Assay of Chikungunya Virus in Cell Monolayers by Immunofluorescence

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Chikungunya virus was quantitatively assayed by counting immunofluorescent foci after infection of BHK21/C13 cell monolayers. The speed and efficiency of virus attachment to cells were markedly enhanced when augmented by centrifugal force. By this procedure, a proportionality was obtained between the number of immunofluorescent foci and the volume of inoculum. Virus penetration into cells was linear and complete within 15 min at 35 C. From observations on the sequential development of viral antigen within cells and immunofluorescent focus counts, foci of infected cells may be enumerated as early as 16 hr after inoculation of cell monolayers. A linear function was demonstrated between immunofluorescent focus counts and relative virus concentration. The immunofluorescent assay was comparable in sensitivity but more precise and rapid than virus assays based on the intracerebral inoculation of suckling mice or on plaque counting. By the immunofluorescent procedure, the 50% neutralizing end point of antiviral serum was rapidly and quantitatively determined.

Quantitative assays of virus infectivity by enumeration of cells containing immunofluorescent viral antigens have been established for agents representative of almost all major animal virus groups and, recently, the procedure has been successfully employed to assay a plant virus (2). However, immunofluorescent assays have been developed for only four viruses of the large number of agents that comprise the different arbovirus groups, i.e., yellow fever (4), Venezuelan equine encephalomyelitis (VEE; 6), Semliki Forest (1), and Rift Valley fever (RVF) virus (5). The feasibility of extending the immunofluorescent technique for the infectivity assessment of another arbovirus, chikungunya, was investigated because it has been demonstrated that cells infected with the virus are amenable to immunofluorescent staining (7; J. D. White and L. L. Kupferberg, Bacteriol. Proc., p. 128, 1966). This is a report on the development and evaluation of this technique for the quantitative assay of chikungunya virus.

MATERIALS AND METHODS

Virus strain. The Banganike strain of chikungunya virus (12) used in this study was in the form of a 10% suckling mouse brain suspension. Assayed in suckling mice, the suspension had a titer of $10^{8.6}$ LD_{50} per ml by intracerebral inoculation.

Cell lines and cultivation. The principal cell line used for assay of the virus was baby hamster kidney (BHK21/C13) obtained from the American Type Culture Collection, Rockville, Md. This cell line morphologically consists of elongated fibroblastic cells. Nutrient medium consisted of Earle minimal essential medium (MEM) supplemented with 1% glutamine (200 mM), 10% tryptose phosphate broth, 10% fetal calf serum, 0.005% neomycin, and 75 μg of kanamycin per ml. Maintenance medium was equal parts of nutrient medium and MEM. Cells were cultivated on circular cover slips (15-mm diameter) inserted in flat-bottomed glass vials (19 by 65 mm). One milliliter of cell suspension containing from 10^5 to 3×10^5 cells was introduced onto cover slips which were then incubated at 35 C for 24 hr or until a complete cell was formed.

Immunofluorescent virus assay. Determinations were usually made in triplicate. Initially, virus dilutions were prepared in phosphate-buffered saline (PBS) solution (*p*H 7.1) free of calcium and magnesium ions (6), but later heart infusion broth was used. Inoculum in a 0.2-ml volume was introduced directly onto cover slip cell monolayers after their transfer from glass vials to rotor chamber inserts (4). The latter were used because they withstood the high centrifugal force required to sediment the virus. Rotor chamber inserts placed in a swinging-bucket (SW 25.1) rotor were centrifuged in a model L Preparative Ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 19,642 to 29,432 × *g* for 15 min at 30 C. The residual inoculum was removed after centrifugation, the cover slip cell monolayers were replaced into glass vials, and 1 ml of maintenance medium was then added to each vial. After incubation at 35 C from 0.5 to 1 hr, the main-

tenance medium was replaced with 1 ml of a 1/15 dilution of monkey viral antiserum in maintenance medium. After further incubation at 35 C from 20 to 22 hr, cover-slip cell monolayers were rinsed twice with PBS solution, fixed with cold (−60 C) acetone, and either prepared immediately for immunofluorescent staining and focus counting or stored at −60 C. Fluorescence of viral antigens in fixed cell cultures was not diminished after storage for several weeks.

