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ASSAY OF VARIOLA VIRUS BY THE FLUORESCENT CELL-COUNTING TECHNIQUE

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ASSAY OF VARIOLA VIRUS BY THE FLUORESCENT CELL-COUNTING TECHNIQUE

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ABSTRACT

A quantitative assay for infective variola virus particles was developed that is based on the enumeration of cells containing fluorescent viral antigen after infection of McCoy cell monolayers. The direct fluorescent antibody technique was employed to stain cells. The efficiency of virus adsorption was markedly enhanced by centrifugation of virus inoculum onto McCoy cell monolayers at 500 x g for 15 minutes. By this procedure, a proportionality was obtained between the number of fluorescent cells and volume of inoculum. Observations on the sequential development of viral antigen within cells and counts of fluorescent cells, showed the optimal time for the enumeration of fluorescent cells was after an incubation period of 16 to 20 hours. A linear function existed between virus concentration and cell infecting units. The assay was shown to be highly sensitive, precise, and reproducible. Fluorescent cells were randomly distributed in infected coverslip cell monolayers. The demonstration that the concentration of variola virus in aerosols can be determined within 24 hours exemplifies the rapidity and applicability of the fluorescent cell-counting technique.

I. INTRODUCTION

Since the introduction of the fluorescent cell-counting technique for the assay of Newcastle disease virus by Wheelock and Tamm,[1] the procedure has been applied successfully for the quantitative titration of adenovirus,[2] human cytomegalovirus,[3] and psittacosis virus.[4] In assaying those viruses, the technique has been shown to be sensitive, precise, reproducible, and rapid (results usually obtained in less than 24 hr). The applicability of the technique has been enlarged further with the development of a fluorescent cell-counting neutralization test to detect and to measure quantitatively psittacosis serum-neutralizing antibodies.* In view of the marked advantages and potential of the fluorescent cell-counting technique in virus studies, the feasibility of using this procedure to assay poxviruses was investigated.

This report describes the standardization of the fluorescent cell-counting technique for the quantitative assay of variola virus.

II. MATERIALS AND METHODS

A. VIRUS

The Yamada strain of variola virus was used throughout this study; its history has been described elsewhere.[5] Virus suspension was prepared by inoculation of the chorioallantoic membrane (CAM) of 11-day chick embryonated eggs with 5×10^3 pock-forming units(pfu) of virus. After incubation at 35 C for 48 hr, infected membranes were harvested, made into a 50% suspension with phosphate-buffered saline (PBS), pH 7.0, and homogenized in a Waring Blendor. The suspension was partially purified by two cycles of differential centrifugation, 780 x g and 8000 x g for 1 hr. After the second cycle, the pellet was suspended in PBS and the suspension distributed in 1-ml portions into glass vials and stored at -60 C. The virus suspension, assayed by the method of CAM inoculation of chick embryonated eggs and enumeration of pfu,[5] contained approximately 4×10^6 pfu per ml.

B. CELL LINE

The established cell line, McCoy, derived from human synovial tissue[6] was used in the fluorescent cell-counting assay for virus. Preliminary experiments employing high multiplicities of virus to cells indicated that more than 99% of the cells were susceptible to infection by variola virus.

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* Hahon and Cooke, unpublished results.

C. CELL CULTIVATION

Nutrient medium for the McCoy cell line consisted of mixture 199 containing 0.5% lactalbumin hydrolyzate, 10% heat-inactivated calf serum, 50 μg streptomycin, and 75 μg kanamycin. Cells were maintained in mixture 199 and 5% calf serum. For virus assay, cells were cultivated on circular coverslips (15-mm diameter) inserted in flat-bottomed glass vials (18 by 100 mm). One ml of cell suspension, containing 1×10^5 to 3×10^5 cells, was introduced onto coverslips that were then incubated at 35 C for 24 hr or until a complete cell monolayer was formed. Coverslip cell cultures were washed twice with 2 ml of maintenance medium prior to the addition of virus inoculum.

