A Plaque Assay for Respiratory Syncytial Virus. (28111)

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The plaque assay first described for animal viruses by Dulbecco(1), and since applied to a large number of viral agents(2), has the advantage of greater accuracy than is obtainable by endpoint dilution methods. It also provides a highly reproducible and sensitive method for quantitation of specific neutralizing antibody. The development of such a method for respiratory syncytial virus seemed of importance because of recent interest in the problem of differentiating the consequences of reinfection in humans from those of primary infection with this agent(3-6). The present communication describes a plaque assay for respiratory syncytial (RS) virus in a continuous human epithelial cell line, HEp-2.

Materials and methods. Virus. The virus used was the Long strain of respiratory syncytial virus, first isolated from the throat of a patient with bronchopneumonia(7). It had been passed 6 times in tissue culture of KB cells, twice in Chang liver epithelium and once in HEp-2 cells. Virus pools used in all experiments were obtained after either one or two further passages in HEp-2 cells.

Tissue culture. HEp-2 cells derived from a strain initially obtained from Microbiological Associates, Bethesda, Md., were grown as monolayers in 32 oz medicine bottles. The cells were grown for 6 consecutive passages in Eagle's minimum essential medium (MEM) (8) in Hanks' salt solution containing 10% unheated calf serum, 0.01 mg/ml Achromysin® and 0.1 mg/ml Kanamycin®. Thereafter they were subcultured without antibiotics. Tests for presence of pleuropneumonia-like organisms(9) in the cells were repeatedly negative when carried out both aerobically and in an atmosphere of 5% CO_2 and 95% nitrogen.

The cells were removed from the glass by treatment with a solution containing 0.2 mg/ml Versene in a phosphate buffer(10) at 37°C for 15-30 minutes. The number of viable cells was determined by counting in a haemocytometer those cells which failed to stain with 1% trypan blue. A total of 1.2×10^6 cells suspended in 5 ml of Eagle's MEM in Earle's salt solution, containing 10% calf serum, .02 M glutamine, 250 units/ml penicillin and 0.25 mg/ml streptomycin were dispensed into 60 mm glass Petri dishes with a Cornwall automatic syringe fitted with an 18 gauge needle. Following inoculation, the dishes were incubated for 36-48 hours at 36.5°C in a humidified chamber filled with 5% carbon dioxide in air, by which time confluent monolayers were regularly observed. Prior to use, the growth medium was drawn off and the confluent cell sheets washed once in Hanks' balanced salt solution and once with MEM containing 5% heat inactivated chicken serum.

Adsorption of virus. Virus dilutions were prepared in Eagle's MEM containing 5% inactivated chicken serum. Petri dishes were washed, the fluids were aspirated and 0.2 ml of dilution fluids or virus-serum mixtures were pipetted into the centers of the dishes. Adsorption was carried out for a period of 2 hours at room temperature (20°-23°C) during which time the dishes were gently tipped at 15 minute intervals better to distribute the inocula. Although the pH of the inocula rose to pH 8.8 after 2 hours at room temperature, these conditions were chosen for adsorption as a matter of convenience, since the results did not differ significantly from those obtained when adsorption was carried out at 36.5°C in the CO_2 incubator.

Plaque overlay medium. The formula for the medium used, obtained through the courtesy of Dr. H. Liebhaber consisted of equal volumes of a 2% solution of Noble Agar (Difco) in distilled water and of a solution composed as follows:

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	ml
1. Distilled water	51.0
2. 20 × concentrate Earle's solution A	10.0
3. 10 × " " " B	20.0
4. 4% yeast extract (Difco)—4% bovine albumin	5.0
5. 1 molar sodium bicarbonate	4.0
6. Chicken serum, inactivated (56°C, 30 min)	10.0

The components of the solution were mixed in the order listed. Penicillin 250 u/ml, streptomycin 0.25 mg/ml and amphotericin 2.5 mg/ml were incorporated into the medium. The sodium bicarbonate solution was added slowly and with constant stirring to minimize precipitation, although the development of some turbidity due to precipitated phosphate had no adverse effect on the system. The amount of bicarbonate used was calculated to produce an agar overlay having a pH of approximately 7.5 in an atmosphere of 5% carbon dioxide. The double strength medium and the agar solution were individually warmed to 42°-44°C in a water bath and mixed just prior to use.

