Varicella-Zoster Plaque Assay and Plaque Reduction Neutralization Test by the Immunoperoxidase Technique

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A new plaque assay for the quantitation of varicella-zoster virus and a plaque reduction neutralization test for the determination of neutralizing antibody titer have been developed using the indirect immunoperoxidase technique. As compared with the classical plaque assay using a solid overlay, the test gives earlier results since plaque counting can be performed on day 3 after the inoculation of cell cultures. In six patients with zoster infection, neutralizing antibody titers ranged from 1:20 to 1:40 before the onset of infection and reached high levels (1:320 to 1:5,120) during the convalescent phase of the disease. Complement-fixing (CF) titers were all negative ($<1:8$) in pre-zoster serum samples from the same patients and ranged from 1:128 to 1:2,048 in the convalescent-phase sera. In the two cases in which late serum samples were available, neutralizing antibody titers matched the preillness levels, whereas CF titers dropped to undetectable levels. Neither neutralizing nor CF antibody was detected in two sera from individuals with no history of varicella-zoster infection. No differences in virus titers or neutralizing antibody titers were observed between the immunoperoxidase and the classical plaque assays. The appropriate characterization of reagent specificity is required before routine application of the test.

Varicella-zoster (V-Z) quantitation studies are commonly performed by using the classical plaque assay under a solid overlay. Since V-Z is a strictly cell-associated virus, the plaque assay was initially employed for the measurement of infected cells (13). Subsequently, the test has been successfully used for the quantitation of cell-free V-Z virus obtained by the sonic treatment of infected human amnion or thyroid cells (4, 5) or human embryo fibroblasts (2). Using cell-free virus, a neutralization test was developed (4, 14). However, thus far, the determination of the immune status to V-Z virus and the evaluation of the protective activity of gamma globulin preparations have been hampered by difficulties encountered in the titration of neutralizing antibody.

In this report, the immunoperoxidase (IP; indirect method) technique is shown to be a very sensitive and specific test for the detection of V-Z-infected cell foci (plaques) within 72 h after infection. This test has been used for the quantitation of V-Z virus (plaque assay) and for the determination of neutralizing antibody titer (plaque reduction neutralization test). As compared with the classical plaque assay, results are obtained several days earlier (on day 3 after the inoculation of cell cultures), and no differences in either virus or antibody titers were observed.

MATERIALS AND METHODS

Cell cultures. For specificity controls and for titration of reagents, 75-cm² tissue culture flasks containing WI-38 cells were inoculated with V-Z-infected cells at an approximate ratio of one infected cell to three uninfected cells. After incubation at 35°C for 6 to 8 h, cell cultures were trypsinized and then suspended in Eagle minimal essential medium (MEM) supplemented with 10% fetal calf serum at a concentration of 10⁵ cells/ml. This preparation was inoculated into each of eight wells of a chambered tissue culture slide (Lab-Tek, Naperville, Ill.). After incubation in a CO₂ incubator at 35°C for 3 to 4 days, the slides were washed with phosphate-buffered saline (PBS), fixed in cold acetone, and then stored at -70°C. The same procedure was used for the preparation of human cytomegalovirus (CMV)- and herpes simplex virus (HSV)-infected and uninfected cell culture slides.

For the preparation of cell-free virus, V-Z was propagated in 150-cm² WI-38 tissue culture flasks at passage 22 to 24 (Flow Laboratories, Rockville, Md.). The medium consisted of Eagle MEM supplemented with 2% fetal calf serum.

Virus strains. A reference V-Z strain (Ellen, American Type Culture Collection, Rockville, Md.) was employed for initial experiments. A strain of V-Z (VR 841) isolated in our laboratory from a skin vesicle of a cancer patient with an apparent zoster infection and identified by complement fixation (CF) was routinely used in parallel with the reference strain for further experiments. Titers of two strains are reported below. A reference CMV (AD 169, 10⁵

50% tissue culture infective dose/ml) and HSV (McIntyre, $10^{7.5}$ 50% tissue culture infective dose/ml) strain was used to infect WI-38 cell cultures for specificity controls.

