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Ex Vivo Protocol for Testing Virus Survival on Human Skin: Experiments with Herpesvirus 2

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We report an ex vivo method, which uses pieces of human skin excised during routine plastic surgery, for testing survival of hazardous pathogens. Using this procedure, we compared the survival of human herpesvirus 2 on human skin and on metal disks. At the physiological skin temperature of 32°C, the half-life of the virus on skin was 1.44 h while on metal disks it was 0.36 h. Even at ambient temperature (22°C), the virus lost infectivity faster (half-life = 0.96 h) on metal disks than on the skin at 32°C. The method described could be used to assess the survival of other human pathogens on skin and to evaluate the germicidal activity of handwashing agents and other topicals.

We have previously reported the use of fingerpads of adult volunteers to assess quantitatively the ability of viruses and bacteria to survive on human skin (1). Since ethics and safety considerations limit the application of the method to use with relatively safe infectious agents, we describe here an ex vivo method for testing the survival of more hazardous pathogens on human skin. This method, which uses pieces of human skin excised during routine plastic surgery, was evaluated with human herpesvirus 2, and parallel tests were run on stainless steel disks to compare virus survival on animate and inanimate surfaces.

A seed culture of Vero cells was obtained from K. Wright of this department. The cells were grown in 75-cm^2 plastic cell culture flasks (Corning, Corning, N.Y.) using minimum essential medium (GIBCO, Grand Island, N.Y.) supplemented with 0.113% sodium bicarbonate (BDH Chemicals, Toronto, Ontario, Canada), 1.5 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; GIBCO), 7% fetal bovine serum (GIBCO), 1% nonessential amino acids solution (GIBCO), 146 μg of L-glutamine (GIBCO) per ml, and 50 μg of gentamicin sulfate (Cidomycin; Hoechst Marion Roussel Canada, Montreal, Quebec, Canada) per ml.

Strain 333 of human herpesvirus 2 was acquired from J. R. Smiley of McMaster University, Hamilton, Ontario, Canada. Stock virus was prepared by infecting 90% confluent Vero cell monolayers in 75-cm^2 tissue culture flasks at a multiplicity of infection of 0.1. Cells 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 infected monolayer. After approximately 60 h, virus cytopathology was 80 to 90% complete. The monolayers were then scraped to collect the virus-cell mixture, which was centrifuged at $190 \times g$ for 10 min. After the supernatant was removed, the pellet was resuspended in Earle balanced salt solution and then sonicated in a water bath (Bransonic Ultrasonic Corp, Danbury, Conn.) for six 30-s bursts. After a second similar centrifugation, the viral supernatants were pooled, dispensed in 0.1-ml aliquots, and stored at −70°C. The titer of the herpesvirus 2 stock was approximately 10^7 PFU/ml.

Virus quantitation 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 three wells. Cell controls were maintained on each plate, and virus controls were performed for each batch of assays. After virus adsorption in a CO_2 incubator at 37°C for 90 min, 2 ml of an overlay, containing maintenance medium with 0.05% human gamma globulin (Miles Inc., Etobicoke, Ontario, Canada), was added to each well. The plates were then incubated at 37°C in a CO_2 incubator. After 3 days, 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 were stained for 15 min with a 1% crystal violet solution (J. T. Baker Chemical Co, Phillipsburg, N.J.). The mean titer was determined from a minimum of three wells.

Fragments of human skin used were those removed from normal adult females by a local plastic surgeon during breast reductions (reduction mammoplasty). Informed and written consent was obtained from each donor prior to the collection of the tissue samples. Sterile cell culture maintenance medium, without serum, was used as the collection and transportation fluid for the tissue samples. All specimens were processed within 4 h of removal from the donor. The tissues were prepared by removing any excess subcutaneous fat and then secured onto a dissecting board. Patches (approximately 2.3 cm in diameter) were then punched out of the skin. The details of the setup of the ex vivo apparatus are given in Fig. 1. After mounting, the tissue holder was inverted with the exposed (subcutaneous) side kept immersed in cell culture medium. Thus, the skin serves as the bottom of a well, such that the viral inoculum and then the eluent can be placed into the well. Using previously described biochemical (measurement of lactate production) and histopathological (anatomically normal) methods with freshly excised human skin bathed in tissue culture medium (10a), we have shown that we can maintain the skin viable in this system for at least 8 h. Stainless steel disks (1-cm diameter) represented hard inanimate surfaces (13) as described previously (11).