Five ml of overlay medium were delivered gently into each Petri dish without previous removal of the inoculum and allowed to set firmly at room temperature for 15-20 minutes.

Incubation. All dishes were inverted and incubated in a humidified chamber containing 5% carbon dioxide in air. The temperature was 36.5° ± 0.3°C unless otherwise noted. A second 5 ml overlay of the mixture described above was added to each dish on day 4. A last 5 ml overlay containing sufficient neutral red to produce a final concentration in each overlaid dish of 1:40,000 was added on the ninth day after inoculation. The dishes were then replaced in the incubator, shielded from light, and serial plaque counts performed beginning on the 9th or 10th day after inoculation.

Tube titration. Stoppered tubes containing monolayers of HEp-2 cells at the same passage level as those used to prepare the plaque assay dishes were seeded with 100,000-120,000 cells contained in 1.5 ml Eagle's MEM in Hanks' balanced salt solution containing 10% inactivated calf serum, penicillin and streptomycin, and incubated at 36.5°C. Prior to use the monolayers were successively washed once with 1.5 ml Hanks' balanced salt solution and 1.5 ml MEM containing glutamine, penicillin and streptomycin, and 0.1% additional $NaHCO_3$. A volume of 0.2 ml of the appropriate virus dilution was added to each tube without prior removal of the medium, and incubated at 36.5°C. Media were changed at 3-day intervals after inoculation and endpoints determined by examination of tubes for the presence of characteristic RS cytopathic effects (7) for a period of 10 days.

Serology. The sera to be tested in plaque reduction neutralization tests were inactivated at 56°C for 30 minutes prior to use unless otherwise noted, diluted in Eagle's MEM-5% chicken serum, and incubated for 1 hour at room temperature with an equal volume of virus-containing fluid calculated to contain approximately 100 PFU/0.1 ml. Three HEp-2 monolayers in 60 mm Petri dishes were then inoculated with 0.2 ml of the serum-virus mixture. Adsorption and incubation of the inoculated Petri dishes were carried out as described and the number of plaques observed at time of maximal counts was compared with the number produced in 3 dishes inoculated with 0.2 ml of mixture of equal parts of virus and Eagle's MEM containing 5% inactivated chicken serum. Plaque counts in dishes receiving serum-virus mixtures were compared with the counts observed in dishes inoculated with the virus-blank control mixture. Endpoint dilution virus neutralization tests in roller tubes were carried out as described elsewhere(3), and the endpoints expressed as the reciprocal of initial serum dilutions in virus-serum mixtures which completely inhibited viral cytopathic effect.

Acute and convalescent sera from infants with naturally occurring RS virus infection associated with febrile illness and pneumonia were kindly provided by Dr. Robert M. Chanock and Dr. Albert Z. Kapikian. Antibody-containing sera prepared by intranasal infection of ferrets with RS virus were obtained through the courtesy of Dr. Helen V. Coates.

Results. A. *Description of lesions in agar-overlaid HEp-$_2$ monolayers.* Uninfected control cell sheets showed excellent survival under a total of three 5 ml agar overlays for