Chikungunya antiserum conjugate and immunofluorescence staining. Antiviral serum was obtained from rhesus monkeys that had been injected intravenously with 10^7 immunofluorescent focus units (IFU) of virus in a 1-ml volume. Animals were injected twice during a period of 1 month. Two weeks after the last injection, the animals were bled. Antiserum was conjugated with fluorescein isothiocyanate by the method of Riggs et al. (9). Conjugated globulin was passed through a column of Sephadex G-50 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) to remove unbound dye. To reduce nonspecific fluorescence, 5 ml of conjugated globulin was diluted with an equal volume of PBS solution and adsorbed twice with 200 mg of acetone-dried mouse liver powder by the procedure of Coons and Kaplan (3).

The direct fluorescent-antibody method was used to demonstrate immunofluorescence of viral antigens in infected cells. Fixed cell monolayers were washed once with PBS solution and stained with conjugated globulin for 30 min at room temperature. Cover-slip cell monolayers were then rinsed in two changes of PBS solution and mounted in a semipermanent medium (10).

Fluorescence microscopy and focus counting. Cover-slip cell cultures were examined with an American Optical microscope equipped with a Fluorolume illuminator (model 645), Corning no. 5849 and Schott BG-12 exciter filters, and an E. K. no. 2A barrier filter. With this optical system at a magnification of 430×, the number of microscopic fields contained in the area of a 15-mm cover slip was 1,064. On occasion when cell monolayers contained numerous immunofluorescent foci, 200× magnification was employed; the number of fields per cover slip was 226. For each cover slip cell monolayer, 50 microscopic fields were examined for immunofluorescent foci. To calculate the number of IFU of virus per milliliter, the average number of IFU per field was multiplied by the number of fields per cover slip, the reciprocal of the dilution of virus inoculum, and a volume factor (for conversion to milliliters).

Suckling mouse virus assay. One suckling mouse litter was inoculated for each virus dilution; each litter consisted of eight 1- to 2-day-old mice. Appropriate virus dilutions were inoculated intracerebrally in 0.02-ml volumes into each mouse. Deaths occurring within 24 hr after inoculation were considered nonspecific. Mice were observed for lethality daily for 10 days. Virus titers were calculated by the Reed and Muench (8) formula and expressed as suckling mouse intracerebral median lethal dose ($SMICLD_{50}$).

Plaque virus assay. Monolayers of guinea pig lung, BHK21/C13, or chick embryo fibroblast cells grown in 25-cm² flasks (Falcon Plastics, Los Angeles, Calif.) were inoculated with 0.1 ml of appropriate virus dilutions and then incubated at 37 C for 1 hr. Five milliliters of overlay medium was added to each flask. This medium consisted of basal medium Eagle (Earle base), 2% fetal calf serum, 2% Noble agar (Difco), 100 μg of diethylaminoethyl (DEAE)-dextran (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), 100 units of streptomycin, and 100 μg of penicillin per ml. Guinea pig lung cell monolayers were incubated at 33 C for 72 hr; BHK21/C13 and chick embryo fibroblast cells were incubated at 33 C for 48 hr. The second overlay was then applied; it was similar to the first except that it contained 0.01% neutral red but no DEAE-dextran. After additional incubation of cell monolayers at 33 C from 18 to 24 hr, plaques were counted.

Determination of virus attachment. Virus attachment was measured by observing and recording the disappearance of virus from inoculum after its addition to cell monolayers. Cover-slip cell cultures to be subjected to centrifugation or stationary incubation at 35 C received 0.2 ml and 0.4 ml of inoculum, respectively. After designated incubation intervals, residual inoculum was removed, and the cell monolayers were immediately washed twice with PBS solution. Residual inoculum was then introduced onto fresh cell monolayers to measure unattached virus. For this, the residual inoculum was attached onto cell cultures by centrifugation at 19,642 to 29,432 × g for 15 min. Cover-slip cell monolayers exposed to initial or residual inocula were then treated in the manner described earlier for assay of virus. The amount of virus that was attached to cells at a given time was expressed as a percentage of the virus input. The latter was the sum of the amounts of attached and residual virus.

