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DEVELOPMENT OF AN *EX VIVO* MODEL TO STUDY THE SURVIVAL AND INACTIVATION OF PATHOGENS ON HUMAN SKIN

A Thesis Submitted to the
School of Graduate Studies and Research
University of Ottawa

In Partial Fulfillment of the Requirements for
the Degree of Master of Science
Department of Microbiology and Immunology
Faculty of Medicine

by

Mary Louise Graham

ABSTRACT

Topicals are generally tested for their safety and efficacy using inanimat surfaces, animals and human volunteers. Ethical considerations restrict the us of human volunteers and animals, and data from *in vitro* tests do not accurate reflect the product's behaviour *in vivo*. This project outlines a novel method fo testing the survival and inactivation of viral pathogens on viable human tissu carriers.

First, a flow-through diffusion cell system was used to assess the *ex viv* viability of fresh and refrigerated human skin fragments removed from the face breasts and abdomen during plastic surgery by a local surgeon. Dermatome skin sections were mounted in flow-through diffusion cells and perfused from th dermis side with cell culture medium (32°C) saturated with 5% CO_2. Samples c the effluent were collected at 4-hour intervals. The metabolic activity, and henc the viability of the skin cells, was measured through anaerobic glucose utilizatio by the accumulation of lactate in the effluent. Lactate was quantitated using a automated Abbott Bichromatic Analyzer. Histopathological examination of th skin fragments over time provided a visual confirmation of viability of the multi layered skin sections. The results showed that human skin cells from the thre body sites could be kept viable in this system for at least 48 hr. However, th rate of metabolic activity was lower in skin samples stored at 4°C for 24 hr prio to the viability tests. Similarly, when a static model was used, viability of the ski sections was maintained over the test period of 8 hr.

Many topical agents are commonly found in products such as ski disinfectants, ophthalmic solutions, and spermicides. The two topically applie chemical agents selected for testing in the *ex vivo* model were chlorhexidin digluconate (CD) and benzalkonium chloride (BC). CD is manufactured as 20% (weight/volume) aqueous solution, is a cationic molecule effective a ambient temperature and is commonly used in healthcare handwas preparations at 0.0025-0.01% (25-100 ppm). BC is commonly used in surgery urology and gynecology as aqueous and alcoholic solutions and is an agent c choice in ophthalmic solutions. BC is commonly used at concentrations of 100 t 500 ppm in solutions to be used on the mucosa of the eye and up to 5000 ppr in cosmetics applied to the skin. In our cytotoxicity tests using confluent Vero ce monolayers, the addition 100 μL of 1000 ppm of BC to the monolayer for 90 mi resulted in complete cytotoxicity and with CD a concentration of 50 ppm after similar contact time, 100% cytotoxicity was found.

The *ex vivo* method was developed to use animate carriers: pieces c human skin excised during routine plastic surgery for the stratum corneum an umbilical cords as mucous membranes. The umbilical cords were used as th mucosal membrane of choice as they could be obtained frequently and withi two hours of delivery of the infant and would provide many carriers from on cord. With both specimen types, the tissue was mounted on a cutting board an 2.3 cm diameter patches were then made with a cutting tool. Each patch wa mounted over the top of a specially designed holder and secured with an O-ring The animate carriers were then ready for use in the *ex vivo* protocol. The *ex viv*

model was evaluated with Human Herpesvirus 2 (HHV 2) strain 333 and Adenovirus 4 (AD 4) strain RI-67 with parallel tests run on the tissues and stainless steel disks to compare virus survival and inactivation on animate and inanimate surfaces. HHV 2 and AD 4 virus pools were made by infecting confluent Vero and 293 cell monolayers, respectively. Throughout the study infectivity for both viruses was measured by plaque assay in Vero cell monolayers.

Using this procedure, the survival of HHV 2 and AD 4 was compared on human skin, umbilical cord and on metal disks. At the physiological skin temperature of 32°C, the virus half-life was 1.93 hr and 5.78 hr on skin, 1.05 hr and 3.85 hr on the umbilical cord while on metal disks it was 0.46 hr and 1.05 hr, for HHV 2 and AD 4, respectively.

To determine the virucidal activity of BC and CD, 10 μL of the test virus (about 10^5 PFU) was allowed to dry for 60 min at 22±2°C and 100 μL of the test germicide was then applied to each contaminated surface for 1 min. At 1000 ppm of BC, there was only about a 50% reduction in HHV 2 infectivity on all types of carriers. At this same concentration of BC against AD 4, the titer dropped to 50% on the animate carriers and with no virus surviving on the metallic disks. By increasing the concentration of BC to 5000 ppm, complete inactivation of both viruses on all carriers was observed. In contrast, with 200 ppm of CD, complete inactivation of HHV 2 was found on the metallic and skin carriers but infectivity was reduced to only 40% on the umbilical cord. For AD 4

even at 1000 ppm complete inactivation was not achieved on any of the carriers, with 2-5% of infectious virus remaining detectable.

The observed differences in virus survival and the behaviour of the chemicals on the animate and inanimate carriers indicate that an *ex vivo* model could provide a more relevant system to (a) study pathogen survival on human skin and mucous membranes, (b) assess the germicidal potential of antiseptics, (c) determine the antimicrobial activity of new compounds, (d) investigate possible interactions between chemicals and infectious agents and (e) facilitate proper regulation of topicals.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AD	Adenovirus
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
ARD	acute respiratory disease
ASTM International	American Society for Testing and Materials
BC	benzalkonium chloride
CD	chlorhexidine digluconate
cm	centimeter
CPE	cytopathic effect
ds	double stranded
EBSS	Earle's balanced salt solution
EKC	epidemic keratoconjunctivitis
ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
FBS	fetal bovine serum
FDA	Food and Drug Administration
g	gravity
hr	hour(s)
H&E	hematoxylin and eosin
HEPES	N-2-hydroxyethylpiperazine-N-2ethanesulfon acid

HHV	Human Herpes Virus
HIV	Human Immunodeficiency Virus
Ki	rate of virus inactivation
L	liter
MEM	minimum essential medium
mg	milligram
min	minute
mL	milliliter
mm	millimeter
NEAA	non-essential amino acid
nm	nanometer
PFU	plaque forming units
ppm	parts per million
s	second
SEM	standard error of the mean
$TCID_{50}$	50% tissue culture infective dose
µg	microgram
µL	microliter
U.S.	United States
U.K.	United Kingdom
UV	ultraviolet

LIST OF FIGURES AND TABLES

INTRODUCTION AND RATIONALE

Significant economic costs to the health care industry and work force are caused by human infections (Sattar et al. 1994). The majority of human infections are transmitted through mucosal surfaces, such as the eye, mouth, or genital tract, and hands are an important vehicle in transferring these infectious agents. For example, this typically involves spread of infections through sexual transmission, a fecal-oral route or from hands in contact with fomites. In order to comprehend the potential for disease transmission one needs to understand the survival and inactivation characteristics of pathogens on these relevant surfaces.

Both animate and inanimate surfaces have been implicated in the spread of infections (Sattar et al. 1987; Jacob 1994; Sattar et al. 1994). For instance, Hepatitis A virus and rotaviruses have been shown to survive for several days on environmental surfaces (Sattar et al. 1986; Mbithi et al. 1991) and for several hours on human hands (Ansari et al. 1988; Mbithi et al. 1992). Thus, both of these surfaces are a concern with respect to infection and the transferring of surviving virus to susceptible body sites. As such, an effective means of preventing and controlling this spread of infectious agents is required in hospitals and other healthcare settings, and in the community at large. With this in mind, there is a public need for topical antiseptics with proven effectiveness. And unfortunately, there is presently a lack of understanding of bacterial and virus-eliminating efficiencies of these antimicrobials due to the fact that there are no existing relevant standardized testing methods which directly address this.

Many topical products, such as antiseptic handwashes or barrier antimicrobials, are marketed as means to interrupt transmission of pathogens. Currently available methods to examine the efficacy of these antiseptic products are not necessarily relevant as they are not tested for pathogen inactivation on cutaneous or mucosal surfaces of the human body. In general, the testing methods to examine the efficacy of disinfectants have been either suspension tests, where a suspension of microorganisms comes into contact with a disinfectant solution for a set time (often 10 min) and temperature, versus carrier tests in which the microorganisms are first allowed to dry on the surface of a carrier and then are exposed to the disinfectant solution for a set time (i.e. 1 and 10 min) and temperature (Sattar et al. 1994). With the differences in testing protocols and conditions used between laboratories it is very difficult to make direct comparisons between the findings of many published studies. Also, tests conducted in suspension often overestimate the germicidal efficacy of products on contaminated surfaces, as it has been found that organisms dried onto surfaces are much more difficult to inactivate (Woolwine et al. 1995; Bellamy 1995; Springthorpe et al. 1986; Lloyd-Evans et al. 1986). The ability of a virus to survive on a carrier surface is also determined by the nature of the surface material (i.e. stainless steel, plastic, glass, fingerpads of a human volunteer); the composition of the virus suspending medium; the relative humidity; the temperature; etc. (van Klingeren 1995; Woolwine et al. 1995; van Klingeren 1995).

One should also question the applicability of these suspension and *in vitro* tests. For instance, a previous study examined the inactivation of HIV using an *in vitro* antiseptic suspension test and a cell-free virus preparation (Wainberg et al. 1990). From this data one still cannot reasonably predict the behaviour of the product in the field. Even when carrier tests are performed, discrepancies have been found between the results of these *in vitro* tests and those of *in vivo* methods with the use of fingerpads of adult volunteers to assess quantitatively the ability of viruses and bacteria to survive on human skin (Woolwine et al. 1995; Ansari et al. 1988). One can only extrapolate from suspension tests or inanimate carrier tests to *in vivo* antiseptic efficacy. Results from fingerpad tests have been found to be comparable with the whole-hand protocol (Ansari et al. 1989; Mbithi et al. 1993).

The lack of data for virus survival on skin is not surprising considering the difficulties and ethical considerations involved in conducting relevant experiments with human volunteers (Ansari et al. 1988). Similarly for inactivation, there is a lack of published information on the *in vivo* efficacy of commonly used hand-washing agents and other topicals (Ansari et al. 1991). In one study, it was found that the efficacy of hand-washing agents to remove virus from hands depended on the type of virus used (Mbithi et al. 1993). In order to avoid risks to human volunteers, some earlier studies used pig skin as a test substrate for evaluating topical antimicrobial activity against bacterial and viral pathogens (Bush et al. 1986; Woolwine et al. 1995). With respect to the testing

of topical products, animals are generally ruled out not only because the skin will react differently to products but also because human bacterial ecology is unique (Leyden et al. 1979). Also, it is certainly more relevant to use human tissue carriers with human specific disease causing agents.

As there are significant potential health hazards from ineffective disinfection, the safety and efficacy of disinfectants and the tests used to determine efficacy data should be redesigned. There has previously been little antiseptic testing done with suspension or carrier tests with viruses which may be due to the fact that there are still no standard methods to determine virucidal activity. For example, the U.S. Food and Drug Administration (FDA) does not have approved testing methods to determine antiviral activities of topical antiseptics (Woolwine et al. 1995). Whereas the Association of Official Analytical Chemists (AOAC) has protocols for evaluating bactericidal, tuberculocidal and sporicidal efficacy claims of disinfectants, there are still no approved AOAC methods for assessing virucidal activity of germicides. ASTM International (formerly the American Society for Testing and Materials) has a suspension and a carrier test using poliovirus, herpesvirus, adenovirus type 2, influenza virus, rhinovirus and vaccinia virus (Bellamy 1995). The ASTM International has recently approved the first *in vivo* fingerpad protocol for efficacy testing of disinfectants. This fingerpad protocol was developed through previous work in the laboratory of Dr. S.A. Sattar (Ansari et al. 1989; Mbithi et al. 1993). The U.S. Environmental Protection Agency (EPA) recommends using a carrier

test for activity against viruses but does not specify any surrogate virus(es) to be included in the challenge. The EPA requires that for each virucidal claim all viral pathogens in the claim must be tested (Sattar et al. 1991). So in summary there are currently no North American or global standardized methods for the assessment of hand or skin disinfection against viruses.

Therefore, there is an urgent need to develop a standard test methodology for topicals which are going to be used against pathogens on human skin and mucosal surfaces, specifically, one that can be reasonably expected to predict field efficacy. As such, this research was undertaken to develop an *ex vivo* model to study the survival and inactivation of pathogens on viable human tissues. To do this, it was first necessary to select model agents and examine both their survival and their inactivation in the newly developed system.

DEVELOPMENT OF THE MODEL.

There were five areas to develop in the production of this new *ex vivo* protocol:

1. **Human tissue carriers:** Selection of appropriate freshly available human tissue for use as carriers. To validate the *ex vivo* model representing an *in vivo* system, the maintenance of tissue viability would first have to be demonstrated.
2. **Chemical agents:** Selection of relevant topically applied products commonly manufactured and used in Canada and elsewhere.
3. **Pathogens:** Viruses to be selected for relevance to transmission by skin or mucosal tissues, as well as representative of different virus families.
4. **Equipment:** Select or manufacture tools necessary to support the interactions of 1-3.
5. **Protocols**: Development of the protocols in order to create an *ex vivo* method which is fully reproducible and represents *in vivo*-like conditions.

REVIEW OF THE LITERATURE

CARRIERS

Topicals and drug products are generally tested for their safety and efficacy using human volunteers, whole animals, fragments of animal skin, cultured skin cells, cadaveric skin, skin flaps and environmental surfaces. Ethical considerations restrict the use of human volunteers to testing of non-hazardous agents only. Experimentation using animals is also subject to many restrictions and results generated using *in vivo* and *ex vivo* animal models may not reflect those based on human tissues (Bronaugh 1989; Wester et al. 1989; Hawkins et al. 1986). Cultured keratinocytes could represent a carrier type but are not readily available, costly, and the integrity of their stratum corneum is inconsistent (Riviere 1991; Collier et al. 1992). The viability, barrier integrity and biochemical functions of cadaveric skin are known to be compromised (Jensen 1984; Hiernickel 1985; Bronaugh et al. 1986). The use of isolated perfused skin flaps is arduous and requires a relatively large skin surface with an intact blood circulation (Riviere et al. 1987; Riviere et al. 1986; Hiernickel 1985; Riviere 1991). And lastly, the germicidal activity of antiseptics and handwashing agents on inanimate surfaces may not reflect the product's behaviour on human skin (Collier et al. 1992; Bronaugh et al. 1991; Collier et al. 1989; Riviere et al. 1986; Hiernickel 1985). Thus, it is ideal to use only viable human tissues when testing for efficacy of topically applied disinfectants against human pathogens.

The outer covering of the body consists of keratinized stratified squamous epithelium, e.g. skin, and non-keratinized stratified squamous epithelium, e.g. lining of body openings such as the oral cavity or vagina. The skin acts as a mechanical barrier and support, a temperature regulator, an insulator, a neurosensory transmitter, and a metabolizer (Ross et al. 1995; Monteiro-Riviere 1991; Collier et al. 1992; Riviere 1991). By comparison to animals, human skin is unique in its stratum corneum thickness and hair follicle density (Bronaugh 1989; Wester et al. 1989). It should be noted that many authors consider pig skin to be the best animal model for human skin because of similarity in structure and function, though differences include different densities of hair follicles and the presence of only apocrine sweat glands in swine and eccrine sweat glands in humans (Riviere 1991).

Because umbilical cord is freshly and frequently available it has been selected as the mucosal membrane of choice for this protocol. The outer amnion covering of the umbilical cord consists of a layer of simple cuboidal epithelium and is structurally similarly to cervical mucosa which is made up of simple columnar epithelium (Ross et al. 1995).

Thus, *ex vivo* human skin and umbilical cord were used in the development of the model for testing the survival and inactivation of human pathogens. To assess the validity of the *ex vivo* model representing an *in vivo* system, the viability of the skin tissue was determined first. Earlier methods to determine the health of the tissue depended on successful grafting on dogs as

proof of viability (Jensen 1984). To eliminate all use of animals, a Flow-Through Diffusion Cell System previously used to demonstrate viability of fragments of fuzzy rat and hairless guinea pig skin (Bronaugh et al. 1990) was employed. This thesis describes the extension of this approach to human skin sections whereby both biochemical measurement of ongoing metabolism along with histopathological evaluation demonstrated maintenance of *ex vivo* human skin viability. Stainless-steel disks were used as carriers to represent non-porous inanimate environmental surfaces and parallel tests were run on the disks to compare virus survival and inactivation on animate vs. inanimate surfaces.

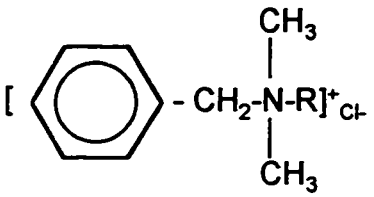
DISINFECTANTS

To assess the efficacy of topical antimicrobials one must consider a variety of factors: are there immediate and residual microbicidal actions on resident and transient flora; are there cumulative effects from frequent use; is it effective in the presence of proteins such as blood, semen, etc. (Denton 1991). It is difficult to select an appropriate and effective disinfectant as current products have diverse efficacy claims. Benzalkonium chloride (BC) and chlorhexidine digluconate (CD) were selected for their relevance as topically applied products which are commonly manufactured and used in Canada (Table 3A and B, Appendix II and III) and elsewhere. The two active agents BC and CD have modes of action that physically disrupt the protein or lipid structures of microorganisms (Merianes 1991; Prince et al. 1991).

Previous studies were performed to determine the anti-HHV 2 activity of non-ionic surfactants in pure form or as found in commercially available formulations. The authors also sought to find whether this activity results from damage to the viral envelope (Asculai et al. 1978). Cytotoxicity of eight nonionic surfactants was determined by observation microscopically after 5 and 60 minutes incubation at 35°C, with concentrations of the agents ranging 0.1%, 0.2%, 1%, 10% to undiluted. 0.5 mL of each dilution of surfactants were added to confluent monolayers of cortical rabbit kidney (RK) cells in 60-mm plastic petri dishes. The authors found that treatment of the RK cell line with the surfactants at dilutions less than 0.2% resulted in loss of the cellular membrane (Asculai et al. 1978). They speculated that loss of the viral envelope occurs in a similar fashion. Next, a 1 mL of $1x10^6$ PFU/mL of HHV 2_{333} was added to 1 mL of the active agent and incubated in a water bath for 1 minute at 37°C. In electron micrographs of HHV 2 after incubation with 5% nonoxynol-9 for 10 s, no envelopes were observed and the nucleocapsids were disrupted (Asculai et al. 1978). It would be of interest to do this *in vitro* suspension test with 500 ppm (or 0.5%) of BC and 50 ppm (or 0.005%) of CD after 90 min contact time where both viral tests demonstrated a 30% reduction in viability of HHV 2. Electron microscopy could reveal if some or all of the viral particles have damaged envelopes. For the non-enveloped AD 4, one could repeat the assay to see if the capsid is affected or not.

