Variables Affecting Viral Plaque Formation in Microculture Plaque Assays Using Homologous Antibody in a Liquid Overlay

A. S. RANDHAWA,* G. J. STANTON, J. A. GREEN, AND S. BARON

Department of Microbiology, The University of Texas Medical Branch, Galveston, Texas 77550, and Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014*

Received for publication 6 January 1977

A liquid antibody microculture plaque assay and the variables that govern its effectiveness are described. The assay is based on the principle that low concentrations of homologous antibody can inhibit secondary plaque formation without inhibiting formation of primary plaques. Thus, clear plaques that followed a linear dose response were produced. The assay was found to be more rapid, less cumbersome, and less expensive than assays using agar overlays and larger tissue culture plates. It was reproducible, quantitative, and had about the same sensitivity as the agar overlay technique in measuring infectious coxsackievirus type B-3. It was more sensitive in assaying adenovirus type 3 and Western equine encephalomyelitis, vesicular stomatitis, Semliki forest, Sendai, Sindbis, and Newcastle disease viruses than were liquid, carboxymethylcellulose, and methylcellulose microculture plaque assays. The variables influencing sensitivity and accuracy, as determined by using coxsackievirus type B-3, were: (i) the inoculum volume of virus; (ii) the incubation period of virus; and (iii) the incubation temperature.

Simple, rapid, accurate, reproducible, and economical microtechniques for viral assays are needed. Viral assays based on plaque formation under semisolid overlays are considered more accurate and reproducible than those depending on the development of cytopathic effect (CPE). In the past, semisolid and liquid overlays have been commonly used in the forms of agar, agarose (6-9, 13, 14, 15), methylcellulose (8, 11, 15, 21, 24, 29), carboxymethylcellulose (9, 23), liquid medium (1, 4, 5, 17-20, 26, 28, 30, 31), and starch (8, 10) in semimicro- and microassays for different viruses.

Some viruses may be plaque-assayed under liquid overlay because of: (i) predominance of direct cell-to-cell spread of virus (herpesviruses and poxviruses); (ii) early plaque formation before spread of virus to form secondary plaques (1, 18); (iii) selected conditions such as virus type, cell type, and temperature of incubation (12); and (iv) the localizing effect of homologous antiserum (1, 16, 23, 28). The specific identification of multicellular foci of infection under liquid overlay containing homologous fluorescent antibody also has been accomplished for vaccinia (5), hog cholera (16), and measles (21) viruses. Inherently simple liquid overlay techniques have not been routinely used, however.

In this investigation particular attention was given to improving conditions for a simple, liquid antibody overlay plaquing technique that would be applicable for rapid, accurate, sensitive, and quantitative assays of a variety of viruses in a microculture system.

MATERIALS AND METHODS

Viruses. Coxsackievirus type B-3, Nancy strain (Cox B-3), was obtained from M. W. Rytel, University of Wisconsin, and passed twice through the hearts of mice *in vivo*. Viruses other than Cox B-3 were obtained from the American Type Culture Collection. Virus suspensions were prepared by standard techniques from infected cell cultures or embryonated eggs and stored frozen at -70°C . Adenovirus type 3 and Semliki forest (SFV), Sendai, and Newcastle disease viruses were passed twice in HEp-2 (human epidermoid carcinoma) cells before use. Chicken embryo cell pools of Western equine encephalomyelitis, Sindbis (SIN), and vesicular stomatitis (VSV) viruses, as well as mouse L cell pools of encephalomyocarditis virus, were used directly.

Cell culture. Most experiments were performed in HEp-2 cells propagated to confluency in 96-well micro-tissue culture plates (no. 3040, Falcon Plastics) and maintained at 37°C , using the media described below and a humidified incubator with a 5% CO_2 atmosphere. RK-13 rabbit cells and mouse L cells were propagated similarly.