Determination of virus penetration. Virus penetration into cells was measured by the insensitivity of attached virus to antiserum. Cell cultures were washed twice with PBS solution, overlaid at designated times with 1 ml of a 1/15 dilution of viral antiserum, and then incubated at 35 C for 20 hr. The quantity of virus that penetrated into cells at a given time was expressed as a percentage of the input virus.

Calculation of attachment and penetration constants. The attachment and penetration rate constants (K) were calculated from the relationship, 2.3 log $(Vo/V_t)/nt$, where Vo = the input virus concentration, V_t = unattached or unpenetrated virus at time t, and n = the number of cells per cubic centimeter determined by resuspension of cover-slip cell cultures after trypsin treatment and enumeration of cells in a hemocytometer.

RESULTS

Virus attachment. The rate of chikungunya virus attachment to BHK21/C13 cell monolayers was determined for both centrifugation and stationary incubation (35 C). Virus inputs for the former and the latter were 740 and 1,480 IFU, respectively. The concentration of cells per cover-slip culture was 4.0×10^5 per ml. Aided

by centrifugal force, approximately 95% of the virus inoculum was attached within 10 min; after stationary incubation for 2 hr, only 14% of virus was attached (Fig. 1). The attachment rate con-
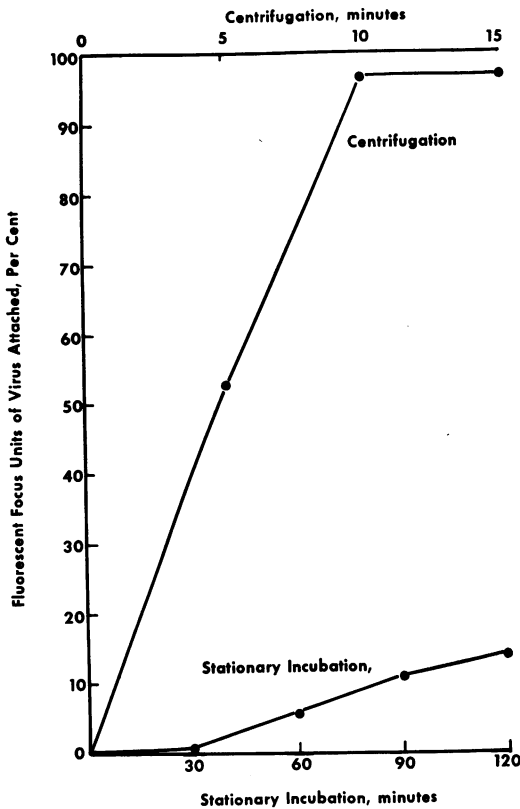

FIG. 1. *Attachment of chikungunya virus onto coverslip BHK21/C13 cell monolayers by centrifugation (19,642 to 29,432 × g at 25 C) and stationary incubation (35 C).*

TABLE 1. *Relationship between volume of inoculum and immunofluorescent focus units (IFU) of chikungunya virus: centrifugation versus stationary incubation*

Vol	IFU per 50 microscopic fields	
	Centrifugation[a]	Stationary incubation[b]
ml		
0.1	33	11
0.2	60	23
0.5	159	19
1.0	332	29

[a] Virus inoculum centrifuged at 19,642 to 29,432 × g for 15 min into BHK21/C13 cell monolayers at 25 C.

[b] Stationary incubation of virus inoculum at 35 C for 2 hr.

TABLE 2. *Effect of different virus diluents on attachment of chikungunya virus onto BHK21/C13 cell monolayers*

Virus diluent	IFU[a] per 50 microscopic fields	Virus titer
		IFU/ml
PBS[b]	43	4.2×10^8
PBS + 0.0009 M CaCl₂ + 0.0005 M MgCl₂	42	4.2×10^8
Cell maintenance medium[c]	42	4.2×10^8
Heart infusion broth	56	5.8×10^8

[a] Immunofluorescent focus units of virus.

