IMMUNOFLUORESCENCE IN THE STUDY OF VIRUSES IN TISSUE CULTURE

by

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"Fluorescent antibodies, whatever their scientific merits, are very attractive under the microscope. They shine in the dark, a brilliant greenish-yellow. Like pebbles in the moonlight, they weave a pattern in the forest which leads the weary children home."

- A.H. Coons (1960).

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GENERAL INTRODUCTION

The imperfect nature of biology as a science is mainly due to a basic complexity of all forms of living entities. But since viruses are relatively simpler forms of animate beings, it is the hope of many that a great deal about life in general will be unravelled by their patient and systematic pursuit. Such hopes seem well-founded in view of the fact that viruses have already become indispensible as objects of study in genetics, biochemistry and molecular biology. As a result, these sciences now constitute the most rapidly advancing fronts in modern biology.

While the importance of this aspect of virus research cannot be over-emphasized, it tends to overshadow the role viruses play as agents of disease. Virus diseases still represent the most frequent of all microbial infections (Horsfall, 1965). Until recently, this situation was simply regarded as hopeless. Apart from certain prophylactic measures, very little could be done to treat or suppress ailments caused by viruses. However, this gloomy picture is now changing as continued efforts to find anti-viral therapeutic agents are slowly being rewarded (Westwood, 1966). Virus chemotherapeutic agents are emerging "from the realm of laboratory curiosities to a position in which they are being used to treat diseases of man" (Kaufman, 1965).

Being able to treat virus diseases necessitates their prompt laboratory diagnosis. The present techniques employed for this purpose are slow and cumbersome. As a result, in the majority of the cases, laboratory diagnosis of virus diseases is nothing more than an academic exercise, the delayed outcome being of little value to physician or patient. This situation, however, is undergoing a rapid change, and in the not too distant future, a quick identification of the etiologic agent could mean the difference between recovery from the disease and death or permanent disability. Therefore, the need for rapid and reliable techniques for laboratory diagnosis of virus diseases is being seriously felt.

Because of faster air travel, certain areas with susceptible human and animal populations are in constant danger of being exposed to infectious diseases from places where such diseases are endemic. Among virus diseases of animals, African swine fever has, in recent years, spread from its established home in Africa to become a serious problem in other parts of the world with highly susceptible swine populations (Frank, 1967). It is imperative to have accurate and rapid methods available for the identification of such infective agents to forestall explosive epidemic situations.

Immunofluorescence has proved particularly promising in the rapid laboratory diagnosis of infectious diseases. The wide acceptance it has gained in recent years in the diagnosis of bacterial, mycotic and parasitic diseases is a testimony to its promise and efficiency in these fields. Reports have also appeared concerning its successful application in laboratory diagnosis of virus diseases. Encouraging as these may be, diversity of techniques used by the various investigators makes it very difficult to generalize these findings and conclusions. Moreover, there is not enough known about the relative sensitivity and reliability of this technique to support its general acceptance as a diagnostic tool. Such knowledge is important before it could be fully trusted for the diagnostic analysis of specimens suspected of containing viruses. The work to be reported here was initiated to fill this gap in our knowledge.

The experimental approach was aimed at:

(1) standardizing the fluorescent antibody technique, so that a uniform procedure could be adopted for the study of many different viruses of medical importance;

(2) testing suspensions of known viruses at varying concentrations to determine the earliest time at which specific fluorescence could be used as an index for ascertaining the presence and identity of the infecting agent in tissue culture.

The present study also included experiments on:

(3) the development of a tissue culture system for this laboratory;

(4) the use of plastic chambers for the cultivation of cells on standard microscope slides;

(5) development of a system for microscopic scoring of immunofluorescent cells or immunofluorescent foci in virus-infected tissue cultures;

(6) use of goat milk antibodies in the direct fluorescent antibody staining of mumps virus in infected tissue culture.

DEVELOPMENT OF EXPERIMENTAL METHODS

(A) DEVELOPMENT OF A TISSUE CULTURE SYSTEM

SUMMARY

A tissue culture system has been established for this laboratory. It has been shown that a medium based on pancreatic digest of beef, termed "tryptic meat broth," forms a suitable basis for the cultivation and maintenance of established and primary cell cultures. Use of this medium has resulted in considerable simplicity in working with cells and viruses, since this medium could be prepared in the laboratory and could be used as a growth, maintenance and overlay medium simply by altering its concentration and ratio with the serum.

In comparative tests, this medium has been shown to be superior to medium 199 in the cultivation and maintenance of HeLa, KB and BS-C-1 cells.

INTRODUCTION

A convenient and dependable system for the cultivation and maintenance of cells *in vitro* is an essential prerequisite for studies of virus growth and replication. Whereas the choice of a system is dictated by the specific problem under consideration, for routine and large scale investigations, economy, simplicity, versatility and efficiency are among the important criteria to be considered.

Even though considerable progress has been made in recent years in the development of chemically defined media for cell culture (Healey and Parker, 1966a; 1966b), they can only be used either as maintenance media or as basal media that are rendered more adequate by the addition of serum or other protein supplements. Moreover, so far there is no indication that cultures of primary cells can be grown in any of the synthetic media in common use (Paul, 1965).

While the use of such media may be necessary for nutritional and biochemical studies, it is still convenient to depend on naturally occurring substances for the cultivation and maintenance of cells *in vitro*. Many such media are not only readily available, but they are inexpensive and free from contaminating viruses and natural antibody (Parker, 1962).

Westwood *et al*. (1957) reported such a medium based on Hartley's pancreatic digest of beef; they found it superior to any commercial naturally occurring or synthetic product tested for giving maximal cell replication and high susceptibility to virus infection. In establishing a tissue culture system for this laboratory it was therefore decided to re-evaluate and compare the merits of this medium with a variety of natural and chemically defined media available commercially.

This medium will be referred to as tryptic meat broth (TMB) throughout this report.

Comparative tests were conducted to determine the suitability of TMB as:

1) a growth medium in comparison with other media,
2) a maintenance medium, and
3) an overlay medium for virus-infected cultures.

Besides these studies, the work reported in this section also includes experiments carried out to determine the effect of heat inactivation of serum and serum concentration on the growth of cells.

Since the object of this work was to establish an efficient medium as rapidly as possible, exhaustive comparisons and repetitions of clear-cut results were not always performed; however, most results were confirmed at least once.

MATERIALS AND METHODS

CELLS: HeLa (Gey *et al*., 1952), KB (Eagle, 1955) and BS-C-1 (Hopps *et al*., 1963) cells were obtained through the courtesy of Dr. A.C. Laing of the Defence Research Board of Canada. Medium 199 with 10% calf serum was being used for the cultivation of these cells at the time of their receipt in this laboratory.

Primary African green monkey (*Cercopithecus aethiops*) kidney cells were purchased as a weekly shipment from Connaught Medical Research Laboratories, Toronto, Ontario. These cells were received as a suspension in lactalbumin hydrolysate + 2% calf serum.

The detailed procedures for the passage and maintenance of these cells will be described elsewhere in this section.

MEDIA: Earle's Balanced Salt Solution (EBSS): EBSS was purchased as a dried powder from Grand Island Biological Co. (GIBCO), 3175 Staley Road, Grand Island, New York 14072. It was made up to contain 1.4 g per liter of sodium bicarbonate.

Medium 199 (Earle's base): This medium (Morgan *et al*., 1950) was also purchased in a powdered form (GIBCO) and was reconstituted to contain 1.4 g/liter of sodium bicarbonate.

<u>Tryptic Meat Broth (TMB)</u>: Initially this medium was prepared in our own laboratory according to the procedure outlined in the 'Appendix'. Since the medium has now become commercially available, the last few batches were purchased from Qualicum Labs., Ottawa, Ontario.

Preliminary tests showed that this medium was best suited for cell cultivation when used at 3% concentration.

<u>Calf Serum</u>: In earlier experiments, the serum used was processed in this laboratory from calf blood obtained through the courtesy of Canada Packers Ltd. Later on, however, this was replaced with fetal calf serum purchased from GIBCO.

<u>Commercial Digests</u>: Tryptic Digest Broth (TDB), Multipeptone (MP) and Aminozyme (AZ) were purchased from Fisher Scientific Co., 8505 Devonshire Road, Montreal 9, P.Q. They were made up according to the manufacturer's instructions and used as 'additives' to give media with the following general composition:

Difco's Yeast extract (2% solution) ----	5.0 ml.
Additive (1% solution) ----------------	5.0 ml.
Calf serum ----------------------------	10.0 ml.
EBSS ----------------------------------	to 100.0 ml.

Antibiotic Mixture: A thousand times concentrated stock solution of a mixture of penicillin and streptomycin (Nutritional Biochemical Corp., Cleveland 28, Ohio) was prepared. This antibiotic mixture was added to all media prior to their use to give a final concentration of 100 units of penicillin and 100 µg of streptomycin/ml.

Trypsin-Versene Solution: A mixture of trypsin (Nutritional Biochemical Corp.) and Versene (Fisher) was made up in calcium-magnesium free phosphate buffered saline (pH 7.2). The exact formula and the method of preparation of this mixture are given in the 'Appendix'.

All through these experiments this mixture was used to detach cells from the glass.

METHODS:

One ounce Brockway bottles were used for all the experiments on comparison of media. Approximately 100,000 cells, suspended in 3.0 ml. of medium, were introduced into each bottle. Using a hemocytometer, cell counts were carried out on replicate cultures at appropriate intervals. Cultures were also examined daily under the microscope to note their appearance.

EXPERIMENTAL AND RESULTS

COMPARISON OF TMB-YEAST EXTRACT MEDIUM AND COMMERCIAL DIGESTS AS GROWTH MEDIA FOR KB CELLS:

In this series of experiments, TMB, TDB, MP and AZ were compared as growth media for KB cells. The results obtained are summarized in Table TC-I. As is evident from the figures, TMB is at least 3 to 4 times more effective than the other media tested in supporting the replication of KB cells.

EFFECT OF HEAT INACTIVATION ON THE CELL GROWTH PROMOTING PROPERTIES OF CALF SERUM:

It is common practice to heat 'inactivate' serum used as a supplement in tissue culture media. This practice is partly traditional and partly a serum toxicity-removing measure of doubtful efficacy (Paul, 1965; Cailleau and Kirk, 1957). However, the following set of experiments indicate that heating the serum is deleterious to its cell growth promoting properties.

A series of calf serum aliquots were heated in an accurately controlled water bath for three different periods of time (15, 30 and 60 minutes) at three different temperatures (52, 56 and 60°C). Nine different test media were thus prepared by adding each serum sample at 10% concentration to aliquots of HeLa cell suspension in a basal medium

TABLE TC-I

COMPARISON OF THREE COMMERCIAL DIGESTS WITH TMB FOR THE GROWTH OF KB CELLS

(Basal Medium - 0.1% yeast extract and 10% calf serum in EBSS)

Days of Incubation	Mean Count ($X10^5$)/Culture (Initial Inoculum 1.2 X 10^5 cells/culture)			
	Medium Supplements			
	TMB	TDB	MP	AZ
1	1.78	1.50	1.20	1.60
2	3.37	1.95	2.47	2.88
5	14.10	3.80	4.98	4.28
8	10.85	1.28	2.34	2.75

TMB - Tryptic Meat Broth

TDB - Tryptic Digest Broth

MP - Multipeptone

AZ - Aminozyme

(3% TMB and 0.1% yeast extract in EBSS). After three days of incubation (36°C), the cells from replicate cultures of each set were counted. The figures are presented in Table TC-II. Increase in both time and temperature affected the growth promoting properties of calf serum, as indicated by a progressive decrease in cell counts.

On the basis of these results, all future experiments were conducted using uninactivated calf serum.

<u>EFFECT OF SERUM CONCENTRATION ON THE GROWTH OF HeLa CELLS</u>:

Since calf serum was used as an essential supplement of TMB-yeast extract medium, it was of interest to determine the serum concentration which would give the maximum rate of cell replication.

Sets of replicate cultures were put up containing 0, 1, 2.5, 5, 7.5 and 10% of uninactivated calf serum. After four days of incubation, cell counts were made on representative cultures. The results are shown in Fig. TC-1. The curve suggests that a further increase in the rate of cell replication could be achieved with even higher concentration of serum than 10%; however, the mean doubling time for HeLa cells at this concentration of calf serum was 46.5 hours which was considered satisfactory for the purpose of the present investigation.

TABLE TC-II

EFFECT OF HEAT INACTIVATION ON THE CELL GROWTH PROMOTING PROPERTIES OF CALF SERUM

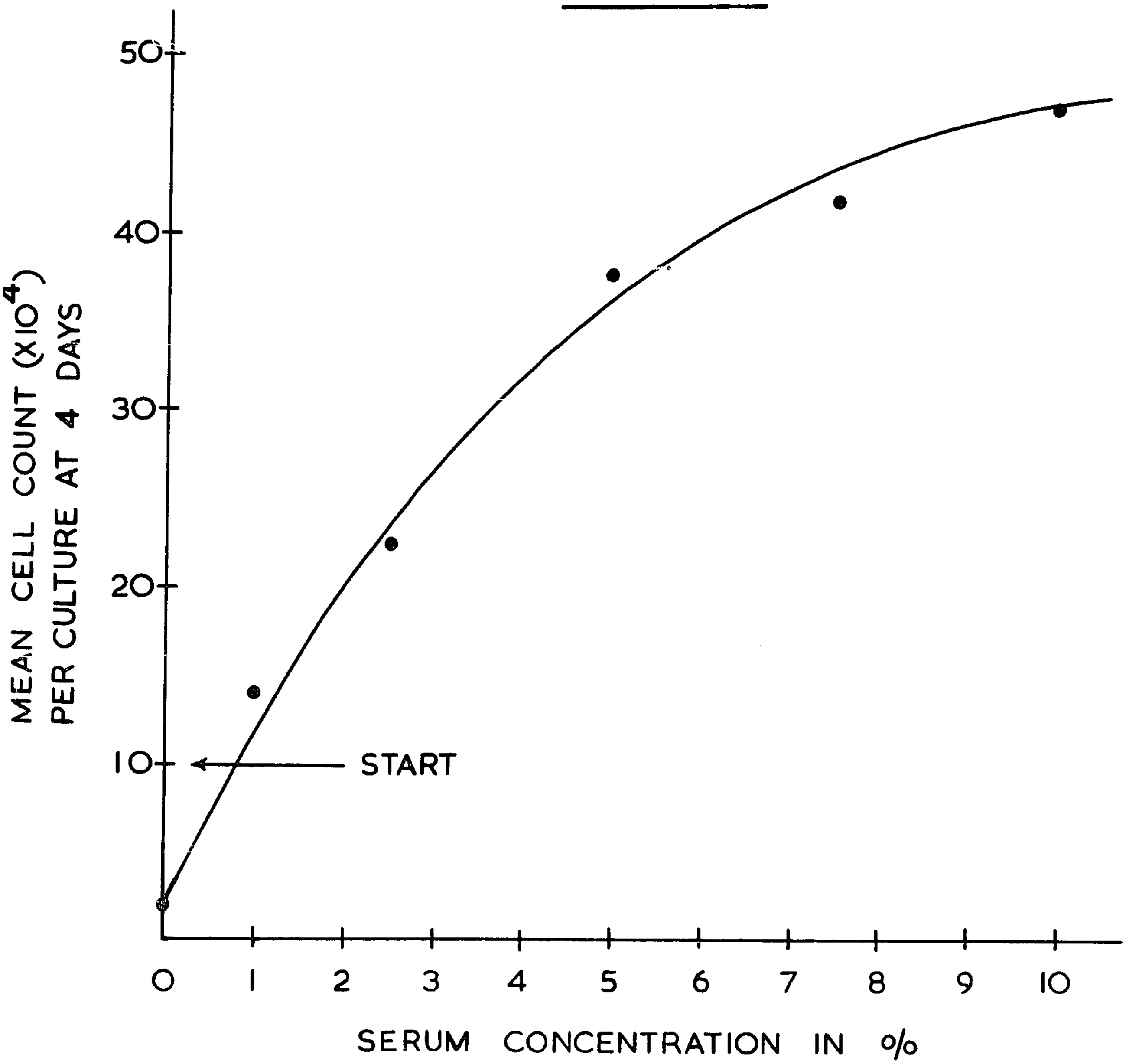
(Basal Medium 3% TMB + 0.1% Yeast Extract)

Inactivation Temperature (°C)	Mean count ($X10^4$)/culture at 3 days (Initial inoculum 10 X 10^4 cells per culture)				
	Time of heating (minutes) 15	30	60	Total	Mean
52	40.0	35.0	32.8	107.8	35.9
56	36.5	29.4	28.1	94.0	31.3
60	27.0	28.6	28.1	83.7	27.9
Total	103.5	93.0	89.0	285.5	-
Mean	34.5	31.0	29.7	-	31.7

FIG. TC-1

EFFECT OF CALF SERUM CONCENTRATION ON REPLICATION OF HeLa CELLS

FIG. TC-1



MEAN CELL COUNT (X10⁴) PER CULTURE AT 4 DAYS
50
40
30
20
10
START
0
1
2
3
4
5
6
7
8
9
10
SERUM CONCENTRATION IN %

EFFECT OF THE INDIVIDUAL COMPONENTS OF TMB-YEAST EXTRACT MEDIUM ON THE REPLICATION OF KB CELLS:

The following experiment was conducted to assess the contribution of the individual components of this medium in promoting the growth of KB cells.

Cultures in separate components of the medium, alone and in various combinations, were set up as shown in Table TC-III. Cell counts were recorded after four days of incubation.

These results suggest, when considered with those shown in the next experiment, that, though yeast extract is a useful addition to a medium consisting of EBSS and serum alone, it is not as good an additive as TMB. In the presence of TMB, yeast extract appeared to be either ineffective or toxic to the cells. This observation was checked and further clarified in the next experiment.

EFFECT OF YEAST EXTRACT ON THE REPLICATION OF KB CELLS:

In earlier experiments yeast extract was included in the growth medium, along with 3% TMB and 10% calf serum, as a source of accessory factors. Its value as a supplement to this medium was tested directly in the following experiment.

Yeast extract was added in various concentrations (0-0.2%) to KB cells suspended in 3% TMB + 10% calf serum. After four days of incubation, cell counts were made on replicate cultures from each set. The results are recorded

TABLE TC-III

EFFECT OF THE INDIVIDUAL COMPONENTS OF TMB-YEAST EXTRACT MEDIUM ON THE REPLICATION OF KB CELLS

Medium	Mean count ($X10^3$)/culture at 4 days (Initial inoculum 30 X 10 cells/culture)
1. EBSS alone	0
2. EBSS + 10% calf serum	80
3. EBSS + 3% TMB	6
4. EBSS + 0.1% yeast extract	1
5. EBSS + 3% TMB + 0.1% yeast extract	3
6. EBSS + 3% TMB + 10% calf serum	204
7. EBSS + 0.1% yeast extract + 10% calf serum	116
8. EBSS + 3% TMB + 0.1% yeast extract + 10% calf serum	97

in Table TC-IV. These results suggest that in fact yeast extract makes no possible contribution to the medium and is actually toxic above a concentration of 0.025%. This finding is in agreement with the results shown in the previous experiment.

Therefore, yeast extract was omitted from this medium, and as a consequence the final growth medium adopted for further experimentation consisted of:

TMB ----------------------------- 3.0 ml.

Calf serum (uninactivated) ------ 10.0 ml.

EBSS -------------------------to 100.0 ml.

COMPARISON OF TMB AND 199 AS GROWTH MEDIA FOR KB, HeLa AND BS-C-1 CELLS:

The work so far served to define a simple growth medium which would support cell replication better than any of the commercial digests tested. The second phase of this investigation involved the comparison of this medium with a commercially available synthetic medium. Medium 199 was chosen for this purpose, since the cell lines tested were already adapted to grow in this medium supplemented with 10% calf serum.

More refined synthetic media have now been reported (Healey and Parker, 1966a; 1966b), but 199 is still one of the most commonly used media for the cultivation of a variety of established cell lines. Apart from being

TABLE TC-IV

EFFECT OF YEAST EXTRACT ON THE REPLICATION OF KB CELLS

(Basal Medium - 3% TMB + 10% calf serum)

Yeast extract (concentration in per cent)	Mean count ($X10^3$)/culture at 4 days (Initial inoculum 30 X 10^3 cells/culture)
Nil	204
0.0125	146
0.025	193
0.05	139
0.1	97
0.2	19

expensive, this medium, like the majority of chemically defined media, is incapable of supporting the growth of primary cells.

Before the efficiency of these two media could be compared, a portion of the cells to be tested were adapted to grow in a mixture of 3% TMB and 10% calf serum by serial passage for at least 8-10 generations.

Using 1 oz. Brockway bottles, cultures of KB, HeLa and BS-C-1 cells were put up separately in these two media. For a total period of six days, cells counts were made at appropriate intervals on representative bottles; they were also examined periodically for their microscopic appearance.

The results obtained are presented graphically in Figs. TC-2 (KB cells), TC-3 (HeLa cells) and TC-4 (BS-C-1 cells). It is evident from these that TMB gave a better rate of replication of all these three cell lines tested. Moreover, when examined microscopically, cells grown in this medium appeared normal and free from granulation.