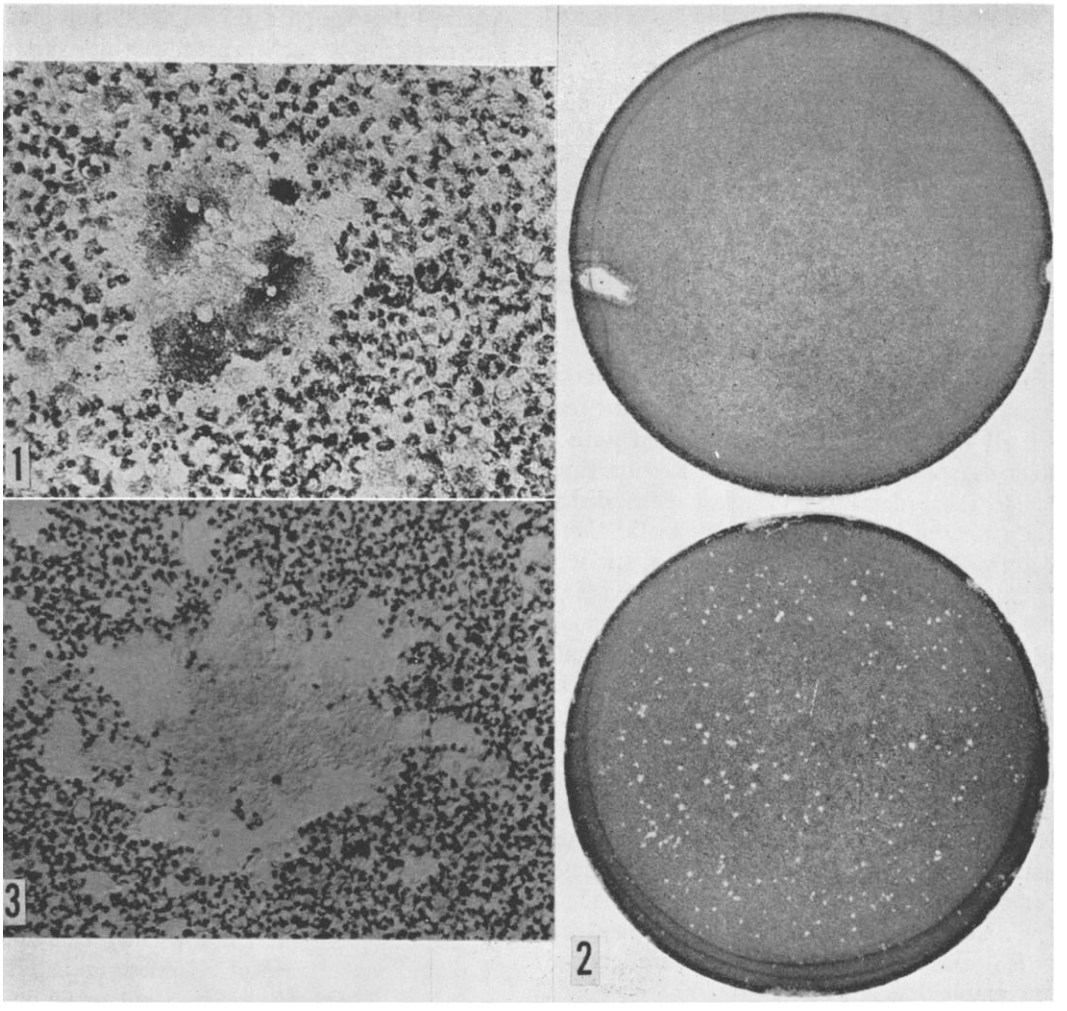

FIG. 1. "Red plaque" produced by RS virus in HEp-2 cell agar-overlaid monolayer. 9th post-infection day. Magnification 100 ×. Neutral red stain taken up in center of syncytium.

FIG. 2. *Top:* Uninfected agar-overlaid HEp-2 monolayer in 60 mm Petri dish. *Bottom:* Similar dish infected 10 days earlier with RS virus showing numerous plaques.

FIG. 3. "White plaque" produced by RS virus in HEp-2 cell agar-overlaid monolayer. 10th post-infection day. Magnification 60 ×. Dead infected cells no longer contain neutral red stain.

periods up to 15 days. Cultures inoculated with RS virus also appeared both macroscopically and microscopically normal for a period of 8 days.

On the ninth day, macroscopically visible, red-staining foci 0.1-0.5 mm in diameter were observed in virus inoculated monolayers. Examination of these "red plaques" under the microscope at a magnification of 100 × revealed them to consist of single, or on subsequent days, multiple syncytia (Fig. 1). The centers of these syncytia were intensely stained with neutral red. No similar foci were seen in uninoculated control dishes. Reexamination of the monolayers after reincubation for 24 hours revealed the presence of minute, fairly uniform, round to irregular plaques having sharply delineated borders and unstained white centers 0.5-1.5 mm in size (Fig. 2). The number of plaques observed gradually increased and tended to approach a maximum on the 11th or 12th day. The diameter of the plaques did not increase appreciably with time and did not exceed 2 mm,

TABLE I. Variation in Number of Plaques Counted in Replicate Petri Dishes.