Sera and peroxidase-labeled antibody preparation. After testing several sera by CF, a zoster convalescent serum with a titer of 1:256 to V-Z, but free from CF antibody at a 1:4 dilution against CMV and HSV, was selected. Similarly, a human serum with antibody to CMV (1:256) and a serum with antibody to HSV (1:1,024), but with no detectable antibody to the other two members of the herpes group, were selected, as previously reported (7).

A goat anti-human immunoglobulin G (IgG) antibody was purified and coupled to horseradish peroxidase (type VI, Sigma Chemical Co., St. Louis, Mo.) by the Avrameas technique (1). The procedure for the preparation of the conjugate and its application has been previously described (8).

Controls for specificity and titration of reagents. Using uninfected as well as V-Z-, CMV-, and HSV-infected WI-38 cells, cross-reactivity controls among the three herpes group viruses were performed as reported (7). Moreover, V-Z-infected cells were treated with: (i) V-Z-positive serum absorbed with V-Z-infected cells or cell-free virus and then conjugate; (ii) V-Z-positive serum, unlabeled and then labeled anti-human IgG antibody; (iii) conjugate only; (iv) PBS only in both steps (control for endogenous peroxidase cell activity).

The absorption of V-Z-positive serum was carried out by incubating equal amounts of a 1:10 serum dilution and V-Z-infected cells of undiluted cell-free virus preparation at 37°C for 1 h and at 4°C overnight with gentle shaking. After a final centrifugation at $100,000 \times g$ for 1 h, the serum was stored at -70°C.

The optimal dilution of the conjugate was selected through a checkerboard titration against the V-Z-positive serum as the highest dilution giving 3+ staining of V-Z-infected cells with the highest serum dilution, without producing any background staining of uninfected WI-38 cells. The optimal dilution of V-Z-positive serum to be employed in plaque assays was selected to contain four peroxidase-labeled antibody units. Dilutions of conjugate and V-Z-positive serum were prepared by using PBS.

Indirect IP method for plaque assay and plaque reduction neutralization test. Cell-free virus for use in V-Z plaque assays was prepared as described by Brunell (2), using both strains of V-Z virus. Our strain (VR 841) was used at passage 15 to 20 on WI-38 cell cultures. When the infected WI-38 cell cultures showed 70 to 90% cytopathic effect, the cells from a 150-cm² flask were removed with a rubber policeman and evenly suspended in 1 ml of Hanks balanced salt solution supplemented with 10% sorbitol and 2% fetal calf serum. After sonic treatment for 25 s using 35-W power (Blackstone model BPO, Blackstone Ultrasonics, Inc., Sheffield, Pa.), the virus preparation was clarified by centrifugation at 3,000 rpm for 20 min and then titrated or frozen at -80°C. At different times after freezing, cell-free virus was thawed and titrated using Hanks balanced salt solution supplemented with 2% fetal calf serum

as a diluent. The sonic treatment procedure yielded cell-free virus titrating 3×10^3 to 5×10^3 plaque-forming units (PFU)/ml for Ellen strain and 5×10^4 to 8.5×10^4 PFU/ml for VR 841 strain. These titers did not drop more than 0.5 log₁₀ after 6 months of storage at -80°C.

The plaque assay for virus titration by the indirect IP technique was routinely performed on 25-cm² WI-38 tissue culture flasks 72 h after infection. Cell monolayers were fixed with absolute alcohol for 10 min and then covered with 1 ml of the working dilution of the V-Z-positive serum. Incubation for 30 min at 37°C was followed by three washings of 5 min each with PBS; thereafter, cells were covered with 1 ml of the optimal dilution of the conjugate and incubated for an additional 30 min. Enzymatic activity was detected after three washings with PBS, according to the method of Graham and Karnovsky (11), using diaminobenzidine reagent with 0.01% H₂O₂. After staining for 10 to 15 min and washing three times with PBS, plaques were counted against a white background.