Benzalkonium chloride (BC).

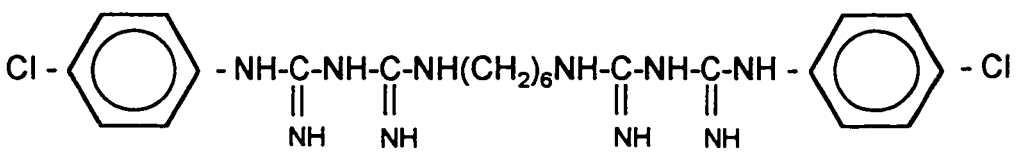
The chemical structure of benzalkonium chloride, a cationic surfactant, is, (Anonymous 1989):



BC, a quaternary ammonium compound, is commonly used in surgery, urology and gynecology as aqueous and alcoholic disinfectant solutions. It is an agent of choice in ophthalmic solutions and is commonly used as a 0.05% solution in eye irrigation and 0.01% preservative in eye drops (Fassihi 1991; Denton 1991). BC is considered safe at a concentration of 0.5% in cosmetics applied to the skin, with a maximum concentration of 0.02% for use in cosmetics around the eyes (Merianos 1991). It was found that a 0.1% BC solution in either water or 70% ethanol is toxic and produced damage when introduced into the middle ear of guinea pigs for a minimum of 10 minutes (Merianos 1991).

Chlorhexidine digluconate (CD).

The chemical structure of chlorhexidine is, (Anonymous 1989):



Chlorhexidine, a biquanide antiseptic, (Anonymous 1992) first made in 1950, is manufactured as a 20% (weight/volume) aqueous solution. This cationic molecule is effective at ambient temperature (Fassihi 1991). It is a known effective and safe topical agent when used as directed and in the past 30 years

of use few adverse reactions have been reported worldwide. Absorption through the skin from topical use in humans has been found to be negligible with no evidence of carcinogenicity. CD has a strong affinity for skin and has been found to produce persistent/residual germicidal effect on the applied surfaces (Denton 1991).

CD is commonly used in healthcare handwash preparations at 0.0025-0.01%. At concentrations of 0.005 to 0.01% CD is considered a bacteriostatic agent for ophthalmic solutions (Fassihi 1991). Higher concentrations (0.1% and 0.5%) may cause eye damage, and greater than 2% is toxic to corneal epithelium and conjunctiva (Denton 1991). CD has been found to be toxic to nerve tissue and its use during ear surgery is not recommended as it could result in deafness (Denton 1991).

PATHOGENS

While it would be relevant to examine all topically-acquired infectious agents in this model, the initial development and testing of the *ex vivo* protocol was limited to two viral agents.

Ideally, topicals chosen for a particular use should be able to inactivate all types of pathogens that may be present on the surface to be treated. However, the specific pathogens present in such cases are often not known and in product evaluations one has to use a model virus to represent any pathogen that may be encountered there. The selection and use of a model infectious agent for germicidal testing can only be done providing that the selected agent's

resistance to disinfection is comparable to most if not all pathogens to be represented. Generally, enveloped viruses have a high sensitivity to disinfectants, whereas non-enveloped ones are moderately sensitive (Prince et al. 1991; Merianos 1991; Denton 1991). The enveloped HHV 2 and non-enveloped AD 4 were selected here for their relevance as important pathogens in human disease; their transmission by skin and mucosal tissues; as well as representative of different virus families.

HUMAN HERPESVIRUS 2

Family. Eight human herpesviruses are currently known: Human Herpesvirus (HHV) types 1 and 2, Varicella-Zoster virus, Epstein-Barr virus, Cytomegalovirus, and Human Herpesvirus 6, 7 and 8. HHV 1 is usually isolated from labial, facial, ocular, or brain lesions; and HHV 2 from genital lesions and tissues of newborns who became infected at birth. Both serotypes can cause either genital or oral-facial infections, and the type involved cannot be identified on clinical grounds alone (Smith et al. 1992).

Morphology. Enveloped HHV particles are 120 nm to greater than 200 nm in diameter (Feldblum et al. 1988). The envelope has 10 nm long surface projections which are thought to mediate cell attachment (Palmer et al. 1988). After fusion with the host cell, herpesvirus uncoates and its DNA is released into the cytoplasm (Palmer et al. 1988). The Herpesvirus genome consists of a linear double stranded (ds) DNA which encodes approximately 70 polypeptides. The genome has two components, L and S, and each element contains inverted

repeats which can thus lead to four possible isomers (Feldblum et al. 1988). The core contains the ds DNA and is packaged in an icosahedral capsid. The nucleocapsid, with a diameter of 100 to 105 nm, is assembled in the nucleus and the envelope is derived from the nuclear membrane as the virus leaves the nucleus.

Transmission. Transmission of herpesvirus between humans has been known to occur since before the 18th century when herpetic lesions were linked with genital infections (Whitley 1990). Studies indicate that 10% of the U.S. population is seropositive for HHV 2, and approximately 500,000 individuals per year are newly infected. This leads to an estimate 40-60 million cases of infected individuals in the U.S. (Whaley et al. 1994; Whitley 1990).

HHV must come in contact with mucosal surfaces or abraded skin for infection to be initiated and the main route of exposure is through intimate person-to-person contact (Whitley 1990). First exposure to either herpes type leads to a "primary infection" from which a state of latency can develop. "Recurrent infections" can be either symptomatic or asymptomatic and the mechanisms of recurrence have not yet been elucidated (Whitley 1990). HHV 1 and 2 can be excreted in the absence of symptoms and therefore provide a silent reservoir for transmission of infection. There is an 80-90% risk of developing genital herpes for a woman exposed to an infected male (Whitley 1990; Smith et al. 1992).

With respect to nosocomial infections, vehicles for HHV transmission have been inadequately studied. Vigorous hand-washing procedures and continuing education of personnel (i.e. in newborn nurseries) may have contributed to the low frequency of transmission in the hospital setting. In examining the role of fomites in non-sexual transmission of HHV 1 and 2, it has been found that for transmission to occur there must be direct contact with infected material and exposed skin or susceptible mucous membranes (Springthorpe et al. 1990). In one previous study, the authors sought to correlate an association of HHV 2 linked to genital ulcers with an increased risk for HIV-1 infections in the heterosexual population (Hook, 3d et al. 1992). It was found that the prevalence of antibodies to HHV 2 was greater in patients with than without HIV-1 infection (Hook, 3d et al. 1992) which even further demonstrates the need for approved anti-viral topicals.

Pathogenesis. Herpetic lesions can occur anywhere on the skin or mucous membranes but most frequently appear in the oral and genital areas (Whitley 1990; Smith et al. 1992). In mucous membranes, shallow ulcers occur as a result of the rupturing of vesicles which have formed due to the thinning of the cornified epithelium (Whitley 1990). Both cell-mediated and humoral immunity responses are required for recovery from HHV infections. Cell-mediated immunity is involved in clearing the virus at the primary site of infection whereas humoral immunity is important in limiting the spread of infection (Eis-Hubinger et al. 1993).

HHV 2 symptoms last for about 3 weeks (Whitley 1990). The virus is acquired at an abraded site, where macules and papules, followed by vesicles, pustules, and ulcers, may appear. Primary infection is associated with > 10^6 viral particles of virus replicating in the genital tract and a period of viral excretion which may persist for an average of 3 weeks. Primary infections can be associated with fever, dysuria, localized linguinal adenopathy, and malaise (Whitley 1990). With HHV 2, transmission is normally via the genital route where sacral ganglia will then become colonized for latency. In women, lesions may be associated with vulva, buttocks, cervix and/or vagina. In the male, primary genital HHV most often is associated with the glans penis or the penile shaft (Whitley 1990). In recurrent infections, the virus is shed for an average of only 2-5 days and at lower concentrations of 10^2-10^3 per 0.2 mL of vesicular fluid (Whitley 1990). The frequency of reactivation is influenced by the anatomic site and the type of virus. Genital infections of HHV 2 are twice as likely to be reactivated (reasons for reactivation still unknown) and recur 8 to 10 times as frequently as genital infection with HHV 1 (Smith et al. 1992).

Neonatal HHV infection is associated with high morbidity and mortality rates. Infection in the newborn is usually acquired by exposure to virus in the maternal reproductive tract during delivery (Smith et al. 1992).

Diagnosis. Virus isolation in cell culture remains the definitive diagnostic method for herpesvirus (Whitley 1990; Smith et al. 1992). Cytologic, immunologic, electron microscopic, and DNA hybridization methods are also

used for rapid diagnosis of HHV 2. Tests for measurement of herpes antibodies are: complement fixation, passive hemagglutination, neutralization, immunofluorescence, and ELISA assays (Whitley 1990; Smith et al. 1992). Many of the commonly available serologic assays are unable to differentiate between HHV 1 and HHV 2 (Smith et al. 1992). In addition to skin vesicles, sites from which virus may be isolated include the cerebrospinal fluid, stool, urine, throat, nasopharynx, and conjunctivae.

Treatment. Acyclovir (Zovirax), a nucleoside analogue, is very active against HHV 1 and 2 (Watts 1992). The drug is well tolerated in pregnancy, and no toxicity was seen in mothers or neonates (Watts 1992). The drug can be administered topically, orally, or intravenously. The emergence of viral strains resistant to acyclovir has been described (Smith et al. 1992). Other chemotherapies in use have been found to have serious side-effects: Ganciclovir (cellular toxicity) (Watts 1992), Foscarnet (renal toxicity) (Watts 1992).

Survival in the Environment. *In vivo* human protocols for virus survival consist mainly of the use of fingerpads and whole hands of volunteers but cannot be used with high risk agents (Mbithi et al. 1993; Cutler 1972; Ansari et al. 1989; Ansari et al. 1988). As such, *in vivo* methods using human pathogens such as genital herpes are essentially limited to animal systems. In previous studies looking at survival of herpesvirus on carriers, when virus was incubated at temperatures ranging from 25°C to 40°C on plastic gloves, toilet seats, plastic

(petri dishes), speculum, dry cotton gauze, glass, and the forearms of one researcher, human herpesvirus was found to survive up to 1 hr (Mbithi et al. 1993), 2 hr (Mbithi et al. 1993), 3 - 4.5 hr (Woolwine et al. 1995; Turner et al. 1982), 18 hr (Mbithi et al. 1993), 72 hr (Mbithi et al. 1993), at least 8 weeks (Mbithi et al. 1990), and 2 hr (Turner et al. 1982), respectively. Both the skin and the plastic samples were dry after 30 minutes which correlated with the rapid reduction in titers observed. On cloth, which had a longer viral inoculum drying time (~ 1.3 hours), the titer was found to decline more slowly (Ansari et al. 1988). Therefore, acquisition of virus could occur by contact with a variety of moist inanimate or animate surfaces.

Specimens from the hot tubs of two health clubs were tested for the presence of HHV and its ability to survive in water and on plastic surfaces (Woolwine et al. 1995). This involved spiking 20 mL samples of either spa water, laboratory tap water or deionized distilled water, with HHV 2 strain 333 and incubating at 37°C to 40°C for 0 to 96 hours. At each time interval, samples were removed to determine infectivity titers. In spa water, which had an average 16 ppm chlorine and 28 ppm bromine, no HHV could be isolated. Conversely, in tap water with no bromine and 4.0 ppm chlorine, HHV survived up to four hours and in deionized distilled water, with neither disinfectant, the virus survived up to 24 hours. This suggests that water may act as a vehicle for HHV (Woolwine et al. 1995). However, no such spread has been documented thus far.

Inactivation Studies. One study used an *in vitro* suspension test and an *in vivo* guinea pig test (with live anesthetized animals), to compare virucidal activity of a quaternary ammonium compound and a non-medicated soap against herpesvirus 1. The authors observed approximately one $\log_{10}$ lower virus recovery in the *in vivo* compared to the *in vitro* method (Prince et al. 1994). Other work involved the *in vitro* inactivation of HHV 2 by five chemical contraceptives: Preceptin gel; Conceptrol; Cooper creme; Lorophyn jelly; and Milex crescent jelly. Unfortunately, the list of active ingredients was not given (Singh et al. 1976). The titer of the original suspension of HHV 2 was reduced by the five chemical compounds by 3 to 4 $\log_{10}$ after a 10 minute contact at room temperature (Singh et al. 1976). In another *in vitro* suspension study looking at the inhibitory activity of five spermicides against HHV 2 strain 333, menfegol, a non-ionic surface-active agent, was found to have a four-fold lower activity against the virus than nonoxynol-9 (Jennings et al. 1993). Similarly, benzalkonium chloride was found to have a similar level of activity against HHV 2 as nonoxynols-9 and -11. Sodium docusate was found to have a 2.5 times greater activity against HHV 2 than nonoxynol-9 (Jennings et al. 1993). This study found that a minimum concentration of 0.025% of either nonoxynol-9 or benzalkonium chloride inactivated HHV 2 in 30 seconds (Jennings et al. 1993).

One group has published an *in vivo* study whereby nonoxynol-9 was demonstrated to protect mice against vaginal transmission of genital herpes (Whaley et al. 1993). In the mouse study, a virus inoculum of HHV-2_G (ATCC

VR-734) with a titer of 1000 $TCID_{50}$ was pipetted into the vagina of mice which had previously been treated with a 5% nonoxynol-9 contraceptive jelly (Ramses Contraceptive Jelly; Schmid Laboratories, Little Falls, New Jersey, USA). Control animals received a lubricant jelly containing no spermicidal or virucidal agents. The nonoxynol-9 was found to be effective in the prevention of vaginal transmission of cell-free HHV-2 in mice by protecting 23 out of 24, when 20 out of 24 of the control animals became infected (Whaley et al. 1993). This *ex vivo* method could similarly be used to test the barrier capability of topicals if the agent is first applied to the skin followed by the application of the pathogen. Ideally, for such a model one would try to demonstrate infection and active replication in the amnion layer of the umbilical cord and then show that the barrier agent blocks all virus replication. This is a very important aspect which is particularly significant with pathogens such as HHV 2 where the virus remains in the patient for life.

Another study looked at the effects of a topical application of a purified anti-HHV 2 monoclonal antibody (MAb III-174) in the prevention of HHV 2 infection. The use of passive immunization with an anti-HHV 2 MAb protected mice from 10^3 $TCID_{50}$ dose of HHV 2 applied vaginally 0.3 minutes after the MAb (Whaley et al. 1994). For complete protection, the minimum concentration *in vitro* and *in vivo* was found to be ~50 μg/mL (Whaley et al. 1994). This appears to be the first study which suggests protection against a vaginally associated

sexually transmitted disease through the use of topical passive immunization of the vagina (Whaley et al. 1994).

ADENOVIRUS 4

Family. There are two genera within the family Adenoviridae (AD) with human viruses belonging to the Mastadenoviridae (Shenk 1996). There are 47 serotypes of human adenoviruses making up the 6 subgroups ranging from A to F. Currently, there is no known antigen common to all adenoviruses. Subgroup E contains only the AD Type 4 serotype and is considered to have low or no oncogenic potential in animals (Shenk 1996).

Morphology. These non-enveloped viral particles are 60-90 nm in diameter (Nicholson 1993). The virus has a dense core and an outer capsid from which 12 penton fibers project (Nicholson 1993). The genome consists of a single linear double stranded DNA which contains inverted terminal repeats and thus circularizes upon denaturation (Nicholson 1993). The AD virus is adsorbed onto the host cell surface and phagocytosed over several hours. The virus uncoats in the cytoplasm with release of viral DNA and then the DNA is transported to the nucleus. Replication begins in the nucleus 6-8 hours after infection of the host cell. The virion is assembled in the nucleus followed by lysis and release of progeny virus (Shenk 1996). Homologous recombination between ADs of the same subgroup, when cells are coinfected, has been found to occur with a high frequency. This would add to the evolution and diversity of AD serotypes (Shenk 1996). It has been speculated that the Group AD 4 could have been generated

from a single recombinatorial crossover event between a group B and a group C virus (Shenk 1996).

Transmission. Human adenoviruses do not infect animals and animal adenoviruses have not been isolated from humans. Transmission is commonly by: person-to-person and hand-to-eye contact from respiratory or ocular secretions; fecal-oral route; swimming in public pools; and improperly disinfected ocular equipment (Nicholson 1993; Viswalingam 1993). After infection ADs are shed for days in the stool and are transmissible both by fomites and aerosolized droplets (Schmidt 1989). Worldwide between 1967-1976, there was no observed seasonality for AD 4, but it was more frequently found in the Southern Hemisphere (Schmitz et al. 1983). AD 4 has been implicated in a number of outbreaks and even epidemics (Nicholson 1993) worldwide: in a swimming pool related outbreak of AD 4 pharyngoconjunctival fever in Georgia, U.S., in 1977, AD 4 was isolated from 20/26 cases and from two concentrated pool water samples, where free chlorine levels were below the recommended level of 0.4 mg/L (D'Angelo et al. 1979); from 1979 to 1980 in Japan, there were 33 cases of AD 4 conjunctivitis involving newborn to elderly patients (Aoki et al. 1982); an AD 4 conjunctivitis outbreak occurred in Bristol, U.K., with 113 cases in 7 months in 1980 (Tullo et al. 1980); as one of the causes in Manchester, England, in 1983-84 during a 16-month-long epidemic of respiratory and ocular infections (Bailey et al. 1986); in Japan, AD 4 is the most important agent of epidemic keratoconjunctivitis caused by ADs, with 68 cases in 1985 and 21 cases in 1988

(Itakura et al. 1991); AD 4 conjunctivitis observed in 83 cases in south Australia in 1989, 23 in 1991, and 38 in 1992, with no common epidemiological factors found (Schepetiuk et al. 1993); and in Glasgow, Scotland, a survey from 1981 to 1991 showed that AD 4 became the most frequently isolated type after 1987 (O'Donnell et al. 1993). To further demonstrate the transmission of this agent, in an outbreak of AD 4 in a Chicago hospital, in 1980, nine hospital personnel, who all had direct physical contact with a military recruit with fatal AD 4-pneumonia became infected with the virus (Levandowski et al. 1981). In general, there have been significant numbers of outbreaks of respiratory disease among military recruits (Cooper et al. 1993). Because of the high level of infectivity, ability to survive on environmental surfaces, and a relatively low level of antibody to AD 4 in the general population, this virus along with other ADs, are likely candidates to cause outbreaks of illness with serious health and economic concerns (Schmidt 1989; D'Angelo et al. 1979).