[b] NaCl (0.1 M) buffered by 0.01 M phosphate buffer.

[c] Minimal essential medium, 5% fetal calf serum, and 5% Tryptose phosphate broth.

stants (K) were 3.4×10^{-7} cm³/min with centrifugation and 2.8×10^{-9} cm³/min with stationary incubation. The former attachment rate constant is comparable to those reported for VEE and RVF viruses under similar experimental conditions (5, 6).

The efficiency of centrifugation and stationary incubation for infecting cell monolayers from different volumes of inoculum is shown in Table 1. A proportionality between the number of immunofluorescent foci and volume of inoculum was obtained when centrifugation was employed. With stationary incubation, a similar relationship was not evident.

Because previous studies with another group A arbovirus (VEE) indicated that the efficiency of virus attachment to cells was markedly influenced by the menstruum used to suspend the virus (6), this factor was also investigated for attachment of chikungunya virus. Virus was diluted in each of four different suspending media, pH 7.1 to 7.3. Before virus inoculum was added, each cell monolayer was washed with its respective test diluent. Virus inoculum was centrifuged onto cell cultures; they were then treated according to the described assay procedure. The compositon of the medium used for attachment of chikungunya virus to cell monolayers did not significantly affect assay values (Table 2). Slightly more virus appeared to be attached, however, in the presence of heart infusion broth.

Virus penetration. The rate of virus penetration into cells was followed by determining the insensitivity of attached virus to antiviral serum at designated times. A virus input of 168 IFU was introduced onto cell monolayers. They were then treated as described. Virus penetration at 35 C proceeded at a linear rate without evidence of an initial lag period (Fig. 2). Approximately 50%

of attached virus penetrated into cells within 7.5 min; the process was complete within 15 min. The penetration rate constant (K) of 2.3×10^{-7} cm³/min was faster than that noted with VEE and RVF viruses (5, 6).

Because an antiviral serum overlay is employed in this assay to prevent the extracellular spread of virus from infected cells, this information on the rate of virus penetration is highly relevant. The earliest time that the antiviral serum overlay may be added without neutralizing attached virus was fixe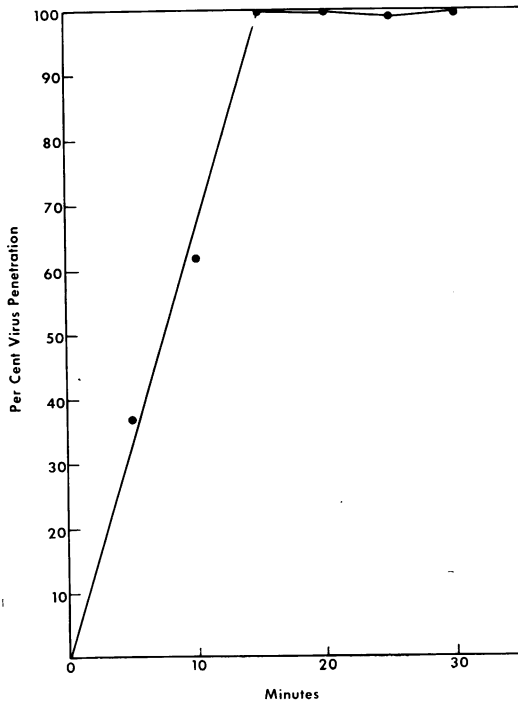d by the results obtained from the preceding experiment. For routine assay of virus, the antiviral serum overlay was added after cell monolayers were incubated at 35 C for 0.5 to 1 hr after virus attachment.

