resistance was unstable (432). The adaptation of *P. aeruginosa* to QACs is a well-known phenomenon (1, 2, 240). Resistance to amphoteric surfactants has also been observed, and, interestingly, cross-resistance to chlorhexidine has been noted (240). This implies that the mechanism of such resistance is nonspecific and that it involves cellular changes that modify the response of organisms to unrelated biocidal agents. Outer membrane modification is an obvious factor and has indeed been found with QAC-resistant and amphoteric compound-resistant *P. aeruginosa* (240) and with chlorhexidine-resistant *S. marcescens* (166). Such changes involve fatty acid profiles and, perhaps more importantly, outer membrane proteins. It is also pertinent to note here the recent findings of Langsrud and Sundheim (274). In this study, resistance of *P. fluorescens* to QACs was reduced when EDTA was present with the QAC (although the lethal effect was mitigated after the cells were grown in medium containing QAC and EDTA); similar results have been found with laboratory-generated *E. coli* mutants for which the MICs of triclosan were increased (318). EDTA has long been known (175, 410) to produce changes in the outer membrane of gram-negative bacteria, especially pseudomonads. Thus, it appears that, again, the development of resistance is associated with changes in the cell envelope, thereby limiting uptake of antiseptics and disinfectants.

Hospital (as for other environmental) isolates of gram-negative bacteria are invariably less sensitive to disinfectants than are laboratory strains (196, 209, 279, 286, 492). Since plasmid-mediated transfer has apparently been ruled out (see above), selection and mutation could play an important role in the presence of these isolates. Subinhibitory antibiotic concentrations may cause subtle changes in the bacterial outer structure, thereby stimulating cell-to-cell contact (109); it remains to be tested if residual concentrations of antiseptics or disinfectants in clinical situations could produce the same effect.

Another insusceptibility mechanism has been put forward, in this instance to explain acridine resistance. It has been proposed (270, 351) that proflavine-sensitive and -resistant cells are equally permeable to the acridine but that resistant cells possessed the ability to expel the bound dye. This is an important point and one that has been reinforced by more recent studies that demonstrate the significance of efflux in resistance of bacteria to antibiotics (284, 330, 355). Furthermore, multidrug resistance (MDR) is a serious problem in enteric and other gram-negative bacteria. MDR is a term used to describe resistance mechanisms used by genes that form part of the normal cell genome (168). These genes are activated by induction or mutation caused by some types of stress, and because they are distributed ubiquitously, genetic transfer is not needed. Although such systems are most important in the context of antibiotic resistance, George (168) provides several examples of MDR systems in which an operon or gene is associated with changes in antiseptic or disinfectant susceptibility; e.g., (i) mutations at an *acr* locus in the Acr system render *E. coli* more sensitive to hydrophobic antibiotics, dyes, and detergents; (ii) the *robA* gene is responsible for overexpression in *E. coli* of the RobA protein that confers multiple antibiotic and heavy-metal resistance (interestingly, Ag^+ may be effluxed [350]); and (iii) the MarA protein controls a set of genes (*mar* and *soxRS* regulons) that confer resistance not only to several antibiotics but also to superoxide-generating agents. Moken et al. (333) have found that low concentrations of pine oil (used as a disinfectant) could select for *E. coli* mutants that overexpressed MarA and demonstrated low levels of cross-resistance to antibiotics. Deletion of the *mar* or *acrAB* locus (the latter encodes a PMF-dependant efflux pump) increased the susceptibility of *E. coli* to pine oil; deletion of *acrAB*, but not of *mar*, increased the susceptibility of *E. coli* to chloroxylenol and to a QAC. In addition, the *E. coli* MdfA (multidrug transporter) protein was recently identified and confers greater tolerance to both antibiotics and a QAC (benzalkonium) (132). The significance of these and other MDR systems in bacterial susceptibility to antiseptics and disinfectants, in particular the issue of cross-resistance with antibiotics, must be studied further. At present, it is difficult to translate these laboratory findings to actual clinical use, and some studies have demonstrated that antibiotic-resistant bacteria are not significantly more resistant to the lethal (or bactericidal) effects of antiseptic and disinfectants than are antibiotic-sensitive strains (11, 88, 89, 319).

TABLE 12. Possible mechanisms of fungal resistance to antiseptics and disinfectants

Type of resistance	Possible mechanism	Example(s)
Intrinsic	Exclusion	Chlorhexidine
	Enzymatic inactivation	Formaldehyde
	Phenotypic modulation	Ethanol
	Efflux	Not demonstrated to date[a]
Acquired	Mutation	Some preservative
	Inducible efflux	Some preservatives[a]
	Plasmid-mediated responses	Not demonstrated to date

[a] Efflux is now known to be one mechanism of fungal resistance to antibiotics (531).

Mechanisms of Fungal Resistance to Antiseptics and Disinfectants

In comparison with bacteria, very little is known about the ways in which fungi can circumvent the action of antiseptics and disinfectants (104, 111, 296). There are two general mechanisms of resistance (Table 12): (i) intrinsic resistance, a natural property or development of an organism (201); and (ii) acquired resistance. In one form of intrinsic resistance, the cell wall presents a barrier to reduce or exclude the entry of an antimicrobial agent. The evidence to date is somewhat patchy, but the available information links cell wall glucan, wall thickness, and relatively porosity to the susceptibility of *Saccharomyces cerevisiae* to chlorhexidine (Table 13) (204–208). Protoplasts of this organism prepared by glucuronidase in the presence of β-mercaptoethanol are lysed by chlorhexidine concentrations well below those effective against "normal" (whole) cells. Furthermore, culture age influences the response of *S. cerevisiae* to chlorhexidine; the cells walls are much less sensitive at stationary phase than at logarithmic growth phase (208), taking up much less [^{14}C]chlorhexidine gluconate (206). Gale (165) demonstrated a phenotypic increase in the resistance of *Candida albicans* to the polyenic antibiotic amphotericin B as the organisms entered the stationary growth phase, which was attributed to cell wall changes involving tighter cross-linking (74). Additionally, any factor increasing glucanase activity increased amphotericin sensitivity.

The porosity of the yeast cell wall is affected by its chemical

TABLE 13. Parameters affecting the response of *S. cerevisiae* to chlorhexidine[a]

Parameter	Role in susceptibility of cells to chlorhexidine
Cell wall composition	
Mannan	No role found to date
Glucan	Possible significance: at concentrations below those active against whole cells, chlorhexidine lyses protoplasts
Cell wall thickness	Increases in cells of older cultures: reduced chlorhexidine uptake responsible for decreased activity(?)
Relative porosity	Decreases in cells of older cultures: reduced chlorhexidine uptake responsible for decreased activity(?)
Plasma membrane	Changes altering CHG susceptibility(?); not investigated to date

[a] Data from references 204 to 208 and 436.

composition, with the wall acting as a barrier or modulator to the entry and exit of various agents. DeNobel et al. (117–119) used the uptake of fluorescein isothiocyanurate (FITC) dextrans and the periplasmic enzyme invertase as indicators of yeast cell wall porosity. Intact *S. cerevisiae* cells were able to endocytose FITC dextrans of 70 but not of 150. A new assay for determining the relative cell wall porosity in yeast based upon polycation-induced leakage of UV-absorbing compounds was subsequently developed. Hiom et al. (206, 208) found that the relative porosity of cells decreases with increasing culture age and that there was a reduced uptake of radiolabeled chlorhexidine gluconate. As the age of an *S. cerevisiae* culture increases, there is a significant increase in the cell wall thickness, with values of 0.19, 0.25, and 0.31 μm recorded for cells from 1-, 2-, and 6-day old cultures, respectively (206).

These findings (Table 13) can provide a tentative picture of the cellular factors that modify the response of *S. cerevisiae* to chlorhexidine. Mannan mutants of *S. cerevisiae* show a similar degree of sensitivity to chlorhexidine as the parent strain (204). The glucan layer is shielded from β-glucuronidase by mannoproteins, but this effect is overcome by β-mercaptoethanol (119). The mannoprotein consists of two fractions, sodium dodecyl sulfate-soluble mannoproteins and sodium dodecyl sulfate-insoluble, glucanase-soluble ones: the latter limit cell wall porosity (119). Thus, glucan (and possibly mannoproteins) plays a key role in determining the uptake and hence the activity of chlorhexidine in *S. cerevisiae. C. albicans* is less sensitive and takes up less [^{14}C]chlorhexidine overall (206), but only a few studies with this organism and with molds have been performed.

Yeasts grown under different conditions have variable levels of sensitivity to ethanol (176, 402). Cells with linoleic acid-enriched plasma membranes are more resistant to ethanol than are cells with oleic acid-enriched ones, from which it has been inferred that a more fluid membrane enhances ethanol resistance (6).

There is no evidence to date of antiseptic efflux (although benzoic acid in energized cells is believed to be eliminated by flowing down the electrochemical gradient [529]) and no evidence of acquired resistance by mutation (except to some preservatives [436]) or by plasmid-mediated mechanisms (426, 436). It is disappointing that so few rigorous studies have been performed with yeasts and molds and antiseptics and disinfectants (see also Miller's [328] treatise on mechanisms for reaching the site of action). Molds are generally more resistant than yeasts (Table 14) and considerably more resistant than nonsporulating bacteria (Table 15). Mold spores, although more resistant than nonsporulating bacteria, are less resistant than bacterial spores to antiseptics and disinfectants (436). It is tempting to speculate that the cell wall composition in molds confers a high level of intrinsic resistance on these organisms.

Mechanisms of Viral Resistance to Antiseptics and Disinfectants

Early studies on the effects of disinfectants on viruses were reviewed by Grossgebauer (189). Potential viral targets are the viral envelope, which contains lipids and is a typical unit membrane; the capsid, which is principally protein in nature; and the genome. An important hypothesis was put forward in 1963 (258) and modified in 1983 (259) in which it was proposed that viral susceptibility to disinfectants could be based on whether viruses were "lipophilic" in nature, because they possessed a lipid envelope (e.g., herpes simplex virus [259]) or "hydrophilic" because they did not (e.g., poliovirus [514]). Lipid-enveloped viruses were sensitive to lipophilic-type disinfectants, such as 2-phenylphenol, cationic surfactants (QACs), chlorhexidine, and isopropanol, as well as to ether and chloroform. Klein and Deforest (259) further classified viruses into three groups (Table 16), A (lipid containing), B (nonlipid picornaviruses), and C (other nonlipid viruses larger than those in group B) and disinfectants into two groups, broad-spectrum ones that inactivated all viruses and lipophilic ones that failed to inactivate picornoviruses and parvoviruses.

Capsid proteins are predominantly protein in nature, and biocides such as glutaraldehyde, hypochlorite, ethylene oxide, and hydrogen peroxide, which react strongly with amino or sulfhydryl groups might possess virucidal activity. It must, however, be added that destruction of the viral capsid may result in the release of a potentially infectious nucleic acid and that viral inactivation would only be complete if the viral nucleic acid is also destroyed.

Unfortunately, the penetration of antiseptics and disinfectants into different types of viruses and their interaction with viral components have been little studied, although some information has been provided by investigations with bacteriophages (307). Bacteriophages are being considered as "indicator species" for assessing the virucidal activity of disinfectants (108) and could thus play an increasing important role in this context; for example, repeated exposure of *E. coli* phage f2 to chlorine was claimed to increase its resistance to disinfection (542).

Thurman and Gerber (509, 510) pointed out that conflicting results on the actions of disinfectants on different virus types were often reported, and they suggested that the structural integrity of a virus was altered by an agent that reacted with viral capsids to increase viral permeability. Thus, a "two-stage"

TABLE 14. Lethal concentrations of antiseptics and disinfectants toward some yeasts and molds[a]

Antimicrobial agent[b]	Lethal concn (μg/μl) toward:		
	Yeast (*Candida albicans*)	Molds	
		Penicillium chrysogenum	*Aspergillus niger*
QACs			
Benzalkonium chloride	10	100–200	100–200
Cetrimide/CTAB	25	100	250
Chlorhexidine	20–40	400	200

[a] Derived in part from data in reference 525.
[b] CTAB, cetyltrimethylammonium bromide.

TABLE 15. Kinetic approach: *D*-values at 20°C of phenol and benzalkonium chloride against fungi and bacteria[a]

Antimicrobial agent	pH	Concn (%, wt/vol)	*D*-value (h)[b] against:				
			Aspergillus niger	*Candida albicans*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Phenol	5.1	0.5	20	13.5	0.94	—[c]	0.66
	6.1	0.5	32.4	18.9	1.72	0.17	1.9
Benzalkonium chloride	5.1	0.001	—[d]	9.66	0.06	3.01	3.12
	6.1	0.002	—[d]	5.5	—[c]	0.05	0.67

[a] Abstracted from the data in references 244 and 245.
[b] *D*-values are the times to reduce the viable population by 1 log unit.
[c] Inactivation was so rapid that the *D*-values could not be measured.
[d] No inactivation: fungistatic effect only.

disinfection system could be an efficient means of viral inactivation while overcoming the possibility of multiplicity reactivation (first put forward by Luria [293]) to explain an initial reduction and then an increase in the titer of disinfectant-treated bacteriophage. Multiplicity reactivation as a mechanism of resistance was supported by the observation of Young and Sharp (546) that clumping of poliovirus following partial inactivation by hypochlorite significantly increased the phage titer. It is envisaged as consisting of random damage to the capsid protein or nucleic acid of clumped, noninfectious virions from which complementary reconstruction of an infectious particle occurs by hybridization with the gene pool of the inactivated virions (298).

Another resistance mechanism also involves viral aggregation, e.g., the persistence of infectivity of formaldehyde-treated poliovirus (458) and the resistance of Norwalk virus to chlorination (249). A typical biphasic survival curve of enterovirus and rotavirus exposed to peracetic acid is also indicative of the presence of viral aggregates (198).

Finally, there remains the possibility of viral adaptation to new environmental conditions. In this context, Bates et al. (28) described the development of poliovirus having increased resistance to chlorine inactivation. Clearly, much remains to be learned about the mechanism of viral inactivation by and viral resistance to disinfectants.

Mechanisms of Protozoal Resistance to Antiseptics and Disinfectants

Intestinal protozoa, such as *Cryptosporidium parvum*, *Entamoeba histolytica*, and *Giardia intestinalis*, are all potentially pathogenic to humans and have a resistant, transmissible cyst (or oocyst for *Cryptosporidium*) (233, 234). Of the disinfectants available currently, ozone is the most effective protozoan cysticide, followed by chlorine dioxide, iodine, and free chlorine, all of which are more effective than the chloramines (234, 264). Cyst forms are invariably the most resistant to chemical disinfectants (Fig. 1). The reasons for this are unknown, but it would be reasonable to assume that cysts, similar to spores, take up fewer disinfectant molecules from solution than do vegetative forms.

Some recent studies have compared the responses of cysts and trophozoites of *Acanthamoeba castellanii* to disinfectants used in contact lens solutions and monitored the development of resistance during encystation and the loss of resistance during excystation (251–255). The lethal effects of chlorhexidine and of a polymeric biguanide were time and concentration dependent, and mature cysts were more resistant than preencystment trophozoites or preexcystment cysts. The cyst "wall" appeared to act as a barrier to the uptake of these agents, thereby presenting a classical type of intrinsic resistance mechanism (163). Acanthamoebae are capable of forming biofilms on surfaces such as contact lenses (186). Although protozoal biofilms have yet to be studied extensively in terms of their response to disinfectants, it is apparent that they could play a significant role in modulating the effects of chemical agents.

Mechanisms of Prion Resistance to Disinfectants

The transmissible degenerative encephalopathies (TDEs) form a group of fatal neurological diseases of humans and other animals. TDEs are caused by prions, abnormal proteinaceous agents that appear to contain no agent-specific nucleic acid (385). An abnormal protease-resistant form (PrP^{res}) of a normal host protein is implicated in the pathological process.

Prions are considered highly resistant to physical and chemical agents (Fig. 1), although the fact that crude preparations are often studied means that extraneous materials could, at least to some extent, mask the true efficacy of these agents (503). According to Taylor (503), there is currently no known decontamination procedure that will guarantee the complete absence of infectivity in TDE-infected tissues processed by histopathological procedures. Prions survive acid treatment, but a synergistic effect with autoclaving plus sodium hydroxide treatment is observed. Formaldehyde, unbuffered glutaraldehyde (acidic pH), and ethylene oxide have little effect on infectivity, although chlorine-releasing agents (especially hypochlorites), sodium hydroxide, some phenols, and guanidine thiocyanate are more effective (141, 309, 503).

With the information presently available, it is difficult to explain the extremely high resistance of prions, save to comment that the protease-resistant protein is abnormally stable to degradative processes.

CONCLUSIONS

It is clear that microorganisms can adapt to a variety of environmental physical and chemical conditions, and it is therefore not surprising that resistance to extensively used antiseptics and disinfectants has been reported. Of the mechanisms that have been studied, the most significant are clearly intrinsic, in particular the ability to sporulate, adaptation of pseudomonads, and the protective effects of biofilms. In these cases, "resistance" may be incorrectly used and "tolerance," defined as developmental or protective effects that permit microorganisms to survive in the presence of an active agent, may be more correct. Many of these reports of resistance have often paralleled issues including inadequate cleaning, incorrect product use, or ineffective infection control practices, which cannot be underestimated. Some acquired mechanisms (in particular with heavy-metal resistance) have also been shown to be clinically significant, but in most cases the results have been spec-

TABLE 16. Viral classification and response to some disinfectants[a]

Viral group	Lipid envelope[b]	Examples of viruses	Effects of disinfectants[c]	
			Lipophilic	Broad-spectrum
A	+	HSV, HIV, Newcastle disease virus, rabies virus, influenza virus	S	S
B	−	Non-lipid picornaviruses (poliovirus, Coxsackie virus, echovirus)	R	S
C	−	Other larger nonlipid viruses (adenovirus, reovirus)	R	S

[a] Data from reference 259; see also reference 444. For information on the inactivation of poliovirus, see reference 514.
[b] Present (+) or absent (−).
[c] Lipophilic disinfectants include QACs and chlorhexidine. S, sensitive; R, resistant.

ulative. Increased MICs have been confirmed, in particular for staphylococci. However, few reports have further investigated increased bactericidal concentrations or actual use dilutions of products, which in many cases contain significantly higher concentrations of biocides, or formulation attributes, which can increase product efficacy; in a number of cases, changes in the MICs have actually been shown not to be significant (9, 88, 89, 319, 428). Efflux mechanisms are known to be important in antibiotic resistance, but it is questionable if the observed increased MICs of biocides could have clinical implications for hard-surface or topical disinfection (423, 428). It has been speculated that low-level resistance may aid in the survival of microorganisms at residual levels of antiseptics and disinfectants; any possible clinical significance of this remains to be tested. With growing concerns about the development of biocide resistance and cross-resistance with antibiotics, it is clear that clinical isolates should be under continual surveillance and possible mechanisms should be investigated.

It is also clear that antiseptic and disinfectant products can vary significantly, despite containing similar levels of biocides, which underlines the need for close inspection of efficacy claims and adequate test methodology (183, 423, 428). In addition, a particular antiseptic or disinfectant product may be better selected (as part of infection control practices) based on particular circumstances or nosocomial outbreaks; for example, certain active agents are clearly more efficacious against gram-positive than gram-negative bacteria, and *C. difficile* (despite the intrinsic resistance of spores) may be effectively controlled physically by adequate cleaning with QAC-based products.

In conclusion, a great deal remains to be learned about the mode of action of antiseptics and disinfectants. Although significant progress has been made with bacterial investigations, a greater understanding of these mechanisms is clearly lacking for other infectious agents. Studies of the mechanisms of action of and microbial resistance to antiseptics and disinfectants are thus not merely of academic significance. They are associated with the more efficient use of these agents clinically and with the potential design of newer, more effective compounds and products.

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Tests with the anaerobes were conducted in Bacto-Anaerobe Medium with Dextrose. Cotton-stoppered tubes were filled with 9 cc. of the medium and placed in a boiling water-bath for twenty minutes and allowed to cool without agitation. To a series of 8 tubes the antibacterial solution was added in amounts to give 0.9, 0.7, 0.5, 0.25, 0.09, 0.07, 0.05 and 0.025 mg. per cc. of medium when all tubes received 1 cc. of a 1:1000 dilution of the organism to be tested in the anaerobe medium.

Bactericidal action was determined by removing 0.2 cc. of the medium from all tubes showing no growth after twenty hours of incubation and adding this to tubes containing 10 cc. of freshly heated and cooled Anaerobe Medium.

Discussion

The newly isolated compound from *Arctium minus* has a relatively low order of antibacterial activity. It is of interest, however, because of its presence in such relatively large quantities in burdock leaves and also from the standpoint of discovering new antibiotic types, a study of which may give evidence of their mode of action. The antibacterial activity is destroyed by treatment with cysteine or N-acetylcysteine but not with S-methylcysteine,[7] and in this respect resembles the behavior of certain other antibacterial agents.[8,9] It is also of interest to observe the frequency of unsaturated lactone structures among antibiotics, notably in anemonin,[10] patulin,[11] penicillic acid,[12] and now apparently in I.

Summary

A new antibacterial agent has been isolated from the leaves of *Arctium minus*. It has a relatively low order of activity against Gram-positive bacteria and is inactive against the Gram-negative group. The compound appears to be a lactone of empirical formula $C_{15}H_{20}O_5$.

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[CONTRIBUTION FROM THE WESTERN REGIONAL RESEARCH LABORATORY[1]]

The Reaction of Formaldehyde with Proteins

BY HEINZ FRAENKEL-CONRAT, MITZI COOPER AND HAROLD S. OLCOTT

The reaction of formaldehyde with proteins is of importance in a number of fields, some of which are the tanning of leather, the hardening of tissues, fibers and plastics, and the preparation of toxoids. Much of the extensive earlier literature on the mode of interaction is cited in recent publications.[2-11]

The confusion existing in this field is emphasized by the fact that, in the four most recent papers, the binding of aldehyde by proteins in acid or neutral solution is attributed to the following groups: (1) all basic and phenolic and aliphatic hydroxyl[6b]; (2) imidazole, possibly amide and peptide, but not primary amino[9]; (3) primary amino and peptide[10]; and (4) primary amide and basic groups.[11] In addition, formaldehyde has been shown to react under certain conditions with the thiol,[12] indole,[13] guanidyl,[3] and disulfide groups.[5]

The present investigation was undertaken when it was observed that gliadin and wheat gluten bound more aldehyde than did other proteins, after treatment with 4% formaldehyde solution at 70° and at *p*H 3 to 7.[14] It was possible to demonstrate that the primary amide as well as the amino groups of proteins bind aldehyde under these conditions. On the other hand, the secondary amides of the peptide chain were found not to react appreciably with formaldehyde. These conclusions were derived from experiments with a series of proteins and protein derivatives, and with synthetic polypeptides and simple model substances.

Experimental

Analytical Methods.—Combined formaldehyde was determined by a method involving hydrolysis and distillation,[15] followed by precipitation of the aldehyde in the distillate with dimethyldihydroresorcinol (dimedon).[16] 100 or 200 mg. of the washed and dried protein derivative and 50 ml. of *N* sulfuric acid were placed in a 1000-ml. round-bottom flask, which was connected to a condenser fitted with a trap and an outlet tube dipping into a mixture of 50 ml. of 0.214% dimedon solution and 75 ml. of *p*H 4.6 acetate buffer (1 part *N* hydrochloric acid and 2 parts *N* sodium acetate). Distillation was continued until

(1) Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture. Article not copyrighted.

(2) Theis and Ottens, *J. Am. Leather Chem. Assoc.*, **35**, 330 (1940).

(3) Highberger and Salcedo, *ibid.*, **35**, 11 (1940); **36**, 271 (1941).

(4) Brother and McKinney, *Ind. Eng. Chem.*, **30**, 1236 (1938).

(5) Middlebrook and Phillips, *Biochem. J.*, **36**, 294 (1942).

(6) (a) Carpenter and Lovelace, *Ind. Eng. Chem.* **34**, 759 (1942); (b) **36**, 680 (1944).

(7) Hegman, *J. Am. Leather Chem. Assoc.*, **37**, 276 (1942).

(8) Gustavson, *Kolloid Z.*, **103**, 43 (1943).

(9) (a) Theis, *J. Biol. Chem.*, **154**, 87 (1944); (b) Theis and Lams, *ibid.*, **154**, 99 (1944).

(10) Nitschmann and Hadorn, *Helv. Chim. Acta*, **27**, 299 (1944).

(11) Wormell and Kaye, *Nature*, **153**, 525 (1944).

(12) Ratner and Clarke, THIS JOURNAL, **59**, 200 (1937).

(13) Ross and Stanley, *J. Gen. Physiol.*, **22**, 165 (1938).

(14) The use of elevated temperature for the protein-formaldehyde reaction has been suggested by Ferretti (French Patent 853,123; March 11, 1940), Middlebrook and Phillips,[5] and others.

(15) Highberger and Retzsch, *J. Am. Leather Chem. Assoc.*, **33**, 341 (1939). The modification proposed by Nitschmann (*Helv. Chim. Acta*, **24**, 237 (1941)) was not found suitable for maximal recovery of the aldehyde bound by the casein at 70°, in agreement with a later paper from the same laboratory (Nitschmann, Hadorn and Lauener, *Helv. Chim. Acta*, **26**, 1069 (1943)).

(16) Yoe and Reid, *Ind. Eng. Chem., Anal. Ed.*, **13**, 238 (1941).

sulfuric acid fumes began to fill the distillation flask. Any precipitate adhering to the outlet tube was washed into the receiver, the content of which was filtered after four to forty-eight hours. The precipitate was washed, dried for sixteen hours at 50° or for three hours at 70°, and weighed.[17]

Free amino groups were determined by a fifteen-minute reaction period in the Van Slyke manometric apparatus[18] with a blacked-out chamber.[19] Total basic groups of untreated proteins were determined by the recently developed dye method.[20] The primary amide groups of untreated proteins were determined by partial hydrolysis (forty minutes at 120° in 1.2 *N* sulfuric acid), followed by distillation of the ammonia. Neither of the latter two methods permitted a differentiation between free and aldehyde-reacted basic or amide groups because of the lability of the N-methylol bond. The method of determining the sum of primary amide, amino and guanidyl groups by the Plimmer technique[21,22] proved applicable for this purpose.

Method of Preparation of Formaldehyde Derivatives.—In most experiments 1 g. of protein or polypeptide[23] was mixed with, or dissolved in, 8 ml. of water.[24] To this was added 1 ml. of buffer and 1 ml. of 37–38% formaldehyde solution. 3 *M* acetate buffer of *p*H 3.5 was used in the acid region and 3.4 *M* phosphate buffer of *p*H 8.2 in the neutral region. The *p*H values measured at the end of the reaction period were usually 3.8 and 6.8, respectively. The samples, in stoppered flasks, were kept in an oven at 70° for four days and shaken at intervals. After this treatment all of the proteins studied, with the exception of gelatin,[25] were sufficiently insoluble in water to permit centrifugation and thorough washing. They were usually washed ten times, each time with 40 ml. of water, and then dehydrated with alcohol and ether. An alternate method of washing, applicable also to soluble aldehyde-treated proteins, consisted in dialyzing the solutions or suspensions for three days against running tap water and one day against distilled water. The aldehyde contents of the final products were found to vary by no more than 10% with the different techniques of isolation.

Effect of Experimental Conditions on Extent of Reaction of Proteins with Formaldehyde.—A reaction period of four days at 70° resulted in products containing maximal amounts of formaldehyde; 50% of the final amount of aldehyde was bound within eight hours, and 90% within twenty-four hours.

The effect of the *p*H of the reaction mixture on the amounts of formaldehyde bound depended upon the nature of the protein groups involved in the reaction. Lysozyme bound about 37% more at *p*H 6.8 than at *p*H 3.8; egg white protein, 18% more; gluten, 10% more; zein, the same amount at both *p*H levels; and polyglutamine, 38% less at the higher *p*H.

The aldehyde concentration and the reaction temperature were found to influence considerably the maximal amount of formaldehyde that could be introduced. Thus gluten bound about 2% of its weight of formaldehyde when treated either at room temperature with 3.8% formaldehyde solution or at 70° with 0.75% formaldehyde. The use of 18% aldehyde solution at 70° (*p*H 3.8) led to the introduction of 7% of the reagent, as compared to the 6% introduced from 3.8% aldehyde solution.

Stability of Bound Formaldehyde.—The formaldehyde retained by the proteins after the usual washing procedure was comparatively stable during further prolonged contact with water at room temperature. After seven days of dialysis against running tap water, the aldehyde contents of gluten and gliadin were about 10% lower than after three days of dialysis. Steam distillation caused the release of most of the bound aldehyde. Holding the dry materials at 100° for seven days reduced the aldehyde contents by about 60 to 70%; at 150°, by 85% in three days.

Dialysis of suspensions of washed aldehyde-treated gluten against 1% sodium sulfite caused the loss of 50 to 60% of the aldehyde in three days; the residual aldehyde was stable to further treatment with sulfite. The aldehyde bound by polyglutamine was split off in sodium sulfite, but not in bisulfite solution. Thus exhaustive "washings" of aldehyde-treated proteins with sulfite solution cannot be regarded as a technique suitable for the removal of the unbound aldehyde alone, for which it has been advocated.[3,7]

Preparation of Polyglutamic Acid, Polyglutamic Methyl Ester, and Polyglutamine.—The polypeptide of *d*(−)-glutamic acid was prepared by the method of Bovarnick[26] with the coöperation of J. C. Lewis of this Laboratory. This material is elaborated by a particular strain of *B. subtilis*,[27] and is isolated by the precipitation of its copper salt from the medium. After the removal of foreign material and inorganic ions as described by Bovarnick,[26] the residual solution was dried from the frozen state.

Anal. Calcd. for $(C_5H_7O_3N)_x$: N, 10.85; amino N (after hydrolysis), 10.85; glutamic acid (after hydrolysis), 114; equiv. wt., 129. Found: N, 10.6; amido N (after hydrolysis), 10.5; glutamic acid,[28] (after hydrolysis) 110; equiv. wt., 130.5.

In order to prepare the methyl ester, the acid polypeptide was esterified with diazomethane in ether or, preferably, by the method of Freudenberg and Jacob.[29] A dialyzed aqueous solution of the ester had a *p*H of 6.6, in contrast to the original polypeptide which, in aqueous solution, is at *p*H 2.8. Titration with 0.1 *N* sodium hydroxide and phenolphthalein indicated that over 97% of the carboxyl groups had been esterified. The solution was dried by lyophilization.

Anal. Calcd.: N, 9.8; methoxyl, 21.6. Found: N, 9.7; methoxyl, 20.8.

The polyamide was obtained as follows: 1 g. of polyglutamic acid methyl ester was suspended in approximately 50 ml. of liquid ammonia in a Dewar flask. A small amount of ferric chloride was added as catalyst. After

(17) The soluble formaldehyde reaction products of simple amides and diketopiperazines were found to be stable under the conditions of dimedon precipitation. It was thus possible to determine the amount of formaldehyde bound by these compounds by comparing the free aldehyde, as determined in an aliquot by direct addition of dimedon and buffer, with the total aldehyde as determined in another aliquot by hydrolysis and distillation. The same technique could not be used with amines or amino acids, the formaldehyde reaction products of which appeared unstable in the presence of dimedon at *p*H 4.6. This was indicated by the finding that the losses in the primary amino nitrogen of these compounds due to reaction with formaldehyde were greatly in excess of the amounts of aldehyde which they retained in the presence of dimedon. A similar phenomenon was recently described by Neuberger, *Biochem. J.*, **38**, 309 (1944).

(18) Van Slyke, *J. Biol. Chem.*, **83**, 425 (1929).

(19) Fraenkel-Conrat, *ibid.*, **148**, 453 (1943).

(20) Fraenkel-Conrat and Cooper, *ibid.*, **154**, 239 (1944).

(21) Plimmer (*J. Chem. Soc.*, **127**, 265 (1925)) has shown that these groups liberate nitrogen quantitatively upon treatment with nitrous acid in the presence of mineral acid. This method has recently been applied to proteins.[22]

(22) Fraenkel-Conrat, Cooper and Olcott, THIS JOURNAL, **67**, 314 (1945).

(23) The proteins and the nylon and polyglycine preparations were the same as those used in previous studies.[20,22] The preparation of polyglutamic acid and polyglutamine is described below.

(24) In the case of gliadin, 50% ethanol was a more suitable reaction medium than water.

(25) Gelatin set to a gel at 70° after a few hours of aldehyde treatment. After twenty-four hours the reaction mixture had liquefied and the protein remained soluble in cold water even after dialysis. The aldehyde content did not increase appreciably during this transformation.

(26) Bovarnick, *J. Biol. Chem.*, **145**, 415 (1942).

(27) The organism used was No. B-571 of the Northern Regional Research Laboratory stock culture collection.

(28) Determined by the method described by Olcott, *J. Biol. Chem.*, **153**, 71 (1944).

(29) Freudenberg and Jacob, *Ber.*, **74B**, 1001 (1941). The application of this method to the esterification of proteins will be described in a future publication.

two days, the excess ammonia had evaporated. The product was taken up in water, dialyzed and lyophilized. Analyses indicated that about 80% of the ester groups had been transformed to amide groups.

Anal. Calcd. for mixed amide ester $((C_5H_8O_2N_2)_4(C_6H_9O_3N))_x$: N, 19.2, amide N, 8.6. Found: N, 18.9, amide N, 8.9.

Results

Protein Groups Responsible for the Binding of Formaldehyde.[30]—When proteins were treated with 4% formaldehyde at 70° and at *p*H 3.5 to 4.0, the resultant products, after thorough washing, contained amounts of formaldehyde that ranged from 7% for gliadin to 0.7% for silk fibroin. The amino nitrogen contents of the treated proteins were reduced to 10 to 20% of those of the starting materials.[31] Yet the amounts of aldehyde bound by most of the proteins were greatly in excess of those equivalent to the amino or even to the total basic groups. However, it was possible to demonstrate a correlation between the sum of the basic and the amide groups of proteins and their capacity to bind formaldehyde (Table I). These findings were regarded as suggestive evidence that the primary amide groups, together with the basic groups,[32] are responsible for a great part of the aldehyde bound by proteins under the conditions used.

This conclusion was supported by a comparison of the aldehyde-binding capacities of proteins or polypeptides modified or prepared in such a manner as to contain maximal or minimal numbers of the reactive groups (Table I). Thus proteins, the amide and basic groups of which had been decreased by treatment with nitrous acid in 2 *N* hydrochloric acid[21] or through reaction with phenyl isocyanate,[22] bound considerably less aldehyde than did the original proteins. Polypeptides of glutamic acid or glycine and the polyamide, nylon, also bound very little aldehyde, which indicates that the carboxyl, and peptide or secondary amide groups do not react to an appreciable extent. On the other hand, a polypeptide containing many amide groups (polyglutamine) combined with more formaldehyde than did any other macromolecular material.

A direct demonstration of the loss in free primary amide groups in proteins through combination with formaldehyde was possible by Plimmer's technique.[21,22] The decreases which were observed after aldehyde treatment of proteins rich in amide groups (Table II) indicate clearly that these groups participate in the reaction.[33]

TABLE I

CORRELATION OF THE AMOUNT OF FORMALDEHYDE BOUND AT *p*H 3.5 TO 4 AND 70° BY PROTEINS, DERIVED PROTEINS AND POLYPEPTIDES WITH THEIR REACTIVE GROUPS[a]

	Groups per 10^4 g.				Moles of aldehyde bound per 10^4 g.
	Primary amino[b]	Total basic	Primary amide	Basic + amide	
Polyglutamine	2.4	2.4	60	62.4	47
Gliadin	1.3	4.3	30	34.3	23
Gluten	1.3	5.3	21	26.3	20
Lysozyme	3.6	12.5	10	22.5	13
Zein	1.2	1.9	18	19.9	15
Casein	4.8	6.8	10	16.8	12
Hoof powder	4.5	8.8	7.6	16.3	12
Egg white proteins	4.5	8.2	7.1	15.3	11
Wool keratin	1.8	8.2	7.9	16.1	11
Egg albumin (cryst.)	4.3	8.8	7.8	16.6	9
Feather keratin	1.5	5.1	7.4	12.5	8
Zein, partly deamidated[c]	1.0	0.3	11.3	11.6	8
Gelatin	3.5	6.0	2.9	8.9	6
Casein, partly deamidated[c]	0.7	2.2	6.6	8.8	6
Gliadin, phenyl isocyanate treated[d]	0.0	1.1	7	8	9
Egg white, phenyl isocyanate treated[d]	0.0	0.3	7	7	4
Gluten, partly deamidated[c]	1.7	1.1	3.0	4.1	4
Gluten, phenyl isocyanate treated[d]	0.2	0	4	4	5
Polyglycine[d]	2.4	2.8	0	2.8	3.0
Polyglutamic acid	1.9	1.9	0	1.9	2.6[e]
Silk fibroin	0.7	1.3	0.4	1.7	2.3
Nylon	0.1	0.6	0	0.6	0.3

[a] Treated with 3.75% formaldehyde solution for four days. The lysozyme, crystalline egg albumin; and silk fibroin were kindly furnished by H. L. Fevold, E. F. Lindquist, and M. Bergmann, respectively. For method of preparation of polyglutamic acid and polyglutamine, see text. [b] After aldehyde treatment, the amino nitrogen of all proteins was reduced by 80 to 90%. [c] By an application of the Plimmer technique[21] to a preparative scale, which did not lead to as complete deamidation as did the analytical procedure. [d] The preparations were obtained as previously described.[22] The amide nitrogen of these samples was determined by the Plimmer technique,[21] since methods based on partial hydrolysis cannot be used with phenyl isocyanate-treated proteins.[22] [e] This reaction took place at *p*H 6.8. When heated at *p*H 3.8, polyglutamic acid became dialyzable. Polyglutamine bound 31 moles of aldehyde at *p*H 6.7.

Discussion

The amounts of formaldehyde bound by a num-

(30) While the present paper was being prepared for publication, the preliminary note of Wormell and Kaye[11] came to our attention. Our data indicate that the reaction of amide groups with formaldehyde does not require the presence of mineral acid and saturated calcium chloride as used by Wormell and Kaye. Thus zein bound 4.5% of its weight of formaldehyde in acetate buffer at *p*H 3.8; 4.1% under the conditions used by these investigators.

(31) These results cannot be attributed to the insolubility of tanned proteins, since it was possible to obtain consistent values for insoluble products such as keratins and for denatured or derived proteins used as powders of varying particle size. The water-soluble aldehyde derivative of gelatin also had a low amino nitrogen content.

(32) No attempt was made to ascertain the extent of participation of the imidazole and guanidyl groups of the various proteins in the formaldehyde reaction. Several previous investigators have indicated that the former but not the latter groups are involved below *p*H 9. However, evidence that guanidyl groups in proteins bind formaldehyde even below *p*H 7.0 will be reported later.

(33) The reaction of simple amides with formaldehyde was studied in detail by Einhorn, *Ann.*, **343**, 207 (1905); **361**, 113 (1908); and more recently by Noyes and Forman, THIS JOURNAL, **55**, 3493 (1933). Under the conditions used by us with proteins, N-methylol acetamide was formed from acetamide and formaldehyde. Methylene diacetamide resulted from the decomposition of N-methylol acetamide at 150°. These compounds were found to yield 70 to 90% of their nitrogen on treating with nitrous acid in the presence of mineral acid. If it is assumed that part of the amido-methylol groups of proteins may also be hydrolyzed under these conditions, the extent of blocking of amide groups may be actually greater than indicated by the data in Table II.

TABLE II

EFFECT OF FORMALDEHYDE ON THE LABILITY OF THE AMIDE, AMINO AND GUANIDYL GROUPS OF PROTEINS TOWARD NITROUS ACID[a]

	Nitrogen liberated by treatment with nitrous + hydrochloric acid[a]		Nitrogen in starting material	
	Formaldehyde derivative, %	Starting material, %	Amide,[b] %	Amide + amino + guanidyl,[b] %
Gliadin	3.4	5.4	4.3	5.1
Zein	1.9	3.5	3.0	3.4
Polyglutamine	1.7	10.4[c]	8.9	9.2

[a] Reacted in volumetric Van Slyke apparatus for twenty-four hours at room temperature, in 2 *N* hydrochloric acid (*cf.* footnotes 21 and 22). [b] The amide and amino nitrogen values were determined; the guanidyl-nitrogen values were calculated from data in the literature. [c] Polyglutamic acid is partially hydrolyzed under these conditions. A similar lability of the peptide links of polyglutamine may explain the observed high value.

ber of proteins under controlled conditions, and released by acid hydrolysis, have been determined. While it is well recognized that the reactions involved are largely reversible and therefore dependent upon conditions of both treatment and washing, it is noteworthy that the data obtained agree in general with comparable data in the literature.

The identity of the protein groups involved in this reaction has been more frequently the object of conjecture than of exhaustive study. Thus, on the basis of titration curves, Theis recently concluded[9] that the primary amino groups of proteins react only above *p*H 9.5. This conclusion was not supported by amino nitrogen determinations. Nitschmann and Hadorn,[10] however, observed marked decreases in the amino nitrogen of casein after formaldehyde treatment at *p*H 5 to 6. The present study has revealed consistent losses of 80 to 90% of the primary amino nitrogen of proteins upon aldehyde treatment in the range of *p*H 3.5 to 7.0.

It has recently been assumed by Carpenter and Lovelace[6a,b] that, in addition to all basic groups, aliphatic hydroxyl groups bind formaldehyde through acetal linkages, and tyrosine binds formaldehyde through addition in the 2,6 positions. These claims were based on the observation that the amount of aldehyde maximally introduced into one protein, casein, at room temperature and at high aldehyde concentration agreed with calculations based on such assumptions. To obtain similar agreement between "expectation" and analytical results in the case of deaminated casein, the further assumption was made that the nitrous acid caused 2,6 substitution of all phenolic groups present, thus preventing the tyrosine in deaminated casein from binding formaldehyde. In contrast to these assumptions, Ross and Stanley[13] and Kassanis and Kleczkowski[34] found that the indole ring of tryptophan, but not the phenolic ring of tyrosine, loses its ability to react with Folin's phenol reagent after aldehyde treatment. While the present investigation was not primarily concerned with this problem, the fact that silk fibroin binds very little formaldehyde also casts doubt upon the reactivity of either aliphatic[35] or phenolic hydroxyl groups, since fibroin is rich both in serine and tyrosine. The failure of an attempt to introduce formaldehyde into *p*-cresol under the conditions used in this study is further evidence that the phenolic ring of proteins is not reactive.[36]

Reaction of the amide groups and of the peptide nitrogen of proteins with formaldehyde has often been hypothesized.[6a,8,9,37,38] That the primary amide groups actually participate in the reaction in acid solution has recently been shown by Wormell and Kaye[11] and by the present study. The relative reactivity of polyglutamine at *p*H 3.5 and 6.8 indicates that amide groups react with formaldehyde more extensively in acid than in neutral solution, in agreement with the conclusions of Wormell and Kaye.[11] On the other hand, the reactivity of basic groups is known to be favored by decreasing acidity of the solution.[2,7,8,9] Thus the ratio of amide to basic groups determines the effect of *p*H on the amount of aldehyde bound by various proteins.

Carpenter and Lovelace[6] concluded that amide groups do not bind formaldehyde, from their observation[39a,b] that asparagine bound no more formaldehyde than did aspartic or glutamic acid. It appears doubtful that asparagine is a suitable model system for the study of the reaction of protein amide groups, since it contains a reactive amino group in proximity to the amide group. Levy and Silberman[40] had previously suggested an interpretation of the asparagine–formaldehyde reaction, in which one molecule of formaldehyde closed a ring involving both the amino and the amide groups. This reaction mechanism may explain the equal amounts of formaldehyde bound by asparagine and aspartic acid That the two reaction products differ in the mode of linkage of formaldehyde is evident from their stability in the presence of dimedon. Carpenter and Lovelace, reporting data obtained by this technique only in their earlier paper on asparagine,[39a] found this substance to bind one equivalent of formaldehyde in a stable manner in alkaline solution. We

(34) Kassanis and Kleczkowski, *Biochem. J.*, **38**, 20 (1944).

(35) Under the conditions described in this paper polyvinyl alcohol bound 2% formaldehyde at *p*H 3.0, which is equivalent to only 5% of the available hydroxyl groups. At *p*H 7.0, the amount of formaldehyde bound was negligible.

(36) 1% solutions of *p*-cresol were treated with 4% formaldehyde both at *p*H 3.8 and 6.7. There was no aldehyde bound after four days at 70°. The added formaldehyde was quantitatively recoverable either by distillation or by direct precipitation with dimedon. The chromogenic value (by the Folin phenol test) of the aldehyde-treated sample was the same as that of a similarly treated control solution.

(37) Holland, *J. Int. Soc. Leather Trades Chem.*, **23**, 215 (1939).

(38) Waldschmidt-Leitz, *Die Chemie*, **55**, 62 (1942).

(39) Carpenter and Lovelace, (a) THIS JOURNAL, **64**, 2899 (1942); (b) **65**, 1161 (1943).

(40) Levy and Silberman, *J. Biol Chem.*, **118**, 723 (1937).

found that the stable addition product is formed also at *p*H 3.8. However, when the dimedon technique was applied to a comparable reaction mixture of aspartic acid with formaldehyde, the product was labile, as were most other amino-methylol compounds investigated.[17] The experiments of Carpenter and Lovelace thus do not constitute evidence against the reaction of protein amide groups with formaldehyde.

The ability of glycine anhydride to bind formaldehyde[41] is frequently quoted in support of the assumption that a similar reaction occurs with the amide nitrogen of the peptide chain. That diketopiperazines are more active than are chain peptides is well known and has been supported by recent evidence.[22] The difference between the two types of —CO—NH— linkage is evident also from the results of the present study in which it is shown that nylon, straight-chain peptides and silk fibroin bind little formaldehyde under conditions which favor the reaction of glycine anhydride with formaldehyde.[42] The contrast between the inactivity of the chain peptide link and the reactivity of the primary amide group is clearly illustrated by the behavior of polyglutamic acid and polyglutamine upon treatment with aldehyde. The former bound less than 1 aldehyde molecule for 100 glutamic acid residues; the latter bound as much as 88 aldehyde molecules for 100 amide residues.

Nitschmann and Hadorn[10] recently restated the theory that the peptide nitrogen of casein is mainly responsible for the amount of fixed formaldehyde, in excess of that bound to lysine. They arrived at this conclusion by the elimination of other protein groups, with the surprising omission of the primary amide groups from their discussion. In support of their conclusion, they mention the reactivity of glycine anhydride. Further, these authors refer to an earlier study of the reaction rate that indicated two separate reaction types. They now interpret the rapid one as involving the amino groups. The slow reaction was greatly dependent upon aldehyde concentration and appeared to continue indefinitely at room temperature. This led Nitschmann and Hadorn to conclude that it must be due to groups present in relatively large numbers in proteins, namely, the peptide groups.

In the present study the reaction of primary amide groups with formaldehyde was found to be slow and greatly dependent upon aldehyde concentration and reaction temperature. Nitschmann and Hadorn's data can thus also be interpreted as indicating the reaction of the amide groups. The difference between the two studies lies mainly in the reaction temperature. At the elevated temperatures that we used, equilibrium was reached within a reasonable time. The amount of aldehyde bound at this point did not exceed the number of reactive side chains, and it was therefore not necessary to hypothesize that the peptide groups were involved. This factor, as well as the use of many different proteins, facilitated the recognition that the primary amide groups and not the peptide bonds are responsible for the slow fixation of formaldehyde.

The results do not exclude the possibility that a few particularly reactive peptide bonds participate in the reaction. Nor is the possibility of a secondary condensation of the amino-methylol groups with the peptide chain, as suggested by Nitschmann and Hadorn[10] disproved. However, this condensation reaction may also occur with other reactive hydrogen atoms.

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Summary

In 4% solution, at *p*H 3 to 7 and 70°, formaldehyde combined with both the primary amino and the primary amide groups of proteins. In contrast to interpretations of other investigators, the secondary amide linkages of the peptide chain, and the phenolic groups, were found not to bind appreciable amounts of formaldehyde.

These findings were confirmed with protein derivatives and synthetic polypeptides containing a maximal or minimal number of reactive groups.

The preparation of a polypeptide rich in primary amide groups from polyglutamic acid is described. This material bound more formaldehyde than did any of the proteins investigated.

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(41) Cherbuliez and Feer, *Helv. Chim. Acta*, **5**, 678 (1922); Bergmann, Jacobson and Schotte, *Z. physiol. Chem.*, **131**, 18 (1923).

(42) The formation of N,N'-dimethylol diketopiperazine from glycine anhydride and formaldehyde was found to occur under our experimental conditions. Alanine anhydride and higher molecular weight diketopiperazines appeared to react to a progressively decreasing extent.

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Avian influenza virus (H_5N_1); effects of physico-chemical factors on its survival

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Abstract

Present study was performed to determine the effects of physical and chemical agents on infective potential of highly pathogenic avian influenza (HPAI) H5N1 (local strain) virus recently isolated in Pakistan during 2006 outbreak. H5N1 virus having titer $10^{8.3}$ ELD_{50}/ml was mixed with sterilized peptone water to get final dilution of 4HA units and then exposed to physical (temperature, pH and ultraviolet light) and chemical (formalin, phenol crystals, iodine crystals, CID 20, virkon®-S, zeptin 10%, KEPCIDE 300, KEPCIDE 400, lifebuoy, surf excel and caustic soda) agents. Harvested amnio-allantoic fluid (AAF) from embryonated chicken eggs inoculated with H5N1 treated virus (0.2 ml/egg) was subjected to haemagglutination (HA) and haemagglutination inhibition (HI) tests. H5N1 virus lost infectivity after 30 min at 56°C, after 1 day at 28°C but remained viable for more than 100 days at 4°C. Acidic pH (1, 3) and basic pH (11, 13) were virucidal after 6 h contact time; however virus retained infectivity at pH 5 (18 h), 7 and 9 (more than 24 h). UV light was proved ineffectual in inactivating virus completely even after 60 min. Soap (lifebuoy®), detergent (surf excel®) and alkali (caustic soda) destroyed infectivity after 5 min at 0.1, 0.2 and 0.3% dilution. All commercially available disinfectants inactivated virus at recommended concentrations. Results of present study would be helpful in implementing bio-security measures at farms/hatcheries levels in the wake of avian influenza virus (AIV) outbreak.

Introduction

Poultry industry in Pakistan is facing various managemental problems along with infectious diseases including avian influenza (AI). This disease of highly pathogenic type was first reported in Pakistan in 1995, caused by subtype H7N3. Since then, various outbreaks of H7N3, H9N2 have been reported in various parts of the country which have inflicted heavy losses to the commercial poultry enterprises [[1,2] and [3]]. In February 2006, avian influenza virus (AIV) subtype H5N1 was for the first time found in two isolated commercial flocks in this country. Biosecurity measures, controlling poultry movements and inactivated vaccines were devised to combat the spread of newly introduced HPAIV H5N1 [4].

Avian influenza viruses by virtue of their infective potential pose a significant threat to human health. AIV subtypes, namely H5, H7 and H9, currently endemic in

poultry in some regions of the world, have been shown capable of infecting humans [[5,6] and [7]]. Therefore, AI infections represent risk factors either for direct infection of humans from the avian host or for the consequences of genetic reassortment between a mammalian and an avian influenza virus, which could become the basis for a generation of a new pandemic virus for humans [8].

It is of crucial importance that AI infections in poultry are controlled to eradicate. International organizations have issued a list of recommendations aiming to control the AI in Asia [9]. The recommendations include implementation of risk reduction interventions such as restriction policies, stamping out, and under certain circumstances appropriate vaccination programmes. Secondary spread of AI is mainly caused through human-related activities such as the movement of staff, vehicles, equipment, and other fomites along with restocking of birds in establishments without following adequate biosecurity measures. It therefore implies that if disinfection of premises, footwear and clothing, vehicles, crates, farm equipment and other materials is not carried out properly, infection will persist in the avian population and the concurrent damage to the poultry industry and the public health threat will not be halted. For this reason, cleaning and disinfecting must be considered an essential part of AI control programmes.

The possibility of reoccurrence of the AI outbreaks in Pakistan is still there because vaccination against the AIV is not rigorously practiced. This threat of the avian influenza has necessitated the pervasive use of disinfectants effective against wide range of viruses, bacteria and fungal spores. There is a wide variety of disinfectants available in market which are claimed to be effective against pathogens. The information about the efficacy of physical and the chemical (disinfectants) agents is scanty. This study, therefore, was designed to evaluate the efficacy of various physical (temperature, Ultraviolet light and pH), and chemical (commercially available disinfectants) agents against local strain of AIV H5N1. The results of this study would be helpful in implementing effective bio-security measures at the farm and hatcheries level.

Methods

Source of Virus

Avian influenza virus was isolated from infected poultry flocks during recent AI outbreaks in and around Rawalpindi/Islamabad area of Pakistan during 2006 at Disease Section of Poultry Research Institute (PRI), Rawalpindi, Pakistan. Subtyping as H5 was performed by haemagglutination (HA) and haemagglutination inhibition (HI) tests using specific antiserum against H5N1 (Weybridge, UK) as described by Olsen *et al.* [10]. Molecular characterization as H5N1 was carried out at National Reference Laboratory for Poultry Diseases, Animal Sciences Institute, National Agricultural Research Centre, Islamabad, Pakistan. The virus cultivated in 9–11 day-old embryonated chicken eggs was subjected to virus titration by the method of Reed and Muench [11]. The amnio-allantoic fluid (AAF) having virus titer of $10^{8.3}$ ELD_{50}/ml was stored in aliquot at -70°C till further use.

Treatment of AIV H5N1 with physico-chemical agents

The preserved virus was cultivated in 9 to11-day-old embryonated chicken eggs. Harvested amnio allantoic (AAF) fluid was titrated on the basis of haemagglutination (HA) potential. Peptone water was prepared, autoclaved and incubated at 37°C for 24 h to check sterility. AAF was diluted in peptone water to have 4 HA unit titer. It was divided into aliquots in sterilized glass vials with 4 ml each. Each vial with H5N1 virus suspension was exposed to 4, 28 and 56°C, ultraviolet light, and different pH values (1, 3, 5, 7, 9, 11 and 13) for different time intervals. The disinfectants used for inactivation of the H5N1 virus included Formalin (Formaldehyde; Merck), Phenol crystals (Merck), Iodine crystals (Merck), CID 20 (CID LINES®, Belgium), Virkon®-S (Antec™ International, UK), Zeptin 10% (Nawan laboratories, Pakistan), KEPCIDE 300 (KEPRO B.V., Holland), and KEPCIDE 400 (KEPRO B.V., Holland), which were mixed with peptone water to attain the required concentration. Each disinfectant product was put in contact with virus suspension at initial concentration of 4 HA units in a ratio of 1:2 at 28°C for 15, 30, 45 and 60 minutes. Effect of soap, detergent and alkali on infectivity of H5N1 virus was also determined using Lifebuoy (Uniliver Pakistan Ltd.), Surf Excel (Uniliver Pakistan Ltd.) and Caustic Soda (Sodium hydroxide, Merck) respectively with the aforementioned protocol.

Inoculation in chicken embryos

Each of the virus suspension exposed to physical factors or disinfectants was filtered through 0.22 μm filter (Millipore™, Millipore corp., Bedford USA) and four chicken embryonated eggs (9 to11 day-old) were inoculated with 0.2 ml of each of the filtrate through allantoic route. Embryonated eggs were also inoculated with untreated AIV H5N1 suspension (4HA titer) and normal saline as positive and negative control respectively. Eggs were incubated at 37°C and were candled after every 24 h for consecutive 72 h. The allantoic fluid was harvested from each of the egg and tested by HA and HI as described by Olsen *et al.* [10]. The inactivation of the virus by physical and chemical treatment was indicated by the survival of the embryo and lack of HA activity of the AAF.

Results

Avian influenza virus H5N1 retained its infectivity at 4°C for more than 100 days although HA activity was decreased. Virus lost its infectivity after 24 h when kept at

room temperature (28°C). Virus tolerated 15 min exposure to 56°C however it was inactivated at 56°C after 30 min of exposure. Ultraviolet light had no deleterious effect on the virus replicating ability even after 60 minutes of exposure (Table 1).

It was observed that H5N1 subtype lost its viability when exposed to pH 1, 3, 11 and 13 after 6 h while it remained viable at pH 7 for all contact times (6, 12, 18 and 24 h). It retained its virulence at pH 5 for 18 h but got inactivated after 24 h. Virus retained its infectivity at pH 9 for more than 24 h (Table 2).

The results revealed that AIV H5N1 can be inactivated by disinfectants at the recommended concentrations (Table 3). H5N1 was inactivated with formalin (0.2, 0.4 and 0.6% after 15 minutes), Iodine crystals (0.4 and 0.6% after 15 minutes), Phenol crystals (0.4 and 0.6% after 15 minutes), CID 20 (0.5% after 60 minutes and 1.0% after 15 minutes), Virkon®-S (0.2% after 45 minutes, 0.5 and 1.0% after 15 minutes), Zeptin 10% (0.5% after 45 minutes, 1% after 30 minutes and 2% after 15 minutes), KEPCIDE 300 (0.5% after 30 minutes and 1% after 15 minutes) and KEPCIDE 400 (0.5 and 1.0% after 15 minutes) at 28°C. Lifebuoy, Surf Excel and Caustic soda inactivated the virus at 0.1, 0.2 and 0.3% concentration after 5 minutes contact time while a concentration of 0.05% was not enough to kill virus (Table 4).