Pathogenesis. Adenoviruses were first isolated in 1953 from cultures of human adenoidal tissue and subsequently from cases of acute respiratory disease (ARD) (Shenk 1996). Since then AD 4 specifically has also been associated with: gastroenteritis; upper and lower respiratory illness which includes severe pneumonia in young children; central nervous system disease; urinary disease; pharyngitis; fever; and only infrequently associated with epidemic keratoconjunctivits (EKC) (Bailey et al. 1986; Schepetiuk et al. 1993; Schmitz et al. 1983; Hierholzer 1995).

Adenoviruses are relatively stable and can be readily recovered from sites of infection with swab samples (Threlkeld et al. 1993). Most human ADs do not induce acute, clinically apparent disease in common laboratory animals (Schmidt 1989). In general ADs cause self-limiting infections with an incubation period of 2-14 days and with illness lasting 5-7 days. AD 4 viral shedding has been found to last a maximum of 10 days (Levandowski et al. 1981). AD 4 is considered to be endemic (Viswalingam 1993; Levandowski et al. 1981).

AD 4 has been isolated from: respiratory, fecal, and eye exudates and less frequently from urine (Hierholzer 1995; Schmitz et al. 1983). In a survey of human adenovirus infections, only 1/249 total ADs cases reported was fatal due to AD 4 and as such AD 4 is considered to have a low fatality rate (Schmitz et al. 1983).

Diagnosis. Virus isolation in cell culture and direct examination by electron microscopy are used on a routine basis (Aoki et al. 1982). The identification of adenovirus is confirmed using specific neutralizing antibodies (Levandowski et al. 1981). Currently, there have been eight genome types of AD type 4 found using DNA endonuclease restrictions patterns (Li et al. 1988).

Treatment. Treatment currently consists of the use of topical corticosteroids to suppress the inflammation (in ocular infections), though this does not alter the basic pathogenesis of disease. No effective antiviral treatment is known (Viswalingam 1993). In Canada and the U.S., the military use live enteric coated adenovirus types 4 and 7 vaccines (Takafuji et al. 1979). Previously, frequent

cases of ARD, particularly in U.S. military recruits, resulted in fatalities but since routine administration of the AD type 4 and 7 vaccines, cases of infections have been substantially reduced (Schmidt 1989).

Survival in the Environment. ADs in eye clinics and ophthalmologists' offices are often incriminated in iatrogenic and nosocomial infections, probably as a result of the virus' ability to survive in the environment (Schmidt 1989). In a series of *in vitro* environmental surface tests, viral inocula of 20 μL of 2 x 10^3 PFU of AD 5, 7, and 19 were added and allowed to dry on 7 mm plastic and 6 mm aluminum foil disks for a period of up to 7 weeks. The authors recovered greater AD 19 than AD 5 or AD 8 on plastic and found no difference between the recovery of the ADs on metal (Gordon et al. 1993). For all 3 ADs, day 49 was the last day virus was recovered on plastic versus day 28 to 49 on metal. As the virus survived the dried state, it was concluded that it is a potential source of EKC (Gordon et al. 1993). Unfortunately, though the authors mention the drying was at ambient (22°C) temperature, the percent relative humidity was not given nor the initial drying time (Gordon et al. 1993).

Inactivation Studies. In vitro work has shown that adenoviruses are more resistant to disinfectants than enveloped viruses, such as herpesviruses (Threlkeld et al. 1993). Infectious adenovirus is inactivated at 56°C or by immersion in a waterbath of 75°C for 30 s or 60°C for 2 min (Ford et al. 1987; Hierholzer 1995). The virus is similarly inactivated by exposure to 0.025% sodium dodecyl sulfate, free chlorine at 0.5 μg/mL, UV irradiation, or a 1:400 to

1:4000 dilution of formalin (contact times not given) (Hierholzer 1995). For AD 8, a study found no decrease in infectious titer after 5 min exposure to UV irradiation, however the virus was suspended in 5% rabbit serum (used to mimic conjunctival secretions) which may have afforded protection from the UV (Clarke et al. 1972). A study noted that phenylmercuric borate (1 hr soak), isopropyl alcohol, ether, cetrimide, and chlorhexidine gluconate do not inactivate adenoviruses (Nagington et al. 1983; Ford et al. 1987). A soak in 500 ppm sodium hypochlorite did inactivate AD 8 after a 2 min contact time (Nagington et al. 1983)

One study subjected AD 8-spiked tonometer tips to disinfectant wipes (dry, water alone, 70% isopropyl alcohol, 3% hydrogen peroxide, and 1% titratable iodine in povidone-iodine iodophor wipes) and a 5 minute disinfectant soak (water, 3% hydrogen peroxide, 0.016% iodophor and 10% sodium hypochlorite) (Threlkeld et al. 1993). The authors found that drying for 15 min on tonometer tips reduced virus titer by 28-82% compared to the control (Threlkeld et al. 1993). No viable virus as found after the tips were dried and wiped. In the disinfectant soaks, virus was only recovered in the water-only soaks (Threlkeld et al. 1993).

OBJECTIVES

In summary, the main objectives of this study were:

1. to determine that viability of *ex vivo* human skin can be maintained over a minimum of eight hours in a static model and 24 hours in a Flow-Through Diffusion unit.
2. to develop and test a protocol for examining the survival of two viral pathogens on *ex vivo* human tissues
3. to develop and test a protocol for investigating the inactivation of the pathogens on human *ex vivo* animate carriers
4. to compare an inanimate stainless steel carrier with human animate carriers for survival and inactivation of the pathogens

MATERIALS AND METHODS

MATERIALS

CELL CULTURES

Vero Cells. A seed culture of Vero cells (derived from African Green Monkey kidney) was obtained from Dr. K. Wright, Department of Microbiology and Immunology. The cells were grown in 75 cm^2 plastic cell culture flasks (Corning, Corning, NY) using minimal essential medium (MEM; GIBCO, Grand Island, NY) supplemented with 0.113% sodium bicarbonate (BDH Chemicals, Toronto, Ontario, Canada), 1.5 M HEPES (N-2-hydroxyethyl piperazine-N-2ethanesulfonic acid; GIBCO), 7% fetal bovine serum (FBS; GIBCO) which was heat inactivated at 56°C for 30 minutes, 1% non-essential amino acids solution (NEAA; GIBCO), 146 μg/mL of L-glutamine (GIBCO) and 50 μg/mL of gentamicin sulfate (Cidomycin; Hoechst Marion Roussel Canada, Montreal, Quebec, Canada).

293 Cells. A seed culture of 293 cells, a transformed human embryonic kidney cell line that contains and expresses the AD 5 E1A and E1B genes (Shenk 1996), was kindly provided to us by Dr. K. Dimock, Department of Microbiology and Immunology. The cell monolayers were grown using the same conditions listed for Vero cells.

VIRUSES

Human Herpesvirus Type 2. Strain 333 of Human Herpesvirus Type 2 was acquired from Dr. J.R. Smiley of McMaster University, Hamilton, Ontario, Canada. Viral pools and plaques assays were performed using Vero cells (see below).

Human Adenovirus Type 4. The American Type Culture Collection VR4 strain RI-67 (officially recognized as the prototype strain for AD type 4) was used. Virus pools were made in 293 cells and plaque assays were conducted in Vero cells (see below).

CARRIERS

Stainless Steel Disks. Stainless-steel disks (1 cm in diameter), punched out of locally purchased no. 4 finish polished sheets (0.75 mm thickness), were used as carriers to represent non-porous inanimate environmental surfaces, as described previously (Sattar et al. 1987). Before use, the disks were soaked overnight in distilled deionized water containing a 10% solution of 7X (Flow Laboratories, McLean, VA) and then thoroughly rinsed under running distilled deionized water. The disks were then autoclave sterilized and dried overnight in an oven at 56-60°C.

Human Skin. Fragments of human skin were removed from normal adult females by a local plastic surgeon, Dr. M. Bell, during facelifts (rhytidectomy), breast reductions (reduction mammoplasty) and abdominal tucks (abdominoplasty) performed at the Ottawa Civic Hospital, Ottawa, Ontario.

Informed and written consent was obtained from each donor prior to the collection of the tissue samples. Due to hospital policy, information on age, gender, or race could not be obtained from the skin donors. Dr. Bell informed us that a chlorhexidine pre-surgical wipe is commonly used on the skin tissues before surgery. Maintenance medium, without serum, was used as the collection, transportation, and perfusion fluid for the tissue samples. On average, the size of the skin samples obtained from each of the procedures was as follows: four 1.5 × 7.0 cm pieces from facelifts, two 10.0 × 20.0 cm pieces from breast reductions, and one 15.0 × 18.0 cm piece from abdominal tucks. All tissue specimens were prepared within four hours of removal from the donor. To process the skin samples, fatty tissue was removed and the skin was secured onto a dissecting board.

For viability analysis in the Flow-Through Diffusion Cell unit (see below), a dermatome (Padgett Electrodermatome, Kansas City, Missouri) was used to slice a 200-400 μm-thick section. Patches (1 cm in diameter) were then punched out of the dermatomed skin layer (and used as described below).

For viability analysis in the static system, virus survival and inactivation studies, full thickness patches (approximately 2.3 cm in diameter) were punched out of un-dermatomed skin. The details of the setup of the *ex vivo* apparatus are given in Figure 2 A.

Umbilical Cord. Human umbilical cords were obtained from a local hospital within 4 hours of delivery of the infant. Cords were obtained with ethics approval

from the Ottawa General Hospital and were on average between 20 and 40 cm long. The cords were collected and transported in tissue culture fluid, as mentioned for skin tissue. The umbilical cord was first fastened onto the cutting board at each end and then a single lengthwise slit was carefully made with a scalpel such that the umbilical tube could then be opened flat and pinned along its length. The production of the mucosal disks was as noted above for the skin.

DISINFECTANTS

Benzalkonium Chloride (BC). Benzalkonium chloride (Sigma) was obtained in the form of a translucent semi-liquid. Dilutions of BC were performed using serum-free maintenance medium. Concentrations of 50, 100, 250, 500, 1000, and 5000 parts per million (ppm) of BC, respectively, were used. These ranges were selected as they represent concentrations found in commonly manufactured BC topical products.

Chlorhexidine Digluconate (CD). Dilutions of the 20% aqueous chlorhexidine solution (Sigma) were performed in serum-free maintenance medium to a final concentration of: 10, 20, 50, 100, 200, and 1000 parts per million (ppm) of CD, respectively. These concentrations differ from BC as they denote ranges found in topical products containing CD as their active agent.

METHODS

PRODUCTION OF VIRAL POOLS AND PLAQUE ASSAYS

Stock HHV 2 was prepared by infecting 90% confluent Vero cell

monolayers in 75 cm^2 cell culture flasks at a multiplicity of infection of 0.1. Cell monolayers were washed with serum-free maintenance medium and then inoculated with the virus. Following adsorption for 90 min in a 5% CO_2 incubator at 37°C, 20 mL of maintenance medium was added to each inoculated monolayer. After approximately 60 hrs, virus cytopathology destroyed 80-90% of the monolayer. The monolayers were then scraped to collect virus/cell mixture which was centrifuged at 190 × g for 10 min. After the supernatant was removed, the pellet was resuspended in 1.5 mL of Earle's balanced salt solution (EBSS) per three 75 cm^2 cell culture flasks. The virus/cell mixture was then rapidly frozen in an ethanol-dry ice slurry followed by thawing in a 37°C waterbath and then sonicated in a waterbath (Bransonic Ultrasonic Corp, Danbury, Conn) for a 30 sec burst, the whole three-step process was repeated six times. Finally, after a second similar centrifugation, the viral supernatant was dispensed in 0.1 mL aliquots and stored at -80°C. The titer of the HHV 2 stock was approximately 10^7 PFU/mL.

The same process was repeated for AD 4 in 90% confluent 293 cell monolayers in 75 cm^2 cell culture flasks at a multiplicity of infection of 0.1. Cytopathology in the 293 monolayers was 80-90% complete after a period of 5-6 days. The freeze/thaw, collection, and final storage of the virus was as for HHV 2. The titer of the AD 4 stock was also approximately 10^7 PFU/mL.

Quantitation for both viruses was by plaque assay in Vero cells grown in 12-well cell culture plates (Corning). Serum-free maintenance medium was used

for serial dilution and 0.1 mL of each dilution was added to each of three wells. Cell controls were maintained on each plate and virus controls were incorporated for each batch of assays performed. After virus adsorption in a CO_2 incubator at 37°C for 90 min, 2 mL of an overlay, containing either a fluid overlay consisting of maintenance medium with 0.05% human gamma globulin (Miles Inc, Etobicoke, Ontario, Canada) for HHV 2, or a gel overlay made with maintenance medium with a 0.6% agarose (type II, Sigma, St. Louis, MO) and 26 mM magnesium chloride (BDH) for AD 4, was added to each well. The plates were then incubated at 37°C in a CO_2 incubator. After 3 days for HHV 2, and 9 days for AD 4, the monolayers were fixed overnight by adding 2.0 mL of a 3.7% solution of formaldehyde (BDH) in normal saline to each well. The fixative was removed and the cell monolayers stained for 15 min with a 1% crystal violet solution (J.T. Baker Chemical Co, Phillipsburg, NJ). The mean titer was determined from a minimum of three wells.

PROTOCOL FOR SKIN VIABILITY ANALYSIS

Two types of viability analysis were performed. The first was using a dynamic flow-through diffusion cell method which has been commonly used in the pharmaceutical industry, and a second static system which is more applicable for this laboratory use.

Flow-Through Diffusion Cell System. Assessment of tissue viability consisted of measuring anaerobic glucose utilization by the dermatomed skin sections over time with a flow-through diffusion cell system (Bronaugh et al. 1985). This

system utilizes a one-chambered diffusion cell. The chamber beneath the skin serves as a container for the receptor fluid which is continually stirred. The receptor fluid leaving the diffusion cell is automatically collected through the use of a fraction collector. The unit was set up to process seven samples in parallel. The fraction collector was connected to a Manostat Cassette pump (Manostat, New York, NY). Attached to the diffusion unit was a multi-source adapter containing seven Teflon cells with an inner diameter of 9-mm to hold the 1-cm diameter skin sections. Each cell contained a 7.0 mm×2.0 mm micro stirring bar to eliminate air bubbles and ensure the direct contact of the diffusion medium with the skin patch. The diffusion system used a Retriever IV Fraction Collector (Isco, Inc., Lincoln, Nebraska) set to collect 4-hr fractions of the effluent. Also included in the unit was a bath/circulator system to maintain the skin surface temperature at 32°C.

At the beginning of each experiment, pieces of parafilm were placed onto the Teflon cells and the unit was decontaminated by running 70% ethanol (v/v) throughout for a minimum of 15 min. The system was flushed with sterile FBS-free maintenance medium for 15 min order to eliminate any residual ethanol and then allowed to equilibrate with further medium. The prepared 1 cm diameter skin sections were mounted with the stratum corneum side up in the diffusion cells. The dermis underside was perfused with the diffusion medium at a flow rate of ~2 mL/hr.

Static Cell System. Assessment of tissue viability consisted of measuring

anaerobic glucose utilization by the full thickness skin sections over 8 hr with a static cell system and comparing to dermatomed tissue in the Flow-Through Diffusion unit over the same time frame. Mounted skin was placed overtop 5 mm deep cross-sections of tubing (Nalgene) whereby the area beneath the skin served as a container for the receptor fluid. The receptor fluid was collected manually after each 1-hr fraction. Thus, the subcutaneous underside was perfused with approx. 1.0 mL of the diffusion medium.

Monitoring of Ongoing Metabolism. Since lactic acid is produced during anaerobic breakdown of glucose by viable skin cells, the amount of lactate in the effluent fractions was measured as an indicator of metabolic activity (Bronaugh et al. 1985). Sigma Procedure No. 735 (Sigma, St. Louis, MO), which measures the conversion of lactic acid to pyruvate and hydrogen peroxide by the addition of lactate oxidase, was used for this purpose. The test involves a colour reaction between the hydrogen peroxide, a chromagen precursor and horseradish peroxidase (these last two items are part of the Sigma kit) which indicates the presence and relative amounts of lactate in the test sample. An ABBOTT VP Super System Bichromatic Analyzer (ABBOTT Labs., Irving, Texas) was used to measure the colour reaction which produces an absorption maximum at 540 nm and the results are expressed as lactate concentrations in mg/dL. Each set of samples run through the bichromatic analyzer contained two negative controls (water and sterile diffusion medium) as well as four positive controls (two from a Lactate Standard Solution (Sigma) of a concentration known to the investigator,

and two blinded controls). The time frame selected reflects previously published rat skin studies (Bronaugh et al. 1990; Collier et al. 1989).

Histopathology. For histopathology, sections of the skin fragments were taken when it was (a) freshly obtained from the donors, (b) perfused for 24 hr and (c) refrigerated (4°C) for 24 hr and perfused for the following 24 hr. Skin sections, both full thickness and dermatomed, were stored in a buffered formalin fixative before examination. For light microscopy, the fixed tissues were paraffin-embedded, cut, and mounted onto glass slides and then stained with hematoxylin and eosin (H&E). Each H&E slide was reviewed and rated for viability by Dr. M. Senterman, Pathologist, Ottawa General Hospital, in a blind study format.

PROTOCOL FOR DETERMINING CYTOTOXICITY OF CHEMICAL AGENTS

Ascertaining the concentrations of BC and CD which are cytotoxic to Vero cell monolayers was performed to demonstrate any toxicity for the cell cultures which must be used for virus plaque assay and to determine if there was any correlation between cytotoxicity and virucidal levels. Also, these products are manufactured to be used on the skin or mucosa and I wanted to visually see any cytotoxicity which may occur.

Cytotoxicity Test 1. To determine which concentrations of the tested chemical agent are cytotoxic to the cell line, 100 μL of the 0.5% concentration was added to eleven wells in a 12-well cell culture plate with confluent Vero cell monolayers, with one well kept as a control. The process was repeated for each

concentration 0.1%, 0.05%, 0.025%, 0.01%, and 0.005%. The plates were then incubated for times of 1, 10, 30, 60 and 90 min in a humid 37°C CO_2 incubator. After each time period, the plates were examined microscopically and any degenerative effects observed visually were noted. The inoculum was then removed with three × 1 mL washes of neutralizer (maintenance media with 7% FBS). For all tests with cell monolayers, the cytotoxic effects were determined using the following scale: 0: no cell damage evident; 1: 25%; 2: 50%; 3: 75%; and 4: 100% of cells affected.

Cytotoxicity Test 2. 100 μL of the chemical agent was added to confluent Vero cell monolayers for a contact time of 90 min (as described above) followed by three × 1 mL washes with culture medium. These "treated" monolayers were then inoculated with normal HHV 2 stock virus at a titer to obtain roughly 100 plaques in 0.1 mL. The plates were then incubated at 37°C for 48 hr. This was to determine the effects of the initial exposure of the cells to the chemical agent on numbers of plaque forming units of HHV 2 detected. As a viral control, a similar aliquot of untreated virus was added to untreated Vero cells monolayers to confirm the original titer.