Susceptibility of cell lines. Chikungunya virus was assayed by the immunofluorescence procedure in different cell lines to determine their susceptibility to infection. The cell lines tested were BHK21/C13, BHK21 (short fibroblastic cells obtained from Microbiological Associates, Bethesda, Md.), KB (Eagle), HEp-2, L-929, guinea pig lung, and McCoy. Virus was inoculated onto cell monolayers in the usual manner and cell cultures were then incubated in the presence of antiviral serum overlay. Virus assayed in the BHK21/C13 cell line was approximately 1.0 log$_{10}$ unit higher in titer than that obtained in the BHK21 cell line (Table 3). In both cell lines, immunofluorescent foci appeared in the presence of antiviral serum; focus counts were the basis of assay values. In the other cell lines, the assay was based on individual infected cell counts, because immunofluorescent foci did not appear in the presence of the antiviral serum overlay. The BHK21/C13 cell line was the most susceptible to virus infection of the cell lines tested and was subsequently employed for the standardization of the assay.

Incubation period. Incubation period is defined as the time interval between virus inoculation and the development of recognizable quantities of fluorescent viral antigens in infected cells. The period was established from sequential observations of infected cell monolayers and immunofluorescent focus counts. Two groups of BHK21/C13 cover-slip cell monolayers were inoculated with 2×10^3 IFU in 0.2-ml volume and treated in the manner described for attachment of inoculum. After the prescribed period for virus penetration into cells, antiviral serum overlay was added to one group of cell monolayers; the other group received maintenance medium. The latter group was included to determine whether there was an optimal time period for counting individual infected cells before the appearance of foci. Both test groups were incubated at 35 C for time intervals of 4, 6, 8, 10, 12, 16, 21, and 25 hr. Representative cell monolayers from each group were then processed for fluorescent-antibody staining.

The earliest sign of specific fluorescence of chikungunya virus antigens in cells was seen at 6 hr after infection. Fluorescence was localized exclusively in the cell cytoplasm and was confined to individual cells. At 8 hr, an occasional immunofluorescent focus that consisted of three or more infected cells was noted. Generally, the number of infected cells in a focus increased to

FIG. 2. *Rate of chikungunya virus penetration into BHK21/C13 cells at 35 C as measured by insensitivity of attached virus to antiviral serum.*

TABLE 3. *Assay of chikungunya virus in different cell lines*

Cell line	Virus titer
	ml
BHK21/C13...............	3.1×10^8 IFU[a]
BHK21..................	2.1×10^7 IFU
L-929..................	3.3×10^7 CIU[b]
Guinea pig lung........	1.8×10^7 CIU
McCoy.................	5.8×10^4 CIU
KB (Eagle).............	1.0×10^4 CIU
HEp-2.................	2.1×10^3 CIU

[a] Immunofluorescent focus units of virus.
[b] Cell-infecting units of virus.

10 or more with prolonged incubation times (Fig. 3). Individual infected cells were mostly seen in cell monolayers incubated with maintenance medium; immunofluorescent foci were predominate in cell cultures incubated with antiviral serum. The increase in numbers of immunofluorescent foci and individual infected cells during incubation of the two test groups of cell monolayers is shown in Fig. 4. In the presence of antiviral serum, immunofluorescent foci
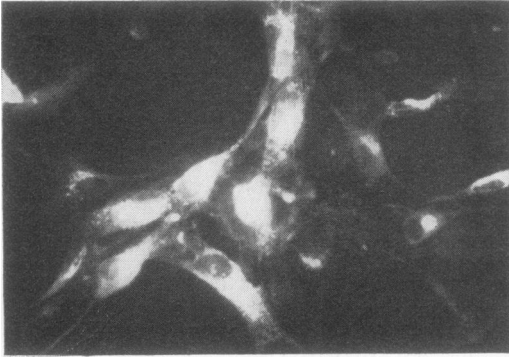

FIG. 3. *Immunofluorescent focus of chikungunya virus infection in BHK21/C13 cells in the presence of antiviral serum.* × 225.

increased during the first 12 hr of incubation, and thereafter the number remained constant. Individual infected cell counts in cell monolayers incubated with maintenance medium increased almost linearly throughout the 25-hr incubation period, which made it difficult to delineate the primary from secondary cycle of infection. In view of these findings, the assay of chikungunya virus was based on immunofluorescent focus counting. Foci could be enumerated as early as 16 hr after inoculation of cell monolayers to obtain an estimate of virus titer.