Exp No.	No. of replicate dishes	Mean No. PFU/0.2 ml	Standard error of mean
1	12	8.5	± .7
2	21	65.0	± 3.3

Microscopic examination of these "white plaques" revealed them to consist of "ghosts" of the syncytia previously noted (Fig. 3).

Data showing the variation in number of plaques observed in replicate HEp-2 plaque assay dishes in several experiments are presented in Table I and are consistent with a high degree of reproducibility. Results similar to those described for glass Petri dishes were obtained with disposable plastic tissue culture Petri dishes (Falcon Plastics, Inc.).

B. *Linearity of plaque counts.* Aliquots of 0.2 ml of serial $10^{-0.5}$ dilutions of stock pools of RS virus were inoculated onto 3 replicate Petri dish HEp-2 monolayers, overlaid as described, and plaque counts carried out on the 11th post-infection day. Fig. 4 shows graphically the results of 2 such experiments. The data indicate that plaque formation by RS virus is linear over the range of dilutions employed and suggest that a single particle is responsible for the initiation of infection.

C. *Comparison of plaque and endpoint dilution assays.* Plaque assays were carried out employing 3 Petri dishes per specimen and the results compared with endpoints obtained in tube titrations employing 4 tissue culture tubes for each 0.5 $\log_{10}$ dilution step. The results of 4 such experiments are shown in Table II. When the data are corrected for the fact that titers expressed in terms of ID_{50}/ml will be 1.44 × higher than those expressed as PFU/ml(2) it is apparent that over the range of virus dosages tested the PFU/ID_{50} ratio was very nearly 1.

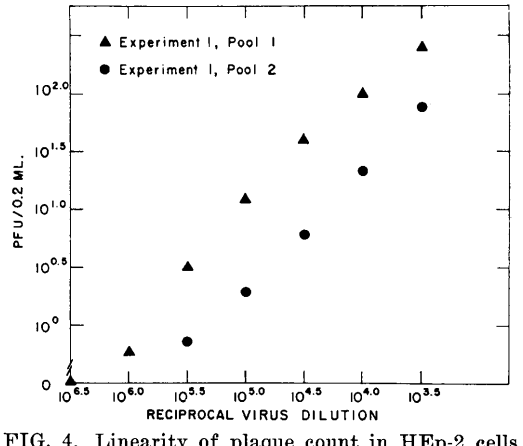

FIG. 4. Linearity of plaque count in HEp-2 cells by respiratory syncytial virus.

The fact that the plaques formed by RS virus under the conditions described are small in relation to the diameter of the assay dishes used makes it possible to count up to 200 such plaques in a single such dish with high precision. Overlapping of plaques was minimal. Results of high accuracy may thus be obtained with far greater economy than is possible by using standard endpoint dilution methods.

TABLE II. Comparison of Plague and End Point Dilution Assays.

Sample	$\log_{10}$ PFU/0.2 ml*	Log TCD_{50}/0.2 ml†
A	.6	.6
B	1.4	.9
C	1.7	1.6
D	2.2	2.1

* 11th day. Expressed as log of average number of plaques counted on 3 Petri dishes.
† 4 titration tubes per 0.5 log dilution of sample.

D. *Specificity.* The virus specificity of the plaques observed was confirmed by carrying out plaque reduction neutralization tests. The results of a typical experiment employing acute and convalescent phase sera from 2 naturally infected infants and from a ferret experimentally infected with the Long strain of RS virus by the intranasal route are shown in Table III.

The data indicate that post-infection ferret serum as well as convalescent phase human serum containing specific complement fixing and virus neutralizing antibody produced specific reduction in the number of plaques observed in the system described. Control pre-infection serum from the same ferret at a 1:10 dilution produced no reduction in plaque count compared with control values. The marked inhibition of plaque formation by acute phase serum #3896, having a tissue culture tube neutralizing antibody titer of less than 4, suggests that the plaque reduction neu-

TABLE III. Specificity of Plaque Formation by RS Virus.