USE OF TMB AS A GROWTH MEDIUM FOR PRIMARY AFRICAN GREEN MONKEY KIDNEY (GMK) CELLS:

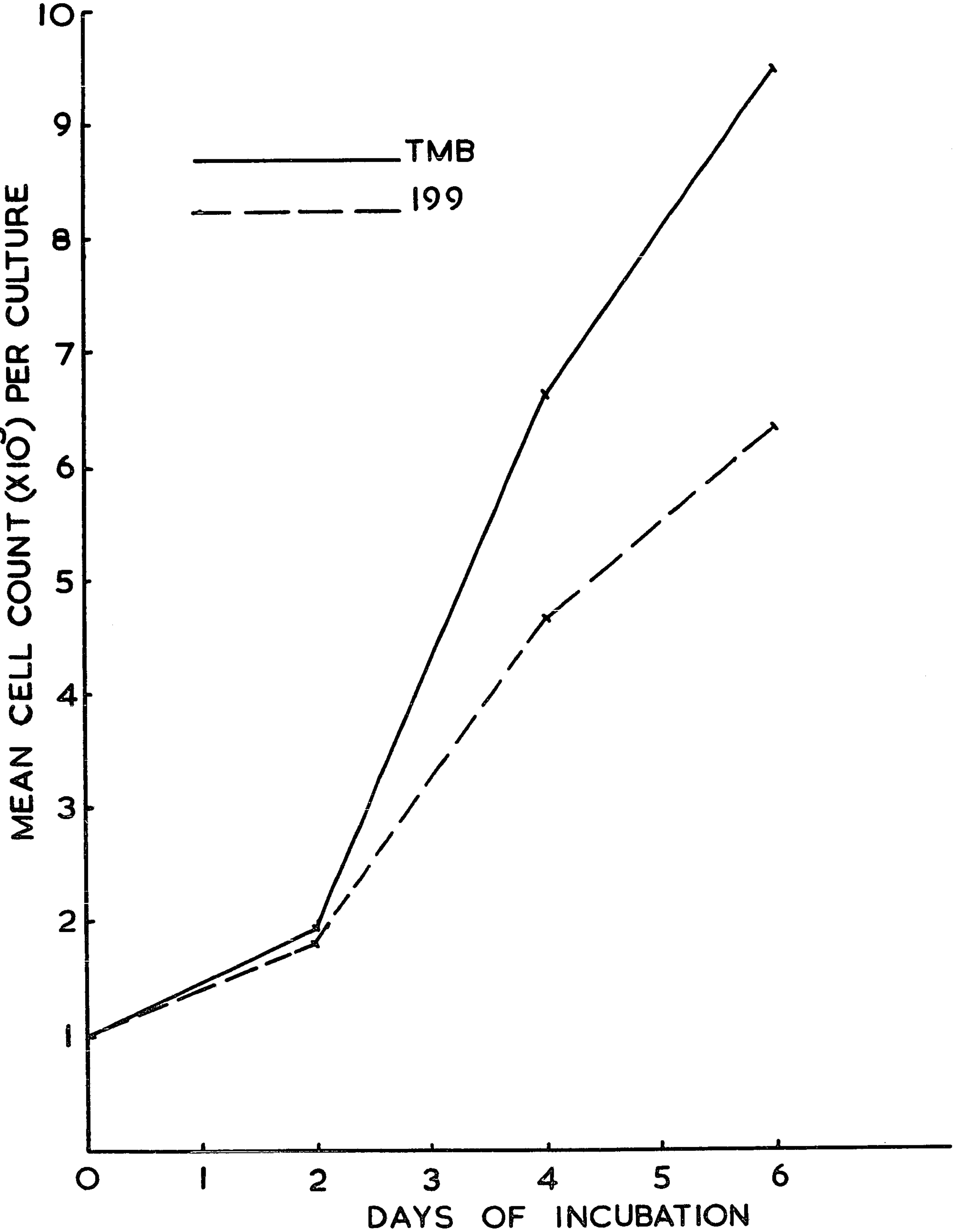
While experiments were being conducted for the establishment of a tissue culture system in this laboratory using established cell lines, primary GMK cells were used in conducting a major portion of the actual experimentation with viruses to be reported in this presentation. These cells not only supported the growth of all the viruses used in this

FIG. TC-2

COMPARISON OF TMB AND 199 AS

GROWTH MEDIA FOR KB CELLS

FIG. TC-2



TMB
199
MEAN CELL COUNT (X10^5) PER CULTURE
DAYS OF INCUBATION
0
1
2
3
4
5
6
7
8
9
10

FIG. TC-3

COMPARISON OF TMB AND 199 AS
GROWTH MEDIA FOR HeLa CELLS

FIG. TC-3

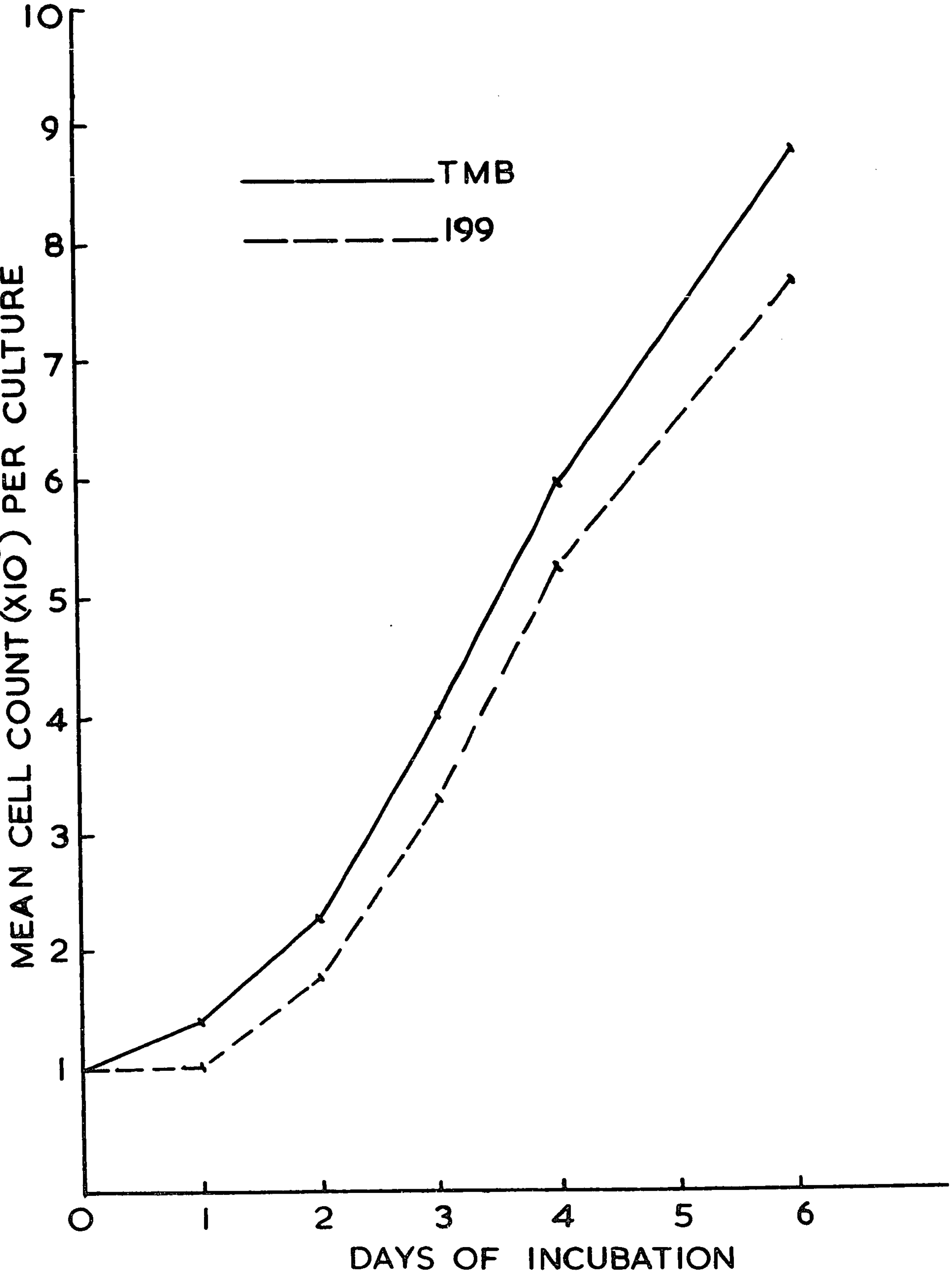
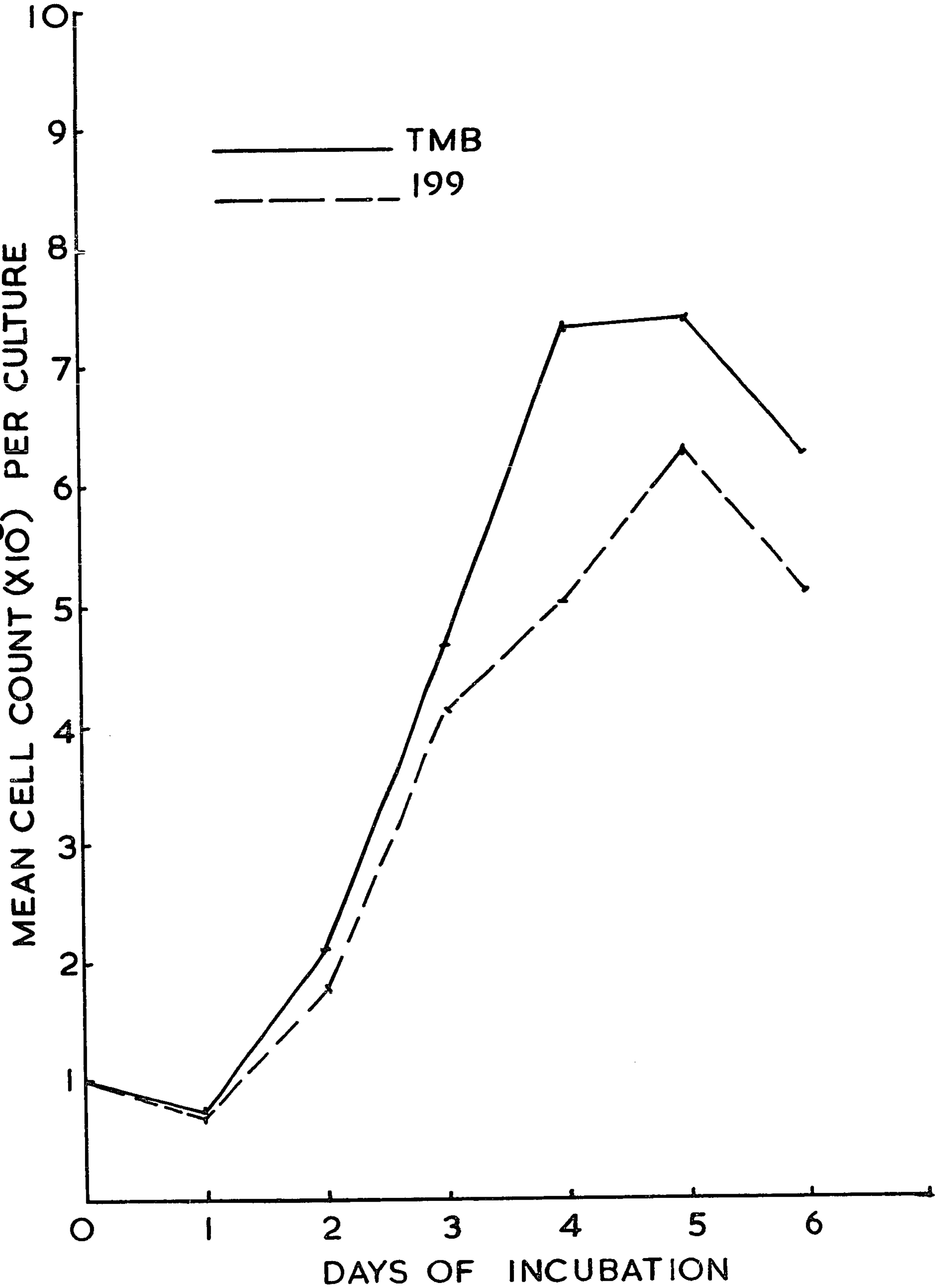

TMB
199
MEAN CELL COUNT (X10^5) PER CULTURE
DAYS OF INCUBATION
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6

FIG. TC-4

COMPARISON OF TMB AND 199 AS
GROWTH MEDIA FOR BS-C-1 CELLS

FIG. TC-4


TMB
199
MEAN CELL COUNT (X10^5) PER CULTURE
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
DAYS OF INCUBATION

study, but receiving these cells as a regular supply from a commercial source greatly facilitated the planning and conducting of experiments while the tissue culture facilities in this laboratory were still in the developmental stages.

Primary monolayers formed by GMK cells were uneven and therefore unsuited for any quantitative studies of viruses. To overcome this difficulty, GMK cells were used as second generation monolayers, which gave a more uniform and controlled growth.

The procedure generally used for handling these cells was as follows:

On receipt, antibiotics were added to the cell suspension and it was dispensed into 32 oz. Brockway bottles. After four days of incubation, the original growth medium (LAH and 2% calf serum in Hanks' base) was replaced with a mixture of 3% TMB and 2% calf serum. On the seventh day after seeding, the cultures showed confluent monolayers. These cell sheets were stripped with trypsin-Versene and after suspension in the TMB growth medium (3% TMB and 2% calf serum) the cells were redistributed as second generation test cultures.

Although no detailed quantitative and comparative tests were carried out on these cells in TMB, the procedure outlined above yielded cultures which were most satisfactory for virus study.

USE OF TMB AS A MAINTENANCE MEDIUM FOR GMK CELLS:

For many purposes, it is essential that monolayers of cells be maintained in culture for prolonged periods. The ideal maintenance medium should keep the individual cells of a confluent monolayer in a healthy, metabolically active but non-multiplying state. In practice, a low rate of cell division is acceptable, and such media are usually devised by diluting the constituents of growth media to the point where they are just adequate to sustain cell replication.

In order to establish a maintenance medium for second generation GMK monolayers, TMB was tested at 1, 2 and 3% concentration without any serum and in the presence of 1, 2 and 3% calf serum.

Replicate secondary GMK monolayers were grown to confluence and then transferred to the various TMB-serum combinations. The cultures were incubated (36°C) for a total period of three weeks with weekly cell counts on representative bottles. The results obtained are presented in Table TC-V.

One per cent TMB and 1% calf serum appeared to be the best combination among those tested. Therefore, this combination was adopted as the standard maintenance medium for GMK cells. The same medium has also been found satisfactory for maintaining HeLa and BS-C-1 cells.

TABLE TC-V

TMB AS MAINTENANCE MEDIUM FOR SECOND GENERATION GMK CELL MONOLAYERS

(Initial Inoculum - 240 X 10^3 cells/culture)

Medium	Mean cell count (10^{-3})/culture on		
	4th day	11th day	18th day
1% TMB	222	160	75
1% TMB + 1% calf serum	277	324	354
2% TMB	236	166	discarded
2% TMB + 2% calf serum	258	213	150
3% TMB	170	156	discarded
3% TMB + 3% calf serum	247	209	137

USE OF TMB AS AN OVERLAY MEDIUM FOR GMK CELLS:

This represents a special aspect of a maintenance medium. A medium may be able to maintain cultures in an apparently normal state for prolonged periods, but it might reduce the susceptibility of these cells to virus infection.

No indication has been obtained that TMB interferes in any way with virus multiplication in the infected cultures. On the other hand, it was observed that cytopathogenic effects due to adenovirus 3 appeared at least 24 hours earlier and with greater intensity in cells maintained in TMB than those kept in lactalbumin hydrolysate-serum mixture. However, the latter progressed slowly so that, in titrations, both media gave the same virus end point.

At various stages of this investigation, this medium has been successfully used, with and without agar, methyl cellulose, starch and antiserum, in overlaying herpes simplex virus, vaccinia, adenovirus 3, poliovirus I and influenza virus infected cultures.

This medium has also been compared with a synthetic medium (1393-5) supplied by Dr. R.C. Parker of Connaught Medical Research Labs.; it contains a serum fraction but no native serum. Herpes simplex virus was chosen for this comparative study. A dilution of the virus known to give countable plaques was introduced simultaneously into cultures overlayed with these two different media. The plaque counts (average of five bottles) obtained after 60 hours of incubation were as follows:

TMB overlay medium ---------------- 314.0 plaques

Parker's medium ------------------ 314.5 plaques

The size of the plaques obtained in Parker's medium was slightly larger than the ones in TMB; there was virtually no difference in the total number of plaques obtained.

DISCUSSION

The experiments reported in this section were aimed at establishing a tissue culture system for virus study. It has been shown that TMB, a medium based on pancreatic digest of beef, forms a suitable basis for the cultivation and maintenance of established and primary cultures. This has resulted in considerable simplicity in working with cells and viruses. This medium which could very easily be prepared in our own laboratory could be used as a growth, maintenance and overlay medium by simply altering its concentration and ratio with the serum. Several batches of this medium have been prepared and used during the course of this study and no noticeable variation in the cell growth promoting activity has been observed in these different lots. On the basis of these results, interest was expressed for the commercial production of this medium; as a consequence, it has now become commercially available through Qualicum Laboratories, Ottawa, Ontario.

This medium, because of its simplicity, economy and versatility, is particularly suited to work in a virus diagnostic laboratory where one deals with a variety of cell types and viruses. Its successful use at the virus diagnostic laboratory of the Ottawa Civic Hospital (Mr. P. Phipps, personal communication) provides a striking example.

(B) DEVELOPMENT OF PLASTIC CHAMBERS FOR THE CULTIVATION OF CELLS ON MICROSCOPE SLIDES

SUMMARY

To overcome the disadvantages inherent in the use of coverslip cultures, plastic chambers were designed and constructed for culturing cells on standard microscope slides. Use and handling of these chambers in studying viruses in tissue culture is described.

Cell monolayers grown in this fashion could be treated in much the same way as tissue sections on slides, without affecting the quantities of media and reagents involved.

These chambers have been successfully used in carrying out immunofluorescent focus titrations and macroplaque assays (with and without semi-solid overlays) of a number of viruses.

INTRODUCTION

During the initial stages of the studies on virus quantitation in tissue culture using immunofluorescence focus assay technique, glass coverslip cultures proved highly unsatisfactory. Owing to their fragility, the handling of coverslips in large numbers was cumbersome. It was also difficult to be sure, throughout the staining and mounting procedures, as to which side of the coverslip carried the cell monolayer. To overcome these limitations, it was decided to adapt standard microscope slides for cell culture, as these could then be treated in much the same way as tissue sections on slides. To achieve this, simple plastic ('Plexiglas', Rohm & Haas Co., Independence Mall W., Philadelphia, Pa.) chambers were designed and constructed in this laboratory (Sattar and Westwood, 1967b). The present section describes the use and handling of these in studying viruses in tissue culture.

CONSTRUCTION AND HANDLING

The tissue culture chamber, as shown in Fig. SC-1, consists of a plastic ring, a nontoxic rubber (Ronthor Reiss Corp., Little Falls, New Jersey, U.S.A.) gasket and two stainless steel clips; the plastic ring with the rubber gasket could be fastened to a standard microscope slide with the use of the clips (Fig. SC-2). Three of these assembled chambers could easily be accommodated in a 100 X 100 X 15 mm.

FIG. SC-1

DIAGRAMMATIC REPRESENTATION OF THE PARTS OF A CHAMBER

(A) TOP VIEW OF THE PLASTIC RING

(B) SIDE VIEW OF THE PLASTIC RING

(C) SILICONE RUBBER GASKET

(D) STAINLESS STEEL CLIP

FIG. SC-1

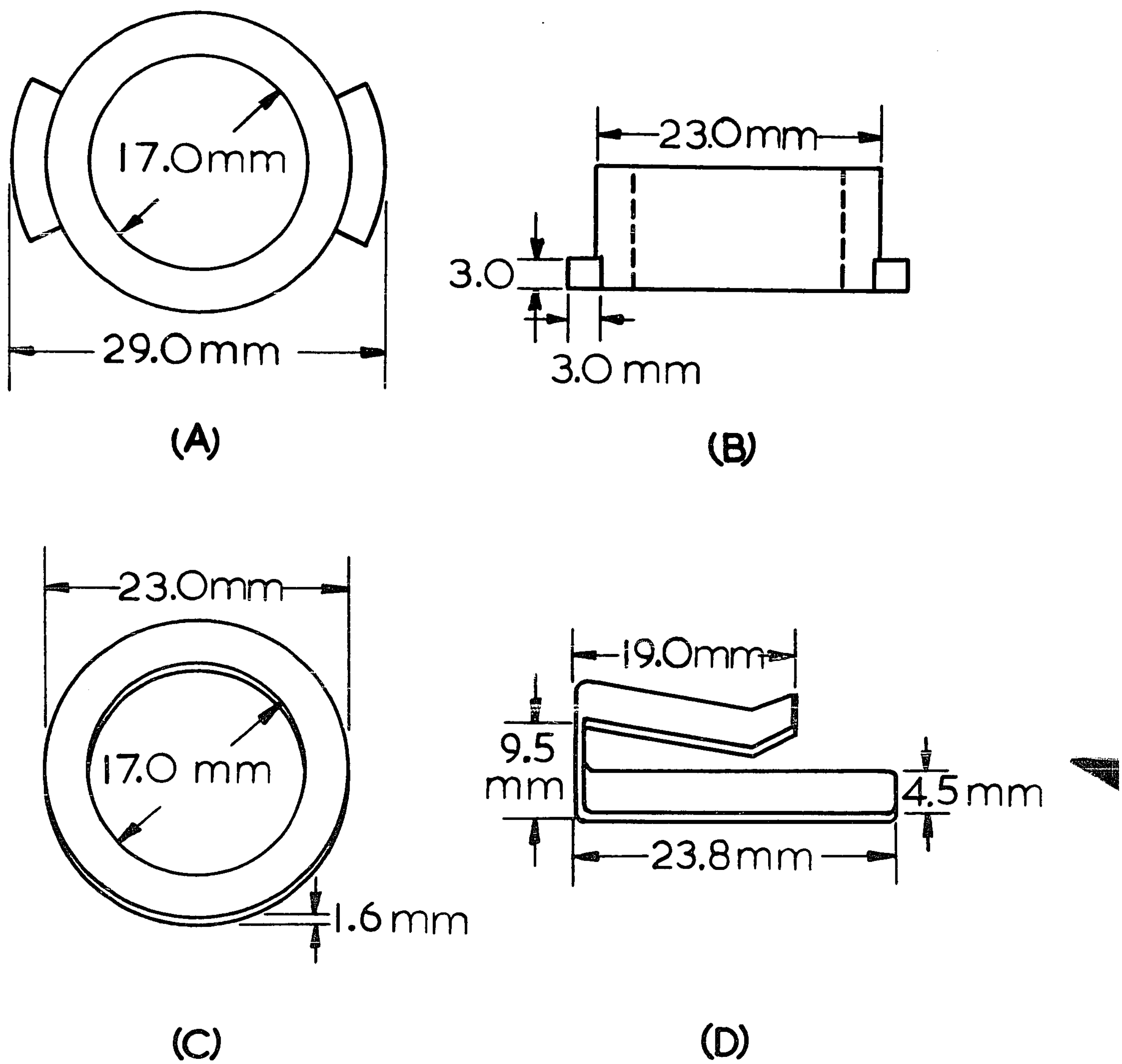
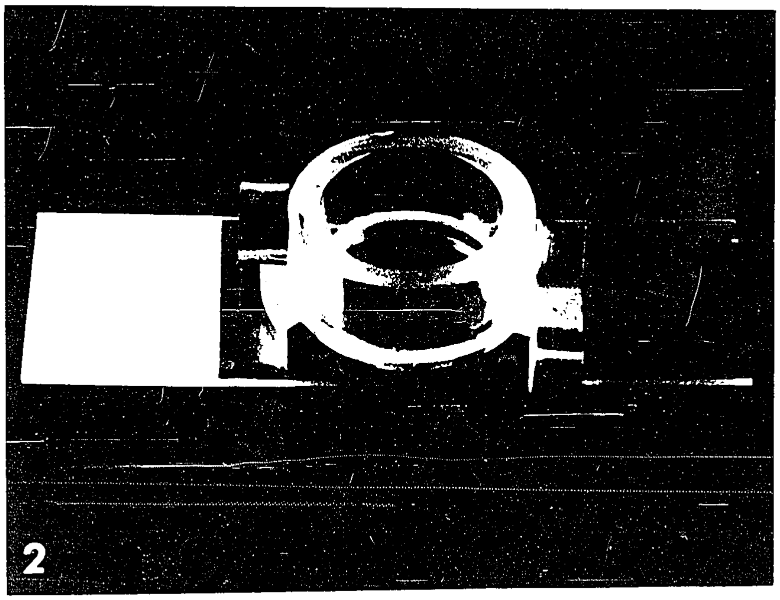

17.0mm
29.0mm
(A)
23.0mm
3.0
3.0 mm
(B)
23.0mm
17.0 mm
1.6mm
(C)
19.0mm
9.5
mm
4.5mm
23.8mm
(D)

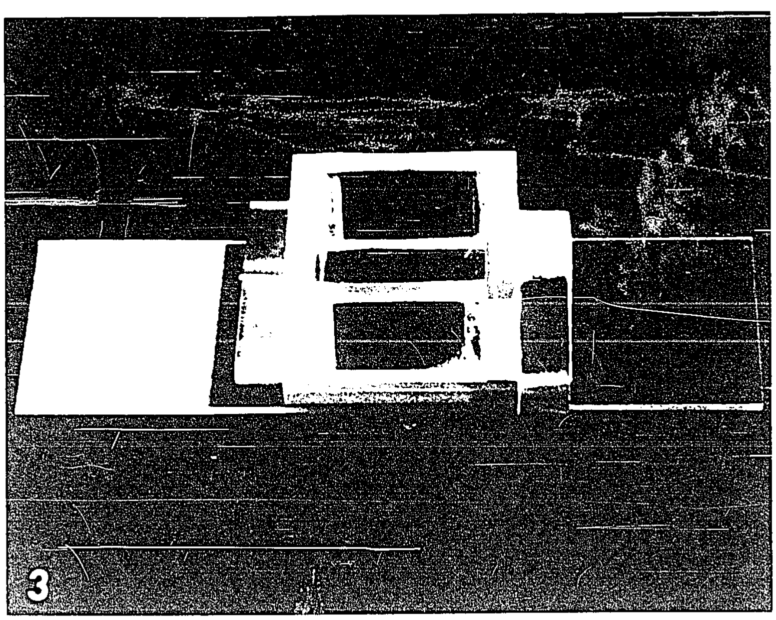
FIG. SC-2

PHOTOGRAPH SHOWING AN
ASSEMBLED RING CHAMBER

FIG. SC-3

PHOTOGRAPH SHOWING AN
ASSEMBLED SQUARE CHAMBER


2


3

square disposable plastic petri dish (Falcon Plastics). It has also been found possible to place two of these chambers on the same slide, so that of the two resulting cell sheets, one could be infected and the other left as a control; when ready, both of these received identical treatment throughout the washing, fixing and staining procedures.