Quantitative evaluations of the assay. A linear relationship was obtained between the number of immunofluorescent focus units and relative virus concentration throughout the inoculum range of 1.2 $\log_{10}$ units (Fig. 5). Each immunofluorescent focus appeared to result from infection by a single infective virus particle or aggregate not divisible by dilution.

Ten determinations were performed in a single experiment to estimate the precision of the assay. Cover-slip cell monolayers were infected with 0.2 ml of a 10^{-5} virus dilution and then treated in the prescribed manner. The number of IFU per milliliter of virus inoculum ranged from 5.2 × 10^8 to 6.3 × 10^8 with a mean of 5.8 ×
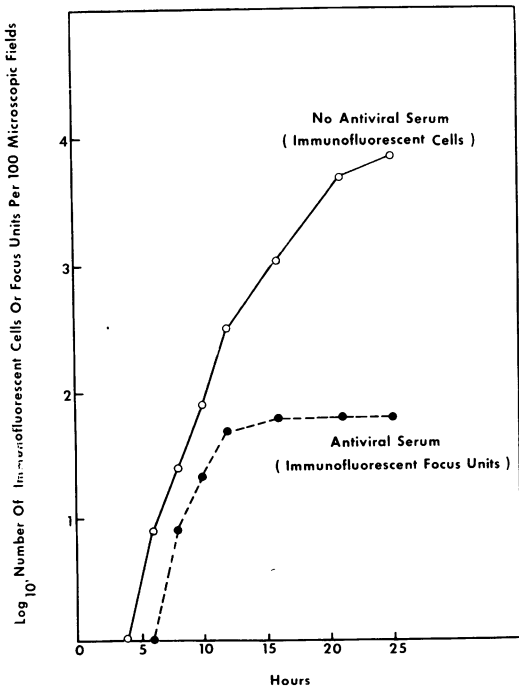

FIG. 4. *Effect of the length of the incubation period (35 C) on the number of infected BHK21/C13 cells in the presence and absence of antiviral serum.*
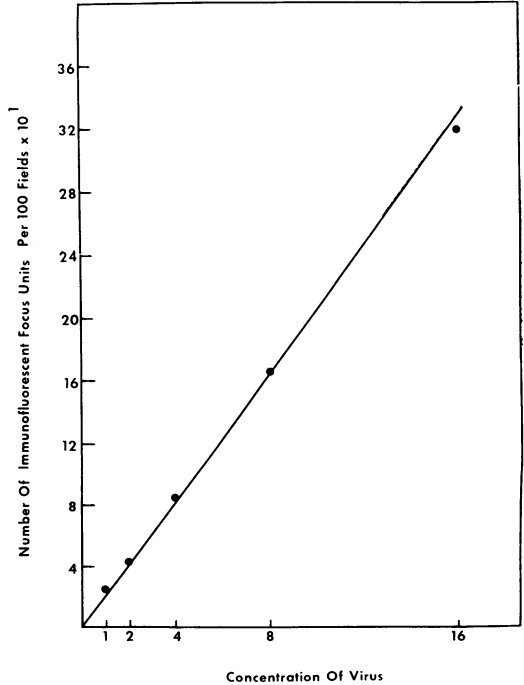

FIG. 5. *Linear function between the number of immunofluorescent focus units and relative concentration of chikungunya virus.*

TABLE 4. *Assay of chikungunya virus by different methods*

Assay no.	IFU[a] per ml	SMICLD$_{50}$ per ml[b]	BHK21/C13 cells (PFU/ml)[c]	GPL cells (PFU/ml[c])
1	5.3×10^8	9.5×10^8	7.6×10^8	3.6×10^8
2	5.8×10^8	10.0×10^8	7.0×10^8	3.2×10^8
3	5.3×10^8	12.0×10^8	7.6×10^8	3.6×10^8
4	5.1×10^8	0.63×10^8	8.8×10^8	2.0×10^8
5	5.1×10^8	10.0×10^8	10.0×10^8	3.6×10^8
6	4.8×10^8	10.0×10^8	7.8×10^8	4.7×10^8
Mean	5.2×10^8	8.6×10^8	8.1×10^8	3.4×10^8
SD[d]	$\pm0.34 \times 10^8$	$\pm4.0 \times 10^8$	$\pm1.1 \times 10^8$	$\pm0.87 \times 10^8$
SE[e]	$\pm0.13 \times 10^8$	$\pm1.63 \times 10^8$	$\pm0.45 \times 10^8$	$\pm0.35 \times 10^8$

[a] Immunofluorescent focus units of virus determined in 20 hr.