Discussion

Persistence of AIV H5N1 is inversely proportional to temperature and it is evident from the data presented in this study. Virus could survive more than 100 days at 4°C but was inactivated after 24 h at 28°C and after 30 min at 56°C. Results from the two highly pathogenic avian influenza (HPAI) H5N1 viruses from Asia indicated that these viruses did not persist as long as the wild-type AIVs. The persistence of HPAI H5N1 viruses from Asia provided some insight into the potential for these viruses to be transmitted and maintained in the environments of wild bird populations [12]. There is variation in thermo stability of H5N1 viruses. Therefore quite contentious results from various parts of the world are reported. Songserm *et al.* [13] studied the stability of H5N1 HPAI virus isolated in Thailand determining the survival of the infectious virus (initial titer of $10^{6.3}$ ELD50/ml) mixed with chicken faeces under different environmental conditions. It was concluded that virus completely inactivated within 30 min after direct sunlight exposure at an environmental temperature of 32 to 35°C but infectivity was still retained after 4 days in shade at 25 to 32°C. They further reported inactivation of same virus after exposure for 3 min at 70°C. Beard *et al.* [14] incubated wet faeces from naturally infected hens during the HPAI (H5N2) 1983–1985 Pennsylvania outbreak at 4 and 25°C. At 4°C infectivity could still be detected after 35 days but after incubation at 25°C only after 2 days.

Table 1: Effect of temperature and ultraviolet light on the survival of avian influenza virus H5N1 subtype

Physical factors (n = 2)	Exposure time (minutes) 15	30	45	60
Temperature (56°C)	++++	----	----	----
Ultraviolet light	++++	++++	+++-	++--

++++ = AAF from all four inoculated chicken embryos showed haemagglutination (HA) and haemagglutination inhibition (HI) tests positive;
------- = AAF from all four inoculated chicken embryos showed undetectable haemagglutination (HA) activity.

Table 2: Effect of pH on the survival of avian influenza virus H5N1 subtype

pH Values (n = 6)	Exposure time (h) 6	12	18	24
1	----	----	----	----
3	----	----	----	----
5	++++	++++	+++-	----
7	++++	++++	++++	++++
9	++++	++++	++++	++++
11	+---	----	----	----
13	----	----	----	----

++++ = AAF from all four inoculated chicken embryos showed haemagglutination (HA) and haemagglutination inhibition (HI) tests positive;
------ = AAF from all four inoculated chicken embryos showed undetectable haemagglutination (HA) activity.

Effect of heat treatment on HPAI virus (A/chicken/Korea/ES/2003, H5N1 subtype) in chicken meat was investigated by Swayne [15]. The initial titers of infected thigh and breast meat with the H5N1 strain were $10^{6.8}$ and $10^{5.6}$ ELD50/g, respectively. After exposure at 30, 40, 50 and 60°C (1 min), the titer in both types of meat sample remained unchanged. Complete inactivation was only reached after exposure at 70°C (1 sec) and at 70°C for 5 sec in the breast and thigh meat, respectively. The exact mechanism of heat mediated virus inactivation is not known. It is however expected that physical factors like temperature are responsible for decreasing the polymerase activity of the virus which ultimately affects its replication activity [[16] and [17]].

Previously ultraviolet radiation (UV) light has not been proven to inactivate AIVs in a timely manner, as data have shown that 45-min exposure to a UV source was not sufficient for absolute inactivation of HPAI strain A/chicken/Pakistan/94 (H7N3) at an initial concentration of 4 HA units in peptone water at pH 7 [18]. Similar results were obtained by Chumpolbanchorn *et al.* [19] who studied

Table 3: Effect of chemical factors on the survival of avian influenza virus H5N1 subtype

Disinfectant	Concentration (%)	Exposure time (minutes)			
		15	30	45	60
Formalin	0.2	----	----	----	----
	0.4	----	----	----	----
	0.6	----	----	----	----
Iodine crystals	0.2	++++	++++	++++	++++
	0.4	----	----	----	----
	0.6	----	----	----	----
Phenol crystals	0.2	++++	++++	++++	++++
	0.4	----	----	----	----
	0.6	----	----	----	----
CID 20	0.2	++++	++++	++++	++++
	0.5	++++	++++	++++	----
	1.0	----	----	----	----
Virkon®-S	0.2	++++	++++	----	----
	0.5	----	----	----	----
	1.0	----	----	----	----
Zeptin 10%	0.5	++++	++++	----	----
	1.0	++++	----	----	----
	2.0	----	----	----	----
KEPCIDE 300	0.2	++++	++++	++++	++++
	0.5	+++	----	----	----
	1.0	----	----	----	----
KEPCIDE 400	0.2	++++	+++	++++	++++
	0.5	----	----	----	----
	1.0	----	----	----	----

++++ = AAF from all four inoculated chicken embryos showed haemagglutination (HA) and haemagglutination inhibition (HI) tests positive;
------ = AAF from all four inoculated chicken embryos showed undetectable haemagglutination (HA) activity.

Table 4: Effect of soap, detergent and alkali on the survival of avian influenza virus H5N1 subtype

Disinfectant	Concentration (%)	Exposure Time (minutes)			
		5	15	30	45
Surf Excel®	0.05	++++	++++	++++	++++
	0.1	----	----	----	----
	0.2	----	----	----	----
	0.3	----	----	----	----
Life buoy®	0.05	++++	++++	++++	++++
	0.1	----	----	----	----
	0.2	----	----	----	----
	0.3	----	----	----	----
Caustic soda	0.05	++++	++++	++++	++++
	0.1	----	----	----	----
	0.2	----	----	----	----
	0.3	----	----	----	----

++++ = AAF from all four inoculated chicken embryos showed haem-agglutination (HA) and haem-agglutination inhibition (HI) tests positive;
------ = AAF from all four inoculated chicken embryos showed undetectable haemagglutination (HA) activity.

the effect of UV light on infectivity of avian influenza virus (H5N1, Thai field strain) in chicken fecal manure. AIV at initial concentration of $2.38 \times 10^{5.25}$ ELD50 was exposed to ultraviolet light at 4–5 microw/cm^2 at room temperature. UV light could not destroy the infectivity of the virus completely even after exposure for 4 h. Distance from the source of light and shallowness of the exposed suspension are also contributing factors in UV mediated viral destruction. Therefore, only microbes on the surface of material and in the air are killed by UV light [20].

Orthomyxoviridae are considered to be sensitive to acid pH values, although their retention of infectivity is dependent on degree of acidity and virus strain [21]. The mechanism by which AIVs infectivity is lost has been well studied. It has been reported that incubation of Influenza

virus at pH 5 favors virus fusion with host cell membrane [22]. A low pH affects haemagglutinin protein which allows fusion with host cell membrane. The conformational change is reversible between pH 6.4 and 6 but irreversible below pH 5 [23]. Results of present study are partially in agreement with Sato *et al.* [23] as H5N1 virus lost its infectivity at pH below 5 (1 & 3) but remained viable even after 18 h at pH 5. Conducting similar studies, Mittal *et al.* [24] calculated the pKa (the pH value at which 50% of HA is activated) and the pKi (the pH value at which 50% of HA is inactivated) and have shown that the pKa was 5.6–5.7 and the pKi was 4.8–4.9 for H1N1 and H2N2 respectively. Hence it can be assumed that haemagglutinin of H5N1virus under investigation could not attach itself to host (Embryo) cell membrane at pH below 5 and ultimately did not replicate to survive. Similarly, Lue *et al.* [25] observed that LPAI subtypes of H7N2 lost 100% infectivity at pH 2 after 5 min, but exposure to pH 5, 7, 10 and 12 for 15 min had no effect on the infectivity of the isolates. The threshold pH, at which the infectivity is lost, depends on the haemagglutinin (HA) subtype of the virus strain. Strains with noncleaved HA are much more stable when compared to strains with cleaved HA. These observations might explain why duck influenza viruses spread well by lake water, while highly pathogenic strains with cleaved HA do not [26].

Commercially available disinfectant products are usually composed of aldehydes, oxidizing agents, phenol compounds, quaternary ammonium compounds (QACs) and alcohols. Each commercial preparation is the result of careful formulation and any modification can reduce the efficacy. Disinfectants evaluated in this study including CID-20, Virkon®-S, Zeptin 10%, KEPCIDE 300 and KEPCIDE 400 were effective in completely destroying H5N1 virus at recommended dilutions of 1.0, 1.0, 2.0, 1.0 and 1.0% respectively after 15 min at 28°C. Virkon®-S and KEPCIDE 400 were equally good in inactivating the virus at half (0.5% after 15 min) of the recommended dilution. Disinfectant induced inactivation of AIV has been reported by various researchers all over the world. Muhammad *et al.* [18] reported the efficacy of Virkon-S against H7N3 subtype and found that 0.5% dilution was able to inactivate AIV fully after 90 min while 1% and 2% concentration achieved virucidal activity in just 30 min. They further described that phenol crystal at 0.2% and 0.4% dilution required 18 and 12 h respectively to kill the same virus which is contrary to present study findings where phenol crystal at 0.4% took only 15 min to kill H5N1 at 28°C. Ito *et al.* [27] has reported the effect of six povidone iodine products at 2, 0.5, 0.25, and 0.23% concentrations on HPAI A/crow/Kyoto/T2/04 (H5N1). The results showed virucidal activity at all concentrations reducing the virus infectious titers to levels below the detection limits of virus isolation only after 10 s at 25°C. It is not in agreement with our finding where Iodine crystals at 0.2% dilution were not able to inactivate H5N1 virus even after 60 min but 0.4 and 0.6% inactivated after 15 min at 28°C. Conducting similar studies, King [28] drew a conclusion that formalin at low concentration such as 0.04% and 0.1% was able to inactivate HPAI and LPAI viruses (H5N2, H5N9 and H9N2) after 16 h at 37°C. Similar results were obtained by Muhammad *et al.* [18] who reported that 0.06% and 0.12% concentration of formalin was not sufficient to inactivate AIV H7N3 after 6 h however at a concentration of 0.24% no virus was detected by virus isolation. A time span of 12 h was necessary to inactivate AIV at all tested concentrations. These time kill studies have revealed that an inverse relationship exists between formalin concentration and required time to kill AIV of any subtype as it is evident from present study that a high concentration (0.2, 0.4 and 0.6%) of formalin killed H5N1 only after 15 min at 28°C. However, the extent of virus infectivity to be destroyed by disinfectants also depends upon the strain of the virus, exposure time, quantity of the virus and nature of the medium used.

Specific studies on the efficacy of soap, detergents and alkalis are not available in the literature. This is perhaps the first report on the efficacy of soaps, detergents and alkalis against AIVs as disinfectant. Soap and detergents are surfactants and have effect on lipid envelop of viruses which make them good disinfectant [29]. In present study, soap (Life buoy) and detergent (Surf Excel) at 0.05% concentration could not kill H5N1 virus after 45 min contact time but inactivated after 5 min at 0.1, 0.2 and 0.3% concentrations. Presence of hydroxide ion (OH^-) in alkalis make the basis for their disinfectant activity as protein denaturation occurs. Their efficacy in denaturing protein is related to environmental temperature and is low at low temperature but increases proportionally by increasing both temperature and concentration [30]. In present study, 0.05% concentration of Caustic soda at 28°C was not sufficient in killing H5N1 virus but increasing concentrations (0.1, 0.2 and 0.3%) inactivated the virus within 5 min contact time at the same temperature (28°C).

This study describes the effects of physical and chemical agents on infectivity of AIV H5N1. It is therefore inferred that H5N1 virus can be inactivated in the poultry farms/hatcheries using high temperature (e.g. 56°C or above), low (1 and 3) or high (11 and 13) pH of the material to be disinfected. However, it may not be practically feasible for the farmers. Use of disinfectants seems more appropriate and practicable. Consequently there is no need to depopulate the poultry sheds after AIV outbreak for long period of time before arrival of new stock if disinfectants are used appropriately.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MAS and MA participated in the design of the study and performed the investigation, analysis and interpretation of data. MAS and SHas conceived of the study, and participated in its design and coordination. MAS, MA and SHam drafted the manuscript while MA also working as corresponding author. All authors read and approved the final manuscript.

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Influenza Virus Inactivation for Studies of Antigenicity and Phenotypic Neuraminidase Inhibitor Resistance Profiling▽

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Introduction of a new influenza virus in humans urges quick analysis of its virological and immunological characteristics to determine the impact on public health and to develop protective measures for the human population. At present, however, the necessity of executing pandemic influenza virus research under biosafety level 3 (BSL-3) high-containment conditions severely hampers timely characterization of such viruses. We tested heat, formalin, Triton X-100, and β-propiolactone treatments for their potencies in inactivating human influenza A(H3N2) and avian A(H7N3) viruses, as well as seasonal and pandemic A(H1N1) virus isolates, while allowing the specimens to retain their virological and immunological properties. Successful heat inactivation coincided with the loss of hemagglutinin (HA) and neuraminidase (NA) characteristics, and β-propiolactone inactivation reduced the hemagglutination titer and NA activity of the human influenza virus 10-fold or more. Although Triton X-100 treatment resulted in inconsistent HA activity, the NA activities in culture supernatants were enhanced consistently. Nonetheless, formalin treatment permitted the best retention of HA and NA properties. Triton X-100 treatment proved to be the easiest-to-use influenza virus inactivation protocol for application in combination with phenotypic NA inhibitor susceptibility assays, while formalin treatment preserved B-cell and T-cell epitope antigenicity, allowing the detection of both humoral and cellular immune responses. In conclusion, we demonstrated successful influenza virus characterization using formalin- and Triton X-100-inactivated virus samples. Application of these inactivation protocols limits work under BSL-3 conditions to virus culture, thus enabling more timely determination of public health impact and development of protective measures when a new influenza virus, e.g., pandemic A(H1N1)v virus, is introduced in humans.

Host switching of viruses from animals to humans may result in an epidemic among humans and can be particularly dangerous for the new, immunologically naïve host. Examples are the introduction of human immunodeficiency virus, severe acute respiratory syndrome coronavirus, and pandemic influenza A viruses in humans. In particular, avian influenza A virus subtypes H5N1, H9N2, and H7N7 have been transmitted directly to humans in the past decade, exhibiting the zoonotic potential of influenza viruses (4, 11, 19, 25). Moreover, the recent introduction of swine origin influenza A(H1N1)v virus in humans initiated the first influenza pandemic of the 21st century (16, 35). Introduction of a new influenza virus in humans urges quick analysis of its virological and immunological characteristics to assist in the determination of the impact on public health and the development of protective measures. At present, however, the necessity of executing pandemic influenza virus research under biosafety level 3 (BSL-3) high-containment conditions hampers timely characterization of such viruses.

Several virological and immunological assays are used for the characterization of a virus and the immune response induced. For antigenic characterization of influenza viruses, hemagglutination assays and hemagglutination inhibition (HI) assays are the "gold standard" tests. In addition, since the global emergence of antiviral-resistant influenza viruses is becoming an increasing problem, the characterization of influenza virus susceptibilities to the neuraminidase (NA) inhibitors (NAIs) oseltamivir and zanamivir is a clinical necessity (2, 9, 13, 17, 23). For investigating the immune response against influenza viruses, the HI assay determines protective humoral responses (8). Finally, in addition to HI assay results, assessment of the human T-cell responses against influenza virus infection has been reported previously to provide an important marker of protection (3, 10, 22). Until now, these assays have been performed mostly by applying live virus, hence necessitating the use of BSL-3 conditions for studying (potential) pandemic influenza virus. Although numerous studies of virus inactivation, e.g., by means of virucidal compounds, UV light, or gamma irradiation treatment, have been performed, these studies have not comprehensively documented the preservation of influenza virus protein function and antigenic characteristics following inactivation (5–7, 14, 18). Specifically, these studies have not addressed whether inactivated virus can be used for

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phenotypic determination of susceptibilities to NAIs and for characterization of T-cell responses.

In this study, we evaluated the inactivation of influenza viruses of human, avian, and swine origins by heat, formalin, Triton X-100, or β-propiolactone (β-PL) and the retention of hemagglutinin (HA) and NA glycoprotein functions and antigenic integrity. The optimal procedures have been used to demonstrate the proof of principle in antiviral susceptibility assays, antigenic characterization, and T-cell response assays with both seasonal and pandemic influenza A(H1N1) viruses.

MATERIALS AND METHODS

Inclusion of donors and isolation of PBMC. Buffy coats from healthy individuals were retrieved from the Sanquin Blood Bank North West Region in accordance with human experimental guidelines (project number S03.0015-X). In addition, peripheral blood mononuclear cells (PBMC) were retrieved from two previously healthy individuals (a 51-year-old female and a 55-year-old male) with laboratory-confirmed influenza A(H1N1)v virus infection 13 and 19 days after the start of symptoms, respectively. Both participants provided written informed consent before the start of the study. The study was approved by the Medical Ethical Committee of the Utrecht University Medical Center. Human PBMC were isolated by density centrifugation and were cryopreserved at −135°C in a solution of 90% fetal calf serum (FCS; HyClone, UT) and 10% dimethyl sulfoxide (Sigma-Aldrich, MO) until analysis.

Influenza antiviral drugs. Oseltamivir carboxylate Ro64-0802 (GS4071) and zanamivir (GG167) were kindly provided by Roche Diagnostics (Germany) and GlaxoSmithKline (The Netherlands), respectively.

Virus expansion. Human influenza virus isolate A/Wisconsin/67/2005 (H3N2) and low-pathogenicity avian influenza virus isolate A/Mallard/NL/12/2000 (H7N3) were used for initial evaluation of virus inactivation protocols. Both virus isolates were cultured on Madin-Darby canine kidney (MDCK) cells until cytopathic effects (CPE) were observed. The culture flasks were subjected to one freeze-thaw cycle, and the culture supernatant was centrifuged (5 min at 1,500 rpm) to clear it of cell debris. The resulting supernatant was stored at −80°C until further analysis.

Final inactivation experiments were performed using supernatants from cultures of pandemic influenza A(H1N1)v viruses A/California/04/2009, A/Paris/2590/2009, and A/Netherlands/602/2009 and seasonal influenza A(H1N1) viruses A/New Caledonia/20/99 and A/Netherlands/268/2008 on MDCK cells, prepared as described above.

$TCID_{50}$ determination. The determination of the 50% tissue culture infectious dose ($TCID_{50}$) was carried out using 96-well plates containing confluent MDCK cell monolayers. The MDCK cells were incubated with serial 10-fold dilutions of influenza virus culture supernatant in infection medium (Dulbecco's modified Eagle medium [DMEM; Gibco] with antibiotics, nonessential amino acids [Gibco], and 2.5 μg/ml trypsin and without FCS) at 37°C for 60 min. After 1 h, the monolayer was rinsed with phosphate-buffered saline (PBS), overlaid with infection medium, and incubated at 37°C for 5 days. To identify influenza virus-positive wells, the hemagglutination assay was performed. The log $TCID_{50}$ per milliliter was calculated using the Reed-Muench method as described previously (29).

Heat inactivation. Influenza virus culture supernatants (400 μl) were incubated for 30 min at room temperature (22°C) and at 35.0, 38.3, 43.7, 49.6, 55.6, 61.3, 66.7, and 70°C in 0.5-ml vials in a T-Gradient thermal cycler (Biometra, Göttingen, Germany) and subsequently stored at −80°C until analysis.

Formalin inactivation. Influenza virus culture supernatants were incubated for 18 and 72 h at 37°C with a 0.02% final concentration of formalin (Merck, Darmstadt, Germany) in PBS. Immediately after inactivation, formalin was removed by dialysis using Dispodialyzers according to the instructions of the manufacturer (Spectrum Laboratories, CA). The samples were dialyzed against 50 ml DMEM at room temperature on a roller bank two times for 2 h each and then overnight with fresh DMEM each time. The dialyzed samples were stored at −80°C until analysis.

Triton X-100. For initial experiments, 450-μl influenza virus culture supernatants cleared of cell debris were incubated for 1 h at room temperature after being subjected to a thorough vortex step for 30 s with 50 μl of Triton X-100 (BDH Chemicals, Poole, United Kingdom) to yield final concentrations of 0, 0.1, 0.2, 0.5, and 1.0% (vol/vol) Triton X-100. In the final experiments with a final concentration of 1% Triton X-100, 450 μl of influenza virus culture supernatant cleared of cell debris was mixed in a thorough vortex step for 30 s with 50 μl of freshly prepared 10% (vol/vol) Triton X-100 in infection medium prior to incubation for 1 h at room temperature. Subsequently, for hemagglutination titer determination and virus culture, Triton X-100 was removed from the supernatants by using the column-based absorption Detergent-OUT kit according to the instructions of the manufacturer (Calbiochem, CA), and the flowthrough was stored at −80°C until analysis.

β-PL. Influenza culture supernatants were incubated for 16 h at 4°C with and without a final concentration of 0.094% β-PL (ACROS Organics, Geel, Belgium). The supernatants were subsequently incubated for 2 h at 37°C to facilitate hydrolysis of β-PL and were stored at −80°C until analysis.

Validation of inactivation by culturing on MDCK cells. Both control and treated influenza virus culture supernatants were subjected to a maximum of three blind passages on MDCK cells. Confluent MDCK cells were incubated with 50 μl culture supernatant and 5 ml infection medium in 25-cm^2 tissue culture flasks at 37°C. After a 10-day incubation period, the flasks were subjected to one freeze-thaw cycle. In the absence of CPE, the harvested culture material was used as an inoculum (50 μl) for a subsequent passage on MDCK cells. All harvested culture supernatants were stored at −80°C until further analysis.

Validation of inactivation by matrix gene PCR analysis. Total nucleic acid (50 μl) was extracted from 200 μl influenza virus culture supernatant with the MagNA Pure LC total nucleic acid isolation kit on a MagNA Pure LC (version 2.0) extraction robot (Roche, Mannheim, Germany). For semiquantitative analysis of influenza virus negative-sense genomic RNA in the culture material, reverse transcriptase PCR (RT-PCR) was performed using avian myeloblastosis virus RT (Promega, WI) and the sense matrix gene primer 5′ AAG ACC AAT CCT GTC ACC TCT GA 3′ (M-Fw) (40) to generate cDNA. To detect positive-sense viral RNA transcripts in the culture material, total RNA was transcribed into cDNA by using recombinant *Tth* DNA polymerase (Applied Biosystems, CA), matrix gene antisense primer 5′ CAA AGC GTC TAC GCT GCA GTC C 3′ (M-Rv) (40), and treatment with RNase H (Roche) and RNase A (Sigma-Aldrich) to remove all RNA. Subsequently, a 94-bp matrix gene fragment was amplified using the LightCycler 480 real-time PCR system (Roche), the LightCycler TaqMan master (Roche) with primer pair M-Fw and M-Rv, and amplicon-specific probe 5′ TTT GTG TTC ACG CTC ACC GTG CC 3′ labeled with 6-carboxyfluorescein/Black Hole Quencher 1.

Hemagglutination assay. Serial twofold dilutions of live and inactivated influenza virus culture supernatants in PBS were prepared and incubated in quadruplet with 0.25% turkey erythrocytes in PBS at 4°C. After 60 min, the hemagglutination titer, expressed as the reciprocal of the highest dilution producing complete hemagglutination, was read.

HI assay. The inhibition of hemagglutination was assessed with ferret antisera raised against seasonal influenza A(H1N1) viruses A/Texas/36/91, A/New Caledonia/20/99, A/Solomon Islands/03/06, and A/Brisbane/59/07 (kindly provided by the WHO Collaborating Centre, London, United Kingdom). To remove nonspecific hemagglutination activity from the sera, 1 volume of ferret antiserum was mixed with 5 volumes of cholera filtrate, incubated at 37°C for 16 h, and then incubated for 1 h at 56°C to inactivate the receptor-destroying activity of the cholera filtrate. Subsequently, 25-μl twofold serial dilutions of ferret antisera in PBS were prepared and incubated with 25 μl of influenza A virus (4 hemagglutinating units) for 30 min at 37°C, after which 50 μl of 0.5% turkey erythrocytes in PBS was added. The inhibition of hemagglutination was read after 1 h of incubation at 4°C and expressed as the reciprocal of the highest serum dilution producing 100% inhibition of hemagglutination.

NA assay. The NA activities and NAI (oseltamivir and zanamivir) susceptibilities of the culture supernatants were determined using a fluorescence-based NA inhibition assay as described previously (17, 27). The assay was based on the detection of the fluorescent product 4-methylumbelliferone, released after hydrolysis of the substrate 2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA; Sigma-Aldrich) by NA. Final NA inhibition data were supplemented with data generated using the NA-*Star* influenza NAI resistance detection kit according to the instructions of the manufacturer (Applied Biosystems). The NAI susceptibility was expressed as the concentration of NAI needed to inhibit the NA enzyme activity by 50% (the 50% inhibitory concentration [IC_{50}]).

PBMC stimulation and flow cytometric analysis. PBMC were infected at a multiplicity of infection of 2 with live influenza virus or pulsed with an equal amount of formalin-inactivated virus (as confirmed by three blind passages in tissue culture) and cultured in RPMI medium containing 10% FCS (HyClone), penicillin, streptomycin, and L-glutamine (Gibco BRL, NY) at 10^6 cells per well in a 48-well plate. Occasionally, where indicated, twofold-larger amounts of inactivated virus were used. Culture supernatant from uninfected cells was formalin inactivated, dialyzed, and used as a negative control. As a positive control, *Staphylococcus aureus* enterotoxin B (Sigma-Aldrich) was used. The cells were incubated for various times at 37°C in 5% CO_2. Phycoerythrin-conjugated anti-

TABLE 1. Confirmation of A(H3N2) and A(H7N3) influenza virus inactivation by phenotypic and molecular tests[a]

Influenza virus	Treatment	Posttreatment		Passage 1			Passage 2			Passage 3		
		vRNA	mRNA	CPE	vRNA	mRNA	CPE	vRNA	mRNA	CPE	vRNA	mRNA
A(H3N2)	Heat (22°C; control)	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	Heat (35°C)	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	Heat (38.3°C)	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	Heat (43.7°C)	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	Heat (49.6°C)	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	Heat (55.6°C)	+	+	−	+	−	−	−	−	−	−	−
	Heat (61.3°C)	+	+	−	+	−	−	−	−	−	−	−
	Heat (66.7°C)	+	+	−	+	−	−	−	−	−	−	−
	Heat (70°C)	+	+	−	+	−	−	−	−	−	−	−
	Control	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	Formalin (18 h)	+	+	−	+	−	−	−	−	−	−	−
	Control	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	Formalin (72 h)	+	+	−	+	−	−	−	−	−	−	−
	Triton X-100 (0.0%; control)	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	Triton X-100 (0.1%)	+	+	−	−	−	−	−	−	−	−	−
	Triton X-100 (0.2%)	+	+	−	+	−	−	−	−	−	−	−
	Triton X-100 (0.5%)	+	+	−	+	−	−	−	−	−	−	−
	Triton X-100 (1.0%)	+	+	−	+	−	−	−	−	−	−	−
	Control	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	β-PL (overnight)	+	+	−	+	−	−	−	−	−	−	−
A(H7N3)	Heat (22°C; control)	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	Heat (35°C)	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	Heat (38.3°C)	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	Heat (43.7°C)	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	Heat (49.6°C)	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	Heat (55.6°C)	+	+	−	+	−	−	−	−	−	−	−
	Heat (61.3°C)	+	+	−	+	−	−	−	−	−	−	−
	Heat (66.7°C)	+	+	−	+	−	−	−	−	−	−	−
	Heat (70°C)	+	+	−	+	−	−	−	−	−	−	−
	Control	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	Formalin (18 h)	+	+	−	+	−	−	−	−	−	−	−
	Control	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	Formalin (72 h)	+	+	−	+	−	−	−	−	−	−	−
	Triton X-100 (0.0%; control)	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	Triton X-100 (0.1%)	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	Triton X-100 (0.2%)	+	+	−	+	−	−	−	−	−	−	−
	Triton X-100 (0.5%)	+	+	−	+	−	−	−	−	−	−	−
	Triton X-100 (1.0%)	+	+	−	+	−	−	−	−	−	−	−
	Control	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	β-PL (overnight)	+	+	−	+	−	−	−	−	−	−	−

[a] Virus specimens are noted as being positive or negative for a CPE and influenza virus matrix gene RNA and mRNA. Control samples were subjected to the same protocol as the treatment specimens except for the treatment itself: control treatments were heat at 22°C and the absence of formalin, Triton X-100, or β-PL. vRNA, viral genomic RNA; ND, not determined.

bodies specific for CD107a (BD Biosciences, CA) were added 16 h before the end of culture. For the detection of intracellular cytokine production, monensin (GolgiStop; BD Biosciences) was added during the last 16 h of culture. After incubation, PBMC were harvested and stained with anti-CD4 Pacific Blue antibody (BioLegend, San Diego, CA), anti-CD8–peridinin chlorophyll protein–Cy5.5 (BD Biosciences), anti-gamma interferon–allophycocyanin (BD Biosciences), anti-interleukin-2 (anti-IL-2)–fluorescein isothiocyanate (eBioscience, CA), anti-tumor necrosis factor alpha–phycoerythrin–Cy7 (BD Biosciences), and LIVE/DEAD fixable dead cell stain (Invitrogen, Paisley, United Kingdom) according to the manufacturers' procedures and were acquired using a FACSCanto II system (BD Biosciences). At least 10^5 viable lymphocytes were acquired based on forward-side scatter characteristics and analysis of viability staining. The results were analyzed using FACSDiva software (BD Biosciences).

RESULTS

Seasonal influenza virus A(H3N2) and avian influenza virus A(H7N3) inactivation. Since we wished to determine the effect of virus inactivation on viral infectivity and viral protein resilience, the procedures were first executed using human and avian influenza virus strains with completely different HA and NA subtypes.

Influenza virus stocks A/Wisconsin/67/2005 subtype H3N2 ($10^{4.8}$ $TCID_{50}$/ml) and A/Mallard/NL/12/00 subtype H7N3 ($10^{5.3}$ $TCID_{50}$/ml) were treated separately with heat, formalin, Triton X-100, and β-PL. Virus infectivity was completely absent after inactivation by temperatures of ≥55.6°C, formalin, Triton X-100 concentrations of ≥0.2%, and β-PL, as confirmed by the absence of CPE, the absence of sense viral mRNA, and the reduction of antisense viral genomic RNA in three subsequent cell culture passages (Table 1). Influenza virus mRNA is not present in the virus itself but is produced only in the infected cell and is therefore a clear measure of virus replication. In contrast to residual virus genomic RNA that is protected by the viral nucleoprotein, posttreatment residual virus mRNA is rapidly degraded in tissue culture. Specimens from cell culture passages showing incomplete virus inactivation,

based on phenotypic (CPE) and molecular data, were not passaged further. The combined viral culture and matrix RNA detection data demonstrate that these procedures result in complete inactivation of $10^{4.8}$ and $10^{5.3}$ $TCID_{50}$/ml human and avian influenza virus strains, respectively.

HA and NA characteristics of inactivated influenza A(H3N2) and A(H7N3) viruses. As reported above, virus inactivation may be attained by each of the four described inactivation techniques. To address whether the methods of virus inactivation affected the virological properties, we determined the HA and NA characteristics (Fig. 1) after virus inactivation by each treatment.

Successful heat inactivation coincided with the loss of HA and NA characteristics for both viruses; however, the human A(H3N2) virus lost HA activity at a slightly higher temperature than the avian A(H7N3) virus (difference in temperature [ΔT], 4°C). In contrast, avian A(H7N3) virus lost NA activity at a higher temperature than the human A(H3N2) virus (ΔT, 14°C). β-PL inactivation did not have a major effect on the avian A(H7N3) virus HA and NA characteristics but reduced the hemagglutination titer and NA activity of the human A(H3N2) virus by 10-fold or more.

Treatments with Triton X-100 and formalin resulted in less significant reduction of HA and/or NA activity than heat and β-PL treatments. Although the log $TCID_{50}$ of infectious virus was not adversely affected by passing of supernatants over the detergent removal column [log $TCID_{50}$s of A(H7N3) virus (means ± standard deviations), 5.0 ± 0.25/ml precolumn treatment and 4.75 ± 0.35/ml postcolumn treatment], both the hemagglutination titers and NA activities of the viruses were reduced by subjection to the column step without the addition of Triton X-100 (mock treatment) compared to those of the stock viruses (values were similar to those for the 1-h heat treatment at 22°C) (Fig. 1). Triton X-100 treatment resulted in inconsistent HA activity compared with that of the control, depending on the virus subtype. However, compared to those of the controls, the NA activities of both viruses were enhanced consistently by the addition of Triton X-100 (Fig. 1C). In addition, without the removal of Triton X-100, the NA activities of these viruses were enhanced even further beyond those of the stock viruses (see Fig. 3A). Therefore, we further investigated the use of Triton X-100 treatment in NAI susceptibility assays.

Nevertheless, formalin treatment best retained both HA and NA activities (Fig. 1) but was more complex to perform than Triton X-100 treatment without detergent removal.

Seasonal influenza A(H1N1) and pandemic influenza A (H1N1)v virus inactivation and HA and NA characteristics. Based on the results obtained from human A(H3N2) and avian A(H7N3) virus inactivation using four different protocols, Triton X-100 and formalin inactivation protocols were chosen to demonstrate the proof of principle using seasonal and pandemic A(H1N1) viruses. To this end, we validated virus inactivation by Triton X-100 and formalin by using two seasonal A(H1N1) viruses, A/Netherlands/268/2008 ($10^{3.8}$ $TCID_{50}$/ml) and A/New Caledonia/20/99 ($10^{5.0}$ $TCID_{50}$/ml), and three pandemic A(H1N1)v virus strains of porcine origin, A/Netherlands/602/2009 ($10^{4.1}$ $TCID_{50}$/ml), A/California/04/2009 ($10^{4.9}$ $TCID_{50}$/ml), and A/Paris/2590/2009 ($10^{8.0}$ $TCID_{50}$/ml). Both 1% Triton X-100 and 18-h 0.02% formalin inactivation protocols effectively inactivated the viruses, as demonstrated by the absence of CPE in three consecutive passages of the treated viruses (Table 2). The results of the RT-PCRs for virus matrix gene RNA and mRNA confirmed that virus replication was absent (Table 2). These data demonstrate that 1% Triton X-100 and 18-h 0.02% formalin inactivation protocols result in complete inactivation of both seasonal A(H1N1) and pandemic A(H1N1)v virus strains at concentrations up to $10^{8.0}$ $TCID_{50}$/ml.

Characterization of the inactivated viruses showed that Triton X-100 treatment again resulted in inconsistent hemagglutination titers (Fig. 2A). After formalin treatment, seasonal influenza A(H1N1) virus hemagglutination titers were retained but A(H1N1)v hemagglutination titers were reduced twofold (Fig. 2C). The NA activities of seasonal influenza A(H1N1) viruses were reduced less than twofold by both inactivation protocols (after Triton X-100 removal) (Fig. 2B and D). Although NA activities of live influenza A(H1N1)v viruses could not be evaluated for technical reasons (no equipment was available in the BSL-3 laboratory), the other viruses showed consistently increased NA activities after treatment with Triton X-100 without removal of the detergent compared to the NA activities of the virus stocks (Fig. 3A). Based on the combined HA and NA characteristics, we concluded that formalin treatment best retained HA and NA activity. In addition, the results confirmed the usefulness of Triton X-100 inactivation without removal of the detergent for the determination of NAI susceptibility.

Analysis of NA inhibition after influenza A(H1N1) virus inactivation. Before the implementation of routine NAI susceptibility testing using inactivated virus culture supernatants, preservation of NA enzyme characteristics after 1% Triton X-100 or 0.02% formalin treatment needed further evaluation. Therefore, oseltamivir-sensitive and -resistant seasonal influenza A(H1N1) virus isolates were inactivated prior to NAI susceptibility testing with the MUNANA assay. The IC_{50} values of oseltamivir for live and inactivated virus isolates remained within the 95% confidence interval of interassay variability for the MUNANA assay, demonstrating that NA enzyme inhibition by oseltamivir was not affected by Triton X-100 or formalin treatment (Fig. 3B and C).

Since Triton X-100 treatment is a rapid and easy-to-use inactivation protocol that in addition enhances the NA activities of influenza virus isolates (Fig. 3A), Triton X-100-treated supernatants were subsequently tested with the commercial NA-*Star* assay. The NA-*Star* assay performed similarly to the MUNANA assay, demonstrating that NAI susceptibility testing using 1% Triton X-100-treated supernatants works well with both assays (Fig. 3C). Subsequent parallel NAI susceptibility testing of 12 Triton X-100-treated A(H1N1)v virus isolates in the MUNANA and NA-*Star* assays showed the NAI-sensitive phenotype of current A(H1N1)v viruses, for which IC_{50} values of both oseltamivir and zanamivir were <1 nM (Fig. 3D and Table 3).

B-cell responses to inactivated influenza A(H1N1) and A (H1N1)v viruses. To verify the preservation of B-cell response antigenic epitopes after influenza virus inactivation, we used the HI assay. Due to the reduction of HA activity after Triton X-100 treatment, only the formalin-treated influenza viruses, except for formalin-treated influenza A(H1N1)v viruses

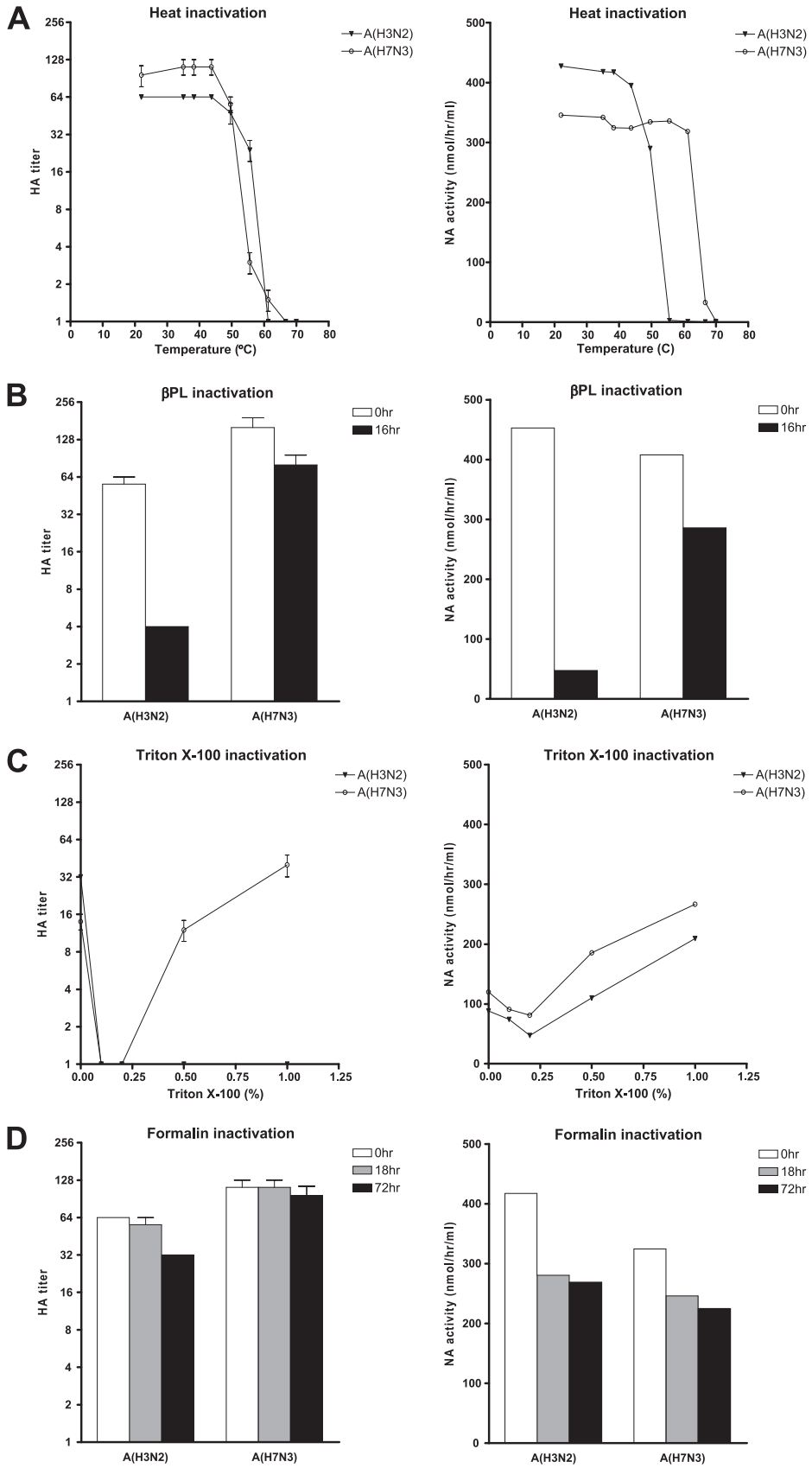

FIG. 1. Hemagglutination titers (left) and NA activities (right) of human influenza A(H3N2) virus and avian influenza A(H7N3) virus after treatment with heat (A), β-PL (B), Triton X-100 (C), or formalin (D). Controls were subjected to the same procedures as the treatment specimens except for the treatment itself: they were heated at 22°C and had no β-PL, Triton X-100, or formalin added. Hemagglutination titers and NA activities after Triton X-100 treatment were determined after the removal of the detergent. Hemagglutination titers of <2 are expressed as 1.

TABLE 2. Confirmation of seasonal and pandemic A(H1N1) influenza virus inactivation by phenotypic and molecular tests[a]

Virus	Treatment or sample status	Posttreatment		Passage 1			Passage 2			Passage 3		
		vRNA	mRNA	CPE	vRNA	mRNA	CPE	vRNA	mRNA	CPE	vRNA	mRNA
Influenza A/Netherlands/268/2008 (H1N1) virus	Control	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	0.02% formalin for 18 h	+	+	−	+	−	−	−	−	−	−	−
	1% Triton X-100	+	+	−	−	−	−	−	−	−	−	−
Influenza A/New Caledonia/20/99 (H1N1) virus	Control	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	0.02% formalin for 18 h	+	+	−	+	−	−	−	−	−	−	−
	1% Triton X-100	+	+	−	+	−	−	−	−	−	−	−
Influenza A/Netherlands/602/2009 (H1N1)v virus	Control	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	0.02% formalin for 18 h	+	+	−	+	−	−	−	−	−	−	−
	1% Triton X-100	+	+	−	−	−	−	−	−	−	−	−
Influenza A/California/04/2009 (H1N1)v virus	Control	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	0.02% formalin for 18 h	+	+	−	+	−	−	−	−	−	−	−
	1% Triton X-100	+	+	−	−	−	−	−	−	−	−	−
Influenza A/Paris/2590/2009 (H1N1)v virus	Control	+	+	+	+	+	ND	ND	ND	ND	ND	ND
	0.02% formalin for 18 h	+	+	−	+	−	−	−	−	−	−	−
	1% Triton X-100	+	+	−	−	−	−	−	−	−	−	−

[a] Virus specimens are noted as being positive or negative for a CPE and influenza virus matrix gene RNA and mRNA. Controls were the stock viruses prior to treatment. vRNA, viral genomic RNA; ND, not determined.

A/Netherlands/602/09 and A/Paris/2590/09 (Fig. 2A and C), demonstrated sufficient hemagglutination titers to be used in the HI assay. The HI titers of four ferret antisera for live- and formalin-treated seasonal influenza A/New Caledonia/20/99 and A/Netherlands/268/08 viruses and pandemic influenza A/California/04/09 virus were similar, illustrating that epitopes on HA to which B-cell responses are directed are preserved after formalin treatment (Fig. 4). In contrast to A/California/04/09 virus, pandemic A/Netherlands/602/09 and A/Paris/2590/09 viruses did not show any cross-reactivity with ferret sera against recent seasonal A(H1N1) viruses.

T-cell responses to inactivated seasonal influenza virus. Since the highest degree of preservation of HA and NA activities was achieved by inactivating influenza A(H3N2) and A(H7N3) viruses using formalin, the preservation of T-cell epitope antigenicity was assessed using formalin-inactivated influenza viruses. To determine the magnitudes and kinetics of T-cell responses to formalin-treated influenza viruses, PBMC from six donors were infected with live influenza A/Wisconsin/67/2005 (H3N2) virus or pulsed with formalin-treated A/Wisconsin/67/2005 (H3N2) virus or a negative-control sample, after which T-cell responses were measured by flow cytometry at 24, 48, 72, and 168 h poststimulation.

Infection of PBMC with live influenza A/Wisconsin/67/2005 (H3N2) virus resulted in a significant increase ($P < 0.05$; Newman-Keuls analysis of variance) in $CD4^+$ and $CD8^+$ T cells expressing CD107a and IL-2, indicating cytotoxic activity and cellular proliferation, respectively (Fig. 5). A similar expression pattern among PBMC pulsed with formalin-treated influenza A/Wisconsin/67/2005 (H3N2) virus was observed. The highest percentages of $CD107a^+$ and $IL\text{-}2^+$ T cells in response to formalin-treated virus were observed approximately 24 h later than the highest level of the response to live virus. Furthermore, the proportions of $CD107a^+$ and $IL\text{-}2^+$ T cells found after pulsing with formalin-treated virus were lower than those found after infection with live virus, but the difference was not statistically significant ($P > 0.05$). Pulsing PBMC with twice the amount of formalin-treated virus did not result in significantly higher T-cell responses ($P > 0.05$). Similar results were obtained with A/New Caledonia/20/99 (H1N1) virus (Fig. 6). Together, these results indicate that T-cell epitope antigenicity of seasonal influenza viruses is preserved after formalin treatment.

T-cell responses to inactivated 2009 pandemic influenza A(H1N1)v virus. Since formalin treatment effectively inactivated seasonal influenza viruses while preserving T-cell epitope antigenicity, we determined whether the T-cell epitope antigenicity of the pandemic A(H1N1)v virus was also preserved. To this end, (H1N1)v virus-specific T-cell responses by PBMC isolated from two individuals with laboratory-confirmed A(H1N1)v virus infections were evaluated. *In vitro* stimulation of these PBMC with inactivated influenza A/Paris/2590/2009 (H1N1)v virus demonstrated increases in the percentages of $CD107a^+$ T cells in the $CD4^+$ and $CD8^+$ T-cell subsets from both donors (Fig. 7). As a control, PBMC were infected with live seasonal influenza A/New Caledonia/20/99 (H1N1) virus or pulsed with formalin-treated A/New Caledonia/20/99 (H1N1) virus, showing a similar increase in $CD107a^+$ cells. Furthermore, live and formalin-treated seasonal influenza A(H1N1) virus samples and formalin-treated influenza A(H1N1)v virus led to increases in $IL\text{-}2^+$ T cells in PBMC from donor A, which were most apparent in the $CD8^+$ T-cell subset. The stimulation of PBMC from donor B did not produce an increase in $IL\text{-}2^+$ T cells in both the $CD4^+$ and $CD8^+$ T-cell subsets, indicating donor variability in the T-cell responses.

Together, these data demonstrate that the influenza A(H1N1)v virus retains T-cell-stimulatory capacity after formalin treatment, indicating that T-cell epitope antigenicity is also preserved for this pandemic influenza virus.

DISCUSSION

Our study provides validated virus inactivation protocols that allow implementation of phenotypic NAI susceptibility

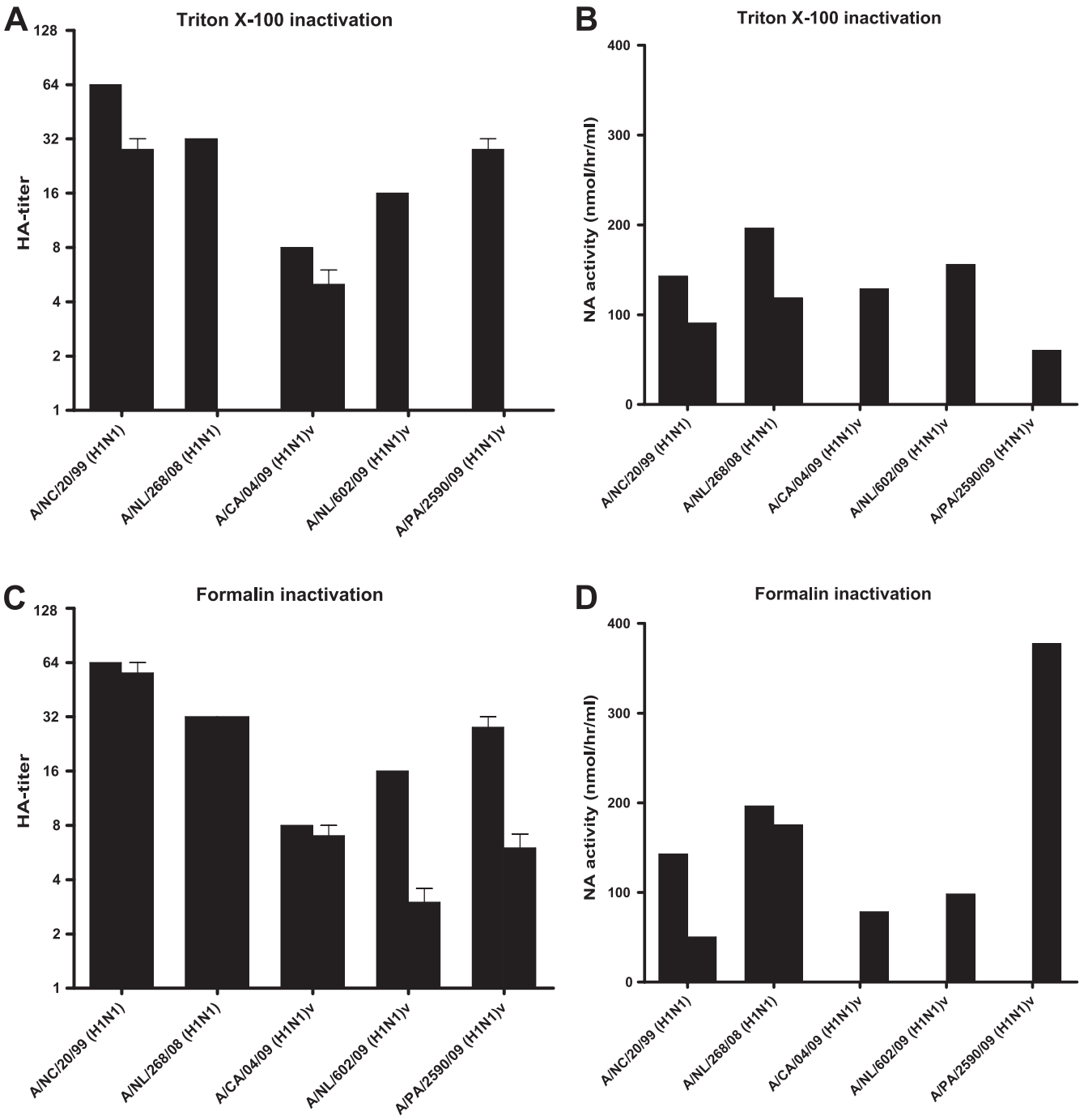

FIG. 2. Hemagglutination titers (left) and NA activities (right) of seasonal A(H1N1) and pandemic A(H1N1)v viruses after inactivation using Triton X-100 (A and B) or formalin (C and D). The left bars show the results for the original stock viruses, and the right bars show the results for the viruses after inactivation. Hemagglutination titers and NA activities after Triton X-100 treatment were determined after the removal of the detergent. Hemagglutination titers of <2 are expressed as 1. NA activities of live A(H1N1)v viruses were not determined and are therefore not shown for these viruses in panels B and D.

testing, HI assessment (for serology as well antigenic characterization of viruses), and T-cell response characterization using avian, swine, and human influenza viruses under BSL-2 containment conditions. Using pandemic influenza A(H1N1)v virus strains, we illustrate the ease of carrying out Triton X-100 and formalin virus inactivation protocols prior to the assessment of A(H1N1)v virus susceptibility to antivirals and the characterization of B- and T-cell responses, respectively, outside the BSL-3 high-containment facility. These inactivation protocols facilitate the diagnostic examination of pandemic influenza viruses by applying standard laboratory conditions at BSL-2.

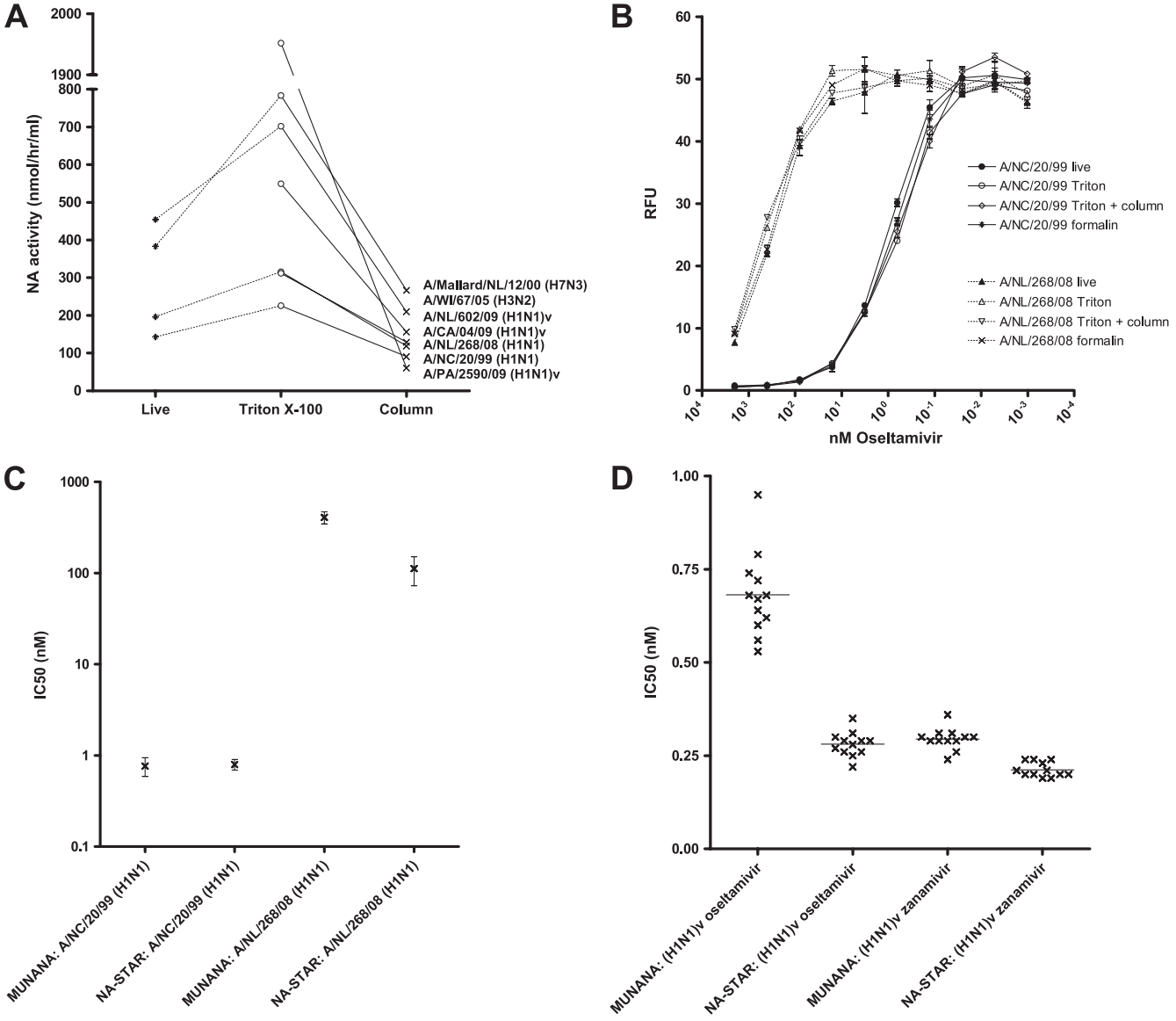

FIG. 3. Results obtained using inactivated influenza viruses in NA inhibition assays. (A) The NA activity increases after 1% Triton X-100 treatment but is subsequently reduced by column-based detergent removal. Lines connect results from experiments with the same virus strain. (B) Effects of 1% Triton X-100 and formalin treatments on the performance of the MUNANA NA inhibition assay with oseltamivir-sensitive and -resistant influenza viruses. Results obtained after column-based detergent removal (Triton + column) are indicated. RFU, relative fluorescence units. (C) IC_{50} values obtained with the MUNANA assay (for live and Triton X-100-treated virus samples, virus samples treated with Triton X-100 and subjected to detergent removal, and formalin-treated virus samples) and the NA-*Star* assay (for live and Triton X-100-treated virus samples and virus samples treated with Triton X-100 and subjected to detergent removal) for NA inhibition with oseltamivir-sensitive and -resistant seasonal influenza A(H1N1) viruses. (D) NAI susceptibilities of 12 1% Triton X-100-treated influenza A(H1N1)v virus isolates as measured with the MUNANA and NA-*Star* NA inhibition assays.

TABLE 3. Phenotypic NAI susceptibilities of 12 clinical A(H1N1)v virus isolates[a]

NAI assay	Virus	Mean IC_{50} (nM) (−SD, +SD)[b] of:	
		Oseltamivir	Zanamivir
NA-*Star* assay	A(H1N1)v	0.28 (0.25, 0.31)	0.21 (0.19, 0.23)
MUNANA assay	A(H1N1)v	0.67* (0.58, 0.79)	0.29** (0.27, 0.32)
	A(H1N1)[a]	0.85* (0.68, 1.05)	0.68** (0.49, 0.92)

[a] Baseline NAI susceptibilities of Dutch seasonal A(H1N1) influenza viruses are given (17).

[b] The NAI susceptibilities of pandemic A(H1N1)v virus were greater than those of seasonal A(H1N1) virus. *, $P = 0.002$; **, $P < 0.001$.

Considering results from documented studies and taking ease of use under BSL-3 conditions into account, therefore excluding, e.g., irradiation protocols, we evaluated human A(H3N2) and avian A(H7N3) virus inactivation by means of heat and the virucidal compounds β-PL, Triton X-100, and formalin (5–7, 14, 18). The most optimal virus inactivation protocols were used to demonstrate the proof of principle with seasonal and pandemic A(H1N1) viruses.