Serum	Species	Description	Reciprocal of antibody titer: CF	Roller tube virus neutralization†	Serum dilution tested	Avg PFU/0.2 ml*
4105	Human	"Acute phase" serum. Infant with bronchiolitis. Age 7 mo.	<2	<4	1:10	67
					1:100	188
4192	"	"Convalescent phase" serum from same infant.	4	128	1:10	0
					1:100	15
					1:1000	147
					1:10,000	188
3896	"	"Acute phase" serum. Infant with pneumonia. Age 16 mo.	<8	<4	1:10	0
					1:100	78
3991	"	"Convalescent phase" serum from same infant.	>64	>128	1:10	0
					1:100	1
					1:1000	70
					1:10,000	166
1872	Ferret	Pre-infection serum			1:10	166
4050	"	21 days post-infection. Serum from same ferret.			1:10	0
					1:100	0
None	—	—		—	‡	154

* 10th day. 4 Petri dishes per serum-virus mixture.
† *Vs.* 32-100 $TCID_{50}$.
‡ Virus suspension mixed with equal volume BME, 5% inactivated chicken serum.

tralization tests are a more sensitive measure of anti-RS antibody than the standard tube dilution neutralization tests heretofore employed.

The possibility that the plaque reducing titer of some human acute phase sera might be due to the presence of a non-antibody inhibitor was studied in another experiment by treating the sera in question with periodate and with receptor destroying enzymes using standard methods(11-12); and also by comparing the titer of unheated serum with that of serum inactivated by heating at 56°C for 30 minutes.

Neither pre-infection human serum nor the normal ferret and guinea pig sera tested at a dilution of 1:10 appeared to contain significant amounts of inhibitor sensitive to inactivation by the methods employed. Heating of RS antibody-containing sera at 56°C for 30 minutes did not diminish the plaque reducing titer observed.

E. *Other observations.* Neither sodium dextran sulfate nor DEAE dextran produced any change in size or morphology of plaques when added to agar overlays in concentrations of up to 100 μg/ml. Early experiments suggest that DEAE dextran may produce a small decrease in number of plaques formed by a given inoculum.

The experiments described were carried out with cells free of demonstrable contamination with pleuropneumonia-like organisms (PPLO). To determine whether such contamination might influence plaque formation, HEp-2 cells were inoculated with a broth culture of the PPLO strain isolated from the cell line prior to antibiotic decontamination. After several further subcultures, during which fluids from cells were consistently positive for PPLO, "contaminated" and "clean" HEp-2 cultures were used to prepare assay dishes. No differences were observed in plaque size, morphology or count following inoculation of each set of dishes with RS virus.

Discussion. The plaque assay for enumeration of infectious RS virus particles in agar-overlaid HEp-2 cells described here has been shown to satisfy criteria of specificity, linearity, reproducibility, and sensitivity necessary for its advantageous routine use in quantitative studies. It offers a convenient and accurate method of use in studies of RS virus multiplication, pathogenesis and epidemi-

ology, as well as a means of effecting plaque purification of different strains of this agent.

The success of the method employed was largely dependent on the seemingly fortuitous selection and propagation of a strain of HEp-2 cells which was highly viable under multiple agar overlays for up to 15 days, while retaining a high sensitivity to infection associated with production of characteristic syncytial cytopathic effect. The presence of pleuropneumonia-like organisms did not appear to decrease the viability of the cells or their sensitivity to RS virus. Other strains of PPLO, however, may be found to do so. Certain variables, which were not critically studied, may also have been important in obtaining a HEp-2 cell with the desired characteristics. These included the use of Versene rather than trypsin for removal of cells from glass surfaces, the original treatment of cells with Kanamycin, and the subsequent maintenance of cultures in the absence of antibiotics.

Previously published observations employing the fluorescent antibody technic(13) revealed no evidence of mitotic division by individually infected cells. In view of this, the observation in the present study that each plaque formed by a single infectious particle contained one or more multinucleate giant cells suggests that syncytium formation may result from a process of recruitment of cells by primarily infected ones adjacent to them. A detailed discussion of this phenomenon (polykaryocytosis) has recently been presented by Roizman(14). The finding that syncytium-containing plaques failed to appear prior to the ninth day after inoculation of agar-overlaid plates was consistent with the similar late appearance of CPE in roller tube cultures containing fluid media when very small doses of infectious virus were added.

