JOURNAL OF CLINICAL MICROBIOLOGY, June 1984, p. 880–883
0095-1137/84/060880-04$02.00/0
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Vol. 19, No. 6

Factors Influencing Quantitative Isolation of Varicella-Zoster Virus

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Received 6 December 1983/Accepted 6 December 1984

Optimal conditions are described for the recovery of cell culture-derived varicella-zoster virus (VZV). Of the cells tested, human embryonic lung fibroblasts were the most sensitive. Storage and handling procedures were examined to determine the stability of VZV in viral transport medium. When the initial VZV titer was high (2×10^4 PFU/ml) 40% of the VZV survived room temperature for 24 h and 75% of the VZV remained viable for this long at 4°C. Recovery was 5- to 10-fold less at lower initial VZV titers ($<2 \times 10^3$ PFU/ml). Other factors which influenced VZV recovery included freezing at −20°C, the use of cotton or calcium alginate swabs, and filtration to remove bacterial contaminants. The tissue culture methods described were used in a reconstruction experiment to demonstrate that VZV could be recovered from a laboratory coat or human skin (0.1 to 0.3% of input VZV) or from a stethoscope (19% of input VZV) as late as 30 min after inoculation. During a clinical trial using optimal VZV recovery procedures, 76% of the patients with herpes zoster yielded VZV when first cultured, and 60% remained culture positive for an additional 48 h.

New antiviral agents are available for the treatment of infections caused by herpes simplex virus (HSV) and varicella-zoster virus (VZV). One measure of their efficacy is the rate at which cutaneous lesions and infected fluids are cleared of infectious virus. For example, adenine arabinoside or acyclovir given intravenously significantly shortens the time that virus is shed from skin lesions (1, 8, 9, 11, 14, 16). Similarly, topical and oral acyclovir accelerate the disappearance of HSV from mucocutaneous lesions in immunosuppressed patients and from genital lesions in normal individuals (3, 4, 10, 15). Treatment of herpes zoster infections with topical acyclovir is currently being evaluated, with one measure of efficacy being the duration of VZV shedding from skin lesions after beginning therapy (M. J. Levin, J. A. Zaia, B. J. Hershey, L. G. Davis, and A. C. Segreti, manuscript in preparation). Quantitative recovery of these viruses may be important to fully evaluate drug efficacy and to help answer certain epidemiological questions, such as the significance of virus persisting on contaminated surfaces (T. Larson and Y. Bryson, Ped. Res. **16**:244A, 1982). Although some of the critical factors affecting quantitative recovery of HSV and cytomegalovirus (another herpesvirus) from clinical specimens have been identified (6, 13, 17), optimal methods for quantitative culturing of VZV have not been established. In this report we describe how conventional methods and materials used for VZV isolation influence the quantitative recovery of that virus.

MATERIALS AND METHODS

Tissue culture. Primary human embryonic kidney (HEK) monolayers in tubes (130 by 16 mm; M.A. Bioproducts) were used within 4 weeks of preparation. Human embryonic lung fibroblasts (HELF) obtained from J. Waner (University of Oklahoma, Norman) were used between passages 13 and 21. Human foreskin fibroblasts (350Q) derived by M. Myers (University of Cincinnati, Cincinnati, Ohio) were used between passage 13 and 21. Foreskin fibroblast line FS-9 was obtained from M. Oxman (University of California, San Diego) and used at passage 36.

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Monolayer cultures were prepared in six-well plastic dishes (35-mm-diameter well; Costar) by seeding with 3×10^5 cells in 3 ml of Dulbecco medium supplemented with 10% fetal calf serum and antibiotics (penicillin, 200 U/ml; kanamycin, 100 μg/ml). Confluent cultures were maintained as long as 3 weeks in medium containing 2% fetal calf serum.

Virus. A pool of VZV was prepared in 350Q cells from a clinical isolate. When 75% of the cells demonstrated cytopathic effect, the volume of medium was reduced and the cells were removed by scraping. Approximately 5×10^6 cells in 5 ml were sonicated (Ultrasonic W-200 probe; 10 s at a setting of 3) and then centrifuged at 4°C for 10 min at $600 \times g$. The supernatant fluid was brought to a 7% sorbitol concentration with 50% sorbitol and stored at −70°C. This cell-free VZV preparation contained 3.5×10^6 PFU/ml as assayed on HELF cells.