After assembly, the chambers could be sterilized by exposing them to ultra-violet light (Westinghouse Sterilamp G36T6H) for 15-20 minutes at a distance of 8-10 inches from the lamp. Infected cultures were soaked in a disinfectant solution overnight and boiled in tap water for about 30 minutes. They were thoroughly washed in running tap water prior to rinsing in three changes of deionized water.

Each chamber was seeded with approximately 120 X 10^3 to 130 X 10^3 cells in 1.0 ml. of medium and incubated in an atmosphere of 5% CO_2-95% air mixture at 36°C. Confluent cell monolayers, with an average of 100 X 10^3 cells/chamber, were ready for use within 10-12 hours after seeding. Inoculation of these cultures with viruses and their maintenance were carried out in essentially the same way as that employed for handling cells grown in petri dishes. The cells could be examined directly under the low power objective of a microscope. When ready, they were put through the washing, fixing and staining steps after removing the chambers from the slides. Frosted ends of the slides could be marked with an ordinary lead pencil.

The ring chambers described in the preceding paragraphs were slightly modified to suit the experimental design for the immunofluorescent focus assays. Square chambers (Fig. SC-3) with an inside area of 17 X 17 mm., to conform to the counting grid (see section of Counting Device), were designed and constructed.

COMMENTS

These chambers have been successfully used for the cultivation of a variety of cells including HeLa, BS-C-1, HEp-2 and second generation *Cercopithecus aethiops* kidney cells. Cultivation of cells in this fashion has overcome many of the disadvantages inherent in working with coverslip cultures, without significantly affecting the quantities of media and reagents involved. Cell sheets grown on a known area of the slide could be put through the required manipulations without disrupting them. This was virtually impossible when large numbers of coverslips were to be handled at the same time.

Deinhardt and Dedmon (1965) have reported the construction of a coverslip holder which may render batch handling of coverslips easier. This, however, does not safeguard against damage to the cell sheets.

Stainless steel rings, fastened to glass coverslips with a mixture of "Vaseline" and paraffin, have been used in the cultivation of cells for virus study (Wildy et al., 1961). Similarly, Bergman et al., (1963) employed glass rings fixed on slides with "Vaseline". Use of rubber gaskets, as reported here, eliminates the necessity of having to remove "Vaseline" and paraffin from the cultures before staining. More recently, use of 'Perspex' rings has also been reported (McLeod and Blackburn, 1966). Here the rings were fixed in annuli drilled in the slides, making this procedure technically involved for routine application.

The chambers reported here are inexpensive and easy to construct. Since all parts of the chamber can withstand commonly used disinfectants and boiling for prolonged periods of time, they can be reused. No special skill is required in assembling and handling them. They may be employed for a variety of purposes including virus macroplaques and microplaques (with or without agar, methyl cellulose overlays), plaque purification, cloning of cells, cytochemistry, autoradiography and chromosome studies.

(C) DEVELOPMENT OF AN IMMUNOFLUORESCENT FOCUS COUNTING DEVICE

SUMMARY

A system has been developed for the accurate microscopic scoring of immunofluorescent foci and immunofluorescent cells in virus-infected slide cultures. It is based on a pattern of fifty randomized and standardized points in a 17 x 17 mm. area.

Counts obtained using this system have been shown to be randomly distributed and statistically reliable.

INTRODUCTION

The quantitative nature of these studies called for an accurate scoring of immunofluorescent (IF) foci or IF cells in virus-infected cultures. The commonly used procedure for this purpose is as follows:

Usually fifty randomly selected microscopic fields on coverslip cultures are examined and the number of IF foci or IF cells is counted in each one of these. The mean number thus obtained is multiplied by the total number of microscopic fields in the culture area at the given magnification; this gives the total number of IF foci or IF cells per culture (Wheelock and Tamm, 1961a; Hahon, 1965; Hahon, 1966).

Spendlove and Lennette (1962) scanned each coverslip culture microscopically to determine the total number of IF foci of vaccinia virus.

Random sampling of microscopic fields and complete scanning of cultures both proved time consuming, cumbersome and unreliable in practice. If randomized and standardized fields could be counted using a uniform pattern, this could make scoring easier and could provide statistically reliable data. The present section deals with the construction, use and testing of such a distribution pattern for counting IF foci or IF cells in virus-infected slide cultures.

CONSTRUCTION AND HANDLING

A distribution pattern (Fig. CD-1) of randomized and standardized points in a 17 X 17 mm. area was made available through the courtesy of Microbiological Research Establishment, Porton, England. A counting grid based on this pattern was constructed and attached to the movable stage of a fluorescence microscope (Fig. CD-2). An adjustable pointer (Fig. CD-4) was mounted on the fixed base of the microscope stage to complete the counting arrangement (Fig. CD-3). The fluorescence-stained culture to be examined was placed on the microscope stage and positioned so that a field at the top left corner of the coverslip lay under the objective. The pointer was then centred precisely over the starting spot (arrowed) on the corresponding area of the counting grid and locked into position. Thereafter, the microscope stage could be moved to bring each spot on the counting grid under the pointer and the corresponding field of the culture under the objective. The culture was thus sampled to a statistically randomized pattern and all foci in the selected areas counted. A work sheet was designed (Fig. CD-5) to facilitate the recording of counts.

In these quantitative studies it was always necessary to use complete monolayers of cells since the number of IF foci or IF cells was determined per unit area of the slide culture.

FIG. CD-1

DISTRIBUTION PATTERN OF FIFTY RANDOMIZED AND STANDARDIZED POINTS IN A 17 X 17 MM. AREA

FIG. CD-1

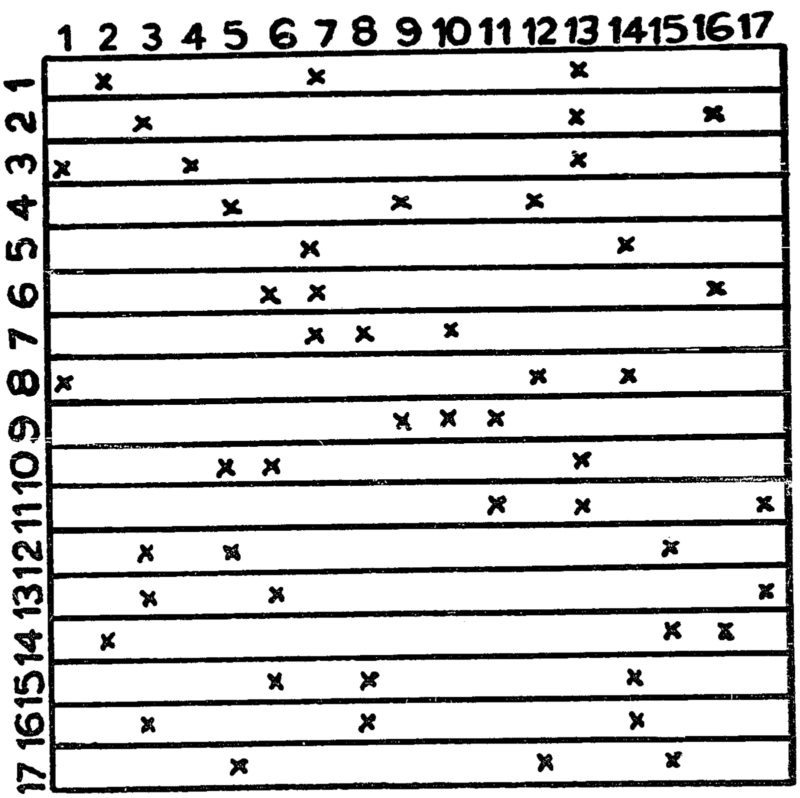
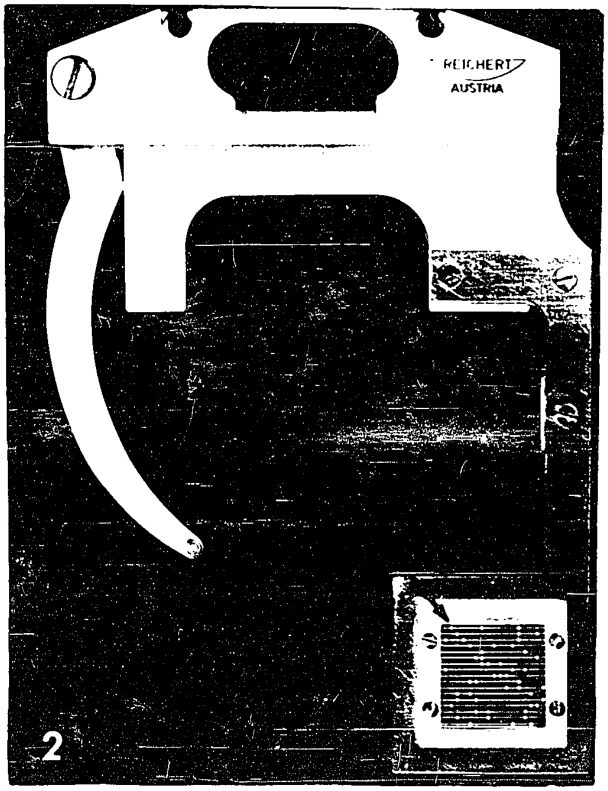


FIG. CD-2

COUNTING GRID ATTACHED TO THE MOVABLE STAGE OF A MICROSCOPE

FIG. CD-3

A CLOSE-UP VIEW SHOWING THE COMPLETE COUNTING ARRANGEMENT


REICHERT
AUSTRIA
2

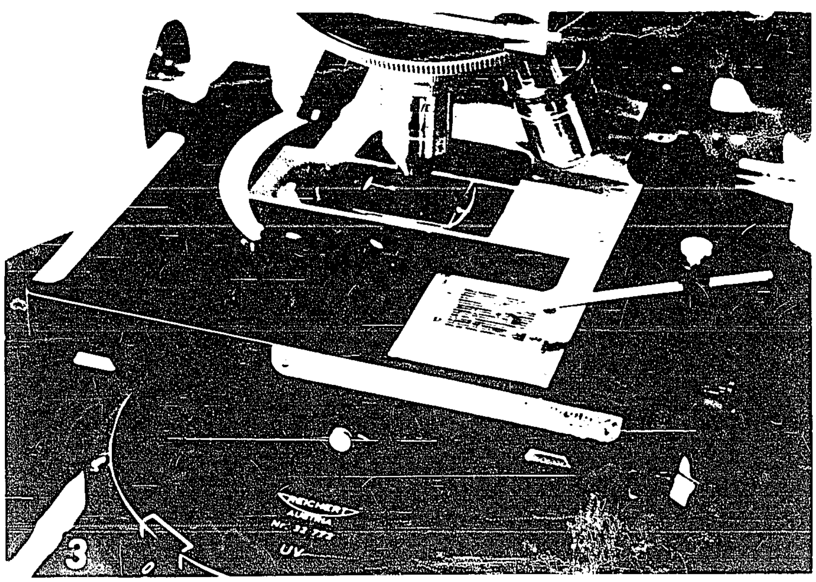

REICHERT
AUSTRIA
UV
3

FIG. CD-4

DIAGRAMMATIC REPRESENTATION OF
THE PARTS OF THE ADJUSTABLE POINTER

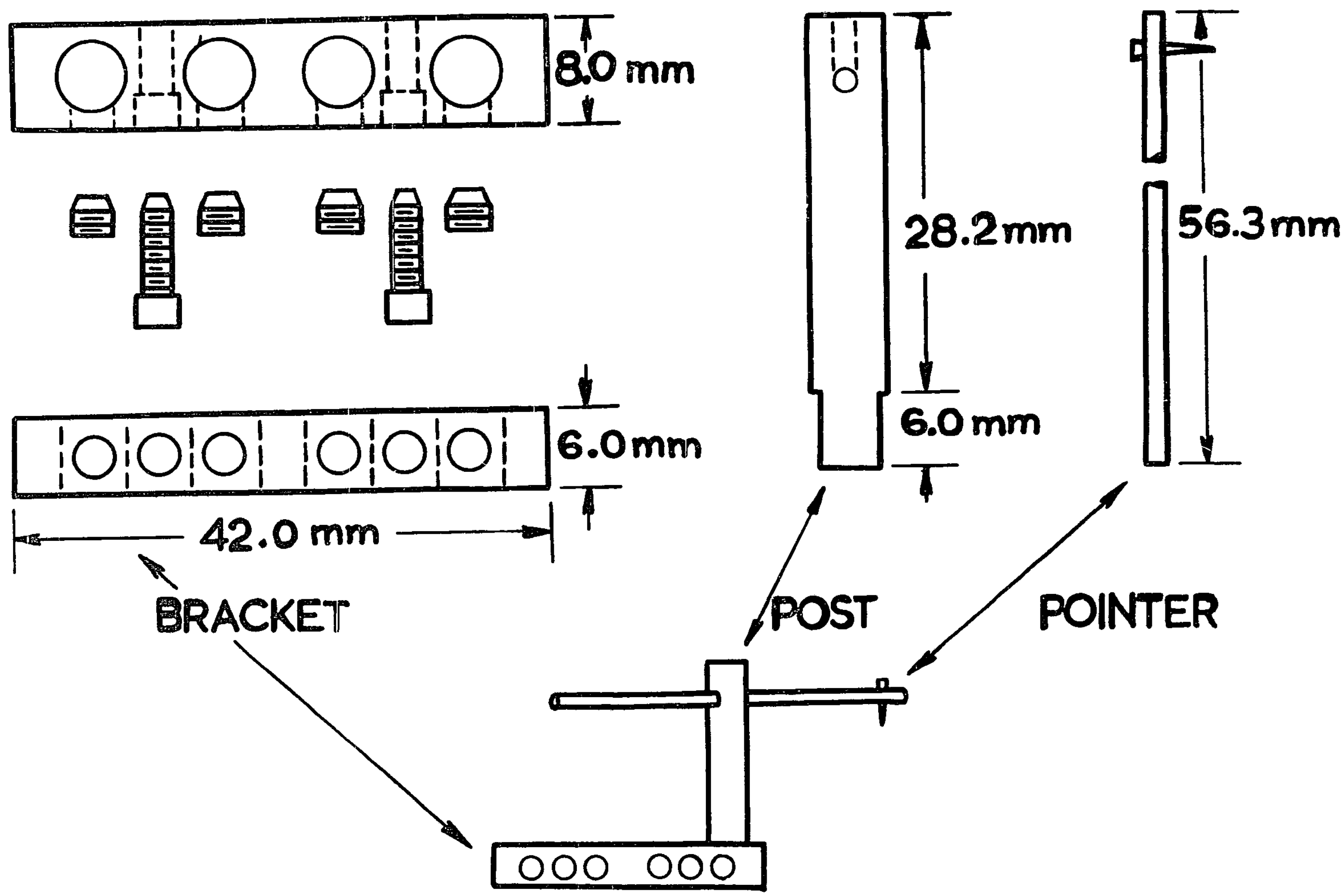


FIG. CD-4

FIG. CD-5

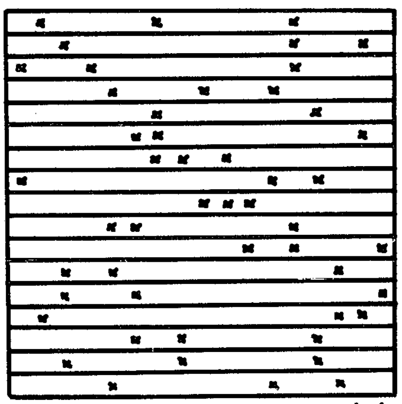
WORK SHEET USED FOR RECORDING

IMMUNOFLUORESCENT FOCUS COUNTS

FIG. CD-5

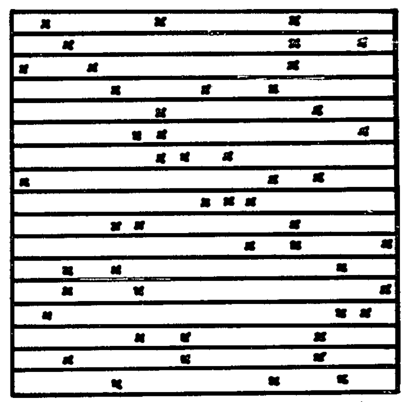
Date ________ Dilution ________

Exp. No. ________ Magnification ________



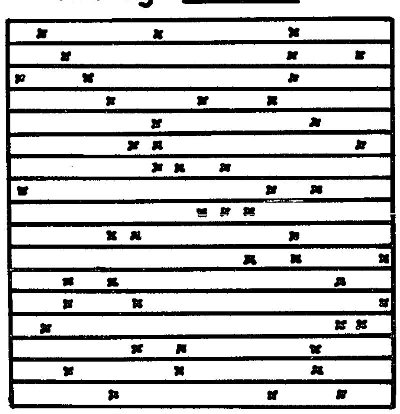
Total ____

Average ________



Total ____

Average ________



Total ____

Average ________

Average of three squares ________

Total no. of IF foci or IF cells/culture ________

Remarks ________________________________

For obtaining IF focus counts (herpes virus and vaccinia), the cell sheets were examined at 100X magnification; whereas for scoring IF cells (influenza virus A/PR8), 400X magnification was used. At these magnifications, the total numbers of microscopic fields in a 17 X 17 mm. area were 229 and 915 respectively. An average count of 50 microscopic fields was multiplied by the total number of fields to get the total number of IF foci or IF cells per slide culture. At least three cultures were examined in this manner for every virus dilution tested.

STATISTICAL ANALYSIS

Reliability of the counting system was determined by the following statistical analysis:

Herpes simplex virus IF focus counts, obtained with the use of the counting grid, were tested against their expected frequency. The data obtained are summarized in Table CD-I. Fig. CD-6 shows a graphic presentation of these data. It is evident from these that there is agreement between the expected and observed frequencies of the focus counts, indicating that the pattern gives a random distribution of counts.

The X^2 test for goodness of fit gives a value of 8.483, and since it is less than the critical value of 11.07 at 5 degrees of freedom and 5% significance, the counting system may be considered statistically reliable.

TABLE ¢D-I

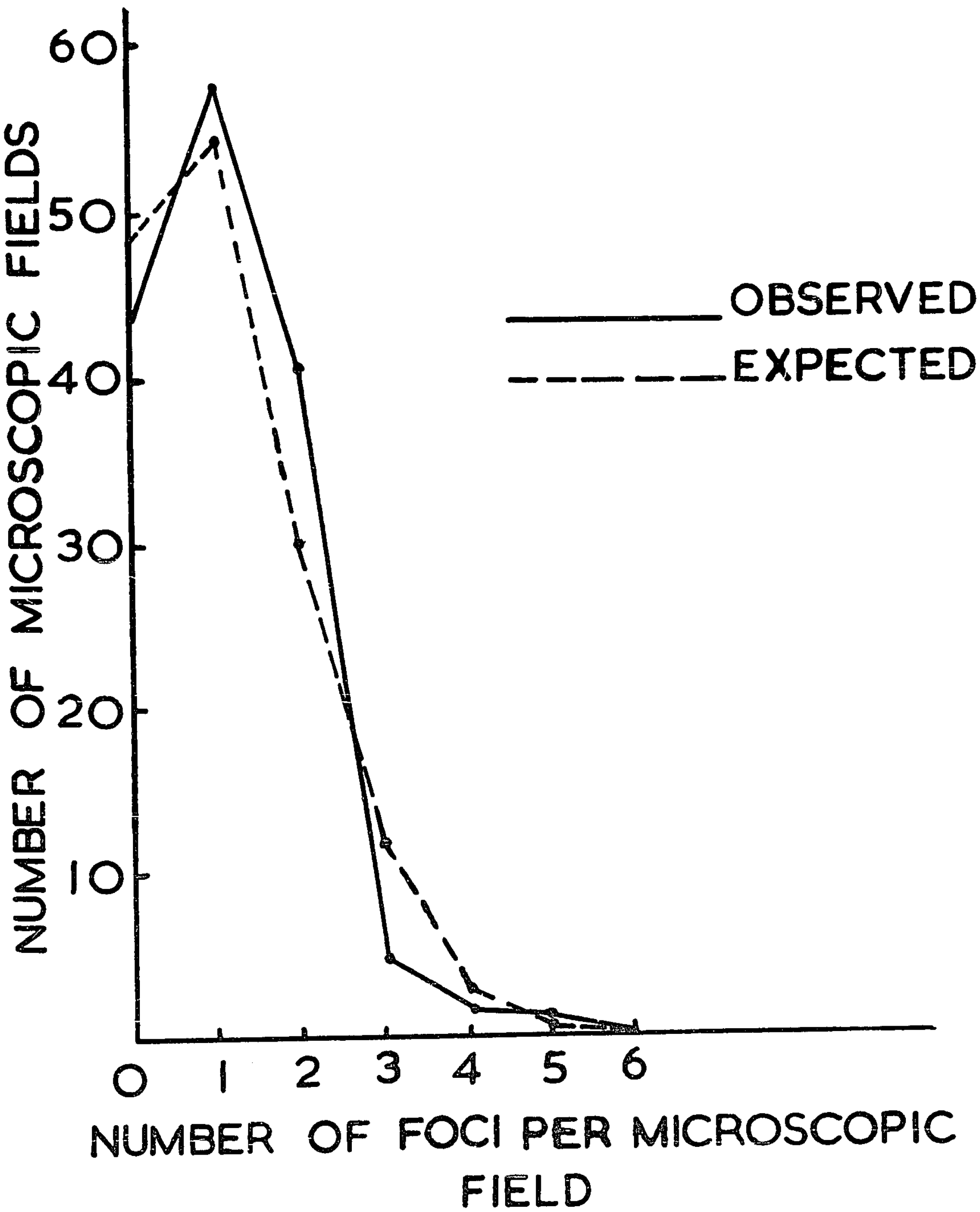
STATISTICAL ANALYSIS OF IF FOCUS COUNTS

Number X	Observed Frequency f	fX	Probability $P(X) = \frac{e^{-m} m^X}{X!}$	Expected Frequency $F = nP(X)$	f-F	$\frac{(f-F)^2}{F}$
0	43	0	0.3263	48.94	-5.94	0.727
1	58	58	0.3654	54.81	+3.19	0.185
2	41	82	0.2039	30.58	+10.42	3.55
3	5	15	0.0760	11.40	-6.4	3.59
4	2	8	0.0210	3.15	-1.15	0.419
5	1	5	0.0047	0.70	+0.3	0.012
Total	150	168	0.9973	149.58	0	$X^2=8.483$

FIG. CD-6

GRAPHIC PRESENTATION OF DISTRIBUTION OF IMMUNOFLUORESCENT FOCUS COUNTS OF HERPES SIMPLEX VIRUS OBTAINED THROUGH THE COUNTING SYSTEM

FIG. CD-6


NUMBER OF MICROSCOPIC FIELDS
60
50
40
30
20
10
OBSERVED
EXPECTED
0
1
2
3
4
5
6
NUMBER OF FOCI PER MICROSCOPIC FIELD

PART I

APPLICATION OF IMMUNOFLUORESCENCE IN THE LABORATORY DIAGNOSIS OF VIRUS DISEASES: A LITERATURE SURVEY

SUMMARY

The fluorescent antibody staining technique employs immune serum globulin labelled with fluorescent dye to locate the corresponding antigen. It combines the sensitivity and specificity of immunology with the precision of microscopy. It has been used for the visualization and identification of viruses, rickettsiae, bacteria, fungi, protozoa and helminths, and also for the detection and identification of serum antibody.