D. VIRUS ASSAY

Assays were usually carried out in duplicate or triplicate. Virus dilutions were prepared in maintenance medium and introduced in 0.2-ml volumes directly into vials containing coverslip cell cultures. Routinely, virus adsorption was carried out by centrifugation at 500 x g for 15 min, at 23 to 25 C. For this procedure, vials were placed in slotted cups containing tube adapters, sealed with a screw-dome cover, and mounted on a 4-place, pin-type head. Centrifugation was performed in an International centrifuge,* size 2, model V. Coverslip cultures were rinsed twice with maintenance medium following adsorption of virus; 1 ml of the medium was added then to each vial. After incubation for 16 to 20 hr at 35 C, coverslips were rinsed twice with cold PBS, fixed with cold (-60 C) acetone, and either prepared immediately for immunofluorescent staining and cell-counting or stored at -60 C for subsequent examination.

In addition to the fluorescent cell-counting technique, virus was assayed by the methods of CAM inoculation of embryonated eggs[5] and in roller tube cell cultures.[7] In this study, the latter procedure was slightly altered; adsorption of virus inoculum onto McCoy cell monolayers was carried out with tubes maintained in a stationary position at 35 C for 2 hr. After an incubation period of 6 days, the tissue culture 50% infective dose ($TCID_{50}$), based on cytopathic changes produced in cell monolayers by virus inoculum, was calculated by the method of Reed and Muench.[8]

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* International Equipment Company, Nedham Heights, Massachusetts.

E. VARIOLA ANTISERUM CONJUGATE AND TECHNIQUE OF IMMUNOFLUORESCENT STAINING

For the preparation of antiserum, rabbits* were immunized initially by multiple scarification with variola virus. When the cutaneous lesions had healed, rabbits were inoculated intraperitoneally with 1 ml of virus containing 5×10^7 pfu in a series of four injections given at two-week intervals. The animals were bled approximately one month after the last injection. Antiserum was conjugated with fluorescein isothiocyanate by the method of Riggs et al.[9] The conjugate was adsorbed twice with mouse liver powder and used undiluted for staining.

The direct fluorescent antibody technique was employed to demonstrate immunofluorescence in infected cells. Fixed cell cultures were washed three times with PBS and stained with the conjugate for 30 min. The coverslip cell cultures were then rinsed in three changes of PBS to remove excess conjugate and mounted in 10% glycerol in PBS.

F. FLUORESCENCE MICROSCOPY

Coverslip cell cultures were examined with an American Optical microscope equipped with a Fluorolume illuminator, model 645, Corning 5840 and Schott BG-13 exciter filters, and an E.K. 24 barrier filter.

G. FLUORESCENT CELL-COUNTING AND CALCULATIONS

At 430 X magnification, 1064 microscopic fields were contained in the area of a 15-mm coverslip with the optical system employed. For each coverslip cell culture, 50 microscopic fields were examined for fluorescent cells. To calculate the number of cell-infecting units (CIU) of virus per ml, the average number of fluorescent cells per field was multiplied by the number of fields per coverslip, the reciprocal of the dilution of virus inoculum, and a volume factor (for conversion to ml).

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* In conducting the research described in this report, the investigators adhered to the "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

III. RESULTS

A. VIRUS ADSORPTION

The applicability of centrifugal force for promoting adsorption of virus onto cell monolayers was investigated since previous studies with psittacosis virus confirmed the efficiency of centrifugation for this purpose.[4] An experiment was performed to determine the rate of virus adsorption onto cell monolayers during stationary incubation (35 C) and centrifugation (500 x g, 25 C). Because comparable fluorescent cell counts were obtained with the use of centrifugal force of 500 x g for 30 min or 1250 x g for 15 min during virus adsorption, the former condition of centrifugation was selected for this test. To vials containing coverslip cell cultures, 0.2 ml of a 10^{-3} dilution of virus suspension was added. Vials were divided into two groups; each group was subjected to a different condition of virus adsorption. At designated intervals during the adsorption period, vials were removed and cell cultures were then treated in the manner described for virus assay.

The per cent of virus adsorbed during each interval with each procedure is shown in Figure 1. Within 15 min, virus adsorption carried out with the aid of centrifugation appeared to be complete, whereas approximately 60% was adsorbed during stationary incubation at 35 C for 2 hr. Since the efficiency and rapidity of virus adsorption onto cell monolayers carried out by the use of centrifugal force was clearly superior to results obtained with stationary incubation, centrifugation at 500 x g for 15 min at room temperature was adopted as the standard procedure for virus adsorption.