VZV titration. VZV was diluted in viral transport medium, and 0.2 ml was added to confluent monolayers in 35-mm-diameter wells. Viral transport medium used to dilute virus was either Hanks balanced salt solution containing double the amount of antibiotics (HBSS) or veal infusion broth containing 0.5% gelatin and antibiotics (VIB). After the adsorption period, 3 ml of medium was added and the cultures were incubated for 5 days at 37°C in a 6% CO_2–85% humidity atmosphere. Plates were fixed with a 3:1 mixture of 95% methanol and glacial acetic acid and stained with 1% crystal violet. Typical VZV plaques were enumerated with an inverted microscope at 50-fold magnification. Control wells contained 50 to 75 plaques.

Virus titers in clinical specimens were expressed as $\log_{10}$ VZV per milliliter of vesicle fluid. When vesicles were no longer present, swabs of skin lesions were tested only for the presence or absence of VZV.

Patient population and clinical specimens. Immunosuppressed patients with herpes zoster were entered into a double-blind, placebo-controlled trial of a topical antiviral agent within 72 h after the onset of skin lesions. Vesicle fluid was obtained with a calibrated capillary tube and placed in 1.0 ml of cold VIB. This was quickly taken to the laboratory and cultured quantitatively. If vesicles were no longer present, the skin lesions were vigorously rubbed with a cotton

TABLE 1. VZV titer in three fibroblast cell lines

Expt	Cell line	VZV titer (PFU/ml × 10^5)[a]
1	HELF	6.32 ± 0.38
	350Q	0.83 ± 0.38
2	HELF	16.75 ± 0.25
	350Q	2.38 ± 0.81
3	350Q	2.45 ± 0.58
	FS-9	0.35 ± 0.10

[a] Cell cultures were 5 or 6 days old when infected with the VZV inoculum, which previously assayed as 35 × 10^5 PFU/ml on HELF cells. VZV titer is the mean of duplicate or quadruplicate cultures ± standard deviation. The differences in yield between the cell lines in each experiment were significant ($P < 0.01$).

swab moistened with VIB. This was agitated in 1.0 ml of cold VIB for 1 min and rolled against the side of the tube, and 0.3 ml of this specimen was cultured in duplicate on HELF monolayers. Results are reported only for patients who received the placebo.

Other materials. Cotton swabs were obtained from Kenwood. Calcium alginate swabs were obtained from Falcon Plastics (2047). Laboratory coat material was rinsed in 10 exchanges of distilled water and autoclaved. Some samples were filtered by the Swinex System (Millipore Corp.) and a 0.45-μm filter (M. A. Bioproducts).

Statistical methods. Student's *t* test for equal or unequal samples was used to evaluate the difference in yield obtained with various conditions.

RESULTS

Three fibroblast lines were compared for sensitivity to VZV (Table 1). In experiments comparing HELF and 350Q cells, the former were approximately eightfold more sensitive; 350Q cells were more sensitive than FS-9 cells. Although it is not practical to routinely use primary HEK cell cultures for titration of clinical specimens, we did compare HEK cultures with the firbroblast lines in a tube titration experiment (experiment not shown). The titer was 2 × 10^5 PFU/ml with HELF cells but only 10^4 PFU/ml with 350Q cells and 5 × 10^3 PFU/ml with HEK cells. This difference in sensitivity was constant over an observation period of 11 days.

HELF cells were used in subsequent experiments to study parameters influencing recovery of VZV. The age of the cells did not alter their sensitivity over 3 to 19 days. Furthermore, there was no change in sensitivity as the cell passage number increased from 13 to 21. The virus titer was not altered by lowering the incubator temperature from 37 to 35°C. Adsorbing the virus inoculum in a small volume (0.2 ml) for various times, either at room temperature or at 37°C, did not give a higher titer than when adsorption was omitted (Table 2).