In the diagnostic laboratory, immunofluorescence now occupies an important place. It is being successfully employed in the routine examination of clinical specimens from a number of infectious diseases. It has proved particularly promising in the laboratory diagnosis of diseases caused by viruses.

To assess the present status of immunofluorescence as a technique in the laboratory diagnosis of virus diseases, the present review attempts to analyse the pertinent literature. It also surveys certain recent findings which may have a direct bearing on the use of immunofluorescence as a

INTRODUCTION

Among the basic tools employed in the study of microorganisms, microscopy and serology occupy two very prominent positions. Methods using fluorochrome-labelled antibodies have brought about a highly desirable combination of these two basic techniques. It has "bridged the gap between the world of the microscope and the world of immunological specificity." (Coons, 1960). Following the observations of Heidelberger et al. (1933) and Marrack (1934) that colored compounds could be conjugated to protein molecules without damaging their specificity, Coons et al. (1941) successfully coupled certain fluorochromes to antibodies for the microscopic localization of antigens. This application of fluorescent dyes as tracers has introduced a great deal of visual advantage over non-fluorescent compounds, since much smaller quantities of the former can be detected microscopically.

The fluorescent antibody staining technique (FAST) or immunofluorescence can be employed in several different ways. Brief descriptions of commonly used modifications follow: In the direct method, the presence of an antigen is demonstrated by using a fluorochrome-labelled specific antibody (Coons et al., 1941). In the indirect method, the antigen is first exposed to unlabelled homologous antiserum and then treated with labelled antibodies against the globulin of the particular species from which the specific antiserum was obtained (Weller and Coons, 1954). In the complement staining method of Goldwasser and Shepard (1958), complement (guinea pig origin) is applied to the antigen after the antigen has been treated with unlabelled homologous antiserum; the complement that is taken up by this antigen-antibody complex is then stained with an anti-guinea pig globulin conjugate.

The relative advantages and disadvantages of these modifications are summarized in Table RL-I.

The usefulness of this technique became evident soon after the demonstration (Coons et al. 1942) that antigens could be detected in the phagocytic cells of the mouse using specific fluorescein-labelled antibodies. Differentiation of virus-infected from non-infected cells, and localization of viral antigens within the infected cells, formed the basis of most of the earlier experiments using this technique (Coons, 1959). It was soon expanded to entail basophilic viruses (Surman et al. 1967), rickettsiaeae (Hahon and Cooke, 1966), bacteria (Cherry and Moody, 1965), protozoa (Collins, et al. 1967) and fungi (Vogel, 1966), thus opening new and important avenues to the study of most of the common

TABLE RL-I

COMPARISON OF THE ADVANTAGES AND DISADVANTAGES OF THE MODIFICATIONS USED IN FLUORESCENT ANTIBODY STAINING

Direct Staining	Indirect Staining	Complement Staining
A single labelled antiserum cannot be used to detect different antigens.	A single labelled antiserum, e.g., goat anti-rabbit globulin can be used to stain any antigen for which one possesses rabbit antisera.	Only one labelled antiserum against guinea pig globulin can be used to stain all systems which fix complement.
In some systems this staining does not give adequate brightness.	Because of the 'layering effect', it increases the brightness and sensitivity of the reaction.	It produces a similar layering effect resulting in increased brightness.

Easier to perform when labelled antisera are available.	More steps involved in performing the test.	Method cumbersome because of more steps involved.
Needs limited controls.	Number of controls increases, since non-specific reactions may occur at both steps of the procedure.	Needs more controls because of more steps involved.
Can be used for testing antibodies in sera ("blocking test").	Can be used for testing the presence of antibody, but is limited to testing sera only from the species for which one has labelled antiserum.	Can be used for detecting antibodies in sera from many different species.
Low titered antisera not suited because they are further diluted when labelled.	Can locate antigen and antibody in low titered antisera used in the middle layer.	Can detect antigen and antibody with low titered sera.

human and animal pathogens (Cherry et al. 1960; Poetschke, 1961; Nairn, 1964; Harris, 1966). Bacteria, by virtue of their high antigenicity, relative freedom from autofluorescence and tolerance to a wide variety of methods of fixation and storage, have been particularly favoured as objects of study in this field (McEntegart, 1964). On the other hand, auto-fluorescence of fungal mycelia and spores has considerably retarded investigations in mycology using immunofluorescence (Cherry, 1960).

There is a large and ever increasing number of problems to which immunofluorescence has been applied successfully in more recent years. Some of these applications include; study of antinuclear factors (Holborow and Johnson, 1964), testing the concentration of ferritin conjugated antibody (Hsu, 1963), determining the purity of virus suspensions (Kudo, 1962), titration of suspensions of cytopathogenic and non-cytopathogenic viruses (Wheelock and Tamm, 1961a; Rapp et al. 1963; Hahon, 1966; Sattar and Westwood, 1967a), differentiation of blood groups (Myhre, 1965), detection of typhoid carriers (Thomason and McWhorter, 1966) and epidemiological investigations (Stulberg et al. 1966).

In spite of its extensive potential, the possibilities of immunofluorescence as an aid in the rapid diagnosis of virus diseases have not yet been fully realized. While the attempts made in this direction have been encouraging, they have also brought to light some of the problems involved in its application as a diagnostic tool. Along with the inherent virtues of being able to detect and identify non-viable organisms and non-precipitating antibody, this technique faces certain serious limitations. These are in most part due to a lack of understanding of some of the basic factors involved in its application. Although there has been a steady evolution of simpler and better techniques within the last ten years, presence of equivocal results may also be due to a diversity of techniques employed and the unavailability of reliable standard reagents. This has prompted investigators in this field to suggest that it be used as a supplement rather than as a replacement for the conventional methods of virus diagnosis, and as such only in the case of a few diseases (Fraser, 1964; Marmion, 1963; Harris, 1966). For a better appreciation of its promise and short-comings in the diagnosis of virus diseases, it is essential to review and critically analyse the data available at present.

The present review will be concerned only with (A) reports of the diagnostic application of immunofluorescence and (B) recent findings which may have a direct bearing on the use of immunofluorescence as a diagnostic technique. Experimental studies on immunofluorescence of viruses will be referred to elsewhere.

(A) DIAGNOSTIC APPLICATIONS

1) Myxovirus Group: Demonstration (Liu, 1956) of influenza virus in nasal washings from patients suspected of influenza was the first attempt in applying immunofluorescence to diagnostic virology. Though the results were obtained the same day the specimens were collected, the technique proved to be less sensitive than the hemagglutination-inhibition tests in virus identification. Blaškovič et al. (1963) found the direct and indirect techniques to be equally sensitive, but again both of these less sensitive than the conventional diagnostic tests in the detection and identification of influenza virus. Carski et al. (1962) obtained a direct correlation between the results of the indirect technique and those of virus isolations in a survey of human respiratory diseases.

Baratawidjaja et al. (1963) employed the indirect technique for the rapid diagnosis of influenza in throat washings. The technique was compared with the egg inoculation method, and in 73 specimens tested there was good agreement between the results obtained by these two techniques. When immunofluorescence was used results could be reported within 4-5 hours after the arrival of the specimen in the laboratory. In their experience, although there were cross reactions between strains within the type, the strain-specific reaction could easily be distinguished by a greater intensity of fluorescence. Hinuma et al. (1962), on the basis of experimental studies, have shown the complement-staining technique to be more sensitive than the indirect method in detecting antibodies and antigens of certain myxoviruses; it also showed a brighter fluorescence, and the antibody titers obtained by this method were similar in magnitude to those found in complement fixation tests. Application (Hers, 1963) of this technique in the diagnosis of myxovirus infection confirms these observations.

During an epidemic of influenza A_2 virus in Japan, Tateno et al. (1965a; 1965b; 1965c) obtained rapid diagnosis (within 60 minutes in some cases) in a high percentage of patients by using the direct staining technique on nasal smears. These investigators have emphasized the importance of choosing the right animal species for the production of immune serum in such studies. In their experience antisera produced in guinea pigs were most satisfactory. They further reported that the buffy coat of heparinized blood and eye secretions may serve as useful sources of influenza virus-containing cells in the immunocytological diagnosis using immunofluorescence.

More recently, Lyarskaya et al. (1966) have applied this technique in the detection of viral antigens in cells from the nasal mucosa of influenza patients and volunteers given influenza vaccine intranasally. They also examined preparations from the tracheae and lungs of mice experimentally infected with the virus. On the basis of their results, they recommended this technique for the routine diagnosis of influenza virus infections.

There are very few reports available concerning the use of immunofluorescence in the diagnosis of infections caused by other members of the myxovirus group. Liu et al. (1961) studied parainfluenza virus-infected cultures by the direct technique using antisera prepared in monkeys. No cross-reactions between the members of this group were observed. Viral antigens could also be detected in cells of nasal epithelium, trachea, bronchi and lungs of intranasally infected hamsters. Leššo and Vaškebová (1965) demonstrated mumps virus in infected chorio-allantoic membranes and found the technique to be more sensitive and rapid than hemagglutination tests. Cohen et al. (1955) applied the technique to agents isolated in tissue culture from three cases of measles. Using the indirect technique, measles antigen could be detected in the infected cells before specific complement-fixing antigens appeared in the fluid phase.

2) Rubella Virus: Within the last three years a number of attempts have been made in the application of immunofluorescence in the rapid detection of rubella virus and rubella antibodies in clinical specimens. The development of such a test for this disease would be helpful, (a) because the virus does not yield itself readily to the conventional methods of cultivation and diagnosis, and (b) because of its ability to cause congenital malformations, thus making a quick assessment of suspected cases valuable.

Brown et al. (1964) successfully used the indirect FAST in the detection of rubella antibodies in human sera. Using a chronically infected cell line (Maasaab and Veronelli, 1966) as a source of rubella virus antigen, positive reactions were obtained with sera from convalescent patients or persons who had been exposed to the virus before; sera from patients in the acute phase of the disease and those from unexposed individuals were negative. Lennette et al. (1966) have reported a modified indirect method for the assay of rubella antibodies. Instead of infected cell monolayers, smears of tissue culture cells infected with rubella virus at a high multiplicity were used as antigen. During this investigation they also showed the indirect FAST to be somewhat more sensitive than either the neutralization or complement-fixation tests in demonstrating significant antibody titer rises in sera from individuals with German measles.

Schmidt et al. (1966) have shown the indirect FAST to be highly specific and sensitive in the identification of rubella virus isolates in tissue cultures. Woods et al. (1966) have also demonstrated specific viral antigens in rubella virus-infected tissue culture cells using the indirect FAST. When the same technique was employed, they could also show fluorescent foci in the heart and skeletal muscles of an infant with the rubella syndrome.

In a more recent study (Estela, 1967), hyperimmune sera from rabbits and roosters were compared using the direct and indirect FAST of rubella specimens. A total of 120 throat washings were tested. By the interference test, 109 of these were positive, and 101 gave a positive reaction by the direct FAST. The eight negative specimens with the direct staining test gave positive fluorescence with the indirect FAST. The remaining 11 throat washings were negative by all three tests. In this study, rooster antiserum was found superior to rabbit antiserum.

3) Arbovirus Group: These viruses are also difficult to identify by conventional methods of cultivation and diagnosis. Recent investigations using immunofluorescence have been encouraging. Atchison et al. (1966) have successfully employed the complement-staining modification of the FAST in the detection of dengue virus. When mouse brain

smears were used, the technique could also detect inapparent dengue infections in early (blind) passages of the unadapted virus strains. Emmons and Lennette (1966) have found the direct FAST reliable and rapid in the laboratory diagnosis of Colorado tick fever. They stained smears made from patient's blood clot or brain of mice inoculated with a suspension of the blood clot. Positive staining was confirmed by virus isolation studies. In the 18 cases studied in this investigation, no false positive or false negative reactions were encountered.

4) Rabies: This disease presents the diagnostician with a serious problem. The majority of the specimens submitted are accompanied with excitement and urgency, and a demand for a rapid and reliable laboratory diagnosis. Perhaps in no other disease has the use of immunofluorescence been found to suit such exacting situations so admirably.

The complement-staining technique (Goldwasser and Kissling, 1958; Goldwasser et al. 1959) has been tested in the laboratory for the diagnosis of rabies in experimentally and naturally infected animals. These investigations yielded results which showed the FAST to be more sensitive than the conventional stains used for detecting Negri bodies. McQueen et al. (1960) have emphasized the usefulness of this technique in the routine diagnosis of rabies in a public health laboratory. In their experience, though the results of this technique were in complete agreement with the mouse inoculation results, nevertheless the use of glycerol-saline in preserving specimens almost always resulted in a decrease in its efficiency. Töpleminova (1961) obtained a rapid and specific diagnosis of rabies with the indirect FAST using antisera of avian origin. Use of equine antisera (Etchebarne et al., 1960) was also found useful in the direct staining of brain smears from infected dogs and mice.

Thomas et al. (1963) have evaluated the value of indirect immunofluorescence in the detection of rabies antibody in human sera. It was found to be more sensitive than the serum neutralization test. They indicate its acceptibility as a screening procedure for the detection of rabies antibody.

In an extensive study of the comparison of immunofluorescence with the conventional methods of rabies diagnosis, Beauregard et al. (1965) found immunofluorescence to be as sensitive as the mouse inoculation test. Besides, the results with immunofluorescence could be obtained with considerable rapidity. This technique is now routinely used in their laboratory (Animal Diseases Research Institute in Hull, Quebec) for the diagnosis of rabies.

5) Poxvirus Group: Murray (1963), using the indirect FAST, detected smallpox and vaccinia viruses in pathological material. Apart from having the advantage of speed and specificity, the technique was found to be as sensitive as the other serological methods used, and could be applied even when insufficient material was available for which other procedures could not be used. Kirsh and Kissling (1963) obtained a positive laboratory diagnosis of smallpox by the FAST with specimens collected from a 14 year old boy suspected of having the disease. Pus and scab smears were unsatisfactory due to non-specific fluorescence of leukocytes, but vesicular material revealed specific fluorescence. Extension of these studies to ten additional cases of questionable smallpox gave three positives by the FAST and also by inoculation of chorio-allantoic membrane of embryonated eggs. The remaining seven were negative for smallpox by immunofluorescence and egg inoculation studies; one of these cases showed herpes virus on CAM.

Avakyan et al. (1961), in a comparative study of immunofluorescence and the conventional methods of smallpox diagnosis, found that the FAST permitted detection of variola virus much earlier and was more sensitive than the other tests. Kratchko et al. (1964) applied the direct FAST to the identification of vaccinia virus in infected tissue culture cells. Examination of specimens from human and animal sources demonstrated the technique to be sensitive and reliable; its use in the routine diagnosis of this disease is proposed by these investigators.

6) Enterovirus Group: Kalter et al. (1959) applied immunofluorescence to laboratory diagnosis of poliovirus in stool specimens. Stool specimen-inoculated monkey kidney cells showing cytopathic effects (CPE) were smeared and fixed in acetone. They were stained with conjugated anti-poliovirus sera produced in monkeys. Out of a total of 33 specimens containing the virus, 30 were typed correctly by this technique. In a similar study, Hatch et al. (1961) correctly identified poliovirus in 34 out of 38 specimens known to contain the virus. Reprocessing the four negative specimens by the same technique revealed the presence of poliovirus in two. Identification of Coxsackie and ECHO virus isolates using immunofluorescence was also reported by Hatch (1963). Out of a total of 70, correct typing was obtained in all but 4 cases. In spite of the presence of definite CPE, one Coxsackie and two ECHO-9 isolates could not be identified. However, when the staining was repeated, two out of these three previous failures were correctly typed. The fourth case showed a false positive reaction with ECHO-4 antiserum, since the isolate was actually ECHO-9. This reaction with ECHO-4 antiserum was also noticed in uninoculated coverslip cultures of rhesus monkey kidney cells

used in this study; absorption of the antiserum with packed cells of this batch removed the non-specific reaction.

The above mentioned studies with enteroviruses reveal two important points in the use of immunofluorescence in diagnostic virology. The first shows the importance of reprocessing the specimens giving negative results; the second refers to the presence of cross-reacting latent viruses in primary monkey kidney cells, which may give false positive staining reactions.

Shaw _et al_. (1961) have introduced an adaptation of the scheme of antiserum pools (Lim and Benyesh-Melnick, 1960) for the rapid identification of enteroviruses using the direct FAST with rabbit antisera. Coxsackie and ECHO virus antisera were divided into smaller sets. Sera from each set were pooled and conjugated with fluorescein isothiocyanate. The results obtained with unknown isolates and known stock cultures were valid when compared with concurrent isolation studies. The problem of non-specific fluorescence in this study was substantially reduced by rhodamine counterstaining of cultures (Smith, 1959).

Riggs and Brown (1962) have compared the efficiency of the direct and indirect techniques in the detection of enteroviruses and titration of their antibodies. The direct technique gave satisfactory results in the identification of enteroviruses in infected tissue culture cells. The indirect method was found to be even more satisfactory, since it made possible the detection and titration of virus specific antibodies acquired actively following clinical or sub-clinical infection or from vaccination.

7) <u>Herpesvirus Group</u>: Herpes simplex virus was successfully detected in clinical specimens (Biegeleisen *et al.* 1959) using the direct FAST with conjugated rabbit antisera. Eight of the fifteen specimens tested revealed the presence of the virus, and these results were confirmed by egg inoculation studies. Kaufman (1960) accomplished the diagnosis of herpes simplex in corneal scrapings employing the direct FAST with both human and rabbit antisera. Out of 17 specimens tested, six were positive both by immunofluorescence and virus isolation studies. In one specimen from an almost certain case of herpes, virus isolation attempts gave negative results, whereas specific fluorescence could be successfully demonstrated. This indicates that immunofluorescence may surpass conventional methods of herpes virus diagnosis in its sensitivity and specificity.

Griffin (1963) has found immunofluorescence reliable in the diagnosis of skin lesions caused by the virus. Examination of stained smears from skin lesions gave positive reactions in 54 out of 56 specimens; virus isolation studies confirmed these results. Thirty-seven cases of aphthae were also examined in this study, with negative results. Uchida et al. (1965) have successfully demonstrated herpes simplex virus in conjuctival smears using immunofluorescence.

Schmidt et al. (1965) have investigated the use of immunofluorescence in the diagnosis of varicella-zoster infections. Smears of virus-infected tissue culture cells were fixed and stained directly with conjugated monkey antisera. The results showed immunofluorescence to be as sensitive and efficient as complement-fixation tests in the diagnosis of chickenpox and zoster.

8) Other viruses: In addition to the studies cited above, there have been reports of successful application of immunofluorescence in the diagnosis of virus diseases of animals (Coffin and Liu, 1957; Nakamura, 1964; Braune and Gentry, 1966; Wright and Burns, 1966).

Although immunofluorescence has been used in experimental studies of adenoviruses (Shimojo et al.,1967) and reoviruses (Spendlove et al., 1963; Oie et al., 1966), no significant findings are available regarding the application of this technique in the diagnosis of diseases caused by these viruses.

On the basis of the results obtained in these varied, though limited studies, it could be noted that immunofluorescence has been very promising in many cases, whereas its application has been less encouraging in others. Since variations of the basic technique were used by different investigators, this lack of uniformity makes it difficult to generalize the conclusions. For a technique to be useful in a diagnostic laboratory it should be, among other things, adaptable. Can immunofluorescence be applied to the diagnosis of most if not all the important human and animal virus diseases with least possible modifications? Would it prove equally sensitive and specific in detecting viruses in a variety of clinical specimens? Obviously, for these questions to be answered satisfactorily, standardization of current methods, followed by their testing on actual clinical specimens, is required.

B. FACTORS INFLUENCING DIAGNOSTIC USE OF IMMUNOFLUORESCENCE

Before any such attempts can be made, a review of the following recent, but seemingly unrelated, reports may prove helpful. It may also explain some of the possible reasons for the unreliability of immunofluorescence methods in certain previous studies.

In the application of this technique to virus diagnosis it should also be remembered that detection of specific antigens, rather than intact virus particles, suffices in many cases. The exact location of specific antigens in an infected cell is often unnecessary too.

Accessory Factors in Normal Sera: Liu (1961), during an immunofluorescence study of primary atypical pneumonia noted the presence of a factor in normal sera which enhanced the reaction between the virus and homologous convalescent serum. This factor was reported to be different from properdin and complement. As has been suggested, it might act as a catalyst to increase the deposition of antibody on antigen and might also bind them together more firmly. Carski (1960) has reported a similar enhancement of fluorescence during studies of simian foamy virus in the presence of fresh normal guinea pig serum.