The efficiency of centrifugation for infecting cell monolayers from different volumes of inoculum is given in Table 1. The results revealed a proportionality between the number of fluorescent cells and volume of inoculum. The rate of cell-virus contact appeared to be independent of the volume of inoculum employed in this experiment when virus adsorption was carried out with the aid of centrifugal force.

B. INCUBATION PERIOD

As early as 6 hr after the introduction of viral inoculum, a faint fluorescent intracytoplasmic center was seen in a few cells. At 8 hr, foci of fluorescence could be distinguished readily at the periphery of the nucleus (Fig. 2). Examination of cell cultures at 16 and 20 hr revealed that the amount and intensity of fluorescence in cells had increased markedly. Fluorescence was diffused throughout the entire cell cytoplasm (Fig. 3), but none was discernible in the nucleus. Lengthy cytoplasmic extensions were common. Fluorescent cells could be easily enumerated during this period. At 24 hr, several rounded cells were present. Fluorescence in these and other cells was dense and granular in appearance at the

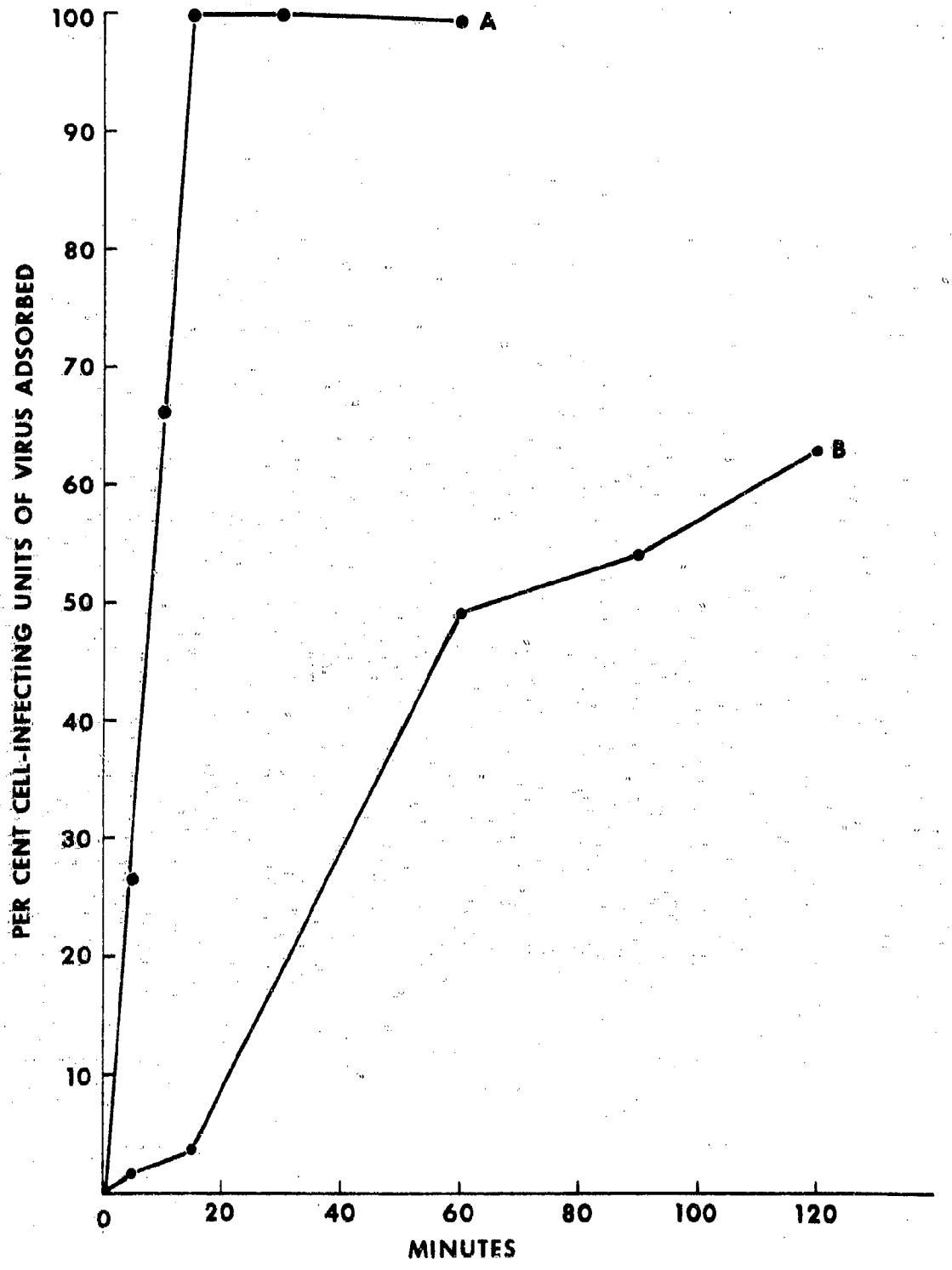

Figure 1. Adsorption of Variola Virus onto Coverslip Cultures of McCoy Cells by (A) Centrifugal Force (500 x g, 25 C) and at (B) Stationary Incubation (35 C).

periphery of the nucleus (Fig. 4). Rounded fluorescent cells were more numerous at 30 hr (Fig. 5) and various stages of constriction of cytoplasmic extensions were seen. At 48 hr, cytoplasmic extensions were generally absent. Rounded cells were abundant at that time and their aggregation tended to form fluorescent foci. Occasionally, minute fluorescent extracellular particles were noted and some cells possessed a fluorescent intracytoplasmic center that suggested a second cycle of virus infection. In general, the cell monolayer at 48 hr was disorganized.