[b] Suckling mouse intracerebral median lethal units of virus determined in 10 days.

[c] Plaque-forming units (PFU) of virus in BHK21/C13 cell monolayers determined at 3 days, in guinea pig lung (GPL) cell monolayers at 4 days.

[d] Standard deviation.

[e] Standard error of mean.

10^8, standard deviation of ±0.17, and coefficient of variation of 2.9%.

To determine the reproducibility of the immunofluorescent focus-counting assay, a vial of virus was randomly selected at approximately 2-week intervals from the standard pool stored at -60 C and assayed. In four such trials, assay values ranged from 5.2×10^8 to 6.3×10^8 IFU per ml with a mean of 5.8×10^8.

The sensitivity of the immunofluorescent focus-counting assay of chikungunya virus was compared with that of the methods of intracerebral inoculation of suckling mice and virus plaque counting. Chick embryo fibroblasts, guinea pig lung, and BHK21/C13 cell monolayers were each employed for virus assay by the plaque procedure. Six determinations were made with each assay; virus from a standard pool was used. Virus assays in either suckling mice or by counting virus-induced plaques in BHK21/C13 cell monolayers appeared to be comparable in sensitivity (Table 4). The mean assay value obtained by immunofluorescent focus counting was only 0.2 $\log_{10}$ unit less than that obtained by the former procedures. The mean assay value derived from plaque counts using guinea pig lung cell monolayers was 0.2 $\log_{10}$ unit less than that obtained by the immunofluorescent focus-counting method. In chick embryo fibroblast cell monolayers, plaques were not formed with the virus dilutions inoculated. The immunofluorescent focus counting assay, however, exhibited less variability than that of the other virus assay methods tested and, in addition, had the singular advantage of rapidity (less than 24 hr).

Serum neutralization test. A preliminary test was made to determine whether the immunofluorescent focus counting assay of chikungunya
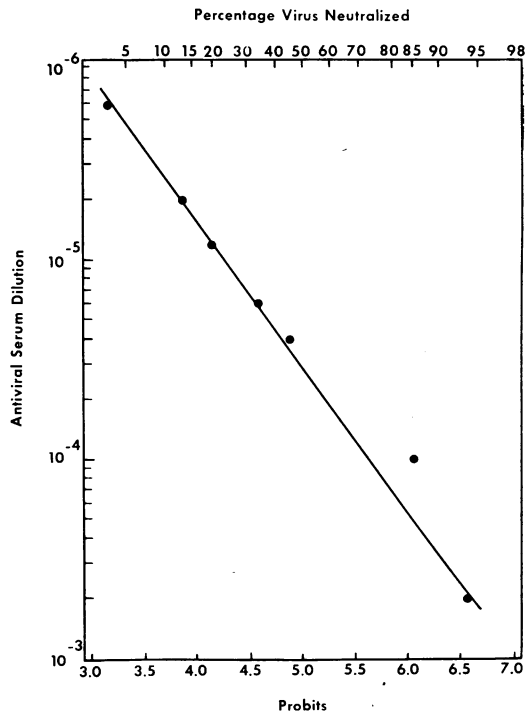