The plaque reduction neutralization test was performed by incubating equal amounts of virus (50 to 200 PFU) and serial twofold dilutions of test serum (inactivated at 56°C for 30 min) at 35°C for 30 min. One milliliter of virus-serum mixture was then inoculated into 25-cm² WI-38 tissue culture flasks. After 30 min of incubation, medium (Eagle MEM supplemented with 2% fetal calf serum) was added. Virus controls (either containing no serum or a known negative serum) as well as test serum (at a 1:5 dilution) and cell (uninfected cell cultures) controls were tested simultaneously. Again, 72 h after inoculation, cell cultures were stained by the IP technique and plaques were counted. A serum dilution giving a plaque reduction of 50% or more was considered positive for neutralizing antibody.

Both virus and antibody titrations were performed in parallel using the classical plaque assay under an agar overlay. This consisted of 0.9% Noble agar in Eagle MEM with 5% fetal calf serum. After incubation for 6 to 9 days at 35°C, a second overlay containing neutral red sufficient to give a final concentration of 1:10,000 was added and plaques were counted on the next day.

CF test. The CF test was performed by the Laboratory Branch CF procedure applied to the microtiter system (6) and employing antigen from V-Z infected MA-184 cells (Microbiological Associates, Bethesda, Md.).

Sera examined. The IP plaque reduction neutralization test was performed on sera from two adults with no history of V-Z infections and on sequential sera from six leukemic patients with clinical zoster, which were taken at different times before and after the onset of infection. All patients had varicella during childhood.

RESULTS

IP staining pattern of V-Z-infected cells. V-Z-infected cells stained by the IP technique showed both nuclear inclusions and diffuse cytoplasmic staining (Fig. 1A). Absorption of V-Z-

positive serum with V-Z-infected WI-38 cells or cell-free virus resulted in the disappearance of nuclear inclusions as well as cytoplasmic staining (Fig. 1B). Foci of 10 to 20 infected cells could be easily detected by gross observation on day 3 after infection when stained by the IP technique. Microscopic examination of nonstained cell cultures on the same day failed to reveal any cytopathic effect. Plaque morphology was mainly circular or linear according to the cell orientation in the monolayer.

Controls for specificity and reagent titration. The specificity of the IP plaque assay was demonstrated by: (i) inability of V-Z-positive serum to stain V-Z-infected cells after absorption with V-Z-infected cells or cell-free virus; (ii) inability of V-Z-positive serum to stain V-Z-infected cells when treatment with labeled anti-human IgG antibody followed previous treatment with unlabeled anti-human IgG antibody; (iii) inability of V-Z-positive serum to stain CMV- and HSV-infected WI-38 cells when used at a 1:16 or greater dilution against a dilution of the conjugate $\geq 1:20$; (iv) inability of

CMV- and HSV-positive serum to stain WI-38 cells infected with V-Z virus when used at a dilution $\geq 1:16$ against the same dilution of the conjugate; (v) inability of V-Z-negative serum to stain V-Z-infected WI-38 cells with a conjugate diluted $\geq 1:20$.

No endogenous peroxidase was detected by the specific histochemical reaction either in infected or uninfected WI-38 cell cultures. In the checkerboard titration nonspecific staining of V-Z-infected cells was observed through a 1:10 dilution of the conjugate, as shown by staining produced by the conjugate alone. The optimal dilution of the conjugate was 1:160. The optimal dilution of V-Z-positive serum was found to be 1:512, and the working dilution for the plaque assay (containing 4 antibody units) was 1:128.