Counts of fluorescent cells were comparable between 16 to 24 hr after infection; the number of fluorescent cells was twofold to threefold higher at 30 and 48 hr, respectively. That the fluorescence noted in cells was specific for viral antigen was confirmed by the serum-neutralization reaction. Fluorescent cells were sparse or absent when mixtures of virus and antiserum were inoculated onto cell monolayers; numerous fluorescent cells were noted when a mixture of virus and normal serum was used as inoculum. Based on the sequential development of viral antigen within infected cells and fluorescent cell counts, the optimal period for incubation of inoculated cell monolayers was established as 16 to 20 hr.

TABLE 1. PROPORTIONALITY BETWEEN VOLUME OF INOCULUM AND CELL-INFECTING UNITS OF VARIOLA VIRUS

Volume, ml	Fluorescent Cells per 50 Fields	CIU,[a] 10^7 per ml
0.1	80	1.7
0.2	169	1.5
0.4	334	1.7
0.5	426	1.8
0.8	677	1.7
1.0	884	1.8

a. Cell-infecting units of virus.

Figure 2. Fluorescent Variola Virus Antigen in Infected McCoy Cell Monolayers (500X). Minute Areas of Fluorescence Seen 8 hr after Infection.

Figure 3. Fluorescent Variola Virus Antigen in Infected McCoy Cell Monolayers (500X). Single Cell Exhibiting Lengthy Cytoplasmic Extensions with Diffuse Fluorescence Seen 16 hr after Infection.

Figure 4. Fluorescent Variola Virus Antigen in Infected McCoy Cell Monolayers (500X). Numerous Fluorescent Cells Seen 24 hr after Infection. Note Dense and Granular Appearance of Fluorescent Viral Antigen.

Figure 5. Fluorescent Variola Virus Antigen in Infected McCoy Cell Monolayers (500X). Rounded Fluorescent Cells Commonly Seen 30 hr after Infection. Two Brightly Fluorescent Cells Are in the Center of the Field.

C. QUANTITATIVE EVALUATIONS OF THE ASSAY

Results in Figure 6 reveal a linear relationship between twofold dilutions of virus over a range of 1.8 log units and the number of cell-infecting units of virus. These data suggest that each fluorescent cell was the consequence of infection by a single infective virus particle.