PROTOCOL FOR TESTING VIRUS SURVIVAL

After mounting of the skin or umbilical cord patches, the tissue holder was inverted with the exposed (subcutaneous) side kept immersed in cell culture medium. Thus, either the stratum corneum or amnion layer served as the bottom of a well, such that the viral inoculum and then the eluent could be placed into

the well. The flow diagram of the *ex vivo* method to test virus survival on human skin, umbilical cord and stainless steel disks is given in Figure 3. To estimate the maximum elution efficiency of the virus placed on either the skin or umbilical cord surface, 10 μL (~2×10^5 PFU) of the test virus suspension was placed onto the stratum corneum or the amnion side of three sample patches and eluted immediately by placing 990 μL of the eluent on each patch (Time 0). Elution consisted of flushing the surface of the carrier with the maintenance medium eluent. To compare virus survival on animate with that on inanimate carriers, the survival protocol was repeated using stainless steel disks.

Plaque assays were used to confirm the original viral titer and to determine the titer of virus recovered at each sampling time. All observations were normalized to the zero minute value as 100%. Each test represents a minimum of three replicates.

PROTOCOL FOR TESTING VIRUS INACTIVATION

The flow diagram of the *ex vivo* method to test virus inactivation on inanimate and animate carriers is given in Figure 4. To determine the virucidal activity of benzalkonium chloride (BC) and chlorhexidine digluconate (CD), the virus inoculum was first allowed to dry for 60 min at 22±2°C. Following drying, 100 μL of the test germicide or control solution was then applied to each contaminated surface for 1 min. The virus/chemical was then eluted and neutralized immediately by flushing the surface of the carrier with 890 μL of neutralizer. To compare virus inactivation on animate versus inanimate carriers,

the protocol was repeated using stainless steel disks. Plaque assays were used to confirm the original viral titer and to determine the titer of virus recovered at each sampling time. All observations were normalized to the sixty minute negative control (i.e. no chemical agent added) value as 100%. Each test represents a minimum of three replicates. The data in the animate skin carrier experiments were obtained from skin patches provided by a single donor.

STATISTICAL ANALYSIS

Statistical analysis (95% confidence level) using ANOVA followed by the Student-Newman-Keuls multiple range test was performed using SigmaPlot/Stat ver 2.0/1.0 (Jandel Corporation, San Rafael, CA) and Microsoft Excel ver 5.0 (Microsoft Corporation) on an IBM-compatible PC.

To calculate virus half-lives over 90 minutes, first order linear-regression lines were generated and fitted to the log-linear plots ($\log_{10}$) using the same program. The rate of virus inactivation (*Ki*) was obtained from the gradient (or slope) of the regression lines as loss in virus infectivity titer in $\log_{10}$ PFU/hour. The relationship between *Ki* and half-life is given by: $ln\ (N_o/N) = Ki\ t$, where *ln* = natural logarithm, N = amount of virus at the beginning of experiment and N_o is the amount of virus remaining at time *t*. Thus, the half-lives $t1/2 = 0.693/Ki$.

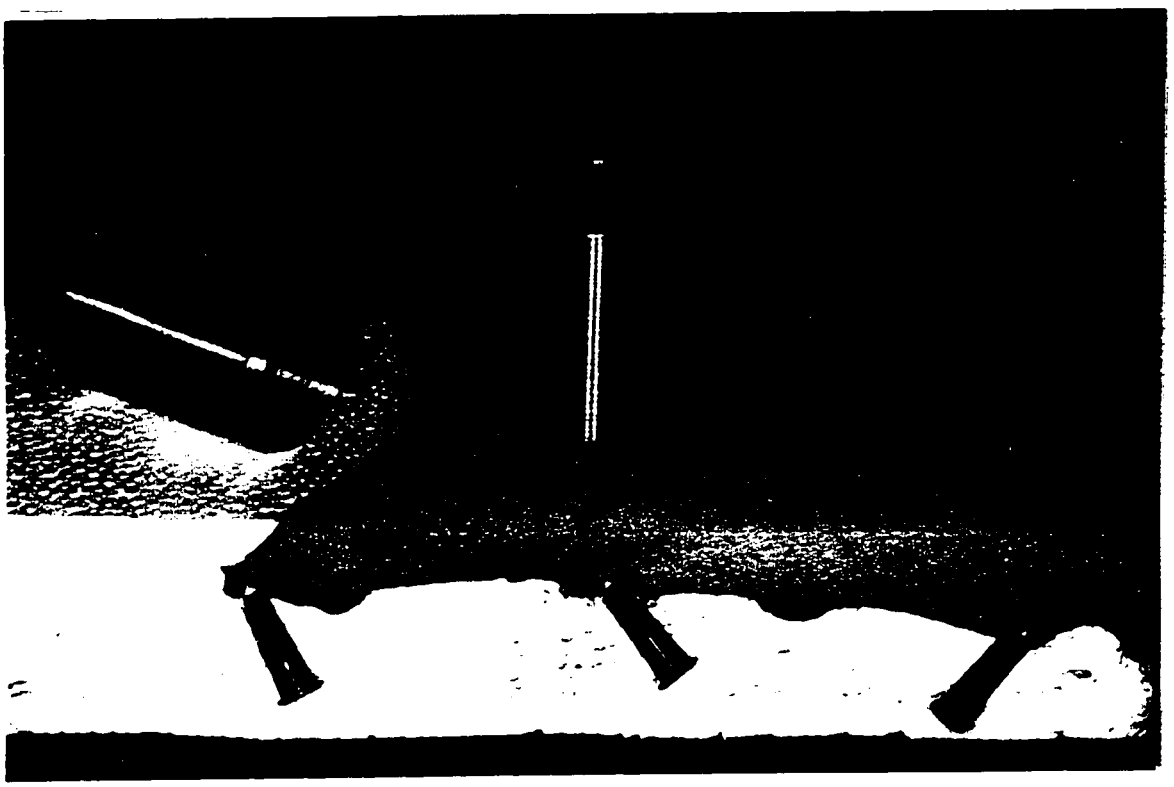


Figure 1. Skin specimen prepared by removing underlying tissue, mounting on cutting board, and then skin patches made with cutting tool.

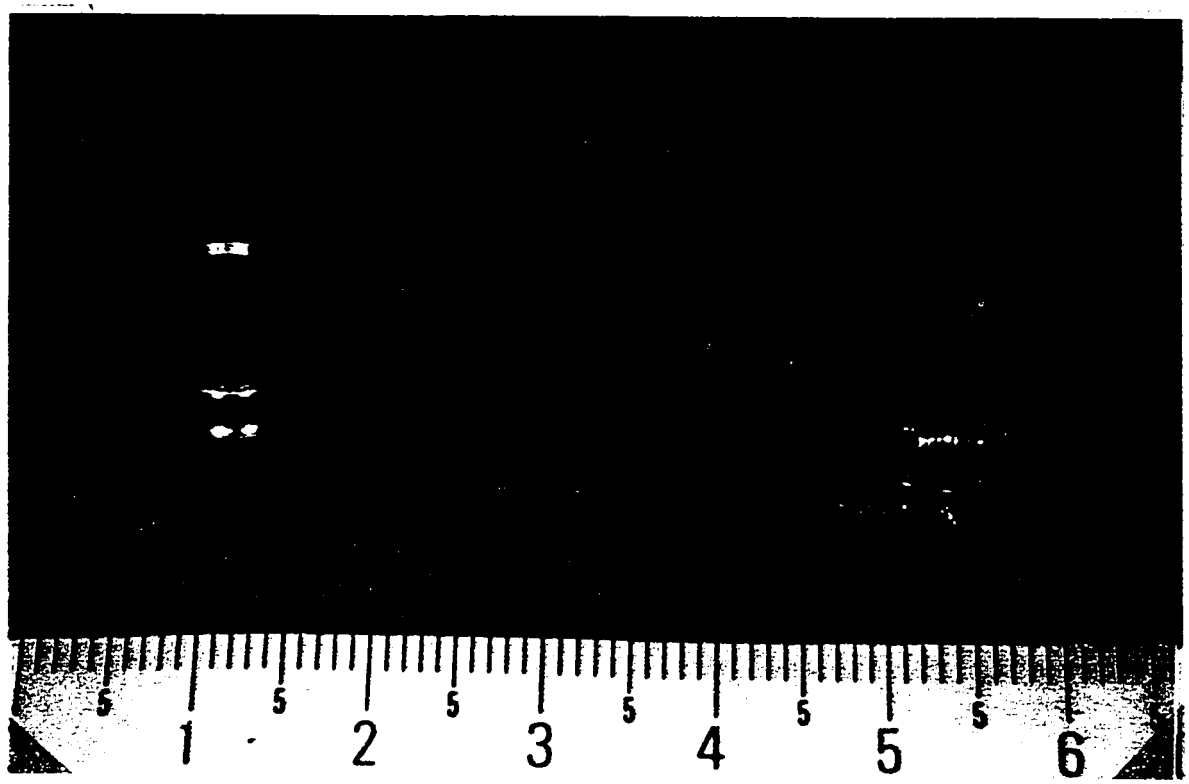


Figure 2A. Apparatus for testing virus survival and inactivation on *ex vivo* human skin and umbilical patches. Each skin patch is mounted over the top surface of the holder with the stratum corneum facing inwards. The patch is secured with an O-ring.

Figure 2B. For testing virus survival and inactivation, an inoculum (10 uL) of virus is aliquoted directly onto the stratum corneum of the mounted *ex vivo* skin patch.

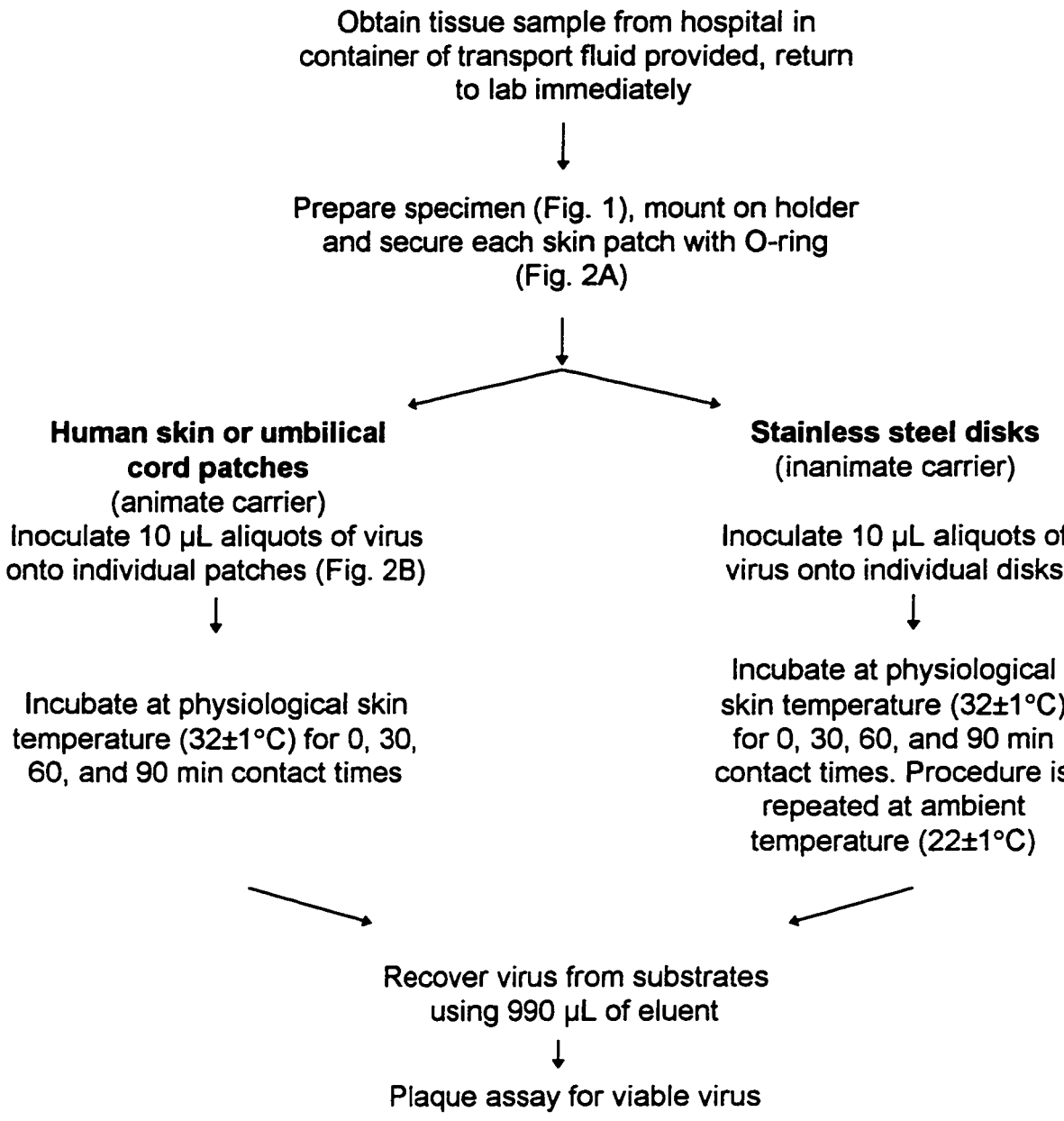


Figure 3. Procedure and apparatus for testing virus survival on *ex vivo* human tissue patches and stainless steel disks. **Note**: In addition, a 10 µL input virus control is inoculated directly into 990 µL of eluent and the number of infectious units in the input inoculum is determined.

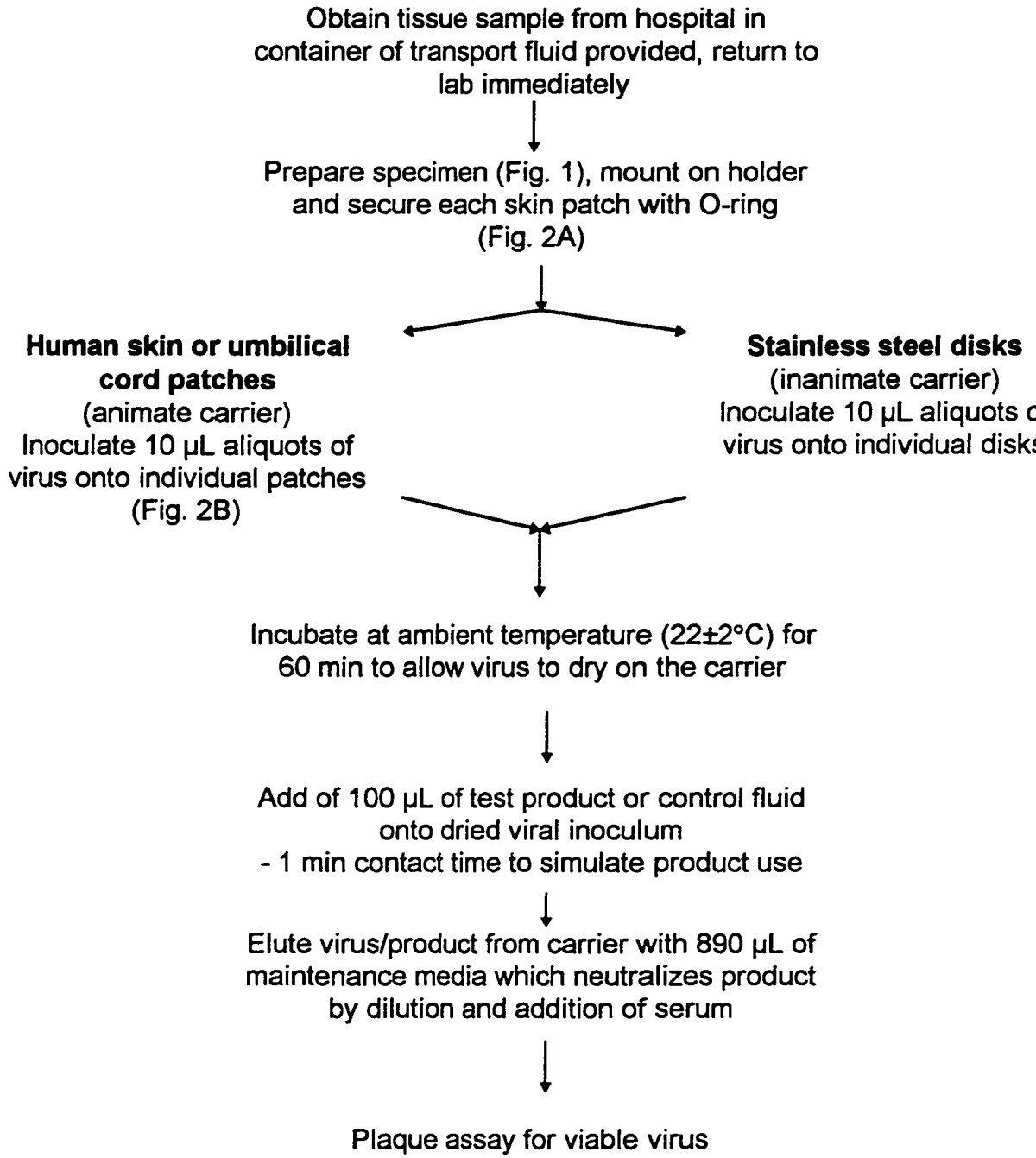


Figure 4. Procedure and apparatus for testing virus inactivation on *ex vivo* human tissue patches and stainless steel disks. **Note**: In addition, a 10 µL input virus control is inoculated directly into 990 µL of eluent and the number of infectious units in the input inoculum is determined.

RESULTS

SKIN VIABILITY ANALYSIS

To determine the viability of fresh skin samples, the skin sections were loaded onto the flow-through unit and fractions of the diffusion medium were collected for 24 hrs. The test was repeated three times, once for each of the three body sites; breast, abdomen, and face. The levels of lactate present in each of the 4-hr fractions are shown in Figure 5. The rates and levels of lactate production by abdominal and breast tissues were comparable and consistent over the test period while there was a decline in the metabolic activity in the sections surgically removed from facial tissue. This may be due to the fact that the donated facial skin fragments from which the disks were made were considerably smaller than those from the other two body sites. They also showed more incision trauma around the edges and it was difficult for us to obtain the disks without avoiding the damaged edges. Therefore, it is likely that sections of facial skin tested here contained portions with injured or dead cells and hence the relatively lower and faster decline in the metabolic activity observed. Lactic acid is produced as a metabolic end product following a series of enzymatic steps (Jensen 1984). This process reflects maintenance of cellular function of the various skin layers. Loss of viability would result in undetectable levels of lactate. This has previously been demonstrated using receptor medium where viability could not be maintained, and the levels of lactate in the receptor fluid became virtually undetectable (Nathan et al. 1990).