Culture media. Growth medium consisted of Dulbecco's modified Eagle medium (Grand Island Biological Co., Grand Island, N.Y.) containing 10% fetal bovine serum and antibiotics (streptomycin, 100 mg/ml; penicillin, 100 U/ml; and Mycostatin, 5 μ g/ml). For maintenance medium, the fetal bovine serum in the growth medium was reduced to 2%. Serial dilutions of stock viruses were made in maintenance medium.

Agar overlay media. A sterile 2% solution of agar in distilled water was prepared from purified agar (Difco Laboratories, Detroit, Mich.) repeatedly washed with saline (0.85% NaCl). The final concentration of agar was 1% and was prepared by mixing equal volumes of melted agar and double-strength maintenance medium at 45°C. The secondary overlay consisted of 1% agar overlay medium containing 1/10,000 (wt/vol) neutral red.

MC overlay medium. The methylcellulose (MC) overlay medium was prepared as described by Schulze and Schlesinger (24). A final concentration of 0.5% MC (Methocel, 4,000 CP, Fisher Scientific Co., N.J.) in maintenance medium was used as the overlay medium.

CMC overlay medium. The carboxymethylcellulose (CMC) overlay medium was prepared in the same manner as that for MC. The final concentration of CMC (Nutritional Biochemicals Corp., Cleveland, Ohio) in the overlay medium was also 0.5%.

Liquid overlay medium. Maintenance medium, without specific antiserum, was used for the liquid overlay.

Antibody overlay medium. Specific hyperimmune sera for use in antibody overlay media for Cox B-3 (bovine), Western equine encephalomyelitis (mouse), and Newcastle disease (guinea pig) viruses were obtained from the National Institute of Allergy and Infectious Diseases, Research Reference Reagents Branch, whereas hyperimmune sera to adenovirus type 3 and VSV, SIN, SFV, and Sendai viruses were prepared in rabbits by standard methods. The antiserum was applied in maintenance medium for the antibody overlay.

Crystal violet stain. All cultures, except those under agar overlay, were stained for plaque counting with crystal violet 1:100 (wt/vol) in 20% methyl alcohol in water.

RESULTS

Selection of optimal concentration of antibody for plaque formation under liquid overlay. The minimal concentration of antibody for maximum production of discrete plaques was determined by an optimal proportion titration ("grid" titration), using one microtiter plate. In this test 25 μ l each of 0.5 log₁₀ dilutions of stock virus suspension were inoculated into eight replicates of microtiter culture wells. The cultures were incubated for 2 h at 37°C, and the inoculum was removed. Amounts of 0.1 ml of serial 0.5 log₁₀ dilutions of antibody were then applied in duplicate in the opposite direction on

the microtiter plate. After a 48-h incubation period, the overlay medium was removed, and the cell sheets were stained with crystal violet. The highest dilution of antibody that resulted in production of discrete plaques and an obviously linear plaque dose response was then selected for use in future assays. In practice, hyperimmune serum prepared in rabbits in the laboratory could be diluted to from 1/100 to 1/10,000, depending on the potency of the antiserum.

Effects of volume of virus and incubation temperature on plaque formation. To determine the effects of virus volume and incubation temperature on plaque formation, a standard suspension of Cox B-3 was inoculated in varying volumes (10 to 100 μ l) onto microtiter cultures of HEp-2 cells. Virus was adsorbed for 2 h at 37°C and removed, and 0.1 ml of antibody overlay medium was added to each culture. The microcultures were then incubated at 30, 33, 35, 37, 38, or 40°C. The results are shown in Fig. 1. More significant increases in virus titers were routinely observed at 38°C. No plaques were formed at 30 (not shown) or 40°C. The highest titers of virus were consistently found using 10 or 25 μ l of virus inoculum.