In a single experiment, ten determinations were made to estimate the precision of the fluorescent cell-counting procedure for variola virus. Cell cultures were infected by a standard quantity of virus inoculum and incubated in the prescribed manner. The number of cell-infecting units of virus per ml of inoculum ranged from 1.0×10^7 to 1.5×10^7 with a mean of 1.2×10^7. The standard deviation of 0.21 compared favorably with that obtained in studies with psittacosis virus.[4]

The sensitivity of the cell-counting procedure was compared with two other methods for estimating the concentration of variola virus — that of enumerating pock-forming units on the CAM of chick embryonated eggs, and that of observing cytopathic changes produced in roller tube cultures as an index of virus infectivity. The results presented in Table 2 indicate that the sensitivity of the fluorescent cell-counting technique, within the limits of the doses employed, was superior to the other two procedures of assay. In addition, less variation occurred among determinations by the cell-counting procedure. One other assay technique for variola virus was employed that is based on the enumeration of hyperplastic foci.[10] The assay, carried out by Pirsch and Purlson of USABL, gave an average titer of 6.8×10^8 focus-forming units per ml for three determinations.

The reproducibility of the fluorescent cell-counting technique using twofold dilutions of virus inoculum in each of four determinations is shown in Table 3. The results attest to the reproducibility of the assay at the concentration levels employed.

The mode of distribution of fluorescent cells on a coverslip cell monolayer previously infected with virus after 20 hr of incubation was determined by examining 300 random microscopic fields. Observed frequencies of fluorescent cells corresponded closely to theoretical frequencies (Fig. 7). Since there was no significant departure from the theoretical Poisson distribution ($X^2 = 3.218$ with $df = 5$), the distribution of fluorescent cells on coverslip cultures was random.