Because of its high sensitivity the plaque reduction neutralization test offers a useful tool for detection of amounts of antibody too minute to be measured accurately by standard tube neutralization tests. The demonstration of the presence of specific anti-RS antibody in sera in which such antibody had not previously been detectable may prove useful in more accurately defining the age incidence of natural infection with RS virus in children and the role played by pre-existing low levels of antibody in determining the outcome of subsequent RS infection both with regard to the type and severity of the illness produced and the magnitude of the resulting antibody response. Such data may ultimately prove to be of importance for development and evaluation of an immunizing vaccine.

Preliminary data from recent studies employing convalescent sera of animals infected intranasally with various strains of RS virus suggest that distinct serotypes of RS virus exist(15). The plaque reduction neutralization test provides a sensitive tool for quantitation of such serologic differences between strains of RS virus isolated from different locations and at various times.

Summary. An accurate and sensitive method for enumeration of infective plaque forming units of respiratory syncytial virus, utilizing agar-overlaid HEp-2 monolayers in Petri dishes, is described. Small macroscopic plaques were observed 9 days after infection, and attained a final diameter of 2 mm and maximum number by the 11th or 12th day. By use of the technic of plaque reduction, it was possible to detect amounts of neutralizing antibody to RS virus hitherto not measurable by the use of standard endpoint dilution methods.

The authors are indebted to Dr. Robert M. Chanock for helpful advice and criticism, and to Mrs. Lena J. Wetherell for invaluable technical assistance.

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Received November 20, 1962. P.S.E.B.M., 1963, v112.

Field Studies of Protection from Infection by Experimental Trachoma Virus Vaccine in Preschool-Aged Children on Taiwan.* (28112)

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Following isolation and serial growth in quantity of trachoma virus in a number of places, there has been great interest in the possibility of development of a trachoma vaccine in the hope of hastening eradication of this disease(1). Steps in development and testing of experimental trachoma vaccines on Taiwan have been reported(2-6). These have included preparation of experimental trachoma vaccines, their test in monkeys and then humans for antibody response and protection. Experimental trachoma vaccines have been shown to affect favorably the clinical course and response to antibiotic treatment of experimentally infected human volunteers(7). After 2½ years of observation in a field trial of experimental trachoma vaccine in children on Taiwan, a significant difference between vaccine and control groups has appeared. This report presents the results.

* This study was supported in part by funding under Public Law 480, Section 104(c), in part by Office of Naval Research contract with University of Chicago, and in part by Public Health Service research grant with Univ. of Washington from Inst. of Neurologic Dis. and Blindness, N.I.H. The opinions and assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or Naval Service at large.

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Materials and methods. Vaccines. Two Taiwan trachoma virus strains were used in preparation of the vaccines. These were TRIC-Taiwan-1 isolated in 1958 (formerly called TW-10) and TRIC-Taiwan-3 isolated in 1959 (formerly called TW-29). Of 29 Taiwan strains tested for cross-protection in the mouse toxicity prevention test, all have fallen into 2 groups of which TW-1 and TW-3 are the prototypes(8). Vaccine tested in the Lungching area was monovalent containing only the TW-3 strain. Vaccine tested in Ta-an was bivalent containing equal amounts of TW-1 and TW-3. In both cases, one ml of vaccine contained virus particles remaining after purification procedure from 0.4 g (40% by weight) of original yolk sac suspension. Details of method of purification of elementary bodies from yolk sac suspensions have been presented(9). Following this purification procedure, which employs trypsin digestion, 3 cycles of high and low speed centrifugation, and polymixin precipitation, the elementary body suspension was killed and stabilized by addition of 0.02% concentration of formaldehyde. Aluminum hydroxide particles were suspended in a 1:200 solution of human gamma globulin. Particles were then sedimented and gamma globulin discarded. When formalized elementary bodies were mixed with coated alum particles, many bodies stuck to the particles. This final aluminum hydroxide vaccine contained about 10