In a more recent report, Takemoto et al. (1966) have demonstrated the presence of a heat-labile factor in hamster sera. This factor was required for the immunofluorescence demonstration of polyoma tumor antigens in tumor cells. Unheated tumor serum gave specific fluorescence, but when this serum was used after heating (at 56°C. for 20 minutes) very poor or no specific fluorescence could be observed. The activity of such heated serum could be restored simply by the addition of fresh normal unheated hamster serum.

Smorodintsev and Yabrov (1963) have reported the presence of non-specific thermolabile stimulators of antibodies in mammalian sera. The addition of an active dose of normal serum was shown to improve considerably the antibody titers of influenza antisera in hemagglutination-inhibition tests. Studies into the mechanism of action of this thermolabile factor on hemagglutination-inhibition and virus neutralization tests revealed it to be connected with the enhancement of avidity of antibodies. In the presence of this factor, specific antibodies showed a rapid, firm and irreversible reaction with antigens. Such findings may represent a regular immunological phenomenon and further investigations in this line may open new and interesting possibilities in improving the sensitivity of antigen-antibody reactions in general.

Differences in Antibodies: Nadel and Carski (1964), while comparing the efficiency of two commercial (a product from Italy and another from Lederle Labs.) horse antirabies sera in preparing fluorescent antibody conjugates for the diagnosis of rabies, found the Italian product to be consistently superior in giving a brighter and more specific fluorescence. Furthermore, they noticed that the neutralizing titer of the Italian serum was invariably inferior to that of the Lederle serum. This indicates that neutralizing antibody titer of a serum may not be a true index of its suitability in immunofluorescence studies. Attempts at finding the reasons for the better performance of the Italian product in immunofluorescence studies of rabies have been without success.

Riggs and Brown (1962), during a study of actively and passively immunized infants against poliomyelitis, have found that active antibody development registered a marked increase in fluorescence, whereas most of the sera from infants possessing high titers of passively acquired antibody failed to show specific fluorescence. No experimental evidence has been gathered to explain the difference in the behaviour of these actively and passively acquired antibodies against poliovirus.

This situation becomes even more complex when one considers the report by Cherry and Freeman (1959). They failed to obtain positive results in immunofluorescence studies of _Bacillus anthracis_ using sera from five individuals who had been recently actively immunized against anthrax.

Non-specific Staining: Perhaps the most serious obstacle in the general acceptance of immunofluorescence as a routine diagnostic procedure is the presence of non-specific reactions (NSR). They can be attributed to a variety of reasons, but owing to the fact that the causes which give rise to these reactions are not fully understood, attempts at their elimination have been mostly empirical. However, recent findings have clarified some of the basic reasons for the presence of these reactions. This may result in the availability of better and more reliable reagents, and thus make a wider acceptance and application of this technique possible.

(1) NSR Due to Protein Interactions: Curtain (1958) has demonstrated that electrophoretically separated, serologically inactive components of antisera could give rise to non-specific staining. Fluorescein- and rhodamine-labelled normal serum globulins and albumins, as well as labelled egg albumin have been shown to produce NSR (Louis, 1958). According to Nairn (1964), conjugation increases the net negative charge on serum proteins at pH 7.0, which now behave as acid dyes and electrostatically bind themselves to positively charged tissue proteins.

Holborow *et al*. (1959) have suggested the use of unconjugated normal sera for blocking these receptor sites before conjugated antiserum is applied to the specimen. Theoretically at least, the components of this conjugated normal serum may not have the same affinity for tissue proteins; secondly, those molecules which may have blocked the receptor sites could in turn provide receptors for the conjugated proteins in the antiserum.

Mayersbach (1959) has shown that freeze-drying, storage of frozen specimens, formalin fixation and pre-treatment with alkaline buffers abolishes or substantially minimizes NSR. However, there is evidence (Beutner, 1961) to suggest that formalin treatment and exposure to alkaline buffers may damage certain antigenic components of specimens under examination. Beutner (1961), working on the concept that non-specific binding of proteins is different from binding of specific serologic reactions, has reported to have reduced NSR by extracting the preparations with 50% glycerol prior to staining.

Absorption of conjugated antisera with tissue powders and tissue homogenates (Kaplan, 1958) is most commonly employed for removing NSR. The efficiency of this method varies greatly depending upon the type of specimens under study and the tissues used for making powders and homogenates. Leucocytes show an affinity for NSR and this persists in conjugated antisera in spite of repeated absorptions even with marrow powders (Danaher _et al_., 1957). On the other hand, red blood cells have been found to be most refractory to NSR (Cohen _et al_., 1960). This fact has been made use of by Ewy and Liu (1962) in staining and titrating influenza virus adsorbed to chicken erythrocytes.

Non-specific staining due to protein interactions can be reduced substantially in many cases by using only the conjugated globulin for staining purposes. However, such attempts by Goldstein et al. (1961) and Myers et al. (1965) in reducing NSR from rabbit sera have met with little success. A further fractionation of antibody globulin has been suggested by Wolf et al. (1963). They report high specificity of staining when only the 7S fraction of gamma-globulin was used in the indirect staining technique. Conjugated gamma-globulins or more purified fractions of antibody molecules can be used at a higher dilution than the whole antiserum, and this in itself has been found to be an excellent means of reducing NSR due to protein interactions (Nairn, 1964).

(2) NSR Due to Excessive Fluorescent Groupings: Conjugation of fluorochromes to proteins is not a uniform process. This results in a marked variation in the number of fluorescent groupings attached per protein molecule. Sub-optimally conjugated sera result in a decrease in specific staining, whereas excessive fluorochrome groups give rise to NSR (Goldstein et al., 1961). This disparity in ratio renders it impossible to assess the quality of different batches of conjugated antisera.

The fluorochrome:protein (F:P) ratio is very critical in giving conjugates which do not produce non-specific fluorescence. Frommhagen (1965) obtained optimally conjugated antibody molecules when F:P ratios of 1:80 or less were used. Clark and Shepard (1963) reported to have accomplished uniform labelling by allowing the fluorochrome to diffuse into a dialysis sac containing the globulin fraction to be conjugated, instead of mixing the two before and dialysing to remove the unconjugated dye. They obtained conjugates of rabies antiserum which showed bright specific fluorescence and no non-specific staining.

(3) NSR Due to "Natural" Antibodies: Antibodies due to previous infections and to "impurities" (e.g. cellular components, serum and proteins used in tissue culture media) in the immunizing material may result in NSR. Absorption of antisera and conjugates with uninfected homologous cells may remove most of the anticellular antibodies. As has been pointed out (Nairn, 1964), this absorption may also remove some of the NSR due to non-specific protein interactions.

Presence of antinuclear factors in sera from systemic lupus erythematosus patients is well documented (Klein et al., 1967). Antinuclear antibodies have been experimentally produced in rabbits (Barnett and Vaughan, 1966). Little information is available regarding the presence and distribution of such factors in other animal species. Since they react specifically with nucleo-proteins from different sources, their presence in antisera and conjugates could result in non-specific staining of virus-infected cells under study. It is conceivable that absorption of sera and conjugates with tissue powders and homogenates removes these antinuclear factors.

Use of Antibodies from Sources Other Than Serum: One possible approach in solving the problem of non-specific fluorescence lies perhaps in the use of antibodies from sources other than serum. Specific antibodies have been shown to exist in body secretions and excretions (Page and Remington, 1966).

Mouse ascitic fluid has been shown to be a good source of specific anti-viral antibodies (Kasel, 1959). Recently, Sartorelli et al. (1966) have obtained specific antibodies from mouse ascites to several different antigens including arboviruses, human serum proteins and sheep erythrocytes. Improvements in techniques (Lieberman et al., 1960; Sartorelli et al., 1966) have made it relatively easy to produce and maintain ascites in mice, thus providing a continuous supply of large quantities of specific antibodies. Antibody titers in immune ascitic fluid were reported (Lieberman et al., 1960) to be higher than those in immune sera from the same animals, although the latter contained 40% more albumin and globulins.

Ascitic fluid antibodies have now been successfully used in immunofluorescence studies of viruses. Larson (1962) found conjugated Coxsackie A14 globulin from immune ascitic fluid to be specific and free from non-specific staining. Ascitic fluid antibodies have also been used in immunofluorescence studies of polyoma virus (Levinthal et al., 1962).

Another promising source of specific antibodies is immune milk. Mitchell et al. (1958) have demonstrated that highly specific milk antibodies could be obtained following instillation of viruses in the mammary gland of ruminants. These antibodies have been shown to possess complement-fixing, hemagglutination-inhibiting and virus neutralizing activities (Mitchell et al., 1967).

Conjugated goat milk antibodies have been used in the direct FAST of mumps virus in infected tissue culture cells (Sattar et al., 1967). These antibodies gave a highly specific reaction and the absence of non-specific fluorescence was striking.

Fixation of Antigens: Fixation of virus antigens for immunofluorescence has been mostly empirical (Fraser, 1964). Improper methods of fixation may lead to erroneous results and may drastically reduce the efficiency of the technique. Coons et al. (1950) found alcohol, formalin and Zenker's reagent to be unsuitable for this purpose; Liu and Coffin (1957) reported that 95% ethyl alcohol resulted in the destruction of canine distemper virus antigens. Fixation by drying is all that is needed in certain cases, but Hinuma and Hummler (1961) have found that drying of poliovirus-infected cultures prior to fixation resulted in total conversion of N (native) to H (heat stable) antigen. Acetone has been found suitable as a general purpose fixative for immunofluorescence studies (Fraser, 1964).

Collection and Preservation of Specimens: Proper collection of specimens is another rather important factor in the application of immunofluorescence to laboratory diagnosis of virus diseases. Specimens should be collected before there is local antibody production; this has been shown to interfere with the staining of certain clinical specimens of smallpox (Coons, 1959). Secondary bacterial invasion may result in non-specific cross reactions and auto-fluorescence (Marmion, 1963). Blood in specimens may interfere with fluorescence because of the quenching effect of hemoglobulin (Nairn, 1964).

Quick preservation of specimens is necessary in many cases to avoid loss of antigens. Preservation of specimens by refrigeration may be helpful. Usefulness of other preservatives for general use is questionable as McQueen et al. (1960) have found glycerol-saline preserved rabies specimens to be unsuitable.

<u>Other Findings of Interest</u>: Apart from the studies referred to in the preceding pages, there have appeared reports in the literature which deserve consideration. In some of these cases the significance of the findings becomes obvious immediately, whereas in others, further work will be needed to assess their true potential.

The complement-staining technique (Goldwasser and Shepard, 1958) has the advantage that it needs only one antibody conjugate directed against complement. In the indirect FAST, one needs separate anti-gamma-globulin conjugates for each species providing antibodies for the primary stage of the staining reaction. The studies of Henle and Henle (1965) have shown that the species limitation of the indirect technique is not absolute. Antibody derived from one species of animal can be stained by labelled antibodies to gamma-globulins not only of the homologous species but of heterologous species as well. Fluorescein-labelled rabbit antibodies to guinea pig or human gamma-globulin were found to be capable of staining the characteristic mumps virus inclusions in infected HeLa cells previously treated with virus-specific antibodies from heterologous animal species.

The complement-staining method has been preferred to the indirect method because of the species limitations of the latter. However, the complement-staining method has its own drawbacks. It is subject to interference by anti-complementary factors (Carpenter, 1965), and not all antigen-antibody reactions fix complement (Rice, 1948). Use of broadly reactive conjugated anti-gamma-globulins in the indirect technique may overcome these limitations and thus facilitate a wider acceptance and application of immuno-fluorescence.

Schneck _et al_. (1966) have reported the synthesis of radioactive fluorescein isothiocyanate. This makes possible the conjugation of proteins with a marker containing two types of labels, thus combining both the rapidity and resolution of fluorescent tracing, and the sensitivity and stability of radioactivity. Since radioactivity, unlike fluorescence, would not be modified by the physical and chemical state of the conjugated protein, the radioactive tracer could serve as an internal standard in quantitative fluorimetry.

There are reports of improvements in the equipment used in fluorescence microscopy. Better and more efficient light sources (Bals and Velculescu, 1966; Young and Armstrong, 1967) are now available, which overcome the disadvantages inherent in high-pressure mercury vapour lamps. Equipment for measuring brightness of fluorescent antibody reactions (Goldman, 1967), and for their automatic scanning and quantitation (Mansberg and Kusnetz, 1966) has been reported.

However, these devices, besides being specialized in use and expensive, are still in the experimental stages of development.

Conclusions: An attempt has been made to analyse some of the facts relating to the application of immunofluorescence in diagnostic virology. Emphasis on this particular aspect may have made the review appear lacking in other respects. However, some of the more recent findings have been referred to which, when further investigated, may add to the specificity and sensitivity of this technique in general.

Immunofluorescence has not only withstood the test of time up to now, but during this short period following its introduction, it has added substantially to our knowledge in many respects.

PART II

EXPERIMENTAL STUDIES ON THE DETECTION OF VIRUS SPECIFIC FLUORESCENCE IN TISSUE CULTURE

SUMMARY

Rapid and sensitive techniques, based on immunofluorescence, have been developed for the titration of herpes simplex, vaccinia and influenza viruses. These titration techniques have been shown to give reproducible results, and their sensitivity compares favourably with the conventional methods of virus quantitation.

The experimental arrangement employed for virus titration was also suited to studies of the dynamics of virus- specific fluorescence in infected cell cultures.

A relationship between the multiplicity of infection and the time of appearance of detectable specific fluorescence has also been established for herpes, polio, vaccinia and influenza viruses. There was a corresponding decrease in the time of appearance of detectable virus-specific fluorescence in tissue cultures with an increase in the amount of virus inoculated.

INTRODUCTION

There exists a definite relationship between the multiplicity of infection and the time of appearance of intact virus particles in infected cells. Cooper (1958) has shown that the latent period of vesicular stomatitis virus could be progressively shortened by repeatedly doubling the multiplicity of infection in chick embryo cells.

In virus-infected cells, appearance of intact virus particles is preceded by the production of specific antigens. These antigens can be demonstrated, using immunofluorescence, making an early identification of infecting virus possible. Since the time of appearance of intact particles in virus-infected cultures is progressively shortened with repeated increases in virus dose, the earliest time at which specific fluorescence becomes detectable is also reduced correspondingly (Cairns, 1960; Wheelock and Tamm, 1961b; Carter, 1965).

It was of interest to investigate this relationship between inocula of varying virus concentration and the time of appearance of detectable specific fluorescence in infected tissue cultures. Establishment of such a relationship for viruses of medical importance would provide a solid foundation for the application of immunofluorescence in the routine analysis of specimens suspected of containing viruses.

Application of immunofluorescence in titrating virus suspensions was first suggested by Dulbecco (1952). It has now been successfully used in the quantitation of a number of viruses (Rapp et al., 1959; Spendlove and Lennette, 1962; Vogt and Rubin, 1963; Yohn et al., 1964; Philipson, 1961; Hahon, 1965 and 1966; Wheelock and Tamm, 1961a; Sattar and Westwood, 1967c; Carter, 1965).

These virus-assay techniques have been reported to be faster and more sensitive in comparison to the conventional methods of virus titration.

Since it was necessary to have sensitive techniques for the titration of viruses to be used in the present study, assay systems, based on immunofluorescence, were first developed. Besides being rapid, sensitive and reproducible, the experimental arrangement employed for these titration techniques was also suited for studies involving direct observations on cells for the detection and localization of specific fluorescence.

Herpes simplex virus was used as a prototype for the development of experimental procedures to be reported here. After the establishment of these procedures, the investigation was expanded to entail other viruses.

MATERIALS AND METHODS

Viruses: Herpes simplex virus used in this study was isolated in this laboratory from a case of herpetic stomatitis. The virus was subsequently characterized by neutralization in tissue culture using a standard herpes simplex antiserum (Microbiological Associates), and on the basis of its typical cytopathology, inclusion formation and pock morphology.

The strains of vaccinia virus, poliovirus I and influenza (A PR8) virus were obtained through the courtesy of Mr. Larry Guerin of this Department.

Production of Antisera: All virus antisera were produced in rabbits. Five weekly intravenous injections (1.0 ml. in each) were administered and blood was collected by ear puncture two weeks after the last injection. In the indirect fluorescent antibody staining technique, these antisera were normally used without dilution or inactivation.

Details of the virus pools used for the immunization of rabbits and the titers of the antisera produced are presented in Table VS-I.

The goat anti-rabbit serum used in the initial experiments was obtained from a commercial source (Difco), but that used in the later work was produced and conjugated

TABLE VS-I

PRODUCTION OF VIRUS ANTISERA IN RABBITS

Virus (Antigen)	Host System Used for Antigen Production	Titer of Virus Pool	Titers of Antisera Produced
Herpes simplex	Primary human amnion cells	$10^{-4.5} TCID_{50}$/0.1 ml.	1/250 against 100 $TCID_{50}$ of the virus
Vaccinia	Primary chick embryo cells	$10^{-6.0} TCID_{50}$/0.1 ml.	1/300 against 100 $TCID_{50}$ of the virus
Poliovirus I	Human embryonic kidney (HEK) cells	$10^{-6.0} TCID_{50}$/0.1 ml.	1/600 against 100 $TCID_{50}$ of the virus
Influenza (A PR8)	Allantoic fluid	$10^{-6.5} TCID_{50}$/0.1 ml.	1/512 against 4 hemagglutinating units of the virus

in this laboratory as follows.

Commercial (Hyland Labs., 4501 Colorado Blvd., Los Angeles 39, Calif.) powdered FII fraction of rabbit serum was reconstituted in distilled water. Freund's complete adjuvant (Difco) was mixed with an equal volume of this antigen solution and injected intramuscularly into a goat. Four weekly injections (4.0 ml. in each) were given and blood was collected from the jugular vein three weeks after the last injection.

An agar immunodiffusion test was performed to determine the presence of specific antibodies in this serum.

Fluorescein Isothiocyanate (FITC): FITC used for the conjugation of the goat antiserum was purchased from Baltimore Biological Co.

Conjugation of Goat Anti-rabbit Gamma-globulin serum: The goat serum was fractionated by precipitation with saturated ammonium sulphate. Protein determinations on the globulin fraction thus obtained were made in a Beckman DB Spectrophotometer at a wave-length of 280 mμ with Lab-trol (LT23YE, Dade) as the standard. Conjugation with FITC was carried out by the method of Riggs *et al*. (1958); for every 80 mg of protein, one mg of the dye was used (Frommhagen, 1965). Unconjugated dye was removed from the reaction mixture by passing it through a column of 'Sephadex' (G-25, Medium, Pharmacia Ltd.). The conjugate was absorbed twice with mouse liver powder before it was used in staining.

The detailed procedure for conjugation is included in the 'Appendix'.

Virus Concentration by Ultra-centrifugation: In the case of herpes simplex, vaccinia and influenza viruses, concentrated virus suspensions were obtained by subjecting the virus pools to ultra-centrifugation ('Spinco', Model L-2, Beckman). The length of spinning times, the centrifugation speeds used and the concentrations achieved are listed in Table VS-II.

Inoculation of Bottle Cultures for Virus Plaque Assays: Second generation GMK cells were cultivated in one ounce Brockway bottles. Cultures showing complete cell sheets were washed once with 5.0 ml. of EBSS. These were then inoculated with 1.0 ml. of the appropriate virus dilution per bottle; at least three cultures were used for each virus dilution tested. After two hours of incubation at 36°C, excess inoculum was removed and 3.0 ml. of overlay medium was added to each bottle and they were placed back in the incubator.

When plaques were ready to be counted, overlay medium

TABLE VS-II

CONCENTRATION OF VIRUSES BY ULTRA-CENTRIFUGATION

Virus	Speed (r.p.m.)	Centrifugal Force (xg)	Time (minutes)	Concentration Achieved
Herpes simplex	15,000	22,600	120	50X
Vaccinia	15,000	22,600	120	30X
Influenza A PR8	18,000	35,000	120	10X

was removed and the monolayers were fixed in 10% foamalin. After washing in tap water, they were stained with Loeffler's methylene blue for five minutes.

Inoculation of Slide Cultures: Growth medium was first removed from these cultures using a pasteur pipette. Virus inoculum volumes of 0.3 ml. were used for each culture, and at least three cultures were inoculated with each virus dilution tested; control cultures received an equivalent amount of the diluent. Use of 0.3 ml. volumes was found necessary in order to get a uniform distribution of the inoculum on the monolayers. Excess inoculum was removed after an appropriate period for virus adsorption. Overlay medium was added, and the cultures were placed back in a CO_2 incubator.

Mouse Liver Powder: Acetone-dried mouse liver powder (Nairn, 1964) was prepared in this laboratory according to the procedure outlined in the 'Appendix'.

Photography: Black and white pictures were taken with 'Kodak' High Speed Ektachrome (Daylight) film. Color prints were obtained from these transparencies.

Fluorescent Antibody Staining of Slide Cultures: Cultivation of cells on microscope slides (Sattar and Westwood, 1967b) has considerably simplified the steps involved in their handling. However, the use of standard staining jars was still not feasible because of the limited amounts of antisera and conjugates available for staining. This necessitated the construction of a slide holder which would permit batch handling of slides without affecting the quantities of reagents involved.

A simple plastic holder was constructed for this purpose (Fig. VS-1). It consisted of a plastic frame, a foam rubber-cushioned plastic strip and three thumb screws. After fixation, slides were arranged on the plastic frame, and then fastened into position by using the foam rubber-cushioned plastic strip and the thumb screws. The cultures could now be handled as a unit and could be put through staining, washing and mounting procedures while still on the holder.

A spreader was also constructed to help in the uniform spreading of the drops of antiserum and conjugates on cultures to be stained (Fig. VS-2).

Fixation and staining of cultures were carried out in the following steps.

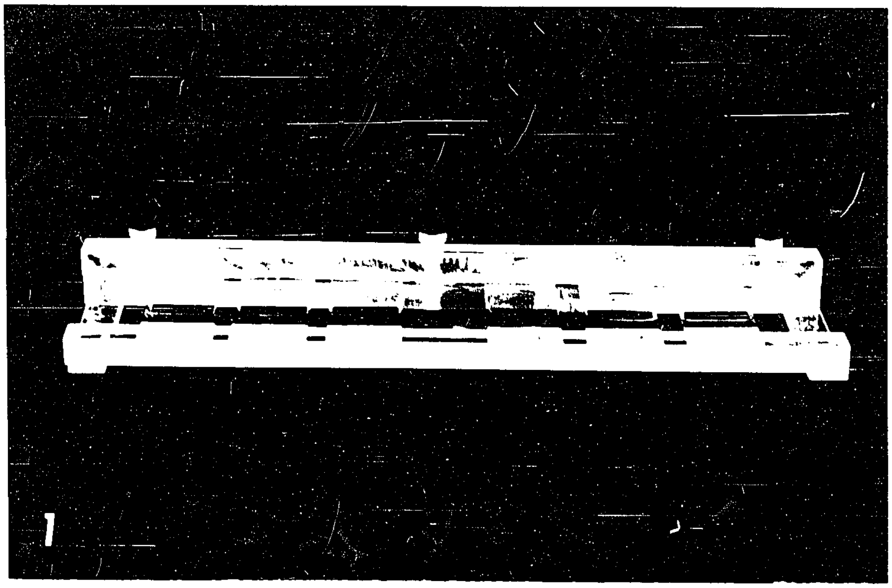
1) After removing the chambers, cultures were washed in three changes of phosphate buffered saline (PBS) at pH 7.4.