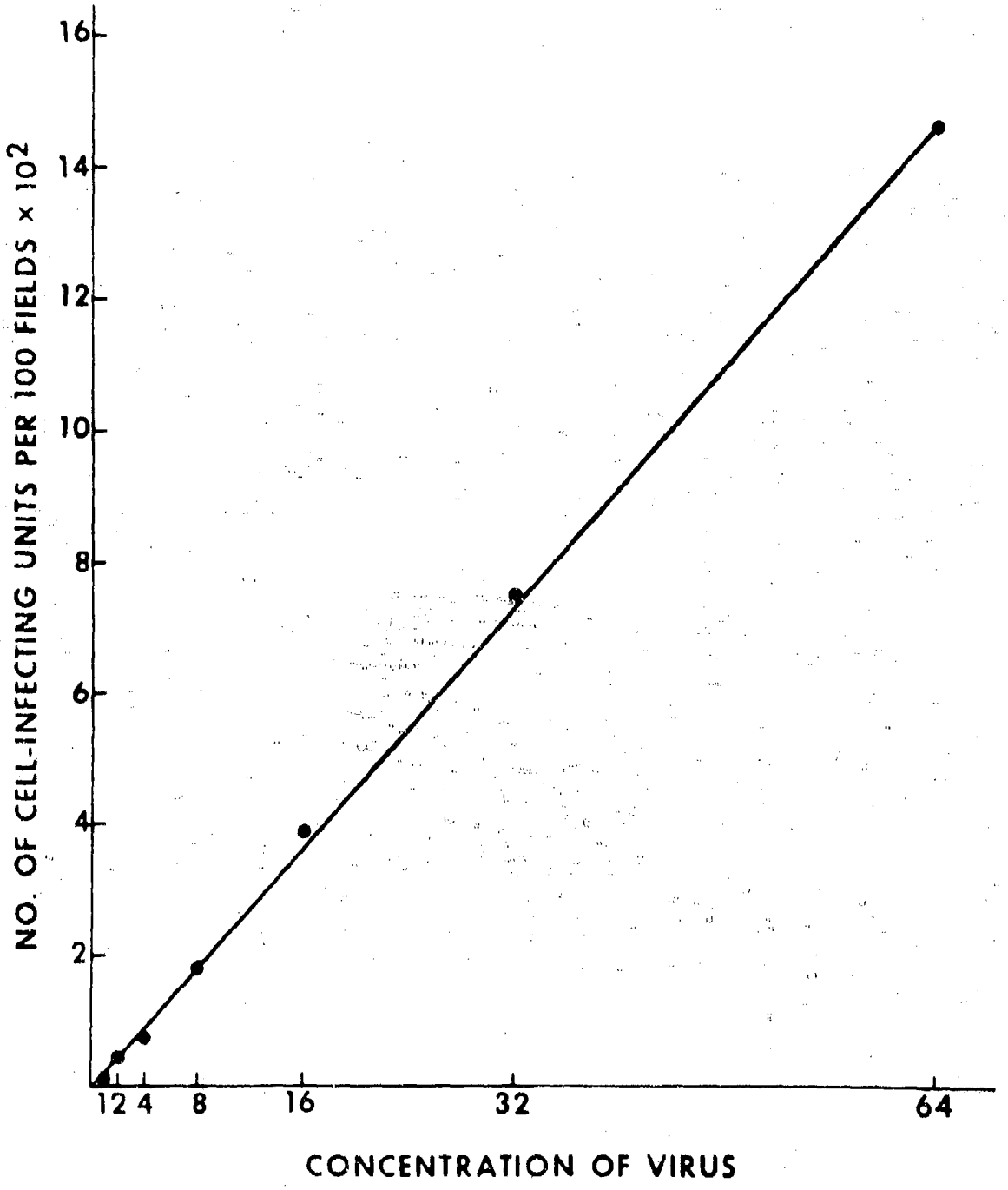

Figure 6. Linear Function Between the Number of Cell-Infecting Units and the Concentration of Variola Virus.

TABLE 2. COMPARISON OF DIFFERENT METHODS FOR THE ASSAY OF VARIOLA VIRUS

Assay	CIU,[a] 10^6 per ml	pfu,[b] 10^6 per ml	$TCID_{50}$,[c] 10^6 per ml
1	10.0	3.9	5.2
2	11.0	2.5	10.0
3	10.0	3.0	5.2
4	15.0	3.4	3.0
5	10.0	2.3	7.9
6	12.0	3.2	10.0
7	14.0	4.8	3.0
8	15.0	4.6	2.5
9	14.0	4.0	1.6
10	10.0	3.0	1.6
Mean	12.0	3.4	5.0
Standard deviation	±0.21	±0.84	±3.26
Standard error of mean	0.15	0.29	0.57

a. Cell-infecting units of virus determined by fluorescent cell-counting technique at 20 hr.
b. Pock-forming units of virus determined by CAM inoculation of chick embryonated eggs, 3-day incubation period.
c. Tissue culture 50% infective dose determined by cytopathic changes in roller tube cultures of McCoy cells, 6-day incubation period.

TABLE 3. REPRODUCIBILITY OF ASSAY FOR VARIOLA VIRUS BY THE FLUORESCENT CELL-COUNTING TECHNIQUE

Assay	10^2 fluorescent cells per 50 fields: Dilution of virus in inoculum					CIU,[a] 10^7 per ml
	5.1×10^{-2}	2.56×10^{-2}	1.28×10^{-2}	6.4×10^{-3}	3.2×10^{-3}	
1	10.47	5.64	2.49	1.32	0.43	2.1
2	10.84	5.48	2.74	0.85	0.46	2.0
3	10.20	5.79	2.53	0.98	0.48	1.9
4	9.98	5.62	2.10	0.96	0.40	1.8
Mean	10.37	5.63	2.46	1.02	0.44	1.9

a. Cell-infecting units of virus.