Heat inactivation of influenza A virus can be very efficient, as demonstrated by studies of thermal processing of meat from

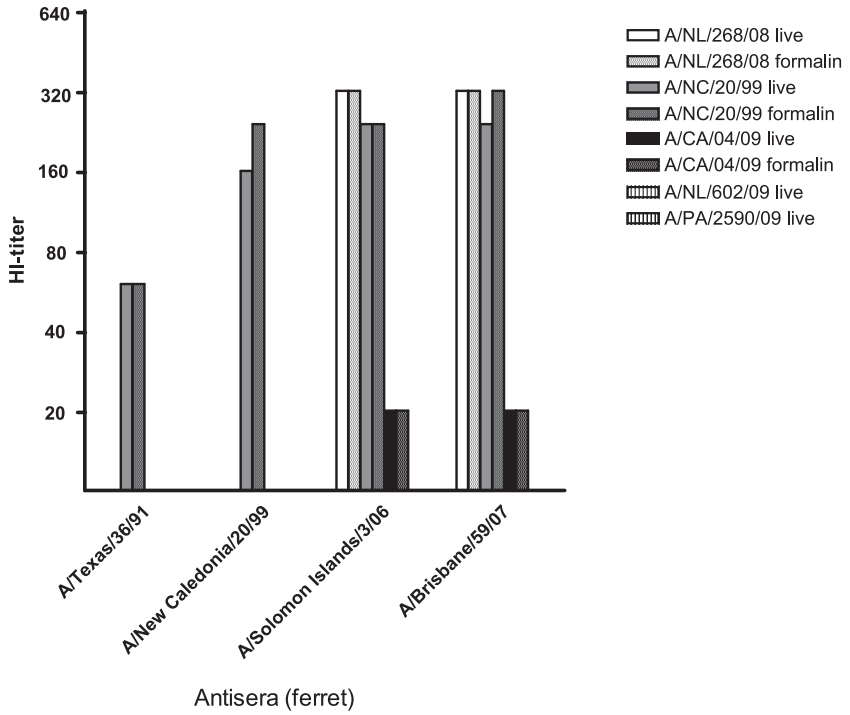

FIG. 4. HI titers of four ferret antisera with live or formalin-treated influenza A(H1N1) viruses. Only one of the A(H1N1)v pandemic strains (A/California/04/09) showed some cross-reactivity with ferret antisera against recent seasonal A(H1N1) influenza viruses. Formalin-treated A/Netherlands/602/09 and A/Paris/2590/09 viruses had too-low hemagglutination titers to be included in the HI assay (Fig. 2C). HI titers of <20 are not shown.

influenza virus-infected chickens (36, 39), and can be achieved in less than 5 s at temperatures above 70°C (38). Reproducible heat inactivation, however, should be carried out under controlled conditions, e.g., by using small volumes in a thermocycler (37). We used a 30-min incubation period with temperatures below 70°C in an effort to preserve protein function. Unfortunately, infectivity and glycoprotein function disappeared almost simultaneously at temperatures above 55.6°C. Interestingly, the avian A(H7N3) virus NA enzyme demonstrated better heat resistance than the human A(H3N2) virus NA (ΔT, 14°C). Nevertheless, heat treatment appeared not to be suitable for our purposes.

Although β-PL treatment resulted in complete inactivation of influenza virus, it negatively affected both HA and NA functions of the tested A(H3N2) virus, as was demonstrated previously by Goldstein and Tauraso (14). During β-PL hydrolysis, the pH can decrease, resulting in a conformational change in HA (28, 31). Furthermore, β-PL is highly reactive with nucleic acids and proteins and can alter RNA/DNA structure, as well as form RNA/DNA-protein complexes (24, 26). As pH measurements during β-PL treatment demonstrated that the pH remained at physiologic values (data not shown), we hypothesize that the formation of RNA/DNA-protein complexes compromised glycoprotein function of the tested A(H3N2) virus. Even though β-PL is widely used by the vaccine industry for vaccine preparation (1), our results indicate that β-PL inactivation is preferentially not to be used in combination with virological and immunological assays.

Treatment with 1% Triton X-100 appeared to be an easy-to-perform procedure for complete influenza virus inactivation and subsequent determination of NAI susceptibility. However, for validation of virus inactivation in cell cultures by Triton X-100 treatment, the detergent had to be removed by passing the supernatants over a detergent-absorbing column to prevent solubilization of MDCK cell membranes in the cell monolayer. As the capacity of the column used is 15 mg detergent and the amount of Triton X-100 at 1% in 500 ml is about 5 mg, complete removal of Triton X-100 was assumed and culture validation results were eventually not affected by residual Triton X-100. Although the column itself had no significant effect on log $TCID_{50}$, the removal of Triton X-100 resulted in variable functional glycoprotein recovery depending on the Triton X-100 concentration used (Fig. 1). Without the column-based removal of Triton X-100, however, NA activity increased, allowing the straightforward application of phenotypic NA inhibition assays (the NA-*Star* assay and the MUNANA assay) (Fig. 3A). These effects may be explained by the mode of action of Triton X-100, solubilization of the lipid membrane of influenza virus and formation of micelles in which the HA and NA proteins are trapped. The concentration of Triton X-100 in a 1% (vol/vol) solution (17 mM) is well above the critical micelle concentration (0.2 to 0.9 mM) at which detergent molecules begin to accumulate in a lipid bilayer membrane, guaranteeing complete solubilization and, therefore, inactivation of influenza virus. However, a note of caution should be made. As the critical micelle concentration of Triton X-100 depends on temperature and complete solubilization at a given concentration of Triton X-100 depends on the amounts of membranes and protein in the solution, our protocol is validated for the given temperature and duration (room temperature for 1 h)

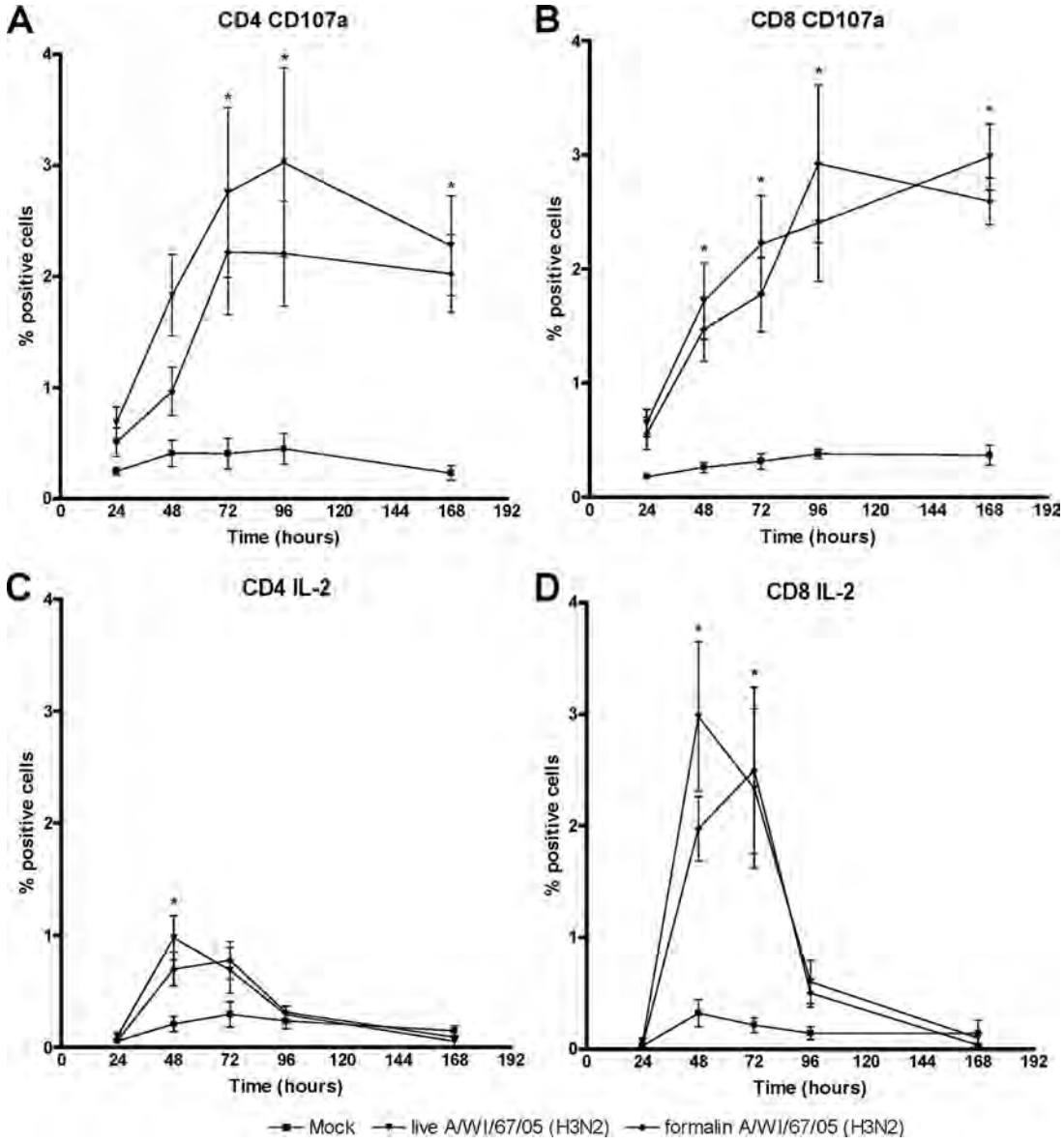

FIG. 5. T-cell response kinetics after PBMC stimulation with live or formalin-treated influenza virus A/Wisconsin/67/05 (H3N2). (A) Percentages of $CD4^+$ $CD107a^+$ T cells; (B) percentages of $CD8^+$ $CD107a^+$ T cells; (C) percentages of $CD4^+$ $IL\text{-}2^+$ T cells; (D) percentages of $CD8^+$ $IL\text{-}2^+$ T cells. Mock, culture supernatant from uninfected cells; *, $P < 0.05$ for live and formalin-inactivated A(H3N2) virus samples compared to mock-treated samples.

and for tissue culture supernatants containing infection medium without FCS that have been cleared of cell debris. The use of higher-protein or lipid-content matrixes, e.g., allantoic fluid with influenza virus, may adversely affect inactivation, and a higher concentration of Triton X-100 should probably be used. Increased NA activity in 1% Triton X-100 solution may be explained by better access of the substrate to the NA in micelles than in the intact virus. The removal of Triton X-100 will cause clumping of NA and HA molecules as well, due to the hydrophobic action of the tails of NA and HA, which are normally located in the lipid bilayer of the virus membrane, resulting in decreased NA activity and inconsistent hemagglutination.

Nevertheless, using 1% Triton X-100 inactivation without detergent removal, we demonstrated that the baseline susceptibilities of A(H1N1)v virus to both oseltamivir ($P = 0.002$; Student's t test) and zanamivir ($P < 0.001$; Student's t test) were greater than those of seasonal influenza A(H1N1) viruses (Table 3). However, since phenotypic NAI susceptibility assays such as the MUNANA and NA-*Star* assays are conducted with virus isolates, this approach still obstructs rapid antiviral susceptibility profiling of an influenza virus infection. The NA activity-enhancing mechanism of Triton X-100 treatment may increase the sensitivity of phenotypic NAI susceptibility assays and may allow direct testing of low-protein-content clinical specimens without the need of virus isolation in the near future.

Formalin treatment appeared to be superior to the other

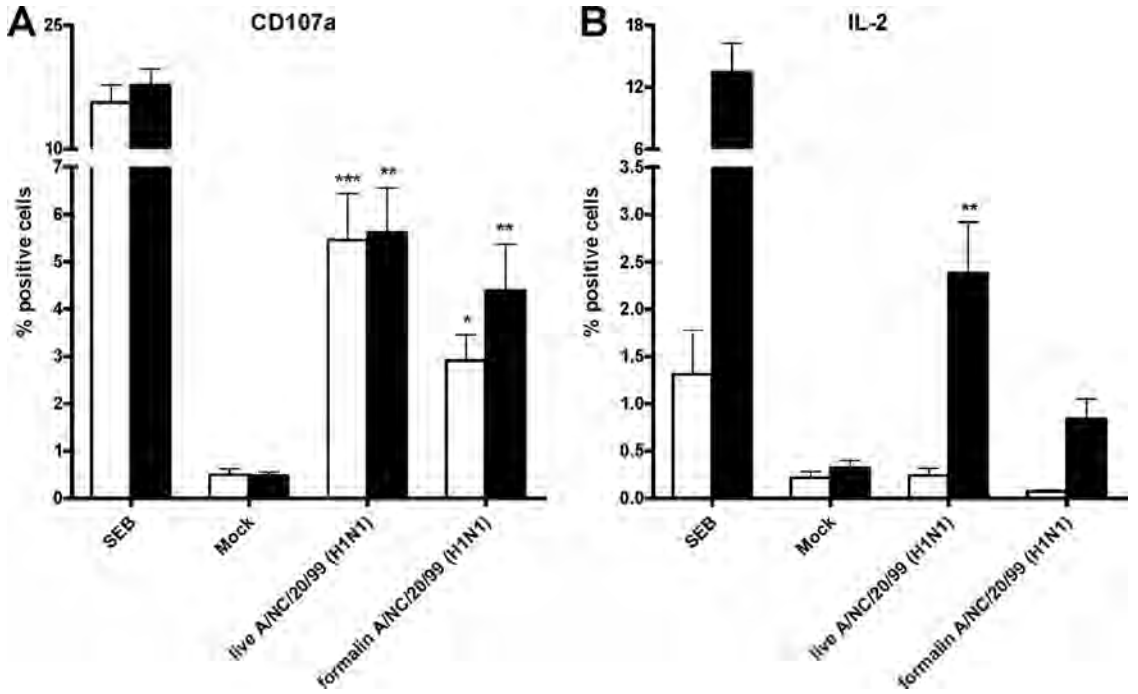

FIG. 6. Detection of T-cell responses against live or formalin-treated A/New Caledonia/20/99 (H1N1) virus demonstrates significant T-cell epitope antigenicity 72 h poststimulation. (A) Percentages of $CD4^+$ $CD107a^+$ T cells (white bars) and $CD8^+$ $CD107a^+$ T cells (black bars); (B) percentages of $CD4^+$ IL-2^+ T cells (white bars) and $CD8^+$ IL-2^+ T cells (black bars). SEB, positive control using *S. aureus* enterotoxin B; mock, culture supernatant from uninfected cells. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ for live and formalin-inactivated A(H1N1) virus samples compared to mock-treated samples.

treatments for antigenic characterization and measurement of B- and T-cell responses. The assessment of the B-cell epitope integrity of formalin-treated influenza virus by the HI assay showed similar HI titers for live and formalin-treated influenza virus samples. Although the ferret antiserum HI assay is able to distinguish major antigenic variants, minor changes in antigenicity are difficult to define reliably (34). Nevertheless, we demonstrated that HA-specific antibodies still bind to formalin-inactivated viruses, suggesting preservation of B-cell epitopes. The HI titers further confirmed the major antigenic difference between recent seasonal influenza A(H1N1) viruses and pandemic A(H1N1)v viruses, corresponding with published data (12). Additionally, we detected antigenic differences among A(H1N1)v viruses. Significant HI titers of the ferret sera for recent seasonal A(H1N1) viruses were found by using live and formalin-inactivated influenza A/California/04/09 H1N1v virus samples, while HI titers of the same sera tested using live A/Netherlands/602/09 H1N1v and A/Paris/2590/09 H1N1v viruses were <20. Influenza virus sequence data obtained from GenBank demonstrates the presence of amino acid substitutions P83S, I191L, T197A, and I321V in HAs of A/Netherlands/602/09 and A/Paris/2590/09 viruses, compared to HA in A/California/04/09 virus. As HA substitutions P83S, I191L, and T197A are located on HA1 antigenic sites, this may explain the differences in HI titers obtained with the three influenza A(H1N1)v viruses. Whether this antigenic change was induced by human immune pressure and correlates with B-cell escape is unclear.

Furthermore, we have demonstrated that formalin treatment of both seasonal and pandemic influenza viruses preserves significant T-cell epitope antigenicity for the detection of cellular immune responses. Upon infection of PBMC with live influenza virus, the numbers of cells expressing CD107a and IL-2 increased rapidly. Interestingly, the responses induced by formalin-treated influenza virus started to increase after a 24-h delay and were reduced compared to the levels induced by live influenza virus. The delay may be due to decreased HA integrity, which we observed in the hemagglutination assays with some formalin-treated viruses, ultimately resulting in less efficient binding and uptake of virus by antigen-presenting cells and subsequent activation of T cells (20, 33). However, a more likely explanation is the difference in antigen presentation routes using live and inactivated viruses. During a live-virus infection, antigen is presented on major histocompatibility complex class I (MHC-I) molecules mainly via the endogenous pathway, whereas antigens from inactivated virus are presented on MHC-I molecules by the alternative cross-presentation pathway, which may affect the induction of $CD8^+$ T-cell responses (15). Furthermore, formalin-inactivated influenza virus is not able to replicate and cannot lead to *de novo* synthesis of viral particles (30). This may result in fewer viral particles and a lower magnitude of the T-cell response (20). However, the amount of inactivated virus did not limit the T-cell response, since pulsing of PBMC with a double amount of inactivated influenza virus did not result in increased T-cell responses (data not shown). Taken together, these findings suggest that differences in antigen processing or activation of different signaling pathways are more likely to affect the T-cell response than the amount of available antigens.

Similarly, differences in IL-2^+ T cells specific for pandemic

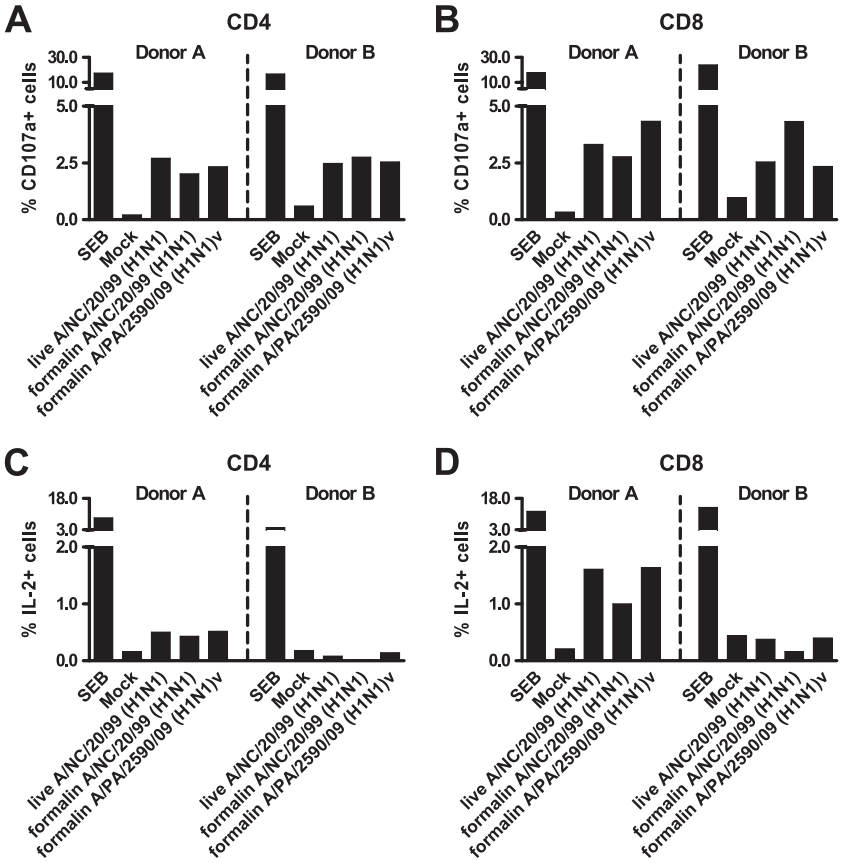

FIG. 7. Detection of T-cell responses against formalin-treated pandemic influenza A/Paris/2590/09 (H1N1)v virus demonstrates significant T-cell epitope antigenicity. (A) Percentages of $CD4^+$ $CD107a^+$ T cells; (B) percentages of $CD8^+$ $CD107a^+$ T cells; (C) percentages of $CD4^+$ $IL\text{-}2^+$ T cells; (D) percentages of $CD8^+$ $IL\text{-}2^+$ T cells. SEB, positive control using *S. aureus* enterotoxin B; mock, culture supernatant from uninfected cells.

influenza A(H1N1)v virus in PBMC from recently infected individuals may depend on the differentiation status of the T cells, since the PBMC from the two donors were isolated at different time points after the start of symptoms, 13 and 19 days (21, 32). Nevertheless, the significance of this finding is that the assay is also applicable to the pandemic A(H1N1)v virus.

In conclusion, we have shown that the standard repertoire of virological and immunological assays using pandemic influenza virus strains can be performed at BSL-2 when appropriate virus inactivation protocols are applied. Depending on the type of assay, different virus inactivation protocols may be preferred. Rapid antiviral susceptibility profiling with inactivated influenza viruses is possible using 1% Triton X-100 treatment, while 18 h of 0.02% formalin treatment and subsequent dialysis are more time-consuming but enable the implementation of a variety of both virological and immunological assays, including the detection of T-cell responses. Both inactivation procedures allow studies of highly pathogenic or pandemic influenza viruses without the requirement for a BSL-3 facility and greatly expand diagnostic and research possibilities. A limiting factor, however, remains the initial isolation and expansion of these viruses at BSL-3. Conclusively, the timely determination of the public health impact and the development of protective measures when a new influenza virus is introduced in humans are assisted by the implementation of our influenza virus inactivation protocols.

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Evaluation of different inactivation methods for high and low pathogenic avian influenza viruses in egg-fluids for antigen preparation

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In view of the emerging avian influenza (AI) viruses, it is important to study the susceptibility of AI viruses to inactivating agents for preparation of antigens and inactivated vaccines. The available information on susceptibility of both the high and low pathogenic AI viruses to different inactivating agents is inadequate and ambiguous. It has been shown that different subtypes of influenza viruses require different physical and chemical conditions for inactivation of infectivity. The present study was undertaken to evaluate the use of beta-propiolactone (BPL), formalin and ether for inactivation and its impact on antigenicity of AI viruses.

A total of nine high and low pathogenic AI viruses belonging to four influenza A subtypes were included in the study. The H5N1 viruses were from the clades 2.2, 2.3.2.1 and 2.3.4. The H9N2 virus included in the study was of the G1 genotype, while the H11N1 and H4N6 viruses were from the Eurasian lineage. The viruses were treated with BPL, formalin and with ether. The confirmation of virus inactivation was performed by two serial passages of inactivated viruses in embryonated chicken eggs.

The infectivity of all tested AI viruses was eliminated using 0.1% BPL and 0.1% formalin. Ether eliminated infectivity of all tested low pathogenic AI viruses; however, ether with 0.2% or 0.5% Tween-20 was required for inactivation of the highly pathogenic AI H5N1 viruses. Treatment with BPL, ether and formalin retained virus hemagglutination (HA) titers. Interestingly ether treatment resulted in significant rise in HA titers ($P < 0.05$) of all tested AI viruses. This data demonstrated the utility of BPL, formalin and ether for the inactivation of infectivity of AI viruses used in the study for the preparation of inactivated virus antigens for research and diagnosis of AI.

1. Introduction

Influenza type A viruses belong to the family Orthomyxoviridae and they are single-stranded, negative-sense RNA viruses, with segmented genome. Influenza viruses are enveloped and around 80–100 nm in diameter. On the basis of the antigenicity of the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) molecules, influenza A viruses are classified into 18 HA subtypes (H1–H18) and eleven NA subtypes (N1–N11) (Knipe and Howley, 2007; Tong et al., 2012, 2013). Wild birds, water-fowl, gulls, shore birds and bats are the natural host and reservoir for type A influenza viruses (Kawaoka et al., 1988; Tong et al., 2013).

Avian influenza (AI) viruses are classified into two forms based on the severity of the illness caused in the poultry. Low pathogenic avian influenza (LPAI) viruses cause no observable or mild clinical symptoms whereas highly pathogenic avian influenza (HPAI) viruses cause severe respiratory illness and death among infected chickens (Alexander, 2000). The outbreaks of HPAI viruses have been reported from Asia, Europe, Africa, America and Australia in the poultry (Oie update on avian influenza, 2014). India also reported outbreaks of HPAI H5N1 virus and the presence of LPAI viruses in poultry (Chakrabarti et al., 2009; Nagarajan et al., 2009; Pawar et al., 2012b). Human infections with AI viruses such as H5N1, H9N2, H7N1, H7N3, and H7N9 have been reported from Asia, Africa, and Europe (CDC information on avian influenza, 2014). This shows that AI viruses are imminent threats to public health as zoonotic pathogens.

The handling of HPAI viruses needs biosafety level-3+ (BSL-3+) facility which limits work on HPAI viruses (Jonges et al., 2010).

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Therefore, the virus inactivation is a necessary step for the preparation of the virus antigens and inactivated vaccines. The inactivated antigens could be used as reagents for various serological assays and diagnosis of AI.

A rational choice of inactivating agent and inactivation conditions is critical for preparation of inactivated vaccines (Budowsky et al., 1993). Beta-propiolactone (BPL), formalin, and ether have been conventionally used for preparation of inactivated vaccines (Chu, 1948; Francis, 1953; Goldstein and Tauraso, 1970; Swayne and Kapczynski, 2008). BPL is an alkylating agent which modifies the structure of nucleic acids inducing nicks in DNA, cross-linking between DNA and proteins as well as between DNA strands in the double helix (Perrin and Morgeaux, 1995). The mechanism with which viral inactivation is achieved by aldehydes is through the alkylation of amino and sulphydrilic groups of proteins and purine bases (De Benedictis et al., 2007). Formalin, which is an aldehyde derivative, cross-links the viral proteins inhibiting the viral replication (Swayne and Kapczynski, 2008). Ether treatment of influenza virus causes disruption of the viral envelope to smaller fragments (Choppin and Stoeckenius, 1964). Generally, less than 0.1% formalin and BPL is used for inactivated vaccine preparations (WHO, 2003).

The available information on susceptibility of AI viruses to different inactivating agents is inadequate and ambiguous (De Benedictis et al., 2007). It has been shown that different subtypes of influenza viruses require different physical and chemical conditions for inactivation of infectivity (Zou et al., 2013). The present study was undertaken to evaluate the use of BPL, formalin and ether for inactivation of high and low pathogenic AI viruses used in the present study and also to investigate its impact on the antigenicity of AI viruses.

2. Materials and methods

2.1. Viruses used

A total of nine representative high and low pathogenic AI viruses comprising four influenza A virus subtypes were used in the study. The tested viruses were isolated from different avian species (chickens, ducks, crows and wild aquatic bird) either during routine surveillance or during the AI outbreaks in different states of India. The viruses generated by reverse genetics (RG) method were also included. The purpose of using H5N1 viruses generated by RG method was that these viruses are used as seed strains for vaccine production and it is necessary to understand the inactivation properties of these viruses. The viruses used were: four isolates of HPAI H5N1 viruses A/chicken/Manipur/NIV9743/2007 (clade 2.2) [accession number: FJ719834], A/crow/Jamshedpur/NIVAN1117307/2011 (clade 2.3.2.1), A/chicken/Navapur/India/33487/2006 (clade 2.2) [accession number: DQ887061], A/crow/WB/NIVAN1117301/2011 (clade 2.3.2.1); two reverse genetically modified H5N1 viruses A/Anhui/2005/PR8-IBCDC-RG-6 (clade 2.3.4), A/India/NIV/2006/PR8-IBCDC-RG-7 (clade 2.2) and three LPAI viruses A/chicken/Pune/India/099321/2009 (H9N2)-G1 lineage, A/duck/WB/India/101006 (H4N6)-Eurasian lineage [accession number: JX310061], A/Aquatic bird/India/NIV-17095/2007 (H11N1)-Eurasian lineage [accession number: CY055175]. These viruses were propagated in 10-day-old embryonated chicken eggs and the virus titers were determined by the hemagglutination (HA) assay using 0.5% turkey red blood cells (TRBCs) (Pawar et al., 2012a; WHO, 2002).

2.2. Inactivation of viruses

2.2.1. Virus inactivation using BPL

BPL (Ferak, Berlin) was diluted in phosphate buffered saline (PBS) and mixed with virus suspension (10 ml) to attain the final concentrations of 0.05% and 0.1%. The preparation was mixed using vortex to form a homogenous mixture and was transferred to a fresh container (15 ml sterile tubes, Tarsons, India) and kept at 4 °C for 16 h in the refrigerator. The preparation was subsequently incubated at 37 °C for 2 h for hydrolysis of BPL (Goldstein and Tauraso, 1970; Jonges et al., 2010). After completion of treatment the virus aliquots were stored at −80 °C until further use.

2.2.2. Virus inactivation using formalin

One percent formalin was prepared in PBS using commercially available formaldehyde solution (Fisher Scientific, India) and mixed with virus suspension (10 ml) to obtain the final concentrations of 0.02%, 0.04% and 0.1%. The preparation was stirred, transferred to a fresh container and incubated at three different time points (16 h at 37 °C, 48 h at 37 °C, 1 week at 4 °C) (Furuya et al., 2010; Jonges et al., 2010; Takada et al., 2003). The virus suspension was stored at −80 °C until further use.

2.2.3. Virus inactivation using ether

Equal quantity of the virus suspension (5 ml) and anesthetic grade ether (5 ml) [Qualigens] were mixed in a 15 ml flat-bottom glass tube with a screw-cap. Adhesive tape was wrapped around the cap to reduce the loss of ether (Andrewes and Horstmann, 1949). The preparation in the tube was stirred for 30 min at room temperature using magnetic stirrer and magnetic bar. The preparation was then centrifuged at 3000 rpm (604 × *g*) for 15 min. The separated aqueous phase was collected in another tube, the residual ether was allowed to evaporate by keeping the preparation open for 1 h in class II B biosafety cabinet, aliquoted and stored at −80 °C until further use (Takada et al., 2003).

Those HPAI (H5N1) viruses which could not be inactivated by ether, were treated with a mixture of ether and Tween-20 (Hi-Media) (Fenters et al., 1970). Tween-20 (1%) was prepared in PBS and added to the virus and ether mixture to obtain the final concentrations 0.05%, 0.1%, 0.2% and 0.5% of Tween-20. The further procedure was similar as above.

2.2.4. Untreated virus controls

The untreated virus controls were subjected to similar experimental conditions as the treatment groups without the addition of inactivating agent. Such controls were included in each experiment.

2.3. Hemagglutination (HA) and hemagglutination inhibition (HI) assays

Titration of untreated and treated viruses was performed in 96-well-V-bottom micro-plates (Tarsons, India). Twofold serial dilutions of viruses were prepared in PBS and incubated with 50 μl 0.5% turkey RBCs (Pawar et al., 2012a). The HA titer was expressed as the reciprocal of the highest dilution giving complete hemagglutination. Allantoic fluids from the eggs inoculated with untreated and treated viruses were harvested and tested by HA assay (WHO, 2002). HI assay was performed to determine the reactivity of the inactivated viruses with antibodies. The HI assay was performed using influenza A antisera raised in fowl (*Gallus gallus*) against the viruses used in the study. The antisera were treated with the receptor destroying enzyme (RDE) (Denka Seiken, Japan) at 37 °C for 16 h and then incubated for 30 min at 56 °C to inactivate the (RDE) activity. The assay was performed as described in the WHO Manual on Animal influenza diagnosis and surveillance (WHO, 2002).

2.4. Confirmation of virus inactivation by inoculation in embryonated chicken eggs

Treated as well as untreated virus suspensions (200 μl per egg) were inoculated in 10-day-old embryonated chicken eggs to

determine the virus infectivity. The undiluted, 1:10 and 1:100 dilutions of the viruses were inoculated in the allantoic cavity. Embryos were observed for sickness and/or death after intervals of 24 h till 72 h post-infection. The dead eggs were immediately transferred at 4 °C. Allantoic fluids were harvested and tested by the HA assay to check the presence of the virus antigen (WHO, 2002; Zou et al., 2013). One more serial passage was performed following the above method for the confirmation of the virus infectivity.

2.5. Infectious virus titration in embryonated chicken eggs

Virus titration was performed to determine infectious virus titer of treated as well as untreated viruses. This was done by inoculating virus dilutions (10^{-1}–10^{-10}) in 10-day-old embryonated chicken eggs. Each virus dilution (200 μl per egg) was inoculated by the allantoic route in four eggs. The inoculated eggs were observed after intervals of 24 h till 72 h post-infection, kept at +4 °C for overnight. The allantoic fluids from the eggs were harvested and tested by the HA assay. Allantoic fluids showing the absence of titer were considered as negative for virus replication. 50% egg infectious dose (EID_{50}) titers were calculated using the Reed and Muench method (Reed and Muench, 1938).

2.6. Immunogenecity of inactivated viruses in BALB/c mice

The inactivated virus suspension (50 μl) was mixed with the Freund's complete adjuvant for the first dose and was inoculated in 6–8-week-old male BALB/c mice, in the intra-peritoneal cavity. Freund's incomplete adjuvant was used for the second dose. Mice were bled after 10 days post-inoculation. Serum was separated, treated with RDE (WHO, 2002) and tested by HI assay for determination of antibody titers using inactivated virus antigens. All viruses inactivated using BPL were used for immunization. AI H9N2, H11N1, H4N6 and two strains of H5N1 RG viruses inactivated by 0.01% formalin and ether were used for immunization.

2.7. Biosafety control measures

The LPAI viruses were handled in class II A2 biosafety cabinet (BSC) in the BSL-2 laboratory and HPAI viruses were handled in class II B2 BSC in BSL-3+ laboratory.

2.8. Statistical analysis

The difference between HA titers of untreated and treated viruses were analyzed by the Mann–Whitney test using the Statistical Package for the Social Sciences (SPSS) (IBM, USA).

3. Results

The infectivity of all the tested viruses was eliminated using 0.1% BPL, 0.04 and 0.1% formalin with 16 h incubation at 37 °C and 1-week incubation at 4 °C; 0.02, 0.04 and 0.1% formalin with 48 h incubation at 37 °C. 0.05% BPL did not inactivate two LPAI viruses (Table 1). Ether inactivated all LPAI viruses including H5N1 viruses generated by the reverse genetics method. The infectivity of three HPAI H5N1 viruses namely A/crow/Jamshedpur/NIVAN1117307/2011, A/chicken/Navapur/India/33487/2006 and A/crow/WB/NIVAN1117301/2011 could not be completely eliminated with ether. However, there was significant decrease ($P < 0.05$) in infectious virus titers (EID_{50}) as compared to the untreated viruses, which showed that all tested AI viruses were sensitive to ether treatment (Table 2). For HPAI H5N1 viruses, the virus inactivation by ether was performed as described by Fenters et al. (1970), in which two parts of ether and one part of virus suspension were mixed and incubated for an increased time period (overnight), but the above three HPAI H5N1 viruses could not be inactivated. All HPAI H5N1 viruses were inactivated when 0.2 or 0.5% Tween-20 was mixed with ether (Table 2).

The HA titers of the viruses treated with BPL, formalin and ether were determined before and after the treatment. There was no significant drop ($P > 0.05$) in the HA titers of the viruses after treatment with BPL, ether, and formalin (Table 3). Interestingly, ether treatment significantly increased HA titers ($P < 0.05$) of all AI viruses (Table 2). The inactivated viruses were immunogenic. The antibody titers against these viruses were in the range of 20–320, as determined by the HI assay.

4. Discussion

As per the WHO recommendations, concentration of BPL for inactivation shall not exceed 0.1% (WHO, 2003). In the present study, 0.1% BPL treatment inactivated both HPAI and LPAI viruses in allantoic fluids and the inactivated virus retained the HA activity. 0.04% formalin at 37 °C for 48 h and 0.1% formalin inactivated all tested AI viruses. 0.02% formalin did not inactivate four AI viruses. It has been shown that formalin at low concentrations, such as 0.04% and 0.1%, inactivated AI H5N2, H5N9 and H9N2 viruses after 16 h at 37 °C, retaining their HA titers (King, 1991). Three different dilutions of formalin (0.06%, 0.12% and 0.24%) at different incubation times (6, 12, 18 and 24 h) against the H7N3 subtype have been tested. After 6 h, AI virus was inactivated by 0.24% formalin. A time-span of 12 h was necessary to inactivate AI viruses at all the tested concentrations of formalin (Muhmmad et al., 2001). It has been shown that AI H5N1 virus was inactivated using 0.2, 0.4

Table 1
Inactivation of avian influenza viruses using different concentrations of BPL and formalin.

Sr. no.	Virus strain/subtype	BPL (%)		Formalin (%)								
				16 h at 37 °C			48 h at 37 °C			1-week at 4 °C		
		0.05	0.1	0.02	0.04	0.1	0.02	0.04	0.1	0.02	0.04	0.1
1	A/chicken/Pune/India/099321/2009 (H9N2)	+	+	+	+	+	+	+	+	–	+	+
2	A/Aquatic bird/India/NIV-17095/2007 (H11N1)	–	+	+	+	+	+	+	+	–	+	+
3	A/duck/WB/India/NIV-101006/2009 (H4N6)	+	+	–	+	+	+	+	+	+	+	+
4	A/Anhui/2008/PR 8-IBCDC-RG-6 (H5N1)	–	+	–	+	+	+	+	+	+	+	+
5	A/India/NIV/2006/PR-8-IBCDC-RG-7 (H5N1)	+	+	–	+	+	+	+	+	–	+	+
6	A/chicken/Manipur/NIV9743/2007 (H5N1)	+	+	+	+	+	+	+	+	+	+	+
7	A/crow/Jamshedpur/NIVAN 1117307/2011 (H5N1)	+	+	+	+	+	+	+	+	+	+	+
8	A/chicken/Navapur/NIV33487/2006 (H5N1)	+	+	+	+	+	+	+	+	+	+	+
9	A/crow/WB/NIVAN 1117301/2011 (H5N1)	+	+	–	+	+	+	+	+	–	+	+

+: complete inactivation: absence of virus replication, in embryonated chicken eggs.
–: no inactivation: virus replication in embryonated chicken eggs.

Table 2
Avian influenza virus inactivation using only ether and ether + Tween-20.

Sr. no.	Virus strains/subtypes	Ether + virus (1:1)		Ether + virus	Ether + virus + Tween-20				HA titers	
		EID_{50} titers			Tween-20 conc.					
		Untreated virus	Treated virus		0.05%	0.1%	0.2%	0.5%	Untreated virus	Treated virus
1	A/chicken/Pune/India/099321/2009 (H9N2)	$10^{9.33}$	No titer	+	ND	ND	ND	ND	512	512
2	A/Aquatic bird/India/NIV-17095/2007 (H11N1)	$10^{8.5}$	No titer	+	ND	ND	ND	ND	1024	>2048
3	A/duck/WB/India/NIV-101006/2009 (H4N6)	$10^{7.5}$	No titer	+	ND	ND	ND	ND	128	>2048
4	A/Anhui/2008/PR 8-IBCDC-RG-6 (H5N1)	$10^{6.29}$	No titer	+	ND	ND	ND	ND	256	384
5	A/India/NIV/2006/PR-8-IBCDC-RG-7 (H5N1)	$10^{6.5}$	No titer	+	ND	ND	ND	ND	512	1024
6	A/chicken/Manipur/NIV9743/2007 (H5N1)	$10^{8.52}$	No titer	+	ND	ND	ND	ND	48	2048
7	A/crow/Jamshedpur/NIVAN 1117307/2011 (H5N1)	$10^{8.33}$	$10^{1.33}$ *	–	–	–	+	+	16	2048
8	A/chicken/Navapur/NIV33487/2006 (H5N1)	$10^{7.33}$	$10^{1.21}$ *	–	–	–	+	+	256	2048
9	A/crow/WB/NIVAN 1117301/2011 (H5N1)	$10^{7.5}$	$10^{2.47}$ *	–	–	–	+	+	512	>4096

+: complete inactivation: absence of virus replication, in embryonated chicken eggs.
–: not inactivated: virus replication in embryonated chicken eggs.
ND – Not done.
* ≥5 log difference between the untreated and ether treated viruses ($P < 0.05$).

Table 3
HA titers of avian influenza viruses before and after treatment with different inactivating agents.

Sr. no	Virus strain/subtype	HA titer before treatment (HA units)	HA titer after treatment (HA units)														
			BPL (%)			Formalin (%) (16 h at 37 °C)				Formalin (%) (48 h at 37 °C)				Formalin (%) (1 week at 4 °C)			
			0.05	0.1	UT	0.02	0.04	0.1	UT	0.02	0.04	0.1	UT	0.02	0.04	0.1	UT
1	A/chicken/Pune/India/099321/2009 (H9N2)	1024	1024	1024	1024	512	512	256	512	1024	256	256	512	512	512	512	512
2	A/Aquatic bird/India/NIV-17095/2007 (H11N1)	1536	1024	1024	1536	1024	1024	1024	1024	1024	1024	512	1024	512	256	192	1024
3	A/duck/WB/India/NIV-101006/2009 (H4N6)	1024	512	512	1024	768	512	256	1024	256	256	128	512	512	512	64	1024
4	A/Anhui/2008/PR 8-IBCDC-RG-6 (H5N1)	1024	512	512	768	1024	768	512	512	512	256	<2	512	1024	512	384	1024
5	A/India/NIV/2006/PR-8-IBCDC-RG-7 (H5N1)	1024	2048	2048	2048	2048	2048	1024	1024	2048	2048	1024	1024	2048	1024	1024	768
6	A/chicken/Manipur/NIV9743/2007 (H5N1)	256	256	256	256	128	128	128	64	128	128	128	64	128	128	128	128
7	A/crow/Jamshedpur/NIVAN 1117307/2011 (H5N1)	512	256	256	256	256	256	256	128	256	256	256	128	256	256	256	256
8	A/chicken/Navapur/7972/2006 (H5N1)	512	256	256	256	128	128	128	512	128	128	128	512	128	32	16	128
9	A/crow/WB/NIVAN 1117301/2011 (H5N1)	512	512	512	512	512	512	512	512	512	512	512	512	512	512	512	512

Note: There was no significant drop ($P > 0.05$) in the HA titers of the viruses after treatment with BPL, ether, and formalin.

and 0.6% formalin, after 15 min treatment (Shahid et al., 2009). The higher concentrations of formalin reduced the inactivation time for AI viruses, but were found to alter the HA titers adversely.

The LPAI viruses including reverse genetically modified H5N1 LPAI viruses were inactivated using equal volume of ether and virus suspension. On the other hand, HPAI H5N1 viruses were not completely inactivated despite using double volume of ether and prolonged virus incubation period with ether (Fenters et al., 1970). However, there was a significant decrease ($\geq$5 logs, $P < 0.05$) in infectious titers of treated viruses as compared to the untreated viruses, which showed that all tested AI viruses were sensitive to ether. All HPAI H5N1 viruses were inactivated when Tween-20 was added in ether. This could probably be due to the possibility of Tween-20 making lipid content of the HPAI virus envelopes accessible for the action of ether. Whether this could be due to the probable differences in the composition of lipid-containing envelopes of HPAI and LPAI viruses, needs further investigation. Andrewes and Horstmann (1949) reported a decrease in the titer for influenza A, influenza B and New Castle disease virus after ether treatment. However, studies showing a rise in the HA titer after treatment with ether also have been reported (Monto and Maassab, 1981; Kendal and Cate, 1983). In the present study, there was significant rise in the HA titers of most of the AI virus isolates after ether treatment.

It has been reported that the inactivating agents cause modification of viral components responsible for antigenicity and immunogenicity (Budowsky et al., 1993). In the present study, the inactivated viruses were immunogenic in BALB/c mice.

The present study suggests that different influenza virus subtypes/strains might react differently to a particular inactivating agent and procedure. Therefore conclusions drawn from inactivation experiments with one virus strain cannot be generalized for the other virus strains. Furthermore, inactivation kinetics may be needed for each strain. In view of the biosafety, virus inactivation is necessary for using them for various purposes. The confirmation of virus inactivation is necessary. The current vaccine production strategies for HPAI viruses involve use of reverse genetically modified viruses. The present study showed that the reverse genetically modified H5N1 LPAI viruses could be inactivated using BPL, formalin and ether suggesting the utility of these inactivating agents for preparation of inactivated vaccines.

The present study has the following limitations; during this study, volumes of virus with inactivating agents ranged from 5 to 10 ml. However, standardization of the virus inactivation using large volumes of virus stocks would be required. The objective of this study was to evaluate the inactivation properties of the AI viruses in allantoic fluids, as allantoic fluids from the inoculated eggs are first inactivated and then subjected for further downstream processing in vaccine production (WHO, 2003). Since only egg-grown viruses have been used in this study; AI viruses grown in the other host system such as cell culture need further evaluation. The possibility of laboratory-to-laboratory variations due to the virus strain, source of the virus and reagents necessitate the optimization of concentrations of inactivating agents in each laboratory.

In conclusion, the present study demonstrated utility of BPL, formalin and ether for inactivation of infectivity of low and high pathogenic AI viruses used in the study.

Conflict of interest

We do not have any conflict of interests.

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Inactivation of *Influenza A virus*, *Adenovirus*, and *Cytomegalovirus* with PAXgene Tissue Fixative and Formalin

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Formalin fixation is known to inactivate most viruses in a vaccine production context, but nothing is published about virus activity in tissues treated with alternative, non-crosslinking fixatives. We used a model assay based on cell culture to test formalin and PAXgene Tissue fixative for their virus-inactivating abilities. MDCK, A549, and MRC-5 cells were infected with *Influenza A* virus, *Adenovirus*, and *Cytomegalovirus*, respectively. When 75% of the cells showed a cytopathic effect (CPE), the cells were harvested and incubated for 15 min, or 1, 3, 6, or 24 hours, with PBS (positive control), 4% formalin, or PAXgene Tissue Fix. The cells were disrupted and the released virus was used to infect fresh MDCK, A549, and MRC-5 cells cultured on cover slips in 24-well plates. The viral cultures were monitored for CPE and by immunocytochemistry (ICC) to record viral replication and infectivity. Inactivation of *Adenovirus* by formalin occurred after 3 h, while *Influenza A* virus as well as *Cytomegalovirus* were inactivated by formalin after 15 min. All three virus strains were inactivated by PAXgene Tissue fixative after 15 min. We conclude that PAXgene Tissue fixative is at least as effective as formalin in inactivating infectivity of *Influenza A virus*, *Adenovirus*, and *Cytomegalovirus*.

Introduction

Manipulating potentially virus-infected human tissue is an occupational hazard pathologists and technicians face on a daily basis. Despite many personal safety precautions such as protective clothing, masks, glasses, and directional air, the risk of viral infection is ever present. Although infection due to seasonal and nonpandemic pathogens are reported amongst nurses and physicians,[1,2] the rise of pandemic viruses such as severe acute respiratory syndrome (SARS) and avian influenza also bring potential biohazards inside pathology departments and morgues.

Not much is known about the stability of viruses in tissue or cytological specimens, but *Influenza A* virus has been found to be infective on smooth surfaces and bank notes for several hours. The duration of infectivity is prolonged up to 24 h when the virus is embedded in protein, such as dried mucus or culture medium.[3] This indicates that virus infectivity is highest when the virus is stored in the host environment. As long as tissue is not chemically fixed, virus infection can occur by exposure to aerosols (e.g., opening a tissue container) or by percutaneous exposure (e.g., scalpel stick accidents during grossing). In the field of vaccine preparation, formalin is widely used to inactivate viruses, although accidents caused by incomplete inactivation have been previously reported.[4] As soon as tissue is thoroughly fixed in formalin, most viruses are fully inactivated.[5] However, tissue fixation takes several hours, depending on the size of the specimen. The average penetration rate of formalin into tissue is 2 mm/h; large organs or specimens can take up to 24 h or longer to be completely fixed. Some viruses are very resilient and are only inactivated after long exposure to glutaraldehyde,[6] indicating that tissue fixation in formalin may not be sufficient to assure a safe working environment for pathologists.

PAXgene Tissue fixation reagent (PAXgene) is a novel, commercially available fixative (provided as ready-to-use solution) that is based on an alcohol/acid mix which results in tissue morphology and antigenicity comparable to that of formalin fixation. The major advance is that macromolecules are preserved in a more native state compared to macromolecules

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derived from formalin-fixed tissue. In proteomic and genomic assays, proteins and nucleic acids derived from PAXgene-fixed, paraffin-embedded (PFPE) tissues react as those derived from fast frozen tissues.[7–10] Recently, Cadoret et al.[11] showed that DNA isolated from PAXgene-fixed tissue of Atlantic salmon gills could be used to detect *Neoparamoeba perurans* DNA by polymerase chain reaction (PCR). Also, conventional histological staining and immunohistochemistry (IHC) could be performed on PAXgene-fixed tissue to visualize the amoeboid parasite in salmon gills.

Although alcohol can be used to decontaminate virus-contaminated surfaces,[12] it is not known if inactivation by alcohol-based tissue fixatives occurs. Since PAXgene fixation leaves macromolecules in their native form,[7–10] it could be that virus could become re-activated upon rehydration. Formalin can be used to inactivate viruses for vaccine production,[4] and recently Kading et al. described inactivation of Rift Valley fever virus during paraformaldehyde fixation of mosquito specimens.[13] To provide a standardized method to monitor virus inactivation, we chose to use this virus cell culture approach to find out whether the novel fixative would inactivate viruses and if the viruses would remain inactive upon rehydration.

We used *Influenza A* virus (RNA virus, *Orthomyxovirus, Influenzavirus A*) and *Adenovirus* (DNA virus, *Adenoviridae, Human adenovirus type 2*) as model viruses in the cell culture virus inactivation assays because these viruses can be transmitted in aerosols that may form during tissue handling,[14,15] and they are known to remain active for hours to days outside the host environment.[3] Human cytomegalovirus (DNA virus, *Herpesvirales*, *Herpesviridae*, *Cytomegalovirus*) was chosen because of its overall prevalence in nearly 60% of the population in the U.S.[16] It has the ability to infect a variety of human cell types such as fibroblasts, smooth muscle cells, macrophages, and cells of the bone marrow.[17] In addition, its ability to establish a lifelong latency after primary exposure in specific types of the myeloid lineage[16,18] makes it relevant for investigating safety matters in tissue processing.

Materials and Methods

Primary infection

A monolayer of MDCK cells (Madin-Darby Canine Kidney Cells, ATCC, CCL-34) in a 75 cm^2 culture flask was incubated with 0.01 multiplicity of infection (MOI) *Influenza A* virus (H3N2, reference strain A/Perth/16/09) containing EMEM medium (Eagle's Minimal Essential Medium, Lonza, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS), 100 IU/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine, 1.5 mg/mL sodium bicarbonate (Cambrex), 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Lonza) and nonessential amino acids (MP Biomedicals Europe, Illkirch, France) for 1 h at 37°C. The medium was removed, the cells were washed with PBS (phosphate buffered salt solution, generic) and new medium was added. After 2 days of incubation 75% CPE (cytopathic effect) was reached.

A monolayer of A549 cells (human lung carcinoma cells, LGC Promochem, United Kingdom, ATCC-CCL-185) in a filter top 162 cm^2 flask was incubated with 0.01 MOI *Adenovirus* type 2 (human clinical isolate from Erasmus MC diagnostic virology – 80°C archive) containing medium (Ham's

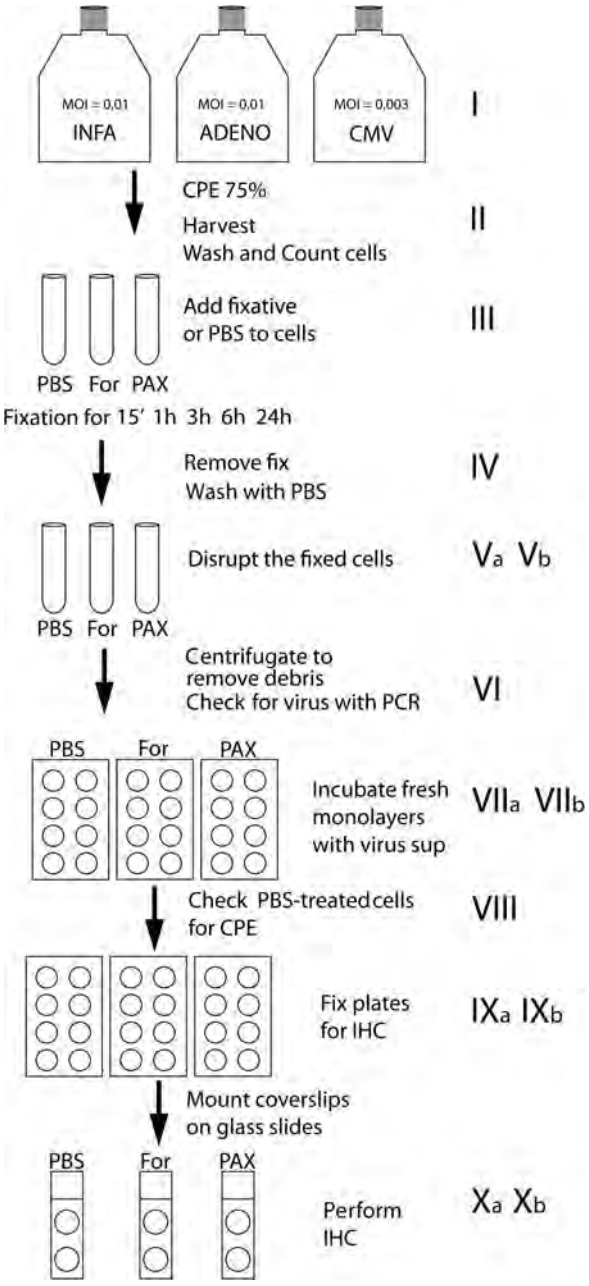

FIG. 1. Experimental workflow. **(I)** Primary culture of virus infected cells. **(II)** When 75% CPE was reached, the cells were harvested and counted. **(III)** Fixatives or PBS (positive control) were added to the virus infected cells. **(IV)** Fixative was removed and cells were washed with PBS. **(Va** and **Vb)** Two different protocols were used to disrupt the different cell types; see Materials and Methods for details. **(VI)** Cell debris was removed by centrifugation, and presence and quantity of virus in the remaining supernatant was detected using PCR. (**VIIa** and **VIIb)** Recipient cells were incubated (either in 24-well plates or in small flasks) with the virus containing supernatant; two different protocols were used depending on cell type; see Materials and Methods for details. **(VIII)** The PBS-treated recipient cells were monitored for CPE. (**IXa** and **IXb)** When 50%–75% CPE was observed, the cover slips were fixed in 80% acetone (influenza A and adenovirus) or in methanol/acetic acid (cytomegalovirus) for ICC. **(Xa and Xb)** ICC was performed to detect virus infected cells (two different visualization steps were used depending on cell type; see Materials and Methods for details).

F12 Medium, Lonza) for 1 h at 37°C, after which 40 mL of medium was added. After 4 days of culture 75% CPE was reached.

MRC-5 cells (human lung fibroblast cells, LGC Promochem, Germany, ATCC #CCL-171) were cultivated in $9 \times 175\,cm^2$ cell culture flasks with EMEM + GlutaMax (Gibco, Life Technologies, UK) supplemented with 10% FCS (Gibco) and 1% Penstrep (Gibco) at 37°C and 5% CO_2 until 60%–70% confluency. Infection was performed in 6 flasks with 2 mL suspensions of human cytomegalovirus AD 169 (HPA #622) (900 pfu/mL) per flask (0.003 MOI). Multiple negative controls without virus infection were performed for every cell line. Cells were continuously cultured undergoing one passage because of high density of cells until 75% CPE was observed (10–14 days) (Fig. 1,I).

Cell harvest, fixation, and disruption

After CPE was observed, cells were harvested using 0.05% Trypsin-EDTA (Gibco). Cells were collected, centrifuged for 5 min at $200 \times g$, washed in PBS and counted. Duplicate aliquots of $1 \cdot 10^5$ cells/mL were made, centrifuged at 200 *g* for 5 min in order to discard excess medium (Fig. 1,II), and fixed at room temperature with 10 mL of formalin (4% buffered formalin, Klinipath, The Netherlands), PAXgene (Paxgene Tissue System, PreAnalytix GmbH, Hombrechtikon, CH), or incubated with PBS (positive control) for 15 min, 1, 3, 6, and 24 h (Fig. 1,III). After fixation or incubation with PBS, cells were centrifuged ($200 \times g$ for 5 min) to remove the fixative/PBS (Fig. 1,IV). For influenza A and adenovirus (Fig. 1,Va), the pelleted cells were resuspended in 3 mL medium and subsequently disrupted by vortexing in glass bead tubes (UTM kit 3 mL, Copan, ITK Diagnostics BV, The Netherlands) to release intracellular virus. For CMV (Fig. 1,Vb), cells were disrupted in a Gentle Macs Dissociator (Miltenyi, Germany), a slightly different procedure. The suspension obtained was centrifuged ($500 \times g$ for 5 min) to remove cell debris (Fig. 1,VI).

Virus quantification and infection of recipient cells

After fixation and disruption of the virus positive cells, PCR was performed to prove presence of virus in the supernatant. Since the same amount of supernatant (derived from the same number of cells) was used for PCR, cycle threshold (Ct) values could be used to show that in all cases the same amount of virus was introduced into the recipient cell culture (Fig. 1, VI).

Influenza A-specific PCR performed on 200 µL of the supernatant confirmed the presence of this virus, with average Ct values of 15.46. Adenovirus-specific PCR[20] performed on 200 µL of the supernatant confirmed the presence of this virus, with average Ct values of 12.96. CMV DNA-specific PCR performed on 200 µL supernatant using QIAamp MinElute Virus Spin Kit for isolation of viral nucleic acids and artus CMV RG PCR Kit (both from Qiagen, Hilden, Germany), according to the protocol provided with the kit, confirmed the presence of CMV in all samples (average Ct 22.88), except the negative controls.

The Influenza A virus supernatant was transferred to fresh MDCK cells (1 mL/well) on cover slips in 24-well plates (in duplicate). Negative control wells were cultured in the presence of culture medium only. The adenovirus supernatant was added to recipient A549 cells on cover slips in 24-well plates (1 mL/well, duplicate wells). Medium without virus was added to the negative control wells (Fig. 1,VIIa). 1.5 mL of each CMV supernatant was used for reinfection of a MRC-5 monolayer with 60%–70% confluency in a $25\,cm^2$ flask (Fig. 1,VIIb). Cells were incubated until 50% CPE was observed in the positive control wells (Fig. 1,VIII).

Detection of virus activity with immunocytochemistry

Medium was removed from the wells, the wells were washed with PBS, and the cover slips were fixed in 80% acetone (influenza A and adenovirus, Fig. 1,IXa) or methanol/acetic acid (CMV, Fig. 1,IXb) for ICC. The influenza A ICC procedure was adapted from the one step fluorescence kit, without further need of optimization. After air drying overnight, the cover slips were incubated for 30 min at room temperature with monoclonal mouse anti-influenza A virus-FITC antibody (Imagen™ Influenza Virus A, REF K610511-2,Oxoid), diluted 1:3 in normal antibody diluent (NAD, ScyTek Laboratories, Logan, Utah).