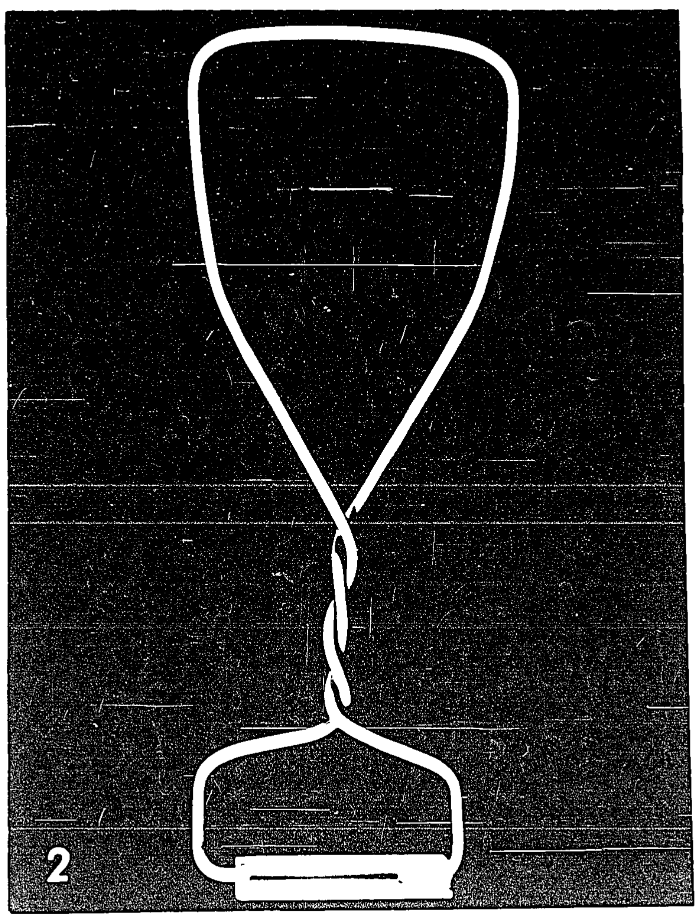
FIG. VS-1

SIDE VIEW OF THE SLIDE HOLDER

FIG. VS-2

SPREADER USED FOR UNIFORM
SPREADING OF STAINING REAGENTS
ON CELL MONOLAYERS





2) They were fixed in acetone at room temperature for ten minutes.

3) Since the indirect method of staining was used all through these studies, cell sheets were first layered with three drops of undiluted antiserum. Following an incubation of 20-30 minutes at room temperature in a moist chamber, slides were washed in three changes of PBS with gentle agitation.

4) Cultures were then layered with three drops of FITC-conjugated goat anti-rabbit globulin. After 20-30 minutes at room temperature, they were washed thoroughly in three changes of PBS and air dried.

5) Coverslips (22 x 22 mm.) were mounted on the cultures using a commercial non-fluorescent mountant (Hartman-Leddon Co. Inc., 60th and Woodland Ave., Philadelphia, Pa.).

All conjugates used for staining were first absorbed twice with mouse liver powder and GMK cell suspension.

Non-specific fluorescence was ruled out by treating, (a) virus-infected cells with normal rabbit serum, (b) un-infected cells with anti-virus serum and (c) infected cells with conjugate alone.

Microscopy: Stained slides were examined using a Reichert Binolux microscope (HBO 200 lamp) with exciter filter E_3 (BG 12/6 mm.) and barrier filter Sp_3 (GG 9/1 mm. + OG 1/1.5 mm.).

Experimental Design to Study the Effect of Multiplicity of Infection on the Time and Rate of Appearance of Specific Fluorescence in Virus-infected GMK Cell Cultures: The term 'multiplicity of infection' refers to the ratio between the number of infectious virus particles in the inoculum and the number of cells in culture. In the following studies virus:cell ratios of 100:1, 10:1 and 1:1 were used.

Separate lots of second generation GMK cell monolayers were inoculated with the three different multiplicities of infection. The inoculated cultures were incubated at 36°C for an appropriate period of time to allow for virus adsorption. At the end of this adsorption period, excess inoculum was removed and the cultures were reincubated after the addition of the plain overlay medium.

For polio, herpes and vaccinia viruses, representative cultures were taken out at two hour intervals, measured from the time of virus inoculation, for a total period of 24 hours. In the case of influenza virus, the first sample was taken out three hours after virus inoculation and from here on the remaining samples were taken every two hours for a total period of 24 hours.

These cultures were fixed, stained and examined qualitatively to determine the earliest time at which specific fluorescence could be detected, and to record the general course of events thereafter.

Cultures representing the three different multiplicities of infection were further examined to determine the percentage of cells showing specific fluorescence at different time intervals after virus inoculation. Twenty different microscopic fields (representing nearly a thousand cells) were scanned and the number of cells showing specific fluorescence (irrespective of its topographical location within the cell) was recorded.

EXPERIMENTAL AND RESULTS

(1) Herpes Simplex Virus

Preliminary Experiments on the Development of Immunofluorescent Foci of Herpes Simplex Virus: In the early stages of herpes simplex virus infection in tissue culture, there is only cell-to-cell spread of the virus without the release of any detectable virus into the surrounding medium. This results in the formation of discrete foci of infection even in the absence of a semi-solid overlay or specific antibody (Farnham, 1958; Kaverin, 1961). Since the use of agar, methyl cellulose or specific antiserum was avoided in the present technique, the early experiments in this series were aimed at the determination of the earliest point at which herpes virus-infected GMK cultures in plain overlay medium (1% TMB + 1% calf serum in EBSS) could be harvested for the determination of immunofluorescence (IF) focus forming units (FFU).

It was found that between 20-22 hours after virus inoculation, the foci were large enough to be visualized and counted microscopically when stained with the fluorescent antibody technique (Fig. VS-3, VS-4 and VS-5). Further incubation did not result in any significant increase in the number of foci, but did lead to coalescence of foci at lower

PHOTOGRAPHS OF GMK CELLS STAINED WITH THE INDIRECT FLUORESCENT ANTIBODY STAINING TECHNIQUE

FIG. VS-3

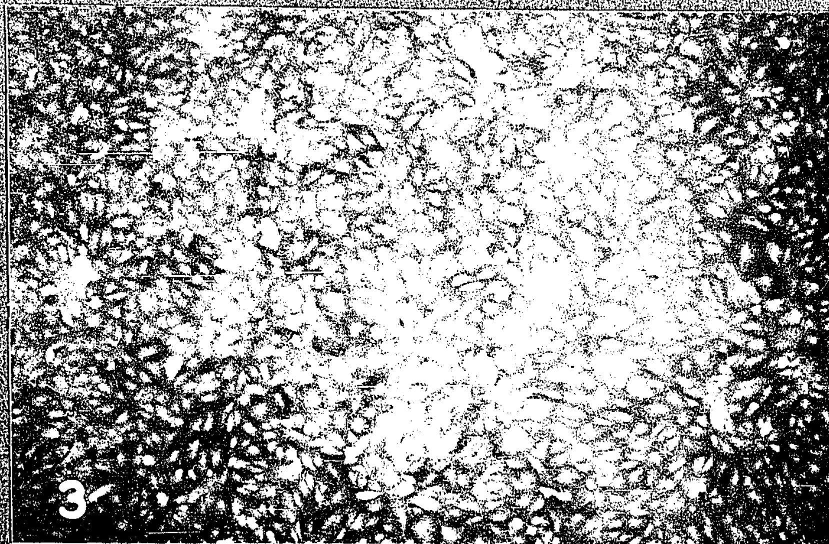
UNINFECTED CELLS (250X)

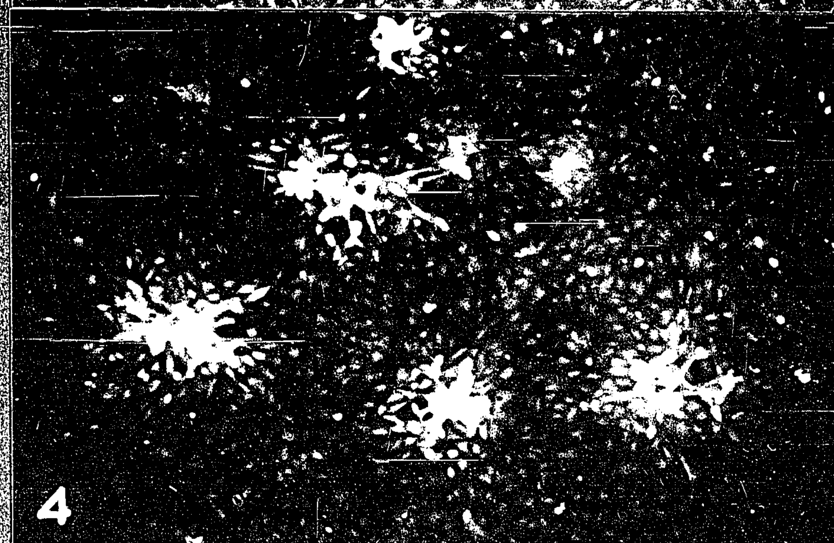
FIG. VS-4

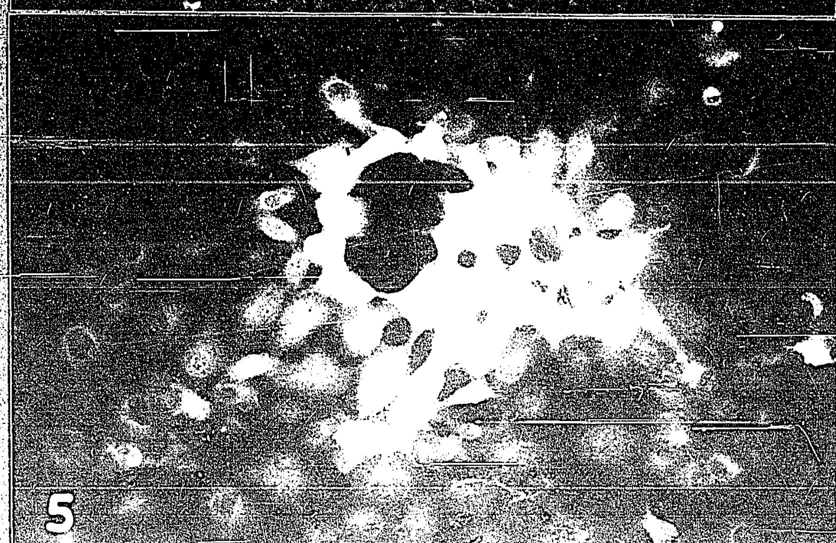
MONOLAYER SHOWING IF FOCI OF HERPES SIMPLEX VIRUS 22 HOURS AFTER VIRUS INOCULATION (250X)

FIG. VS-5

AN IF FOCUS OF HERPES SIMPLEX VIRUS 22 HOURS AFTER VIRUS INOCULATION (500X)


3


4


5

dilutions. Therefore, all focus counts of herpes simplex virus were recorded 20-22 hours after virus inoculation.

Rate of Virus Adsorption: Experiments were conducted to determine the rate of adsorption of the virus to GMK cells from a 0.3 ml. inoculum at 36°C.

GMK cell monolayers in slide chambers were inoculated with a dilution of the virus which was known to give countable foci. These were incubated at 36°C in a 5% CO_2 atmosphere. Three chambers were removed at 30 minute intervals for a total period of 3 hours. Excess inoculum was removed and the cultures were placed back in the incubator after adding the overlay medium.

The excess inoculum from the 3 hour samples was placed in a fresh set of cultures to determine the presence of any residual virus left over even after three hours allowed for adsorption.

The results obtained are presented in Fig. VS-6. As can be seen from the graph, virus adsorption reaches an equilibrium between 90 and 120 minutes after inoculation. Not all the virus present in the inoculum is adsorbed to the cells even after three hours of incubation. This is evident from the presence of nearly 20% residual virus in the excess inoculum from the three hour sample. Therefore, under the

FIG. VS-6

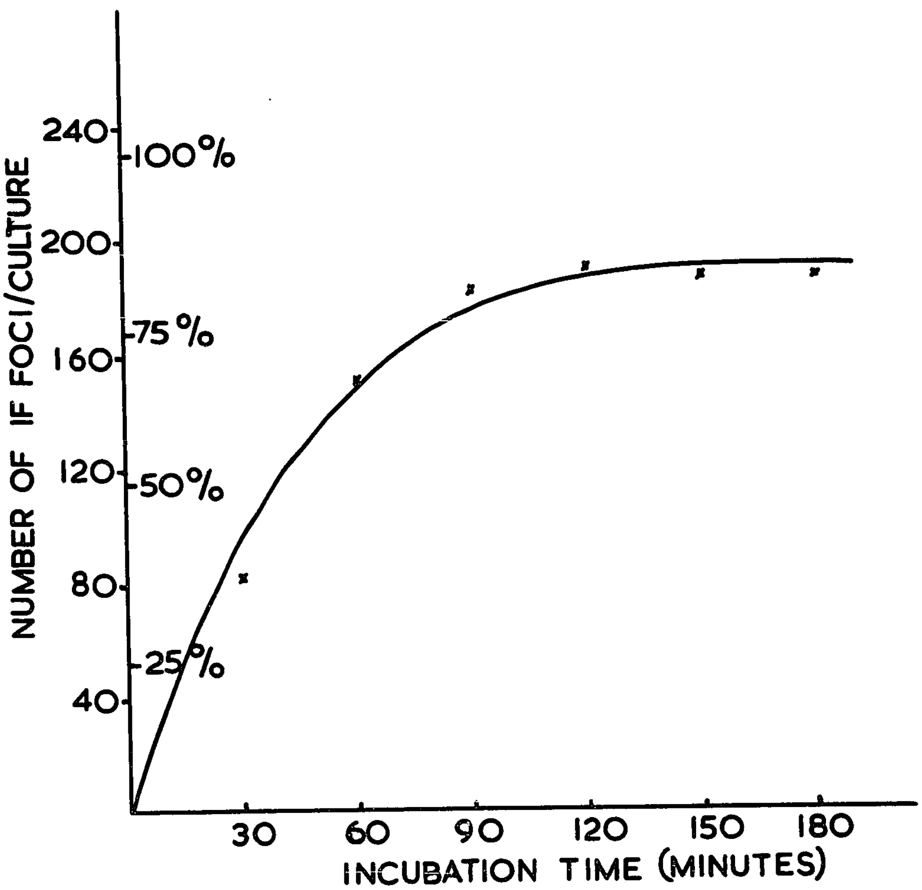
RATE OF ADSORPTION OF HERPES SIMPLEX VIRUS TO GMK CELLS AT 36°C (INOCULUM VOLUME 0.3 ML.)

TOTAL VIRUS PRESENT IN THE INOCULUM = 230 FFU (100%).

VIRUS ADSORBED AFTER THREE HOURS OF INCUBATION = 185 FFU (80%).

RESIDUAL VIRUS = 46 FFU (20%).

FIG.VS-6


NUMBER OF IF FOCI/CULTURE
240
200
160
120
80
40
100%
75 %
50%
25%
30
60
90
120
150
180
INCUBATION TIME (MINUTES)

test conditions, the system gives an adsorption efficiency of about 80%. In all subsequent experiments with herpes virus an adsorption period of two hours at 36° was used.

Similar rates of adsorption have been reported for herpes simplex virus by Farnham (1958) and Newton and Stoker (1958) and for infectious rhinotracheitis virus by Stevens and Groman (1963). Inability of all infectious herpes virus particles in the inoculum to adsorb to susceptible cells has previously been demonstrated by Yoshino and Taniguchi (1956) and Kaplan (1957).

Relationship between Virus Concentration and Number of IF Foci of Herpes Simplex Virus: That a direct relationship exists between the concentration of the virus and the number of IF foci, was demonstrated as follows.

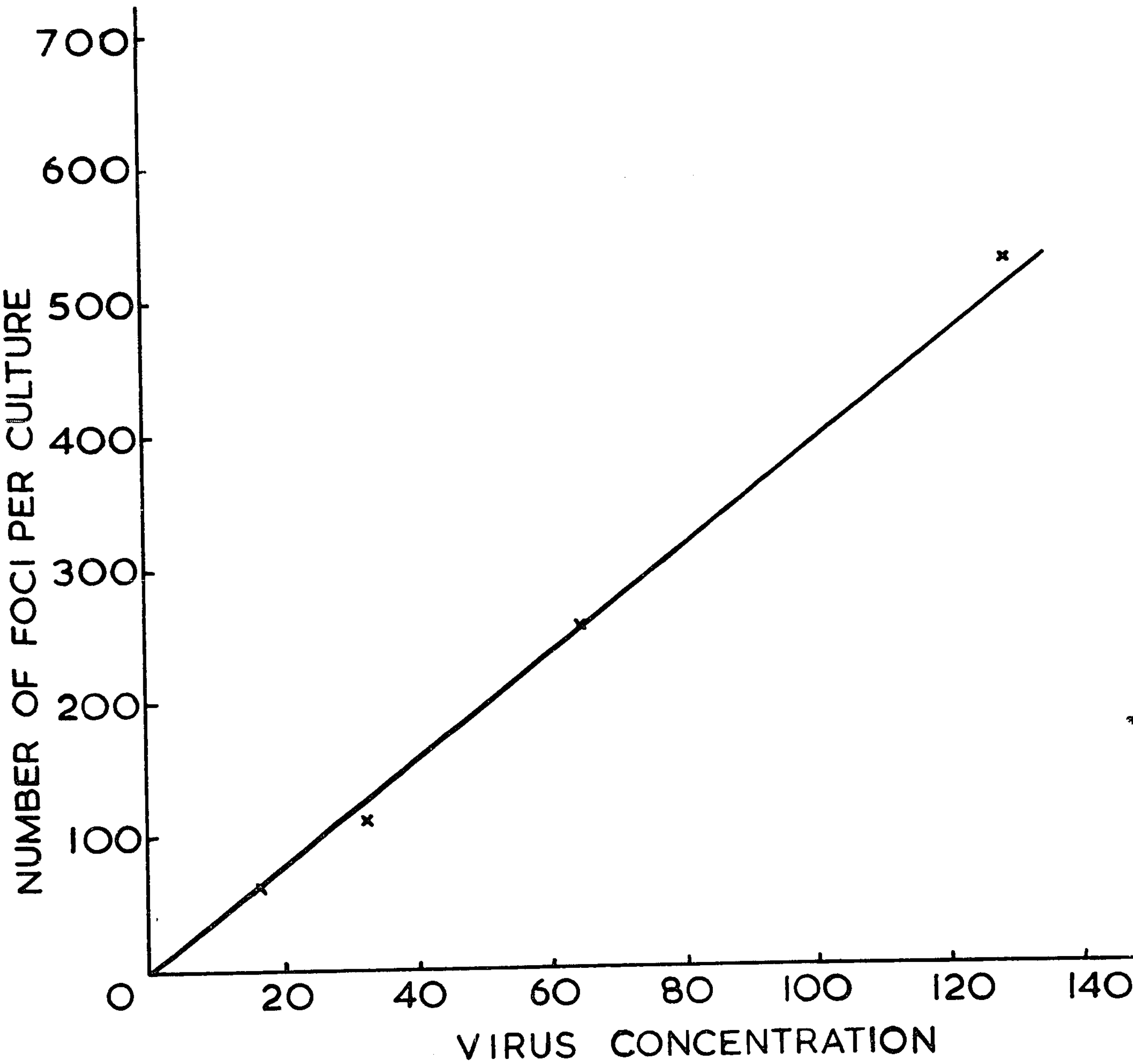
Two-fold dilutions (1/16-1/128) of the virus were inoculated into slide cultures, using three chambers for each virus dilution tested. Following a two hour incubation period at 36°C, excess inoculum was removed, overlay medium was added and the cultures were reincubated.

The results obtained are plotted in Fig. VS-7. A direct relationship between virus concentration and the number of fluorescent foci is clearly demonstrated.

FIG. VS-7

RELATIONSHIP BETWEEN CONCENTRATION AND THE NUMBER OF IF FOCI OF HERPES SIMPLEX VIRUS

FIG. VS-7



700
600
500
400
300
200
100
O
20
40
60
80
100
120
140
NUMBER OF FOCI PER CULTURE
VIRUS CONCENTRATION

<u>Reproducibility of Results</u>: To determine the reproducibility of herpes virus IF focus assay results, three titrations were carried out over a period of 38 days using the same virus pool.

As can be seen from the first two columns of Table VS-III, there was excellent agreement between the results obtained in these three separate experiments.

<u>Comparison of Herpes Simplex IF Focus Assays with Conventional Plaque Assay Technique</u>: When second generation GMK cell monolayers are inoculated with herpes simplex virus, plaques countable with the naked eye appear after about 60 hours of incubation at 36°C in the presence of an antiserum overlay (added 6-8 hours after virus inoculation). Although results could be obtained earlier (within 20-22 hours) using the IF focus technique, the sensitivity of this technique was to be compared with the plaque assay method.

Table VS-III shows the data obtained in three separate experiments using the same virus pool. A comparison of the titers obtained shows that the titers of focus assay experiments were marginally higher in every case, indicating that the sensitivity of the IF focus technique is at least as great as that of the conventional plaque assay.

TABLE VS-III

COMPARISON OF IMMUNOFLUORESCENT FOCUS TECHNIQUE AND PLAQUE ASSAY OF HERPES SIMPLEX VIRUS IN GREEN MONKEY KIDNEY CELLS

Experiment Number	Immunofluorescent Focus Titer/0.3 ml. (20-22 hours)	Plaque Assay Titer/0.3 ml. (60 hours)
6645	5.53×10^5	4.8×10^5
66412	5.7×10^5	4.2×10^5
66513	5.58×10^5	5.1×10^5

No attempt was made to compare the focus assay with pock titration on the chorio-allantoic membrane of embryonated eggs.

Effect of Multiplicity of Infection on the Time and Rate of Appearance of Specific Fluorescence in Herpes Virus-Infected GMK Cell Cultures:

Qualitative Observations: Fig. VS-8 shows the appearance of GMK cells in an uninfected culture. In infected cultures, specific fluorescence was first detected as brightly fluorescent spots in the nuclei of the infected cells (Fig. VS-9). This was followed by an increase in the number and size of these fluorescent spots. There was no apparent relationship between their number inside the nucleus and the multiplicity of infection. In some cells enlargement of these fluorescent spots led to their coalescence, which now appeared as a large homogeneous area of viral antigen inside the nucleus.