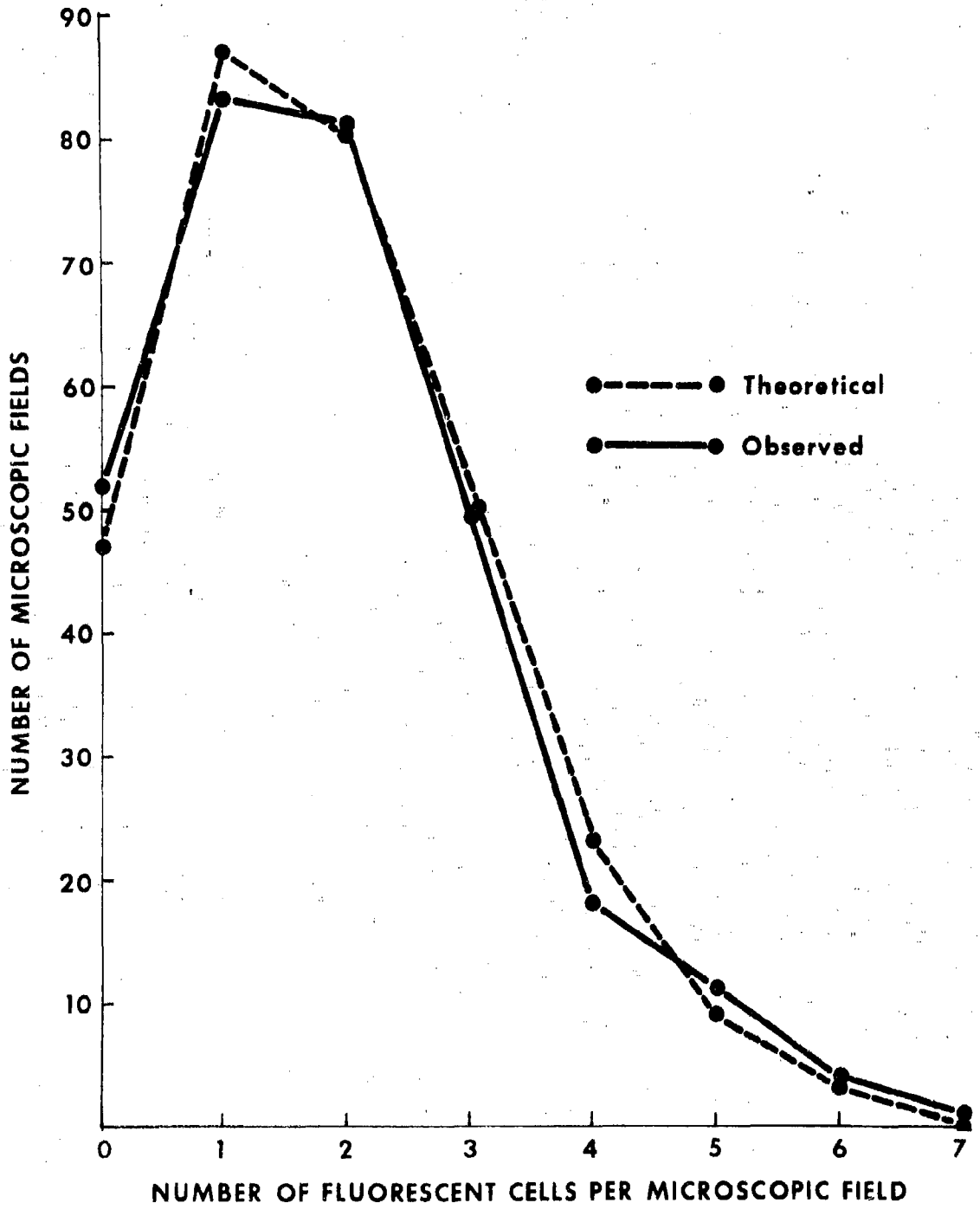

Figure 7. Frequency Distribution of Fluorescent Cells on Monolayers of McCoy Cells Infected with Variola Virus.

D. ASSAY OF VIRUS AEROSOLS

The practicality of the fluorescent cell-counting procedure for estimating the concentration of variola virus in aerosols was investigated. Details and results of four replicate tests are shown in Table 4. These data indicate that the assay was capable of detecting and measuring low concentrations of the virus in aerosols. A singular advantage of the assay was the short time required for assessment. All virus aerosols were assessed within 24 hr.

TABLE 4. ESTIMATION OF THE CONCENTRATION OF VARIOLA VIRUS IN AEROSOLS BY THE FLUORESCENT CELL-COUNTING TECHNIQUE

Test	Concentration per impinger fluid,[a] per cent recovery — Cloud Age, minutes				Decay, per cent per minute (0-60 min)
	0	15	30	60	
1	$\frac{7.4 \times 10^3}{2.5}$[b]	$\frac{6.3 \times 10^3}{2.2}$	$\frac{5.3 \times 10^3}{1.8}$	$\frac{4.2 \times 10^3}{1.4}$	0.92
2	$\frac{9.5 \times 10^3}{3.3}$	$\frac{8.5 \times 10^3}{2.9}$	$\frac{6.3 \times 10^3}{2.2}$	$\frac{6.3 \times 10^3}{2.2}$	0.67
3	$\frac{7.4 \times 10^3}{2.5}$	$\frac{6.3 \times 10^3}{2.2}$	$\frac{6.3 \times 10^3}{2.2}$	$\frac{5.3 \times 10^3}{1.8}$	0.56
4	$\frac{6.3 \times 10^3}{2.1}$	$\frac{5.3 \times 10^3}{1.8}$	$\frac{5.3 \times 10^3}{1.8}$	$\frac{4.2 \times 10^3}{1.4}$	0.67
Mean	2.6	2.2	2.0	1.7	0.70

a. Based on volume of 10 milliliters.
b. Cell-infecting units of virus; volume of inoculum used for assay was 0.2 milliliters.