FIG. 6. *Estimation of chikungunya serum-neutralizing antibodies by the immunofluorescent virus assay procedure. Fifty per cent serum neutralizing end point was interpolated from the percentage reduction of immunofluorescent foci of infected cells by antiviral serum dilutions.*

virus could be adapted for estimating serum neutralizing antibodies against the virus. Appropriate dilutions of monkey antiviral or normal serum (control) were mixed with equal volumes

of a constant quantity of virus (5.0×10^4 IFU per ml). Virus and serum dilutions were prepared in PBS. After test mixtures were incubated at 35 C for 2 hr, 0.2 ml of each mixture was introduced onto one of three cover-slip BHK21/C13 cell monolayers for assay of residual virus infectivity in the manner described previously. To determine the 50% serum-neutralizing end point, the percentage of reduction of immunofluorescent focus counts for each antiviral serum dilution was computed from the control counts. Reduction percentages were then plotted against the logarithm of the corresponding final dilutions of antiviral serum on probability paper. Results (Fig. 6) show that a linear relationship was obtained over a critical range. By interpolation, the dilution of antiviral serum that neutralized 50% of virus was determined.

DISCUSSION

The feasibility of employing the immunofluorescence procedure for the quantitative assessment of chikungunya virus infectivity was established by the studies described here. The assay was highly precise, reproducible, and comparable in sensitivity to other established methods of chikungunya virus assessment. The outstanding feature of the method, in common with immunofluorescent assays described for other arboviruses (1, 4–6), was the ability to assess virus infectivity within 24 hr after inoculation of cell monolayers. The time required to assay chikungunya virus by intracerebral injection of suckling mice or plaque counting was 10 days and from 3 to 4 days, respectively.

The use of centrifugal force for attaching virus onto cell monolayers offers several advantages over stationary incubation. By the former procedure, virus attachment is efficient and rapid, thermal inactivation of virus inoculum is minimized, and the proportionality demonstrated between immunofluorescent focus counts and volume of inoculum facilitates the detection of virus particles from dilute suspensions. With the use of centrifugal force, almost synchronous infection of cells may be achieved, and the stages of virus attachment onto and penetration into cells may be readily delineated. These are highly desirable advantages in studies concerned with the early stages of virus replication.

For attachment of chikungunya virus to cells, the requirements were less demanding than those for VEE virus, another group A arbovirus. Maximal attachment of chikungunya virus occurred in the presence of menstruums of varied composition. With VEE virus, however, maximal attachment to cells occurred only in the presence of PBS free of calcium and magnesium ions. In contrast to the findings with chikungunya virus, heart infusion broth markedly inhibited VEE virus attachment (6). This indicates that there is an inherent difference in the requirements of these two group A arboviruses for effecting maximal attachment to cells.

An attempt to base the assay of chikungunya virus on counts of individual infected cells was hampered by the appearance of foci of infected cells in BHK21/C13 cell monolayers. These foci occurred even when inoculated cell monolayers were incubated in the presence of a potent antiviral serum overlay. When different established cell lines were inoculated and incubated with antiviral serum, virus infection was confined to individual cells. In earlier studies, direct evidence was obtained to show that chikungunya virus infection of BHK21/C13 cell monolayers occurs by cell to cell transmission in the presence of antiviral serum overlay and, also, extracellularly in the absence of the overlay (Hahon and Zimmerman, in preparation). Under similar circumstances, foci of VEE virus-infected cells in BHK21/C13 cell cultures also occurred in the presence of antiviral serum, but infection was limited to individual cells when different cell lines were used. These observations suggest that BHK21 cell lines possess some unique biological membrane structure or physiology that is conducive for cell to cell transmission of virus particles. It may be of some relevance that the cell line has been extensively employed for neoplastic transformation by viruses (11). Because BHK21/C13 cells were the most susceptible of the cell lines tested to chikungunya virus infection, it was the host cell line selected for assay of the virus. The assay was based, therefore, on the appearance of immunofluorescent foci that could be enumerated as early as 16 hr after inoculation of cell monolayers.

In a preliminary test, the 50% neutralizing end point of antiviral serum was determined by reacting chikungunya virus with antiserum. Over a critical range, a linear relationship was obtained between reduction percentages of immunofluorescent focus counts and dilutions of antiviral serum. This finding augurs well for the feasibility of estimating, rapidly and quantitatively, chikungunya serum neutralizing antibodies by the immunofluorescence virus assay procedure.

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