Adsorption period and plaque formation. The effect of adsorption period in relation to the volume of inoculum on Cox B-3 plaque formation in microplate cultures was determined by inoculating different volumes (10, 25, 50, or 100 μ l) of Cox B-3 on HEp-2 cultures and adsorbing for 1, 2, 3, or 4 h at 37°C under 5% CO₂. An overlay containing a 1:4,000 dilution of stock antiserum as determined above was applied, and the plates were incubated for 48 h at 37°C. The plaques were counted after staining. The titer of the virus was calculated and graphed as

FIG. 1. Effect of temperature of incubation and volume of inoculum on Cox B-3 plaque formation in microculture plates. λ = μ l.

plaque-forming units (PFU) per milliliter versus time (Fig. 2). The highest virus titer was obtained using a 2-h adsorption period when the volume of the inoculum used was 10 or 25 μ l.

Effect of different overlay media on time of plaque formation by Cox B-3. An inoculum of 25 μ l of a $10^{-5.5}$ dilution of Cox B-3 was adsorbed on HEp-2 cultures for 2 h at 37°C under 5% CO₂ tension. Four wells of microplate cultures were used for each type of overlay medium. Supernatant fluids were removed, and overlays of antibody, liquid, MC, or CMC were applied. The cultures were incubated for a 20-, 24-, 40-, or 48-h period before staining and counting.

Plaques of Cox B-3 were not observed up to 20 h postinfection under any overlays (Fig. 3). At 24 h, a mean of 12 plaques per culture was observed under the antibody overlay and 17 plaques per culture under the liquid overlay,

FIG. 2. Effect of volume of inoculum and time of adsorption on plaque formation of Cox B-3 in microculture plates. $\lambda = \mu$ l.

FIG. 3. Effect of overlays of liquid, antibody, CMC, and MC on time of development of countable plaques in microculture plates. $\lambda = \mu$ l.

but no plaques were seen under CMC or MC. At 40 h postinfection, cultures overlaid with antibody medium showed 16 plaques, whereas those overlaid with liquid medium showed confluent lysis. No plaques were detected under CMC or MC. Forty-eight-hour cultures overlaid with antibody, CMC, and MC showed mean plaque counts of 17, 10, and 3, respectively. Thus, Cox B-3 plaques could be counted a day earlier and were higher in number under antibody overlay medium than under MC or CMC. In addition, the mean number of plaques formed under antibody overlay medium remained relatively unchanged from 24 to 48 h. In summary, plaques formed under antibody overlay appeared rapidly, persisted for a convenient length of time, and were higher in number than those formed under CMC or MC (Fig. 4 and 5).

In other virus-cell systems the rapidity of plaque formation did not always parallel the above results. For example, plaques of VSV on mouse L cells appeared earlier (20 h) under MC than did those under antibody overlay (40 h). In RK-13 cells infected with VSV and overlaid with antibody, microplaques developed at 24 h, but no comparison was made with other overlays. Using encephalomyocarditis virus in mouse L cells overlaid with antibody, plaques first developed at 48 h.

Cox B-3 plaquing under agar overlay. To determine the plaquing efficiency of Cox B-3 in a macro-agar method and compare it to the efficiencies obtained in microculture assays, Cox B-3 was plaqued on monolayer cultures of HEp-2 cells by the standard agar overlay plaquing technique in shell tissue culture vials (2), using 1 ml of agar overlay medium, followed by 1 ml of the second agar overlay medium at 24 h postinfection. The titer of the virus determined by plaque counts 24 h later was $10^{8.5}$ PFU/ml. The agar plaquing method was more sensitive and reproducible than MC, CMC, and liquid overlay microculture plaque assays, whereas it was as sensitive as the antibody overlay and CPE techniques. In addition to being cumbersome, the agar overlay plaquing method took 24 h longer for its completion than the antibody overlay technique.

Relationship between plaque count and virus dilution. Replicate cultures were inoculated with 25 μ l of serial 0.5 log₁₀ dilutions of Cox B-3. After 2 h of adsorption, the fluid was removed, and 0.1 ml of either antibody overlay or liquid overlay was applied. Plaques were stained and read 48 h postinoculation. The plaque count was plotted against the dilution to determine whether there was a linear dose response (Fig. 6).