IP plaque assay for V-Z titration. Initial experiments were carried out to determine whether a difference in plaque count could be detected between the IP plaque assay (counting on day 3) and the classical plaque assay under a solid overlay (counting on day 7 to 10). Since no solidifying overlay was used for the IP technique, the appearance of secondary foci could make the technique inapplicable for quantitation purposes. No significant difference in plaque counts was observed between the IP technique (at day 3 or 4) and the classical agar overlay technique at day 7 to 10. A linear relationship between plaque counts and virus dilutions was obtained (Table 1 and Fig. 2) when plaque counting was done within 96 h after infection. After this time, secondary foci began to appear in tissue cultures since no solidifying overlay was used.

IP plaque reduction neutralization test. Some examples of actual plaque counts in neutralization tests performed on two sera from individuals with no history of V-Z infections and six convalescent-phase sera taken from leukemia patients with clinical zoster are reported in Table 2. All six convalescent-phase sera showed a high titer of V-Z neutralizing antibody, ranging from 1:320 to 1:5,120, whereas the

FIG. 1. WI-38 V-Z-infected cells stained by the indirect IP technique (A) before and (B) after absorption of the specific immune serum with V-Z-infected cells. $\times 1,100$.

TABLE 1. V-Z virus plaque assay by the indirect IP technique (reading on day 3) and the classical procedure using an agar overlay (reading on day 7) for V-Z virus titration

Dilution of cell-free virus (VR 841)	IP assay			Agar overlay assay		
	Plaque counts	Mean	PFU/ml	Plaque counts	Mean	PFU/ml
10^{-1}	TMTC ^a			TMTC		
10^{-2}	TMTC			TMTC		
10^{-3}	65, 77, 73, 69	71	7.1×10^4	65, 70, 76, 61	68	6.8×10^4
10^{-4}	4, 6, 6, 8	6	6.0×10^4	8, 5, 6, 7	6.5	6.5×10^4
10^{-5}	0, 0, 0, 0	0		0, 0, 0, 0	0	

^a TMTC, Too many to count.

FIG. 2. Plaques stained by the indirect IP technique 3 days after inoculation of WI-38 cells with cell-free V-Z virus (VR 841). (Top) Dilution of 10^{-4} ; (Middle) dilution of 10^{-3} ; (Bottom) dilution of 10^{-2} . $\times 0.9$.

TABLE 2. Results of the plaque reduction neutralization test by the indirect IP technique on six convalescent-phase sera from patients with zoster infection and two sera from individuals with no history of V-Z infections

Serum	Plaque counts/serum dilution ^a												Virus control ^b	50% Titer
	5	10	20	40	80	160	320	640	1,280	2,560	5,120	10,240		
P.G.	0	0	0	0	0	9	27	45	60	83	91	90	90	640
W.R.	0	0	0	0	0	0	0	7	27	67	86	92	90	1,280
O.H.	0	0	0	0	0	0	0	9	5	35	48	60	65	1,280
D.P.	0	0	0	0	0	0	0	0	9	15	29	45	65	5,120
L.L.	0	0	0	0	0	16	62	115	163	178	190	182	184	320
H.H.	0	0	0	0	0	14	58	104	152	175	186	193	184	320
S.P.	91	95											90	<5
G.H.	89	94											90	<5

^a Reciprocal of serum dilutions.

^b Each number is the average of the plaque counts on two different tissue culture flasks.

two sera from individuals with no past clinical V-Z infections did not show any detectable neutralizing antibody.