The adenovirus ICC (peroxidase-DAB) procedure was also adapted from the one-step fluorescence kit. Background staining, predominantly found in dividing cells, was eliminated by diluting the primary antibody until negative control cells were free of any background. Adenovirus ICC was performed with a 1:160 diluted monoclonal mouse anti-adenovirus-FITC antibody (Imagen™ Adenovirus, REF K610011-2. Oxoid). Slides were repeatedly washed with PBS/0.05%Tween, and the primary antibody was detected by peroxidase-labeled EnVision (Chemvision, Dako, Denmark). The peroxidase label was visualized with DAB (Chemvision) and the cells were counterstained with haematoxylin (Fig. 1,Xa).

For CMV, contrary to the other assays, the cover slips were not air dried overnight but washed in PBS prior to the

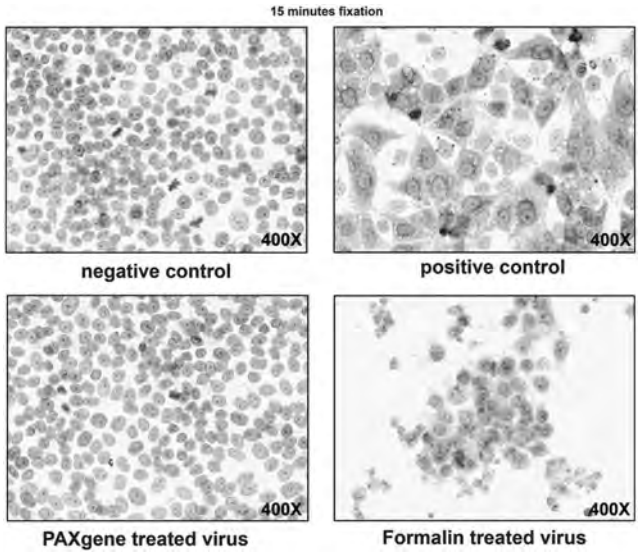

FIG. 2. Inactivation of Influenza A, The *upper panel* shows the negative and positive control MDCK cells stained for *Influenza A* virus. The *lower panel* shows staining of monolayers inoculated with virus that was fixed for 15 min with PAXgene and formalin. Original magnification 400X.

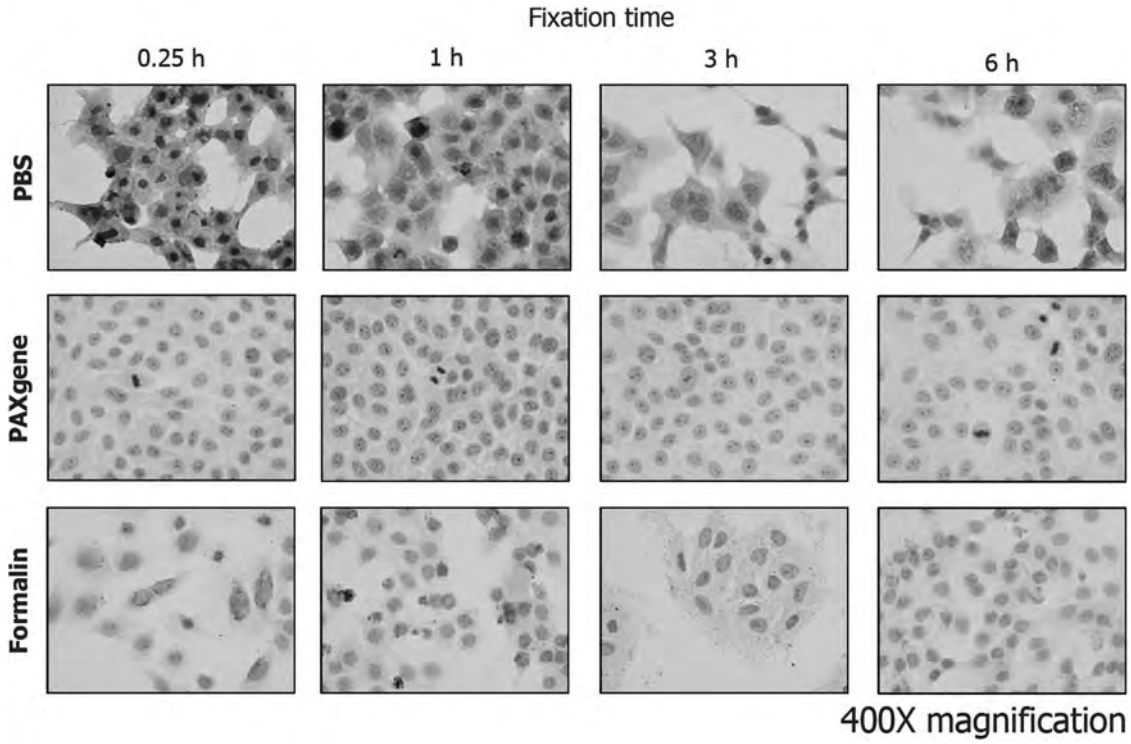

FIG. 3. Inactivation of Adenovirus. The *upper row* shows the positive control A549 cells stained for *Adenovirus*. The *middle row* shows staining of cells inoculated with PAXgene-fixed virus, and the *bottom row* shows staining of cells inoculated with formalin-fixed virus. Cells inoculated with virus that was fixed with formalin for 3 h show weak staining. After 6 h of formalin fixation, no staining is observed. Original magnification 400X.

ICC procedure. 1:50 diluted monoclonal mouse anti-CMV (M085401, Dako) was incubated for 60 min. After repeated washing, the second antibody EnVision Ready-To-Use (Dako) was incubated for 30 min, washed again, and staining was performed with AEC Substrate System (Ready-To-Use) (Dako, 3-amino-9-ethylcarbazole that forms a red end-product at the site of the target antigen). The reaction was stopped with water, and slides were counterstained with hematoxylin for 1 min. Slides were covered with Aquatex (Merck, Germany) (Fig. 1,Xb).

Results

Figure 2 shows formalin- and PAXgene-inactivated influenza A virus within 15 min of fixation. No viral activity was found after 1, 3, 6, and 24 h of fixation (data not shown). The positive control showed a strong signal, while the negative control showed no virus-specific signal, nor any background staining. The monolayer of MDCK cells to which formalin-fixed virus was applied was no longer intact and most cells looked damaged and deformed. The deformed cells were virus negative. However, while viable cells were also detected, there was no specific ICC signal indicating absence of active virus.

When A549 cells infected with *Adenovirus* were fixed with formalin, total inactivation was observed only at 6 and 24 h of fixation. PAXgene inactivated *Adenovirus* within 15 min (Fig. 3). Human CMV was inactivated by both formalin and PAXgene after 15 min incubation (Fig. 4). The MRC-5 monolayer showed a mild degree of cell damage after formalin-treated cell supernatant was applied. The amount of damaged cells seems to be dependent on the duration of formalin incubation. PBS control samples showed strong signals with immunohistological staining. No signals of active CMV by immunohistological staining were seen after PAXgene and formalin fixation from 15 min onwards (data not shown).

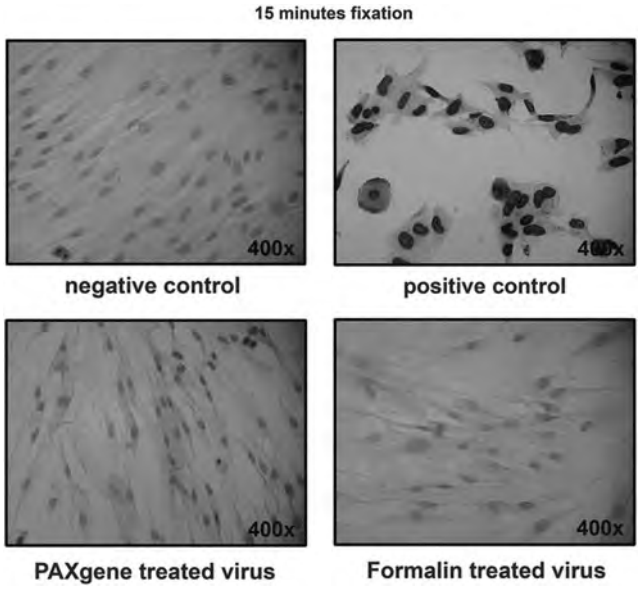

FIG. 4. Inactivation of Cytomegalovirus. The *upper panel* shows the negative and positive control MRC-5 cells stained for *Cytomegalovirus*. The *lower panel* shows staining of monolayers inoculated with virus that were fixed for 15 min with PAXgene and formalin. Original magnification 400X.

Discussion and Conclusion

The aim of this study was to test the virus inactivating properties of formalin and PAXgene in a virus culture assay. Since we found no literature with evidence or insight regarding inactivation of virus in tissue samples at the time these experiments were planned, we designed a cell culture model to investigate whether formalin and PAXgene would inactivate several viruses. This cell culture model does not resemble solid tissue fixation precisely, but it does allow us to gain insight into how different virus strains react to tissue fixatives while actively infecting a cell layer. Any extrapolation of this cell culture data to solid tissue fixation is based on known dynamics of solid tissue fixation and commonalities between cell fixation and solid tissue fixation. All model viruses remained infective for at least 24 h in a PBS solution at room temperature. Therefore, we may conclude that virus also remains infective for at least 24 h in unfixed tissue specimens. The penetration rate of formalin is around 2 mm per hour. Large clinical tissue specimens are almost always cut open to enhance fixation, but some parts of the specimen could remain too thick to allow thorough fixation overnight. Our observation that the time needed to inactivate viruses differs between type of virus and type of fixative, argues for vigilance when working with large solid organs, even after overnight gross fixation.

We have chosen ICC instead of PCR as the virus replication read out system. With PCR, differentiation between infective and inactivated virus is not possible. PCR was only used to show that virus was present in the medium used for re-infection of the recipient cells. As the PCR kits are not validated for use with cell culture material, calculating copy numbers could be misleading. Since we have taken equal amounts of material for PCR, the Ct values do serve as a measure for the amount of virus put into the recipient culture and confirm that equal amounts of virus were actually present in the supernatants.

To avoid false positive ICC results (i.e., detection of fixed virus-positive donor cells), the fixed cells and control cells were disrupted before the inoculation of fresh recipient cells. The culture was ended when the positive control wells showed 50%–75% CPE. Prolonged culture would have resulted in complete lysis of the positive control cells, which would eliminate positive ICC because of lack of cells. The damaged cell layers observed after treatment with formalin-fixed virus could be explained by a cytotoxic effect of residual formalin cross links in the virus homogenate. The number of affected cells seems to increase in correlation with formalin fixation/inactivation time, indicating that an increase of cross links or any chemical alteration in the cells may lead to loss of cells in the culture. Nevertheless, we did find viable, healthy looking cells in these wells; both these and the affected cells were virus negative. It is safe to conclude that, although a cytotoxic effect may have taken place, the virus was unable to infect the remaining cells and therefore was inactivated by formalin fixation.

Inactivation of *Adenovirus* by formalin seems to be a slower process than inactivation by PAXgene. This may be due to the fundamental difference between the two methods of fixation and the different virus structures. In the case of influenza A and CMV, *t*he virus envelope provides more molecules for formalin cross links. The non-enveloped adenovirus is more resilient to formalin inactivation as fewer surface molecules are available for cross linking.[21] Formalin forms cross links between proteins and nucleic acids, whereas alcohol-acetic acid-based fixatives like PAXgene precipitate macromolecules. Since the standard formalin solution contains only 4% formaldehyde, the number of molecules in the aqueous solution may be insufficient to form cross-links in small virus particles in a short period of time. The high alcohol concentration (not disclosed but at least 50% to 70%) in PAXgene may deliver an abundance of molecules to precipitate the virus proteins. More research in the field of virus-induced pathology is needed to explore the benefits of PAXgene for this specific subject. However, previously published articles show that histological techniques as well as proteomic and genomic techniques work with PAXgene-fixed tissues.[7–10] The possibility of detecting parasite DNA in DNA extracted from PFPE salmon gills[11] suggests that in future experiments, virus RNA, DNA, and proteins may be easily detected in PFPE tissues.

In conclusion, our experiments show that the viruses investigated, *Influenza A*, *Adenovirus,* and *Cytomegalovirus,* can be inactivated by PAXgene tissue fixative and formalin. When virus was rehydrated after PAXgene fixation (i.e., resuspension in medium), re-activation did not occur. Therefore, PAXgene is, at least regarding inactivation of these three viruses, a safe replacement of formalin in clinical pathology laboratories.

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Author Disclosure Statement

No competing financial interests exist.

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Herpes Simplex Virus Type-1-Induced Activation of Myeloid Dendritic Cells: The Roles of Virus Cell Interaction and Paracrine Type I IFN Secretion[1]

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Herpes Simplex Virus Type-1-Induced Activation of Myeloid Dendritic Cells: The Roles of Virus Cell Interaction and Paracrine Type I IFN Secretion[1]

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Adaptive cellular immunity is required to clear HSV-1 infection in the periphery. Myeloid dendritic cells (DCs) are the first professional Ag-presenting cell to encounter the virus after primary and secondary infection and thus the consequences of their infection are important in understanding the pathogenesis of the disease and the response to the virus. Following HSV-1 infection, both uninfected and infected human DCs acquire a more mature phenotype. In this study, we demonstrate that type I IFN secreted from myeloid DC mediates bystander activation of the uninfected DCs. Furthermore, we confirm that this IFN primes DCs for elevated IL-12 p40 and p70 secretion. However, secretion of IFN is not responsible for the acquisition of a mature phenotype by HSV-1-infected DC. Rather, virus binding to a receptor on the cell surface induces DC maturation directly, through activation of the NF-κB and p38 MAPK pathways. The binding of HSV glycoprotein D is critical to the acquisition of a mature phenotype and type I IFN secretion. The data therefore demonstrate that DCs can respond to HSV exposure directly through recognition of viral envelope structures. In the context of natural HSV infection, the coupling of viral entry to the activation of DC signaling pathways is likely to be counterbalanced by viral disruption of DC maturation. However, the parallel release of type I IFN may result in paracrine activation so that the DCs are nonetheless able to mount an adaptive immune response. ***The Journal of Immunology,*** **2004, 173: 4108–4119.**

Herpes simplex virus type-1 infection resolves in the periphery predominantly as a result of local $CD4^+$ and $CD8^+$ T cell responses (1). In common with most other T cell responses, this HSV response is believed to be initiated by dendritic cells (DCs)[3] from the injured site (2, 3). Several pieces of evidence point to a role for these cells in HSV-1 infections in vivo, including an inverse correlation between DC number and HSV-1-induced skin lesion severity (4). The Th1-skewed T cell response that is important in clearing HSV-1 from the periphery is dependent on IL-12 (5), which is known to be a cytokine secreted by DC during the early phase of Ag presentation (6).

In the local lesion sites, at the time of herpetic infection, the majority of professional APCs in the skin are epidermal Langerhans cells and dermal DC. Both of these cell types are known to be of myeloid origin (2). Plasmacytoid DCs (PDC) (7) are not present in the human skin under these conditions (8). Therefore, following cutaneous infection by HSV, the virus will initially interact with either Langerhans cells or dermal DC. Several recent studies have therefore focused on the interaction between HSV-1 and human myeloid DC (9–12). These studies, however, have predominantly focused on the negative effects of HSV on DC function, identifying disruption of several critical aspects of DC physiology, including their morphology, cytokine secretion, and T cell stimulation (9, 12). Nevertheless, HSV infection is generally accompanied by strong cellular as well as humoral immunological responses (1). Therefore, the question arises as to the nature of the "danger" signals induced by HSV, which trigger DC activation and migration and which are necessary for an adaptive immune response to develop.

A number of observations suggested that the infection of DC by HSV-1 did indeed induce some aspects of DC activation (12). Firstly, infected DCs release a soluble factor with a paradoxical effect, activating neighboring uninfected DC and priming them to secrete elevated levels of IL-12. Secondly, HSV-1-infected DC showed a more mature phenotype than uninfected DC, characterized by elevated MHC class II and CD86 expression. This upregulation was, however, unable to render the DC potent T cell stimulators, due to the ability of the virus to disrupt the DC maturation process.

In this study we dissect two pathways activated by HSV-1 infection of DC, which when unchecked by the products of viral transcription, can both stimulate DC maturation. The first pathway is mediated via the release of a soluble factor, identified in this study as type I IFN, which can act as paracrine activator of bystander uninfected cells. In addition, HSV-1 can induce DC activation directly, via binding of glycoprotein D (gD) to the DC surface and activation of NF-κB and p38 MAPK. This pathway, however, is inhibited by expression of one or more HSV-1 gene

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[3] Abbreviations used in this paper: DC, dendritic cell; PDC, plasmacytoid DC; MDDC, monocyte-derived DC; MOI, multiplicity of infection; MFI, mean fluorescence intensity; IFNAR, type I IFN-α receptor; VHS, virion host shutoff; HVEM, herpesvirus entry mediator; WT, wild type; gD, glycoprotein D.

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products. We suggest that the balance between activation via these two pathways and inhibition by viral products is a key factor in determining the immunological outcomes of HSV-1 infection.

Materials and Methods

Antibodies

The following mAbs were used: CD3 (supernatant mouse mAb UCH T1, IgG1, gift of Prof. P. Beverley, Edward Jenner Institute for Vaccine Research, Newbury, U.K.); CD2 (mouse mAb MAS 593, IgG2b; Harlan SeraLab, Loughborough, U.K.); CD19 (supernatant mouse mAb BU12, IgG1, gift of D. Hardie, Birmingham University, Birmingham, U.K.); HLA-DR (supernatant mouse mAb L243, IgG2a, gift of Prof. P. Beverley); CD14 (supernatant mouse mAb HB246, IgG2b, gift of Prof. P. Beverley); CD1a (supernatant mouse mAb NA1/34, IgG2a, gift of Prof. A. McMichael, John Radcliffe Hospital, Oxford, U.K.); CD86 (supernatant mouse mAb, BU63, IgG1, gift of D. Hardie); HLA-ABC (W6/32; Serotec, Oxford, U.K.); CD13-FITC (mouse mAb WM15, IgG1; DPC Biermann, Bad Nauheim, Germany); IgG1 isotype control Ab to *Aspergillus niger* glucose oxidase (mouse mAb DAK-GO1, IgG1; DAKOCytomation, Ely, U.K.); HSV-VP16 (supernatant mouse mAb LP1, IgG1, gift of Prof. T. Minson, Cambridge University, Cambridge, U.K.); neutralizing HSV-gD (supernatant mouse mAb LP2, IgG1, gift of Prof. T. Minson); non-neutralizing HSV-gD (supernatant mouse mAb AP7, IgG1, gift of Prof. T. Minson); human IFN-α (mouse mAb MMHA-11, IgG1; IDS, Boldon, U.K.), which is an Ab that recognizes largest number of IFN-α subtypes; IgG2a isotype control Ab anti-mouse MHC class I (TIB92, IgG2a; American Type Culture Collection, Manassas, VA); and type I IFN-α receptor (IFNAR) chain 2 (MMHAR-2, mouse IgG2a; EMD Biosciences, San Diego, CA).

Cell preparation

Monocyte-derived DC (MDDC) were prepared from 120 ml fresh whole blood from healthy volunteers. Mononuclear cells separated on lymphoprep (Nycomed Pharma, Oslo, Norway) (400 g, 30 min) were incubated in six-well tissue culture plates for 2 h at 37°C/5% CO_2 in RPMI 1640 (Invitrogen Life Technologies, Paisley, U.K.) supplemented with 10% FCS (PAA Laboratories, Linz, Austria), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (all from Clare Hall Laboratories, Cancer Research U.K., London, U.K.) as complete medium. Nonadherent cells were removed and the adherent cells were cultured in fresh medium with 100 ng/ml human recombinant GM-CSF and 50 ng/ml IL-4 (both gifts from Schering-Plough Research Institute, Kenilworth, NJ). After 4 days incubation, loosely adherent cells were collected and any remaining lymphocytes removed by incubation with CD3, CD2, and CD19 mAb, followed by anti-mouse IgG-coated immunomagnetic Dynabeads (Dynal Biotech, Merseyside, U.K.). The DCs were cultured for a further 3 days in complete medium with fresh GM-CSF and IL-4 at a concentration of 5×10^5 DC/ml and then used for infection (see below). In some experiments DCs were stimulated with LPS (100 ng/ml; Sigma-Aldrich, Poole, Dorset, U.K.) and/or recombinant human IFN-α2a (Roche, Basel, Switzerland) for 16 h.

Virus preparation

HSV-1 was derived from HSV-1 strain 17+ containing a GFP cassette, as previously described (12). The virus was propagated on confluent baby hampster kidney BHK cells and harvested by freeze-thaw. Cellular debris was removed by centrifugation at 3500 rpm for 45 min at 4°C. The supernatant was then immediately removed, filtered through a 5-μm followed by a 0.45-μm filter and spun at 12,000 rpm for 2 h at 4°C. The supernatant was discarded and the subsequent viral pellet was gently resuspended in 1 ml HBSS. The resuspended pellet was then sonicated three times for 10 s in a water bath sonicator and stored at −70°C. The virus stock used in this entire study had a titer of 1×10^9 PFU/ml as determined by plaque assay. This virus preparation is referred to as wild-type HSV (WT-HSV). The dilution of the virus during preparation makes it unlikely that another danger sensation was active at the time of initial HSV exposure. For UV inactivation, the HSV-1 preparation (10^7 PFU/ml) was exposed for 20 min to UV-C light source at a distance of 6 cm delivering 20 mW/cm^2. This virus preparation is referred to as UV-HSV. HSV-1 was formaldehyde inactivated by incubating HSV-1 (10^9 PFU/ml) with equal volume of 2% formaldehyde (BDH, Poole, U.K.). The virus stock was then diluted to 10^7 PFU/ml with RPMI 1640 and excess formaldehyde neutralized by adding 0.175% sodium bisulphite (Sigma-Aldrich) solution (final concentration). This virus is referred to FIX-HSV. Exposure of DC to the equivalent concentrations of fixative and neutralizing agent (formaldehyde and sodium bisulphite) did not alter DC surface phenotype, excluding indirect effects on the DC by the viral treatment protocol (data not shown). HSV-1 was incubated in equal volumes of virus (at 2×10^7 PFU/ml) and LP2 Ab ± 12.5 μg/ml heparin (C. P. Pharmaceuticals, Rexam, U.K.) at 37°C for 15 min and then added to DC directly. AP7 non-neutralizing anti-gD Ab was used as a control. All virus stocks tested negative for the presence of mycoplasma.

Infection of DCs

DCs were infected with HSV-1 as previously described (12) at a multiplicity of infection (MOI) of 1 (or equivalent for inactivated viruses) unless otherwise stated. For all virus infections, uninfected groups received RPMI 1640 treated in the same manner as the virus. Previous experiments had determined that mock viral preparations had equivalent effects on DC as RPMI 1640 (data not shown) (12).

Supernatant transfer studies

In some experiments, the effect of supernatant of infected cells on normal uninfected DCs was tested. Supernatants from uninfected and infected DCs were taken at 16 h incubation, centrifuged in a minifuge at $16{,}000 \times g$ for 30 min, and passed through a 0.2-μm pore-size filter (Sartorius, Goettingen, Germany) to remove any viral particles and cell debris, before adding to autologous DC. Direct assay of these supernatants found no remaining live virus, both by plaque assay and by GFP expression. Supernatant-treated DCs were then incubated for 16 h and examined for surface phenotype changes.

Flow cytometric analysis

DCs were stained for surface markers by incubation first with the relevant mAb (30 min, 4°C) followed by 1/25 diluted PE-conjugated goat anti-mouse Ig (Jackson ImmunoResearch Laboratories, West Grove, PA) (30 min, 4°C). Detection of opsonized HSV-1 particles was achieved by staining infected cells only with PE-conjugated goat anti-mouse Ig (Jackson ImmunoResearch Laboratories). For intracellular IFN-α staining DCs were cultured in complete medium supplemented with 2 μM monensin for a maximum of 12 h, fixed in 4% formaldehyde (10 min, 4°C) at a concentration of $\leq 10^6$ cells/ml. This was followed by a wash in HBSS supplemented with 2% goat serum (FACS buffer). All subsequent washes and incubations were conducted in FACS buffer supplemented with 0.5% saponin (Sigma-Aldrich). Nonspecific Ab binding was blocked with 10% goat serum solution (15 min, 4°C) before addition of the relevant primary mouse mAb (30 min, 4°C). This was followed by PE-conjugated goat anti-mouse Ig (30 min, 4°C). Staining of other intracellular Ags was conducted as previously mentioned in the absence of monensin, at the times previously detailed. The stained cells were examined by flow cytometry immediately, using a FACScan flow cytometer (BD Biosciences, Oxford, U.K.) and analyzed with WinMDI software. Results are expressed as median fluorescence intensity (MFI).

Cytokine measurement

Secreted type I IFN was measured using a bioassay as described (13). Secreted IL-12 was measured by ELISA: IL-12 p40 (R&D Systems, Abingdon, U.K.) and IL-12 p70 (eBiosciences, San Diego, CA).

Microscopy

DCs were analyzed by confocal microscopy Bio-Rad Confocal microscope (Hercules, CA). GFP fluorescence was recorded by a 488 nm excitation laser and detected in the fluorescence channel by a 522 ± 32 nm emission filter. The images were analyzed using Confocal Assistant and Adobe Photoshop software.

Cell viability

DC viability was assessed by MTT reduction assay as previously described (12).

Proliferation assays

T cell proliferation was assessed by culturing DC, either uninfected or infected for 16 h with allogeneic T cells (10^5/well) as previously described (12). Results are expressed as counts per minute.

Western blotting

DCs were incubated in complete medium containing 0.5% FCS v/v for at least 24 h before treatment. The levels of phospho-p38, total p38, and IκB-α were assayed as previously described (14), using rabbit polyclonal Abs to phospho-p38 (9211; Cell Signaling Technology, Frankfurt am Main, Germany), total p38 (9212; Cell Signaling Technology), and IκB-α

(sc-371; Santa Cruz Biotechnology, Santa Cruz, CA). Densitometry analysis of the blots was performed using GeneSnap software (Syngene, Frederick, MD). The ratios of the densities of the phospho-p38 to total-p38 were calculated to give an indication of the proportion of p38 phosphorylated in each condition.

Statistical analysis

Where appropriate, the means of paired groups were analyzed by a two-tailed Student's *t* test.

Results

Characterization of MDDC and HSV-1 infection

The MDDC cultures used in this study contained predominantly myeloid DC as shown by high CD1a, low CD14 expression and >99% expression of both CD11c and CD13 (Fig. 1*A*). HSV-1 infection of MDDC was previously shown to inhibit their Ag-presenting capacity (9, 12). However, using an HSV-1 strain engineered to express GFP, we observed that following infection, two markers of DC maturation, CD86, and HLA-DR, were elevated in both GFP^- and GFP^+ DC, relative to uninfected DC (Fig. 1*B*). GFP may not be expressed in all infected DC, thus underestimating infection efficiency. However, almost 100% of DC expressing GFP following infection also expressed the late HSV proteins VP16 (intracellular) and gD (extracellular) at high levels. There was no significant population of VP16 or gD expressing DC that did not express GFP, confirming that this transgene is indeed expressed in all infected DC in this system and can be used as a parameter for gating on the direct effects of HSV infection (Fig. 1, *C* and *D*).

Type I IFN secretion by MDDC

The elevated expression of CD86 and HLA-DR in the GFP^- DC population, suggested that uninfected DCs in these cultures were receiving a soluble maturation stimulus. Indeed, culturing autologous DC in the supernatants from HSV-infected DC cultures

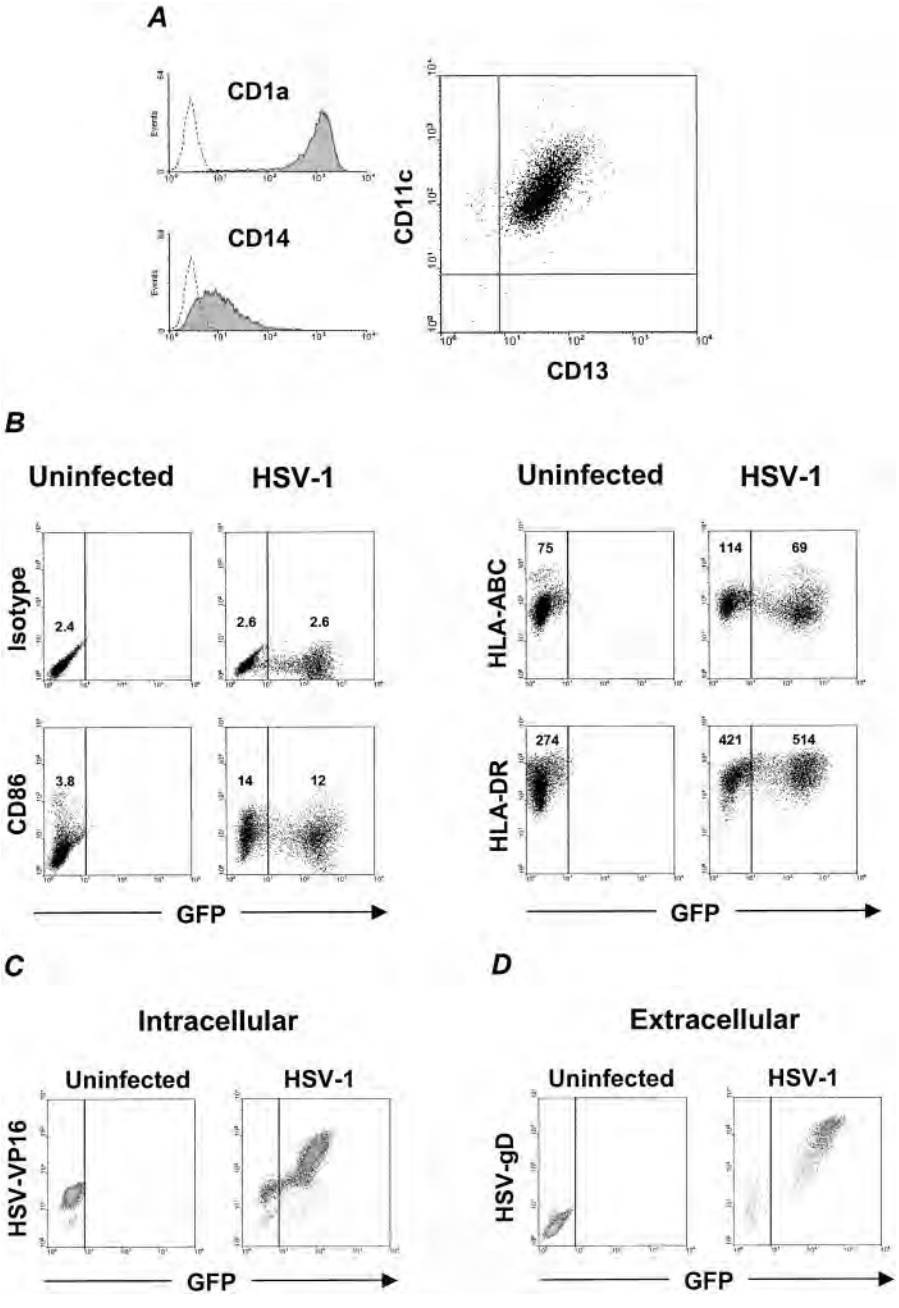

FIGURE 1. Characterization of MDDC infection by HSV-1. *A*, The expression of myeloid markers by the MDDC was assessed by flow cytometry. MDDC were infected with HSV-1 and incubated for 16 h. The expression of GFP transgene was assessed together with the expression of CD86 (*B*), HLA-DR (*B*), HLA-ABC (*B*), HSV-1 VP16 (*C*), and HSV-1 gD (*D*). Numbers in dot plot quadrants refer to MFI expression of Ab on *y*-axis. Representative of three independent experiments.

(supHSV-DC), but not supernatant from uninfected DC (supCON-DC), induced up-regulation of costimulatory and MHC class II molecules, albeit less than that observed with LPS stimulation (Fig. 2*A*). SupHSV-DC did not contain significant levels of the proinflammatory cytokines IL-1, IL-6, TNF-α, or IFN-γ (Y. McGrath, unpublished observations and data not shown). In contrast, HSV-1 infection induced the release of significant levels of biologically active type I IFN (Fig. 2*B*).

Despite the purity of the myeloid DC population used in this study (Fig. 1), human PDC are known to secrete large quantities of type I IFN in response to viral stimulation (7). Therefore, it was important to demonstrate that the type I IFN released following HSV-1 infection did not derive from small numbers of contaminating PDC. This was confirmed by intracellular staining for IFN-α, in which the increase in IFN staining was mediated by a shift in fluorescence of the entire DC population, not by a subset of IFN-secreting DC (Fig. 2, *C* and *D*). As the type I IFN secretion system responds to a well-described autocrine- and paracrine-positive feedback loop (15), DCs in these experiments were infected at a high MOI of 3. The resulting infection efficiency was near 90%, reducing to a minimum the number of uninfected DC, and ensuring that the stimulus for IFN secretion was coordinated and originated directly from the viral infection.

Type I IFN-mediated activation of DC

Analogous to the effects of supHSV-DC, recombinant IFN-α was a maturation stimulus for DC, up-regulating CD86 and HLA-DR molecules in a dose-dependent manner (Fig. 3*A*). Of note, in the concentration range of type I IFN secreted following HSV infection ($\sim$250 IU/ml), the maturation was submaximal relative to that of LPS, analogous to that seen when DCs were cultured in supHSV-DC (Fig. 2*A*).

We have previously shown that supHSV-DC primed DC for increased IL-12 p40 secretion following LPS stimulation (12) and this priming effect is also observed for the secretion of the functional heterodimer of this cytokine, IL-12 p70 (data not shown). To assess whether type I IFN could reproduce this effect, DCs were cultured in the presence of recombinant IFN-α and stimulated with LPS. Consistent with the type I IFN activity in supHSV-DC, IFN-α itself was not a stimulus for DC secretion of IL-12 p40 but could prime DC for increased LPS-mediated IL-12 p40 secretion (Fig. 3*B*). This synergistic action was also observed for IL-12 p70 secretion (Fig. 3*C*). Furthermore, IFN-α increased the IL-12 p70:p40 ratio (the proportion of functional heterodimer secreted) (Fig. 3*D*), suggesting a regulatory role for IFN-α also on the IL-12 p35 subunit.

Role of type I IFN in HSV-1-mediated DC maturation

To confirm that the type I IFN in the supHSV-DC was responsible for the maturation effects observed, we attempted to neutralize its activity in the supernatants by preventing binding of IFNAR. Culturing DCs in supHSV-DC in the presence of a neutralizing mAb to one of the two IFNAR chains (IFNAR2) abrogated the ability of the supernatant to up-regulate CD86 (Fig. 4*A*) and HLA-DR (data not shown), confirming that type I IFN secreted after infection was the major active component in supHSV-DC.

As shown in Fig. 1*B*, DCs infected with HSV-1 also displayed a more mature phenotype. To investigate whether this could be attributed to the autocrine activity of type I IFN, we infected DC in the presence of anti-IFNAR2 Abs. HSV-1 induced up-regulation of CD86 (Fig. 4*B*) and HLA-DR (data not shown) on DC was not abrogated by this treatment. Thus, we concluded that HSV-1 infection of DC resulted in a maturation process independent of the autocrine activity of type I IFN secreted.

In summary, Figs. 2, 3, and 4 show that following HSV-1 infection, DCs of myeloid origin can secrete biologically significant amounts of type I IFN to induce partial maturation of neighboring uninfected DCs but this is not required for the maturation observed in infected DC.

UV-inactivated HSV-1 infection of MDDC abolishes inhibition of WT-HSV and induces maturation

We investigated which step of the virus infection process resulted in DC maturation. This strategy also allowed us to identify the level at which HSV-1 exerts its main inhibitory effects on DC function. To remove the effects of de novo viral gene expression, we inactivated the virus using UV light. Intracellular staining for the HSV-1 tegument protein, VP16, 2 h postinfection confirmed that UV-inactivated HSV-1 (UV-HSV) was able to infect DC with equal efficiency to WT-HSV (Fig. 5*A*). Inactivation of the viral genome was confirmed by the absence of GFP expression following overnight culture (Fig. 5*B*). In contrast to WT-HSV, DC viability was not compromised following UV-HSV infection (Fig. 5*C*) and the strong defect in T cell stimulation observed with WT-HSV was also lost in the absence of HSV gene expression (Fig. 5*D*). Similarly, DC morphology was not altered following UV-HSV infection, and the DC showed increased dendrite formation in response to LPS, like uninfected cells. In contrast, WT-HSV-infected DC lost dendrites and became rounded up both in the presence and absence of LPS (Fig. 5*E*).

UV-HSV infection of DC resulted in similar changes in surface phenotype to those seen with WT-HSV, up-regulating CD86 (Fig. 6) and HLA-DR (data not shown) expression. However, in contrast to WT-HSV, LPS stimulation of UV-HSV infected DC resulted in further up-regulation of CD86 and MHC class I (Fig. 6).

In summary, Figs. 5 and 6 show that HSV-1 gene expression is responsible for the deleterious effects of HSV-1 infection on DC, rendering DC morphology and phenotype refractory to further stimulation by LPS stimulation, and also inducing cell death and preventing efficient T cell stimulation. However, the changes in phenotype also demonstrate that viral receptor binding and/or entry into DC is a maturation stimulus.

HSV interaction with DC surface up-regulates CD86 and activates p38 MAPK and NF-κB

To identify this activation event in more detail, we modified WT-HSV particles further. Formaldehyde fixation of HSV virions (FIX-HSV) rendered them unable to fuse with the cell membrane and therefore noninfective, as determined by the absence of VP16 staining 2 h postinfection (data not shown) and lack of GFP expression after overnight culture (Fig. 7*A*). However, the virus' ability to bind to the DC surface remained intact, as determined by flow cytometry by the presence of HSV-1 gD on the surface of DC 1 h after infection (Fig. 7*B*). DC exposed to FIX-HSV up-regulated the surface marker, CD86 (Fig. 7*B*) similarly to WT-HSV. Furthermore, like UV-HSV, FIX-HSV-infected DCs were not refractory to further LPS stimulation, again in contrast to the effects of WT-HSV (Fig. 7*B*).

HSV-1 infection has been shown to activate NF-κB and p38 MAPK previously (16, 17), but the role of early viral interaction with receptors on the cell surface in initiating this activation has not been documented. Therefore we assessed the ability of viral infection to induce the degradation of IκB-α, a cytosolic inhibitor of the NF-κB subunits. Western blotting showed that WT-HSV infection of DC induced significant IκB-α degradation within 30 min after infection (Fig. 8*A*). This is regarded as indicative of

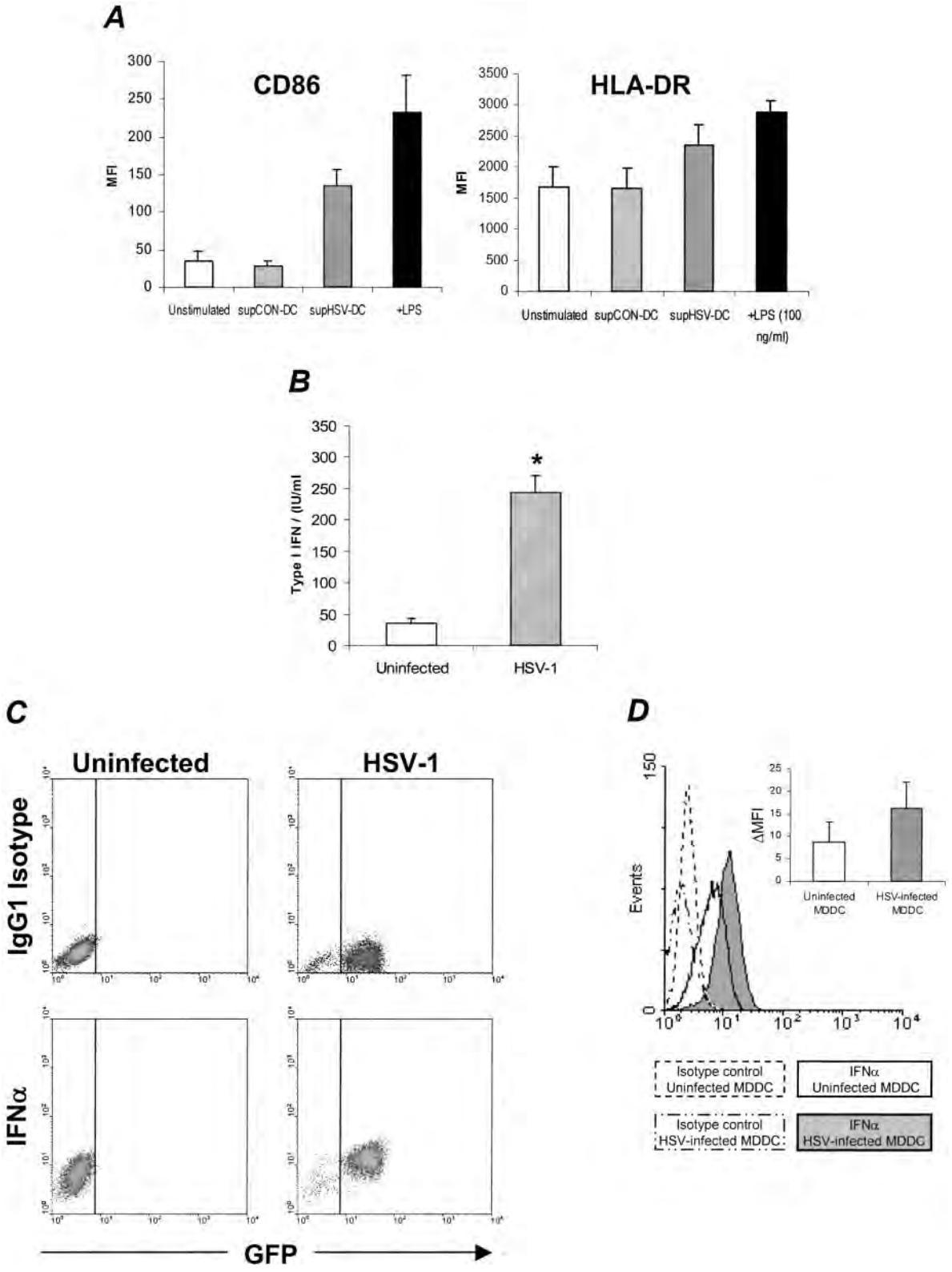

FIGURE 2. Type I IFN secreted by MDDC infected with HSV-1. *A*, Supernatants from uninfected and HSV-1 infected cultures in Fig. 1 (supCON-DC and supHSV-DC, respectively) were harvested and used to culture autologous DC for 16 h. Surface markers of DC maturation were assessed. The data are the mean of three independent experiments. *B*, Supernatants from uninfected and HSV-1 infected DC were harvested 16 h after infection and type I IFN secretion determined by an antiviral bioassay. The data are the mean of four independent experiments. Error bars represent SEM; $*$, $p < 0.01$ relative to uninfected DC. *C*, MDDC were infected with HSV-1 at MOI of 3 and incubated for 12 h in the presence of 2 μM monensin. Production of IFN-α was assessed by intracellular staining. Representative of three independent experiments. *D*, MDDC as in *C*, but displayed as frequency histogram. Representative of three independent experiments. Bar chart (*inset*) represents mean ΔMFI IFN-α staining of three independent experiments. Error bars represent SEM.

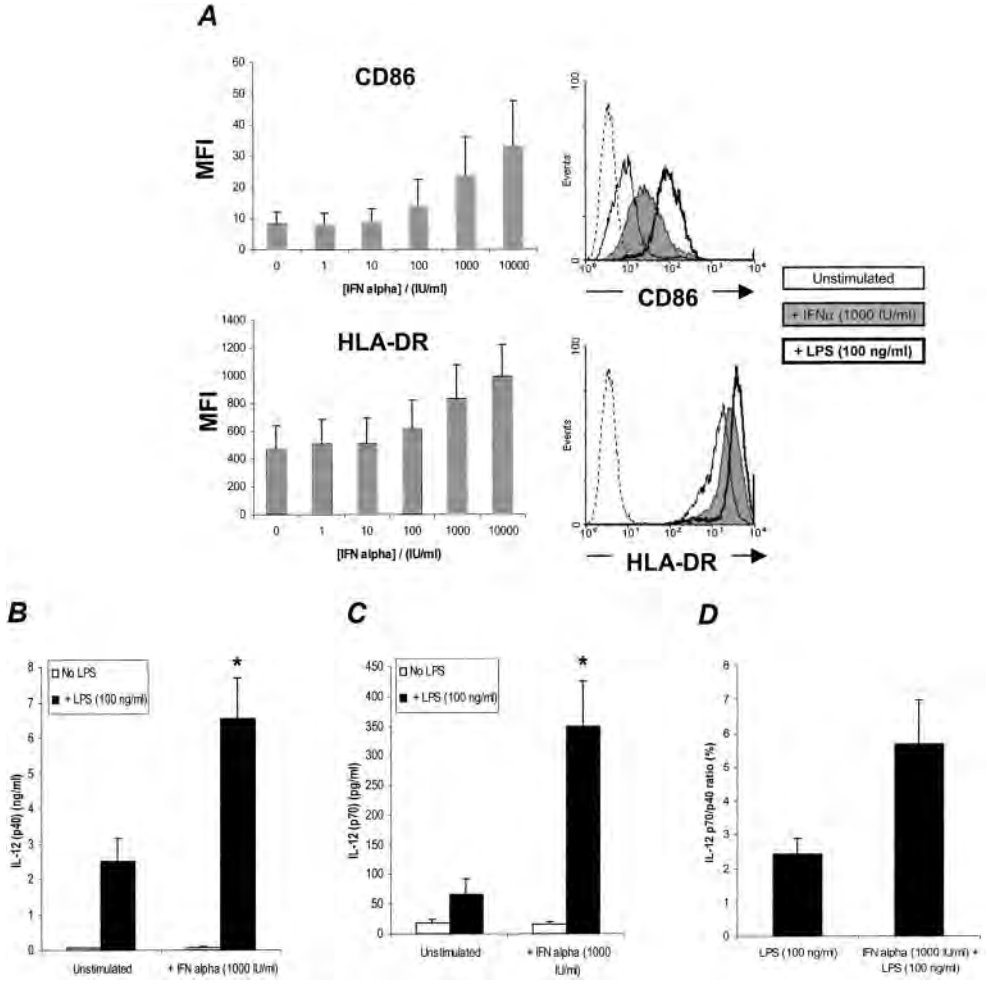

FIGURE 3. Type I IFN is responsible for maturation of bystander DC but not for infected DC. *A*, MDDC were cultured for 16 h in a range of concentrations of recombinant IFN-α (*left*) and changes in surface phenotype assessed. Data are mean MFI of three independent experiments. Error bars represent SEM. MDDC were cultured for 16 h in 1000 IU/ml IFN-α or 100 ng/ml LPS (*right*) and changes in surface phenotype were assessed. Representative of three independent experiments. *B*, MDDC were cultured in the presence or absence of recombinant 1000 IU/ml IFN-α $\pm$ 100 ng/ml LPS for 16 h. Supernatants were assayed for IL-12 p40 or IL-12 p70 (*C*) by ELISA. Results are mean of three independent experiments. Error bars represent SEM; $*$, $p < 0.01$ relative to LPS only stimulated DC. *D*, Bar chart of IL-12 p70:IL-12 p40 ratio calculated from bar charts *B* and *C*.

NF-κB activation, as loss of the IκB-α protein permits NF-κB transcription factor subunits to translocate to the nucleus and induce transcription of NF-κB controlled genes, such as CD86 (18). As the activation of the p38 MAPK pathway, which is another important modulator of DC maturation (19–21), is also induced in DC, the same membranes were also assayed for p38 phosphorylation. WT-HSV induced significant phosphorylation of p38 after infection (Fig. 8*A*). This was quantified relative to total p38 by densitometry (Fig. 8*B*). Interestingly, LPS mediated greater p38 activation than WT-HSV, correlating with the degree of maturation seen with LPS relative to the (submaximum) maturation observed following HSV-1 infection (Fig. 7*B*) (12).

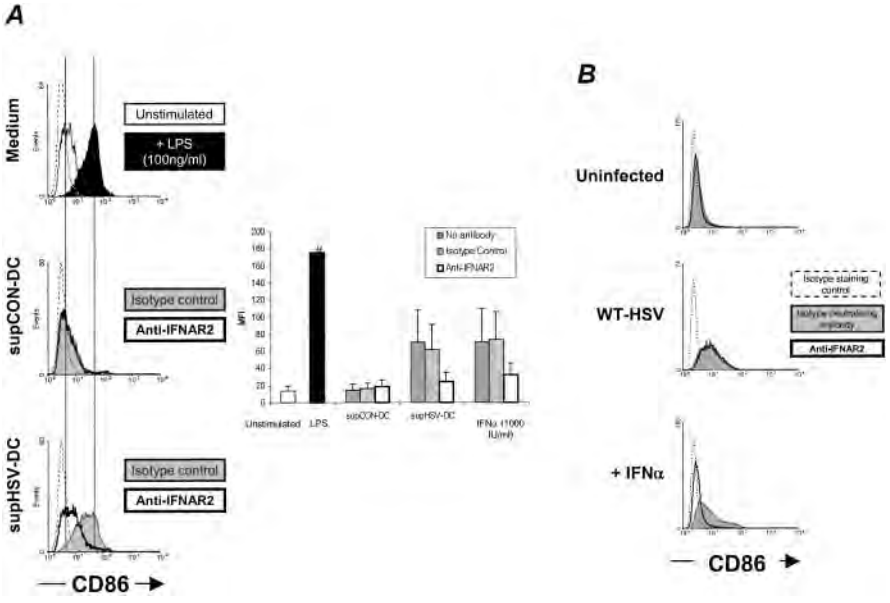

FIGURE 4. Culturing DCs in supHSV-DC in the presence of a neutralizing mAb to one of the two IFNAR chains. *A*, MDDC were cultured in supCON-DC, supHSV-DC or 1000 IU/ml IFN-α for 16 h in the presence of anti-IFNAR2 mAb or an isotype control and changes in surface phenotype were assessed. Representative experiment (*left*) and MFI (*right*) of three independent experiments are shown. Error bars represent SEM; #, SEM $\pm$ 100 MFI. *B*, DC were infected with HSV-1 at MOI of 1 or treated with 1000 IU/ml IFN-α in the presence of anti-IFNAR2 mAb or an isotype control Ab and expression of CD86 assessed. WT-HSV data gated on GFP$^+$ DC. Representative of three independent experiments.

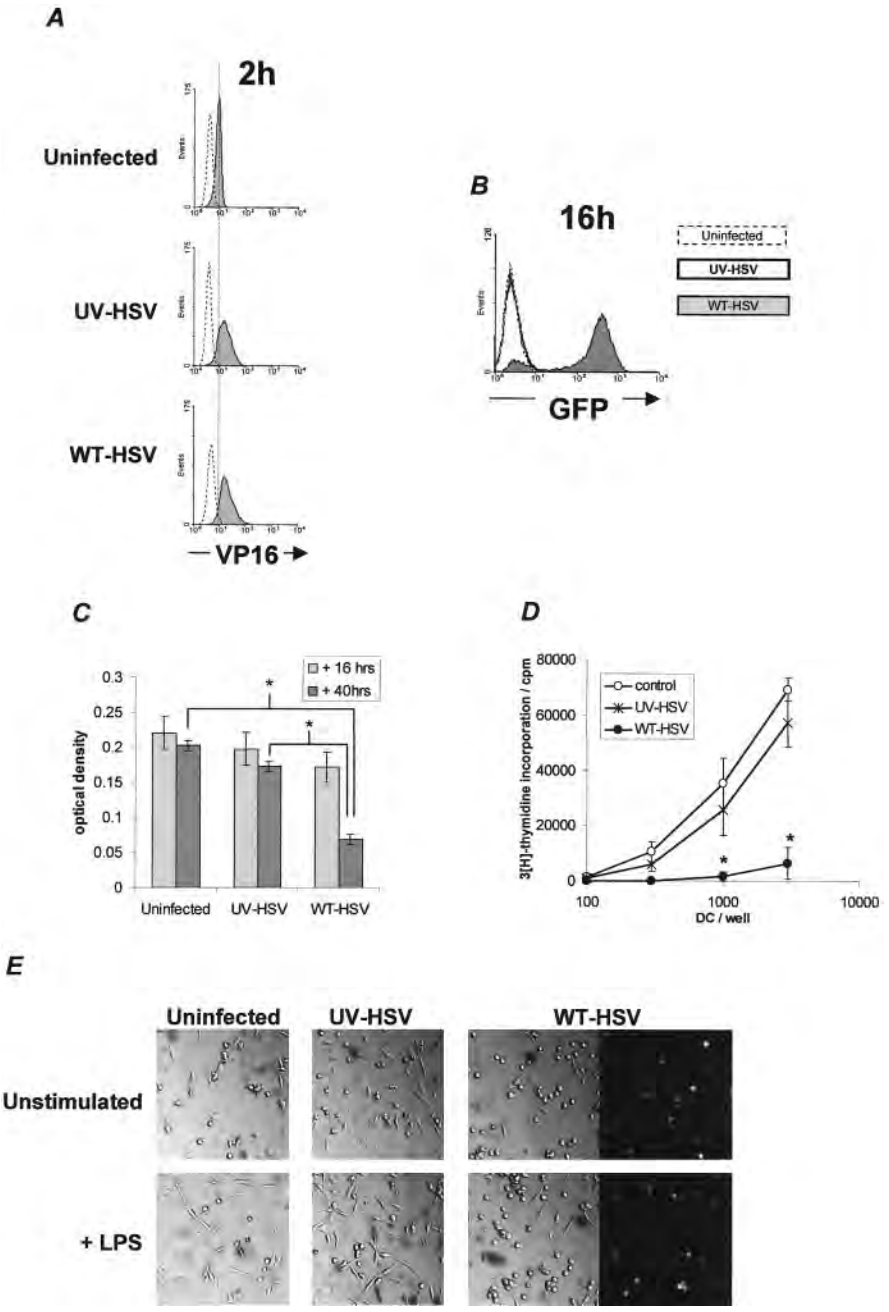

FIGURE 5. Effects of viral gene expression on HSV-1-mediated changes in DC viability, function, and morphology. *A*, DC were infected with UV-HSV or WT-HSV for 2 h and stained for intracellular HSV-1 VP16. Results are representative of three independent experiments. *B*, DCs infected with UV-HSV or WT-HSV were analyzed for GFP expression 16 h after infection. Representative of at least three independent experiments. *C*, Viability of DC infected with UV-HSV or WT-HSV was analyzed by MTT reduction assay 16 and 40 h after infection. Data shown as mean OD of three independent experiments. Error bars represent SEM. *D*, DC infected with UV-HSV or WT-HSV were used to stimulate allogeneic T cell proliferation. Data are mean [^{3}H]thymidine incorporation of three independent experiments; $*$, $p < 0.01$ between WT-HSV and either UV-HSV or uninfected DC. *E*, DC adherent to fibronectin-coated glass coverslips were infected with UV-HSV or WT-HSV in the presence or absence of 100 ng/ml LPS. Morphology of DC was assessed 8 h after infection. Phase contrast and GFP fluorescence displayed in separate panels for WT-HSV. Representative fields of at least three independent experiments.

Glycoprotein D neutralization of HSV-1 abolishes CD86 up-regulation and type I IFN secretion

HSV-1 entry into cells is mediated by glycoprotein B and glycoprotein C attachment to cell surface heparan sulfate, followed by gD-mediated entry via one of the entry receptors (22). Therefore, to determine the viral factors responsible for the up-regulation of CD86, we neutralized FIX-HSV and WT-HSV virions with a mAb to HSV-1 gD (LP2 clone) and observed that the stimulus required for the up-regulation of CD86 expression was abrogated (Fig. 9*A*). The effect on FIX-HSV confirmed that extracellular binding of gD to a cell surface receptor was a sufficient activating signal to induce partial DC maturation seen following HSV-1 infection. To exclude the possibility that the presence of Abs on the viral envelope could sterically hinder the interaction of viral ligands with the cell surface, HSV particles were also precoated with a non-neutralizing anti-gD mAb (AP7 clone) before addition to DC. As determined by GFP expression, this Ab did not prevent infection to any significant degree, in contrast to LP2 (Fig. 9*B*). Furthermore, staining for the presence of mouse IgG on the surface of DC 16 h after infection demonstrated that DC infected with AP7 coated HSV (i.e., the GFP$^+$ population), expressed mouse IgG on the cell surface. This is likely to have originated from non-neutralized viral envelope that fused with the DC membrane.

DC exposed to FIX-HSV coated in AP7 demonstrated an equally mature phenotype as virus treated with an isotype control Ab, in contrast to the neutralizing effect of LP2. Thus Fig. 9, *B* and *C* demonstrate that the ability of neutralizing anti-gD Abs to prevent DC maturation is likely to occur by preventing specific sites on gD from interacting with activatory surface receptors, rather than through nonspecific steric effects or possibly by interacting with inhibitory FcγR on DC.