For each test, a virus cloud was formed by disseminating 10 milliliters of a 20% infective CAM suspension in heart infusion broth with an FK-8 gun into a 1500-liter rotating drum (3.5 rpm). The total quantity of virus disseminated was 1.2×10^7 cell-infecting units. One virus pool was used for all tests.

The internal environment of the drum was 27 C and 20% RH. Clouds were sampled with miniature Shipe impingers for two minutes using an orifice with flow rate of 18 liters per minute. Impinger fluid consisted of McCoy cell culture medium with one to two drops of sterile olive oil.

IV. DISCUSSION

Although both the direct and indirect fluorescent antibody techniques have been employed successfully for diagnostic purposes to demonstrate viral antigen in smears from smallpox lesions,[11-14] the techniques have not been utilized for the quantitative determination of infective virus particles. The development of an assay for variola virus by the fluorescent cell-counting technique that is highly precise, sensitive, and reproducible extends the scope of the technique for the assay of additional viruses. An outstanding feature of the procedure is the ability to obtain estimates of virus infectivity in less than 24 hr. That the technique may be applicable to other poxviruses is suggested from preliminary experiments made in the course of this investigation. Fluorescent viral antigen was noted in McCoy cell monolayers after infection with either cowpox, monkeypox, alastrim, vaccinia, or rabbitpox viruses. The similar appearance and distribution of fluorescent viral antigen in the cytoplasm of cells infected by these poxviruses, however, precludes their differentiation from one another on the basis of immunofluorescence.

The use of centrifugal force in the fluorescent cell-counting procedure for promoting adsorption of virus inoculum onto cell monolayers, demonstrated in this study and, previously, with psittacosis virus,[4*] proffers several advantages over adsorption carried out in a stationary position at 35 C for several hours. By the former procedure, adsorption is efficient and rapid (complete within 15 min at 25 C), the effect of thermal inactivation on infective virus is minimized, and the ability to detect virus particles from dilute suspensions may be enhanced by increasing the volume of inoculum. These features contribute to the sensitivity of the assay. Virus adsorption augmented by centrifugation also provides for the synchronous infection of cells by virus. This is a desirable circumstance in studies to characterize the various developmental stages involved in the formation of mature virus particles.

The incubation period is an integral part of the fluorescent cell-counting technique. In this study, it is defined as the interval between virus inoculation and the development of recognizable quantities of fluorescent viral antigen in cells. Ideally, this period should be terminated before newly synthesized virus particles are released (to prevent the secondary infection of cells) but should be sufficient to insure the formation of substantial amounts of viral antigen in primary infected cells. The concomitant increase in density of fluorescent viral antigen, and the uniformity of fluorescent cell counts from 16 to 20 hr after infection established this interval as the incubation period. The time sequence of the development of viral antigen, visualized by fluorescent antibody staining, was in agreement with the observations reported on the formation of variola virus antigen in monkey kidney cells[11] and, in general, on the development of a related poxvirus, vaccinia.[15-17]

* Hahon and Cooke, in press.

In addition to the assay of variola virus and other poxviruses, the fluorescent cell-counting technique is applicable to other facets of research with these viruses. Preliminary tests to determine the viral specificity of fluorescent cells revealed that the neutralization of variola virus infectivity by immune serum resulted in a significant reduction of fluorescent cell counts. A quantitative linear relationship between the reduction of fluorescent cells and dilution of immune serum has been reported previously.[4] These findings augur the feasibility of developing a rapid fluorescent cell-counting neutralization test to measure poxvirus serum-neutralizing antibodies. Based on the same principle of a reduction of fluorescent cell counts, the usefulness of the technique may be extended also to the quantitative assay of interferon that is produced by viruses.

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