Based on measurements of lactate concentrations in the effluent, the metabolic activity of the skin sections in these experiments was maintained for at least 24 hrs.

Often it may not be possible to utilize skin samples soon after collection. Therefore, the *ex vivo* viability of human skin samples was tested following their 24-hr storage at 4°C, such that at the end of the viability testing the skin had been kept in the lab for a total of 48 hr. Fragments of tissue from the same donor were used with and without refrigeration to reduce variability between donors. While the refrigerated abdominal and breast skin fragments appear to produce lactate for 24 hr after storage at 4°C, their metabolic activity was lower than that of the fresh tissue. The results for the two body sites are presented in Figure 6. As mentioned earlier, facial skin fragments in general were considerably smaller, and this did not permit us to obtain enough skin sections from the same donor to run comparative tests with fresh vs. refrigerated skin.

These results show that, whereas it may be preferable to use the skin in an *ex vivo* model soon after its removal from the donor, proper refrigeration of the samples maintains the viability of the skin cells, thus allowing their subsequent use.

In Figure 5 in the fresh samples of the breast and abdominal skin, and in Figure 6 with the refrigerated specimens, there appears to be an increase in the rate of lactate production from 4-8 hr. This phenomenon has also been observed in earlier studies on rat skin (Collier et al. 1992) and more work is needed with

increased sampling between 1-8 hr to better address this issue.

For the static model, assessment of tissue viability consisted of measuring anaerobic glucose utilization by full thickness and dermatomed skin sections over 8 hr with a Static Cell System and comparing the results to those determined using fresh and stored dermatomed tissue in the Flow-Through Diffusion Unit. As shown in Figure 7, the full thickness skin in the static model remained viable for at least 8 hrs. In comparison to the dermatomed skin at the same time frame, the full thickness skin sections followed the same pattern as for the dermatomed skin but the amount of lactate produced by the thicker skin was at a much higher concentration.

Skin samples were prepared for light microscopy both before and after each 24 hr time course. Presence of cellular integrity was confirmed microscopically in all cases and a histopathological evaluation determined the maintenance of a viable multi-cell layer (Figures 8 A and B). Though histopathology confirms anatomically normal epidermal and dermal layers by gross morphology, monitoring of metabolic activity may be better as a quantitative indicator of skin viability.

The data show that skin sections can be kept viable *ex vivo* for at least 24 hrs in a diffusion system with or without prior storage at 4°C and for at least 8 hrs in a static model.

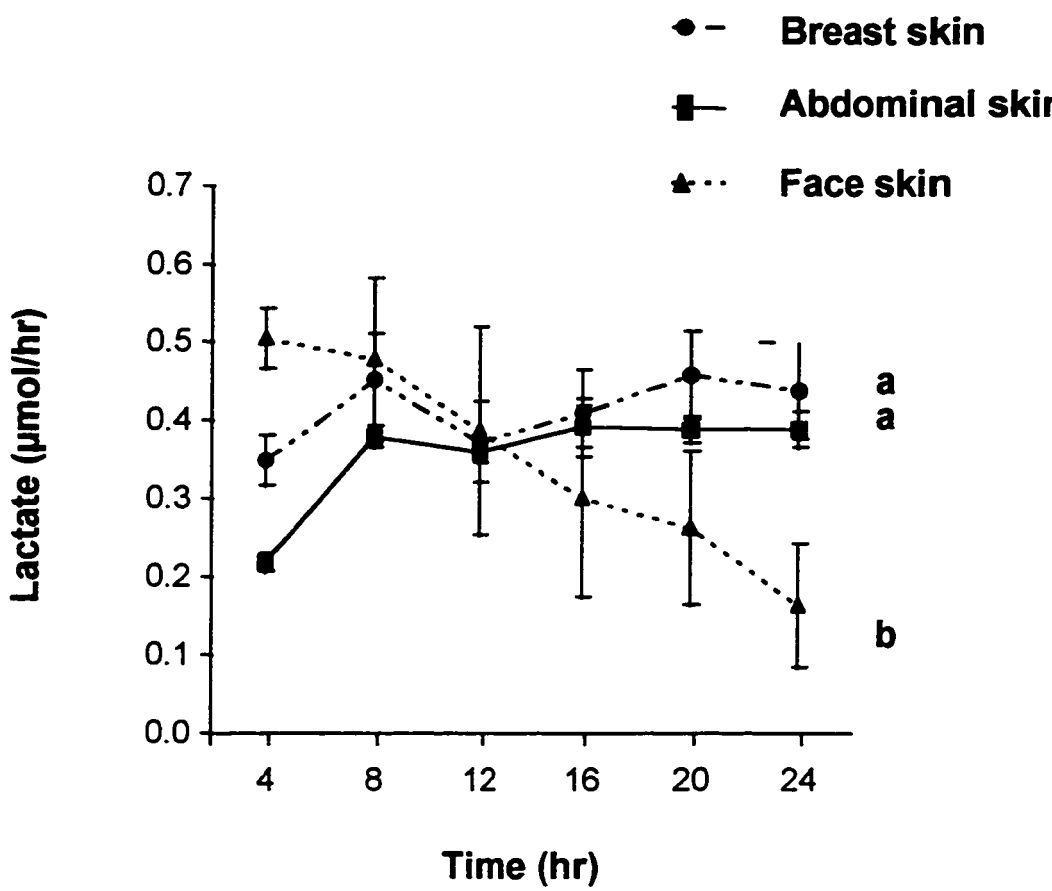


Figure 5. *Ex vivo* viability of human skin sections from three body sites determined by measuring the rate of lactate in the diffusion medium released from ongoing metabolism of the skin. Values are means ± SEM; where breast n=6; abdomen n=7; and face n=5. [ab] Statistically significant differences ($P \leq 0.05$) between the body sites at 24 hr.

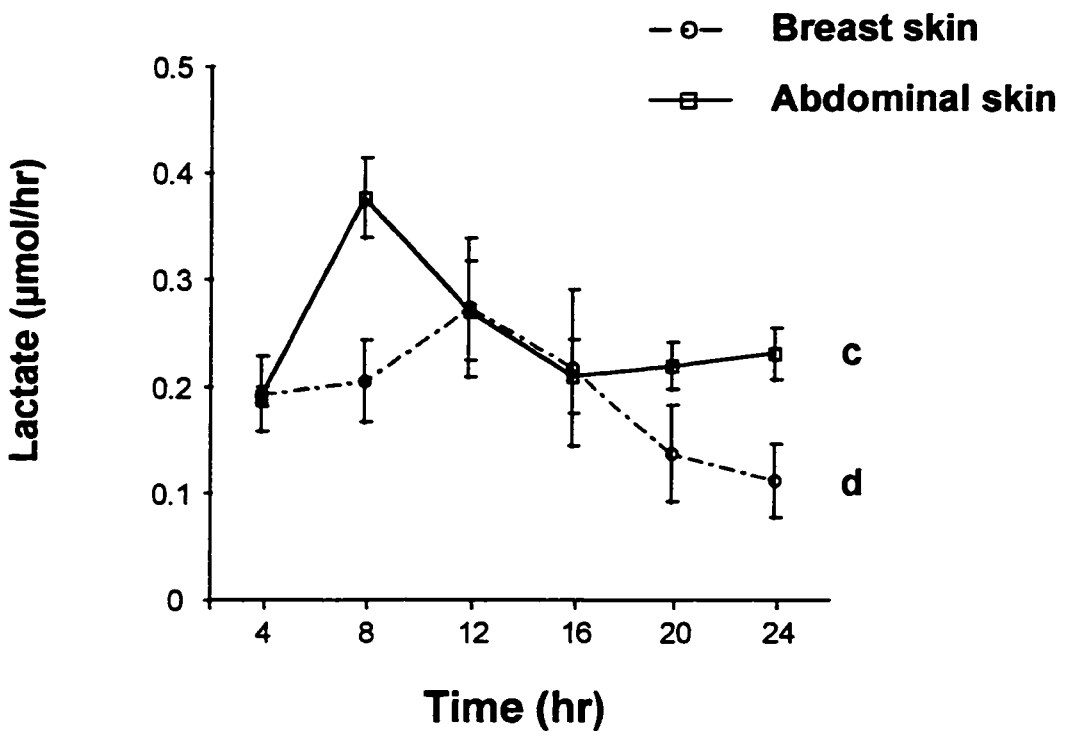


Figure 6. The rate of lactate released into the diffusion medium from breast and abdominal skin sections which had first been refrigerated at 4°C for 24 hr. Values are means ± SEM; where breast n=6; and abdomen n=7. [cd] Statistically significant differences ($P \leq 0.05$) between the body sites at 24 hr.

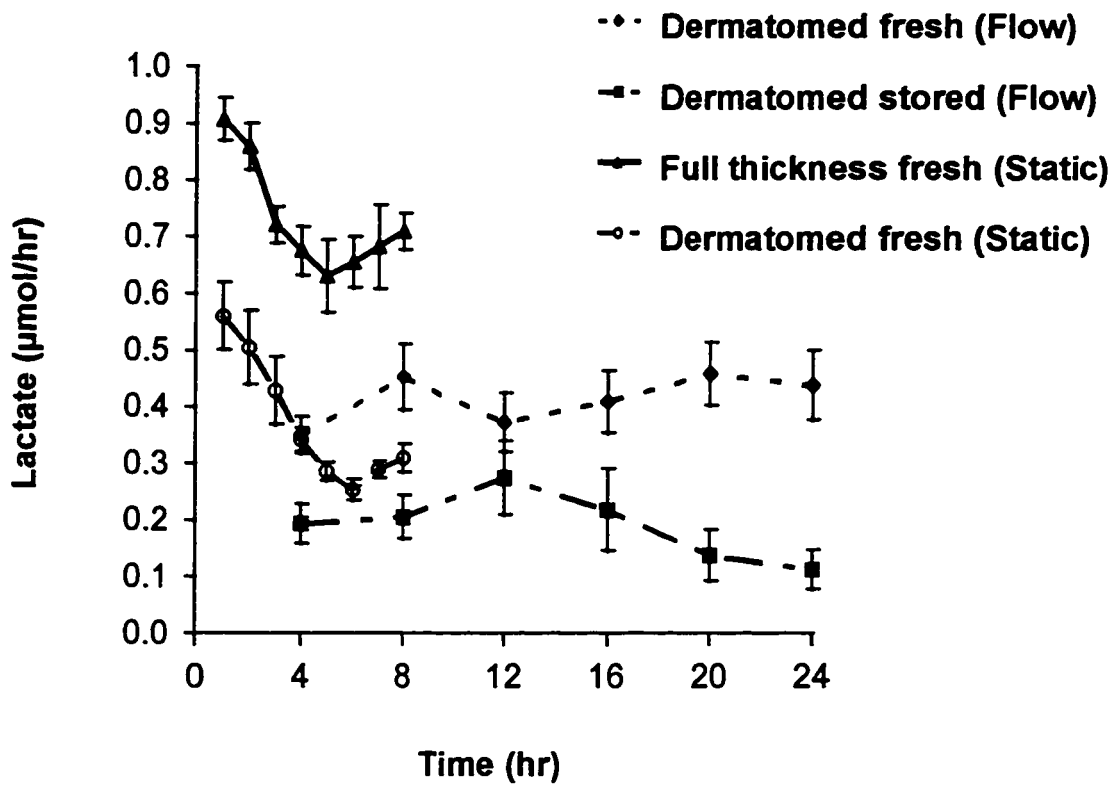


Figure 7. Comparison of *Ex vivo* viability of breast skin sections determined by measuring the rate of lactate in the diffusion medium from fresh full thickness and dermatomed skin in a Static Cell over 8 hr versus fresh and stored dermatomed skin in the Flow-Through Diffusion Unit over 24 hr. Values are means ± SEM.

Figure 8A. Histopathology of a skin section from breast tissue. Light micrograph with H&E staining showing anatomically normal epidermis and dermis in skin after perfusion for 24 hr. *Bar, 30 μm.*

Figure 8B. Histopathology of a skin section from abdominal tissue. Light micrograph with H&E staining showing anatomically normal epidermis and dermis in skin after perfusion for 24 hr. *Bar, 30 μm.*

CYTOTOXICITY

These tests determined if any of the concentrations of BC and CD which are used in the virus inactivation assays are cytotoxic to Vero cell cultures (Cytotoxicity Test 1) and to demonstrate if there were any correlation between cytotoxicity and virucidal levels (Cytotoxicity Test 2).

Cytotoxicity Test 1. The rating system for the observed microscopic cytotoxicities is given in Figure 9. All confluent monolayers were examined microscopically and any degeneration present was noted using this visual scale (0 through 100%).

It was observed that for BC approximately 50% of the Vero cell monolayer showed degeneration at 1000 ppm after a 1 minute contact time (Figure 10 A). Only with 5000 ppm of BC and after a 60 min contact time was 100% degeneration demonstrated. Two concentrations, 50 and 100 ppm, showed no degeneration whatsoever, even after a contact time of 90 min.

For CD, the observed cytotoxicity, where 50% or greater of the cell population is affected, was first found with 50 ppm after a 10 min contact time, (Figure 10 B). All of the Vero cells were affected after 60 min with this same concentration of CD. The two lower concentrations, 10 and 20 ppm, caused no visible degeneration over a 90 min contact time.

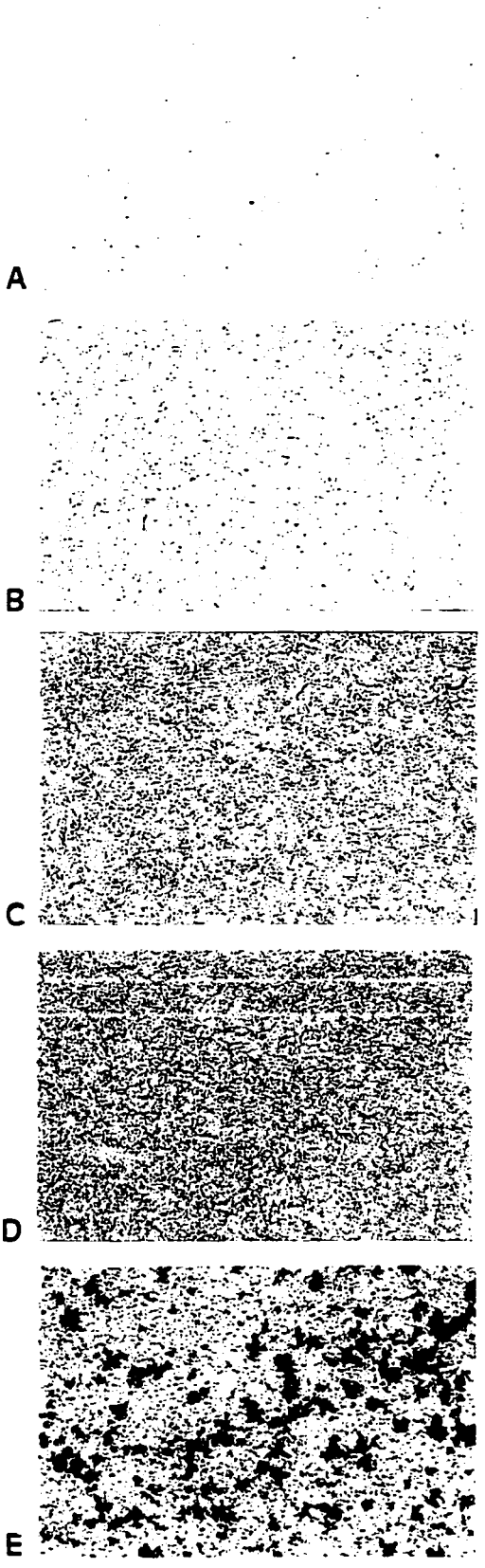
Cytotoxicity Test 2. This test examined confluent Vero cell monolayers after 90 min of contact with either chemical agent followed by one or three 10 min washes with 1 mL of neutralizer (i.e. maintenance medium). These "treated"

monolayers were then inoculated with 0.1 mL of HHV 2 stock virus diluted to a titer of 1000 PFU/mL.

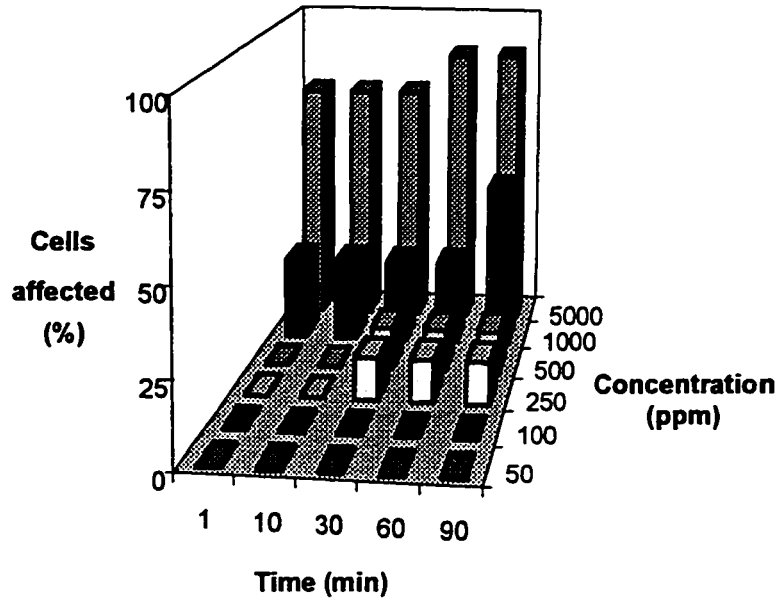
At 50 and 100 ppm of BC, where no cytotoxicity was observed (Figure 10 A), no effects on the number or size of the virus plaques were found (Figure 11A). At both 250 and 500 ppm, with one wash, the number of infectious virus particles surviving was reduced more than 80% and the plaques obtained were smaller in size than for controls. In contrast, at the same concentrations but after three washes with neutralizer, there was only a 20% reduction in the amount of virus surviving and no difference in the size of the plaques was observed following treatment with 250 ppm. With either one or three washes following exposure to 1000 ppm of BC there was no viable virus remaining.

Unlike with BC, with CD, where no cytotoxicity was observed at 10 and 20 ppm even after 90 min, the number of virus PFU was reduced by 25 to 45% as compared to the negative controls (Figure 11B). Thus CD had effects on cells that were not observed visually. This indicates that the viral test is a more sensitive assay to demonstrate cytotoxicity. By 50 ppm with either one or three washes, no viable virus was detected which correlated with the visual observation that at 50 ppm after 90 min 100% cytotoxicity was found (compare Figure 10B and 11B).