A linear response was observed when anti-

FIG. 4. Assay (48 h postinfection) of Cox B-3, using MC, CMC, antibody (Ab B-3), and liquid (medium) overlays. Absence of staining indicates CPE due to Cox B-3. From left to right as the virus dilution increases, a complete CPE, incomplete CPE, and then individual plaques are seen. The last two rows on the right side are cell controls (CC). B-3 line shows 0.5 log₁₀ dilutions of Cox B-3.

FIG. 5. Assay (72 h postinfection) of Cox B-3, using MC, CMC, antibody (Ab B-3), and liquid (medium) overlays. Absence of staining indicates CPE due to Cox B-3. From left to right as the virus dilution increases, a complete CPE, incomplete CPE, and then individual plaques are seen. The last two rows on the right side are cell controls (CC). B-3 line shows 0.5 log₁₀ dilutions of Cox B-3.

body overlay was used (Fig. 6). There was no linear response when liquid overlay was applied, indicating that secondary plaques had formed at 48 h in the absence of antibody.

Effect of antibody overlay on virus content of liquid overlay. To determine the relative ability of the antibody overlay to inhibit secondary plaque formation, fluids from four cultures infected with Cox B-3 and overlaid with either liquid or antibody overlay medium were harvested and pooled at 24 and 48 h postinfection. Virus content of the pools was determined by the antibody overlay technique. The quantity of virus detectable in antibody-containing fluids harvested at 24 and 48 h postinfection was $10^{3.3}$ and $10^{5.8}$ PFU/ml, respectively, whereas the concentration of virus in the liquid overlay medium where secondary plaques developed was $10^{5.6}$ and $10^{6.7}$ PFU/ml, respectively, at 24 and 48 h postinfection. Thus, the retardation of secondary plaque formation under antibody overlay was correlated with decreased virus titers in the supernatant of this overlay.

Comparison of CPE ($TCID_{50}$) and plaque (PFU) assays. The CPE end point and plaque assays were done on monolayers of HEP-2 cells in microplates. For CPE assays, 0.1 ml of serial $\log_{10}$ virus dilutions of each virus were inoculated into eight replicate wells. The microplates were then incubated at 37°C. The CPE was read after 120 and/or 168 h of incubation and the mean tissue culture infective dose ($TCID_{50}$) per milliliter was calculated using the Karber method (22). For plaque assays, cultures were inoculated with 25 μ l of serial $\log_{10}$ dilutions of

each virus. The supernatant medium was removed after 2 h of adsorption, and the cultures were overlaid with 0.1 ml of antibody or liquid overlay medium, or with 0.05 ml of MC or CMC overlay medium. This 0.05-ml volume of semi-solid overlay was determined to be optimal in preliminary experiments. The microcultures were incubated for 48 h at 37°C. The plates containing MC and CMC were then kept for 1 h at 4°C for liquefaction before washing off the overlays with cold phosphate-buffered saline (pH 7.2) and staining with crystal violet. Cultures overlaid with antibody or liquid medium were stained at 48 h. Plaques were read, and the titers were expressed as $\log_{10}$ PFU per milliliter. The postinfection incubation period used was 5 or 7 days for the CPE technique, whereas it was only 2 days in all the overlay assays except agar (3 days). Mean comparative titers of Cox B-3 (Table 1) as determined by using the CPE, liquid, antibody, agar (not shown), CMC, and MC overlays were 8.9, $\approx$ 8.7, 8.4, 8.5, 7.7, and $\approx$ 6.5, respectively.

To determine the applicability of the antibody overlay method for plaquing other cytopathic viruses, the relative titers of adenovirus type 3, Western equine encephalomyelitis, VSV, SFV, Sendai, SIN, and Newcastle disease viruses were determined by a 5-day CPE assay and by a 2-day microculture plaque assay, using antibody, liquid, CMC, and MC overlays. The results (Table 1) show that the $TCID_{50}$ method was equally or more sensitive for assaying the indicated viruses than any of the other assays. However, the $TCID_{50}$ assay required 3 additional days for completion. The liquid antibody assay was as sensitive as the $TCID_{50}$ assay for Cox B-3, SFV, Sendai, SIN, and Newcastle disease virus determinations. The liquid antibody assay was more sensitive in plaque-assaying Cox B-3, adenovirus type 3, Western equine encephalomyelitis, SFV, and Sendai viruses than the CMC method and was as sensitive for assaying SIN. All the viruses indicated were only approximately assayable by the liquid overlay and MC plaque techniques at 48 h because of the formation of secondary and/or non-discrete plaques. This became more evident at 72 h postinfection (Fig. 5).