Specific fluorescence in the cytoplasm was first restricted to the periphery of the nucleus, but it soon diffused out into the rest of the cytoplasmic area. In the terminal stages of the infection some cells showed a gradual decrease in the intensity of nuclear fluorescence, and specific fluorescence was evident only in the cytoplasm. Whether

PHOTOGRAPHS OF GMK CELLS STAINED WITH THE INDIRECT FLUORESCENT ANTIBODY STAINING TECHNIQUE

FIG. VS-8

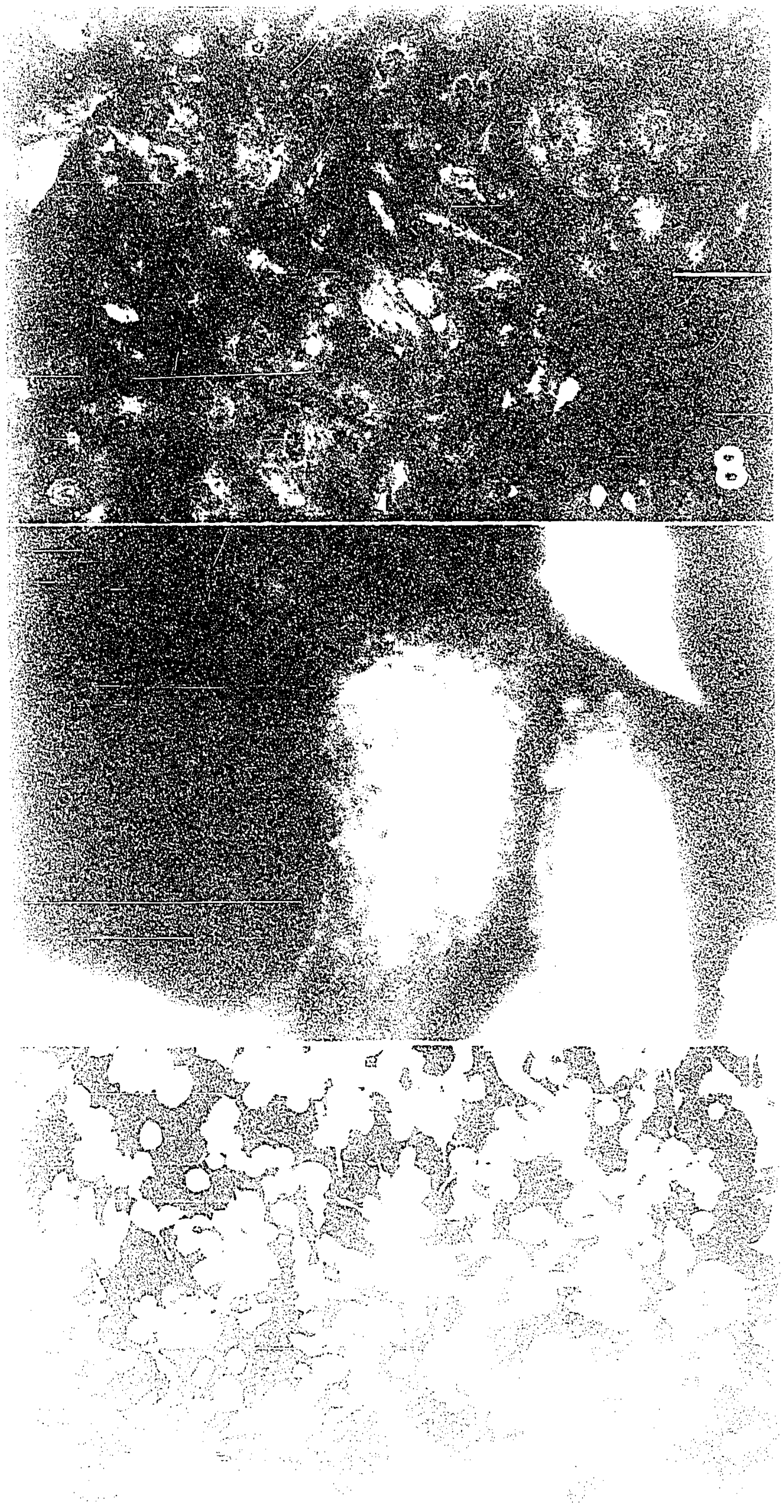
UNINFECTED CELLS

FIG. VS-9

HERPES VIRUS-INFECTED CELLS SHOWING VIRUS SPECIFIC FLUORESCENCE AS BRIGHTLY FLUORESCENT SPOTS IN THE NUCLEUS

FIG. VS-10

HERPES VIRUS-INFECTED CULTURE SHOWING ADVANCED CYTOPATHIC EFFECTS WITH VIRUS SPECIFIC FLUORESCENCE


8

this was generally true of all the cells at this advanced stage of the infection was difficult to assess because of the superimposition of the bright cytoplasmic fluorescence over the nuclear area in most of these cells.

Typical cytopathic changes were also evident in these cultures (Fig. VS-10). Rounded cells and lengthy cytoplasmic projections were commonly seen.

These observations are in essential agreement with the earlier reports on the development of specific fluorescence in herpes simplex virus-infected cells (Lebrun, 1956; Nii et al., 1961; Ross and Orlans, 1958; Roane and Roizman, 1966).

Quantitative Observations: Cultures representing the three multiplicities of infection were examined to determine the percentage of cells showing specific fluorescence at different time intervals after virus inoculation. The experimental design used for this purpose is outlined in detail in the section on 'Materials and Methods'. The results obtained are presented in Table VS-IV and Fig. VS-11.

At the highest multiplicity of infection (100:1), specific nuclear fluorescence could be detected in nearly 20% of the cells as early as four hours after virus inoculation. Fourteen hours after virus inoculation almost all the cells even in the cultures with the lowest multiplicity

TABLE VS-IV

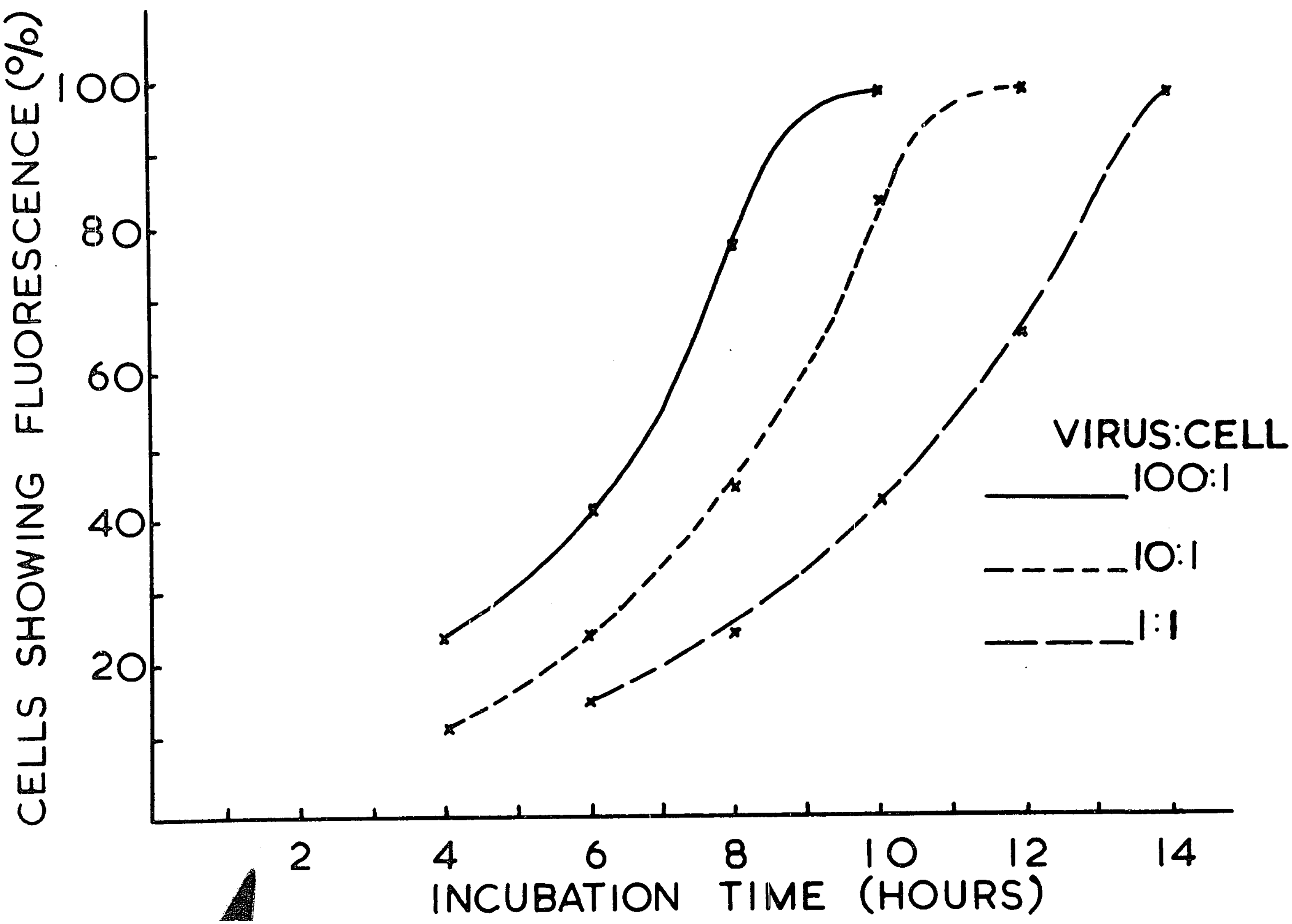
HERPES SIMPLEX VIRUS-INFECTED CELLS SHOWING SPECIFIC FLUORESCENCE AT DIFFERENT STAGES AFTER VIRUS INOCULATION

Time after virus inoculation (hours)	Cells showing specific fluorescence (%) virus:cell		
	100:1	10:1	1:1
4	24	10	-
6	40	23	14
8	77	43	25
10	100	82	41
12	-	100	64
14	-	-	100

FIG. VS-11

RELATIONSHIP BETWEEN MULTIPLICITY OF INFECTION AND THE RATE OF APPEARANCE OF SPECIFIC FLUORESCENCE IN HERPES SIMPLEX VIRUS-INFECTED GMK CELL CULTURE

FIG. VS-11



CELLS SHOWING FLUORESCENCE (%)
100
80
60
40
20
2
4
6
8
10
12
14
INCUBATION TIME (HOURS)
VIRUS:CELL
100:1
10:1
1:1

(1:1) showed specific fluorescence. Since all the cells in these cultures could not have been infected simultaneously using a 1:1 ratio of virus to cells, presence of specific fluorescence in all the cells at fourteen hours indicates more than one cycle of virus replication.

Times at which 50% of the cells would show fluorescence at these different multiplicities were determined from Fig. VS-11 and were as follows.

Virus:cell	Time (hours)
100:1	7
10:1	9
1:1	11

Discussion: In spite of the ability of herpes simplex virus to grow readily in a variety of cell types (Kaplan, 1957; Farnham, 1958; Crandell, 1959; Osterhout and Tamm, 1959; Russell, 1962), development of a simple and sensitive plaque assay of this virus offers certain difficulties. Farnham (1958) has reported the ability of uninfected as well as herpes virus-infected HeLa cells to assimilate neutral red stain. The same phenomenon has been observed in our laboratory using GMK cells. Ronat (1959), on the other hand, noted the occurrence of decolorization of neutral red stain from HeLa cell monolayers, rendering the scoring of herpes virus plaques difficult.

Use of specific antiserum is known to stop the development of secondary plaques (Black and Melnick, 1955; Wheeler and Canby, 1959), and it has been substituted in the overlay to overcome the toxic effects of agar. The use of antiserum overlays is not entirely satisfactory, since it has to be added in a separate step after the addition of the rest of the medium to avoid neutralization of a fraction of the adsorbed virus which remains accessable to the action of specific antibodies (Watkins, 1960; Russell, 1962). Addition of specific antiserum even after 24 hours of herpes virus inoculation has been found to result in a reduced plaque count (Farnham, 1958).

Although methyl cellulose has been found to improve herpes virus plaque counts (Tytell and Neuman, 1963), its use makes the technique difficult and time consuming (Hotchin, 1955; Rapp et al., 1959; Spendlove and Lennette, 1961). Moreover, Rapp and Benyesh-Melnick (1963) found it inferior to agar when employed in herpes zoster plaque overlays.

The assay system for herpes simplex described in the foregoing pages precludes the need for agar, methyl cellulose or specific antiserum in the overlay medium. Besides being rapid, it compares favourably in its sensitivity and reproducibility with the conventional plaque assay using the same host system; it is also suited for the study of the dynamics of virus-specific changes at the cellular level. Using the same experimental arrangement, the relationship between the virus dose and the time of appearance of specific fluorescence has been studied.

(2) Vaccinia Virus:

Preliminary Experiments on the Development of Immunofluorescent Foci of Vaccinia Virus: In the early stages of poxvirus infection in tissue culture, there is only cell-to-cell spread of the virus without the release of any detectable virus into the surrounding medium (Joklik, 1966). This results in the formation of discrete foci of infection even in the absence of a semi-solid overlay or specific antibody (Hahon, 1965; Carter, 1965). Since the use of agar, methyl cellulose or specific antiserum was avoided in the present technique, the early experiments in this series were designed to determine the earliest point at which vaccinia virus-infected GMK cultures in plain overlay medium (1% TMB+1% calf serum in EBSS) could be harvested for the determination of IF focus forming units (FFU).

It was found that between 14-16 hours after virus inoculation, the foci were large enough to be visualized and counted microscopically when stained with the fluorescent antibody technique (Fig. VS-12, VS-13 and VS-14). Further incubation did not result in any significant increase in the number of foci, but did lead to their coalescence at lower dilutions. Therefore, all focus counts of vaccinia virus were recorded 14-16 hours after virus inoculation.

PHOTOGRAPHS OF GMK CELLS STAINED WITH THE INDIRECT FLUORESCENT ANTIBODY STAINING TECHNIQUE

FIG. VS-12

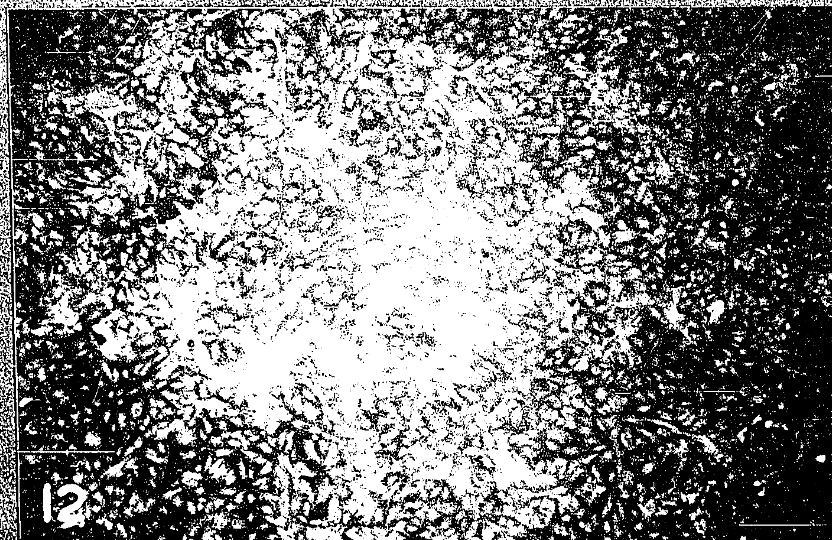
UNINFECTED CELLS (250X)

FIG. VS-13

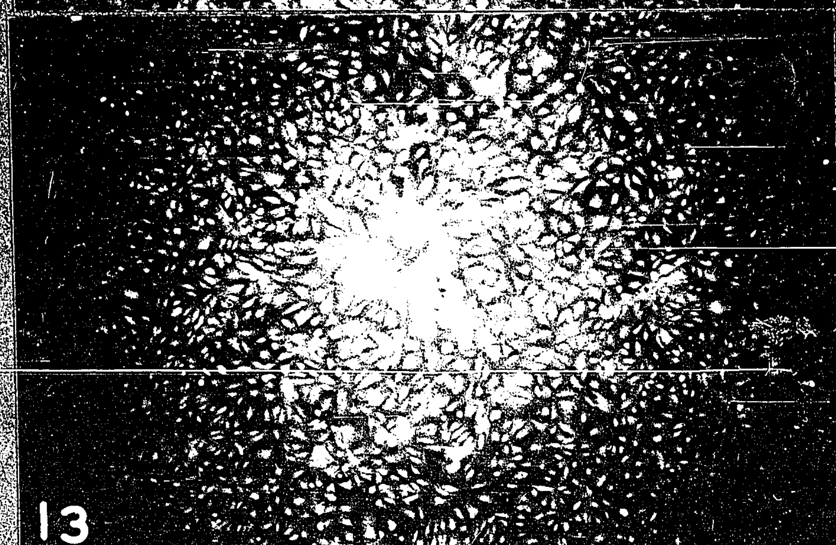
CULTURE SHOWING AN IF FOCUS OF VACCINIA VIRUS 15 HOURS AFTER VIRUS INOCULATION (250X)

FIG. VS-14

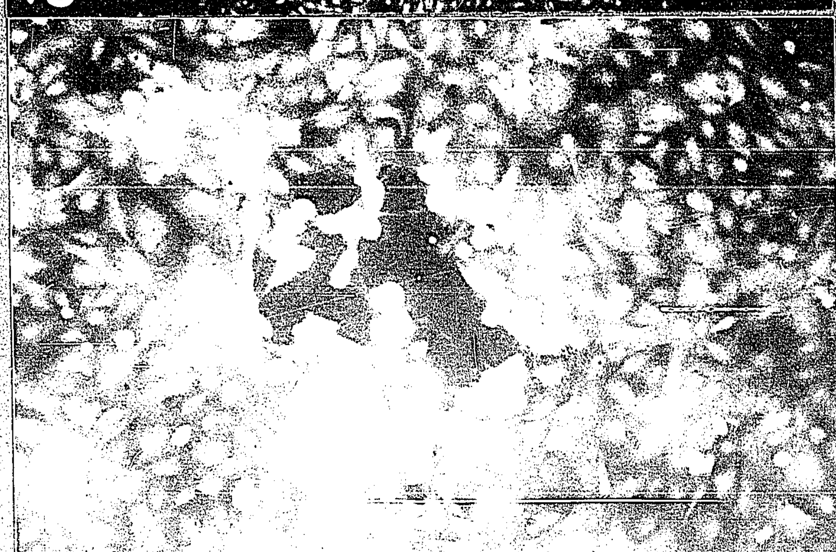
AN IF FOCUS OF VACCINIA VIRUS 15 HOURS AFTER VIRUS INOCULATION (500X)



12



13



Rate of Virus Adsorption: Experiments were conducted to determine the rate of adsorption of the virus to GMK cells from a 0.3 ml. inoculum at 36°C.

GMK cell monolayers in slide cultures inoculated with a dilution of the virus which was known to give countable foci. These were incubated at 36° C in a CO_2 incubator. Three chambers were removed at 30 minutes intervals for a total period of three hours. The excess inoculum was removed and the cultures were placed back in the incubator after adding the overlay medium.

The excess inoculum from the three hour sample was placed in a fresh set of cultures to determine the presence of any residual virus left over in the inoculum even after three hours allowed for adsorption.

The results obtained are presented in Fig. VS-15. As can be seen from the graph, virus adsorption reaches an equilibrium between 90 and 120 minutes after virus inoculation. Similar rates of adsorption for poxviruses have previously been reported (Hahon, 1965). Not all the virus present in the inoculum is adsorbed even after three hours of incubation. This is evident from the presence of nearly 25% residual virus in the excess inoculum taken at the end of three hours of incubation.

FIG. VS-15

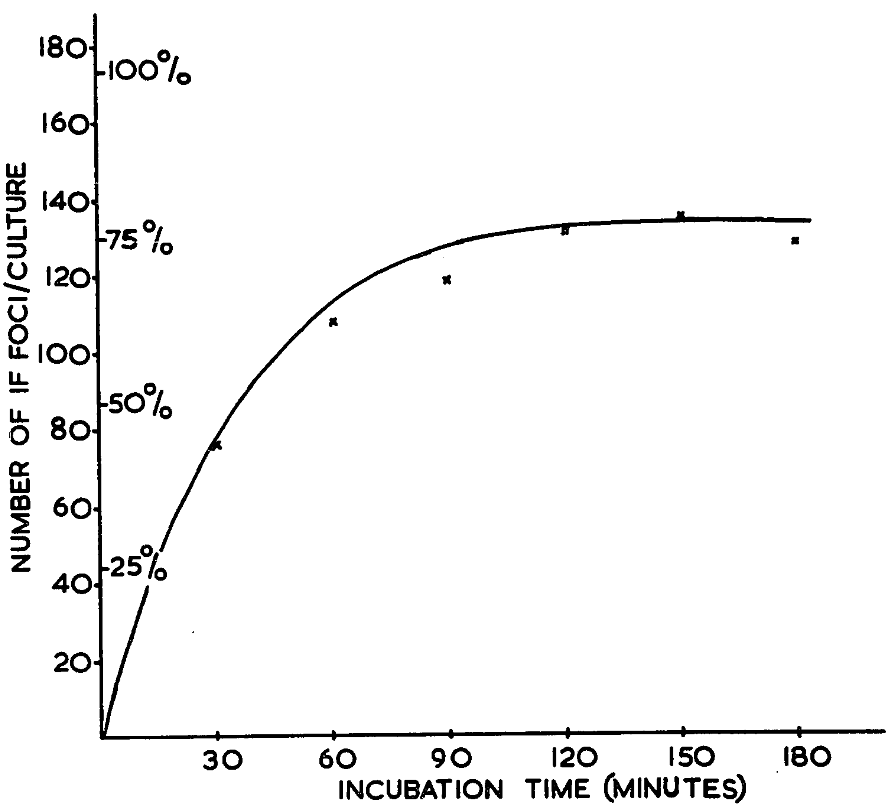
RATE OF ADSORPTION OF VACCINIA VIRUS TO GMK CELLS AT 36°C (INOCULUM VOLUME 0.3 ML.)

TOTAL VIRUS IN THE INOCULUM = 173 FFU (100%).

VIRUS ADSORBED AFTER THREE HOUR OF INCUBATION = 130 FFU (75%).

RESIDUAL VIRUS = 43 FFU (25%).

FIG. VS-15


NUMBER OF IF FOCI/CULTURE
180
160
140
120
100
80
60
40
20
100%
75%
50%
25%
30
60
90
120
150
180
INCUBATION TIME (MINUTES)

Inability of all the infectious vaccinia virus particles in the inoculum to adsorb to susceptible cells has previously been demonstrated (Postlethwaite, 1960).

Under the test conditions, the system gives an adsorption efficiency of about 75%. In all subsequent experiments with this virus, an adsorption period of two hours at 36°C was used.

<u>Relationship Between Virus Concentration and Number of IF Foci of Vaccinia Virus</u>: A direct relationship between virus concentration and the number of IF foci of vaccinia virus was demonstrated as follows.

Two-fold dilutions (1/16-1/128) of the virus were inoculated into slide cultures of GMK cells, using at least three chambers for each virus dilution tested. Following a two hour incubation period at 36°C, excess inoculum was removed, overlay medium was added and the cultures were reincubated. After fourteen hours they were fixed, stained and examined to determine the number of IF foci per culture.