The neutralization of gD did not totally exclude the role of other viral structures in the activation of DC. Two of these are glycoprotein B and glycoprotein C that attach HSV-1 virions to heparan

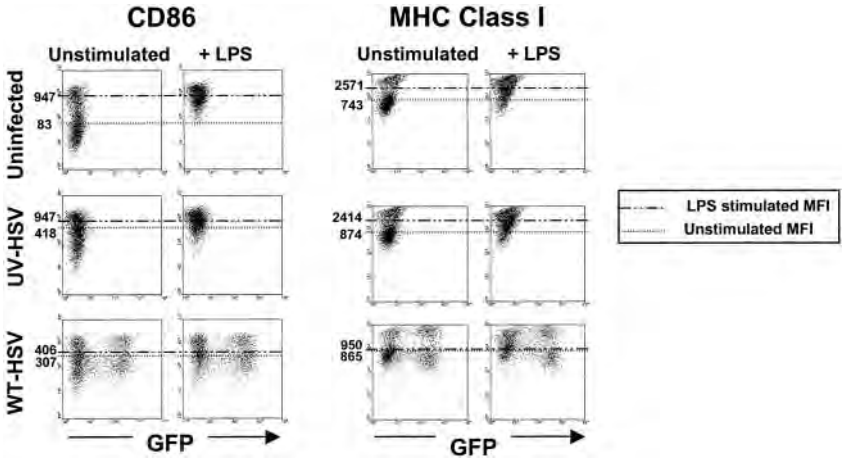

FIGURE 6. Changes in DC surface phenotype after UV-HSV infection. DC infected with UV-HSV or WT-HSV for 16 h in the presence or absence of 100 ng/ml LPS were analyzed for expression of surface molecules. Numbers on dot plot represent MFI. Representative of three independent experiments.

sulfate on the DC surface (22). It was possible that gD neutralization also prevented heparan sulfate binding and that this interaction was an important activation signal in DC, as proposed for CMV (23). However, binding of HSV-1 virions neutralized with LP2 mAb on DC could still be detected by flow cytometry. Furthermore, this binding was sensitive to prior incubation of the neutralized virions with heparin, which can bind glycoprotein B and glycoprotein C, preventing viral attachment to heparan sulfate (Fig. 9*D*). Therefore, gD neutralization of HSV-1 still permitted virion attachment to heparan sulfate on the cell surface, and attachment per se is not an activatory signal in DC.

Similar to the changes in surface phenotype, gD neutralization of WT-HSV with LP2 also abolished DC secretion of type I IFN (Fig. 9*E*). We also observed equivalent IFN secretion as WT-HSV in five of seven individuals' DCs infected with UV-HSV and in two of three individuals' DCs exposed to FIX-HSV. Therefore, HSV-DC surface interaction is important for inducing IFN secretion and viral envelope gD plays an important role in inducing this effect.

Discussion

DCs play a central role in the initiation of T cell-mediated antiviral immune responses (2). This is particularly important in the context of resolving immunity to HSV-1 in vivo (3, 4) and therefore the study of the interaction between this virus and DC has attracted considerable interest.

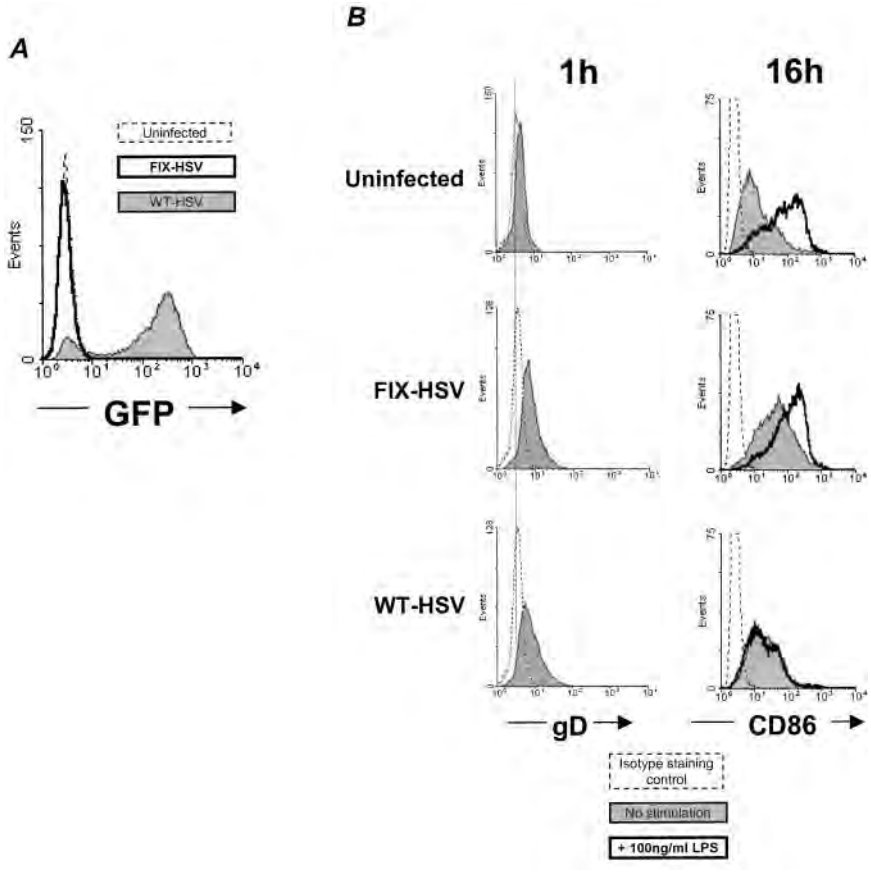

FIGURE 7. Surface phenotype changes on DC exposed to FIX-HSV. *A*, DCs infected with FIX-HSV or WT-HSV were analyzed for GFP expression 16 h after infection. Representative of at least three independent experiments. *B*, DCs infected with FIX-HSV or WT-HSV for 1 h (*left*) or 16 h in the presence or absence of 100 ng/ml LPS (*right*) were analyzed for expression of gD (*left*) or CD86 (*right*). WT-HSV data gated on GFP^+ DCs. Representative of three independent experiments.

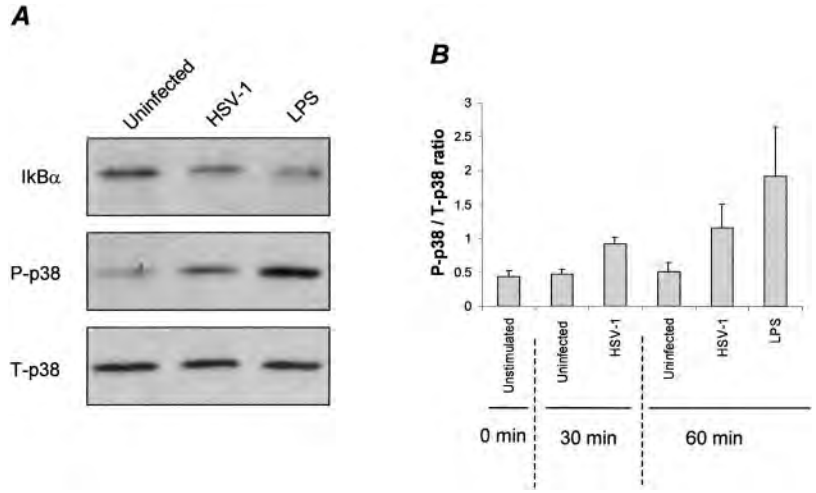

FIGURE 8. Signaling consequences of HSV-1 infection of DC. *A*, MDDC were infected with WT-HSV (MOI = 3) or stimulated with LPS for 60 min. Cell lysates were harvested and assayed by Western blot for IκB-α, phospho-p38 and total p38. Results are representative of three independent experiments. *B*, Data in *A* presented as ratio of band intensity of phospho-p38 to total-p38 MAPK. Results are mean of three independent experiments. Error bars represent SEM.

HSV-1 infection of human DC results in a functional impairment of the infected cells (9, 12). HSV infection, however, is generally associated with humoral and cellular immune responses, implying that DC activation, as well as DC impairment must occur. In this study, therefore, the mechanisms involved in the initial activation have been analyzed in detail. We propose a mechanism by which myeloid DC, the first APC to encounter HSV-1 in vivo, can bypass the block in viral Ag presentation by releasing type I IFN that can mature bystander DC. In parallel, however, DC interaction with HSV envelope results in NF-κB and p38 MAPK activation and direct maturation of infected DC. This dual effect has important implications both for the use of HSV-1 as a gene delivery vector and in vaccine design.

Viral inhibitory mechanisms

De novo synthesis of viral gene products was predominantly responsible for the inhibitory effects seen following WT-HSV infection of immature DC. DC infected with UV-HSV remained viable, could stimulate T cell proliferation efficiently, had normal morphology, and were able to respond to further maturation stimuli through changes in cytoskeletal shape and through up-regulation of CD86 and MHC class I to maximal levels, in sharp contrast to the effects seen with WT-HSV (9, 12) (Figs. 5 and 6). These findings agreed with previous studies showing that UV-HSV infection of mature DC results in no loss of function (10), but it is important to note that mature DC are generally more resistant to viral infection than immature DC (9) and therefore subtler effects of viral proteins might not have been observed.

The specific viral components responsible for the changes observed have not been defined. Recently, a key role has been suggested for the tegument protein virion host shutoff (vhs), which inhibits protein synthesis by accelerating mRNA degradation (24). However, the precise targets for vhs and other candidate viral immunomodulatory molecules remain to be elucidated.

Bypassing the roadblock and HSV-induced production of type I IFN by myeloid DC

Given the importance of DC in the initiation of the antiviral T cell response, mechanisms to by-pass the loss in Ag-presenting function resulting from HSV-1 infection are necessary to develop effective antiviral immunity. Previously, we observed that bystander uninfected DC had a more mature phenotype and that this effect was mediated by soluble factors (12). In this study, we have concluded that this paracrine signal is type I IFN released from infected myeloid DC and that this is responsible for the maturation of bystander DC and can prime DC for increased IL-12 secretion in the presence of LPS (Figs. 2–4).

Type I IFN is a family of cytokines made up of many subtypes that were originally identified for their antiviral properties and that can be secreted by all cell types (15). A subset of DC, PDC secrete particularly high levels of type I IFN when stimulated with certain viruses (7). However, the inhibitory effect of IL-4 on PDC survival (25) and their CD2 surface expression (26) (a marker used to deplete contaminating T and NK cells in our experimental protocol) made the presence of such DCs in our studies unlikely. The combination of myeloid markers expression in >99% of these DC (Fig. 1) and intracellular staining for IFN-α secretion confirmed that the source of type I IFN secreted into the supernatant of HSV infected cultures was undeniably MDDC (Fig. 2). This is consistent with a recent study that showed that myeloid DC can produce as much type I IFN as PDC through a protein kinase R-dependent mechanism, if the viral RNA genome is transfected directly into the cytoplasm (27), but add to it with the observation that significant functionally active type I IFN is also secreted following natural infection of DC by the DNA virus HSV-1. Type I IFN release was observed in the majority of individuals following DC infection with UV- and FIX-HSV, in a gD-dependent manner (Fig. 8), consistent with previous reports (28). The DC receptors involved in inducing this IFN secretion are currently unspecified. Important candidates include chemokine receptors (28) and the mannose receptor, which has been implicated in the secretion of type I IFN by DC in response to HSV-1 (29, 30). Nevertheless, the data presented in this study strongly suggest that the stimulus for type I IFN secretion in MDDC to this DNA virus is independent of viral replication and therefore of the production of viral RNA, and thus differs from the mechanisms proposed for some RNA viruses (27).

DC response to type I IFN

To confirm that the type I IFN in supHSV-DC was responsible for the paracrine effects on DC function, we used recombinant IFN-α and showed that this cytokine had the same effect on DC as supHSV-DC (Fig. 3). Although previous studies have demonstrated that IFN-α induces submaximal DC maturation (31–33), our data show that the response is dose-dependent. Sufficient IFN-α can result in a degree of maturation equal to that seen with LPS (data not shown). It is important to note that the amount of type I IFN secreted following HSV infection (~250 IU/ml) closely reproduced the submaximal up-regulation of CD86 and HLA-DR observed in DC cultured in supHSV-DC. In vivo, the localized

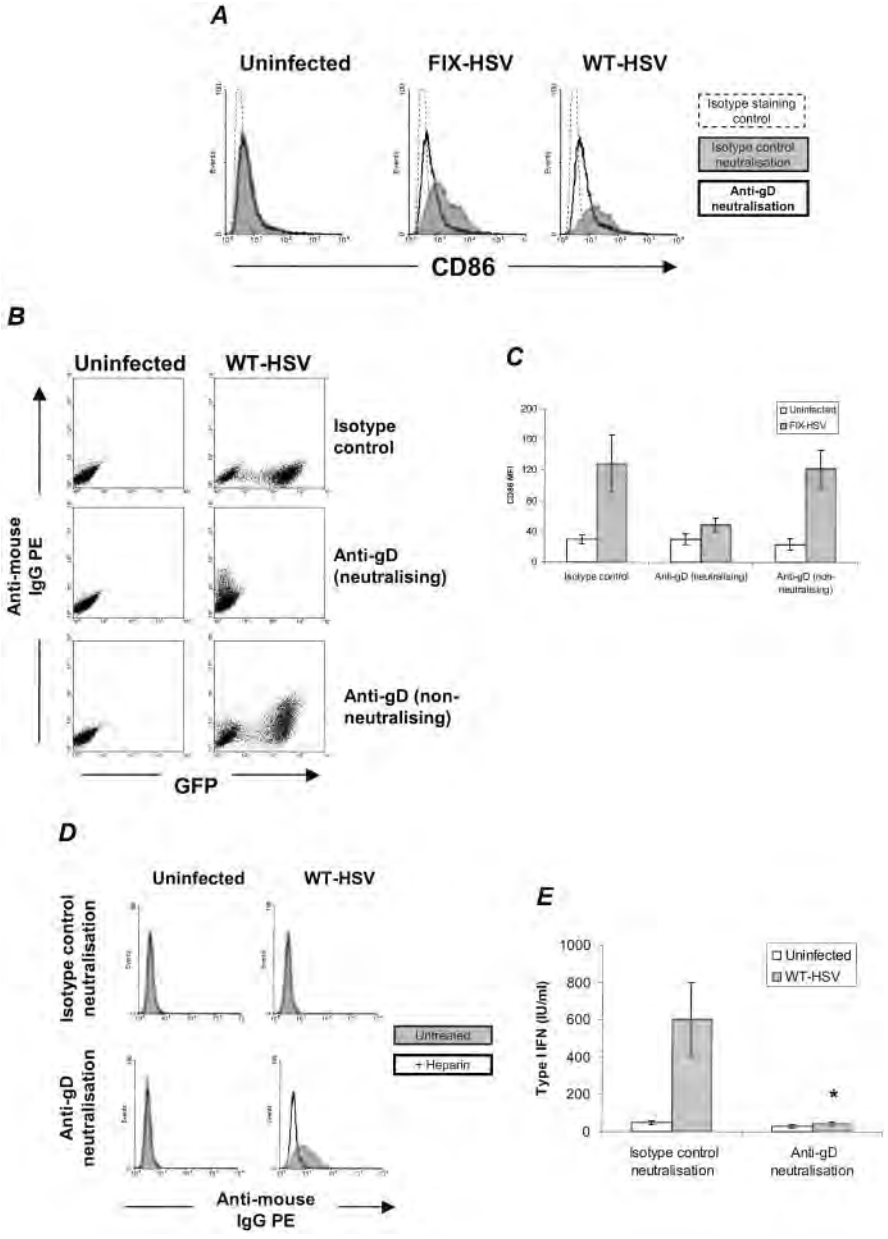

FIGURE 9. The effects gD interaction with DC on CD86 expression and type I IFN secretion. *A*, FIX-HSV or WT-HSV was neutralized with anti-gD mAb (LP2 clone) and then used to infect DC for 16 h. Expression of CD86 was analyzed by flow cytometry. WT-HSV data are gated on GFP^+ DC. Results are representative of three independent experiments. *B*, DC infected with WT-HSV exposed to neutralizing (LP2 clone) or non-neutralizing (AP7 clone) anti-gD mAb for 16 h. Expression of mouse IgG and GFP was assessed by flow cytometry. Representative of three independent experiments. *C*, DC infected with FIX-HSV exposed to LP2 or AP7 clones anti-gD mAb were infected for 16 h and expression of CD86 was assessed by flow cytometry and presented as mean CD86 MFI of three independent experiments. Error bars represent SEM. *D*, WT-HSV was neutralized with anti-gD mAb (*bottom*) and then incubated in the presence or absence of 12.5 μg/ml heparin before infection of DC for 1 h. DC were stained for the presence of opsonized viral particles on the surface with PE-conjugated goat anti-mouse Ab. Representative of three independent experiments. *E*, WT-HSV was neutralized with anti-gD mAb and then used to infect DC. Supernatants were harvested after 16 h and type I IFN secretion determined by an antiviral bioassay. Results are mean of four independent experiments. Error bars represent SEM; $*$, $p < 0.01$ relative to WT-HSV neutralized with an isotype control Ab.

concentration of IFN-α achieved may be much higher, and drive full DC maturation.

We have also shown that type I IFN secreted from HSV-1 infected DCs can overcome the HSV-1 induced block in IL-12 secretion (12) by priming bystander uninfected DCs for enhanced IL-12 secretion. Although the synergistic effects observed with IFN in this study are similar to those reported by others (33, 34), the regulation of IL-12 secretion in human DC by type I IFN remains an area of controversy, in relation to the subtypes of IFN and the precise timing of IFN exposure. In contrast to IFN-α (Fig. 3) (33), IFN-β does not have a priming effect on DC IL-12 secretion (35) and addition of type I IFN during DC differentiation generates more mature DCs with impaired ability to secrete IL-12, consistent with an "exhausted" DC phenotype (36–38).

DC response to HSV-1

Despite the similar effects of IFN-α and HSV-1 on DC phenotype, the maturation of DC infected with WT-HSV was not dependent on the secretion of type I IFN (Fig. 4). The autocrine loop may have been disrupted by HSV-1 interference with type I IFN induced signaling pathways (39). However, even after FIX-HSV infection, in which this disruption presumably does not occur, DC maturation also occurred independent of the autocrine activity of type I IFN (data not shown). HSV-1 is not unique in inducing type I IFN independent activation, as some RNA viruses, such as influenza and Sendai virus, also activate DCs in this way (40). However, in other instances, viral interaction with the DC surface induces autocrine cytokine feedback loops that are necessary to mature DC (41). Therefore, the critical factors in determining direct DC maturation by viral infection are likely to be the viral ligands, the cellular receptors they interact with, and the subsequent downstream signaling events induced.

HSV-1 entry into DCs requires glycoprotein B and glycoprotein C attachment to surface heparan sulfate followed by gD binding to one of two surface receptors, either herpesvirus entry mediator (HVEM) or poliovirus related receptor-1 (22). UV-HSV and FIX-HSV induced CD86 up-regulation on DC was dependent on gD binding to cell surface receptor(s) (Fig. 9). Notably, gD-neutralized HSV virions were still able to attach to heparan sulfate, but did not up-regulate CD86 (Fig. 9), excluding HSV attachment to

heparan sulfate as an activation stimulus for DC, contrary to the effects of CMV (23). Binding non-neutralizing anti-gD mAb to HSV-1 did not prevent the activation, excluding the role of inhibitory FcγR or nonspecific steric inhibition by the mAb. In addition it emphasized that gD interaction with receptors on the DC surface plays a critical role in the activation of DC. This was underlined by the activation 30 min after infection of both p38 and NF-κB (Fig. 8), two pathways that are necessary to complete the full program of DC maturation (18–21).

The ability of gD to bind to the surface of cells to exert functional effects has been previously described (28). However, the receptors that exert its function on DC have not yet been specified. One candidate is HVEM, a member of the TNFR superfamily that is expressed on DCs (9, 42) (data not shown) and has been shown to activate NF-κB upon ligation in other cell types (43). Although a nonviral HVEM ligand, LIGHT, can induce maturation of DC and enhance their function as APC (44), gD and LIGHT bind to different sites on HVEM (45) and the signaling consequences of binding these ligands may differ. HVEM has not been shown to be involved in p38 MAPK activation. Nevertheless, it is interesting that in this study there was correlation between activation of p38 and NF-κB, and the phenotype of HSV-infected DC compared with LPS, which supports the hypothesis that these pathways are important in the maturation of DC following HSV-1 infection.

The ability of HSV-1 to activate DC directly improves the therapeutic prospects of replication incompetent vectors. Infection of DC and expression of the desired transgenes in the context of a mature DC will favor the induction of a potent T cell response (24). Furthermore, identification of the viral proteins that have adjuvant capacity on DC may also identify novel immunogenic vaccine candidates (3).

The direct activation of DC by this virus also raises important issues with regards to host-pathogen recognition. Recent studies have focused on the ability of the innate immune system, of which DCs are a central component, to recognize pathogen-associated molecular patterns and swiftly initiate anti-pathogen responses. In this respect, the ability of DC to recognize conserved envelope structures of HSV-1 is consistent with such a model of pathogen recognition. Further studies are required to elucidate whether gD is the sole stimulatory ligand in this system or whether proteins involved in envelope-membrane fusion are also critical in inducing activating signals in DC (46). Nevertheless, as the activation of both p38 MAPK and NF-κB is required for several facets of DC maturation (18–21), it is perhaps surprising that HSV-1 has conserved structures that can activate both these proinflammatory pathways that could promote antiviral responses by DC. However, HSV-1 activation of both NF-κB and p38 MAPK can enhance the efficiency of viral replication (16, 17). Therefore, the reliance on the activation of these host signaling pathways for efficient viral replication may have provided the driving force for the evolution of viral mechanisms to dampen the function of DC that are activated early in the initial infection process (9, 12). In this way, the ability of DC to mature in response to HSV-1 infection may result primarily from the dependence of the virus to activate certain signaling pathways for its own replicative advantage. Further studies are required to determine whether these events benefit to a greater extent the survival of the virus or the host.

Role of DC in resolution of HSV-1 lesions

This study also addresses important issues regarding the role of DC in a peripheral herpetic skin infection. Signals from the resident DC, in combination with those from neighboring non-APC (e.g., keratinocytes, see Ref. 47), may be required both to initiate the maturation of neighboring uninfected myeloid DC and to limit local viral replication. The important role of type I IFN in the differentiation of monocytes to DC (48) may also result in the selective local recruitment of myeloid DC. Murine models have shown that uninfected myeloid submucosal and lymph node resident DC subsets are responsible for HSV-specific T cell activation after peripheral infection (49–51). The implied reliance on DC cross presentation in these models underlies the importance of innate immune responses to HSV-1. This study proposes that early local release of type I IFN by myeloid DC drives DC maturation, enhances cross-priming by DC and skews toward Th1 responses (52). This tilts the immunological balance from HSV-1 tolerance to HSV-1 immunity (53).

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viruses

MDPI

Article

Virus Inactivation by Formaldehyde and Common Lysis Buffers

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Abstract: Numerous mammalian viruses are routinely analyzed in clinical diagnostic laboratories around the globe or serve as indispensable model systems in viral research. Potentially infectious viral entities are handled as blood, biopsies, or cell and tissue culture samples. Countless protocols describe methods for virus fixation and inactivation, yet for many, a formal proof of safety and completeness of inactivation remains to be shown. While modern nucleic acid extraction methods work quite effectively, data are largely lacking on possible residual viral infectivity, e.g., when assessed after extended culture times, which maximizes the sensitivity for low levels of residual infectiousness. Therefore, we examined the potency and completeness of inactivation procedures on virus-containing specimens when applying commonly used fixatives like formaldehyde or nucleic acid extraction/lysis buffers. Typical representatives of different virus classes, including RNA and DNA viruses, enveloped and non-enveloped, such as adenovirus, enterovirus, lentivirus, and coronavirus, were used, and the reduction in the in vitro infectiousness was assessed for standard protocols. Overall, a 30-minute incubation with formaldehyde at room temperature effectively inactivated all tested enveloped and non-enveloped viruses. Full inactivation of HIV-1 and ECHO-11 was also achieved with all buffers in the test, whereas for SARS-CoV-2 and AdV-5, only five of the seven lysis buffers were fully effective under the tested conditions.

Keywords: virus; biosafety; inactivation

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1. Introduction

When determining the biological safety of inactivation processes, the quantitative assessment of possible residual viral infectivity is critical. This is of particular importance when specimens containing BSL-3 or -4 level pathogens ([1] for Switzerland as well as other countries with similar biosafety regulatory requirements) are transferred as "non-infectious samples" to a lower biosafety level. National guidelines regulate fixation or treatment procedures in a high containment location prior to a transfer to another laboratory of a lower safety level. While manufacturing guidelines principally focus on the complete inactivation and removal of viruses [2–4], treatment of research and diagnostic samples often use inactivation procedures for viruses that aim at retaining properties such as their shape and genomic intactness for downstream analyses in a BSL-2 or -1 environment. Such downgrading critically depends on the reliability of inactivation protocols. One potential hurdle for a simple extrapolation from one virus type to a more general statement is the great heterogeneity between virus classes (DNA vs. RNA or enveloped versus non-enveloped, etc.). But also differences in viral titers or the nature of a specimen from a given diagnostic or research context are likely to lead to varying inactivation success.

This study therefore aimed at providing reliable information on the completeness of given inactivation processes for virus-containing specimens that can inform about potential residual risks of infectiousness of a treated sample.

It is a central fact that viruses can be quite stable in their natural environment. Other groups [5] as well as this study report that even enveloped viruses such as HIV or SARS-CoV-2 can be very stable over time under laboratory conditions [6] and that certain viruses may resist standard chemical detergents for extended periods of time [7,8]. This topic has been extensively discussed, e.g., in a working group of the European virus archiving consortium 'EVA-GLOBAL (www.european-virus-archive.com (accessed on 25 July 2023)). Therefore, a careful evaluation of the methods used for eliminating residual infectivity is deemed necessary, but only little systematic information is currently available for the different virus orders and families. Hence, validation of common lysis buffers for relevant viruses in diverse specimens including protein-containing liquids is needed, which confirms buffer potencies and provides information about possible residual risks of sample infectiousness.

In this study, we simulated standard conditions of laboratory samples and clinical specimens and compared seven common lysis buffers as well as formaldehyde for their respective potencies to inactivate viruses, using representatives of different virus families including enveloped and non-enveloped viruses, such as lentivirus, coronavirus, adenovirus, and enterovirus.

With respect to specific denaturing agents, guanidinium thiocyanate ($C_2H_6N_4S$) or ammonium thiocyanate (NH_4SCN), contained in lysis buffers, are chaotropic agents and therefore act as general protein denaturants that disrupt the hydrogen bonding network between water molecules and reduce the stability of the native state of proteins by weakening the hydrophobic effect. In buffer MC136A, 1-thioglycerol is present as a reducing agent. Formaldehyde (HCHO) is a potent electrophile that can react with biological nucleophiles in proteins and DNA and facilitates the formation of intra-strand and DNA–protein cross-links in vitro [9].

2. Materials and Methods

2.1. Formaldehyde Inactivation Solution

The formaldehyde (FA) inactivation solution was prepared from a 37% stock of formaldehyde (Sigma-Aldrich, Buchs, Switzerland, Cat. No. 33220) diluted 1:10 in PBS (Sigma, D8537), leading to the final FA concentration of 3.7% added to the infected cells (without media) in the inactivation protocol.

2.2. Lysis Buffers

The lysis buffers for nucleic acid extraction in this test panel included: AVL buffer (Qiagen), RLT buffer (Qiagen), and TRIzol Reagent (Invitrogen), and the four lysis buffers A671, MC136A, MC143A, and MC501C (Promega). Details are listed in Table 1.

Table 1. Inactivation buffers.

Name	Company	Extraction Kit/Reference	Active Ingredients	Usage on
AVL	Qiagen	Catalog no. 19073	Guanidinium thiocyanate	viral cell culture supernatant
RLT	Qiagen	Catalog no. 79216	Guanidinium thiocyanate	infected cells
TRIzol	Invitrogen/ Thermo Fisher Scientific	Catalog no. 15596026	Thiocyanic acid, compound with guanidine (1:1) Ammonium thiocyanate	viral cell culture supernatant
A671	Promega	AS1620—Cultured Cells DNA Kit	Guanidinium thiocyanate	infected cells
MC136A	Promega	AS1460—Maxwell®RSC miRNA from Tissue and Plasma/Serum Kit	Guanidine thiocyanate 1-thioglycerol	viral cell culture supernatant
MC143A + A826D	Promega	AS1660—PureFood Pathogen Kit	Guanidinium thiocyanate	infected cells
MC501C	Promega	AS1330—Viral TNA Kit AS1400—Blood Kit	Guanidinium thiocyanate	viral cell culture supernatant

2.3. Viruses

The panel of commonly used virus candidates contains a non-enveloped DNA virus: adenovirus type 5 (AdV-5, Adenovirus), obtained through E. Moissonnier, University Aix-Marseille, Marseille France; a metabolically very stable picornavirus: enteric cytopathic human orphan virus type 11 (ECHO-11, Enterovirus), provided by C. Tapparelle, HUG, Geneva, Switzerland; the enveloped retrovirus HIV-1 (clonal subtype B isolate NL4-3, Lentivirus), originally from M. Martin, NIAID, Bethesda, MD, USA; and a coronavirus (SARS-CoV-2, Wuhan, isolate Muc), provided by G. Kochs, University of Freiburg, Freiburg, Germany. Key properties and the sources of virus strains are summarized in Table 2.

Table 2. Human virus strains used in the study.

Genus	Virus	Characteristics/Features	Strain	Biosafety Level
Adenovirus	AdV-5	Non-enveloped, DNA (ds)	-	BSL2
Enterovirus	ECHO-11	Non-enveloped, RNA (ss, +)	-	BSL2
Lentivirus	HIV-1	Enveloped, RNA (ss, +)	NL4-3	BSL3
Coronavirus	SARS-CoV-2	Enveloped, RNA (ss, +)	Wuhan	BSL3

2.4. Cell Culture and Virus Stocks

All cell types were grown in complete DMEM (cDMEM): DMEM (Sigma, D0819) supplemented with 10% fetal bovine serum (FBS), (Gibco, 10270) and 1% penicillin/streptomycin (Bioconcept AG, Allschwil, Switzerland, 4-01F00H), or in complete RPMI medium (cPRMI): RPMI 1640 (Sigma) supplemented with 10% FBS and 1% penicillin/streptomycin.

Virus infections were inoculated in the corresponding standard growth media, except for SARS-CoV-2, which utilized DMEM with 2% FBS and 1% penicillin/streptomycin. Cells were incubated at 37 °C with 5% CO_2. The term "cell culture media" (CCM) refers to both cDMEM or cRPMI. Initial virus inocula were expanded in susceptible cell lines (Table 3) to provide maximal virus titers and stocks. AdV-5 and ECHO-11 were propagated on 90% confluent cultures of suitable cells in T75 flasks. cDMEM was completely aspirated, and cells were infected in 2 mL of cDMEM plus virus at an MOI = 1 in 100 µL for 1 h at 37 °C. Then, 12 mL of cDMEM was re-added to the cultures. When reaching a full CPE (complete cell detachment), virus was harvested on day 3 (AdV-5) or day 6 (ECHO-11). The cell suspension was transferred to a 50 mL tube, subjected to one freeze/thaw cycle on dry ice, and centrifuged at 4 °C, 2000× *g* for 5 min. Aliquots of 1 mL volume were prepared and stored at −80 °C. SARS-CoV-2 was propagated until a full CPE developed. Virus was then harvested from the cell-free supernatant on days 7–12 without freeze–thawing and centrifuged at 1200× *g*, 4 °C for 10 min. HIV-1 stocks were prepared from the chronically infected clonal HUT4-3 cell line [10] in complete RPMI medium on day 3 after seeding 1.5 × 10^5 cells/mL in 115 mL in T1175 flasks. The cell suspension was transferred to a 50 mL tube and centrifuged (2000× *g*, 5 min). Virus-containing supernatant was aliquoted in 2 mL tubes, which were then high-speed centrifuged for 1 h at 4 °C at 21,100× *g* for virus concentration. After aspirating the top 90% volume from each centrifuged tube, the remaining 10% volume was collected; 1 mL aliquots were prepared and frozen (−80 °C). The propagated virus stocks were used for titer determination by plaque assay (AdV-5, ECHO-11; SARS-CoV-2) or a virus-specific reporter assay (ONPG-conversion for HIV-1, [11]).

Table 3. Propagation and reporter cells.

Virus	Cell Type	Propagation	Readout	Harvest	Final Titer (TCID50)
AdV-5	A549	3 days	day 7	SN + detached cells, freeze/thaw, centrifugation	1×10^8/mL
ECHO-11	Vero	6 days	day 7	SN + detached cells, freeze/thaw, centrifugation	3×10^8/mL
HIV-1	HUT4-3	3 days	-	Remove cells, concentrate virus 10fold by centrifugation	3×10^5/mL
	SXR5&SupT1	-	day 10		
SARS-CoV-2	CaCo2/ VeroE6-T2	12 days/ 4 days	-	SN + detached cells, centrifugation	3×10^6/mL
	VeroE6	-	day 7		

A549: adenocarcinomic human alveolar basal epithelial cells; VeroE6: kidney epithelial cells from African Green Monkey; VeroE6-T2: Vero cells, stably expressing hu-TMPRSS2; HUT4-3: Hut78-derived, stably producing infectious HIV-1; SXR5, HeLa derived human cervical cancer cells; SupT1: human T-cell lymphoblasts; CaCo2: human colon epithelial cells.

2.5. Interfering Substances

In the laboratory setting, viral infections are mostly conducted in defined culture media. However, the composition can be quite different when clinical specimens or samples from animal experimentation are handled. Therefore, we applied also conditions with various degrees of "dirtiness" that attempt to mimic the potentially interfering substances anticipated for such samples. With five distinct conditions, differences in viral inactivation with increasing levels of protein and other interfering components (Table 4) were compared.

Table 4. Interfering substances.

Level of "Dirtiness"	Supplement to DMEM
low dirty condition	0.3 g/L BSA
dirty condition	3.0 g/L BSA
dirty condition + erythrocytes	3.0 g/L BSA + 3.0 mL/L sheep erythrocytes
dirty condition + yeast	10.0 g/L BSA + 10.0 g/L yeast extract
high dirty condition	80.0 g/L BSA

Stock solutions of all interfering substances were produced as 2× concentrates. BSA (Sigma) was dissolved in DMEM (without the addition of FBS or Pen/Strep), and the solution was supplemented according to the level of dirtiness with sheep erythrocytes (Fiebig Naehrstofftechnik, Germany) or yeast extract (Sigma). Solutions were sterile-filtered (0.22 µm; Sarstedt) and kept in the refrigerator.

2.6. Experimental Procedure (Formaldehyde)

The principal inactivation procedure followed four sequential steps: First, cells were infected with the titrated virus inoculum. In the second step, infected cultures were treated with formaldehyde. In the third step, adherent cells were harvested at different time points by scraping. In a fourth step, aiming at the detection of any residual infectivity in the chemically treated samples, aliquots from the cell scrapings were added to growing, sub-confluent cultures. This process was repeated for three consecutive blind cell passages.

In addition, the putative residual virus was serially diluted for blind titration assays. To ensure reproducibility, each inactivation but also the blind passages and virus titrations were executed as three independent experiments, each one in duplicates. For SARS-CoV-2, direct replicates were used.

The formaldehyde inactivation was performed only on cells using a high MOI of the respective virus to test the activity limits of this substance. In our experience, the treatment of cell-free virus supernatant is at least as efficient as the inactivation of the corresponding infected cells. A separate experiment was therefore not included. Incubation times and concentration ranges were chosen as described in common laboratory protocols.

2.6.1. Cell Culture for Titration and Blind Passages

On the day before infection, three 12-well plates were equally seeded with the cell line of interest. In addition, one 96-well plate was seeded for virus titration. The applied seeding densities of the respective cell types are listed in Table 5.

Table 5. Seeding densities for 12-well plates (12wp) for the respective cell types used for inactivation, titration in 96-well plates (96wp), and for blind passaging in 12wp.

Virus	Cells	Culture Media	12wp Treatment	12wp Blind Passages	96wp Titrations
AdV-5	A549	cDMEM	2×10^6/plate	2×10^6/plate	1×10^6/plate
ECHO-11	Vero	cDMEM	1×10^6/plate	1×10^6/plate	1×10^6/plate
HIV-1	SXR5 SupT1	cDMEM	2.5×10^6/plate	1.5×10^6/plate	8×10^5 plate
		cRPMI	5×10^5/plate	5×10^5/plate	2×10^5/plate
SARS-CoV-2	VeroE6	cDMEM (2% FBS)	2×10^6/plate	2×10^6/plate	1×10^6/plate

2.6.2. Infection

The cell culture medium was completely removed from the 12-well plates. Cells were then infected at multiplicities of infection (MOI) of 100 (AdV-5), 300 (ECHO-11), 0.2 (HIV-1) or 2.0 (SARS-CoV-2) in final volumes of 200–300 µL of culture media per well and incubated for 1 h at 37 °C, 5% CO_2. Then, 0.5–2 mL CCM were added per well and incubated for another hour in the same conditions.

2.6.3. Experimental Controls

For solidly verifying a lysis-buffer-driven viral inactivation, various positive and negative controls were included in the same experiments along with the matter of interest, the "treated sample (TC)":

"Stock control (Stock)": A total of 20 µL of crude virus inoculum was used directly in the titration. This control served as "input-reference" for any titer drop in the process, to be compared with the treated samples.

"Infected cells without treatment (CCM+)": Used as mock inactivation, cells were infected and treated with cell culture medium instead of formaldehyde.

"Water control (H_2O+)": Infected cells were lysed only with deionized water. This treatment was included as a control for the input virus content in the culture (liberated as well as intracellular particles). For harvesting virus from this control, the medium was completely removed from the well prior to adding 1 mL of sterile milliQ water. After 20 min of incubation to allow the complete osmotic lysis of the cells, the entire content of the well was collected by repeated up-and-down pipetting and transferred to a 1.5 mL tube for titration. Of note, virus losses that could have occurred during the incubation period were not estimated, and no inhibitors to prevent virus degradation by cellular enzymes were added.

"Cell toxicity control (Ctox)": To assess the impact of any remaining traces of formaldehyde after the cleaning step, uninfected cells were incubated with cell culture medium instead of virus and then treated with formaldehyde for 30 min.

"Negative control (CCM-)": Uninfected cells were treated with complete medium instead of formaldehyde for the indicated 5 to 30 min. This provided information about the overall cell viability of the cultures.

As "positive control (PC)", we used a mixture of cell-free virus with interfering substances, yet without any further treatment.

The "T0 (no lysis) control" was manipulated the same way as the treated samples but using water instead of lysis buffer and without the addition of ethanol.

The "TD control" was similar to T0 but did not undergo a centrifugation step in the removal column. This control is intended to observe any virus loss by the column passage.

The "negative control (IS NC)" determined a possible impact of the interfering substances on the viability of the uninfected cells.

To exclude any possible influence of the buffers used, all control samples were diluted exactly the same way as the treated samples.

2.6.4. Inactivation and Cell Collection

For the treatment procedure, the entire culture supernatant was removed from the cultures in the 12-well plate. Then, 1 mL of formaldehyde 3.7% (FA) or the respective control solutions was added to the corresponding wells.

The incubation times for viral inactivation were either 1, 15, 30, or 60 min (AdV-5, HIV) or 15, 30, 60, and 120 min (ECHO-11) since a higher stability of the latter virus was anticipated. SARS-CoV-2 was incubated for 10, 15, 30, or 60 min. One additional well was treated for 5 min with formaldehyde (5′FA) for each virus.

The treatment solutions FA or CCM were removed at the end of the incubation, and cells were gently washed three times with 1 mL PBS to minimize toxicity from residual formaldehyde. PBS was completely removed after the last washing step. Then, 1 mL CCM was added to the wells and cells were scraped off. The entire content of each well was collected in a 1.5 mL tube for further use in titration experiments or blind passages.

2.6.5. Virus Titration

Cells were seeded one day prior to titration in a 96-well plate as stated in Table 5. The titrations included the following conditions: virus stock (stock), water control (H_2O+), infected cells without treatment (CCM+), and 5 min of formaldehyde treatment (5′FA).

For AdV-5 and ECHO-11, the titration started with an undiluted sample in the first row followed by a serial dilution in log steps (10^{-1} to 10^{-10}). For SARS-CoV-2, the titration started with a 10^{-1} dilution in the first row followed by a serial dilution in half-log steps. The last column served as a negative control without infection or treatment.

For HIV-1, a spinoculation step was introduced to obtain higher infection rates, in which the viral particles are forced onto the cell layer, by centrifuging the plates for 90 min at 800× *g* and 25 °C.

Infections were cultivated for 7 days after infection: for AdV-5 or ECHO-11, in final volumes of 200 µL/well, and for SARS-CoV-2, in 110 µL. HIV-1 infected cultures were incubated for 10 days at 37 °C, 5% CO_2 in a total volume of 220 µL/well.

For the determination of viral loads in the HIV-1 stability experiments (Figure 1), viral reverse transcriptase activity was quantitatively determined as described earlier [12].

HIV titers were determined by a virus-specific reporter assay (ONPG-conversion for HIV-1). Titers of SARS-CoV-2 ($TCID_{50}$/mL) were determined on day 7 as described in the evaluation section using the Spearman–Karber formula [13].

$$TCID_{50} = 10^{X_0 - d/2 + (d \times (\text{sum } r_i)/n_i)}$$

X_0 = positive logarithm of the highest dilution at which all wells are positive

D = dosis distance in log

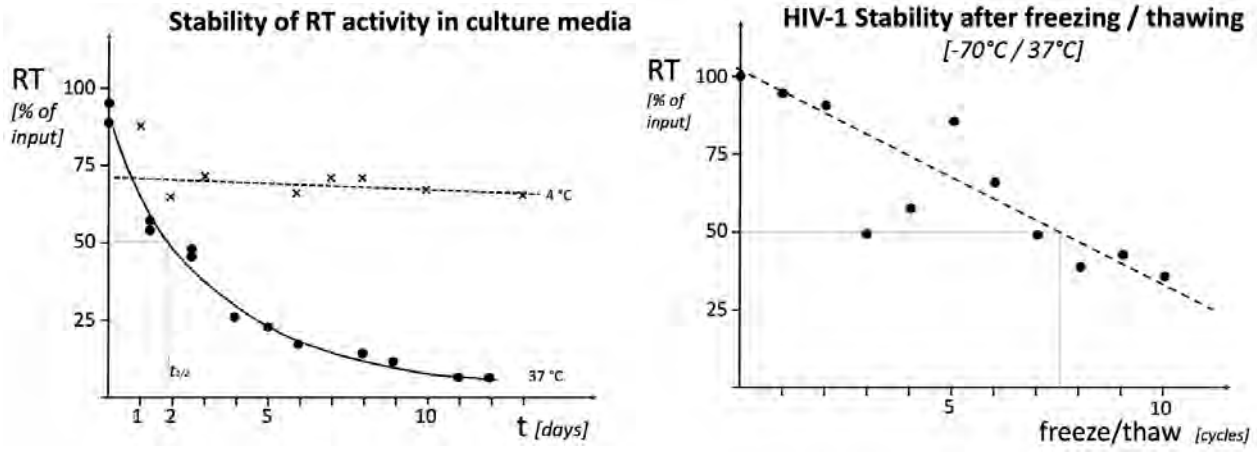
n_i = number of repeats per dilution

r_i = the sum of all positive wells starting from X_0

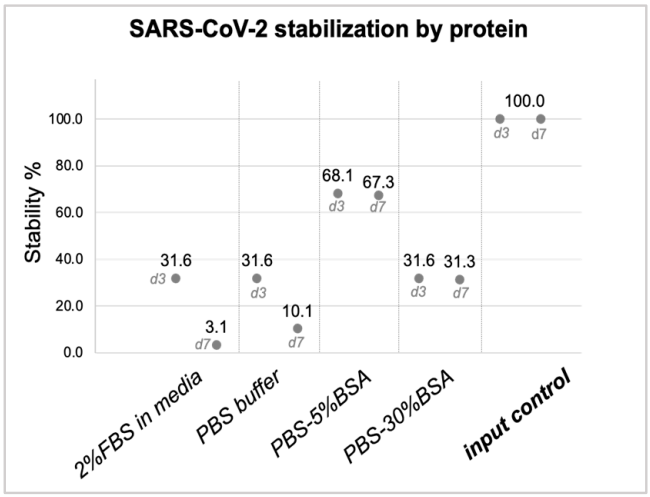
D = dosis distance in log

n_i = number of repeats per dilution

r_i = the sum of all positive wells starting from X_0



(**A**) (**B**)



(**C**)

Figure 1. Stability of the HIV Reverse Transcriptase enzyme (HIV-1 (**A**)), of virus particles (HIV-1 (**B**)) and virus infectivity (SARS-CoV-2, (**C**)) after typical laboratory manipulations. (**A**): HIV-1 containing cell-free culture supernatant, kept for 13 days at 4 °C ("x" and dotted line) or at 37 °C (solid symbols, solid line), or (**B**): subjected to repeated cycles of freezing on dry ice, followed by thawing at 37 °C. (**C**): SARS-CoV-2 virus infectivity after 3 or 7 days of incubation as indicated on the *X*-axis.

2.6.2. Blind Passaging

Cells were seeded one day prior to the start of the blind passages as stated in Table 5. The blind passages included the following conditions: different timepoints of FA treatment and a positive control without treatment (CCM+) on a separate plate.

By microscopic inspection, the development of a cytopathic effect (CPE) was monitored. At day 7, 1 mL of supernatant was transferred to another culture plate with uninfected cells (passage 2) and similarly for a third passage. These successive 7-day passages were performed at 37 °C, 5% CO_2 with the same procedure for all viruses on their respective host cell as indicated.

2.7. *Experimental Procedure (Lysis Buffers)*

2.7.1. Virus Inactivation

For evaluating the buffers AVL, RLT, and TRIzol, the following viruses were used: AdV-5, ECHO-11, HIV-1 as well as SARS-CoV-2. For buffers A671, MC136A, MC143A and MC501C, the viruses AdV-5, HIV-1, and SARS-CoV-2 were tested.

The general inactivation protocol contained four handling steps, which were followed by a stepwise titration for detecting residual infectivity of the viral extracts and by three blind passages to recover residual low titer virus.

In the first step, the virus was mixed with the potentially interfering substance stock solution in equal amounts to create the desired dirty conditions, which were designed to represent specimens with different protein content. In the second step, the respective lysis buffer was added, which was followed by the indicated incubation time. Additional chemicals (e.g., ethanol, chloroform) were added only where required, but the suggested heat or proteinase-K treatment was omitted for buffers MC136A, MC143A, and MC501C. In a third step, the sample was added to an Amicon Ultra-4 column, 100 kDa (Merck AG, Zug, Switzerland) in a final volume of 4 mL, inverted 3 times, and centrifuged (3200× *g*, 15 min) to remove residual traces of lysis buffer and chemicals. Viruses were then recovered from the membrane of the removal column by aspiration as per the manufacturer's instructions after washing the membrane with 1 mL CCM. The virus was diluted to the intended concentration and plated on susceptible cells (to avoid cytotoxicity, these dilutions had been ascertained in a first round of infections). For buffers A671, MC143A, and MC501C, further dilutions were found to be necessary to eliminate residual toxicity: two dilutions (1:10, 1:200) were used for the blind passages to determine the remaining viral infectivity.

2.7.2. Lysis Conditions

AVL—To 100 µL virus, mixed with interfering substance, 400 µL AVL buffer was added and incubated for 10 min; then, 400 µL ethanol, 96% (Roth AG, Arlesheim, Switzerland), was added. The treated sample was transferred to an Amicon removal column as described above. The procedure was repeated with a second column by transferring the 1 mL extract from the first column to a second column and repeating the steps as described above. The recovered column extract was diluted 1:5.

RLT—For sample treatment, the culture supernatant was removed from one well in the 24-well plate, and 700 µL RLT buffer was added to the cells. The cells were then scraped off and transferred to a 1.5 mL screw-cap tube. The suspension was vortexed for 1 min. Then, 700 µL ethanol (70%) was added to the tube and centrifuged (300× *g*, 1 min) to pellet cell debris. The cell-free supernatant was run through an Amicon removal column as described above, and the recovered column extract was diluted 1:10.

MC136A: To 500 µL virus mixed with interfering substance, 230 µL MC136A buffer was added; then, the tube was vortexed for 5 s and incubated for 15 min (37 °C). The treated sample was run through two consecutive Amicon removal columns as described above, and the recovered column extract was diluted 1:10.

MC501C: To 300 µL virus mixed with interfering substance, 330 µL MC501C buffer was added; then, the tube was vortexed for 10 s and incubated for 10 min. The treated sample was run through two consecutive Amicon removal columns as described above and the recovered column extract was diluted 1:10 (and 1:200).

Before applying A671, MC143A, or RLT to the infected cultures as per protocol, cells were infected with the respective virus inoculum. In order to infect the cells, they were trypsinized (Bioconcept), pelleted, resuspended in 5 mL PBS (Sigma), and counted. The suspensions were divided into two 50 mL tubes with 5×10^6 cells each, pelleted again, resuspended in the corresponding volume of virus for the required MOI or the same volume of cDMEM (uninfected control), respectively, and incubated for one hour (37 °C). Afterwards, the cells were pelleted, washed in 9 mL cDMEM, pelleted again, and resuspended in 12.5 mL cDMEM. The cell suspensions were seeded into two 24-well plates (one plate infected cells, one plate uninfected cells) with 0.5 mL/well. The plates were incubated for 24 h. Mock-treated uninfected cells served as negative control (NC).

For TRIzol lysis, 100 µL of virus was mixed with interfering substance prior to adding 300 µL TRIzol reagent and incubating for 5 min; 60 µL chloroform (Merck) was added to the sample, and the tube was centrifuged (12,000× *g*, 2 min) for phase separation. The

upper and interphase were taken off, run through an Amicon removal column as described for AVL, and the recovered column extract was diluted 1:5.

Before treatment with A671, infected cells from one well of a 24-well plate were scraped off and transferred to a 1.5 mL screw-cap tube. Then, 600 µL A671 buffer was added to the cell suspension. The suspension was mixed, and the tube was centrifuged (300× *g*, 1 min) to pellet cell debris. The cell-free supernatant was run through two consecutive Amicon removal columns as described above. The recovered extract was diluted 1:10 and 1:200.

For MC143A lysis, a volume of 200 µL MC143A buffer was added to the infected cell suspensions, and the tube was briefly vortexed and incubated for 4 min at room temperature. Then, 300 µL of the second buffer component (A826D) was added, the tube was vortexed for 5 s, and after, it was centrifuged (300× *g*, 1 min) to pellet cell debris. The cell-free supernatant was run through two consecutive Amicon removal columns as described above, and the recovered column extract was diluted 1:10 and 1:200.

2.7.3. Virus Titration

All virus samples (TC, T0, TD, PC) were titrated in triplicates. Negative controls (IS NC, cDMEM) were included in quadruplicates. Cultures were trypsinized, and cells were counted and seeded in 96-well plates (Table 5) with overnight incubation. Once the respective inactivation protocol was applied, the treated samples and controls could be processed in non-cytotoxic dilutions. Dilutions were made in 96-well plates: the culture media of the 8 wells of column A of the plate were completely removed. For AdV-5 and ECHO-11, 200 µL of the undiluted virus inoculum was added per well of column A. Then, starting from the wells of column A, 20 µL was transferred to the 180 µL of cDMEM pre-dispensed in the wells of column B. After gently mixing by up-and-down pipetting and the repeated transfer of 20 µL of virus-containing media to the next column of the plate, serial 1:10 dilutions covered the range from undiluted virus to a dilution of 10^{-10} in column G. Column H of each 96-well plate served as an uninfected control: the cell culture media was completely removed, and 200 µL of either control (IS NC, cDMEM) was added per well.

To optimize the infections by HIV-1, the respective plates were centrifuged at 800× *g* for 90 min after adding the virus inoculum ("spinoculation").

For SARS-CoV-2, the titration started with a 10^{-1} dilution in column A of the plates, which was followed by a serial dilution in half-log steps.

To ensure a complete cellular infection, the plates were incubated for 10 days for HIV-1 or 7 days for all other viruses.

2.7.4. Blind Passaging

As a functional proof of the effective inactivation of all infectious virus after treatment, sequential passaging of treated (infected) cell suspensions/extracts on susceptible uninfected target cells has been established as a mandatory standard procedure. To this end, three sequential passages of treated virus on susceptible cells were performed.

Cells were trypsinized, counted, and seeded in a 6-well plate the previous day in a volume of 2 mL per well. Then, 1 mL of the treated virus sample (TC) as well as the negative controls (IS NC) was added to one well of the 6-well plate. After the completion of each passage, 1 mL of the supernatant was transferred to another 6-well plate with pre-seeded cells in 2 mL (CCM). For each blind passage, the following conditions were included: extracts from lysis buffer treatment, cell toxicity control (Ctox), and negative control (CCM-). By microscopic inspection, the development of a cytopathic effect (CPE) was monitored. At day 7, 1 mL of supernatant was transferred to another culture plate with uninfected cells (passage 2) and similarly for a third passage. These successive 7-day passages were performed at 37 °C, 5% CO_2 with the same procedure for all viruses on their respective host cell as indicated.

Infected but untreated control cells (CCM+) were added to uninfected host cells in a separate 6-well plate for 2 to 7 days to verify full inoculum infectivity and host-cell susceptibility.

2.8. Readout for Formaldehyde and Lysis Buffer Experiments

For AdV-5, ECHO-11, and SARS-CoV-2, all plates were evaluated by assessing the typical CPE by microscopic inspection: At the end of the incubation period, all titration plates were stained with Crystal Violet (Sigma) for titer evaluation by visualizing viral plaques. Cultures were fixed by adding 80 µL formaldehyde (3.7%; Sigma) per well and incubation for 1 h. Then, formaldehyde was removed, and 50 µL 0.5% Crystal Violet solution (Sigma, C6158) was added per well and incubated for 5 min. The plate was then rinsed with water and evaluated by eye (violet: intact cells; clear: CPE on infected cultures). Residual titers were calculated by applying the Spearman–Karber formula.

The HIV-1-infected plates were examined by microscopically monitoring the formation of syncytia in cultures of HIV-susceptible cell lines (co-culture of adherent SXR5 reporter cells with SupT1 lymphocytes in suspension). In addition, HIV-1 titration plates were evaluated using the in-house established HIV-susceptible LTR-lacZ reporter cell line SXR5 (enzymatic ONPG-to-ONP conversion) [11]. For this, the culture supernatant was removed, and 10 µL Glo Lysis buffer (Promega) was added per well and incubated for 10 min. The chromogenic solution per plate follows: 1 mL buffer H (250 mM sucrose, 20 mM Na monophosphate, pH 7.5), 8 mL buffer Z (10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, 100 mM NaH_2PO_4, pH 7.5) and 2 mL ONPG solution (8 mg/mL in buffer Z) were mixed and 80 µL solution was added to each well. Color conversion in the wells developed within 2 h of incubation and was scanned visually when a clear contrast between the wells with infection (yellow) and those with no HIV infection (transparent) became apparent. At this point, absorbance at 405 nm was quantified using a TECAN reader.

3. Results

3.1. Virus Stability

Applying commercial lysis buffers or approved chemicals in accordance with manufacturers' protocols to clinical specimens or research samples naturally implies the complete inactivation of viral pathogens. On the other hand, it may surprise to find the stability even of enveloped viruses such as HIV or SARS-CoV-2 quite high over time under standard laboratory conditions. We analyzed cell-free, HIV-1-containing culture supernatant for particle stability, and we found that at 37 °C, supernatant RT enzyme activity as an indicator for the intactness of the viral particle had a half-life of about 2 days, while it remained stable for far more than 10 days at 4 °C (Figure 1A). Also, freeze–thaw cycles (−70 °C to 37 °C) affected the enzymatic stability of RT only moderately with ca. 7% loss per cycle (50% activity after 7–8 cycles, Figure 1B).

The decline of viral infectivity was assessed, using SARS-CoV-2, after incubation for 3 and 7 days in different conditions (Figure 1C). While in plain standard cell culture medium or PBS buffer, infectious titers diminished rapidly, the presence of 5 or 30% BSA was able to greatly reduce the titer decline for at least 7 days.

Moreover, the stability of SARS-CoV-2 virus particles under cell culture conditions appears to be remarkably high, even in the presence of viable, growing cells. To test the resilience of SARS-CoV-2 during exposure to non-permissive cells, 10^5 infectious particles were added to a culture of HEK293T cells in complete DMEM at two temperatures of 34 °C and 30 °C. Infectious virus was still obtained until day 13 under either condition. This was quite unexpected, since the activity of cellular proteases and nucleases, e.g., from dying cells, was anticipated to inactivate SARS-CoV-2 particles within a short time. The previously published study by Widera et al. supports the observation that SARS-CoV-2 viruses are stable for several weeks during liquid storage [5].

3.2. Summary of Results for Formaldehyde Inactivation

Older inactivation protocols for FA suggest an incubation time of 5 min with a 3.7% FA solution. Therefore, in the first test, summarized in Table 6, the standard reduction

was determined for the four virus families of Table 2, and the potency of a 5-minute formaldehyde inactivation was expressed as log-titer reduction in the respective virus.

Table 6. Summary of the "log titer " for each virus as indicated for each indicated condition: "stock", water dilution ("H_2O"), dilution in cell culture media ("CCM+"), or after a 5-minute formaldehyde (FA) treatment. The final virus infectivity (as TCID50/mL) is shown. FA-treated samples boxed in gray; nd = values < 1.2 log omitted due to failing control well. ** indicates that complete inactivation was reached only in one out of three repeat experiments. Titer reduction is shown compared to the initial virus stock.

Virus	Condition	Experiment 1 log TCID50/mL	Experiment 2 log TCID50/mL	Experiment 3 log TCID50/mL	Average TCID50/mL	Titer Reduction
AdV-5	Stock	7.2	7.2	7.2	1.58×10^7	
	H_2O+	4.2	5.2	4.2	1.58×10^4	
	CCM+	4.2	4.2	4.2	1.58×10^4	
	FA 5′	1.2	1.2	1.2	$<1.58 \times 10^1$	6.0 $\log_{10}$
ECHO-11	Stock	11.2	11.2	11.2	1.58×10^{11}	
	H_2O+	11.2	11.2	11.2	1.58×10^{11}	
	CCM+	11.2	10.2	10.2	6.32×10^{10}	
	FA 5′	2.2	2.7 **	1.2	2.25×10^2	9.2 $\log_{10}$ **
HIV-1	Stock	5.2	5.2	5.2	1.58×10^5	
	H_2O+	nd	2.2	2.2	1.58×10^2	
	CCM+	nd	2.2	4.2	7.98×10^3	
	FA 5′	nd	1.2	1.2	$<1.58 \times 10^1$	4.0 $\log_{10}$
SARS-CoV-2	Stock	5.8	5.8	-	2.8×10^6	
	H_2O+	4.9	4.9	-	8.1×10^4	
	CCM+	5.4	4.9	-	1.8×10^5	
	FA 5′	0.9	0.9	-	8.1×10^0	4.9 $\log_{10}$

A reduction by >6 $\log_{10}$ was consistently observed for ECHO-11 and AdV-5. For HIV-1 with a lower initial titer of 1.6×10^5 TCID50/mL, and SARS-CoV-2 with a lower input titer of 2.8×10^6 TCID50/mL, the observed full virus reduction can safely be expressed as a titer reduction by >4 $\log_{10}$ (Table 6, Figure 2). The limit of detection in Figure 2 ranged from 0.2 log TCID50/mL for HIV-1 to 1.2 log TCID50/mL for ECHO-11.