The flow diagram of the ex vivo method to test virus survival on human skin is also given in Fig. 1. To estimate the elution efficiency of the virus placed on the skin surface, 10 μl (2×10^5

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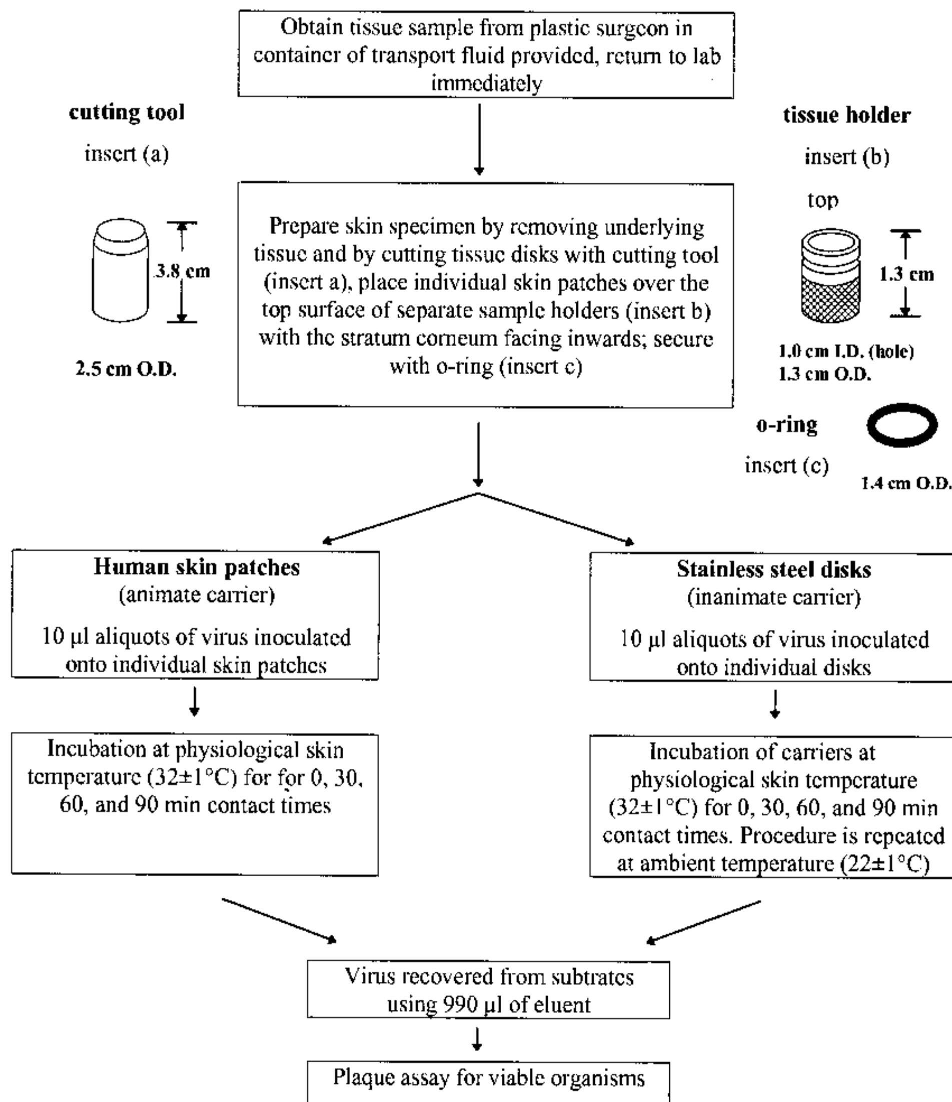

FIG. 1. Procedure and apparatus for testing virus survival on ex vivo human skin patches and stainless steel disks. Note: in addition, a 10-µl-input virus control is inoculated directly into 990 µl of eluent, and the number of infectious units in the input inoculum is determined.

PFU) of the test virus suspension was placed onto the stratum corneum side of three skin patches and eluted immediately by placing 990 µl of the eluent on each patch (time zero). Elution consisted of flushing the surface of the carrier with 990 µl of maintenance medium eluent. To compare virus survival on human skin with that on inanimate carriers, the survival protocol was repeated using stainless steel disk carriers.

Plaque assays were used to confirm the original viral titer and to determine the titer of virus recovered at each sampling time. Thus, the 0-min virus titer (PFU per milliliter) represents an efficiency of recovery of 96.8% ± 25.8% (standard deviation) of the input virus. The virus titer at each sampling is the amount of virus remaining expressed as a fraction of that eluted at 0 min. All observations were normalized to the 0-min value as 100%. Each test represents a minimum of three replicates. The data in the animate carrier experiments were obtained from skin patches provided by a single donor. The results are shown in Fig. 2. Statistical analysis (95% confidence level) using analysis of variance followed by the Student-Newman-Keuls multiple range test was performed using SigmaPlot/Stat ver 2.0/1.0 (Jandel Corporation, San Rafael, Calif.) on an IBM-compatible PC. 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 are minimum values for possible virus survival on skin. Confirmation of elution efficiency at longer contact periods would require the use of a virus preparation labeled with a radioactive isotope. 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 per hour and the time required for a 50% loss of virus titer, which is $0.693/K_i$. The results (Table 1) demonstrate that the virus half-lives were found to be longest on skin (32 ± 1°C), then on metal disks (22 ± 1°C), and finally on disks at 32 ± 1°C.