Figure 9. Rating of cytotoxicity observed visually by light microscopy after exposure to either of BC or CD disinfectants on Vero cell monolayers, panel **A.** control with no cytotoxicity ; **B.** 25% of cells affected; **C.** 50% of cells affected; **D.** 75% of cells affected; **E.** 100 % of cells affected.


A
B
C
D
E

A. Benzalkonium Chloride



B. Chlorhexidine Digluconate

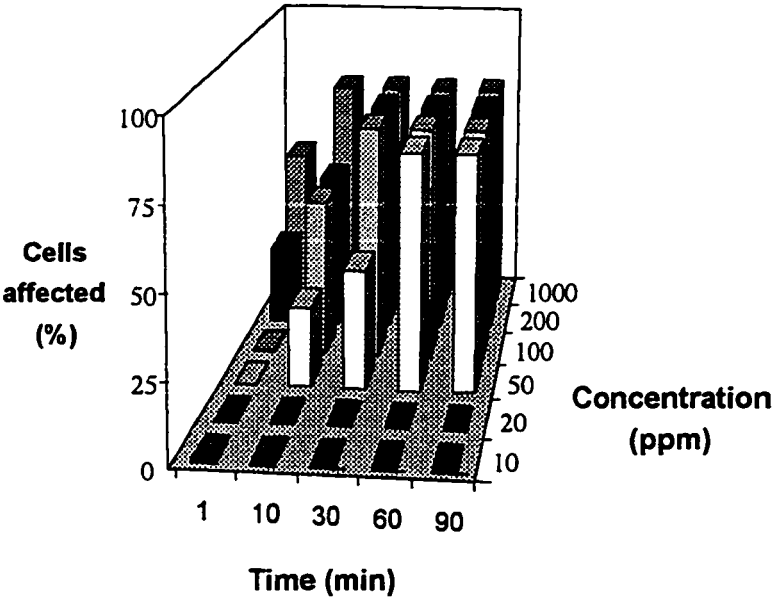
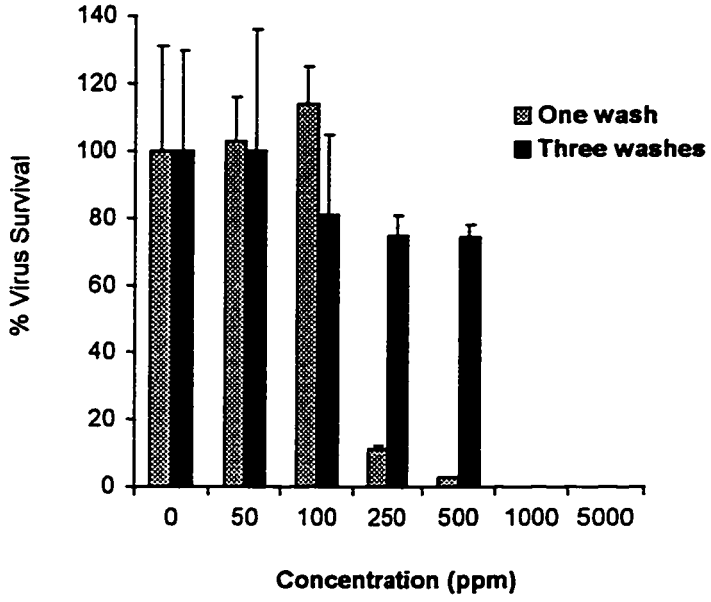


Figure 10. Cytotoxicity observed by light microscopy with disinfectants on Vero cell monolayers, panel **A.** Benzalkonium chloride; **B.** Chlorhexidine digluconate.

A. Benzalkonium Chloride



B. Chlorhexidine Digluconate

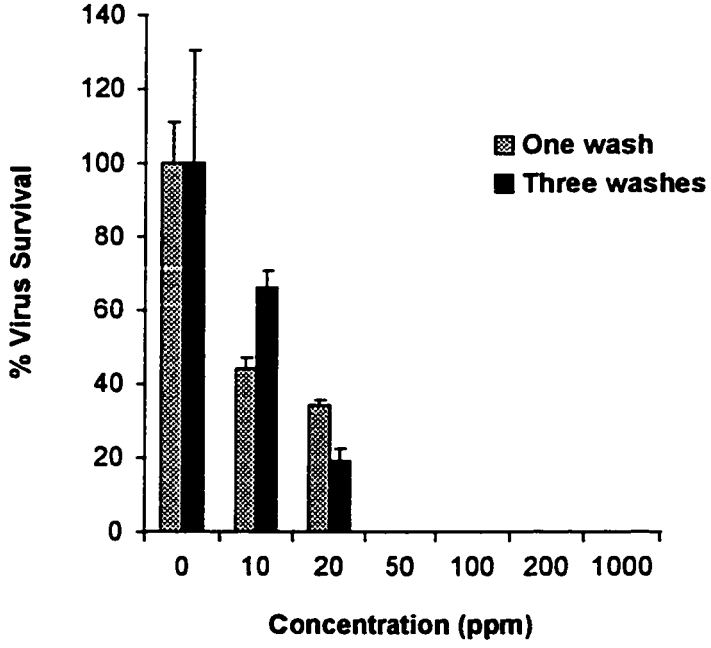


Figure 11. Cytotoxicity test to measure whether chemical agents had any effect on Vero cells. Cell monolayers were treated with 100 μL of either **A.** Benzalkonium chloride or **B.** Chlorhexidine digluconate, at varying concentrations for 90 min followed by either 1 or 3 ten min washes with maintenance medium, and then inoculated with 100 PFU/0.1 mL of HHV 2. Controls (0) were inoculated with virus only with no chemical pre-treatment.

VIRUS SURVIVAL

This test compared virus survival on animate (skin and umbilical cord) with inanimate carriers (stainless steel disks). Plaque assays were used to confirm the original viral titer and to determine the titer of virus recovered at sampling time of 0, 30, 60, and 90 min. The estimated maximum virus elution efficiencies, given in Table 1, are representative of the percentage of the zero-minute virus titer (PFU/mL) to the in-put virus. The virus titer at each sampling (X-value) is the amount of infectious virus recovered expressed as a fraction of that eluted at zero minute. All observations were normalized to the zero minute value which was set as 100%. Each test represents a minimum of three replicates.

The highest efficiency of recovery was on the animate surfaces for the enveloped herpesvirus and on the inanimate carrier for the non-enveloped adenovirus virus. The survival results for HHV 2 are shown in Figure 12 and in Figure 13 for AD 4. Although not unique to skin surfaces, the decrease in virus recovery may be due to a combination of virus adsorption and inactivation over time. Therefore, the results obtained for virus survival represent minimum virus survival. Confirmation of elution efficiency at longer contact periods would require the use of a virus preparation labeled with a radioisotope. Virus survival for each set of conditions was plotted also against time using an exponential model. The log-linear plot was used to determine the inactivation coefficient (K_i) of $\log_{10}$ reduction in virus titer/hr and the time required for a 50% loss of virus

titer ($T_{1/2}$), which is $0.693/K_i$.

The results (Table 2) demonstrate that half-lives of both viruses were found to be longest on skin, followed by umbilical cord, and finally lowest on disks. Specifically, for HHV 2, the half-life was more than four-times longer on skin and two-times longer on the umbilical cord as compared with the survival on metallic disks. With AD 4, the half-life was higher than five times more on skin and more than three times longer on umbilical cord than the survival of AD 4 on metallic disks. In comparing virus survival on skin to umbilical cord, a difference of 54% and 67% higher for HHV 2 and AD 4, respectively, was observed.

Thus, differences in survival of HHV 2 were observed between the three types of carriers. The enhanced survival on the animate carriers further supports the need for this *ex vivo* model in studying the survival of pathogens on human skin and mucous membranes.

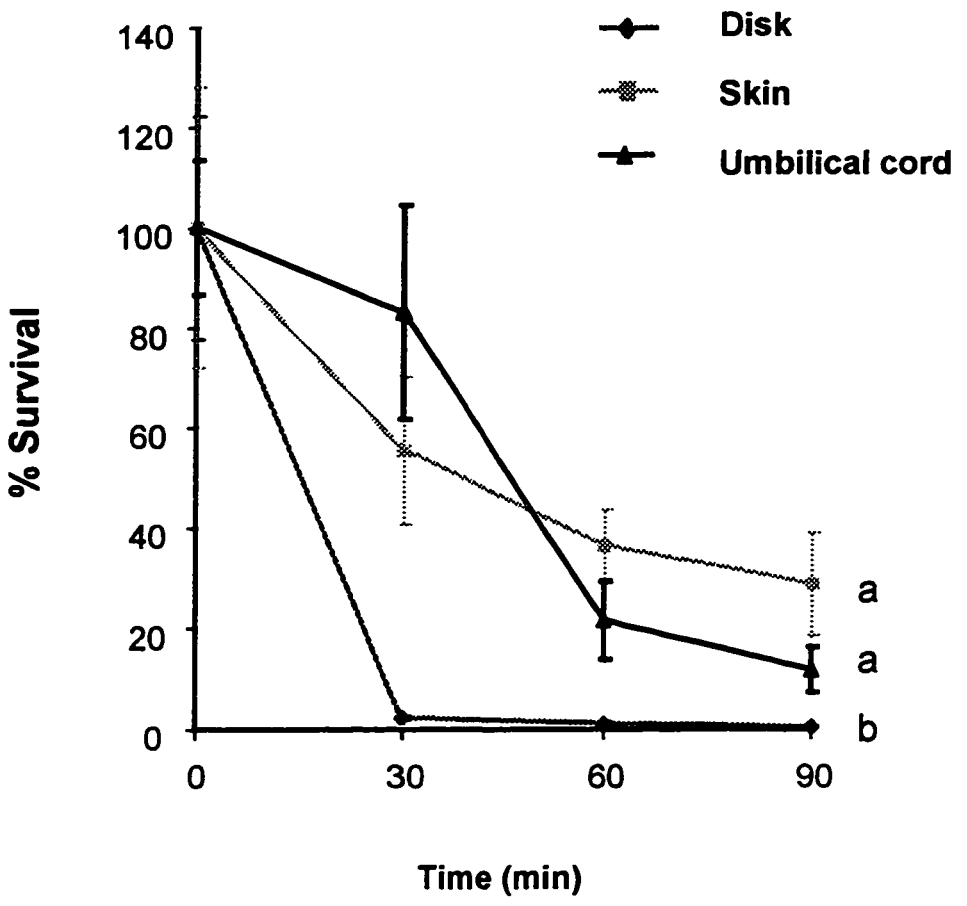


Figure 12. Survival of Human Herpes Virus 2 on inanimate (disks - stainless steel) and animate (skin - stratum corneum; umbilical cord - amnion) carriers at physiological skin temperature (32°C). Results represent a minimum of three repeats and values are means ± SEM. [ab] Statistically significant differences ($P \leq 0.05$) between the inanimate and animate carriers at 90 min.

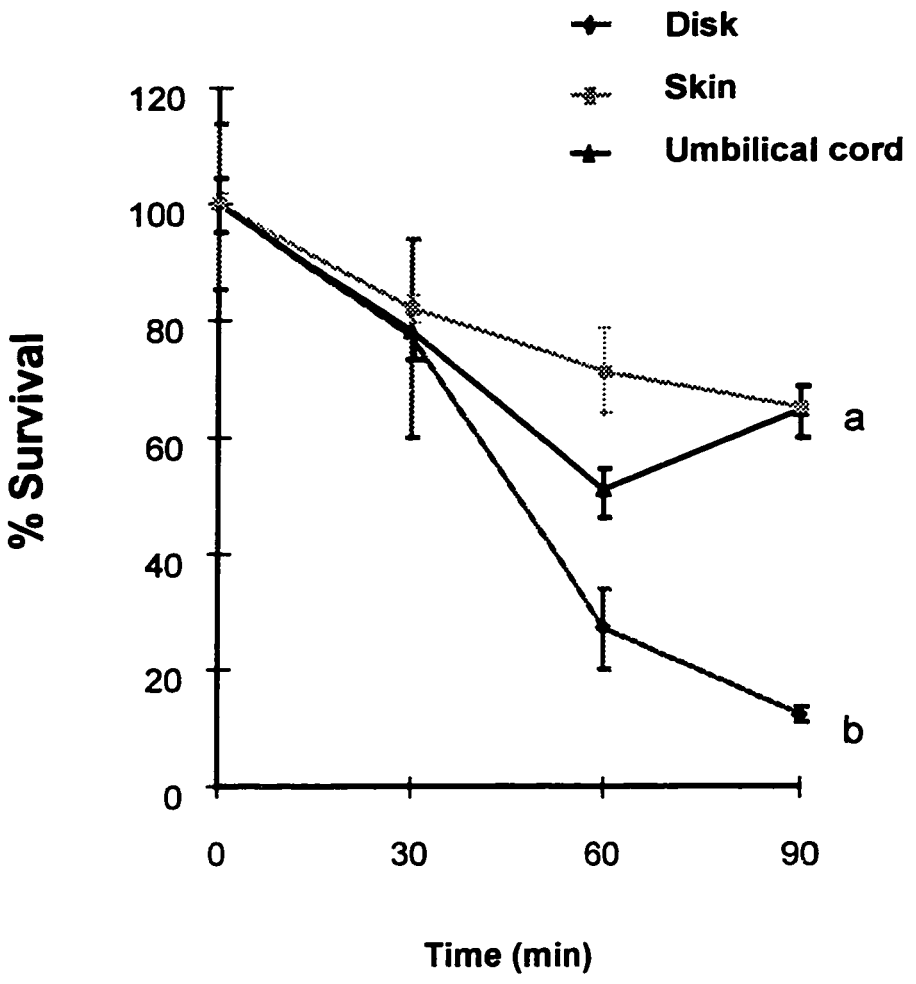


Figure 13. Survival of Adenovirus 4 on inanimate (disks - stainless steel) and animate (skin - stratum corneum; umbilical cord - amnion) carriers at physiological skin temperature (32°C). Results represent a minimum of three repeats and values are means ± SEM. [ab] Statistically significant differences ($P \leq 0.05$) between the inanimate and animate carriers at 90 min.

Table 1. Efficiency of recovery of Human Herpes Virus 2 (HHV 2) and Adenovirus 4 (AD 4) on inanimate and animate carriers. The values of the animate carriers are representative of more than one donor. Values are ±SD.

Carriers	HHV 2	AD 4
Disk	65.89±12.49	84.58±34.98
Skin	166.67±63.25	51.93±2.51
Amnion	89.32±50.83	58.91±7.45

Table 2. Estimated rates of inactivation (*Ki*) and half-lives of Human Herpes Virus 2 (HHV 2) and Adenovirus 4 (AD 4) on inanimate and animate carriers at 32°C and 50%±5% relative humidity.

Carriers	HHV 2			AD 4		
	[a]K_i	[b]Correlation coefficient	Half-lives (hr)	[a]K_i	[b]Correlation coefficient	Half-lives (hr)
Disk	0.025	0.87	0.462	0.011	0.96	1.050
Skin	0.006	0.97	1.925	0.002	0.97	5.775
Amnion	0.011	0.93	1.050	0.003	0.61	3.850

[a]K_i = virus inactivation coefficient as $\log_{10}$ reduction in virus titer PFU/hr
[b]Correlation coefficient refers to the measure of the linear association between two variables

VIRUS INACTIVATION

To determine the virucidal activity of benzalkonium chloride (BC) and chlorhexidine digluconate (CD) the 10 μL virus inoculum was allowed to dry on the carrier for 60 min at 22±2°C and 100 μL of the test germicide was then applied to each contaminated surface for 1 min. The control virus titer (PFU/mL) represents the amount of virus recovered after 60 min contact time with each respective carrier in the absence of disinfectant. All observations were normalized to the 60 minute value as 100%. Each test represents a minimum of three replicates.

Benzalkonium Chloride

At 250 ppm of BC, there was only a 20 to 40% reduction in HHV 2 infectivity on disks and umbilical cord and no reduction on the skin (Figure 14 A). All three types of carriers were found to have at least a 35% reduction in HHV 2 survival at 1000 ppm of BC.

At 100 ppm of BC, a 55% reduction in the titer of AD 4 was observed on the inanimate carrier with little or no reduction on the animate carriers. At 1000 ppm, AD 4 titer dropped to less than 50% on the animate carriers and with no virus surviving on the disks. By increasing the concentration of BC to 5000 ppm, complete inactivation of both viruses on all carriers was observed.

For both viruses, on the skin, a detergent activity but no inactivation effect of BC is observed at 50 ppm, (Figure 14 A & B) (my interpretation). At this low

concentration the chemical agent allows for a better elution of infectious virus from the carrier along with breaking of virus clumps. This is demonstrated by the peak which is observed at 50 ppm, where a greater recovery of viable virus is found compared to the 0 control.

Chlorhexidine Digluconate

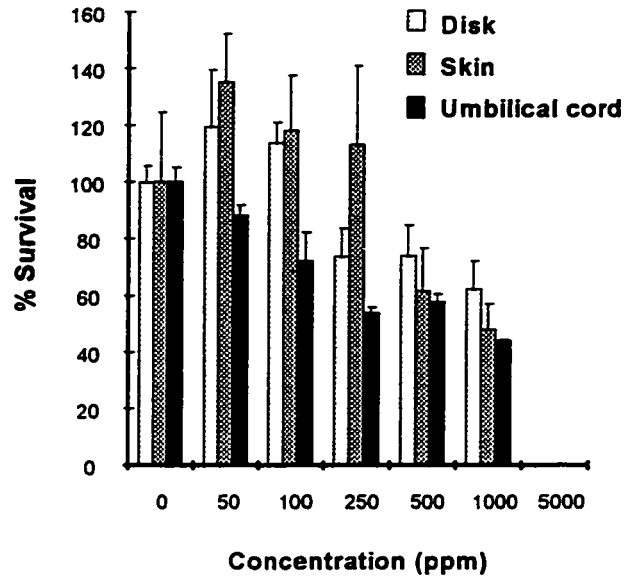
At 20 ppm of CD, nearly 50% of the HHV 2 on the metallic disks and umbilical cord was inactivated with no reduction in virus infectivity on the skin at that concentration (Figure 15 A). Increasing the CD concentration to 50 ppm gave a greater than 60% reduction in HHV 2 infectivity on all the carriers. By 200 ppm the inactivation of HHV 2 was complete on both the disk and skin carriers but was still at approximately 60% on the umbilical cord. Even at 1000 ppm the umbilical cord had about 10% of HHV 2 surviving.