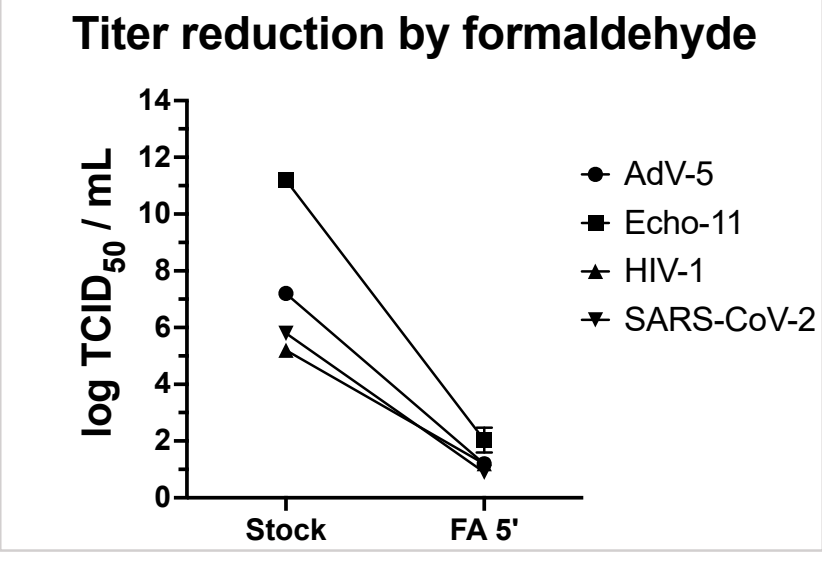


Figure 2. Virus titers (log TCID50/mL) before and after a 5 min exposure to FA is shown for Echo-11 (square symbols), HIV-1 (triangles), AdV-5 (circles), and SARS-CoV-2 (inverted triangle). Independent triplicate experiments (duplicates for SARS-CoV-2) are shown.

3.2.1. Formaldehyde Titration

Virus titrations were performed to verify the TCID50 values of each positive control and the drop of titer in the treated condition.

A virus reduction exceeding 6 $\log_{10}$ was determined for AdV-5 and related to the titer of the initial stock. After FA treatment, the signal fell below the threshold of detection. For ECHO-11, in two out of three experiments, residual infection events remained detectable for the lowest virus dilution.

For HIV, we observed a complete elimination of viral infectivity, translating in a virus reduction by at least 4 logs to levels below the threshold (HIV-1, Figure 2). A higher reduction could not be demonstrated for HIV-1, as the primary virus stock only had a TCID50 of 5 $\log_{10}$. For SARS-CoV-2, a titer reduction of 4.3 $\log_{10}$ was reached.

A "stock control" of the starting sample with a known titer was included as a reference value for which a TCID50 had been determined before. Titers of the controls are from here on referred to as "experimental titers", and they are determined under the same conditions as the investigated samples.

Although a carry-over of cytotoxic substances was not expected since the lysates were thoroughly washed before collection, rare events of cytotoxicity in the first dilutions of the titration were suspected. Consequently, those wells displaying apparent cytotoxic changes were disregarded in the analysis.

One of the HIV-1 repeat experiments was excluded due to the absence of virus progeny in the positive control. When comparing initial titers of AdV-5 and HIV-1 to the titers obtained with CCM+ and H_2O controls, a small difference was observed. This might be explained by effects of the handling process, i.e., during times of incubation, washing, or time needed for sample collection (Table 6, lines "H_2O", "CCM+").

3.2.2. Formaldehyde Blind Passage

In order to confirm the absence of residual infectivity, three sequential blind passages on susceptible cells were performed. During this experimental validation, all positive controls for entero- and adenovirus had produced a clear CPE within 48 h post-infection, as expected. In the cytotoxicity controls, incubating cells with the chemical in the absence of virus, some partial cytotoxicity was observed in two out of six test series. This was also observed for individual wells of treated samples during the first round of blind passaging, suggesting a minimal carry-over of the fixative into the cultures. Nevertheless, cytotoxicity

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could be distinguished from cytopathic effects caused by the respective viruses. This was confirmed in each subsequent passage: upon the further dilution of the cytotoxic agent, no evidence for cytotoxicity was noted in any of the cultures of blind passages 2 or 3.

The absence of viral replication (-) or cytopathic effect (CPE) was judged by microscopic inspection. A more detailed summary of residual virus in every blind passage is given in Table 7. Summarizing the results after three blind passages (BP) for each of the tested incubation periods with formaldehyde (FA), 30 min of FA inactivation eliminated the infectivity of all virus classes in the test.

Table 7. Summary of blind passages in 3 distinct experiments (A–C) after formaldehyde treatment. For each virus, the host cell is given in parentheses. Green boxes indicate the absence of viral replication. Red boxes indicate the formation of a CPE or syncytia (S), which is typical for the respective virus. "-" indicates that the control without FA was stopped after the 1st blind passage.

		----------A----------			----------B----------			----------C----------		
Virus	**Condition**	**BP1**	**BP2**	**BP3**	**BP1**	**BP2**	**BP3**	**BP1**	**BP2**	**BP3**
AdV-5 (A549)	no FA	CPE	-	-	CPE	-	-	CPE	-	-
	1′ FA		CPE	CPE	CPE	CPE	CPE		CPE	CPE
	15′ FA						CPE			CPE
	30′ FA									
	60′ FA									
ECHO-11 (Vero)	no FA	CPE	-	-	CPE	-	-	CPE	-	-
	15′ FA									
	30′ FA									
	60′ FA		-							
	120′ FA									
HIV-1 (SXR5 & SupT1)	no FA	CPE	-	-	CPE	-	-	CPE	-	-
	1′ FA		S	S		S	S		S	S
	15′ FA									
	30′ FA									
	60′ FA									
SARS-CoV-2 (VeroE6)	no FA	CPE	-	-	CPE	-	-	CPE	-	-
	5′ FA			CPE		CPE	CPE		CPE	CPE
	15′ FA									
	30′ FA									
	60′ FA									

For ECHO-11, HIV-1, and SARS-CoV-2, even a 15-minute FA incubation time was sufficient to completely inactivate these viruses. However, for AdV-5, the 15-minute incubation was not able to eliminate infectivity, as in one of the three cultures, a CPE emerged during blind passage 3.

Shorter exposure times to FA are not recommended, since they may not reliably inactivate the virus of interest. For example, the brief exposure (1–3 min) to FA was insufficient for AdV-5 and HIV-1, and a 5-minute incubation was not sufficient to inactivate SARS-CoV-2. For the ECO-11 picornavirus, which has been reported to be rather resistant to FA, exposures shorter than 15 min were not analyzed.

3.3. Summary of Results for Lysis Buffer Inactivation

In order to adequately evaluate viral inactivation by the lysis buffers, both titration plates and the blind passages (for residual virus activity) were analyzed for a comparison of TCID50/mL.

3.3.1. Virus Titration after Lysis Buffer Treatment

The titrations demonstrated a drop in the TCID50/mL for treated samples versus untreated samples in the dilution series. This study had initially aimed for a decrease in viral titer of at least 6 $\log_{10}$ from T0 (no lysis) to the treated sample TC (lysis). This turned out not to be possible for every virus in the test. While T0 values showed some

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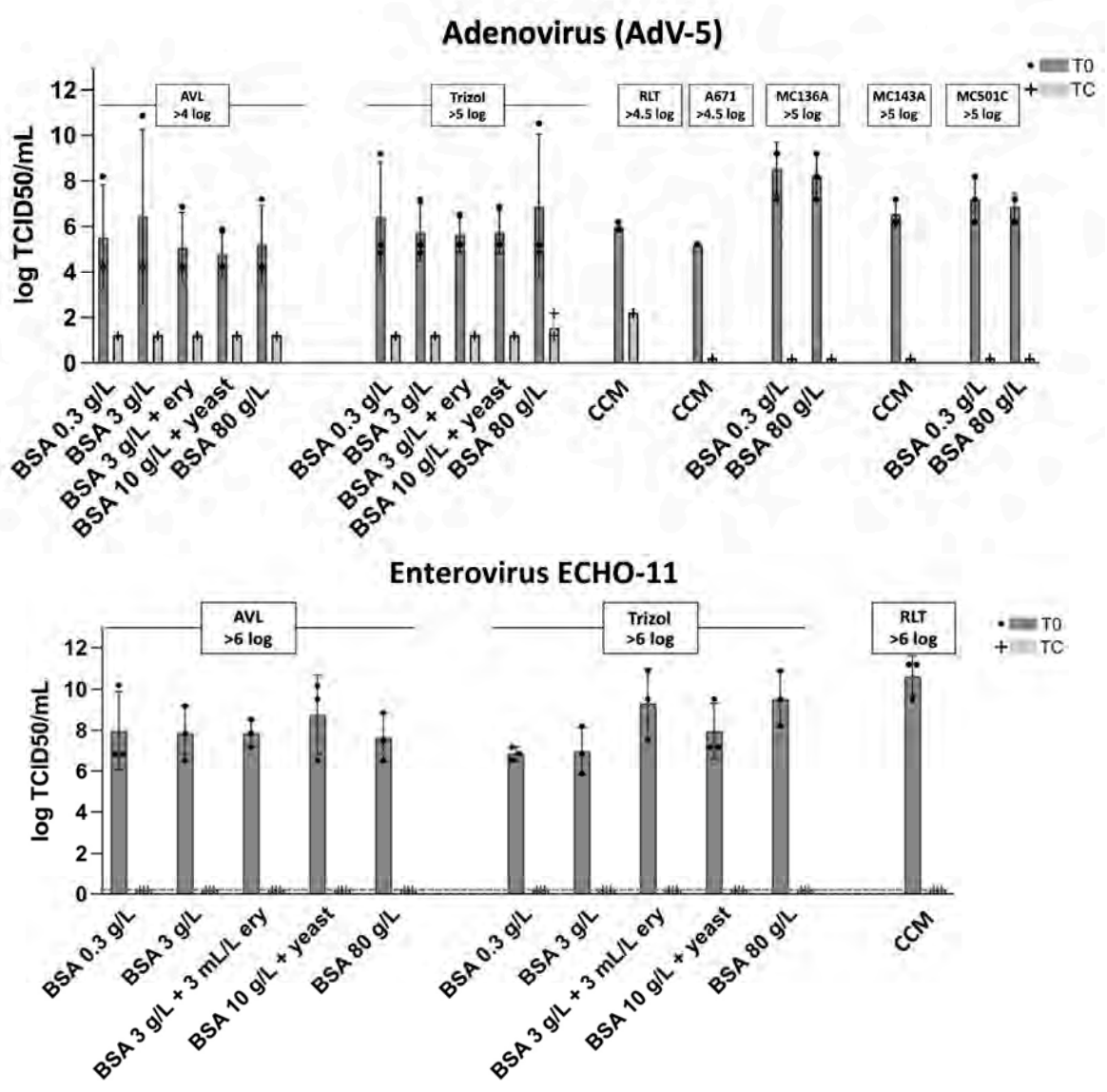

Figure 3. *Cont.*

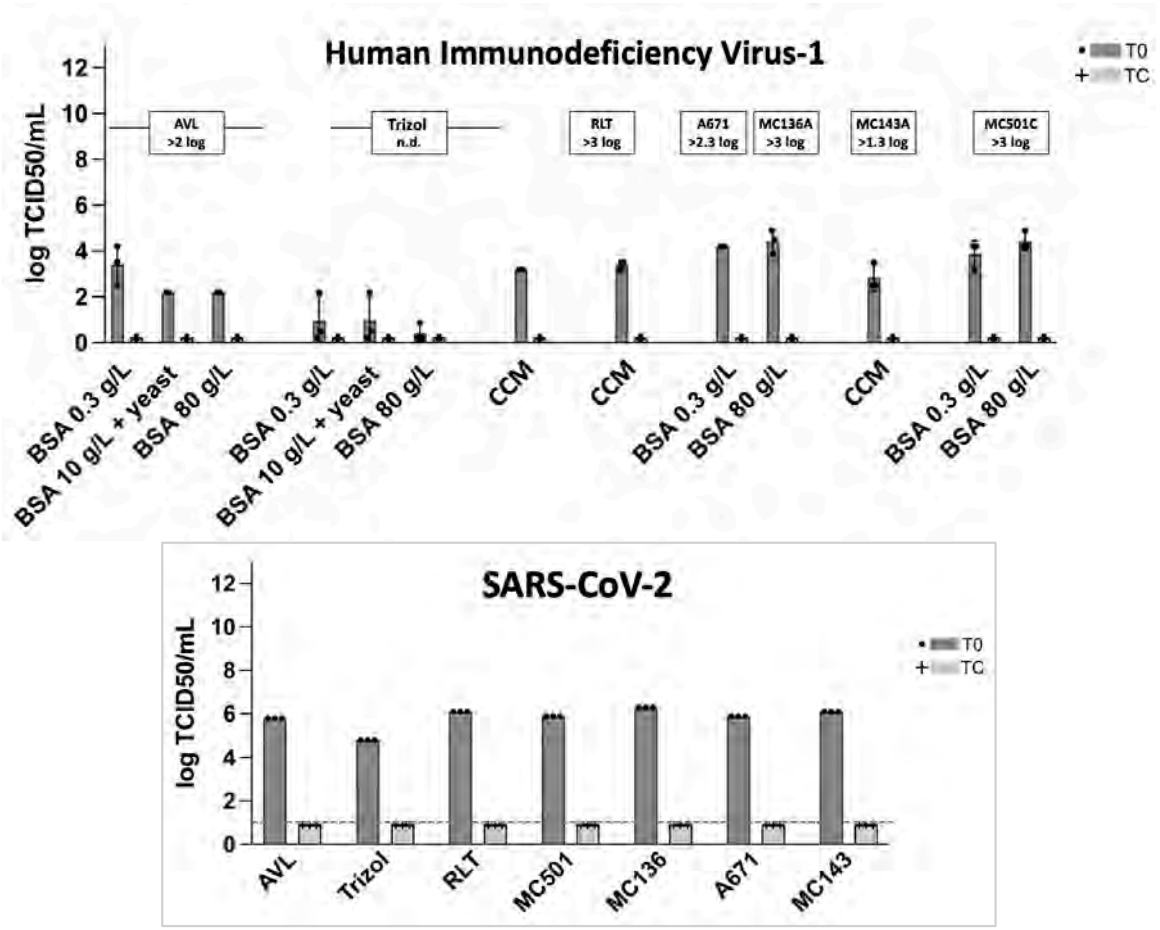

Figure 3. Virus inactivation by lysis buffers. For each of the viruses and conditions, a first aliquot was analyzed prior to adding the test agent (T0, dark gray bars) and a second sample after incubation with the indicated reagent (TC, light gray). For AdV-5, ECHO-11, and HIV-1, also several 'dirty' conditions were analyzed as shown. All tests were completed in three independent replicates. Dotted lines indicate the detection limit in the respective assay.

3.3.2. Blind Passaging after Lysis Buffer Treatment

The definitive absence of virus replication is not explicitly proven after a single replication cycle, but it has been established to include up to three sequential blind passages on susceptible cells. Similarly, Widera et al. also report that "lysis buffers do not always reach a complete infectivity loss and therefore it is advised to use a second inactivation method to guarantee a safe inactivation" [5]. Therefore, a second inactivation agent such as ethanol is already included in some of the manufacturers' protocols, e.g., AVL or RLT buffer. Overall, no cytopathic effect (CPE) that would indicate residual virus replication was detected after passaging the cells for more than three weeks in three consecutive passages (Table 8) of enterovirus or HIV-1. The safe lysis of AdV-5 and no sign of virus recovery after three blind passages could only be demonstrated for buffers A671, MC143A and TRIzol in the presence of at least 3 g/L BSA. In addition, AdV-5 was completely neutralized in two out of three experiments by the treatment with buffers (AVL with at least 3 g/L BSA + Ery) or RLT. Buffers (AVL at 3 g BSA/L or below), MC136A and MC501C (lysis of the viral supernatant only tested in the presence of interfering substances) did not fully inactivate AdV-5. An important note: the manufacturer recommends using the lysis buffers MC136A and MC501C in conjunction with a proteinase K/heat treatment!

Treatment with buffer MC136A (again omitting the recommended proteinase K/heat treatment) was unable to completely inactivate SARS-CoV-2, and for MC143A and MC501C, a CPE for SARS-CoV-2 was noted in one of the three blind passage experiments.

Table 8. Summary of blind passages. To test low residual virus in supernatants from treated specimens, these were used as inocula to set up repeated rounds of incubation. This 'blind supernatant passage' as a standard method to amplify and reveal low traces of infectious virus is repeated in three successive rounds indicated as p1 to p3 for each buffer. Red boxes are used to indicate a strong virus-typical CPE. Yellow boxes indicate a weak CPE. Green boxes indicate the complete absence of CPE. "-" indicates that no experiment was performed for the respective condition.

3.4. Utility of Salt Removal Columns

Any titer reduction that could be caused by the plain passage through an Amicon removal column was assessed by comparing the two positive controls TD (no lysis, no column) to T0 (no lysis, with column passage) samples. Both control samples were similarly treated with water instead of lysis buffer. The reducing effect of the column turned out to be minimal (Figure 4) but tended to depend on the initial virus concentration: while for AdV-5 and HIV-1 (with titers of 1×10^8 TCID50/mL and 1×10^5 TCID50/mL, respectively), we observed only a minimal loss of viral titers after passage through a removal column, the column-passage of ECHO-11 (titer > 1×10^{11} TCID50/mL in experimental titration) caused a reduction of 1–3 $\log_{10}$. Although not experimentally verified, it is possible that this was due to a limited binding capacity of the columns. For SARS-CoV-2, we found no reducing effect over the column for most buffers except for MC501C with a reduction of 2.2 $\log_{10}$. Potential reasons for these differences were not further investigated, but virus-specific differences in the affinity to the resign cannot be excluded.

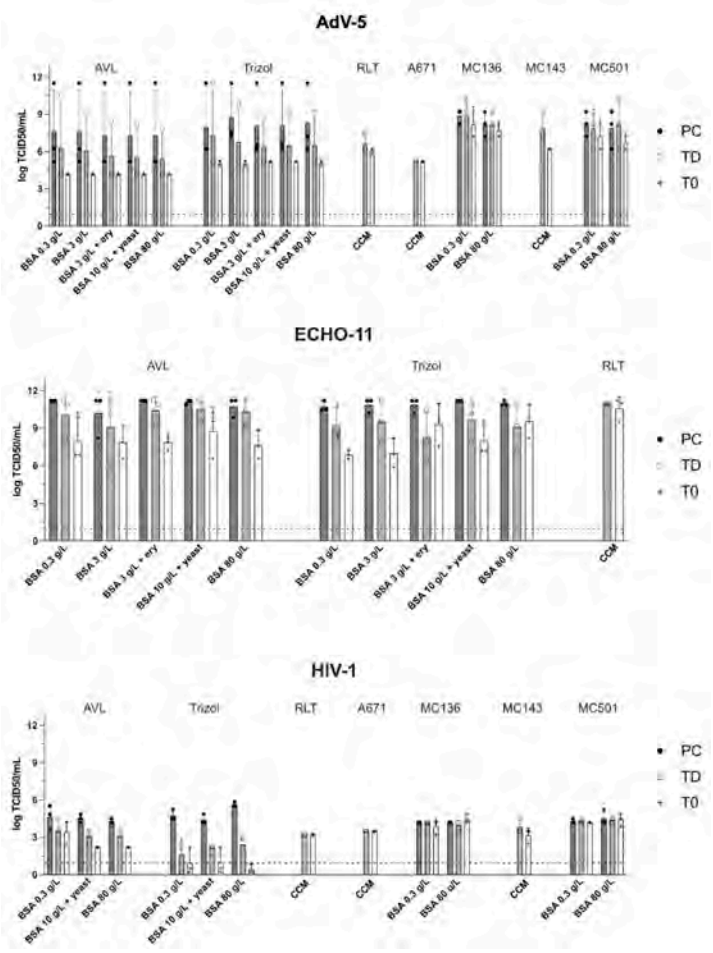

Figure 4. Assessment of the possible influence of the agent-removal column and potential impact of the addition of external protein in "dirty" conditions. "process" indicates, from which procedure the respective samples were taken. Of note, none of the PC, TD or T0 samples had been treated with the indicated reagents. The quantification limit (10^{-2}/mL) is indicated by a dotted line. Abbreviations: BSA—bovine serum albumin; "ery"—erythrocyte concentrate added; "CCM"—complete culture media.

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As expected, for most viruses, only minimal differences between positive control (PC) and TD samples were observed. The virus-reducing effect of the passage through a removal column was more pronounced for ECHO-11 with a titer of (>1 × 10^{11} TCID50/mL) than for AdV-5, HIV, or SARS-CoV-2 (titers 1 × 10^8 TCID50/mL, 1 × 10^5 TCID50/mL and 2.8 × 10^4 TCID50/mL, respectively).

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3.5. Potential Influence of Interfering Substances on Viral Inactivation

No significant difference in the inactivating potential that might have been caused by the presence of interfering substances was observed (Figure 4). For AdV-5 and ECHO-11, five conditions of "dirtiness" were applied in the presence of AVL buffer, RLT buffer, and TRIzol. A minimal experimental variability in the positive control samples of each experiment comparing the different dirty conditions was likely due to the sequential conduct on different days. Moreover, no dose-dependent impairment was found when increasing amounts of BSA or other interfering contents (sheep erythrocytes, yeast extract) were added (Figure 4). Two or three main 'dirtiness' conditions (0.3 g/L BSA; 10 g/L BSA + yeast extract; 80 g/L BSA) were also applied to the lysis of HIV-1, again demonstrating the absence of any activity-reducing effect of high protein concentrations. Based on these negative findings, dirty conditions were not tested for SARS-CoV-2.

Summarizing all results for the lysis buffers (Table 9), no replicative virus was detectable after three successive blind passages for five of seven lysis buffers, confirming the complete virus inactivation by those buffers.

Table 9. Virus reduction by various lysis buffers. Starting titers are indicated for each virus; the "log-reduction" value results from the final virus dilution in the culture. The strength of CPE is given as "-": absent; "+" mild CPE; "++" significant CPE; "+++" full CPE. "?" indicates an indeterminate result. * A heat inactivation step as suggested by the supplier was omitted to test the inactivating properties of the buffers themselves without a thermal contribution.

Buffer	Virus	Stock Titer (TCID50/mL)		CPE Results			Reduction from Mock (TD) $\log_{10}$
		Stock	Experiment	p1	p2	p3	
AVL	AdV	1×10^8	1×10^8	>5.5	?	?	>5.5
	ECHO-11	3×10^8	4×10^{11}	>9.9	-	-	>9.9
	HIV-1	3×10^5	1×10^5	>3	-	-	>3
	SARS-CoV-2	2.8×10^6	2.8×10^6	>4.7	-	-	>4.7
RLT	AdV	1×10^8	1×10^8	>5.3	-	-	>5.3
	ECHO-11	3×10^8	4×10^{11}	>10.6	-	-	>10.6
	HIV-1	3×10^5	1×10^5	>3.9	-	-	>3.9
	SARS-CoV-2	2.8×10^6	2.8×10^6	>5	-	-	>5
TRIzol	AdV	1×10^8	1×10^8	>6.3	-	-	>6.3
	ECHO-11	3×10^8	4×10^{11}	>9	-	-	>9
	HIV-1	3×10^5	1×10^5	>1.9	-	-	>1.9
	SARS-CoV-2	2.8×10^6	2.8×10^6	>4.3	-	-	>4.3
A671	AdV	1×10^8	1×10^8	>5	-	-	>5
	HIV-1	3×10^5	1×10^5	>3	-	-	>3
	SARS-CoV-2	2.8×10^6	2.8×10^6	>6	-	-	>6
MC136A	AdV	1×10^8	1×10^8	>8.5	+	+++ *	>8.5
	HIV-1	3×10^5	1×10^5	>3.9	-	-	>3.9
	SARS-CoV-2	2.8×10^6	2.8×10^6	>6.1	+	+++ *	>6.1
MC143A	AdV	1×10^8	1×10^8	>7.7	-	-	>7.7
	HIV-1	3×10^5	1×10^5	>3.6	-	-	>3.6
	SARS-CoV-2	2.8×10^6	2.8×10^6	>5.3	++	+++ *	>5.3
MC501C	AdV	1×10^8	1×10^8	>8	+	+++ *	>8
	HIV-1	3×10^5	1×10^5	>4.2	-	-	>4.2
	SARS-CoV-2	2.8×10^6	2.8×10^6	>7	++	+++ *	>7

The two lysis buffers MC136A and MC501C were, in the absence of proteinase K/heat, unable to fully inactivate AdV-5; MC143A with no proteinase K/heat did not completely eliminate SARS-CoV-2. Interfering substances mimicking protein conditions in clinical samples did not affect viral inactivation by any of the buffers.

4. Discussion

In recent years and during the course of this study, different viral pathogens became relevant in the laboratory or new ones emerged. This resulted in a certain heterogeneity in the test panels for the various viral pathogens. Beyond this, a comprehensive direct side-by-side comparison of a large array of relevant pathogens was not the main focus of this work. We rather intended to examine in detail the possible impact of a common formaldehyde-based fixation buffer or of commercial lysis buffers that are commonly used in research laboratories on preparations of viruses from representative classes. To better reflect the heterogeneous composition of virus-containing specimens, several typical protein conditions as potentially interfering substances were examined.

A successful inactivation was verified using the sensitive method of three consecutive blind virus passages. As any trace of replication-competent virus in one culture would be amplified during the next blind passage, the absence of infectivity after a 3-week observation may be taken as a safe indicator for complete viral inactivation. This sensitive culture method revealed and confirmed that for adenoviruses and SARS coronaviruses, not every lysis buffer may be suitable.

For a chemical treatment with FA, the results validate the full inactivation of high titer stocks of enveloped (HIV-1, SARS-CoV-2) as well as non-enveloped viruses (AdV-5, ECHO-11) with a 30-minute incubation period at room temperature. As the lipid bilayers of the viral envelopes render these virus particles less stable than the protein-coated capsids of adenoviruses or enteroviruses, this is in full agreement with their complete inactivation within 15 min.

Also, the non-enveloped picornavirus ECHO-11 was effectively inactivated within this time but retained some viability when exposed to FA for only 5 min. For AdV-5, another non-enveloped virus candidate, the period of 15 min was not fully sufficient, as in some cultures, replicative AdV-5 virus could be recovered after passage.

The complete inactivation of the enveloped viruses in the panel is in agreement with the property that lipid bilayers are principally highly sensitive to detergents. Accordingly, HIV-1 and SARS-CoV-2 (enveloped) were readily inactivated by the tested lysis buffers. This was also observed for the non-enveloped Echo-11 virus.

In remarkable contrast, AdV-5 was only incompletely inactivated by AVL, RLT, and Trizol, which was an observation also reported by others [14].

For the buffers MC501, MC143A, and MC136A, the thermal or proteolytic inactivation step of the specimens (proteinase K or 56 °C heat treatment) had deliberately been omitted: as it is known that heat is able to affect and inactivate viruses, this study rather focused on the very properties of the chemical lysis buffer compositions. Consequently, insufficient inactivation cannot be concluded due to not adhering to the manufacturer's protocol. Nevertheless, without the heat- or proteinase-K step, SARS-CoV-2 and AdV-5 are only incompletely inactivated by buffers MC501, MC143A, or MC136A.

This study shows that viral susceptibility to different lysis buffers can greatly vary among virus orders and families. The fact of residual viral infectiousness being likely or possible when using certain lysis buffers/processes must be recognized and taken into account in quality management processes for laboratory work involving viruses.

5. Conclusions

- This study demonstrates the relevance of a thorough assessment of the reliable inactivation of viruses and the risk of residual infectiousness in samples in laboratory and clinical settings.

- We demonstrate that not all buffers or fixatives readily inactivate every virus when applying standard conditions, which indicates that, especially for new pathogens, safe conditions must be verified and validated that may require a prior evaluation of the inactivation system to be used.
- An interference of high protein contents and other potentially interfering supplements as reported by others [2] for inactivation processes was not confirmed with our test panel.

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Conflicts of Interest: The authors declare no conflict of interest.

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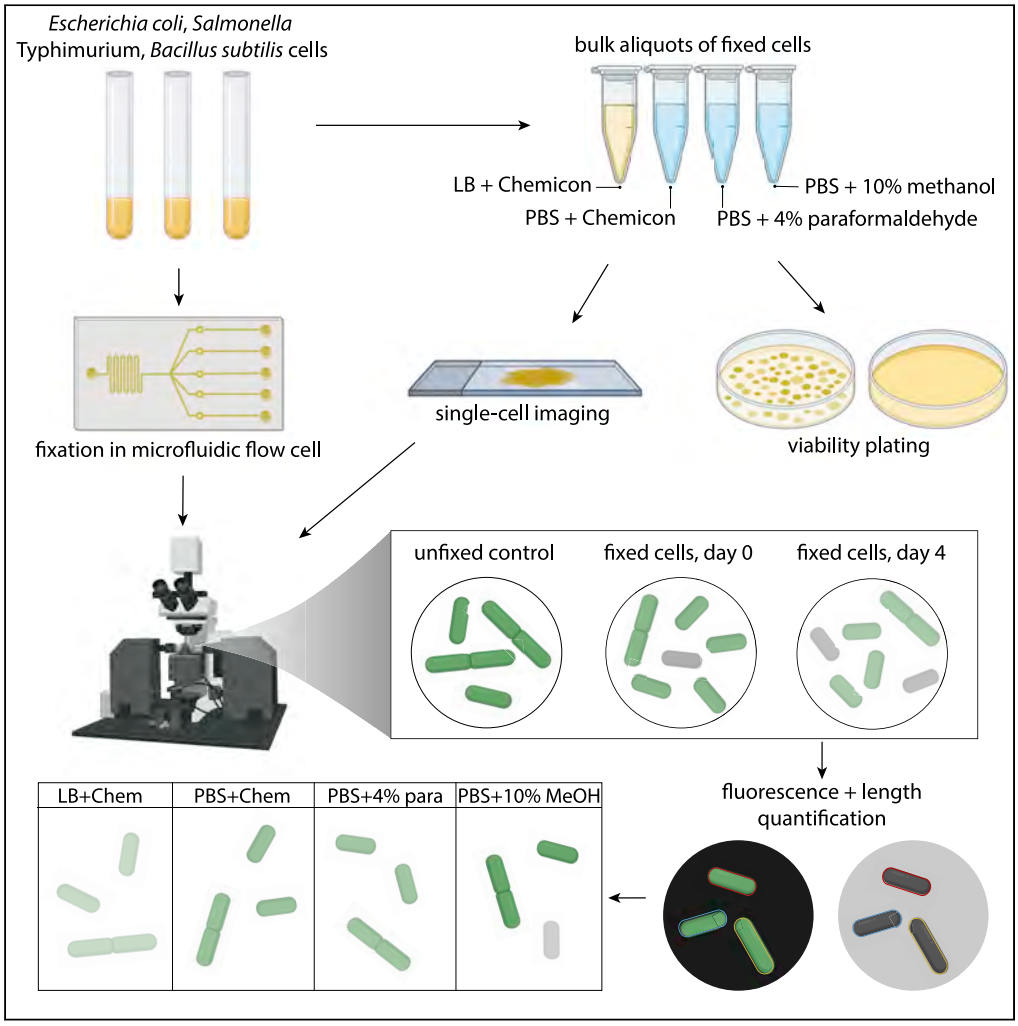

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Highlights

Effects of fixation on cellular dimensions and fluorescence patterns were quantified

Length and cytoplasmic GFP exhibited rapid dynamics during formaldehyde fixation

Methanol preserved fluorescence but did not fully inhibit growth and caused lysis

Extended storage faces tradeoffs of fluorescence maintenance and membrane integrity

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Effects of fixation on bacterial cellular dimensions and integrity

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SUMMARY

Fixation facilitates imaging of subcellular localization and cell morphology, yet it remains unknown how fixation affects cellular dimensions and intracellular fluorescence patterns, particularly during long-term storage. Here, we characterized the effects of multiple fixatives on several bacterial species. Fixation generally reduced cell length by 5–15%; single-cell tracking in microfluidics revealed that the length decrease was an aggregate effect of many steps in the fixation protocol and that fluorescence of cytoplasmic GFP but not membrane-bound MreB-msfGFP was rapidly lost with formaldehyde-based fixatives. Cellular dimensions were preserved in formaldehyde-based fixatives for $\geq$4 days, but methanol caused length to decrease. Although methanol preserved cytoplasmic fluorescence better than formaldehyde-based fixatives, some *Escherichia coli* cells were able to grow directly after fixation. Moreover, methanol fixation caused lysis in a subpopulation of cells, with virtually all *Bacillus subtilis* cells lysing after one day. These findings highlight tradeoffs between maintenance of fluorescence and membrane integrity for future applications of fixation.

INTRODUCTION

Bacterial cell shape is a critical physiological parameter that is connected with a variety of behaviors including adhesion, motility, immune system evasion, and antibiotic tolerance (Young, 2006). Long-term evolution of the Gram-negative bacterium *Escherichia coli* selected for cells with increased cellular volume coupled to increased fitness relative to the parental strain (Lenski and Travisano, 1994), suggesting that cell size is at least correlated with fitness and may be directly selected on. Cell shape and size are defined by the cell wall, a rigid macromolecule formed from glycan strands cross-linked by short peptides (Holtje, 1998). The cell wall is important for maintaining the mechanical integrity of bacterial cells: it resists swelling due to large internal turgor pressures (~1 atm (Deng et al., 2011)), and thus cells that lack a cell wall become osmotically sensitive. Although the cell wall is generally associated with determination of cell shape, recent studies have shown that proteins involved in a variety of other processes such as metabolism (Elbaz and Ben-Yehuda, 2010), DNA replication (Bazill and Retief, 1969; Harry et al., 1999; Hirota et al., 1968; Mann et al., 2017; Yoshikawa, 1970), and translation (Peters et al., 2016) can also play major roles in morphogenesis. Cell shape can therefore be a critical parameter that is informative of the internal cellular state. For example, filamentation can be an indicator of defects in cell division or DNA damage (Schoemaker et al., 1984), and increases in cell width are associated with nutritional transitions (Grover et al., 1980; Woldringh et al., 1980; Zaritsky et al., 1993).

Achieving a deeper understanding of bacterial cell biology will likely benefit from extensive, unbiased screens of genetic libraries and chemical perturbations. Measurements of morphology and the intracellular patterns of DNA and membrane staining have been used to define cytological profiles that revealed the mechanisms of action of novel antibiotics (Martin et al., 2020; Nonejuie et al., 2016). Although high-throughput systems biology has driven advances that enable rapid imaging of large strain libraries (Campos et al., 2018; Kuwada et al., 2015; Shi et al., 2017; Ursell et al., 2017; Werner et al., 2009), in the time required for imaging cells can continue to grow or change physiology, motivating fixation methods to "freeze" the physiological state of cells for sequential imaging.

Fixation is ubiquitous in cell biology. In the late 19th century, formaldehyde was discovered to be an antibacterial agent that hardened tissues and yielded excellent histological staining (Fox et al., 1985). Fixatives denature proteins via coagulation or cross-linking, thereby terminating biochemical reactions

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(Kiernan, 2000). In addition to preserving cells in a particular state, fixation also allows cells to be permeabilized to introduce fluorescent antibodies targeting a protein of interest for immunofluorescence microscopy, which has revealed intracellular organization patterns in bacteria such as localization of the divisome (Den Blaauwen et al., 1999; Pende et al., 2018) and of sporulation proteins (Pogliano et al., 1995). By tagging native proteins, immunofluorescence microscopy avoids the need to construct a version of the protein of interest fused to a fluorescent protein and can be used to validate localization conclusions based on fluorescent protein fusions that may not be completely functional. Importantly, while cells are thought to remain stable after fixation for at least one week (Levin, 2002), the impacts of fixation on cell morphology and on fluorescence levels remain mostly unknown on both short and long timescales. Multiple studies reported decreased cell numbers and altered fluorescence histograms after storage of fixed marine bacteria (Kamiya et al., 2007; Troussellier et al., 1995), and another study found fixative-dependent effects on *Yersinia pestis* cell morphology (Wang et al., 2016). Thus, it is critical to evaluate whether and how fixed cells change over time, especially given that certain labeling protocols (e.g. 16S rRNA fluorescence *in situ* hybridization) require overnight incubation (Schramm et al., 2002). Moreover, even careful fixation can alter samples and potentially introduce artifacts; for example, the mesosome was purported to be an organelle within Gram-positive bacteria (Van Iterson, 1961) but was later shown to be an artifact of fixation (Ryter, 1988).

Fixatives also increase the mechanical strength or stability of tissues (Lee et al., 1989; Talman and Boughner, 1995), yet the mechanical and morphological effects of fixatives on bacterial cells have not been studied. Gram-negative bacteria, which have a relatively thin cell wall compared with Gram-positive bacteria (Holtje, 1998), possess an outer membrane external to the cell wall. The outer leaflet of the Gram-negative outer membrane contains mainly lipopolysaccharide (LPS) molecules instead of phospholipids, and ionic bonds between LPS molecules as well as protein components endow the outer membrane with mechanical stiffness comparable to that of the cell wall (Rojas et al., 2018). The outer membrane also acts as a barrier to many chemicals (Ruiz et al., 2006). In Gram-positive bacteria, the cell wall is intercalated with teichoic acids that could similarly affect envelope stiffness and permeability. Thus, fixation could increase or decrease envelope stiffness depending on its relative effects on the molecules that stabilize each layer, impacting cell size by changing the mechanical balance with turgor pressure and decreasing intracellular fluorescence if some cytoplasmic molecules are lost during fixation. Overall, despite the importance of fixation for drawing meaningful biological conclusions about bacterial structure and physiology, its quantitative effects on bacterial cells have yet to be sufficiently quantified.

To close this knowledge gap, here we systematically tested the effects of several common fixatives on three model rod-shaped bacteria: Gram-negative *Escherichia coli* MG1655 and *Salmonella enterica* serovar Typhimurium 12023 and Gram-positive *Bacillus subtilis* 168. We found that cell size generally decreased as a result of fixation. By tracking single cells in a microfluidic flow cell throughout the fixation protocol, we determined that most steps exert measurable effects on cell length and that cytoplasmic fluorescence rapidly decreased during exposure to the fixative while MreB-msfGFP levels were maintained. We measured the fluorescence, dimensions, and integrity of each species over several days after fixation and discovered that losses in fluorescence and disrupted cell envelope integrity were dependent on the particular fixative used. These findings constitute an important resource for predicting and evaluating the degree to which fixation affects quantitative cell biology measurements.

RESULTS

Fixation impacts cellular dimensions in a variable manner across fixatives and bacterial species

There are a variety of ways in which fixation could impact bacterial morphology, for instance by disrupting the membrane or by changing turgor pressure. We sought to determine the extent to which various common fixatives preserve cellular dimensions in three model bacterial species that differ in the composition of their cell envelopes. *E. coli* and *S.* Typhimurium have an outer membrane and a thin (~2-4 nm (Gan et al., 2008)) cell wall, while *B. subtilis* lacks an outer membrane but has a thick (~30 nm (Misra et al., 2013)) cell wall. Some fixatives have been reported to affect membrane structure (Pogliano et al., 1999), leading us to hypothesize that Gram-negative and Gram-positive species might be differentially affected by fixatives due to their different envelope architectures. Each of the strains we investigated also expressed cytoplasmic green fluorescent protein (GFP, transparent methods), enabling us to measure any loss of fluorescent proteins during fixation.

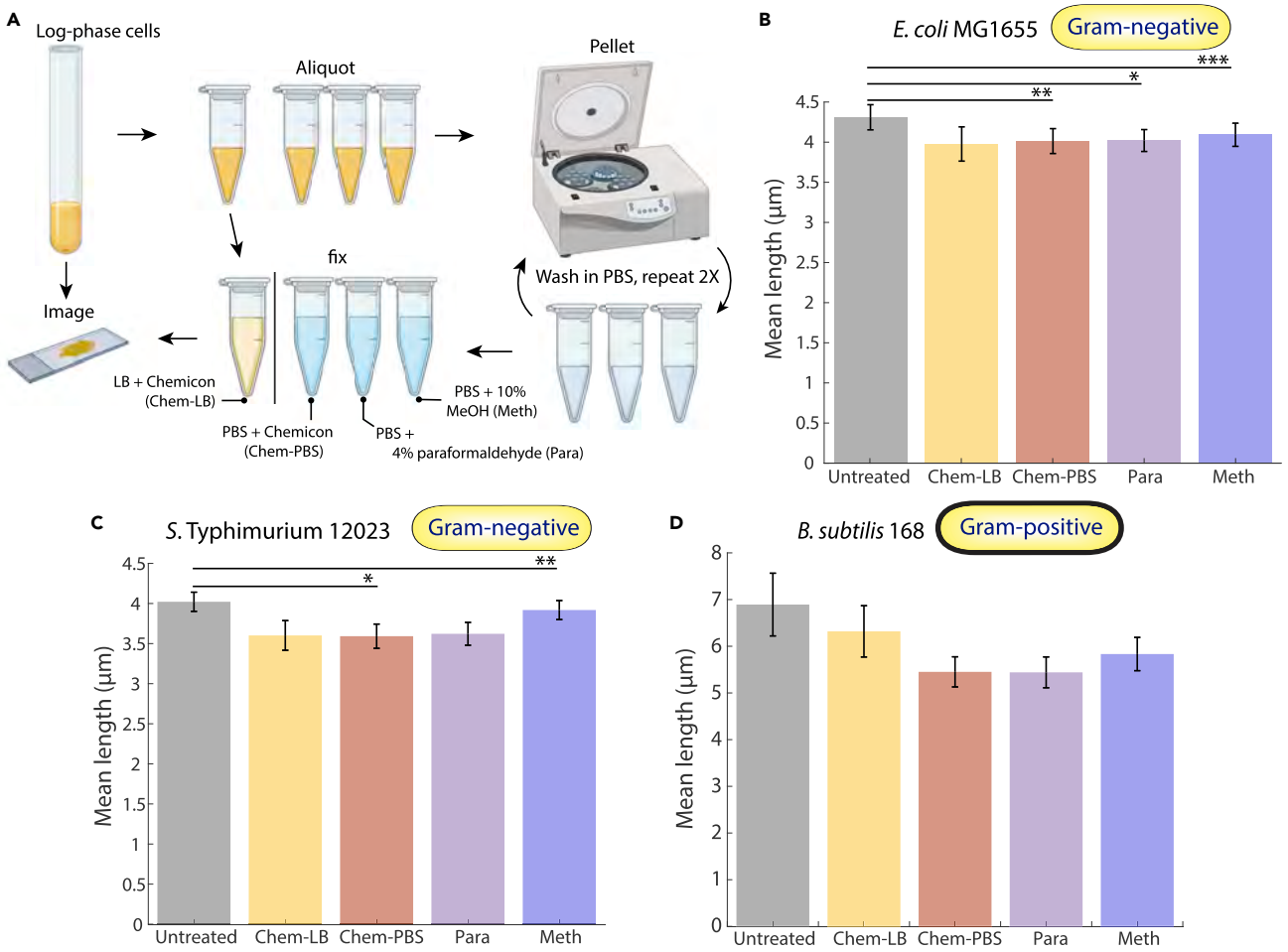

Figure 1. Fixation generally results in decreases in bacterial cell size

(A) Schematic of fixation protocol. A log-phase culture grown in LB was split into several aliquots: one was immediately imaged, one was fixed in Chemicon without washing and then imaged, and the other three were washed twice in PBS and then fixed in Chemicon, paraformaldehyde, or methanol before imaging (transparent methods). Fixed cells were imaged and segmented to quantify cellular dimensions and average fluorescence and compared to unfixed cells.

(B–D) Mean lengths of *E. coli* (B), *S.* Typhimurium (C), and *B. subtilis* (D) cells before (untreated) and after fixation. The fixation protocol resulted in similar length decreases for *E. coli* and *S.* Typhimurium (Gram-negative, thin cell wall) and larger decreases in *B. subtilis* (Gram-positive, thick cell wall), likely due to disruption of chaining. Measurements are means of ≥3 experiments, and error bars represent 1 standard error of the mean. Each replicate experiment involved $n \geq 152$ cells. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ by paired t-test.

For each species, we grew cultures into log-phase growth and split them into several aliquots (Figure 1A). One aliquot was spotted immediately onto 1% agarose pads with lysogeny broth (LB) and imaged to determine the size and dimensions of live cells (transparent methods). We fixed cells in another aliquot by adding 5X Chemicon, a commercial fixation solution containing proprietary amounts of formaldehyde, methanol, and various buffering agents, for a final concentration of 1X Chemicon in LB; this aliquot was to test the necessity of removing the culture medium prior to fixation. The remaining aliquots were spun down and washed twice with phosphate-buffered saline (PBS) before resuspension in PBS with one of three common fixatives: 1X Chemicon, 4% paraformaldehyde, or 10% methanol (transparent methods). Immediately after this protocol, we acquired phase-contrast images of hundreds of cells for each condition on LB agarose pads, segmented cells, and quantified cellular dimensions (transparent methods).

The mean cell length and volume of unfixed *E. coli* MG1655 cells were 4.33 ± 0.16 μm (Figure 1B) and 7.30 ± 1.80 μm^3 (Figure S1A), respectively. Length and volume measurements were highly correlated as expected (Figure S1A); hence, we focused on cell length for simplicity. In all four fixation conditions, cell length decreased (4–7%), with the greatest decrease in Chemicon (Figure 1B). We observed similar levels

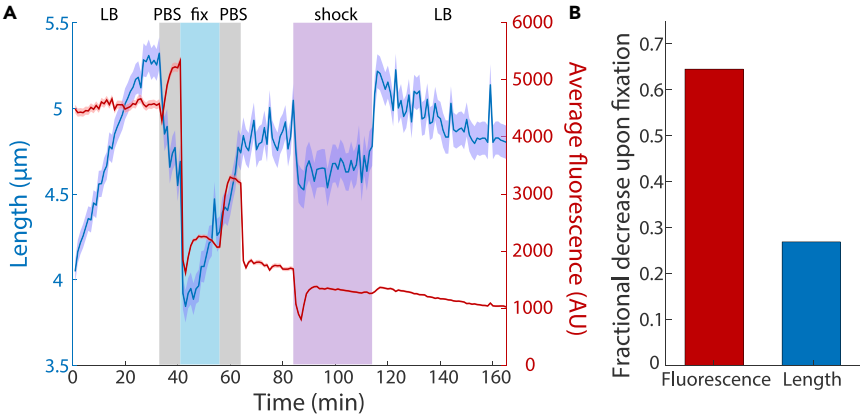

Figure 2. Cell length varies throughout the fixation protocol

(A) Mean cell length (blue) and fluorescence (red) for *E. coli* MG1655 cells expressing cytoplasmic GFP tracked in a microfluidic flow cell (transparent methods) and subjected to the fixation protocol followed by osmotic shocks. Cells were equilibrated for 30 min in LB to achieve exponential growth and then switched to 1X PBS for 8 min to mimic washing. Cells were then fixed in PBS with 1X Chemicon for 15 min and transferred back to PBS for a second wash. To ensure successful fixation, cells were switched to LB for 20 min and no growth occurred. Finally, cells were exposed to a hyperosmotic shock with LB+1 M sorbitol for 30 min, which demonstrated that the membranes still acted as a permeability barrier (Figure S2A). Length varied over many steps of the protocol, while fluorescence decreased dramatically primarily upon fixation. The slow decrease after fixation is likely due to photobleaching. Lines are mean values, and shaded regions represent 1 standard error of the mean (n = 150 cells).

(B) The fractional decrease in cell length and fluorescence between t = 30 min (just before the first PBS wash) and 43 min (just after introduction of the fixative).

of shrinkage in *S.* Typhimurium (3–11%), with Chemicon again resulting in the largest decrease (Figure 1C). For *B. subtilis*, the decrease was much more pronounced for all fixation conditions following washing (15–20%) compared with direct fixation with Chemicon in LB (8%). The enhanced decrease was due at least in part to the disruption of *B. subtilis* chaining during centrifugation; washing of live cells in PBS resulted in a similar ~15–20% decrease in cell length (Figure S1B). Thus, fixation variably impacts measurements of cell morphology, although in a mostly systematic manner.

Cell size is affected by many steps in the fixation protocol

Since fixation protocols generally involve several resuspensions in solutions such as PBS that can have differing osmolality, it was unclear whether the length decreases we observed were directly due to fixation or to physiological changes such as a reduction in turgor pressure. To analyze the shape dynamics of individual *E. coli* cells during fixation, we carried out the entire Chemicon fixation protocol in a microfluidic flow cell (Video S1, transparent methods). We first equilibrated log-phase cells in the flow cell in LB for 30 min (after 1 hr of outgrowth from stationary phase in a test tube) so that they achieved exponential growth, and then rapidly (in <10 s) switched the medium to 1X PBS for 8 min to mimic the time scale of washing in a centrifuge. Immediately after the cells were transferred to PBS, they displayed a rapid decrease in length (Figure 2A), presumably due to hyperosmotic shock since LB has lower osmolality (240–260 mOsm/kg (Rojas et al., 2014)) than PBS (~290 mOsm/kg). Consistent with a reduction in turgor pressure, cell width also decreased upon the transfer to PBS (Figure S2A). Following the shock and initial rapid decrease in length, the cytoplasm gradually shrank by ~6–10% (Figure 2A), consistent with other reports that transfer into a medium lacking carbon causes shrinkage of the cytoplasm away from the cell wall (Shi et al., 2020). After the PBS wash, cells were transferred into PBS with 1X Chemicon for 15 min of fixation. Cells initially shrank even further, reaching a ~27% decrease relative to pre-wash (Figure 2B), but then began to increase in length after ~6 min and partially recovered to near their starting length at the end of the preceding PBS wash (Figure 2A). Upon transfer from the fixative back to PBS for 8 min to mimic a second wash, cell length increased rapidly back to approximately the mean length at the end of the first wash (Figure 2A), presumably due to the hypoosmotic shock induced by transfer from 1X Chemicon to PBS. The final single-cell lengths were consistently lower by ~5–10% than the lengths before fixation, consistent with our bulk culture measurements on LB agarose pads (Figure 1B) and thus indicating that imaging on agarose pads does not itself affect cell size in a fixation-dependent manner.

To isolate the effects of Chemicon on cell length, we repeated the above protocol with LB substituted for PBS, avoiding the wash prior to Chemicon addition. We observed a ~10% decrease in length during fixation that rapidly reversed after cells were returned to LB, indicating that Chemicon addition causes a hyperosmotic shock relative to LB (Figure S2B). These data are consistent with the length decrease in bulk culture measurements, given that we did not wash cells fixed with Chemicon+LB in bulk culture prior to imaging (and hence they would resemble the cells during fixation in the microfluidic flow cell). Taken together, the overall length decrease is a function of both washing in PBS and the added fixative solution.

To ensure that *E. coli* cells were actually fixed by Chemicon (and therefore dead), after the second PBS wash in our initial experiment, we switched the medium in the flow cell to LB for 20 min. We observed an initial, rapid increase in mean cell length (Figure 2A), due to the hypoosmotic shock from the lower osmolality of LB compared with PBS. Subsequently, no cells grew, suggesting that all cells had been effectively fixed.

To determine the extent to which the inner and outer membranes still acted as a permeability barrier after fixation, we applied a hyperosmotic shock with LB+1 M sorbitol to post-fixation cells in the microfluidic device (transparent methods). Cells rapidly shrank by ~10% (Figure 2A), similar to the response of growing *E. coli* cells (Rojas et al., 2014). We then switched the cells back to LB, which caused a hypoosmotic shock that fully recovered mean cell length back to the mean post-fixation length (Figure 2A). Thus, fixation with Chemicon does not completely disrupt the ability of the *E. coli* membranes to act as a permeability barrier.

Chemicon fixative solution causes a rapid decrease in cytoplasmic fluorescence

In our microfluidic flow-cell experiments with *E. coli* cells expressing cytoplasmic GFP, during both PBS washes the average fluorescence intensity (total fluorescence per unit area) increased, by up to ~2-fold (Figure 2A). The increase was coincident with cell shrinking during the first wash but not the second wash (Figure 2A), and no such large increases were observed when the cells were washed in LB rather than PBS (Figure S2B). To investigate whether imaging in PBS resulted in a fluorescence increase relative to imaging in LB, we quantified the fluorescence of live cells and fixed cells without and with washing in PBS. After background normalization, PBS washing of live cells resulted in a 10–15% increase in fluorescence (Figure S3A). PBS washing of fixed cells resulted in a larger, ~2-fold increase (Figure S3B). These increases were approximately consistent with the magnitudes of fluorescence increase in the microfluidic flow cell during the washes before and after fixation (Figure 2A), suggesting that imaging in PBS versus LB itself affects fluorescence levels in a manner independent of the difference in backgrounds.

After the switch to Chemicon solution in the microfluidic flow cell, there was a rapid (within 2 min), dramatic decrease in GFP fluorescence of ~60% (Figures 2A and 2B). This decrease was followed by partial fluorescence recovery during the first 5–10 min, possibly due to maturation of fluorophores, followed by a slight decrease for the remainder of the time in Chemicon (Figure 2A). After the second wash, we surmised that cells were no longer producing GFP as fixation should render them metabolically inactive and that the slow decrease in fluorescence, at a rate similar to that at the end of Chemicon exposure (Figure 2A), was likely due to photobleaching. Relative to live cells, the decrease in fluorescence in *E. coli* due to fixation was >60% (Figure 2B), similar to measurements from bulk cultures in Chemicon (Figures 3A and S3B).

Bulk cultures yielded similar fluorescence decreases in Chemicon+LB and paraformaldehyde+PBS as in Chemicon+PBS, while fluorescence levels were largely maintained with methanol (Figure 3A). *S.* Typhimurium cells experienced similar decreases as *E. coli* (Figure 3B). *B. subtilis* cells maintained cytoplasmic GFP fluorescence in all fixation conditions except direct fixation in LB (Figure 3C), indicating that washing is important for fluorescence quantification in *B. subtilis*. Thus, the large decrease in cytoplasmic GFP fluorescence with formaldehyde-based fixatives likely occurs very rapidly upon introduction of the fixative, while methanol fixation largely avoids fluorescence loss in all three species.

MreB levels do not decrease during fixation

While cytoplasmic GFP levels decreased by >2-fold upon fixation for *E. coli* and *S.* Typhimurium (Figures 2A, 2B, and 3A), many proteins with nonuniform localization patterns bind to the membrane or are membrane associated, which may prevent protein loss during fixation. MreB is an essential protein that forms short, membrane-bound filaments and is required for rod-shaped growth in *E. coli* and many other rod-shaped bacteria (Shi et al., 2018). To study the effects of fixation on MreB levels, we employed an *E. coli* MG1655 strain that expresses a sandwich fusion of MreB to msfGFP (MreB^{sw}-msfGFP) from the

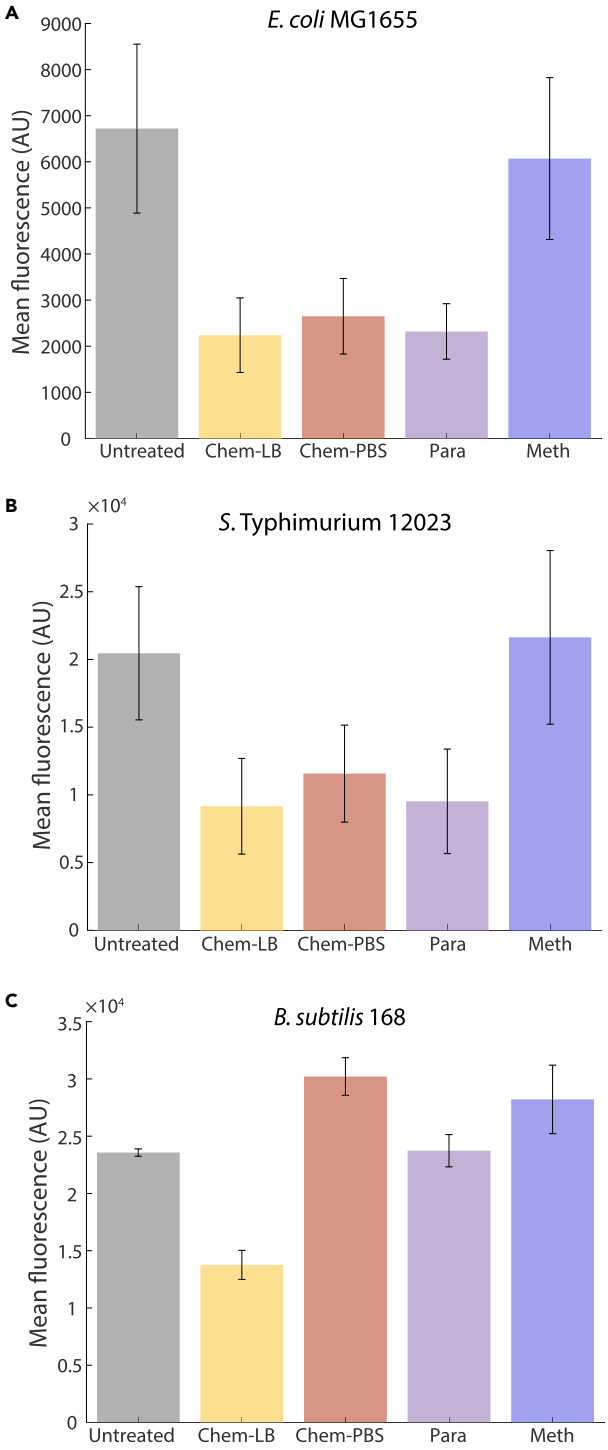

Figure 3. Methanol generally maintains cytoplasmic fluorescence levels, while the effects of formaldehyde-based fixatives are dependent on the species and on washing prior to fixation

(A and B) For *E. coli* (A) and *S.* Typhimurium (B), there was a ~60% loss in intracellular fluorescence directly after the fixation protocol with any formaldehyde-based fixative (but not methanol), presumably due to the sharp decrease we observed 2 min after switching to Chemicon solution in a microfluidic flow cell (Figure 2A).