Sequential sera taken from the same six zoster patients both before and after the onset of infection were available for testing by the IP

neutralization and CF tests. Results are shown in Table 3. All patients showed titers of 1:20 to 1:40 to V-Z virus by the IP neutralization test before the clinical appearance of zoster and a fourfold or greater increase in titer within 1 or 2 weeks after the onset of infection. Titers ranged

TABLE 3. *Determination of V-Z antibody titer by the indirect IP plaque reduction neutralization (Nt) and the CF test on sera taken at different times, with respect to the onset of infection, from six leukemic patients with zoster*

Patient ^a	Time after onset of infection	Titer ^b by	
		Nt	CF
P.G. (35)	11 months before	40	<8
	1 day	40	<8
	4 days	80	8
	2 weeks	320	64
	3 weeks	640	128
W.R. (42)	5 months before	40	<8
	1 week	1,280	256
	2 weeks	2,560	256
	3 weeks	2,560	256
O.H. (53)	0 weeks before	40	<8
	1 week	2,560	1,024
	2 weeks	10,240	1,024
	3 weeks	2,560	2,048
D.P. (27)	2 weeks before	20	<8
	1 week	5,120	1,024
	3 weeks	2,560	1,024
L.L. (28)	7 months before	20	<8
	2 weeks	320	1,024
	45 weeks	20	<8
H.H. (44)	18 months before	20	<8
	2 weeks	320	512
	16 weeks	40	<8

^a Numbers in parentheses indicate the patient's age in years.

^b Titers are expressed as the reciprocal of the serum dilution.

from 1:320 to 1:10,240 during the convalescent phase of the disease. CF antibody usually increased in parallel with neutralizing antibody, but within a year dropped to the same undetectable levels found before the onset of the disease (cases L.L. and H.H.).

Serum antibody titrations were done in parallel using the plaque reduction neutralization assay method with a solid overlay. No differences in titer greater than a twofold dilution were found. Moreover, no differences in serum antibody titer were found using the Ellen strain or our V-Z strain (VR 841) within a range of 50 to 200 PFU.

DISCUSSION

The IP technique described herein is able to detect single V-Z-infected cells 24 h after infection and makes plaques visible as brown spots 0.5 mm in diameter as early as 40 h after the infection of cell cultures. Plaques 1 to 2 mm in diameter may be counted easily after an incubation period of 72 h, when cytopathic effect is not usually detectable by microscopic observation, but V-Z-infected cell foci are darkly stained by the IP technique. This is in contrast to the classical V-Z plaque assay using a solid

overlay, in which plaques produced by destruction of infected cells are detected only after a minimum incubation time of 7 to 8 days.

The staining specificity of V-Z-infected cells has been demonstrated by the disappearance of both nuclear and cytoplasmic staining after absorption of the specific V-Z immune serum with V-Z-infected cells or cell-free virus and by the lack of cross-reactions with human CMV and HSV when V-Z-positive serum and conjugate were used at appropriate dilutions.

Recently, the plaque reduction neutralization test using a solid overlay has been made more sensitive for detecting antibody from past V-Z infections by adding complement and determining the titer of complement-requiring neutralizing antibody (15). Results of the present study confirm the persistence of neutralizing antibody for several years after the primary V-Z infection (17, 18) and the short-lived nature of CF antibody. It reported that CF, as well as fluorescent antibody to V-Z virus, may fall below assayable levels 6 to 12 months after chicken pox (3). Nevertheless, fluorescent antibody to V-Z membrane antigens (10, 20) as well as immune adherence hemagglutinating antibody (9) have recently been reported as valid indicators of immune status to V-Z virus. The correlation between V-Z neutralizing antibody and these two different types of V-Z antibody needs to be established.

The main advantage of the IP plaque assay over classical V-Z virus plaque assays is that of obtaining results earlier. Moreover, no solidifying medium is required. However, a disadvantage of the technique lies in the use of a human serum, which must be selected to contain antibodies only to V-Z virus and not to other human herpesviruses. Several data suggest that V-Z virus and HSV share common antigens (12, 14, 16, 19). Moreover, a good quality, high-titer peroxidase-labeled anti-human IgG serum is required to avoid problems of nonspecific staining resulting from the use of a low working dilution of the conjugate.

In conclusion, the determination of the neutralizing antibody titer by the IP plaque assay represents a valid improvement over methods presently used for measuring neutralizing antibodies to V-Z virus, as far as rapidity of results is concerned.

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