Additional experiments evaluated the effect of conventional virus handling procedures on recovery of VZV. For example, significant delay may occur during transport and between the arrival of VZV specimens (vesicle fluid or swabs of skin lesions) in the clinical virology laboratory and their inoculation into tissue culture. The stability of cell culture-derived VZV is shown in Table 3. When the initial VZV titer was high (>10^4 PFU/ml) plaque counts decreased only 25% in transport medium held for as long as 24 h at 4°C, and the titer was relatively stable even at room temperature. Similar results were obtained when either HBSS or VIB was used for transport medium. When the initial virus titer was

TABLE 2. Effect of adsorption time and temperature on VZV titer

Adsorption time (min)[a]	Adsorption temp (°C)	VZV titer (PFU/ml × 10^5)[b]
0	23	0.22 ± 0.02
30	23	0.22 ± 0.02
	37	0.22 ± 0.01
60	23	0.21 ± 0.01
	37	0.24 ± 0.02
120	23	0.17 ± 0.01
	37	0.21 ± 0.01

[a] Time that 0.2 ml of VZV inoculum (in HBSS) was present on drained monolayer culture before adding the final 3 ml of medium. Culture was agitated every 15 min during adsorption.

[b] Mean of quadruplicate cultures ± standard deviation. *P* value is > 0.10 for the difference in titer between the test conditions and the control (0 time).

TABLE 3. Effect of transport time and temperature on the recovery of VZV

Titer of original specimen (PFU/ml)	Transport time (h)[a]	Temp (°C)[b]	VZV recovery (%)
1.2 × 10^4	1	4	100
	1	23	83
	3	4	100
	3	23	75
	6	4	75
	6	23	50
	24	4	75
	24	23	42
0.35 × 10^3–1.9 × 10^3	1	4	100
	1	23	52
	3	4	96
	3	23	60
	6	4	55
	6	23	33
	24	4	40
	24	23	3

[a] The time that VZV inoculum was held in viral transport medium at the temperature indicated.

[b] Temperature at which VZV inoculum was held before titration.

TABLE 4. Other factors influencing VZV recovery

Factor	VZV recovery (%)[a]
Freezing, −20°C, 24 h	<1
Freezing, −70°C, 24 h	>78 ± 14
Cotton swab, 5 min,[b] 4°C	10 ± 4
Alginate swab, 5 min,[b] 4°C	20 ± 5
Swinex filtration[c]	<1

[a] Initial VZV titer was 1.2 × 10^4 PFU/ml. Data shown are the means of two experiments ± standard deviation.

[b] Time that swabs were present in virus-containing transport medium.

[c] Virus-containing transport medium was passed through a Swinex filter before titration.

TABLE 5. Recovery of VZV from environmental surfaces

Surface	% Recovery[a]
Control[b]	100
Laboratory coat (10 cm^2)[c]	0.1 ± 0.2
Third finger[c]	0.3 ± 0.2
Stethoscope[c]	19 ± 4

[a] Data shown are the means of two experiments ± standard deviation.

[b] Control VZV solution was 2.1×10^4 PFU/ml.

[c] These environmental surfaces were inoculated with 0.2 ml of the VZV solution which remained in place for 30 min (solution evaporated). These surfaces were then rinsed with 3 ml of HBSS or VIB, and the total volume was cultured.

lower (10^2 to 10^3 PFU/ml), the quantity of virus decreased faster, especially at room temperature.

VZV did not survive freezing at −20°C, but 78% was recovered after storage at −70°C (Table 4). The presence of a cotton or calcium alginate swab in virus suspensions held for short periods of time (5 to 10 min) on ice reduced the titer approximately fivefold. When virus suspensions were filtered through a 0.45-μm filter, as is sometimes done when microbial contamination is suspected, more than 99% of the infectivity was lost.

The capacity of VZV to persist on environmental surfaces was investigated by placing a known quantity of VZV on clinically relevant surfaces (Table 5). After 30 min at room temperature, a minimum of 0.1 to 0.3% of the inoculum was recovered from the coat material or from a washed finger, but 19% was recovered from the stethoscope.

Optimal VZV recovery procedures were subsequently used during a clinical trial. VZV was isolated on the first attempt from 16 of 21 immunosuppressed patients with herpes zoster (Table 6). All of these patients had a positive culture at some time after multiple culture attempts, and all were seen within 72 h of the onset of skin lesions. Sixty percent of cultures remained positive over the next 72 h of illness. The mean VZV titer in these clinical specimens did not decrease during the first 48 h in which the patients were followed.