(C) *B. subtilis* cells maintained cytoplasmic fluorescence in all fixatives, as long as cells were washed prior to fixation. Measurements are averages over 3 experiments, and error bars represent 1 standard error of the mean. Each replicate experiment involved $n \geq 152$ cells.

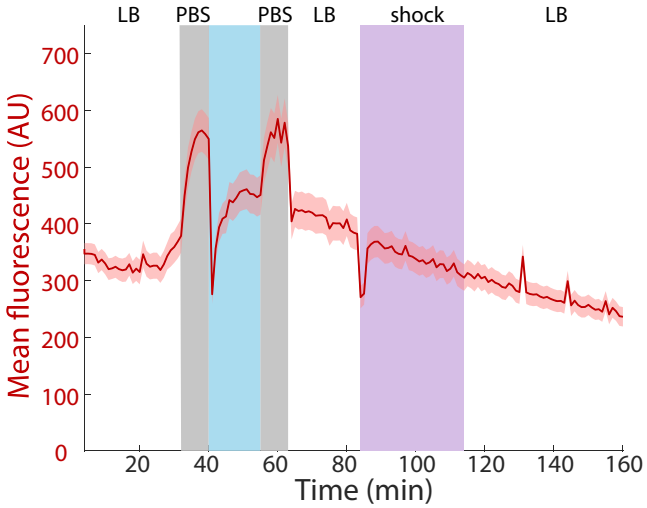

Figure 4. Levels of fluorescent protein fused to MreB were not strongly affected by fixation

Mean fluorescence of *E. coli* MG1655 cells expressing MreB^{sw}-msfGFP subjected to the fixation protocol as in Figure 2A. msfGFP levels were much less affected by fixation than cytoplasmic GFP (Figure 2A). The slow decrease after fixation is likely due to photobleaching. Lines are mean values, and shaded regions represent 1 standard error of the mean (n = 51 cells).

chromosome as the sole copy of *mreB* (Ursell et al., 2014); this strain has approximately wild-type-like growth rate and cell shape (Ouzounov et al., 2016). We performed the fixation protocol in a microfluidic flow cell as above and found that MreB^{sw}-msfGFP fluorescence dynamics exhibited a major difference compared with wild-type cells expressing cytoplasmic GFP (Figure 2A): the decrease in MreB^{sw}-msfGFP fluorescence immediately after Chemicon introduction was not as large, and more importantly, fluorescence levels recovered back to pre-fixation levels with 5 min (Figure 4). These data indicate that the decrease in cytoplasmic GFP is likely due to loss of GFP molecules rather than denaturation and that loss of membrane-bound proteins such as MreB^{sw}-msfGFP may be much less than cytoplasmic proteins.

The treatment time necessary for fixation varies across fixatives

Since *E. coli* cells still exhibited morphological dynamics throughout and after exposure to Chemicon (Figure 2A), we next assessed cell viability over various treatment times in fixative. We treated *E. coli* cells with Chemicon in LB or PBS-washed cells with 1X Chemicon, 4% paraformaldehyde, or 10% methanol and then plated on LB without fixative to test for viable cells (defined as the ability to form a colony; note that any remaining fixative was diluted substantially) at fixation durations of 0 min (exposure to the fixative and then plating within 1 min), 5 min, 15 min, and 60 min. The viability of cells treated with Chemicon or paraformaldehyde was severely attenuated (by >100-fold) within 1 min, and no cells were viable after 5 min (Figure 5A). By contrast, methanol reduced viability by only 1–2 orders of magnitude even for 1 hr of treatment (Figure 5A). Similar behavior was observed for *S.* Typhimurium and *B. subtilis* (Figure S4).

To interrogate the physiological state induced by methanol treatment, we exposed *E. coli* cells to 10% methanol for 15 min and then placed them on LB-agarose pads for 2 hr of single-cell imaging. 33 of 64 cells exhibited growth (Figure 5B, Video S2), roughly consistent with our plating results (Figure 5A). Of the cells that grew, their growth (Figure 5B) was considerably slower than untreated cells, which have doubling times of ~60 min at room temperature (Barber, 1908), indicating that the methanol also affected the ability of cells to grow. Even after 2 hr of methanol treatment, some cells were able to grow and divide (Figure S5A), at a rate only slightly lower than those that grew after 15 min of treatment (Figure S5B). Thus, although typical methanol treatments reported in the literature are for 15–60 min, *E. coli* cells are not necessarily fully killed by this treatment interval.

Fluorescent protein concentration in fixed cells decreases over days in a fixative-dependent manner

Since our microfluidic experiments revealed that fluorescence initially decreased rapidly upon exposure to the fixative but then stabilized for at least tens of minutes (aside from photobleaching, Figure 2A), we next

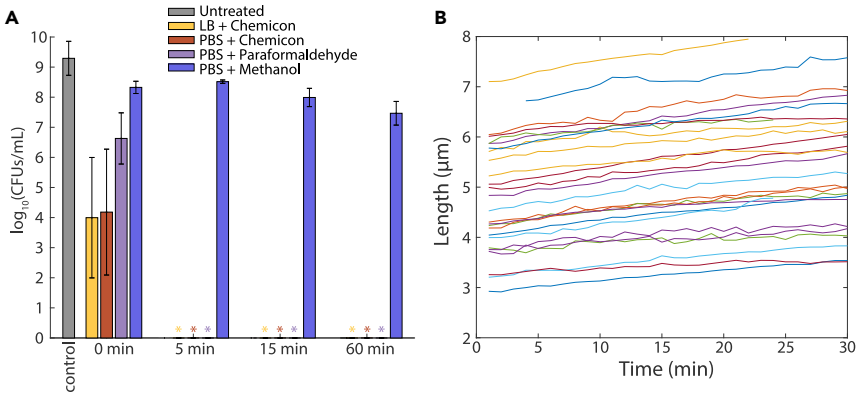

Figure 5. Some *E. coli* cells are still able to grow after one hour of methanol fixation, while other fixatives halt growth almost immediately

(A) Colony forming units (CFUs) of *E. coli* cells treated for varying amounts of time with different fixatives. Cells plated immediately after resuspension in Chemicon or paraformaldehyde (0 min) exhibited reductions of multiple orders of magnitude in colony forming ability, while 60 min of methanol fixation still permitted growth of $>10^7$ CFUs/mL. Asterisks indicate that no colonies were observed. Values are means and error bars represent 1 standard deviation of $n = 3$ independent replicates.

(B) Single-cell growth at room temperature was impacted by 15 min of methanol fixation. Thirty three of 64 cells exhibited growth (26 representative cells shown here) but with a substantial decrease in growth rate relative to untreated log-phase cells (Figure S5B).

asked whether fluorescence levels in fixed cells remain stable over long periods (days) relevant to laboratory experiments. We stored samples of *E. coli*, *S.* Typhimurium, and *B. subtilis* after 15 min of fixation in Chemicon+LB, or Chemicon, 4% paraformaldehyde, or 10% methanol in PBS at 4°C; all samples except Chemicon+LB were washed and resuspended in PBS prior to storage. We imaged an aliquot every 24 hr to quantify cell size and fluorescence. For *E. coli* (Figure 6A) and *S.* Typhimurium (Figure 6B), the mean length of intact cells changed only slightly over 4 days in any of the fixatives, while *B. subtilis* cell length decreased by ~0.5 μm (Figure 6C).

For some fixatives, cytoplasmic GFP fluorescence intensity decreased over time, suggesting loss of intracellular material. For *E. coli* (Figure 6D) and *S.* Typhimurium (Figure 6E), methanol preserved fluorescence intensity better than formaldehyde or Chemicon-based fixation, consistent with our observation that many cells remain alive after methanol exposure (Figure 5B); for *E. coli*, methanol fixation resulted in a ~25% decrease after 1 day, but the overall decrease relative to untreated cells was much less than in formaldehyde or Chemicon-based fixation (Figure 6D). For formaldehyde-based fixation, fluorescence intensity dropped the most (~2-fold) on day 1 (Figures 6D and 6E); as we observed in our microfluidic experiments (Figure 2A), this drop likely occurred almost immediately upon fixation. For all three species (Figures 6D–6F), fluorescence in Chemicon+LB continued to drop over time. To test whether washing Chemicon+LB-fixed cells in PBS would rescue the fluorescence decrease, we fixed *E. coli* cells as described above and split the solution into two aliquots. One aliquot was washed in PBS. Over 4.5 days, the msfGFP intensity of PBS-washed cells still decreased, remaining approximately twice that of unwashed cells (Figure S6).

While methanol exposure better preserved cytoplasmic fluorescence, we also observed an increasing fraction of "ghosts" (lysed cells, as visually assessed by the obvious loss of phase intensity) over time (Figures 6G–6I and S7), suggesting that methanol causes significant membrane damage. For *B. subtilis*, ~40% of cells lysed during fixation, and there were no cells with intact membranes one day later. Envelope integrity was more sensitive to methanol in *E. coli* than in *S.* Typhimurium (Figures 6G and 6H), consistent with the greater decrease in cell length (Figures 6A and 6B) and fluorescence (Figures 6D and 6E) in *E. coli* compared with *S.* Typhimurium cells over time. All lysed cells lacked cytoplasmic fluorescence (Figure S7). While there was a small number of ghosts in all fixatives (Figure S8), paraformaldehyde and Chemicon largely preserved the integrity of *E. coli* and *S.* Typhimurium cells over the course of 5 days (Figures 6H and 6I).

To test whether the fluorescence of membrane-associated proteins decreased in intensity over time after fixation, we processed MreB^{sw}-msfGFP cells with the same growth and fixation protocols as above. Similar

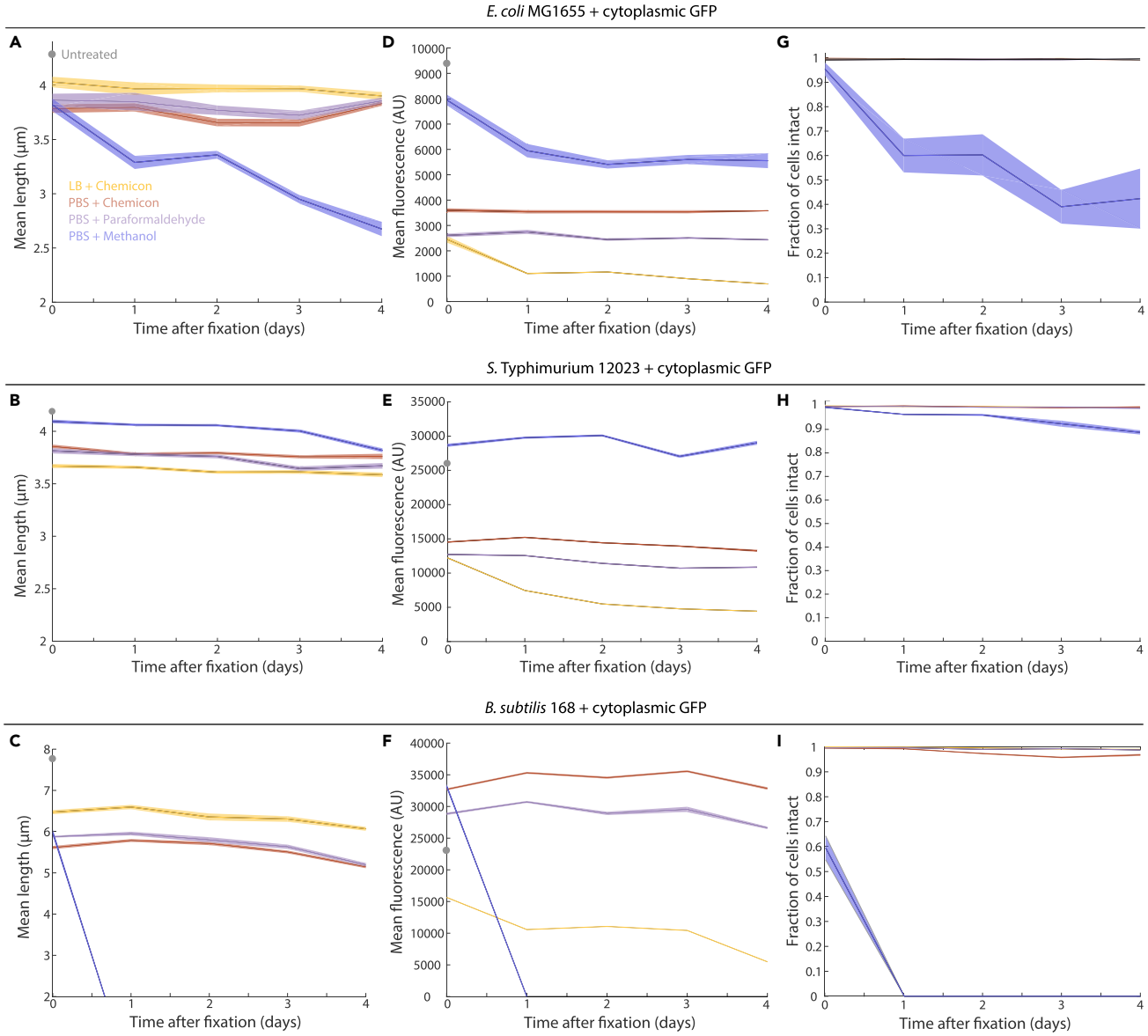

Figure 6. After multiple days of storage, cell length, intracellular fluorescence, and envelope integrity are affected in a fixative-dependent manner

(A–C) The mean cell length of *E. coli* (A), *S.* Typhimurium (B), and *B. subtilis* (C) cells over several days of storage at 4°C. *E. coli* and *S.* Typhimurium cells treated with formaldehyde-based fixatives approximately maintained cell length, while *B. subtilis* cells generally decreased somewhat in length and *E. coli* cells decreased substantially in methanol. No *B. subtilis* cells were intact 1 day after fixation in methanol (I), explaining the sharp decrease in length.

(D–F) For *E. coli* (D) and *S.* Typhimurium (E), intracellular GFP fluorescence intensity was maintained after the initial ~2-fold decrease in Chemicon or paraformaldehyde (Figures 3A and 3B). For *S.* Typhimurium, fluorescence was maintained at pre-fixation levels, while *E. coli* cells lost fluorescence over time after methanol fixation. For *B. subtilis* (F), fluorescence levels continued to decrease after Chemicon fixation in LB.

(G–I) Fixation in methanol led to an increasing number of lysed cells over time, particularly for *E. coli* (G) and *B. subtilis* (I), indicating cell membrane damage and loss of cellular contents.

Measurements are averages over cells and error bars represent 1 standard error of the mean, with $n \geq 152$ cells each. Gray circles are untreated cells.

length decreases were observed upon fixation (Figures 7A and 7B) as for wild-type cells (Figures 1B and 5B). However, in all but Chemicon+LB, msfGFP fluorescence actually increased over time (Figures 7C and 7D), consistent with the modest increase while exposed to Chemicon in our microfluidic experiments (Figure 4) and similar to previous observations of FtsZ-GFP in fixed versus live *B. subtilis* cells (Arjes et al., 2014). Moreover, cell length was essentially maintained in all fixatives, including methanol (Figure 7C); interestingly, the

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Staphylococcus aureus-specific IgA antibody in milk suppresses the multiplication of *S. aureus* in infected bovine udder

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Abstract

Background: Bovine mastitis caused by *Staphylococcus aureus* (*S. aureus*) is extremely difficult to control and new methods for its prevention and management are required. Nasal vaccines may prevent initial bovine mastitis infection caused by *S. aureus*. However, limited information is available regarding induction of mucosal immune response through nasal immunization with antigen and its suppression of *S. aureus* multiplication during bovine mastitis. This study sought to investigate whether induction of immunoglobulin A (IgA) in milk by nasal immunization could suppress multiplication of *S. aureus* in the bovine udder.

Results: Nasal immunization with formalin-killed *S. aureus* conjugated with a cationic cholesteryl-group-bearing pullulan-nanogel was performed. Anti-*S. aureus*-specific IgA antibodies were significantly more abundant in the milk of immunized cows than in non-immunized animals ($P < 0.05$). *S. aureus* counts in the quarter were negative in both non-immunized and nasal-immunized cows 1 week after mock infusion. In *S. aureus*-infused quarters, *S. aureus* multiplication was significantly suppressed in immunized compared with non-immunized cows ($P < 0.05$). Furthermore, a significant negative correlation was found between *S. aureus*-specific IgA antibodies and *S. aureus* counts in infused quarters of both non-immunized and nasal-immunized cows ($r = -0.811$, $P < 0.01$).

Conclusion: In conclusion, the present study demonstrates that *S. aureus*-specific IgA antibodies in milk successfully suppressed the multiplication of *S. aureus* in infected bovine udders. Although the exact mechanism explaining such suppressive effect remains to be elucidated, nasal vaccines that can induce humoral immunity may help prevent initial infection with *S. aureus* and the onset of bovine mastitis.

Keywords: *Staphylococcus aureus*, IgA, Milk, Bovine mastitis

Background

Bovine mastitis refers to inflammation of the mammary gland and can be caused by several bacterial species [1, 2]. It is a complex disease and a major source of economic loss for the dairy industry [1, 3]. *Staphylococcus aureus* is the most common etiologic agent of chronic, contagious, and intractable bovine mastitis [4, 5]. Because *S. aureus*-derived bovine mastitis is extremely difficult to control, new methods aimed at its prevention and containment are required. Vaccination is an effective strategy to prevent inflammation, including bovine mastitis. Even though vaccines are used globally to protect cows from mastitis caused by *S. aureus* [6, 7], they are not sufficiently effective at preventing onset of the disease and minimizing its global economic fallout [8, 9]. Therefore, further research on improved effectiveness of the vaccine is required.

Systemic immunization ensures that adequate amounts of antigen reach peripheral lymphoid tissues and protect against infectious agents, however it is largely ineffective in providing immunity to mucosal surfaces [10]. To this end, mucosal vaccines have been proposed recently as effective control methods [11]. They induce a mucosal immune response by preventing the entry of pathogens into

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the body through the mucosal surface. Mucosal membranes can secrete immunoglobulin A (IgA) antibodies to block or inactivate pathogens [12]. Therefore, mucosal immune responses can function as an important first line of defence on the mucosal surface. Mucosal vaccines have been designed based on the premise that antigen uptake at inductive sites results in the dissemination of IgA-committed plasma cells to remote mucosal tissues and glands [13, 14]. In humans, protective mucosal immune responses are induced most substantially by mucosal immunization through the oral or nasal routes [15]. In the case of ruminants, the nasal cavity is one of the most promising administration sites because the oral route has high digestive enzymatic activity [16, 17] and may dissolve vaccine antigens. Thus, nasal vaccines may help prevent initial infection with *S. aureus* and avoid bovine mastitis. However, limited information is available regarding induction of mucosal immune responses through nasal immunization with antigen and its ability to suppress the multiplication of *S. aureus* in udders with bovine mastitis.

The objective of this study was to investigate whether induction of IgA in milk by nasal mucosa immunization could suppress multiplication of *S. aureus* in the bovine udder. Considering that mucosa-administered antigens are generally less immunogenic and may induce tolerance, potent mucosal adjuvants, vectors, or other special delivery systems are required for successful mucosal vaccination [18]. In this context, the use of toxin-based adjuvants is undesirable as it carries the risk that the toxin may reach the central nervous system [19, 20]. Moreover, following the failure of single antigen vaccine approaches against *S. aureus*, most efforts are now focused towards multiple antigen strategies [21]. To overcome these concerns, we used formalin-killed *S. aureus* (FKSA) supplied via an intranasal vaccine-delivery system with a nanometre-sized hydrogel consisting of cationic type of cholesteryl group bearing pullulan (cCHP) [22]. cCHP nanogels have been reported to interact with proteins and cell membranes through hydrophobic and electrostatic interactions [22]. The same study also suggested that cCHP nanogels formed complexes with FKSA, which could interact with the nasal mucosa. Therefore, to confirm that cCHP nanogel/inactivated *S. aureus* complexes could serve as a delivery system for mucosal immunization, we compared the uptake of inactivated *S. aureus* alone and cCHP nanogel/inactivated *S. aureus* complexes in Waldeyer's ring. cCHP nanogel facilitated early antigen uptake by enhancing delivery and adherence of the vaccine antigen to the nasal epithelium. In a previous study, high-magnification images showed that cCHP/botulinum type A neurotoxin was internalized into the nasal epithelium within 1 h following nasal administration and that it gradually detached from cCHP nanogel in nasal epithelial cells [22]. In addition, ex vivo uptake of microparticles by bovine pharyngeal tonsils revealed macrophages with intracellular beads in the deep regions of the epithelial layer 1 h after administration [23]. Based on the above evidence, we focused on events occurring 1 h after nasal administration. Experimental challenge studies with *S. aureus* have shown an effect of vaccination on the amount of bacterial shedding after the challenge [24]. Therefore, after nasal immunization of cows with inactivated *S. aureus*, we used experimental models of mastitis through mammary infection with *S. aureus* and investigated *S. aureus*-specific IgA antibody and *S. aureus* counts in milk. This study sets the basis for the future development of an efficient nasal vaccine against bovine mastitis.

Results

cCHP nanogel/*S. aureus*-conjugated FITC (SA-FITC) is taken up significantly more than SA-FITC alone in tissues from Waldeyer's ring

In calves given intranasal SA-FITC alone or cCHP nanogel/SA-FITC complexes, $FITC^+$/ $CD45^+$ cells were detected in the pharyngeal tonsil, tubal tonsil, palatine tonsil, and lingual tonsil, but not in the spleen and mesenteric lymph node. In particular, $FITC^+/CD45^+$ cells in the pharyngeal tonsil, tubal tonsil, and palatine tonsil were significantly higher following administration of cCHP nanogel/SA-FITC complexes than SA-FITC alone (mean % was 10.6 vs. 5.0 for pharyngeal tonsil; 7.2 vs. 3.2 for tubal tonsil; and 7.8 vs. 4.9 for palatine tonsil; $P < 0.05$) (Fig. 1d).

Anti-*S. aureus*-specific IgA and immunoglobulin G (IgG) antibodies in milk increases following nasal immunization with FKSA

To confirm increased presence of *S. aureus*-specific IgA antibodies in milk, three cows were repeatedly immunized with FKSA. The optical density (OD) values of anti-*S. aureus*-specific IgA antibodies in composite milk (three cows, $n = 3$) were significantly higher two, five, and 6 weeks after immunization than before immunization (mean ODs were 0.38, 0.61, 0.62, and 0.18, respectively; $P < 0.05$) (Fig. 2a); whereas OD values of anti-*S. aureus*-specific IgG antibodies in composite milk (three cows, n = 3) were not significantly changed (Fig. 2b).

Anti-*S. aureus*-specific IgA antibodies in milk is higher in immunized than in non-immunized cows

On pre-infusion day (day 0), after nasal immunization with FKSA, OD values of anti-*S. aureus*-specific IgA antibodies were significantly higher in composite milk from immunized cows than in milk from non-immunized controls (mean ODs were 0.50 vs. 0.18, respectively; $P < 0.05$) (Fig. 3a); whereas OD values of anti-*S. aureus*-specific IgG antibodies in milk did not differ significantly between non-immunized and nasal-immunized cows (Fig. 3b).

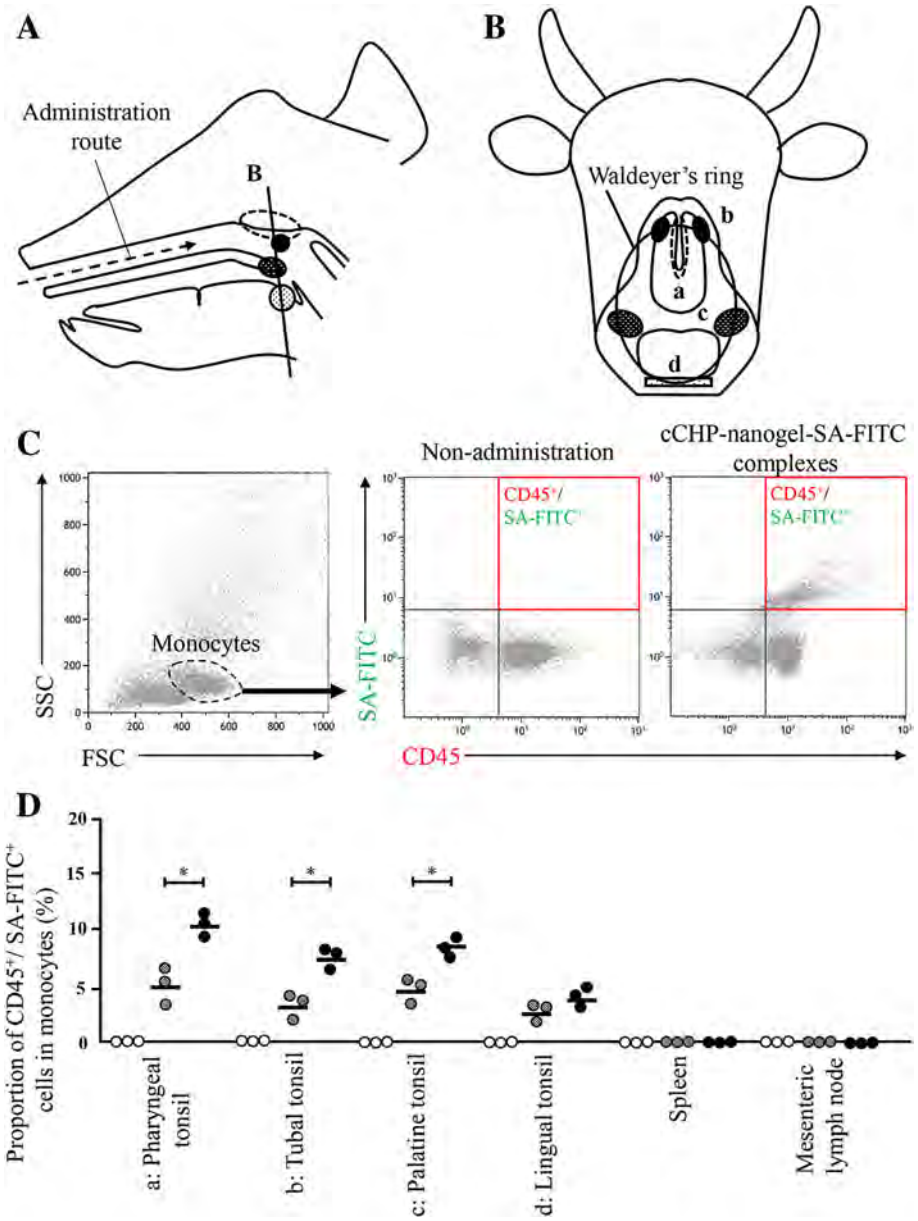

Fig. 1 Distribution of $CD45^+$ cells in calves lymphoid pharyngeal tissues (Waldeyer's ring), 60 min after intranasal administration of *S. aureus*-conjugated FITC. After nasal administration with *S. aureus*-conjugated FITC (SA-FITC) or cCHP nanogel-SA-FITC complexes, $FITC^+/CD45^+$ mononuclear cells of Waldeyer's ring, spleen, and mesenteric lymph node in calves were evaluated by flow cytometry. **a** Transversal section (plane indicated as B) of Waldeyer's ring and route of administration with SA-FITC or cCHP nanogel/SA-FITC complexes. **b** Location of the pharyngeal tonsils (**a**), paired tubal tonsils (**b**), paired palatine tonsils (**c**), and lingual tonsil (**d**). **c** Gating scheme for the identification of $CD45^+$ leukocytes based on forward and side light scatter characteristics of tissue monocytes. **d** Dot plot showing the percentage of $FITC^+/CD45^+$ mononuclear cells in pharyngeal tonsils (**a**), paired tubal tonsils (**b**), paired palatine tonsils (**c**), lingual tonsil (**d**), spleen, and mesenteric lymph node. Black circles correspond to nasal immunization with cCHP nanogel/SA-FITC complexes, grey circles to nasal immunization with SA-FITC, and white circles to non-immunization controls. *Significant difference between the mean of each group ($P < 0.05$)

Anti-*S. aureus*-specific IgA antibodies in milk increases following infusion with *S. aureus* BM1006

After *S. aureus* infusion, somatic cell count (SCC) increased dramatically and peaked at 3 days for composite milk and *S. aureus*-infused quarter; whereas no significant change was detected following mock infusion. No significant differences in SCC could be detected between day 0 and day 7 in non-immunized and nasal-immunized animals (Fig. 4). Three to seven days after *S. aureus* infusion, all the cows exhibited localized signs of clinical mastitis, including clots in the milk, swelling of the udders, and a loss of milk yield. One of the non-immunized cows presented also systemic signs, including elevated rectal temperature and loss of appetite. We then investigated the OD value of *S. aureus*-

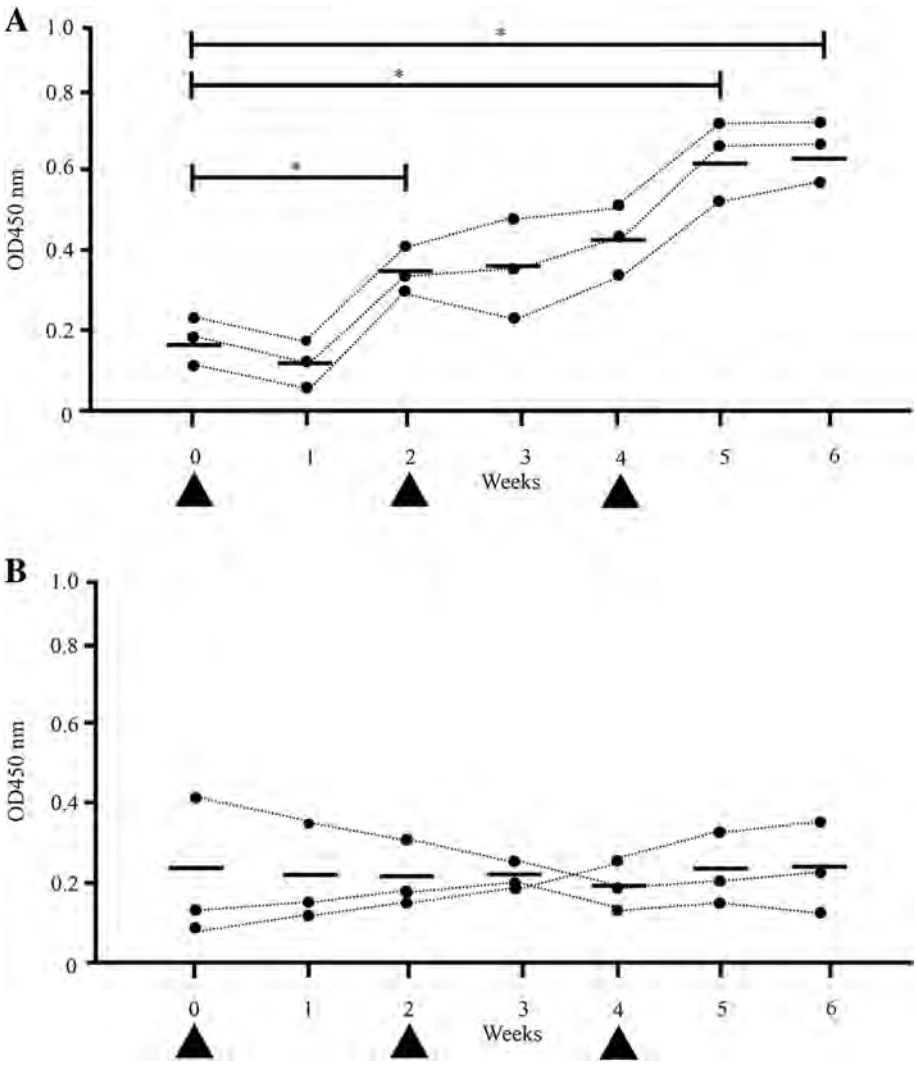

Fig. 2 Anti-*S. aureus*-specific IgA and IgG antibodies in milk from cows immunized with FKSA coupled with cCHP nanogel, administered intranasally in 2 weeks intervals in three subsequent doses (OD values). **a** OD values of anti-*S. aureus*-specific IgA antibodies in composite milk samples (three cows, $n = 3$) from cows that underwent repeated nasal immunization with FKSA three times with a two-week interval. **b** Anti-*S. aureus*-specific IgG antibodies. Each black circle corresponds to a sample and the bar represents the mean. Nasal immunizations are indicated by arrowheads. *Significant difference between the mean of each group on day 0 and from one to 6 weeks after immunization ($P < 0.05$)

specific IgA and IgG antibodies and *S. aureus* counts in milk from these cows (Fig. 5). In non-infused quarters of non-immunized and nasal-immunized animals (three cows, six quarters, $n = 6$, respectively), no significant differences in *S. aureus*-specific IgA antibodies could be detected between day 0 and 7 days post-mock infusion (Fig. 5c). In contrast, in composite milk (three cows, $n = 3$) and infused quarters (three cows, six quarters, $n = 6$) from immunized cows, the OD value increased significantly 7 days after infusion compared with day 0 (mean of composite milk = 0.64 vs. 0.41, mean of *S. aureus*-infused quarter = 0.72 vs. 0.42, respectively; $P < 0.05$) (Fig. 5a and b). The values were not significantly different in non-immunized cows (three cows, six quarters, $n = 6$) (Fig. 5a, b, and c). No significant differences in *S. aureus*-specific IgG antibodies could be detected between day 0 and day 7 in composite milk, post-*S. aureus*- and mock-infused quarters of non-immunized and nasal-immunized animals (Fig. 6a, b, and c).

S. aureus counts in milk from pre-immunized and non-immunized cows decrease 1 week after intramammary *S. aureus* infusion

At pre-infusion and n days 1, 2, 3, and 7 after mock infusion, *S. aureus* counts could not be detected in either non-immunized or nasal-immunized cows (three cows, six quarters, n = 6, respectively, data not shown). However, multiplying *S. aureus* could be detected in infused quarters in both non-immunized and nasal-immunized

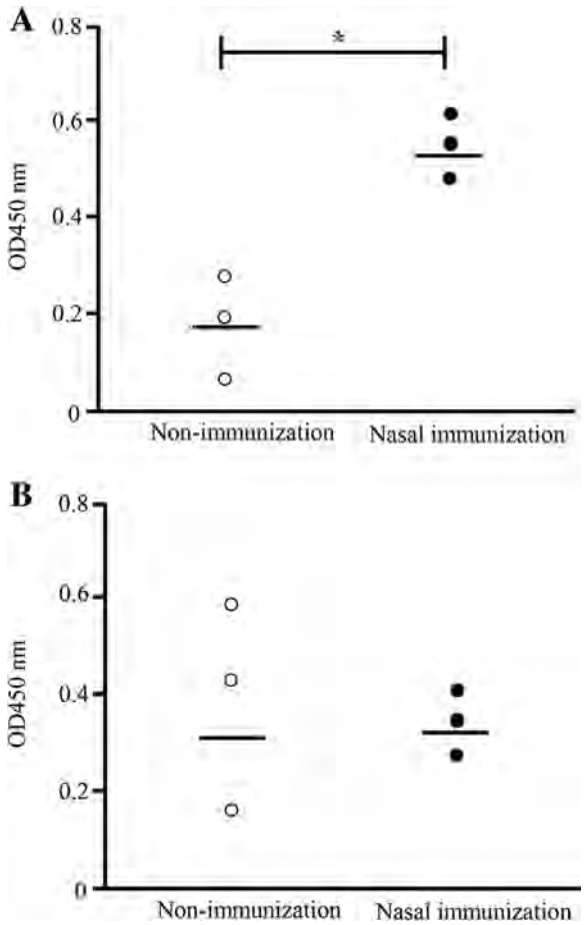

Fig. 3 Anti-*S. aureus*-specific IgA and IgG antibodies in milk from immunized and non-immunized cows (OD values). After 4 weeks following nasal immunization (10 weeks after day 0 of pre-immunization = day 0 of pre-infusion), cows were tested for anti-*S. aureus*-specific IgA and IgG antibodies in milk of immunized and non-immunized cows (**a**) OD values of anti-*S. aureus*-specific IgA antibodies in milk after nasal immunization with FKSA or no immunization. **b** Anti-*S. aureus*-specific IgG antibodies. Each data point represents the OD value for one composite milk sample (three cows, n = 3), and the bar represents the mean. Black circles correspond to nasal immunization samples, white circles to non-immunization controls. *Significant difference between the mean of each group ($P < 0.05$)

cows 7 days after infusion (three cows, six quarters, n = 6, respectively). In particular, *S. aureus* was significantly suppressed in immunized compared with non-immunized cows (mean of *S. aureus* counts expressed as log10 colony-forming units (CFU)/mL was 3.7 vs. 5.2, respectively; $P < 0.05$) (Fig. 7).

S. aureus counts correlate negatively with anti-*S. aureus*-specific IgA antibodies in milk

To evaluate the effect of IgA antibodies on the suppressed multiplication of *S. aureus* in milk, a correlation between the two values 1 week after *S. aureus* infusion was analysed by the Pearson correlation coefficient. A significant negative correlation was found between *S. aureus*-specific IgA antibodies and *S. aureus* counts in infused quarters of both non-immunized and nasal-immunized cows (six cows, 12 quarters, $n = 12$, $r = -0.811$, $P < 0.01$) (Fig. 8a), but no statistical correlation was found for *S. aureus*-specific IgG antibodies (six cows, 12 quarters, n = 12, $r = -0.466$, $P < 0.01$) (Fig. 8b).

Discussion

In this study, we investigated whether *S. aureus*-specific IgA antibodies in milk induced by intranasal *S. aureus* immunization suppressed the multiplication of intramammary *S. aureus*. Initially, we investigated the ability of cCHP nanogel to complex with FKSA. Fluorescence intensity was higher at 0.05 mg/mL than at 0.02 mg/mL cCHP-Rh. cCHP nanogel consists of the cationic molecular ethylenediamine modified cholesteryl pullulan, and previous studies have demonstrated that cationization of nanogel increases the binding constant between nanogels and anionic proteins such as bovine serum albumin [25]. This is due to both hydrophobic and electrostatic interactions between cationic nanogels and bovine serum albumin. The surface of *S. aureus*, like that of most bacteria, has a negative charge [26] and, therefore, is thought to form a complex with the positively charged cationic nanogel.

Antigen proteins complexed with the cCHP nanogel possess a positive charge, possibly promoting adhesion to the negatively charged surface of the nasal mucosa. Kopatz et al. demonstrated that electrostatic interaction on anionic heparan sulphate proteoglycans played an important role in the uptake of cationic PEI/DNA complexes by the mucosal surface [27]. In addition, cCHP/botulinum type A neurotoxin bound electrostatically to the negatively charged nasopharynx-associated lymphoid tissue (NALT) was found to be responsible for the initiation of antigen-specific immune responses [22]. Therefore, cCHP nanogel complexed with inactivated *S. aureus* could adhere continuously to the nasal mucosa. So far, there have been no reports on the interaction of *S. aureus* complexed with cCHP nanogel to the mucosa, including the lymphoid tissues of Waldeyer's ring. In particular, pharyngeal tonsils are considered to be the most important tissue for the immune response due to the presence of microfold cells that take up foreign antigens [23]. Hence, we focused on Waldeyer's ring as an immune response inductive site. Interestingly, flow cytometry detected $FITC^+/CD45^+$ cells only in the tissues of Waldeyer's ring but not in physically distant tissues, such as the spleen and mesenteric lymph nodes (Fig. 1d). Because CD45 is a marker expressed on all leukocytes, which plays an important role in signal transduction in T cells, intranasally administered bacteria were likely taken up by local leukocytes. Our result suggests that

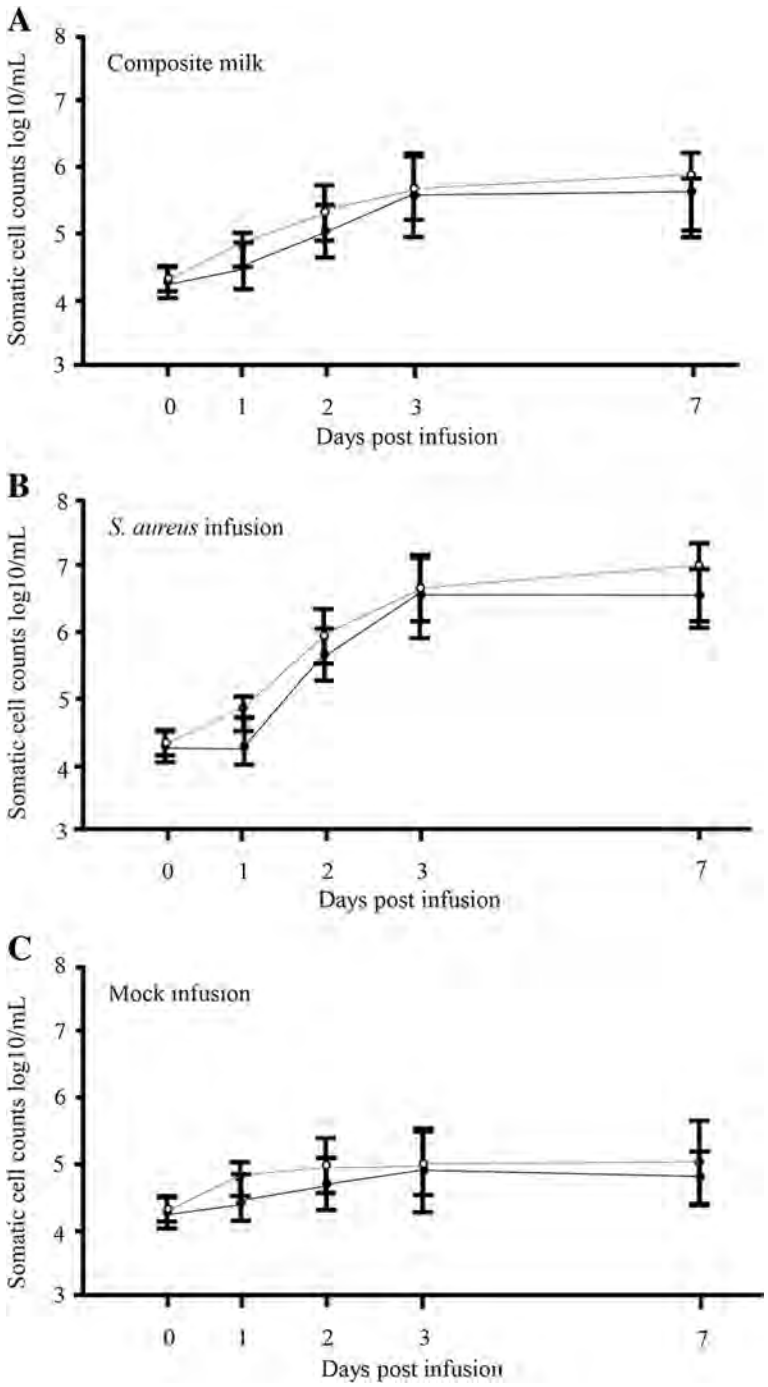

Fig. 4 Somatic cell count in milk following quarters' infusion with *S. aureus* BM1006. Somatic cell counts in composite milk and quarter milk following nasal immunization with FKSA or no immunization in the early stage of lactation, and mock-infusion with PBS or infusion with *S. aureus* BM1006 in the gland cistern. **a** Composite milk (three cows, n = 3). **b** Two quarters infused with *S. aureus* BM1006 in the gland cistern (six quarters, *n* = 6). **c** Two quarters mock-infused with PBS (six quarters, n = 6). Mean cell counts (± standard error) are shown. Black circles correspond to nasal immunization samples, white circles to non-immunization controls

Waldeyer's ring is the first site of recognition of inhaled pathogens. Moreover, $FITC^{+}/CD45^{+}$ cells in the pharyngeal tonsil, tubal tonsil, and palatine tonsil were significantly more abundant when administered as cCHP nanogel/SA-FITC than as SA-FITC alone (Fig. 1d). These results also suggested that the cationization of *S. aureus* by complexing with cCHP nanogel enhanced antigen uptake by leukocytes of Waldeyer's ring.

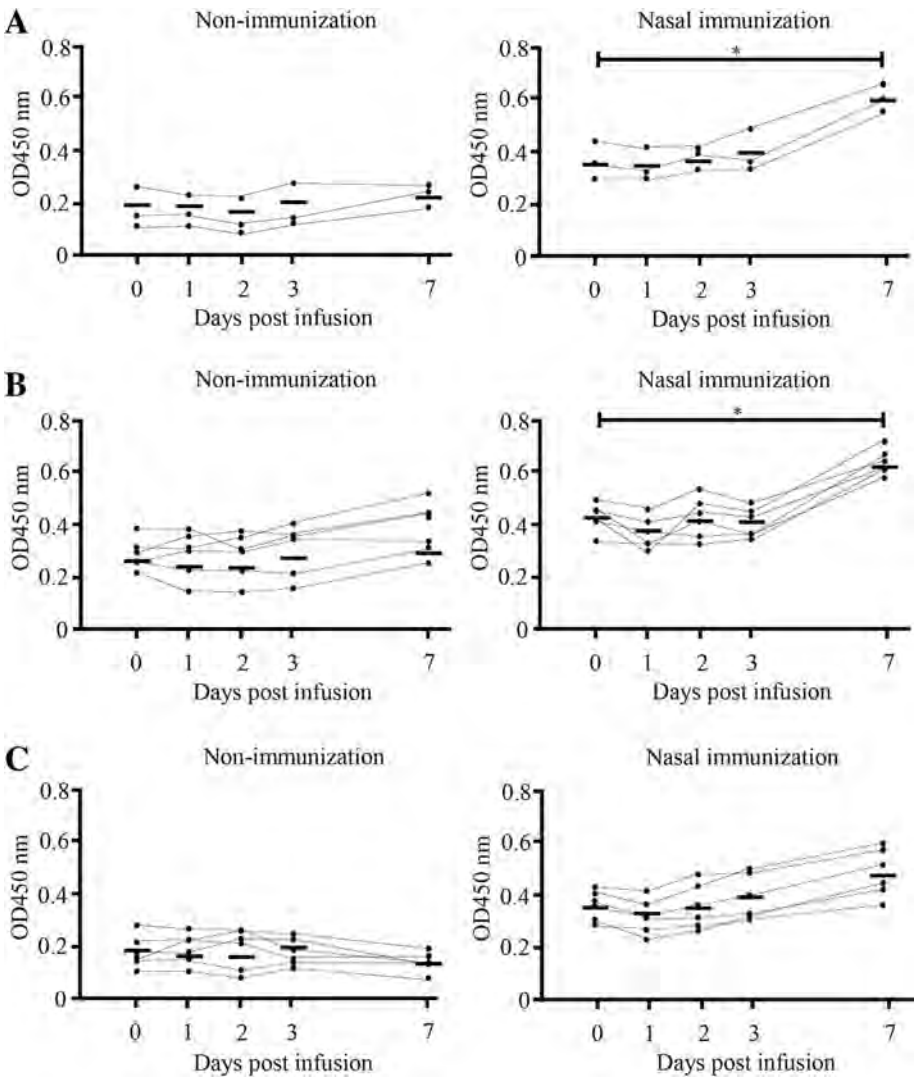

Fig. 5 Anti-*S. aureus*-specific IgA antibodies in milk (OD values) following quarters' infusion with *S. aureus* BM1006. OD values of *S. aureus*-specific IgA antibodies in milk following nasal immunization with FKSA or no immunization in the early stage of lactation. **a** Composite milk (three cows, n = 3). **b** Two quarters infused with *S. aureus* BM1006 in the gland cistern (six quarters, n = 6). **c** Two quarters mock-infused with PBS (six quarters, n = 6). Each data point represents the OD value for one sample, and the bar represents the mean. Black circles correspond to nasal immunization samples, white circles to non-immunization controls. *Significant difference between the mean on day 0 and day 7 after infusion in the same group ($P < 0.05$)

Therefore, cCHP nanogel can represent a suitable delivery system for mucosal immunization, including with nasal immunization antigens. Time-dependent kinetics of $CD45^+/FITC^+$ cells will have to be determined to further characterize the properties of cCHP nanogel.

Next, cows received nasal immunization with FKSA three times with a two-week interval to induce the production of *S. aureus*-specific IgA antibodies. Results demonstrate that the OD value of *S. aureus*-specific IgA antibody in milk was significantly higher in nasal-immunized cows than in pre-immunized control animals (Figs. 2 and 3). Immune responses generated in NALT provide long-term protection against respiratory disease [28, 29]. In the case of cows, inactivated vaccines and modified live virus vaccines are administered via the intranasal route to prevent BHV-1 infections [30–32]. In addition, the nasal cavity is one of the most promising administration sites because the oral route has high digestive enzymatic activity and may dissolve vaccine antigens. Interestingly, a previous study reported that cows, which were immunized via the mucosa (including intranasally) with inactivated *S. aureus* strains, presented *S. aureus*-specific IgA antibodies in the milk [33], confirming that nasal immunization with inactivated *S. aureus* could actually induce *S. aureus*-specific IgA antibodies in the mammary gland. In contrast, mucosal immunization generally induces mucosal-specific IgA

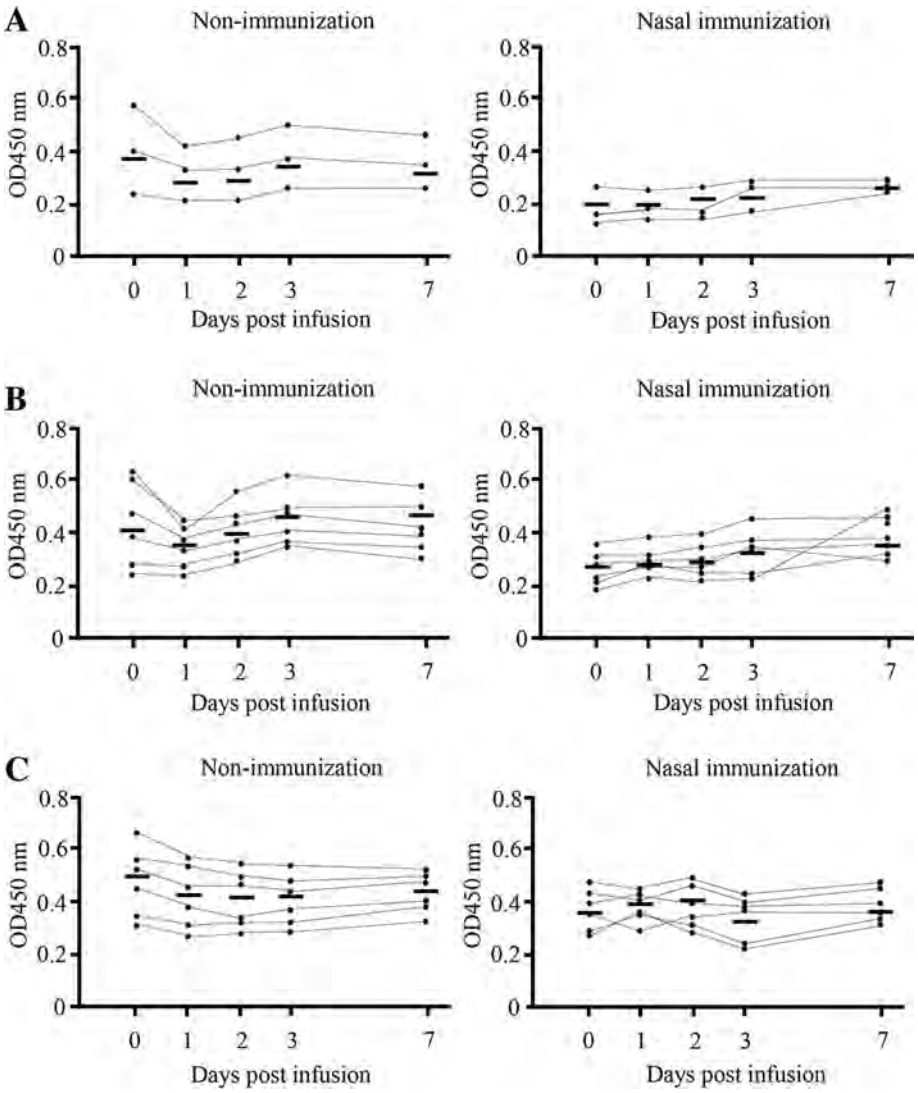

Fig. 6 Anti-*S. aureus*-specific IgG antibodies in milk (OD values) following quarters infusion with *S. aureus* BM1006. OD values of *S. aureus*-specific IgG antibodies in milk following nasal immunization with FKSA or no immunization in the early stage of lactation. **a** Composite milk (three cows, n = 3). **b** Two quarters infused with *S. aureus* BM1006 in the gland cistern (six quarters, n = 6). **c** Two quarters mock-infused with PBS (six quarters, n = 6). Each data point represents the OD value for one sample, and the bar represents the mean. Black circles correspond to nasal immunization samples, white circles to non-immunization controls

antibodies and serum-specific IgG antibodies; however, the OD value of anti-*S. aureus*-specific IgG antibodies in composite milk did not differ significantly in this study (Fig. 2). This phenomenon may be related to differences in adjuvant. Nanoparticulate carriers such as cCHP nanogel provide adjuvant activity by enhancing antigen delivery or activating innate immune responses. Strength and mechanisms of immunostimulation induced by nanocarrier vaccines depend on various factors, such as chemical composition, particle size, homogeneity, and electrical charge [34–36]. Misstear et al. [37] demonstrated that T helper 1- and T helper 17-biased responses elicited by targeted nanoparticles using intranasal vaccination with microfold cell-targeted ovalbumin-loaded nanoparticles provided protection against *S. aureus*, without the induction of any detectable antigen-specific serum IgG. At the same time, Boerhout et al. suggested that intranasal mucosal immunization by spray resulted in a marginal response only, indicating that this route is less suitable for generating humoral protection against *S. aureus* [38]. This discrepancy with respect to our results may be due to the different administration method. Bovine mucosa-associated lymphoid tissues in the upper respiratory tract include NALT and the lymphoid tissues of Waldeyer's ring, which itself encompasses the pharyngeal, palatine, tubal, and lingual tonsils. Even though NALT is located near the nasal

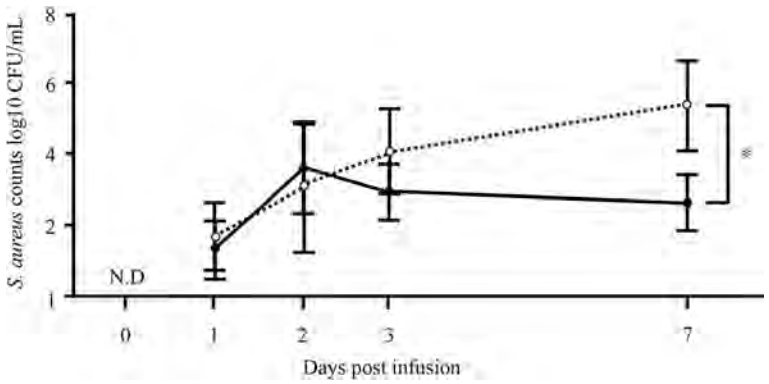

Fig. 7 *S. aureus* counts in milk 1 week after experimental intramammary infection of immunized and non-immunized cows. *S. aureus* counts in milk were determined in immunized and non-immunized cows from pre-infusion on day 0 to day 1, 2, 3, and 7 after *S. aureus* infusion into the quarter (three cows, 6 quarters, n = 6). Mean (± standard error) *S. aureus* counts are shown. Black circles correspond to nasal immunization samples, white circles to non-immunization controls. *Significant difference between the mean of each group ($P < 0.05$). N.D, not detected

cavity, there have been only a few studies on it in cows [39]. Therefore, to make sure that FKSA were delivered to Waldeyer's ring located in the deep part of the nasal cavity, cattle were treated intranasally with a ~ 15-cm feeding needle for rats, rather than a spray commonly used for nasal administration. Future studies should focus on the type of adjuvant to be used for delivery and its ability to stimulate various pathways.

Next, after nasal immunization of inactivated *S. aureus* to cows, we used experimental models of mastitis through mammary infection with *S. aureus* and investigated *S. aureus*-specific IgA and IgG antibodies and *S. aureus* counts in milk. In composite and *S. aureus*-infused quarter milk from immunized cows, IgA antibodies increased significantly on day 7 after infusion compared with day 0 (Fig. 5a and b), but no significant differences in *S. aureus*-specific IgG antibodies could be detected between days 0 and 7 (Fig. 6a and b). In addition, in non-infused quarters, there was no significant difference in *S. aureus*-specific IgA and IgG antibodies between day 0 and day 7, irrespective of immunization (Figs. 5c and 6c). Leitner et al. [40] indicated that IgA antibodies were the major Ig isotype in most *S. aureus*-infected quarters with subclinical chronic mastitis but not in non-infected quarters. These results suggest that production of the IgA isotype is localized to individual quarters and nasal immunization can provide local immunity against *S. aureus* infection. Similarly, average *S. aureus* counts in infused quarters were significantly lower in immunized cows than in non-immunized animals (Fig. 7). Unlike IgGs, the IgA isotype is not among the immunoglobulin classes in milk that promote phagocytosis [41, 42]. However, IgA antibodies may control the severity of the initial infection via immune exclusion, thus preventing bacteria from adhering to the epithelial cells on the mucosal surface [12]. In fact, our present study confirms a significant negative correlation between OD value of *S. aureus*-specific IgA antibodies and *S. aureus* counts (Fig. 8a), but a statistical correlation was not found for *S. aureus*-specific IgG antibodies (Fig. 8b). Accordingly, induction of *S. aureus*-specific IgA antibodies positively affects prevention and control of *S. aureus* infection in the quarter. In addition, our previous study showed a significant correlation between *S. aureus* counts and the proportion of mammary epithelial cells in milk, thus suggesting that *S. aureus* counts in milk reflected exfoliation of mammary epithelial cells related to mammary damage [43]. These reports suggested that reduction of *S. aureus* counts through induction of IgA antibodies could protect cells from initial infection by *S. aureus,* including in cases of mammary damage. Present results suggest that induction of *S. aureus*-specific IgA antibodies by nasal immunization is beneficial for the clearance of *S. aureus* from the udder.