DISCUSSION

The present findings identify conditions required for the assay of a number of viruses by a microculture plaque technique using a simple overlay of homologous antiviral antibody in liquid medium. The technique was first evaluated using Cox B-3 and HEP-2 cells. The conditions required to optimize the assay for Cox B-3 were

FIG. 6. Relationship between plaque count at 48 h and dilution of Cox B-3 inoculum.

TABLE 1. Comparative titers of various viruses, using different microculture assays in HEP-2 cells

Virus	Titer (log ₁₀ /ml)				
	TCID ₅₀ (day 5 or 7 postinoculum)	PFU (day 2 postinoculum)			
		Liquid overlay (=) ^a	Antibody overlay	CMC overlay	MC overlay (=) ^a
Cox type B-3	8.9	8.7	8.4	7.7	6.5
Adenovirus type 3	9.8	8.5	8.3	7.2	7.2
Western equine encephalomyelitis	5.8	6.5	5.3	4.9	4.6
VSV	5.4	4.5	4.2	4.8	4.3
SFV	7.0	5.5	6.9	5.2	4.6
Sendai	8.3	8.5	8.2	7.1	6.6
SIN	5.8	5.8	5.9	5.6	4.9
Newcastle disease	7.9	6.6	7.8	6.2	5.5

^a (=), Approximate titers. In the case of liquid overlay, secondary plaque formation was present, whereas under MC, plaques were indistinct and difficult to enumerate.

(i) a virus inoculation volume of 25 μ l or less, (ii) a virus adsorption time of 2 h before application of the antibody overlay, and (iii) an incubation temperature of 37 to 38°C.

The requirement for a small volume of viral inoculum to maximize sensitivity of the assay is probably due to the requirement for intimate contact between virus and cells to insure rapid adsorption and penetration of virus. The requirement for a 2-h adsorption time is interpreted as the time required for virus to penetrate cells and thereby become inaccessible to the action of antibody in the overlay.

A linear relationship was demonstrated between the plaque count and dilution of Cox B-3 in the liquid antibody overlay system up to 48 h, indicating that the plaque count was directly proportional to the concentration of virus. When liquid overlay without antibody was used, the relationship was not linear at 48 h, suggesting that secondary plaques were formed by that time. Substantially more virus was present up to 48 h in the liquid overlay lacking antibody compared to that containing antibody, and no secondary plaques appeared in the latter, suggesting that the low concentration of antibody in the overlay retarded secondary plaques by decreasing the amount of virus. A linear dose response has been reported previously for two picornaviruses overlaid without antibody in the liquid medium (1, 18). In these systems the plaques had to be read very early, sometimes microscopically, before secondary plaques developed. The same situation appears to occur with VSV in WISH cells at 37°C (12). However, at 33°C in a WISH-cell system there is retardation of secondary plaque formation, which allows a prolonged time period for reading plaques.

The usefulness of liquid antibody overlay for

assaying hog cholera virus (16) has been reported, but the conditions for optimizing the assay were not determined (16). Additional viruses that we could assay by the present technique were adenovirus type 3, Western equine encephalomyelitis, VSV, SFV, Sendai, SIN, and Newcastle disease viruses.

A possible disadvantage of the present technique is the need for antiserum to be used in the overlay. Most virus laboratories, however, should be able to make or secure hyperimmune serum for this purpose. Once prepared, such a serum may be used for an extremely large number of assays. For example, 50 ml of a hyperimmune serum that can be diluted 1:500 for optimal plaquing (as determined by box titration) provides 25,000 ml of overlay medium for assays. Since less than 2 ml of overlay is required for one complete microculture plaque assay (0.1 ml per microtiter well), such a serum pool could be used for 12,500 virus assays.