A single experiment had been attempted to determine if the amnion could be inoculated with virus and if virus replication would occur. 10^5 PFU of HHV 2 were inoculated onto umbilical carriers for 30 min followed by a 1 mL liquid overlay of maintenance medium (similar to Figure 2B). The carriers were incubated at 37°C for 3 days with 100 μL of medium drawn off every 24 hrs. Dilutions of each of the four times, 0 (control), 24, 36 and 72 hrs, were plated onto confluent Vero cell monolayers to determine the amount of infectious virus particles. No increase in virus titer was found and hence it was not possible to demonstrate that replication had occurred. It was found that approximately a 1 log reduction in the titer occurred during each 24 hrs interval such that a total of

3 logs reduction was observed after 72 hrs. Further tests should be conducted to determine if replication can be demonstrated. If replication can be shown then any virus not properly inactivated poses a risk of infection to the host, as in the above inactivation test where 10% of HHV 2 survived on the mucosal surface even after exposure to 1000 ppm of CD

In contrast to HHV 2, the infectivity of AD 4 on the disks very rapidly dropped off such that at 10 ppm CD, a greater than 85% reduction in the survival of the AD 4 virus was observed. At this concentration of CD there was only about 50 to 60% reduction of AD 4 on the animate carriers. For inactivation of AD 4 on the umbilical cord a constant and steady drop in the amount of virus surviving was found as the concentration of CD increased from 10 ppm to 1000 ppm. Even at 1000 ppm complete inactivation of AD 4 was not achieved on any of the carriers with reductions ranging from 95 to 98%.

A. Human Herpes Virus 2



B. Adenovirus 4

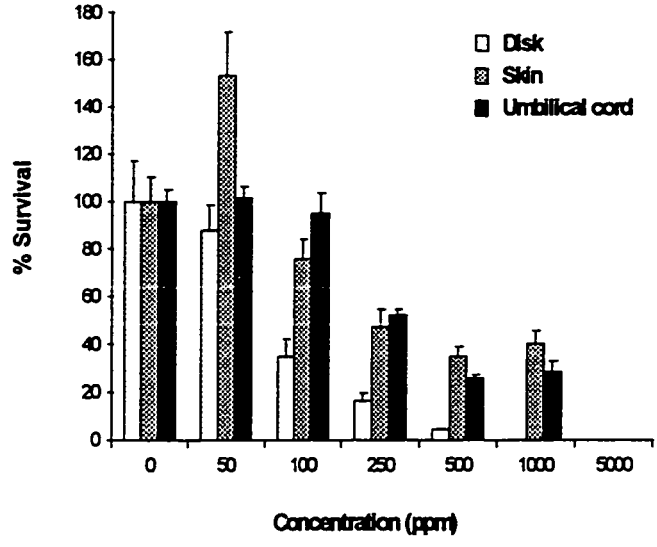
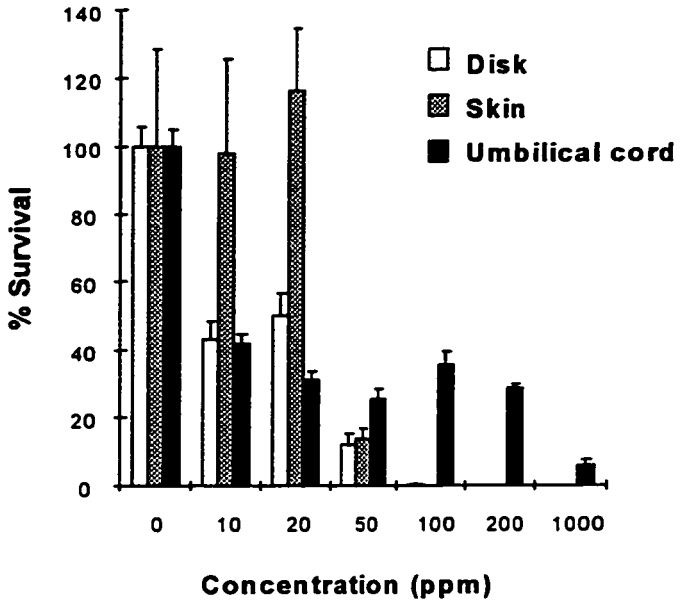


Figure 14. Inactivation of **A.** HHV 2 and **B.** Adenovirus 4 with Benzalkonium chloride on inanimate (disks - stainless steel) and animate (skin - stratum corneum; umbilical cord - amnion). Virus was allowed to dry on each of the carriers for 60 min at ambient temperature (22±1°C) followed by treatment with 100 µL of BC for a contact time of 1 min. The virus/chemical mixture was then neutralized (by dilution and the addition of serum) and eluted and the amount of virus surviving was determined. Values were normalized to the control ((0), the amount of virus remaining after the 60 min drying time, and with no chemical added) value as 100%. Results represent a minimum of three repeats and values are means ± SEM.

A. Human Herpes Virus 2



B. Adenovirus 4

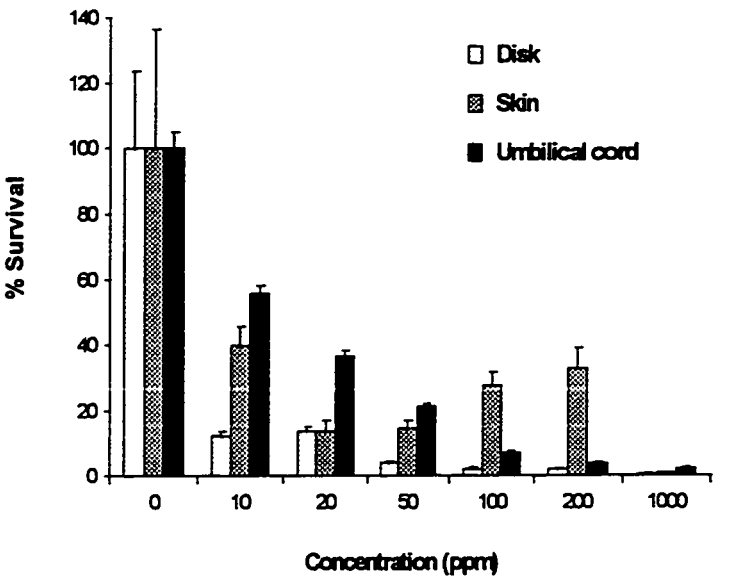


Figure 15. Inactivation of **A.** HHV 2 and **B.** Adenovirus 4 with Chlorhexidine digluconate on inanimate (disks - stainless steel) and animate (skin - stratum corneum; umbilical cord - amnion) carriers. Virus was allowed to dry on each of the carriers for 60 min at ambient temperature (22±1°C) followed by treatment with 100 μL of CD for a contact time of 1 min. The virus/chemical mixture was then neutralized (by dilution and the addition of serum) and eluted and the amount of virus surviving was determined. Values were normalized to the control ((0), the amount of virus remaining after the 60 min drying time, and with no chemical added) value as 100%. Results represent a minimum of three repeats and values are means ± SEM.

DISCUSSION

SKIN VIABILITY ANALYSIS

Although *ex vivo* models for animal skin have been described (Bronaugh et al. 1990; Bronaugh et al. 1985; Riviere et al. 1986; Hiernickel 1985), to my knowledge this is the first report on comparative viability of freshly obtained sections of human skin from three body sites. Lactate output from fragments of fuzzy rat and hairless guinea pig skin, along with histopathological evaluation, has been used previously as a measure of skin viability in animals (Bronaugh et al. 1990). This report describes the extension of this approach to human skin sections. As previously observed for animal tissues, the biochemical data and histopathological analysis reported here show that viability of human skin sections can also be maintained for at least 48 hrs in the flow-through cell system or for at least 8 hrs in a static model. While further experiments are needed to elucidate the reasons for raised lactic acid concentrations in the beginning of the experiment between 4 and 12 hours, such an increased metabolic activity has also been observed in animal skin studies (Bronaugh et al. 1990; Collier et al. 1989). One could compare lactate produced with the activity of a second skin metabolic indicator, for example lactate dehydrogenase (LDH). Because of the high concentration of LDH normally found within intact skin, leakage into the diffusion medium would be a good marker of epidermal integrity. LDH concentrations usually increase in nonviable preparations (Riviere 1991). For instance, if the lactate peak at 4 to 12 hours is due the release of non-viable

cells one would similarly expect a peak in LDH. Further studies would need to be conducted to critically assess this phenomenon.

Due to time limitations, similar testing for the continuance of viability of the umbilical cord patches could not be performed. The conditions demonstrated to maintain viability in skin were also used for fresh cord patches. Viability tests would have to be performed with the amnion in order for this *ex vivo* model to be accepted as a standard testing method. While the umbilical cord would not be the usual route of viral exposure, this was used as the mucosal surface in this study as the tissue was readily available and each cord can on average, produce a minimum of nine carriers.

This *ex vivo* human skin technique is simple and inexpensive for the evaluation of dermal and topical products. It is of interest to note here the other uses this system has with respect to toxicity testing. This method makes it possible to measure the influence of potentially damaging or toxic additives without putting human subjects at risk and yet this system eliminates any variabilities observed with animal skin (Bronaugh et al. 1991; Wester et al. 1989). Similarly, a large number of formulations and different doses can be screened in a short period of time, and at relatively low cost. Effluent samples using the Flow-Through unit can be quickly and easily collected at any designated time points. Using this model one can closely monitor hydration, temperature, pH, and diffusion variables unlike in other models, such as a two-chambered diffusion system (Bronaugh et al. 1985).

The epidermis, in particular the stratum corneum, has often been cited as the principal or rate limiting barrier to skin penetration. This model can further be used to study this barrier function of skin, including techniques used in a skin flap model whereby the outward transdermal movement is assessed (Riviere 1991) or to test for permeation where the integrity of the skin barrier is damaged, for example by abrasion or irradiation (Bronaugh 1989).

Unlike in static models, the flow-through diffusion system allows for a larger sink in which a diffusing chemical can solubilize (Bronaugh et al. 1985; Wester et al. 1989). Because of the potential for build-up of metabolic by-products which are cytotoxic, one might wish to use only shorter durations with the static model. In this system, both static and flow-through cell systems demonstrated similar viability profiles over an 8-hr period. In previous percutaneous absorption studies, comparison of a static cell to a flow-through diffusion cell in rat skin demonstrated comparable results (Bronaugh et al. 1985). Further repeats for longer durations would be necessary to ensure maintenance of viability. Although the goal of earlier animal skin studies was to assess survivability during 24-hr perfusion periods, lactate production was found to remain at its initial rate for 80 hr (Bronaugh et al. 1990).

It has been recommended that data from at least three animal or human subjects be obtained and averaged to allow for biological variation between subjects. In absorption studies, different body sites of human skin also show variability. The scrotum has a twenty-fold higher absorption than either the skin

on the back and abdomen or the arms and legs (Bronaugh et al. 1991). The facial regions also have a four times greater absorption than the trunk region (Bronaugh et al. 1991). In this study, it was not unexpected that the facial skin tissue showed diminished viability by comparison with skin from breast and abdomen considering the available pieces were quite small and hence subjected to a higher degree of trauma per square centimeter.

Other research involved the use of porcine skin as it is one of the animal skin systems which roughly resembles that of humans (Woolwine et al. 1995). However, in one study the processing of the freshly removed pigskin involved washing the hide, followed by dehairing, and then the material was frozen at -20°C. For use in an experiment, the samples were then thawed, destubbled with a sterile disposable razor, cut into squares, and rinsed in warm tap water (Woolwine et al. 1995). The authors did not perform any tests to determine the viability of the porcine tissues used to represent human skin. This may explain the differences they obtained in the recovery efficiency of ø6 bacteriophage from the fingerpads of volunteers compared to the pig hide carriers (Woolwine et al. 1995). Possible causes of impaired barrier properties of skin might include (1) disease or trauma of donor, (2) deterioration of skin between death and sample harvest, (3) damage to skin during harvesting, and (4) skin storage conditions before use. Previous studies with stored cadaveric skin demonstrated that the viability, barrier integrity and biochemical functions were compromised (Bronaugh 1989; Jensen 1984; Hiernickel 1985; Bronaugh et al. 1986).

The time periods selected in this initial development were to demonstrate the appropriateness of the *ex vivo* methodology. For further validation of the model the test period could be lengthened to at least 48 hours for fresh skin and 72 hours for refrigerated skin using the flow-through method, and 24 hours for the static cell system.

In conclusion, based on biochemical and histopathological evaluation, I have shown that human skin sections remain viable in the system described for at least 48 hr. The levels of lactate obtained were relatively consistent between the three body sites. Both the fresh abdominal and breast skin tissue produced similar amounts of lactate over 24 hr. For optimal metabolic activity, fresh skin sections should be employed, as their activity declines with storage at 4°C.

EX VIVO MODEL

In vivo human protocols consist mainly of the use of fingerpads and whole hands of volunteers but cannot be used with high risk agents (Mbithi et al. 1993; Cutler 1972; Ansari et al. 1989; Ansari et al. 1988). As such, *in vivo* methods using human pathogens such as genital herpes are essentially limited to animal systems. One study used an *in vitro* suspension test and an *in vivo* guinea pig test (with live anesthetized animals), to compare virucidal activity of a quaternary ammonium compound disinfectant and a non-medicated soap against herpesvirus 1. The authors observed approximately one $\log_{10}$ lower virus recovery in the *in vivo* compared to *in vitro* method (Prince et al. 1994). Recently, a porcine skin carrier for virucidal tests has been used (Woolwine et al. 1995).

The authors did not perform any tests to determine the maintenance of tissue viability and considering the method of collection (from fresh cadavers), storing (-20°C) and preparing the tissues (destubbling), viability would be compromised and one must question the applicability of this *in vitro* method (Woolwine et al. 1995).

In the *ex vivo* method described in this study clinically relevant conditions with realistic contact times were selected. The enveloped HHV 2 and non-enveloped AD 4 were selected for their relevance to transmission by skin or mucosal tissues, as well as representative of different virus families. The use and selection of a model virus for virucidal testing should only be done providing that the selected virus' resistance to disinfection is comparable to all the viruses to be represented.

BC and CD were chosen as they are relevant active constituents of topically applied products commonly manufactured and used in Canada (Table 3 A and B, Appendix II and III) and elsewhere. Both of the agents are surfactants that have been known to physically disrupt the protein or lipid structures of microorganisms (Merianos 1991; Prince et al. 1991).

The selection of appropriate freshly available human tissue for use as carriers was limited to reduction skin tissues (keratinized stratified squamous epithelium) and umbilical cords (mucous membrane or non-keratinized stratified squamous epithelium) as these tissues are the only ones available fresh and in quantities sufficient to generate many carriers from one donor. The statistical

significance of the study was maintained by using a minimum of three carrier repeats for each test.

VIRUS SURVIVAL

Enhanced survival was observed for HHV 2, with approximately 35% and 15% greater survival on the skin and umbilical cord carriers, respectively, than on the inanimate disk carrier. An even greater difference was found with AD 4, where a 50% greater survival of the virus was found on both the skin and umbilical cord as compared to the disk. The time periods were selected to demonstrate the appropriateness of the *ex vivo* methodology. The test period was kept to 90 minutes for practicality, and for comparison to the inanimate surfaces where virus decay was more rapid. For further validation of the model the test period could be lengthened to at least 8 hours so long as the viability of the animate carrier is maintained.

RH is known to play an important role in virus survival (Sattar et al. 1987) and it is possible that the RH of the animate carriers and possible variability between donors would likewise affect the survival of both HHV 2 and AD 4 as compared to the inanimate carrier. An earlier *in vivo* study measured the water content of stratum corneum in human male volunteers who were in 21% to 78% relative humidity (RH) rooms for 1 hr at a temperature of 23 ± 2°C (Morrison et al. 1988). The researchers trimmed off skin from the back of the hands of the volunteers and immediately used gas chromatography-mass spectrometry to determine the water content of the skin. They found a low of 0.082 mg water/mg

stratum corneum at 21% RH to a high 0.41 mg water/mg stratum corneum at 78% RH. This is equal to 5 times more water/mg skin at 78% than 21 % RH (Morrison et al. 1988). While the carriers were maintained in an environment with a relative humidity of 50%±5%, one could speculate that the water content in the animate carriers could be very variable and that this would have an affect on the viability and possibly on protection of the pathogen. A further study would be to test virus survival on one *ex vivo* animate carrier at a range of RH. Also in the above study, all of the volunteers were male and limited to the 30-44 yrs age group. One would also wish to include females and a broader age. With respect to differences found on the basis of sex, female rats were found to have whole epidermis skin on their backs half as thick (31 µm vs. 61µm) and roughly twice as permeable as male rat skin (Bronaugh 1989). Further work could also be done to test for survival where the integrity of the skin barrier is damaged (i.e. abraded or irradiated skin) (Bronaugh 1989).

More work is required to determine donor to donor variation in survival of herpesvirus or any other agent for which this method is used.

VIRUS INACTIVATION

Earlier studies showed that a 0.02% CD concentration is active against herpesvirus but inactive against Adenovirus Type 2 (Denton 1991). I have demonstrated that a concentration of CD greater than 100 ppm (or 0.01%) was required to inactivate HHV 2 on either the disk or the skin carriers but even at 1000 ppm (0.1%) the virus was not completely inactivated on umbilical cord.

In the *ex vivo* assay with a 1 min contact time, 500 ppm (or 0.05%) of BC was sufficient to decrease the survival of AD 4 by greater than 50% on all three carriers but only about 40% effective against HHV 2. A higher (5000 ppm) concentration of BC was required to inactivate both viruses on all three carriers. In another study it was demonstrated that with a minimum contact time of 10 minutes, BC was active at 700-1000 ppm against Adenovirus 2 (Prince et al. 1991). Conversely, BC was found to lack activity against picornaviruses even at a concentration of 10%. It would be of interest to use the resistant picornaviruses in the *ex vivo* survival and inactivation assays described here.

The concentrations of the two active agents were selected as representative of currently manufactured products (Appendix II & III). One must take into consideration that while the pure forms of the chemicals were tested, the product formulation would also influence the effectiveness of the active ingredients. A combination of active ingredients can have synergistic or additive antiseptic effects.

An important aspect of any inactivation study is to limit the exact timing of the contact between the pathogen and disinfectant. To do this one must remove or neutralize any remaining antiseptic activity. Neutralization of the antiseptics was achieved in two ways: by dilution, whereby the 100 μL chemical inoculum was diluted 10X by the addition of 900 μL of neutralizer, and by inactivation due to the presence of FBS in the eluent.

A single unsuccessful experiment was performed to demonstrate if virus replication could occur in the mucosal covering of the umbilical cord. If replication can be shown, then any virus not completely inactivated poses a risk of infection to the host, for example, as in the inactivation tests where 10% of HHV 2 survived on the mucosal surface even after exposure to 1000 ppm of CD. It is important to note that the low viral infectious dose needed for infection makes complete antisepsis of virus-contaminated material even more significant than for many bacterial pathogens (Weswood et al. 1976).