Based on our results, *S. aureus* in milk was still detected in immunized cows, in spite of a significant negative correlation between *S. aureus*-specific IgA antibodies and *S. aureus* counts (Fig. 8a). This result indicates that although nasal immunization can suppress the multiplication of *S. aureus* and limit new infections, complete control was not achieved. Compared to other bacterial pathogens, *S. aureus* presents several challenges. Targeting specific antigens has been suggested as the most efficient method to develop an effective vaccine. However, other studies suggest that *S. aureus* milk isolates have a large polymorphism and regional patterns, stressing the importance of developing vaccines based on antigens common to different isolates. Accordingly, no optimal vaccine antigen against *S. aureus* could be achieved, making it difficult to control the multiplication of this bacterium. At the same time, it should be noted that previous studies focused mainly on IgG antibodies and, particularly in cows, research on induction of

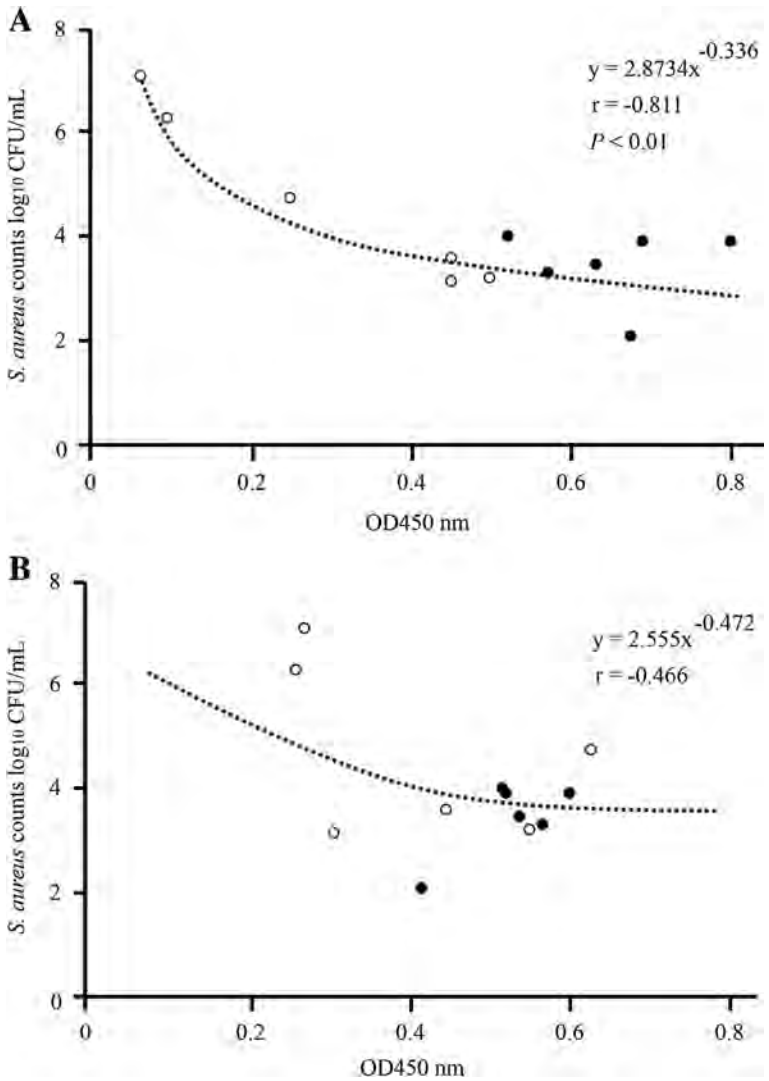

Fig. 8 *S. aureus* counts and anti-*S. aureus*-specific IgA or IgG antibodies in milk a week after experimental intramammary infection of immunized and non-immunized cows. **a** Linear regression analysis showing a statistical correlation between *S. aureus* counts and OD value of *S. aureus*-specific IgA antibodies in infused quarters of non-immunized and nasal-immunized cows (six cows, 12 quarters, $n = 12$). **b** *S. aureus*-specific IgG antibodies. Black circles correspond to nasal immunization samples, white circles to non-immunization controls (three cows, six quarters, n = 6). $P < 0.01$ was considered highly statistically significant

IgA antibodies is still limited. Until recently, anti-*S. aureus* vaccine approaches have focused on induction of neutralizing/opsonizing antibodies by IgG, but there is increasing evidence that cellular immunity may be equally or more important for protective immunity [44]. Indeed, mucosal sites are responsible for the generation of antigen-specific T helper 2-dependent IgA responses and T helper 1- and cytotoxic T lymphocyte-dependent immune responses, which function as the first line of defence on mucosal surfaces. In many cases of mucosal vaccines, the main protective effector function elicited by immunization is stimulation of a secretory local IgA antibody response and an associated mucosal immunologic memory. However, in other instances, although still less extensively studied, there is evidence of an important role also for the cellular arm of the immune response that includes mucosal $CD8^+$ cytotoxic T lymphocytes, $CD4^+$ T helper cells, as well as natural killer cells [45]. Remarkably, Misstear et al. [37] demonstrated that a targeted nasal vaccine promoted clearance of an acute *S. aureus* systemic infection, but also that a purely cellular response was sufficient for this protection in mice. It is of strategic importance that future research focuses on the search for effective vaccine antigens against bovine mastitis, which can be applied via the mucosal route. Moreover, it will be essential to assess functional responses through induction of both humoral and cellular immunity, including T cell-mediated responses.

Conclusion

In conclusion, the present study demonstrates that induction of *S. aureus*-specific IgA antibodies in milk by nasal immunization suppressed multiplication of *S. aureus* in the udder. Although future research will need to clarify the exact mechanism by which IgA antibodies suppress bacterial multiplication, our findings support the ongoing effort to develop a mucosal vaccine against bovine *S. aureus*-induced mastitis and indicate that stimulation of an anti-*S. aureus* humoral immune response in milk might contribute to prevention and control of the disease.

Methods

Cattle

Nine 6 to 10-week-old male calves and six 16 to 28-month-old Holstein primiparous heifers during early lactation were obtained from dairy farms in Hokkaido, Japan. None of the animals used in the present study had any previous history of clinical or subclinical mastitis. Four weeks after parturition, three cows were used for intranasal immunization with FKSA. During the experiment, all cows were kept in a biosafety level 2 animal facility at the Hokkaido Research Station, National Institute of Animal Health (Sapporo, Hokkaido, Japan). The following criteria, which correspond to the Japanese diagnostic standard for bovine mastitis, were used for quarter milk samples: negative for *S. aureus* infection and SCC < 3×10^5 cells/mL.

Preparation of cCHP nanogel/inactivated *S. aureus* complexes

S. aureus strain BM1006 (sequence type 352, clonal complex 97), which causes bovine mastitis [46], was used to prepare FKSA as described in our previous study [47]. Washed *S. aureus* BM1006 was suspended in phosphate-buffered saline (PBS) containing 0.5% formaldehyde, incubated with shaking (50 rpm) at room temperature for inactivation, and then washed three times with PBS. We confirmed the absence of viable cells after 1 h following inactivation (Additional file 1). The inactivation step was then performed overnight to ensure complete inactivation.

cCHP, or cCHP-rhodamine (cCHP-Rh) were synthesized as described previously [22, 25, 48]. cCHP or cCHP-Rh were suspended in PBS. The suspension was sonicated for 15 min with a probe sonicator (BRANSON, Danbury, CT, USA) and centrifuged at 20,000×*g* for 30 min. The obtained supernatant was then filtered through a 0.22-μm filter (Millipore, Billerica, MA, USA). To confirm whether cCHP nanogel reliably complexed with FKSA, we incubated 5×10^9 FKSA cells/mL with cCHP-Rh nanogel (final concentration 0.02, 0.05, or 0.2 mg/mL) for 30 min at room temperature, and then observed the samples under a confocal laser microscope (LSM780, Carl Zeiss, Göttingen, Germany). Images revealed that FKSA was not sufficiently complexed by the cCHP-Rh nanogel at 0.02 mg/mL; however, complexation was successful at 0.05 mg/mL, as indicated by substantially stronger fluorescence intensity of the 0.05 mg/mL sample. Conversely, there was no major difference in fluorescence intensity between this and the 0.2 mg/mL sample (Additional file 2). To ensure that the cCHP nanogel complexed with FKSA, 5×10^{10} FKSA cells were incubated for 30 min at room temperature with cCHP nanogel at a final concentration of 1 mg/mL. The resulting cCHP nanogel/inactivated *S. aureus* complexes were suspended in 2 mL PBS and used for intranasal immunization.

Nasal administration of cCHP nanogel/inactivated *S. aureus*-conjugated FITC to calves

SA-FITC was purchased from Thermo Fisher Scientific (S2851, Waltham, MA, USA). As above, 3×10^9 SA-FITC cells were incubated for 30 min with cCHP nanogel at a final concentration of 1 mg/mL. The resulting cCHP nanogel/SA-FITC complexes, as well as SA-FITC alone (3×10^9 cells/2 mL, respectively), were administered with a ~ 15-cm flexible feeding needle to each of three male calves into one nostril (Fig. 1a). One hour after nasal administration, calves were injected xylazine (Selactar 2% injection solution, Bayer Yakuhin, Tokyo, Japan) as a sedative, general anaesthesia was induced by intravenous sodium pentobarbital (Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan), and calves were sacrificed by exsanguination from the carotid artery. To confirm that the cells had taken up SA-FITC, tissues surrounding Waldeyer's ring (pharyngeal tonsil, tubal tonsil, palatine tonsil, and lingual tonsil) (Fig. 1b), the spleen, and the mesenteric lymph node were collected after sacrifice and analysed by flow cytometry. Three calves not treated with either SA-FITC or cCHP nanogel/SA-FITC were sacrificed and tissues from the same sites were used as negative controls.

Flow cytometry analysis

Flow cytometry was performed on lymphocytes isolated from the above-mentioned tissues. All tissues were finely cut, transferred into PBS containing 1 mg/mL collagenase (034-22363, Wako, Osaka, Japan), and incubated at 37 °C for 10 min in a shaking water bath. The digested tissues were washed with PBS and filtered through a 40-μm stainless steel mesh to separate the cells from the excess tissue debris. To detect leukocytes, 1×10^6 leukocytes were incubated with rabbit anti-CD45 antibody (1:200; Abcam, Cambridge, MA, USA) for 60 min, rinsed, and then incubated with Alexa Fluor 647 donkey anti-rabbit IgG antibody (1:200; Molecular Probes, Leiden, The Netherlands) for 1 h. Finally, the cell suspensions were fixed in 500 μL of 1% formalin in PBS. The number and percentage of $FITC^+/CD45^+$ mononuclear cells in tissue was analysed on a Gallios™ flow cytometer (Beckman Coulter Inc., Fullerton, CA, USA) based on forward

and side light scattering properties (Fig. 1c). A total of 10^4 events per sample were collected, and positive cells were expressed as a proportion of mononuclear cells. Rabbit IgG isotype controls (Abcam) were used to detect non-specific staining and establish the criteria for positive cell populations (data not shown).

Nasal immunization with cCHP nanogel/inactivated *S. aureus* complexes

Three Holstein cows were intranasally immunized three times to one nostril with FKSA-complexed cCHP nanogel (5×10^{10} cells/2 mL) with a two-week interval between each dose. Following nasal immunization, the cows were clinically evaluated to determine their well-being in terms of local reactions of the quarter (quarter uniformity, milk abnormalities, appetite, and milk yield) and systemic reactions (rectal temperature and appetite). No significant SCC in milk (Additional file 3), systemic or local reactions were observed (data not shown). After 4 weeks following nasal immunization, cows were tested for experimental intramammary infusion with *S. aureus*.

Experimental intramammary infusion with *S. aureus*

Six cows (three non-intranasally or three intranasally cows) in the early stage of lactation were intramammary infused with *S. aureus* BM1006. Before infusion, we conducted a bacteriological examination of milk samples, which were found to be completely negative for *S. aureus*. Intramammary infusion with *S. aureus* was performed as described in our previous study [43]. Briefly, *S. aureus* was diluted to ~ 20 CFUs in a total of 5 mL. Two quarters from each cow were selected after evening milking. Teats were allowed to air-dry, and a *S. aureus* suspension was infused into the gland cistern of two quarters, whereas the remaining two quarters were mock-challenged with the same volume of PBS. Clinical signs such as rectal temperature, udder uniformity, milk abnormalities, appetite, and milk yield for each cow were recorded in the morning.

Milk sample collection and *S. aureus* counts

Milk samples were collected from day 0 of pre-immunization every week for up to 6 weeks, and from day 0 of pre-infusion until day 1, 2, 3, and 7 after infusion with *S. aureus.* Samples were collected from each individual quarter and composite milk. Composite milk samples refer to milk aliquots collected from all four quarters into a single sample vial [49, 50]. A composite milk sample (40 mL) was created by mixing milk from each of the four quarters into a sterile test tube. A 1-mL aliquot of each milk sample was spread on a Petrifilm Staph Express Count plate (3 M, Minneapolis, MN, USA) and incubated at 37 °C for 24 h. Then, CFUs were counted. When colony colours other than red-violet were present, the plates were applied to a Petrifilm Staph Express Disk and incubated for 3 h. The colonies that formed pink zones were counted as *S. aureus*, and the results were expressed as CFU/mL. The SCC of the milk was measured using a DeLaval cell counter DCC (DeLaval, Tumba, Sweden) as described by Kawai et al. [51].

The enzyme-linked immunosorbent assay (ELISA) for the detection of specific IgA and IgG antibodies against *S. aureus*

To detect specific IgA and IgG antibodies against *S. aureus* in individual quarter and composite milk, a microtiter plate was directly coated with FKSA as capture antigen. FKSA in PBS was dried in an oven at 5×10^6 cells/well in 96-well ELISA plates (C96 Maxisorpcert, Nunc-Immuno Plate, Thermo Fisher Scientific) overnight at 37 °C. After incubation, wells were washed with Tris-buffered saline-Tween-20 (TBST) and then incubated with 100 μL of milk (diluted 1:100 in PBS) for 90 min at room temperature. After five TBST washes, wells were incubated with horseradish peroxidase-conjugated sheep anti-bovine IgA antibody (diluted 1:30000, Bethyl Laboratories, Inc., Montgomery, TX, USA) or horseradish peroxidase-conjugated sheep anti-bovine IgG antibody (diluted 1:30000, Bethyl Laboratories, Inc.) for 2 h at room temperature. The freshly prepared substrate was added and OD was measured at 450 nm using the 3′,3′, 5,5′-tetramethylbenzidine microwell peroxidase substrate system (KPL, Gaithersburg, MD, USA). All samples were analysed in duplicate and mean values were calculated. Anti-*S. aureus*-IgA or IgG antibodies were calculated by subtracting OD values for the buffer controls (OD450 nm = ~ 0.15), which were included in duplicate in all ELISAs, from specific sample OD values. To standardize and compare results between plates, positive control milk samples (from bovine mastitis caused by *S. aureus*, OD450 nm = ~ 1.5 for specific IgA antibody, OD450 nm = ~ 1.0 for specific IgG) were included in duplicate in all ELISAs. OD values were normalized against those of positive controls.

Statistical analysis

Differences among groups of $FITC^+/CD45^+$ mononuclear cells, SCC, *S. aureus* counts, and the OD value of *S. aureus*-specific IgA and IgG were evaluated using Student's *t*-test. Associations between *S. aureus* counts and antibody OD values were analysed by applying linear mixed models with SAS software (SAS Institute Japan Ltd., Tokyo, Japan). Pearson correlation coefficient and nonlinear regression analysis were used to assess the relationship between *S. aureus* counts and OD value of anti-*S. aureus*-specific IgA or IgG antibodies. $P < 0.05$ and $P < 0.01$ were considered significant and highly significant, respectively.

Additional files

Additional file 1: Survival curve of *Staphylococcus aureus* BM1006 strain inactivated with 0.5% formaldehyde. *S. aureus* BM1006 (5×10^{10} CFUs) was incubated with 0.5% formaldehyde for 0, 15, 30, 180, and 1080 min, and then the number of colonies was counted using a Petrifilm Staph Express Count plate. Data are presented as logarithmic bacterial reduction in log CFU/mL. Black circles represent the mean of five independent experiments, error bars indicate the standard deviation. (TIF 91 kb)

Additional file 2: Confocal fluorescence images of inactivated *Staphylococcus aureus* BM1006 strain incubated with cCHP nanogel. Formalin-killed *S. aureus* BM1006 samples (FKSA, 5×10^9 cells/mL) were incubated for 30 min at room temperature with 0.02, 0.05, or 0.2 mg/mL cCHP-Rh nanogel, and then observed using a confocal laser microscope. Bright-field, cCHP-Rh, and merge images are shown. Scale bars, 5 μm. Arrows indicate FKSA not complexed by the cCHP-Rh nanogel. (TIF 428 kb)

Additional file 3: Somatic cell count in milk following nasal immunization with FKSA. After nasal immunization with FKSA or no immunization, somatic cell count (SCC) in composite and quarter milk was analysed. Each data point represents the SCC for composite milk sample (three cows, $n = 3$) and quarter milk samples (three cows, 12 quarters, $n = 12$); the bar represents the mean. Black circles correspond to nasal immunization samples. (TIF 82 kb)

Abbreviations

cCHP: cationic type of cholesteryl-group-bearing pullulan; CFU: Colony-forming unit; ELISA: Enzyme-linked immunosorbent assay; FKSA: Formalin-killed *Staphylococcus aureus*; IgA: Immunoglobulin A; IgG: Immunoglobulin G; NALT: Nasopharynx-associated lymphoid tissue; OD: Optical density; PBS: Phosphate-buffered saline; SCC: Somatic cell count; TBST: Tris-buffered saline-Tween-20

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Authors' contributions

YN, YK, and TH substantially contributed to conception or design of the study. YN, KS, AH, CK, NK, TN, and SS contributed to acquisition, analysis, or interpretation of data. YN drafted the manuscript. YN, YK, TT, HA, KA, and TH critically revised the manuscript for important intellectual content. All authors have read and approved the final version of the manuscript.

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Availability of data and materials

The dataset supporting the conclusions of this article is included within the article.

Ethics approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All animal handling procedures were in accordance with the Regulations of the Animal Experiment Committee, National Institute of Animal Health, NARO, which also approved the experimental design. All animal experiments were approved by the National Institute of Animal Health Ethics Committee (authorization number: 29–013-2).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Inactivation of the coronavirus that induces severe acute respiratory syndrome, SARS-CoV

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Abstract

Severe acute respiratory syndrome (SARS) is a life-threatening disease caused by a novel coronavirus termed SARS-CoV. Due to the severity of this disease, the World Health Organization (WHO) recommends that manipulation of active viral cultures of SARS-CoV be performed in containment laboratories at biosafety level 3 (BSL3). The virus was inactivated by ultraviolet light (UV) at 254 nm, heat treatment of 65 °C or greater, alkaline (pH > 12) or acidic (pH < 3) conditions, formalin and glutaraldehyde treatments. We describe the kinetics of these efficient viral inactivation methods, which will allow research with SARS-CoV containing materials, that are rendered non-infectious, to be conducted at reduced safety levels.

Keywords: SARS; Coronavirus; Virus inactivation; Tissue culture

1. Introduction

In late 2002, an outbreak of unusual life-threatening respiratory disease of unknown etiology began in Guangdong Province, China. This disease was designated severe acute respiratory syndrome (SARS) and was later determined by Drosten et al. (2003), Ksiazek et al. (2003), and Rota et al. (2003), to be caused by a novel coronavirus, termed SARS-CoV. Since the identification of coronavirus as the infectious agent for SARS, numerous laboratories have begun research on this virus. According to the WHO, 8098 people were diagnosed with SARS and 774 people died of this disease during the initial outbreak of 2003. Due to the severity of SARS disease and the contagious nature of the causal agent, the WHO website (http://www.who.int/csr/sars/biosafety2003_12_18/en/) has provided guidelines for working safely with this coronavirus. The WHO recommends biosafety level 3 (BSL3) as the appropriate containment level for working with live SARS-CoV, and there is a concern that another SARS outbreak could occur following an accidental exposure in a laboratory. Since the end of the SARS epidemic in July 2003, there have been three known cases of SARS in laboratory researchers due to accidental exposure to the virus (Normile, 2004). Successful inactivation of the virus allows the transfer of material from a BSL3 to a BSL2 environment and may reduce the risk of accidental infections through unsafe laboratory practices. Inactivated cell-culture derived viral stocks may also be useful for the development of vaccines and the study of their safety and immunogenicity. We examined the efficiency of several methods of viral inactivation, including methods that may inhibit viral replication or entry.

2. Materials and methods

2.1. Virus and cells

We infected African green monkey kidney (Vero E6) cells with SARS-CoV (Urbani strain) that was kindly provided by Drs. L.J. Anderson and T.G. Ksiazek from the

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Centers for Disease Control and Prevention, Atlanta, GA. Briefly, Vero E6 monolayer cells were infected by inoculating cultures with 50 μl of virus ($10^{6.33}$ $TCID_{50}$ per ml) in a final volume of 5 ml Dulbecco's modified Eagle's medium (DMEM) (Biosource International, Camarillo, CA) in T150 flasks for 1 h at 25 °C. Dulbecco's modified Eagle's medium containing supplements (10% fetal bovine serum, 2 mM/ml L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.5 μg/ml fungizone) (Biosource International) was added to the flask and the cells were incubated at 37 °C for 3 days. Supernatant was collected, clarified by centrifugation, and stored at −70 °C as the viral stock. The Vero cells were maintained in DMEM with supplements. All personnel wore powered air-purifying respirators (3 M, Saint Paul, MN) and worked with infectious virus inside a biosafety cabinet, within a BSL3 containment facility.

2.2. Quantitation of viral titers

Viral titers were determined in Vero cell monolayers on 24 and 96-well plates using a 50% tissue culture infectious dose assay ($TCID_{50}$). Serial dilutions of virus samples were incubated at 37 °C for 4 days and subsequently examined for cytopathic effect (CPE) in infected cells, as described by Ksiazek et al. (2003). Briefly, SARS-CoV-induced CPE of infected cells was determined by observing rounded, detached cells in close association to each other. Evidence of inactivation was determined by absence of CPE in Vero cells, indicating loss of infectivity.

2.3. UV light treatment

Ultraviolet light (UV) treatment was performed on 2 ml aliquots of virus (volume depth = 1 cm) in 24-well plates (Corning Inc., Corning, NY). The UV light source (Spectronics Corporation, Westbury, NY) was placed above the plate, at a distance of 3 cm from the bottom of the wells containing the virus samples. At 3 cm our UVC light source (254 nm) emitted 4016 $\mu W/cm^2$ (where $\mu W = 10^{-6}$ J/s) and the UVA light source (365 nm) emitted 2133 $\mu W/cm^2$, as measured by radiometric analysis (Spectronics Corporation). After exposure to the UV light source, virus was frozen for later analysis by $TCID_{50}$ assay using CPE as the endpoint.

2.4. Gamma irradiation treatment

We prepared 400 μl samples of SARS-CoV and kept them on dry ice during transport. Test samples were subjected to gamma radiation (3000, 5000, 10,000, and 15,000 rad) from a ^{60}Co source, while control samples were protected from exposure. Test and control samples were handled and transported identically, except test samples were exposed to the gamma radiation source. Samples were kept frozen until analysis of inactivation by $TCID_{50}$ assay.

2.5. Heat treatment of virus

We incubated 320 μl aliquots of virus in 1.5 ml polypropylene cryotubes using a heating block to achieve three different temperatures (56, 65 and 75 °C). After heat treatment, samples were frozen for later analysis by $TCID_{50}$ assay using CPE as an endpoint.

2.6. Formaldehyde and glutaraldehyde treatment

Formaldehyde (37%, Mallinckrodt Baker, Inc., Paris, KY) and glutaraldehyde (8%, Sigma, Saint Louis, MO) were diluted 1:10 and 1:40 in sterile PBS. These diluted aldehydes were added to virus samples to achieve final dilutions of 1:1000 and 1:4000 in 400 μl. The final concentrations of formaldehyde were 0.037% (1:1000) and 0.009% (1:4000), and the final concentrations of glutaraldehyde were 0.008% (1:1000) and 0.002% (1:4000). The virus and aldehyde samples were incubated at 4, 25, and 37 °C, for up to 3 days. The samples were mixed briefly with a vortex on each day. The samples were stored at −70 °C until analysis by $TCID_{50}$ assay.

2.7. pH treatment

Virus aliquots were adjusted to the desired pH using 5 M and 1 M HCl or 5N and 1N NaOH. Subsequently, they were divided into three aliquots, incubated at the desired temperature (4, 25, and 37 °C), neutralized to a pH 7, and analyzed for viral titer using the $TCID_{50}$ assay.

2.8. Infectivity of viral RNA and detergent-disrupted virions

Infected Vero cells were prepared by inoculation with 20 μl of virus at a $10^{6.37}$ $TCID_{50}$ per ml of SARS-CoV in a final volume of 2 ml in a T25 flask for 1 h at 25 °C. DMEM with supplements was added to the flask and the cells were incubated at 37 °C for 3 days. The monolayer was washed with 1X phosphate buffered saline (PBS), cells were lysed with the addition of 2.5 ml of a phenol and guanidine isothiocyanate solution (TRIzol Reagent, Sigma), and cytoplasmic RNA was isolated according to the manufacturer's specifications. Vero cells were inoculated with 10 μl of purified RNA in 0.5 ml DMEM. After an hour, DMEM with supplements was added. Additionally, Vero cells were transfected with cytoplasmic RNA using DMRIE-C (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Cells were incubated at 37 °C, and observed for CPE on days 3 and 4.

To examine the infectivity of detergent-disrupted virions, SARS-CoV infected Vero monolayer cells were washed and dissociated with trypsin/versene, pelleted by centrifugation, and washed with PBS. After centrifugation, the pellet was lysed with sodium dodecyl sulfate/nonidet P-40 (SDS/NP-40; 0.1% SDS, 0.1% NP-40, in 0.1x PBS; Sigma), frozen

at −70 °C, thawed, and clarified by centrifugation. The supernatant was used to infect Vero cell monolayers in 6-well plates, such that the final concentration of SDS was 0.002 or 0.018%. Three and four days following the inoculation, cells were observed for evidence of CPE.

3. Results

3.1. Effect of radiation on the infectivity of SARS-CoV

UV light is divided into three classifications: UVA (320–400 nm), UVB (280–320 nm), and UVC (200–280 nm). UVC is absorbed by RNA and DNA bases, and can cause the photochemical fusion of two adjacent pyrimidines into covalently linked dimers, which then become non-pairing bases (Perdiz et al., 2000). UVB can cause the induction of pyrimidine dimers, but 20–100-fold less efficiently than UVC (Perdiz et al., 2000). UVA is weakly absorbed by DNA and RNA, and is much less effective than UVC and UVB in inducing pyrimidine dimers, but may cause additional genetic damage through the production of reactive oxygen species, which cause oxidization of bases and strand breaks (Tyrrell et al., 2001).

To examine the inactivation potential of UVA and UVC, virus stocks were placed in 24-well tissue culture plates and exposed to UV irradiation on ice for varying amounts of time, as indicated in Fig. 1A. Exposure of virus to UVC light resulted in partial inactivation at 1 min with increasing efficiency up to 6 min (Fig. 1A), resulting in a 400-fold decrease in infectious virus. No additional inactivation was observed from 6 to 10 min. After 15 min the virus was completely inactivated to the limit of detection of the assay, which is ≤1.0 $TCID_{50}$ (log_{10}) per ml. In contrast, UVA exposure demonstrated no significant effects on virus inactivation over a 15 min period. Our data show that UVC light inactivated the SARS virus at a distance of 3 cm for 15 min.

A standard procedure to inactivate viruses during the manufacture of biological products is gamma irradiation (Grieb et al., 2002). To investigate the effect of gamma irradiation on SARS-CoV, we subjected 400 μl of SARS-CoV to gamma radiation (3000, 5000, 10,000, and 15,000 rad) from a ^{60}Co source, while control samples were protected from exposure. No effect on viral infectivity was observed within this range of gamma irradiation exposure (Fig. 1B).

3.2. Effect of heat treatment on the infectivity of SARS-CoV

Heat can inactivate viruses by denaturing the secondary structures of proteins, and thereby may alter the conformation of virion proteins involved in attachment and replication within a host cell (Lelie et al., 1987; Schlegel et al., 2001). To test the ability of heat to inactivate the SARS-CoV, we incubated virus in 1.5 ml polypropylene cryotubes at three temperatures (56, 65 and 75 °C) for increasing periods of time.

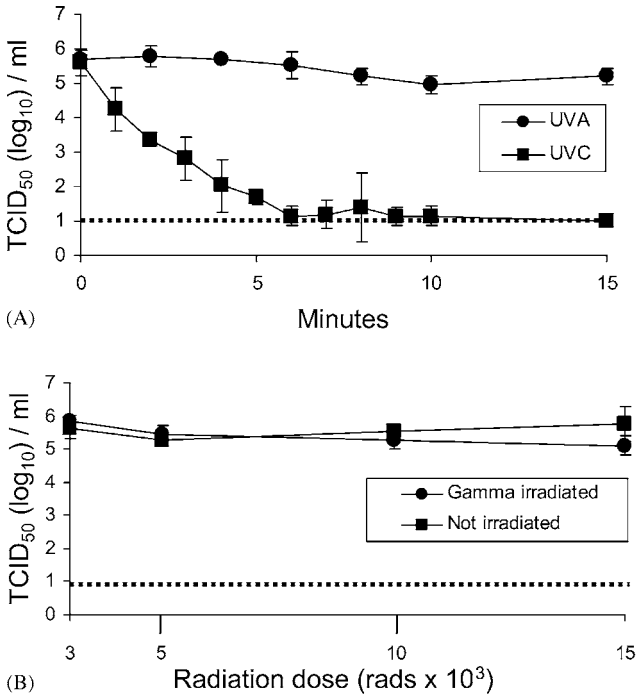

Fig. 1. Effect of radiation on the infectivity of SARS-CoV. (A) UV irradiation. The UV lamp was placed 3 cm above the bottom of 24-well plates containing 2 ml virus aliquots. Samples were removed at each time point, frozen, and titrated in Vero E6 cells. The results shown are representative of three independent experiments. (B) Gamma irradiation. Virus aliquots (400 μl) were placed in cryovials on dry ice and exposed to the indicated dose of gamma irradiation. Control samples were treated identically, without radiation exposure. Samples were titrated in Vero E6 cells in triplicate. The dotted line denotes the limit of detection of the assay.

We found that at 56 °C most of the virus was inactivated after 20 min (Fig. 2A). However, the virus remained infectious at a level close to the limit of detection for the assay, for at least 60 min, suggesting that some virus particles were stable at 56 °C (Fig. 2A and C). At 65 °C, most of the virus was inactivated if incubated for longer than 4 min (Fig. 2B). Again, some infectious virus could still be detected close to the limit of detection for the assay, after 20 min at 65 °C. While virus was incompletely inactivated at 56 and 65 °C even at 60 min, it was completely inactivated at 75 °C in 45 min (Fig. 2C). Surprisingly, at both 56 and 65 °C the virus was inactivated at early time points but at 60 min a small amount of virus was detected. One possible explanation for this result may be the presence and subsequent dissociation of aggregates. Taken together, these results suggest that viral inactivation by pasteurization may be very effective.

3.3. Effects of formaldehyde and glutaraldehyde on the infectivity of SARS-CoV

Formalin (dilute formaldehyde) has been used for a number of years to inactivate virus for use in vaccine products, such as the widely used and very effective polio vaccine (Salk and Salk, 1984). Other attempts at using formalin inactivation for generation of vaccines for respiratory syncytial virus (Kim

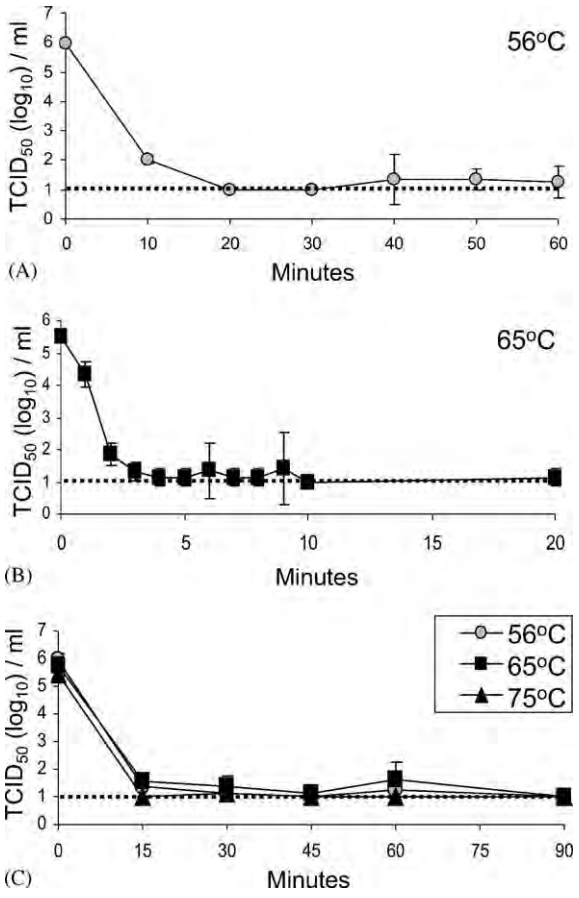

Fig. 2. Effect of heat treatment on the infectivity of SARS-CoV. Virus aliquots (400 μl) were incubated at (A, C) 56 °C, (B, C) 65 °C and (C) 75 °C. Samples were removed at the designated time, frozen, and titrated in Vero E6 cells in triplicate. The dotted line denotes the limit of detection of the assay.

et al., 1969) and measles virus (Fulginiti et al., 1967) were not useful, as they induced an aberrant immune response resulting from formalin-induced perturbations of the viruses. Formalin inactivation occurs when nonprotonated amino groups of amino acids, such as lysine, combine with formaldehyde to form hydroxymethylamine. The hydroxymethylamine combines with the amino, amide, guanidyl, phenolic, or imidazole group of amino acids to create inter- or intramolecular methylene crosslinks (for review, see Jiang and Schwendeman, 2000). Fraenkel-Conrat (1954) observed the absorption spectra of several plant viruses and determined that formalin also binds in a reversible manner to RNA, blocking reading of the genome by RNA polymerase. Glutaraldehyde can also be used to inactivate virus and is used as a disinfecting agent of medical instruments, such as endoscopes (Tandon, 2000), and as a fixative for electron microscopy (McDonnell and Russell, 1999).

We examined formalin and glutaraldehyde inactivation of the SARS-CoV by incubating virus samples with formalin or glutaraldehyde at two different dilutions (1:1000 and 1:4000). Each of the diluted aldehydes was incubated with virus at 4, 25 or 37 °C. Both of the aldehydes exhibited temperature de-

Table 1
Effect of formaldehyde and glutaraldehyde inactivation of SARS-CoV

Virus	Dilution	Day 1			Day 2			Day 3		
		4 °C	25 °C	37 °C	4 °C	25 °C	37 °C	4 °C	25 °C	37 °C
Formaldehyde treatment										
No	1:1000	x[a]	x	x	x	x	x	x	x	x
Yes	1:1000	x	x	x	x	x	x	x	x	x
Yes	1:4000	4.45 ± 0.25[b]	1.31 ± 0.29	1.31 ±0.29	4.02 ± 0.38	1.31 ± 0.29	1.5 ± 0	3.28 ± 0.14	1.14 ± 0.29	1.14 ± 0.29
Glutaraldehyde treatment										
No	1:1000	≤1.0[c]	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0
Yes	1:1000	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0
Yes	1:4000	2.70 ± 0	1.31 ± 0.29	≤1.0	2.26 ± 0.38	≤1.0	≤1.0	2.19 ± 0.25	≤1.0	≤1.0
Yes	No	5.28 ± 0.29	5.09 ± 0.58	4.03 ± 0.14	5.03 ± 0.14	4.86 ± 0.29	3.36 ± 0.29	5.12 ± 0.14	4.1 ± 0.52	2.78 ± 0.14

[a] Formaldehyde treatment fixed the cells in the $TCID_{50}$ assay so virus could not be detected.
[b] Geometric mean of triplicate samples ± S.D.
[c] The limit of detection for the $TCID_{50}$ assay is ≤1.0.

pendence in their ability to inactivate virus (Table 1). Neither formalin nor glutaraldehyde, at a 1:4000 dilution, was able to completely inactivate virus at 4 °C, even after exposure for 3 days (Table 1). At 25 and 37 °C, formalin inactivated most of the virus, close to the limit of detection of the assay, after 1 day, yet some virus still remained infectious on day 3. However, glutaraldehyde completely inactivated the virus by day 2 at 25 °C and by day 1 at 37 °C. This suggests that both formalin and glutaraldehyde inactivation of SARS virus may be efficient methods of inactivation, if proper conditions are met.

3.4. Effect of pH changes on the infectivity of SARS

Weismiller et al. (1990) determined that a pH of 8.0 induces a conformational change in the spike protein of the coronavirus, mouse hepatitis virus (MHV), which enables fusion of the virion with the host cell. However, Xiao et al. (2003) determined that the spike protein of SARS-CoV mediated fusion with the host cell at a neutral pH. These data suggest that different pH conditions affect the spike proteins of coronaviruses, and the activity of the spike protein of the SARS-CoV may be sensitive to changes in pH, possibly by changing the infectious nature of the viral particles. Therefore, we investigated the effect of different pH exposures on the infectivity of SARS-CoV. After exposing SARS-CoV to extreme alkaline conditions of pH 12 and 14 for 1 h, and subsequently reversing the conditions to a neutralized, buffered solution, the virus was completely inactivated (Fig. 3). Moderate variations of pH conditions from 5 to 9 had little effect on virus titer, regardless of the temperature. However, highly acidic pH conditions of 1 and 3 completely inactivated the virus at 25 and 37 °C. At 4 °C, a pH of 3 did not fully inactivate the virus. These data indicate that the infectivity of SARS-CoV is sensitive to pH extremes.

3.5. Infectivity of isolated viral RNA and isolated proteins

Biochemical and molecular biology experiments may require the isolation of nucleic acids or proteins from virus-infected cells. We used a phenol and guanidine isothiocyanate solution (TRIzol, Sigma) to isolate cytoplasmic RNA from SARS-CoV infected Vero cells. After inoculation of Vero cells with the isolated RNA, we determined that SARS-CoV RNA was not able to produce CPE in the cells (data not shown). We also found that transfection of the cells with this RNA, using a liposome-based transfection reagent (DMRIE-C, Invitrogen, as per manufacturer's instructions for RNA transfection), was also not sufficient to cause infection of Vero cells (data not shown).

Additionally, we tested the effectiveness of SDS/NP-40 treatment on inactivation of the SARS-CoV. Briefly, SARS-CoV-infected Vero cells were lysed with an SDS/NP-40 solution, clarified by centrifugation, and the supernatant was used to infect Vero cell monolayers. No CPE was observed in the cells after 3 and 4 days, indicating that SDS/NP-40-induced disruption of the virions was sufficient to prevent survival of infectious particles.

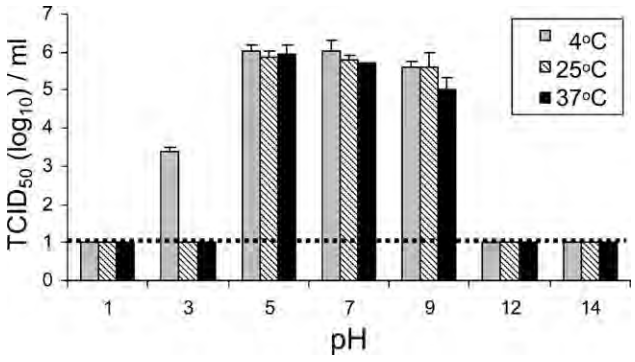

Fig. 3. Effect of pH conditions on the infectivity of SARS-CoV. Virus aliquots (2 ml) were adjusted to the indicated pH condition, divided into triplicate samples, incubated at the designated temperature for 1 h, neutralized, frozen, and titrated. The dotted line denotes the limit of detection of the assay.

4. Discussion

Inactivation of SARS-CoV can be achieved through a number of techniques, given sufficient time and appropriate temperature conditions. We caution that the inactivation procedures discussed above were performed under specific conditions. Due to the grave consequences of a potential SARS-CoV human infection, great care should be taken to ensure that any inactivation procedures used to make the virus safe for BSL2 conditions are effective for each viral stock.

We determined that greater than 15 min of UVC treatment inactivated the virus while UVA light had no effect on viability, regardless of duration of exposure. Duan et al. (2003) examined the effect of UVC light on SARS-CoV at an intensity of >90 $\mu W/cm^2$ and a distance of 80 cm, and determined that inactivation of the virus occurred at 60 min. Inactivation may have occurred more efficiently in our study due to the greater intensity of UVC light and the closer proximity of the light source. We also examined the effect of gamma irradiation on SARS-CoV, and found no decrease in infectivity at the highest dose of 15,000 rad. This result was not surprising, as the Centers for Disease Control and Prevention have used a much higher dose of 2×10^6 rad to inactivate potential SARS-CoV-infected serum specimens for study in BSL2 laboratories (Ksiazek et al., 2003). This dosage is in the same range ($3–4.5 \times 10^6$ rad) that is necessary to inactivate viruses in monoclonal antibody preparations (Grieb et al., 2002) and bone diaphysis transplants (Pruss et al., 2002).

Our experiments showed that heat treatment of SARS-CoV for 45 min at 75 °C resulted in inactivation of the virus, while 90 min at 56 and 65 °C was required for virus inactivation. Laude (1981) determined that thermal inactivation of another coronavirus, transmissible gastroenteritis virus of swine, also occurred faster at higher temperatures, such as 47 and 55 °C, than at the lower temperature of 31 °C. Our data are similar to the findings of Duan et al. (2003), wherein viral

inactivation occurred at 90, 60, and 30 min after incubation at 56, 65, and 75 °C, respectively. Heat is an effective means of SARS-CoV inactivation, however, stocks containing viral aggregates may require a longer duration of heat exposure.

We determined that formalin and glutaraldehyde inactivated SARS-CoV in a temperature- and time-dependent manner. While incubation at 4 °C inhibited the effect of these chemicals, at 37 °C or room temperature, formalin significantly decreased the infectivity of the virus on day 1, while glutaraldehyde inactivated SARS-CoV after incubations of 1–2 days. As glutaraldehyde is commonly used to disinfect medical instruments, especially endoscopes, care should be taken to analyze time, temperature, and concentration requirements necessary for complete SARS-CoV inactivation.

Weismiller et al. (1990) determined that a pH of 8.0 induces a conformational change in the spike protein of the coronavirus MHV that enables fusion of the virion with the host cell. However, Xiao et al. (2003) determined that the spike protein of SARS-CoV mediated fusion with the host cell at a neutral pH. These data suggest that different pH conditions affect the spike proteins of coronaviruses, and the activity of the spike protein of SARS-CoV may be sensitive to changes in pH, possibly by changing the infectious nature of the viral particles. We determined that exposure of SARS-CoV to extreme basic or acidic conditions caused inactivation, while the virus remained stable within a range of neutral pH. The pH of gastric secretions of the stomach ranges from 1.0 to 3.5, while the small and large intestines range from pH 7.5 to 8.0 (Guyton and Hall, 1997). Taken together, these data suggest that ingestion of SARS-CoV would probably result in inactivation of most virions by stomach acid. However, acidic conditions of the stomach may be partially neutralized by a particularly large meal or antacid ingestion, and under these conditions the virus might have a chance to move through the stomach into the slightly basic conditions of the intestines. Leung et al. (2003) have shown enteric involvement of the SARS virus, as evidenced by the presence of active viral replication in intestinal biopsy specimens from five patients, and the isolation of SARS-CoV RNA in stool specimens up to 10 weeks after onset of symptoms. These data, coupled with the previously mentioned stability of the virus to moderate pH conditions, suggest that the SARS virus may survive ingestion and a fecal/oral route of infection may be possible.

Our experiments showed that UVC light, heat, formalin, glutaraldehyde, and extremes of pH, were able to inactivate SARS-CoV. However, gamma irradiation at the doses tested, was not sufficient to inactivate the virus. As expected, neither viral RNA alone nor virions disrupted by SDS/NP-40 were infectious. These conditions were appropriate for our viral stocks as described, however, we caution that researchers need to test viral stocks for complete inactivation before handling the virus at lower safety levels. These data analyze virus samples in tissue culture medium and we are currently testing the inactivation properties required of SARS-CoV in biological (body) fluids. Understanding the ways in which SARS-CoV can be inactivated, will allow the transfer of the virus from BSL3 to BSL2 conditions, and will promote the study of inactivated viral vaccines.

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Recipe

Paraformaldehyde (PFA; 4%)

Reagent	Quantity (for 100 mL)	Final concentration
Paraformaldehyde (PFA)	4 g	4%
Phosphate-buffered saline for immunohistochemistry (10X), diluted to 1X	100 mL	

Slowly dissolve PFA in 1X phosphate-buffered saline over low heat until solution clears. Cool. Use immediately or aliquot and store at −20°C for up to 1 yr.

Paraformaldehyde in PBS

Cold Spring Harbor Protocolscshprotocols.cshlp.org

1. doi:10.1101/pdb.rec9959 *Cold Spring Harb Protoc* *2006. 2006: pdb.rec9959-*

Recipe

Paraformaldehyde, EM grade

PBS (10X)

NaOH (1 M)

HCl (1 M)

For a 4% paraformaldehyde solution, add 4 g of EM grade paraformaldehyde to 50 mL of H_2O. Add 1 mL of 1 M NaOH and stir gently on a heating block at ~60°C until the paraformaldehyde is dissolved. Add 10 mL of 10X PBS and allow the mixture to cool to room temperature. Adjust the pH to 7.4 with 1 M HCl (~1 mL), then adjust the final volume to 100 mL with H_2O. Filter the solution through a 0.45-µm membrane filter to remove any particulate matter. Make the paraformaldehyde solution fresh prior to use, or store in aliquots at −20°C for several months. Avoid repeated freeze/thawing.

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Formaldehyde Prepared From Paraformaldehyde Is Stable

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ABSTRACT. For critical histological investigations, tissue fixation is sometimes carried out in formaldehyde freshly prepared from paraformaldehyde by heating. The purity of formaldehyde produced in this way is superior to that of commercial stock solutions. We studied the stability of freshly prepared formaldehyde solutions by determination of pH and titration of acid, which reflect the formation of formic acid. It was found that very small amounts of acid are produced during the heating of paraformaldehyde. Prolonged heating or storage of freshly prepared formaldehyde for up to 8 days did not significantly increase the amount of acid. It was also found that heating of the paraformaldehyde is not necessary, since depolymerization may take place at room temperature.

We conclude that formaldehyde prepared from paraformaldehyde remains stable for considerable periods of time, and it is therefore unnecessary to prepare it immediately prior to fixation. Also, in many cases, buffering of the fixative may be omitted, since only minor changes in the pH occur during fixation.

Key words: fixation, formaldehyde, histology, liver, paraformaldehyde

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Commercial concentrated formaldehyde is a solution of approximately 37% formaldehyde in water with 10–15% methanol added as a stabilizing agent (Merck Index 1983). The pH of the solution may be strongly acidic. For fixation of histological specimens, the formaldehyde is most often diluted to a 4% (1.3 M) solution and buffered to a neutral pH. For critical studies, fixation is frequently carried out in more pure formaldehyde solutions that have been freshly prepared by heating paraformaldehyde in alkaline water (Robards and Wilson 1993, Hayat 1972, Bancroft and Stevens 1990).

Oxidation converts formaldehyde into formic acid, and reduction produces methanol. It is not clear from the literature how fast these reactions occur in a freshly prepared formaldehyde solution; therefore, many investigators prepare formaldehyde by heating paraformaldehyde immediately before use, a time-consuming procedure which also includes the risk for inhalation of formaldehyde vapor.

For the present study, we depolymerized paraformaldehyde at different temperatures, including room temperature. The stability of formaldehyde prepared from paraformaldehyde was studied by measuring the pH and the amount of acid formed in the solution. In addition, the acidity of formaldehyde solutions prepared from paraformaldehyde was determined before and after use. In this way we determined whether or not buffering is necessary.

MATERIALS AND METHODS

To measure the acidity of freshly prepared formaldehyde solutions, 4 g paraformaldehyde (Sigma, St. Louis, MO) was placed in a 250 ml Ehrlenmeyer flask with 100 ml distilled water and 5 µl concentrated (58%) NH_4OH (Mallinckrodt, St. Louis, MO). After heating to near boiling temperature while stirring with a magnetic bar, the slurry turned clear indicating the depolymerization of the paraformaldehyde into the monomer. The solution was removed from the heat source and allowed to cool at room temperature. It was stored in the laboratory at room temperature in a clear glass vial with a screw cap.

Before heating, immediately after letting the solution cool, and on the eighth day thereafter, 10 ml samples from the vial were removed to measure pH and to titrate to the pH of the original slurry using 0.01 M NaOH. The entire procedure was repeated five times.

In a similar experiment, five vials with paraformaldehyde slurry in water with NH_4OH as above were heated in a water bath to 80 C. Clearing occurred in these vials after approximately 17 min. One vial was removed from the water bath immediately upon clearing, and the remaining four vials were removed at 25, 35, 45 and 60 min, respectively, after the start of the heating. The pH measurements and titrations were carried out as above.

The effects of prolonged heating of paraformaldehyde were studied as well. A vial with paraformaldehyde, NH_4OH and water, as above, was heated to boiling on a hot plate. Immediately upon clearing, a 10 ml aliquot was withdrawn, and subsequently 10 ml samples were obtained 2, 4, 6 and 10 min later. Analyses of pH and titration to the pH of the original slurry were carried out as above.

The depolymerization of paraformaldehyde at room temperature was studied in vials containing 100 ml of paraformaldehyde in water and NH_4OH as above. In some experiments, the amount of concentrated NH_4OH was increased to 20 µl. The time required for clearing of the paraformaldehyde slurry was measured in vials with and without stirring with a magnetic bar.

A sample of commercial 37% formaldehyde from an opened 4-year-old bottle (lot # 920580, Fisher, Fair Lawn, NJ) was analyzed after dilution to 4% with distilled water. The label indicated that the original amount of titratable acid was 0.003 meq/g and that 10–15% methanol had been added.

The influence of tissue specimens on the acidity of the formaldehyde solution was studied separately. For this purpose, 4% formaldehyde was prepared fresh from paraformaldehyde as described above. The solution was divided into two aliquots: one was buffered with 0.1 M sodium phosphate to pH 7.3, and the other aliquot was unbuffered. Samples of fresh rat liver, ranging from approximately 1 to 7 g were put into 20 ml fixative and kept at room temperature overnight. The pH and the amount NaOH required for titration to pH 8.0 were determined for the two fresh fixatives and for each vial after fixation. Samples of the fixed livers were then dehydrated and embedded in paraffin. Sections of 4 µm from the two liver samples were stained with hematoxylin and eosin and compared under a microscope.

RESULTS

In many of the experiments involving the depolymerization of paraformaldehyde, a barely visible layer of the polymer remained on the bottom of the vials. If absent immediately after the depolymerization, the layer nearly always appeared upon storing the vial for a couple of days.

The pH of the paraformaldehyde slurry ranged from 8.33 to 8.94. Immediately after heating and subsequent return to room temperature, the pH ranged from 5.93 to 6.31. Eight days later, the pH ranged from 5.85 to 6.22.

Titration in the 5 vials containing 10 ml of the formaldehyde solution to the pH of

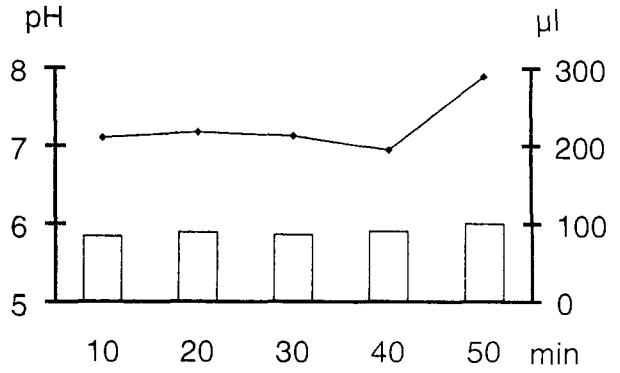

Fig. 1. pH (bars; left ordinate) and volume of 0.01 M NaOH (curve; right ordinate) required for titration of 10 ml aliquots of 4% unbuffered paraformaldehyde heated to 80 C for various periods of time (abscissa) following complete depolymerization of paraformaldehyde (17 min).

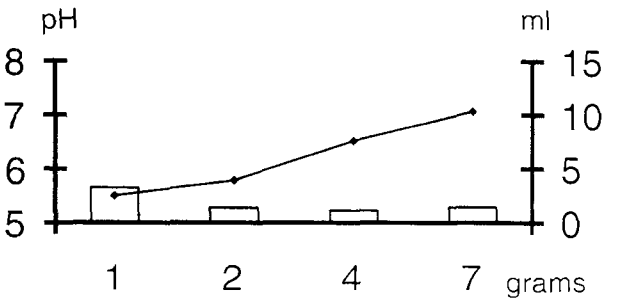

Fig. 3. pH (bars; left ordinate) and volume of 0.01 M NaOH (curve; right ordinate) required for titration to pH 8.0 of 1 ml unbuffered formaldehyde solution previously used for overnight fixation of fresh rat liver specimens of different sizes (abscissa).

the slurry with 0.01 M NaOH required from 137 to 751 µl immediately after heating and cooling. Eight days later, 138–455 µl was required.

There was no significant difference in pH or titrable acidity (Fig. 1) among the solutions heated to 80 C for different periods of time.

Prolonged boiling of the formaldehyde after the slurry had cleared tended to decrease the pH slightly (Fig. 2); however, there was no significant correlation between the amount of titrable acid and the heating period.

The conversion of paraformaldehyde into formaldehyde at room temperature occurred after continuous stirring for 2 days, provided that 20 µl of NH_4OH had been added. At the lower concentration of NH_4OH or in the absence of stirring, depolymerization was slower or did not take place.

The pH of the diluted commercial formalin was 3.87 and the amount of 0.01 M NaOH required for titration of 10 ml to pH 8.5 was 1319 µl.

Tissue specimens in the fixative lowered the pH (Fig. 3) and increased the amount of NaOH required for titration to pH 8.00 (Fig. 4) in proportion to the amount of tissue present. We observed no obvious differences in morphology or staining between the tissue specimens fixed in buffered vs. unbuffered formaldehyde.

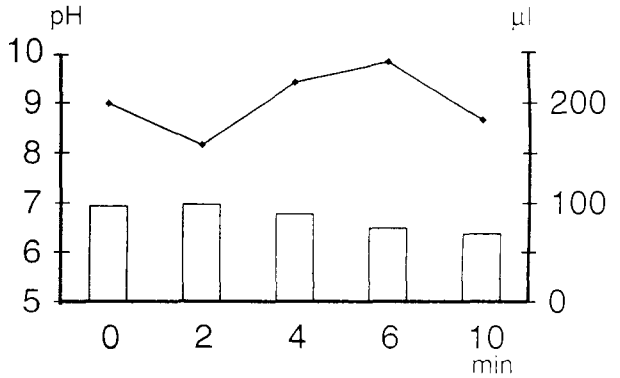

Fig. 2. pH (bars; left ordinate) and volume of 0.01 M NaOH (curve; right ordinate) required for titration of 10 ml aliquots of 4% unbuffered formaldehyde boiled for various periods (abscissa) following the depolymerization of paraformaldehyde.

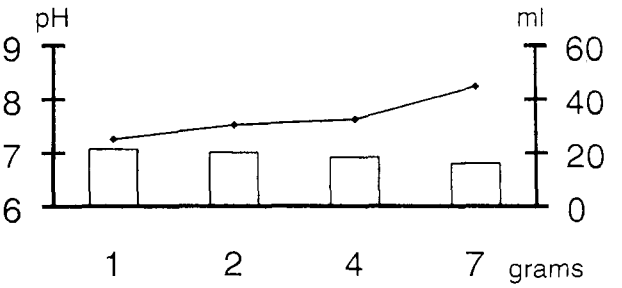

Fig. 4. pH (bars; left ordinate) and volume of 0.01 M NaOH (curve; right ordinate) required for titration to pH 8.0 of 1 ml buffered formaldehyde solution previously used for overnight fixation of fresh rat liver specimens of different sizes (abscissa).

DISCUSSION

The conversion of paraformaldehyde to formaldehyde by heating is a hazardous procedure that carries the risk of inhalation of toxic fumes. Our study has shown that this conversion also takes place at room temperature, albeit at a slow rate. Adopting our procedure will reduce the health hazards considerably.

Our results indicate that some acid is formed during heating of paraformaldehyde in alkaline water. The small amount of NaOH required for titration, however, indicates that the concentration of this acid (presumably formic acid) was very low. Prolonged heating does not produce appreciable additional amounts of acid. Also, no additional acid is formed during storing of the formaldehyde for up to 8 days. Reformation of paraformaldehyde from the monomer appears to be insignificant.

If formaldehyde of high purity is required for fixation, depolymerization of paraformaldehyde provides formaldehyde with only small amounts of contaminating acid. The product is stable for at least 8 days.

Because commercial formaldehyde stock solution may be highly acidic, buffers should be used to bring the pH to the desired level, most commonly 7.2–7.4. With the low acidity of the formaldehyde solutions prepared from paraformaldehyde, buffering may be less important and may even be omitted.

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