Another possible disadvantage of this microculture plaque system is that no more than 50 plaques can be accurately read per well. Thus, assays requiring extremely high precision would require additional replicates for the enumeration of more plaques.

The safety hazard of decanting viral inocula after adsorption should be recognized, and, instead, supernatant fluids should be aspirated into a closed decontamination system.

The small size of the plaques has not caused a counting problem, since the plaque size has been adequate with most viruses. The use of a hand lens or a low-powered dissecting microscope permits the counting of extremely small plaques if they occur. Most plaques reached countable size under liquid antibody overlay by 1 to 2 days. Sensitivity to virus has also not been a problem, since virus titers under liquid

antibody overlay were equal to or greater than those under semisolid overlays. However, the CPE end point assay (TCID₅₀) for Cox B-3 was slightly more sensitive to virus than were the assays under liquid antibody or semisolid overlays. The CPE assay is not quantitative, however, and therefore is less accurate than plaque assays when the same number of cultures are used, and it requires a much longer time for completion.

The present technique may be applicable in a number of laboratories due to its several advantages. These advantages include: (i) simplification due to elimination of semisolid overlay; (ii) rapidity of the assay, perhaps due to the often increased rate of virus replication and plaque formation under conditions of higher oxygenation (3); (iii) the absence of agar or other inhibitors of virus (24, 27); (iv) conservation of materials in terms of indicator cells, media, culture plates, and incubator space; and (v) applicability to a wide range of cytolytic viruses.

It is likely that this technique may also be useful for assays of interferon, antibody, and antiviral compounds. The technique also lends itself to experimental manipulation during plaque formation, unlike systems using semisolid overlays. These possibilities are under study.

ACKNOWLEDGMENTS

A.S.R. was a recipient of a postdoctoral fellowship from the James W. McLaughlin Fellowship Fund, Galveston, Tex. This work was supported by a James W. McLaughlin award for research in infection and immunity and, in part, by grants from the Public Health Service (RR 05427, Division of Research Resources) and the Department of Health, Education, and Welfare (S00170).