Thus, differences in survival of herpesvirus 2 were observed between the two types of carriers. The enhanced survival on the animate skin carrier further supports the need for this ex

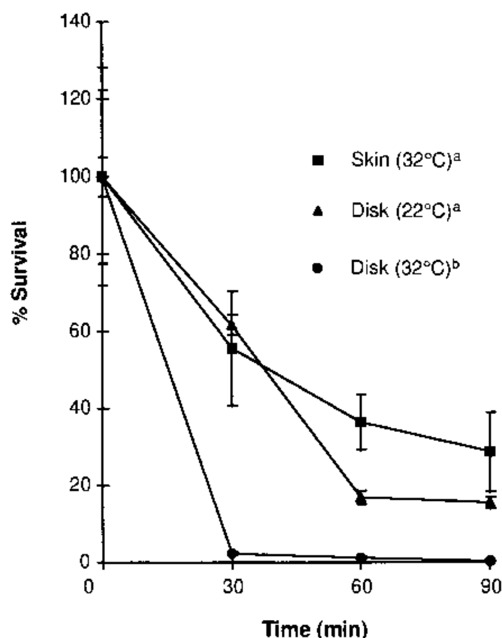

FIG. 2. Survival of human herpesvirus 2 on animate and inanimate surfaces. For in vivo-like conditions, both the skin and disk carriers were studied at physiological skin temperature (32°C). The experiment was similarly repeated on the inanimate surface at ambient temperature (22°C). Results represent a minimum of three replicates (means ± standard errors of the means). [a,b], Statistically significant differences ($P \leq 0.05$) between the carriers at 90 min.

vivo model in studying the survival of pathogens on human skin.

Herpesvirus infections continue to be a significant health concern worldwide. Studies indicate that 10% of the U.S. population is seropositive for human herpesvirus 2, and nearly 500,000 new cases occur annually (15, 16). This leads to an estimated 40 to 60 million cases of infected individuals in the U.S. (16). Herpetic lesions can occur anywhere on the skin or mucous membranes, and as the virus can be excreted in the absence of symptoms it therefore provides a silent reservoir for transmission of infection (12, 16). In previous studies looking at survival of herpesvirus on environmental carriers, when incubated at ranges from 25 to 40°C on plastic gloves, toilet seats, plastic (petri dishes), speculum, dry cotton gauze, glass, and the skin of human volunteers, human herpesvirus was found to survive up to 1 h (9), 2 h (9), 3 to 4.5 h (14, 17), 18 h (9), 72 h (9), at least 8 weeks (8), and 2 h (4, 14), respectively. More work is required to determine donor-to-donor variation in survival of herpesvirus or for any other agent for which this method is used.

In vivo human protocols consist mainly of the use of fingerpads and whole hands of volunteers but cannot be used with high risk agents (1–3, 9). As such, in vivo methods using human pathogens such as genital herpesvirus 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 nonmedicated soap against herpesvirus 1. The authors observed approximately 1 $\log_{10}$ lower virus recovery in the in vivo compared with the in vitro method (10). Other research involved the use of porcine skin as it is one of the animal skin systems which roughly resembles that of humans (17). Unfortunately, in one study the processing of the freshly removed pigskin involved washing the hide, followed by dehairing and then freezing 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 (17). Previous studies with stored cadaveric skin demonstrated that the viability, barrier integrity, and biochemical functions are known to be compromised (5–7). In the study above, the authors did not perform any test 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 the ϕ6 bacteriophage from the fingerpads of volunteers compared with the pig hide carriers (17). Although our previous studies (10a) have indicated the viability of the excised skin for 24 h, our purpose here was to demonstrate the appropriateness of the ex vivo methodology. The test period was kept to 90 min for practicality and for comparison with the inanimate surfaces, where virus decay was more rapid. For further validation of the model the test period could be lengthened to at least 24 h.

The ex vivo human skin model described here uses freshly excised viable skin, and no correlations or extrapolations need to be made. This protocol is similarly being applied to the testing of topicals against hazardous pathogens. There are numerous potential applications of this ex vivo human skin model. The system described could be standardized and may be used to (i) study the survival of pathogens on human skin; (ii) assess the germicidal potential of antiseptics, especially against hazardous agents, and facilitate their regulation; and (iii) determine the antimicrobial activity of new compounds during product development. 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.

TABLE 1. Estimated rates of inactivation (K_i) and half-lives of human herpesvirus 2 on skin and stainless steel disk carriers

Carrier (temp, Rh[a])	K_i[b]	Correlation coefficient[c]	Half-life (h)
Skin (32°C, 50% ± 5%)	0.008	0.36	1.440
Disk (32°C, 50% ± 5%)	0.032	0.66	0.361
Disk (22°C, 35% ± 5%)	0.012	0.84	0.963

[a] Rh, relative humidity.
[b] K_i, virus inactivation coefficient as $\log_{10}$ reduction in virus titer PFU per hour.
[c] Correlation coefficient refers to the measure of the linear association between two variables.

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