This mucosal model also has applicability for testing of survival and inactivation of sexually transmitted pathogens and topically used spermicidal products. It has been found that 0.5% CD in 70% alcohol solution was completely effective against HIV after 15 s (Denton 1991). In an *in vitro* study, BC has been shown to directly destroy or inactivate viral reverse transcriptase activity upon contact with HIV-1 (Wainberg et al. 1990). The authors assessed the susceptibility of MT-4 T lymphocytes challenged with H-9 T-leukocyte derived HIV-1 (1.5×10^4 PFU/mL) which were then exposed to different concentrations of BC over 10 minutes. The cell-associated virus in HIV-infected genital secretions was found to be inactivated by 0.05% BC when exposed for a minimum of 5 minutes. Viral infectivity was monitored by viral transcriptase and a p24 antigen ELISA assay which measured an increase in p24 over 30 days to demonstrate the presence or absence of HIV-infectivity (Wainberg et al. 1990). In another *in vitro* test, 0.05% BC was found to inactivate $HIV\text{-}1_{GB8}$ and at

concentrations >0.05% BC was toxic to the JM cell line used to culture the virus (Jennings et al. 1993). It would be of interest to determine the effectiveness of currently manufactured spermicides against agents such as HIV using the *ex vivo* mucosal model.

LIMITATIONS OF THE MODEL

Skin and umbilical patches are required to be 2.3 cm in diameter in order to be properly mounted in the holders used in this study. Ideally, a smaller, 1 cm diameter, would generate a greater number of patches from tissue of one individual. The mounting procedure of securing the patches on to the holders with an o-ring is difficult to manipulate wearing latex gloves, which are necessary for biosafety. In this *ex vivo* model, there is no way to measure if the skin patches have properties found in the intact *in vivo* organ. The *in vivo* skin properties include an intact stratum corneum, exfoliation, an acid mantle, topically secreted IgA antibodies, and antibacterial compounds are constantly produced by organisms normally found on the skin (Collier et al. 1992).

FUTURE WORK TO FURTHER VALIDATE THE MODEL

The *ex vivo* human skin model described here uses freshly excised viable skin and no correlations or extrapolations need to be made from animal models. One would also wish to apply this *ex vivo* survival and inactivation model to viruses which are more resistant to disinfection: enteroviruses, hepatitis A virus, coxsackieviruses, rotaviruses, etc. Similarly, many bacteria, which are important

skin or sexually transmitted pathogens, should also be tested in this model. Also, organic loads, such as genital secretions, saliva, perspiration or blood, could be added to either the carrier or the pathogen to better reflect the topical product's use *in vivo*.

In this model one would have to incorporate a radiolabeled tracer in the pathogen to fully differentiate between inactivation or a failure to fully recover the organism from the carriers.

Nearly all of the tissues were obtained from female donors only as they make up the majority of plastic and reduction surgery patients. I was also limited to only three body sites. Furthermore, the skin tones from the skin tissues indicated that most of the donors were caucasian. As these are elective surgeries one could surmise that most of the individuals belong to middle or higher income groups. Similarly with the umbilical cord, no information as to the sex or race of the donor was available. These are aspects which one would want to vary in order to validate the model such that the data reflects complete representatives of all types.

This *ex vivo* method would provide more relevant data than previous tests such as *in vitro* suspension tests using HIV-1 in cell culture fluid and in genital secretions where the authors examined virus inactivation by spermicides (Wainberg et al. 1990). Also, new topical products released on the market such as amphotericin B compounds or magainins (Jacob 1994) could be assessed in this *ex vivo* model before use by the public. Similarly, the chemical agents could

include "off the shelf" topical products such as those listed in the Appendix or other commonly used disinfectants such as alcohols.

FURTHER POTENTIAL APPLICATIONS

There are numerous potential applications of this *ex vivo* human skin model. The system could be standardized and may be used to (a) study the survival of pathogens on animate carriers (alone or in mixed inoculum), (b) assess the germicidal potential of antiseptics, especially against hazardous agents, and facilitate their regulation, (c) the interaction and germicidal activity of topicals against infectious agents, (d) determine the antimicrobial activity of new compounds during product development, (e) the efficacy and safety of new bioengineered skin products, (f) cutaneous toxicological, pharmacological, and biochemical effects. Regulatory agencies, manufacturers of pharmaceuticals or specialty chemicals, standard setting organizations, and the public at large would benefit from increased assurance that commercially available topical antimicrobial products will meet their label claims when used as directed.

CONCLUSIONS

1. Viability of *ex vivo* human skin could be maintained over a minimum of eight hours in a static model and 24 hours in a Flow-Through Diffusion unit.
2. A model for examining the survival of two viral pathogens on *ex vivo* human tissues was developed and tested, with survival differences found between the two virus types and between the inanimate and animate carriers.
3. A model for investigating the inactivation of the pathogens on human *ex vivo* carriers was developed and tested, with inactivation differences found between the two virus types, between the inanimate and animate carriers and between the two antiseptics.

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DEVELOPMENT OF AN *EX VIVO* METHOD TO TEST THE VIRUCIDAL ACTIVITY OF ANTISEPTICS AND SPERMICIDES ON SKIN TISSUE

CONSENT

Contact Person: Dr. S.A. Sattar, Professor
Dept. of Microbiology and Immunology
Phone: 562-5800 ext. 8314
Fax: 562-5452

During the course of your operation, a small amount of skin tissue is routinely removed at the surgical site. Normally this tissue is discarded. Rather than discarding it, with your permission, we will use this tissue for experimental studies in our research laboratory, to determine the effect of chemicals to inactivate virus in contact with skin surfaces. Strict patient confidentiality will be observed in these studies. There is no obligation whatsoever to participate in this study, and refusing to participate will in no means prejudice your care during your hospital stay.

I, _____, do hereby consent to skin tissue, which is normally discarded at the time of my operation, being used for research purposes.

Dated this _____ (dd/mm/yy)

Witness signature _____

Patient signature _____

(Valid until December 1, 1996)

451, ch. Smyth
Ottawa (Ontario) K1H 8M5 Canada

451 Smyth Rd.
Ottawa, Ontario K1H 8M5 Canada

Canadian Notified Drug Products Containing Benzalkonium Chloride.

#	Product Name	DIN	Name of Manufacturer	Other Active Agents
1.	ENDBAC 256 - Accumix Liq	00885746	Johnson and Son Ltd.	
2.	J.H.40 Liquid Detergen Sanitizer 2.5%	00891266	Canadian Mill Supply Co., Ltd.	
3.	UNIBAC Liq 4.5%	01924508	Unique Inc. Produits Sanitaires	
4.	Antiseptic Lot 0.1%	01912399	AUI	
5.	Antisol Plus Liq 10%	00310972	Safetex Products Ltd.	
6.	Arkonsol Liq 0.133%	00407283	Laboratoire Romilo	
7.	Bactine First Aid Spray Liq	00607959	Miles Canada Inc., Consumer Healthcare Div.	2) Lidocaine hydrochloride
8.	Band-Aid Medic Fabric 0.4 mg/pad	00754234	Johnson & Johnson Inc.	
9.	Band-Aid Medic Sheer 0.4 mg/pad	00754242	Johnson & Johnson Inc.	
10.	Benzalchlor 1:750 Liq 0.133gm/100mL	00603406	Regal Pharms Div. Bradcan Corporation	
11.	Benzalchlor 50	00168203	Regal Pharms Div. Bradcan Corporation	
12.	Benzalkonium Chloride Antisept Towel	00555983	Professional Disposables Div. Nice-Pak	
13.	Benzalkonium Chloride Towel Pad 0.4%	01984977	Q H P Medical Ltd.	
14.	Deosan Liq	01924494	Diversey Inc.	2) Alkyl ethylbenzyl dimethyl ammonium chloride
15.	Emerdine Liq 0.133%	00321362	Pharmetics Ltd.	
16.	Foot Bath	00505625	Sani-Marc Inc.	
17.	Germiphene Concentrate 13.0%	00109991	Germiphene Corporation	
18.	Irrigation Tray W Benzalkonium Pad 1:750	01945785	Baxter Corporation	
19.	Medi-Quik First Aid Spray	00697753	Mentholatum	1) Lidocaine
20.	Mevon No.72 Liq	02020831	Molnycke Ltd.	2) Alkyl ethylbenzyl dimethyl ammonium chloride
21.	Neo Vagex	00147591	Neolab Inc.	1) Acetarsone, 2) Iodochlorhydroxyquin
22.	Obstetrical Towelette Pad 0.4%	01949969	Q H P Medical Ltd.	
23.	Oragel Mouth-Aid	00687502	Commerce Pharmaceutics Inc.	1) Benzocaine, 3) Zinc chloride
24.	P & W Liquid Antiseptic 0.13%	00788333	Jedmon Products Ltd.	
25.	PMS Benzalkonium Chloride Solution	00785288	Pharmascience Inc.	
26.	Sting Stop 0.1%	00311243	Trafalgar Industries of Canada Inc.	
27.	Sting Stop 0.1%	00319589	Canadian Custom Packaging	
28.	Tanac Liquid	00560758	Commerce Pharmaceutics Inc.	1) Benzocaine, 2) Tannic acid
29.	TRL 131 Swim Pool Algaecide	00531766	Trojan Chemicals	
30.	Zephiran Liq 1.33 mg/mL	02017806	Sanofi Winthrop Canada Inc.	

Canadian Notified Drug Products Containing Chlorhexidine Digluconate.

#	Product Name	DIN	Name of Manufacturer	Other Active Agents
1.	Baxedin Liq 0.05%	00788422	Luvabec Laboratoires Inc.	
2.	Baxedin Liq 20%	00658413	Luvabec Laboratoires Inc.	
3.	Bioprep	00591033	Avmor Ltd.	2) Didecyl dimethyl ammonium chloride
4.	Bioprep	00618934	Amberine Products Ltd.	2) Didecyl dimethyl ammonium chloride
5.	Bioprep	00618888	Sani Professionel Ltee.	2) Didecyl dimethyl ammonium chloride
6.	Bioprep	00618969	Larose & Fils Ltee.	2) Didecyl dimethyl ammonium chloride
7.	Bioprep	00651281	Cartier Chemicals Ltd.	2) Didecyl dimethyl ammonium chloride
8.	Bioprep Antiseptic Hand Soap 1%	00637394	Midwest Supplies (1974) Ltd.	
9.	Bioprep Liq	02004534	J.C. Sani Care Inc.	2) Didecyl dimethyl ammonium chloride, 3) Isopropyl alcohol
10.	Chlorhexidine 0.5% Liq	00804622	Biopharm Inc.	2) Isopropyl alcohol
11.	Chlorhexidine Liq 0.05%	00804614	Biopharm Inc.	
12.	Chlorhexidine-20% Liq	00804606	Biopharm Inc.	
13.	Cida-Stat Foam	02005018	Huntington Laboratories Inc.	2) Isopropyl alcohol
14.	Cida-Stat	00721549	Huntington Laboratories Canada Inc.	
15.	Dexidin 20 sol 20 mg	00832030	Atlas Inc.	
16.	Dexidin Liq 2%	00832138	Atlas Inc.	
17.	Dexidin Liq 4%	00832111	Atlas Inc.	
18.	E-Z Scrub 747 4%	01939947	Becton Dickinson	
19.	Flamazine C	00714917	Smith & Nephew Inc.	1) Silver sulfadiazine
20.	Foam Care Chg Surgical Hand Scrub	00853585	Ballard Medical Products	2) Isopropyl alcohol
21.	Foam Care Chg Surgical Hand Scrub	00853607	Ballard Medical Products	2) Isopropyl alcohol
22.	Germi Stat Gel 2%	01930079	Germiphene Corporation	
23.	Germi Stat Gel 4%	01930087	Germiphene Corporation	
24.	Henry Schein Antimirobial Skin Cleanser	01994042	ABJ Custom Packaging Div., Germiphene Corp.	
25.	Hexifoam-Skin Disinfectant Aer 1%	00803677	Soltec Research Pty Ltd.	2) Alcohol anhydrous
26.	Hibidil Liq 0.05% (single use)	02022192	Zeneca Pharma Inc.	
27.	Hibitane gluconate 20% Solution	02020947	Zeneca Pharmalnc.	
28.	Hibitane Skin Cleanser Liq 4%	01932373	Whitehall-Robins Inc.	

29.	Hibitane Skin Cleanser Liq 4%	00245097	Ayerst Laboratories	
30.	IC-Gel	00887935	Germiphene Corporation	1) Isopropyl alcohol
31.	Luroscrub Antimicrobial Skin Cleanser	00817651	The Dial Corp.	
32.	Mediwipe Pad	00769053	Generation Products SDN BHD	2) 2-Phenoxyethanol
33.	Novoclens Liq 4%	00844624	Novocol Pharmaceutical of Canada Inc.	
34.	Oronine H Ointment 1%	02092913	Classical Remedia Ltd.	
35.	Perioselect Antimicrobial Sol 0.12%	01957864	Sultan Chemists Inc.	
36.	Rouhex G 20%	00608254	Rougier Inc.	
37.	Rouhex G 4%	00608270	Rougier Inc.	
38.	Rouhex G Liq 2%	00614882	Rougier Inc.	
39.	Salvesept Crm	01954091	Protector Canada Inc.	2) Cetrimonium bromide
40.	Savlodil Liq (single use)	02022206	Zeneca Pharma Inc.	2) Cetrimide
41.	Savlon Hospital Concentrate	02021005	Zeneca Pharma Inc.	2) Cetrimide
42.	Scrub Stat 4 foam Liq	01988514	Huntington Laboratories Inc.	
43.	Scrub-Stat IV 4%	00779229	Huntington Laboratories Canada Inc.	2) Isopropyl alcohol
44.	Soft Care Antiseptic Hand Soap	00556327	Johnson and Son Ltd.	2) Didecyl dimethyl ammonium chloride
45.	Spectro Gram 2 Soap 2%	00758485	Spectropharm Inc.	
46.	Stanhexidine 2%	00728799	Stanley Pharmaceuticals Ltd.	
47.	Stanhexidine 2% W Isopropyl Alcohol 4%	01938991	Stanley Pharmaceuticals Ltd.	2) Isopropyl alcohol
48.	Stanhexidine 20%	00728802	Stanley Pharmaceuticals Ltd.	
49.	Stanhexidine 4%	00728810	Stanley Pharmaceuticals Ltd.	
50.	Stanhexidine 4% W Isopropyl Alcohol 4%	01938983	Stanley Pharmaceuticals Ltd.	2) Isopropyl alcohol
51.	Stanhexidine Red Tincture 0.5%	00807753	Stanley Pharmaceuticals Ltd.	
52.	Steri-Stat Antiseptic Gel	01975242	Ingram and Bell Inc.	2) Isopropyl alcohol
53.	Steri-Stat Liq 2.0%	01975218	Ingram and Bell Inc.	
54.	Steri-Stat Liq 20%	01975196	Ingram and Bell Inc.	
55.	Steri-Stat Liq 4.0%	01975226	Ingram and Bell Inc.	2) Isopropyl alcohol

PUBLICATIONS, POSTERS AND AWARDS

PUBLICATIONS

1. **M.L. Graham**, V.S. Springthorpe, S.A. Sattar. 1996. "*Ex Vivo* Protocol for Testing Virus Survival on Human Skin: Experiments with Herpesvirus 2". *Applied and Environmental Microbiology,* 62 (11): 4254-4255.
2. **M.L. Graham**, S.A. Qureshi, S.Bujaki, S.A. Sattar. 1997. "Determination of *Ex Vivo* Viability of Human Skin Using a Flow-Through Diffusion Cell System" to be submitted for publication.
3. **M.L. Graham**, V.S. Springthorpe, S.A. Sattar. 1997. "*Ex Vivo* Protocol for Testing Virus Inactivation on Human Skin: Experiments with Herpesvirus 2 and Adenovirus 4", manuscript under preparation.

POSTERS

Oct 29-30, 1996 — **Ottawa Life Sciences Conference and Exhibition, Ottawa, Ontario.** Poster presentation entitled "*Ex Vivo* Protocol to Test Efficacy of Topicals Against Herpesvirus 2" **M.L. Graham**, V.S. Springthorpe, S.A. Sattar

Oct 27-31, 1996 — **American Association of Pharmaceutical Scientists, Seattle, Washington.** Poster presentation entitled "An Assessment of Viability of Human Skin Sections Obtained From Different Anatoimical Sites Using a Flow-Through Diffusion Cell System" S.A. Qureshi, **M.L. Graham**, S.A. Sattar, I.J. McGilveray

Sep 15-18, 1996 **36th Interscience Conference on Antimicrobial Agents and Chemotherapy, American Society of Microbiology, New Orleans, Louisiana.** To Present a poster entitled "*Ex Vivo* Protocol to Test Efficacy of Topicals Against Herpesvirus 2" **M.L. Graham**, V.S. Springthorpe, S.A. Sattar

Oct 17-18, 1995 **Ottawa Life Sciences Conference and Exhibition, Ottawa, Ontario.** Poster presentation entitled "An *Ex Vivo* Human Skin Model to Evaluate Absorptive, Toxicological and Germicidal Activites of Topicals" S.A. Qureshi, **M.L. Graham**, S.A. Sattar, I.J. McGilveray

AWARDS

Oct 29, 1996 Silver Graduate Student Poster Award, Ottawa Life Sciences Council

June, 1996 ASM Sustaining Member Student Travel Grant Award from the ICAAC Program Committee of the Americal Society of Microbiology

June, 1996 Student Travel Grant Award from the Faculty of Medicine, University of Ottawa to attend ICAAC

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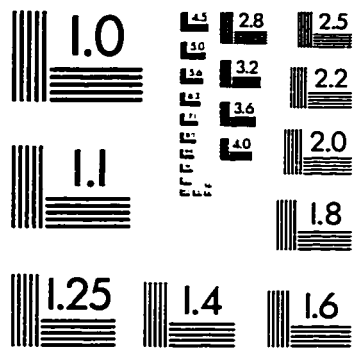

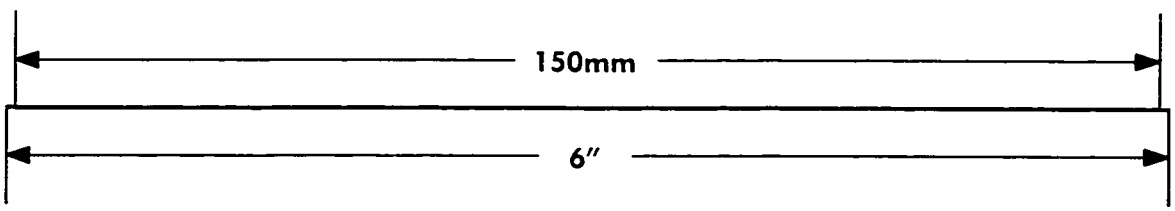

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