LITERATURE CITED

1. Baker, D. A., and L. A. Glasgow. 1969. Rapid plaque assay for encephalomyocarditis virus. *Appl. Microbiol.* 18:932-934.
2. Baron, S., C. E. Buckler, and K. K. Takemoto. 1966. A rack permitting efficient handling of tissue cultures. *Appl. Microbiol.* 14:1042-1043.
3. Baron, S., J. S. Porterfield, and A. Isaacs. 1961. The influence of oxygenation on virus growth. I. Effect on plaque formation by different viruses. *Virology* 14:444-449.
4. Black, F. L., and J. L. Melnick. 1955. Micro-epidemiology of poliomyelitis and herpes-B infections. *J. Immunol.* 74:236-242.
5. Carter, G. B. 1965. The rapid detection, titration, and differentiation of variola and vaccinia viruses by a fluorescent antibody-coverslip cell monolayer system. *Virology* 25:659-662.
6. Coates, H. V., D. W. Alling, and R. M. Chanock. 1966. An antigenic analysis of respiratory syncytial virus isolates by a plaque reduction neutralization test. *Am. J. Epidemiol.* 83:299-313.
7. Cooper, P. D. 1955. A method for producing plaques in agar suspension of animal cells. *Virology* 1:397-401.
8. Daniel, M. D., H. Rabin, H. H. Barahona, and L. V. Melendex. 1971. Herpes virus Saimiri. III. Plaque formation under multi agar, methyl cellulose and starch overlays. *Proc. Soc. Exp. Biol. Med.* 136:1192-1196.
9. DeMadrid, A. T., and J. S. Porterfield. 1969. A simple microculture method for the study of group B arboviruses. *Bull. W.H.O.* 40:113-121.
10. DeMaeyer, E., and E. Schonne. 1964. Starch gel as an overlay for the plaque assay of animal viruses. *Virology* 24:13-18.
11. Dolan, T. M., J. D. Fenters, P. A. Fordyce, and J. C. Holper. 1968. Rhinovirus plaque formation in WI-38 cells with methylcellulose overlay. *Appl. Microbiol.* 16:1331-1336.
12. Green, J. A., G. J. Stanton, J. Goode, and S. Baron. 1976. Vesicular stomatitis virus plaque production in monolayer cultures with liquid overlay medium: description and adaptation to a one-day, human interferon-plaque reduction assay. *J. Clin. Microbiol.* 4:479-485.
13. Henderson, J. R., and R. M. Taylor. 1960. Propagation of certain arthropod-borne viruses in avian and primate cell cultures. *J. Immunol.* 84:590-598.
14. Holland, J. J., and L. C. McLaren. 1959. Improved method for staining cell monolayers for virus plaque counts. *J. Bacteriol.* 78:596-597.
15. Hotchin, J. E. 1955. Use of methylcellulose gel as a substitute for agar in tissue culture overlays. *Nature (London)* 175:352.
16. Mengeling, W. L. 1968. A fluorescent micro-plaque assay for hog cholera virus: localization of infection with homologous antiserum. *Arch. Gesamte Virusforsch.* 23:27-39.
17. Noyes, W. P. 1953. A simple technique for demonstrating plaque formation with virus of vaccinia. *Proc. Soc. Exp. Biol. Med.* 83:426-429.
18. Parsons, P., and D. A. J. Tyrrell. 1961. A plaque method for assaying some viruses isolated from common colds. *Nature (London)* 189:640-642.
19. Plowright, W. 1962. Rinderpest virus. *Ann. N.Y. Acad. Sci.* 101:548-563.
20. Postlewaite, R. 1960. A plaque technique for the titration of vaccinia virus in chick embryo cells and some features of vaccinia infection in this system. *Virology* 10:466-482.
21. Rapp, F., S. J. Seligman, L. Jaross, and I. Gordon. 1959. Quantitative determination of infectious units of measles virus by counts of immunofluorescent foci. *Proc. Exp. Biol. Med.* 101:289-294.
22. Rhodes, A. J., and C. E. Van Rooyen. 1968. Textbook of virology, 5th ed., p. 118-120. The Williams & Wilkins Co., Baltimore.
23. Russell, W. C. 1962. A sensitive and precise plaque assay for Herpes virus. *Nature (London)* 195:1028-1029.
24. Schulze, I. T., and R. W. Schlesinger. 1963. Plaque assay of Dengue and other group B arthropod-borne viruses under methyl cellulose overlay media. *Virology* 19:40-48.
25. Scott, J. F. McN., D. L. McLeod, and J. A. Tokumaru. 1961. A biologic comparison of two strains of Herpesvirus hominis. *J. Immunol.* 86:1-12.
26. Sommerville, R. G. 1959. A microplaque method for counting enterovirus particles. *Virology* 9:701-702.
27. Takemoto, K. K., and H. Liebhaver. 1961. Virus-polysaccharide interactions. I. An agar polysaccharide determining plaque morphology of EMC virus. *Virology* 14:456-462.
28. Tokumaru, T. 1969. Herpesviruses, p. 663. In E. H. Lennette and N. J. Schmidt (ed.), *Diagnostic procedures for viral and rickettsial infections*, 4th ed. American Public Health Association, New York.
29. Tytell, A. A., and R. E. Newman. 1963. A medium free of agar, serum and peptone for plaque assay of Herpes simplex virus. *Proc. Soc. Exp. Biol. Med.* 113:343-346.

30. Wheeler, C. E. 1960. Further studies on the effect of neutralizing antibody upon the course of herpes simplex infections in tissue culture. *J. Immunol.* 84:392-403.
31. Wheelock, E. F., and I. Tamm. 1961. Enumeration of cell infecting particles of Newcastle disease virus by the fluorescent antibody technique. *J. Exp. Med.* 113:301-316.