The time required to observe a culture before VZV cytopathlic effect is evident is shown in Table 7. Thirty-five percent of the cultures were positive within 5 days; 40% did not become positive until more than 10 days elapsed.

DISCUSSION

The persistence of virus in diseased sites has been used as one parameter for evaluating the efficacy of antiviral agents. With influenza A infections (7, 12) and mucocutaneous HSV infections (4, 8, 9, 14, 16) there has been good correlation

TABLE 6. Recovery of VZV from herpes zoster[a]

Days after first culture	Total cultures	No. of positive cultures (%)	VZV titer ($\log_{10}$ PFU/ml) ± SD[b]
0	21	16 (76)	2.9 ± 1.0 (13)
1	21	13 (62)	2.5 ± 1.3 (11)
2	21	13 (62)	3.3 ± 2.1 (9)
3	7	4 (57)	1.7 ± 1.1 (2)
4	10	3 (30)	1.0 ± 0.0 (2)

[a] Patients were initially cultured within 72 h after onset of herpes zoster.

[b] The numbers in parentheses indicate the number of positive cultures which were cultured in a quantitative fashion.

TABLE 7. Time elapsed before VZV is detected in tissue cultures of clinical specimens

Days after inoculation of culture	Cumulative positive cultures (%)
2	3 (9)
3–5	12 (35)
6–10	20 (60)
11–15	34 (100)

between the rapidity with which virus disappears after therapy and clinical measurements of improvement. To apply quantitative virological techniques to trials of therapy for VZV infection, we sought to optimize methods for recovering this virus. The HELF cell line was the most sensitive of the human fibroblast cell lines tested for recovery of VZV. The passage level (13 to 21 passages) and the age of the cell monolayer (3 to 19 days) did not influence the recovery rate. A period of adsorption before feeding inoculated cultures was not required with this cell line.

We confirmed that frozen specimens should be held at −70°C and not −20°C. The viability of VZV in viral transport medium was a function primarily of temperature and initial titer. The latter variable may reflect losses on the walls of test tubes and pipettes used for transfer. For short periods of time, even at room temperature, more than half of the cell culture-derived VZV initially present was recoverable. This was true for as long as 6 h for virus suspensions kept at 4°C. Additional losses occurred when the virus-containing solutions were exposed to cotton or calcium alginate swabs. Decrease in virus titer in the presence of calcium alginate has been reported for HSV (2, 5) and has been attributed both to physical binding to the swab material and to toxic substances within the swab that inactivate virus and are toxic to tissue culture (B. A. Lauer, H. A. Masters, and M. J. Levin, manuscript in preparation). Thus, VZV specimens should be transported at 4°C. If they must be stored, this can be done safely at 4°C for several hours. Swabs should be removed as soon as possible after agitating them and squeezing them against the side of the tube. Specimens should not be sterilized by using the Swinex filtration system since >99% of virus is lost on the filter.

These observations were made on a laboratory-prepared VZV inoculum, but they should help define practical methods for handling and culturing specimens of VZV. Their application to a clinical trial indicated that 76% of culture-proven cases of herpes zoster in immunosuppressed patients yielded VZV in the 72 h after the onset of skin lesions and that the frequency of culture-positive cases exceeded 50% for an additional 72 h. The mean VZV titer in vesicle fluid was $10^{2.9}$ in initial cultures and remained close to this titer for an additional 48 h.

Of potential importance was the finding that 0.1 to 0.3% of VZV could persist at room temperature for at least 30 min on garments and human skin, and 19% of VZV can be recovered after 30 min on the plastic diaphragm of a stethoscope. It is unclear whether virus can be transmitted from these surfaces to susceptible individuals, but this finding lends support to recommendations for careful hand washing and gowning by hospital staff coming in close contact with infected patients.

ACKNOWLEDGMENTS

We thank Brian A. Lauer for suggestions on the manuscript and Carolyn V. Hudspeth for preparing it. G. Davis and A. C. Segreti helped collect and prepare the clinical data.

This work was supported by grants from the Louis and Sydell Bruckner Memorial Fund and by University of Colorado School of Medicine Biomedical Research Support grant BRS 3779-11. The clinical trial was supported by the Burroughs Wellcome Co.

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