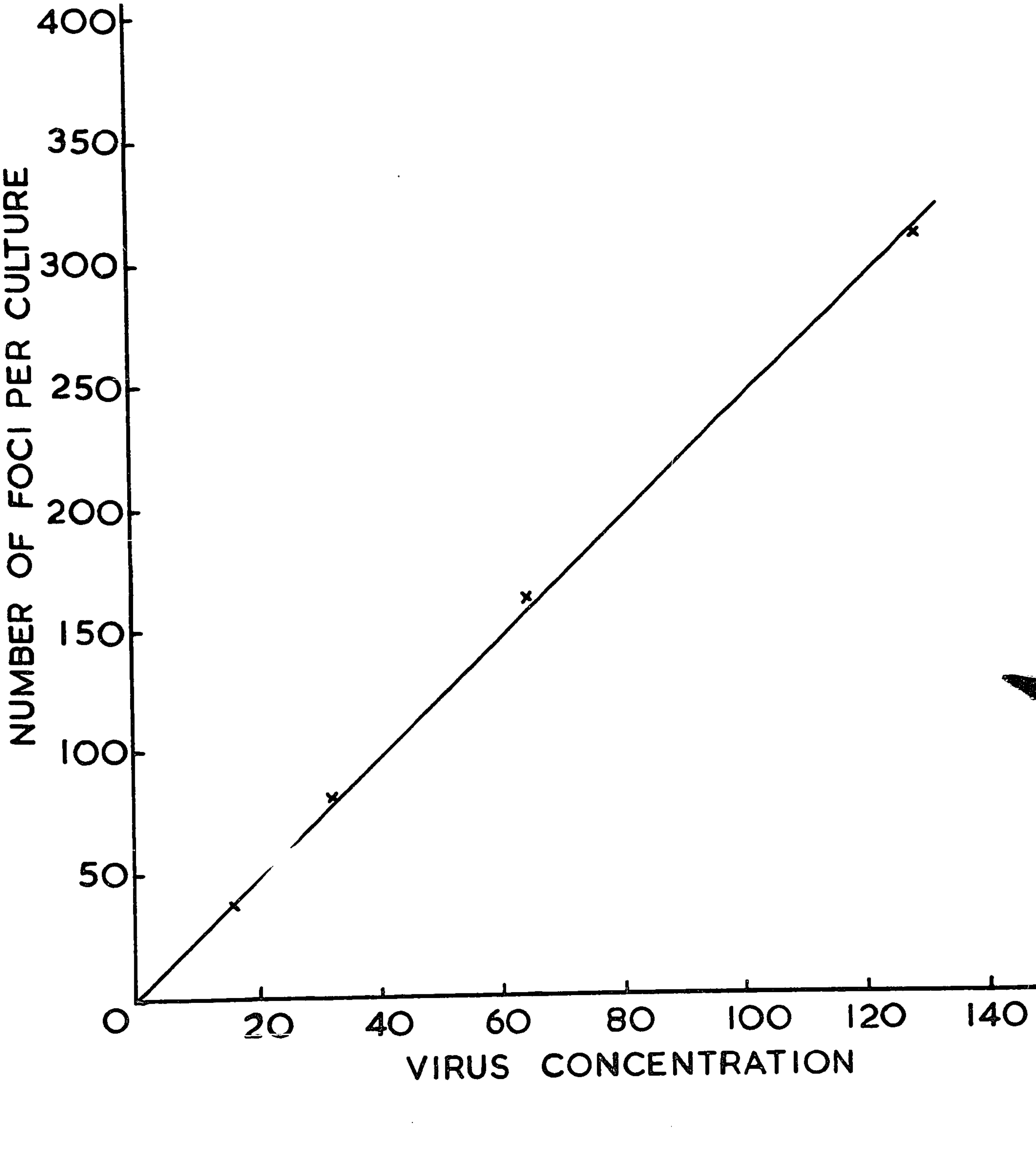
The results obtained are presented in Fig. VS-16. A direct relationship between virus concentration and the number of IF foci is clearly demonstrated.

FIG. VS-16

RELATIONSHIP BETWEEN CONCENTRATION

AND NUMBER OF IF FOCI OF VACCINIA VIRUS

FIG. VS-16



NUMBER OF FOCI PER CULTURE
400
350
300
250
200
150
100
50
0
20
40
60
80
100
120
140
VIRUS CONCENTRATION

Reproducibility of Results: To determine the reproducibility of vaccinia virus IF focus assay results, three titrations were carried out over a period of 18 days using the same virus pool.

As can be seen from the first two columns of Table VS-V, there was excellent agreement between the results obtained in these three separate experiments.

Comparison of Vaccinia Virus IF Focus Assay with Macroplaque Technique in Slide Chambers: In the case of herpes simplex virus, the IF assay was compared with the conventional plaque technique performed in one ounce Brockway bottles. The plaque assay therefore was carried out in a closed system and with a different ratio of inoculum volume (1.0 ml.) to monolayer area than that used in IF focus assays.

Since it was found possible to carry out a macro-plaque assay in the slide chambers, it was decided to compare it with the IF focus assay of vaccinia virus, thus providing a better comparison of the sensitivities of the two techniques.

The following procedure was used for the macro-plaque assay. GMK monolayers in slide chambers received appropriate dilutions of the virus, using at least three cultures at each dilution tested. Following an adsorption period of two

TABLE VS-V

COMPARISON OF IMMUNOFLUORESCENT FOCUS ASSAY AND MACRO-PLAQUE TITRATION TECHNIQUE FOR VACCINIA VIRUS IN SECOND GENERATION GREEN MONKEY KIDNEY CELLS

Experiment number	Immunofluorescent focus titer/0.3 ml. (14-16 hours)	Macro-plaque assay titer/0.3 ml. (38 hours)
6743	2.53×10^6	2.3×10^6
67415	2.41×10^6	2.25×10^6
67421	2.62×10^6	2.10×10^6

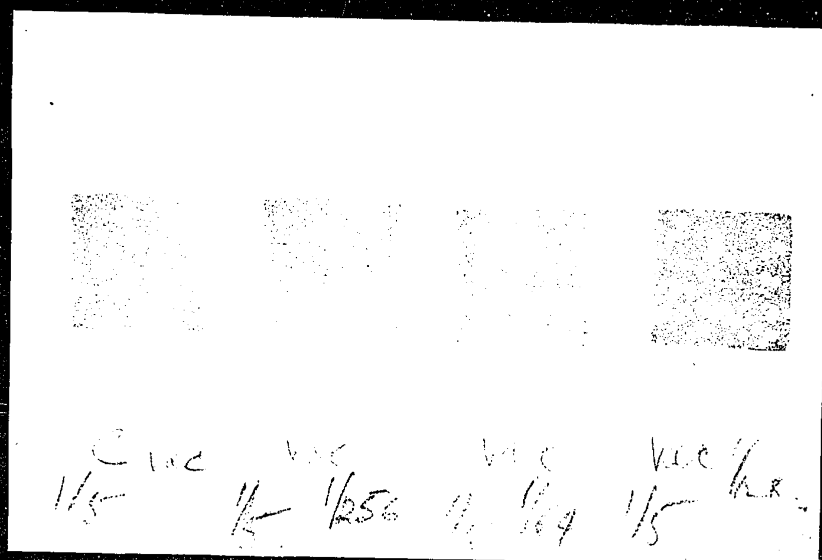
hours at 36°C, excess inoculum was removed and the plain overlay medium was added; the cultures were reincubated. Eight hours after virus inoculation, specific anti-vaccinia rabbit serum (titer 1/300 against 100 $TCID_{50}$ of the virus) was added to the medium at a final dilution of 1/250 - a concentration which had been shown to prevent the formation of secondary plaques without interfering with primary plaque development. After an additional incubation period of 28 hours, the cultures were fixed in 10% formalin and stained with Loeffler's methylene blue. As can be seen from Fig. VS-17, these plaques could be counted with the naked eye. Table VS-V shows the data obtained in three separate experi-metns using the same virus pool. IF focus assay titers were in every case marginally higher than those obtained by the macro-plaque technique, indicating that the focus assay method is at least as sensitive as the latter.

No attempt has been made to compare the IF focus assay with pock titration on chorio-allantoic membranes, since the sensitivity of this titration method is dependent on the degree of adaptation of the virus strain to the embryonated egg and the comparison would not provide a generally applicable measure of the sensitivity of the assay technique.

FIG. VS-17

PHOTOGRAPH SHOWING THE APPEARANCE OF VACCINIA VIRUS PLAQUES IN SLIDE CULTURES OF GMK CELLS 38 HOURS AFTER VIRUS INOCULATION

(CULTURES FIXED IN 10% FORMALIN AND STAINED WITH LOEFFLER'S METHYLENE BLUE)


1/256
1/64

Effect of Multiplicity of Infection on the Time and Rate of Appearance of Specific Fluorescence in Vaccinia Virus-infected GMK Cell Cultures:

Qualitative Observations: Fig. VS-18 shows the appearance of GMK cells in an uninfected culture. In infected cultures, specific fluorescence was first seen in the cytoplasm of the infected cells as a diffused perinuclear halo. It soon extended to cover the rest of the cytoplasm. Its distribution at this stage was granular. When examined at a higher magnification, these granules appeared as cytoplasmic inclusions. As the infection progressed, the granules enlarged in size and eventually coalesced to completely fill the cytoplasm with specific fluorescence (Fig. VS-19).

In the present study, no attempt was made to demonstrate the proportionality between the number of these inclusions and the multiplicity of infection; that a direct relationship exists between these two factors, has previously been demonstrated (Cairns, 1960).

Typical cytopathic changes were also evident in these cultures. Cells in the advanced stages of infection were rounded and slightly enlarged in size in comparison to uninfected ones. Long cytoplasmic strands were abundant along with numerous small projections originating at the

PHOTOGRAPHS OF GMK CELLS STAINED WITH THE INDIRECT FLUORESCENT ANTIBODY STAINING TECHNIQUE

FIG. VS-18

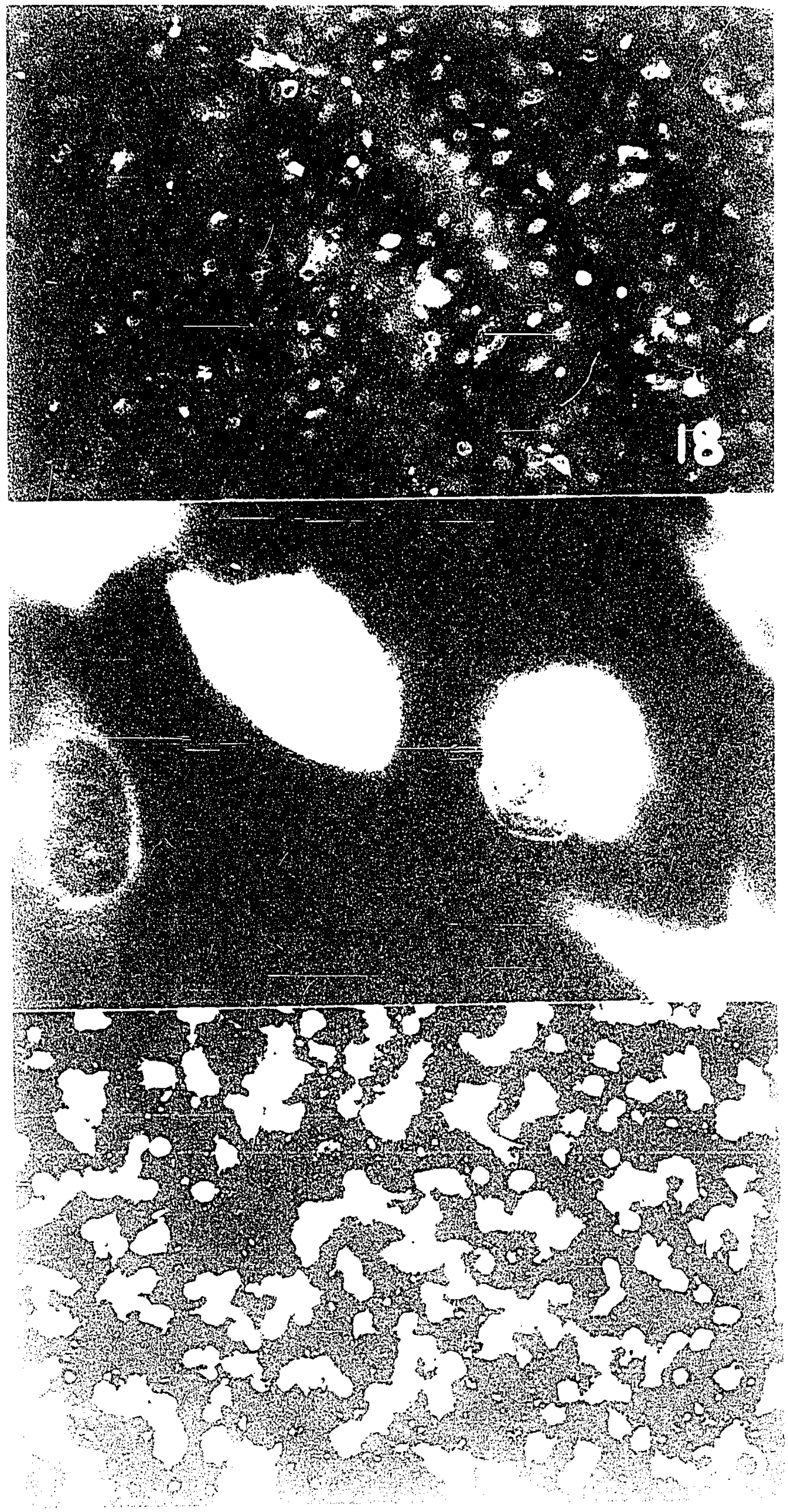
UNINFECTED CELLS

FIG. VS-19

VACCINIA VIRUS-INFECTED CELLS SHOWING SPECIFIC FLUORESCENCE IN THE CYTOPLASM

FIG. VS-20

VACCINIA VIRUS-INFECTED CULTURES SHOWING ADVANCED CYTOPATHIC EFFECTS WITH VIRUS SPECIFIC FLUORESCENCE


18

surface of infected cells. Cultures with extensive cellular destruction also showed minute fluorescent extra-cellular particles (Fig. VS-20). Whether these particles represented fragments of disintegrated cells or aggregates of virus particles could not be established.

These observations are in essential agreement with the previous reports on the development of specific fluorescence in poxvirus infected cells (Loh and Riggs, 1961; Kato _et al._, 1964; Carter, 1965; Hahon, 1965; Joklik, 1966).

Quantitative Observations: Cultures representing the three multiplicities of infection were examined to determine the percentage of cells showing specific fluorescence at different intervals after virus inoculation. The results obtained are presented in Table VS-VI and Fig. VS-21.

At the highest multiplicity of infection (100:1) two hours after virus inoculation, the cultures showed extensive cytopathic effects along with bright specific fluorescence in nearly 50% of the cells. At six hours, there was complete destruction of the cell sheet with fluorescence in almost all the cells that remained attached to the glass.

This rapid and total destruction of the culture at a high multiplicity of infection may have been due to a "toxic" effect of the virus. That poxviruses produce such toxic

TABLE VS-VI

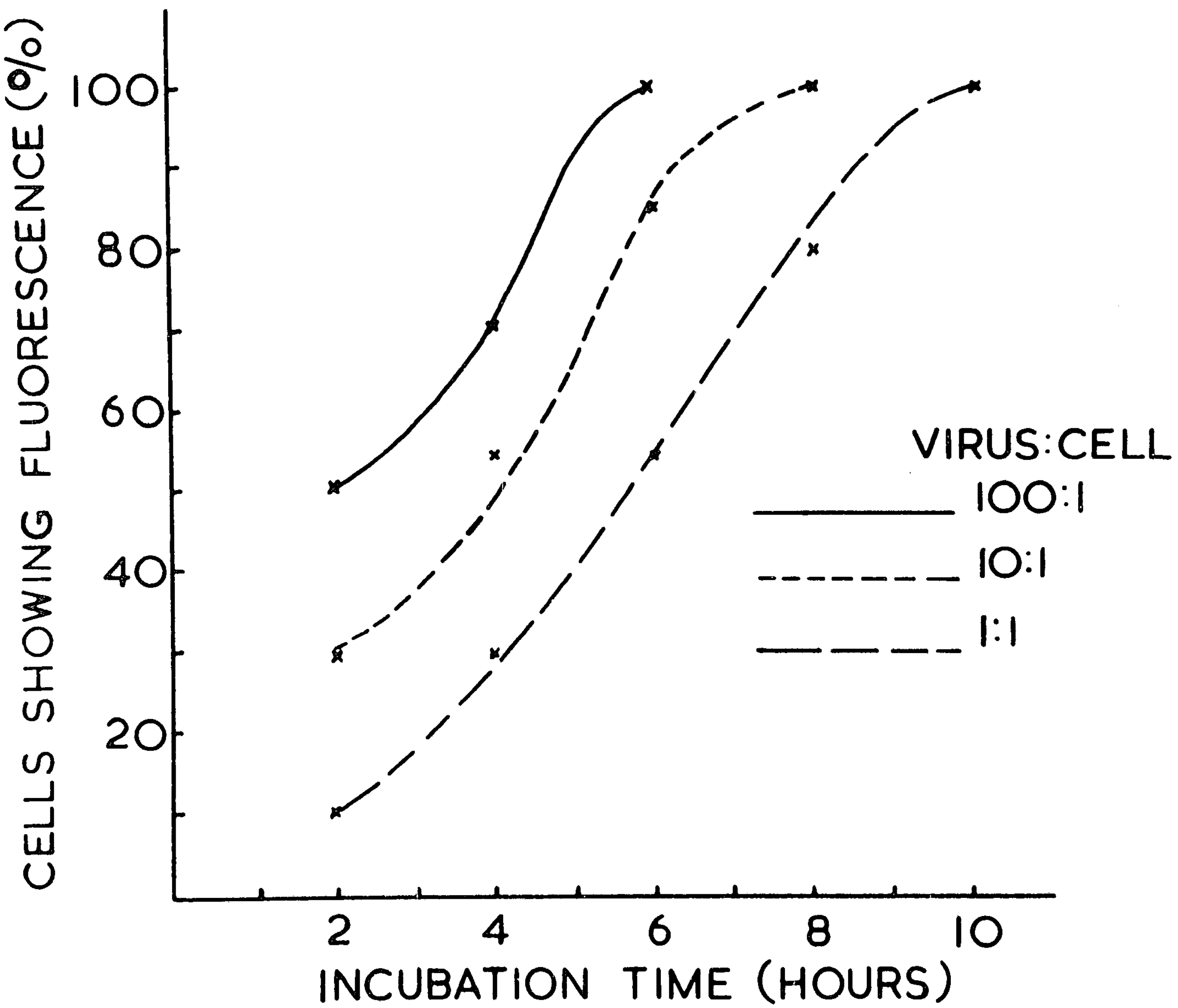
VACCINIA VIRUS-INFECTED CELLS SHOWING SPECIFIC FLUORESCENCE AT DIFFERENT STAGES AFTER VIRUS INOCULATION

Time after virus inoculation (hours)	Cells showing specific fluorescence (%) Virus:Cell		
	100:1	10:1	1:1
2	50	30	10
4	70	55	30
6	100	85	56
8	-	100	80
10	-	-	100

FIG. VS-21

RELATIONSHIP BETWEEN MULTIPLICITY OF INFECTION AND THE RATE OF APPEARANCE OF VIRUS SPECIFIC FLUORESCENCE IN VACCINIA VIRUS-INFECTED GMK CELL CULTURE

FIG. VS-21


CELLS SHOWING FLUORESCENCE (%)
100
80
60
40
20
2
4
6
8
10
INCUBATION TIME (HOURS)
VIRUS:CELL
100:1
10:1
1:1

effects in tissue culture, has previously been demonstrated (Appleyard et al., 1962; Hanafusa, 1962). The factor responsible is the poxvirus particle itself, not a separate "soluble" toxin (Joklik, 1966). The presence of specific fluorescence in these cultures may represent antigens produced as a result of a partial cycle of virus replication (Westwood, 1963).

Cultures with the lowest multiplicity (1:1) of infection showed specific fluorescence in nearly all the cells ten hours after virus inoculation. Since all the cells in these cultures could not have been infected simultaneously using a 1:1 ratio of virus to cells, presence of specific fluorescence in all the cells at ten hours indicates more than one cycle of virus replication.

Times at which 50% of the cells would show fluorescence at these different multiplicities were determined from Fig. VS-21 and were as follows:

Virus:Cell	Time (hours)
100:1	2
10:1	4
1:1	5 1/2

Discussion: Use of specific antiserum is known to stop the development of secondary plaques of poxviruses (Joklik, 1965) and it has been substituted in the overlay to overcome the toxic effects of agar. The use of antiserum overlay is not entirely satisfactory, since it has to be added in a separate step after the addition of the rest of the medium to avoid neutralization of a fraction of the adsorbed virus which remains accessable to the action of specific antibodies. In the presence of specific antibody, plaque formation is delayed (Postlethwaite, 1960).

Poxvirus plaques produced under sodium bicarbonate-agar overlays are small even after 5 days of incubation (Noyes, 1953). Although substitution of Tris buffer for bicarbonate buffer in the agar overlay has been shown to give more satisfactory results (Porterfield and Allison, 1959), there was no significant reduction in the time required for plaque formation.

Successful application of immunofluorescence in the titration of poxviruses has already been reported (Carter, 1965; Hahon, 1965; Yohn et al., 1964). These techniques along with the one reported here, have been found to be sensitive, rapid and reproducible. They preclude the need for agar or specific antiserum in the overlay medium.

The experimental arrangement used for IF focus assay of vaccinia virus is also suited to the study of the dynamics of specific fluorescence in infected cell cultures.

(3) Influenza Virus

Preliminary Experiments on the Development of IF Cells of Influenza Virus: Strains of myxoviruses that have been adapted to grow in embryonated eggs produce an abortive infection in tissue cultures of mammalian cells (Fraser, 1967). In such an infection, the primarily infected cells synthesize both virus specific nucleic acid and proteins, but are incapable of producing infectious progeny virus. The presence of virus-specific antigens can be demonstrated in these cells with the help of immunofluorescence.

White et al. (1965) developed a quantitative hemadsorption test for the titration of influenza virus in abortively infected HeLa cells. They demonstrated that there was no detectable increase in the number of hemadsorbing cells after 16 hours, and that the final number of hemadsorbing cells was directly proportional to the input dose of the virus.

In the present study, an IF cell assay is described for influenza virus. It is based on the observation that abortively infected GMK cells showed bright and specific fluorescence when stained by the fluorescent antibody technique.

In preliminary studies, it was found that between 12-13 hours after infection, a majority of the infected cells showed bright fluorescence both in the cytoplasm and the nucleus (Fig. VS-22). Since at this stage they could be easily distinguished from uninfected cells, all IF cell counts for cell infecting units (CIU) of influenza virus were made 12-13 hours after virus inoculation. Further incubation did not result in any significant increase in the number of fluorescent cells, but did make their detection and counting difficult because of a marked decrease in the intensity of the fluorescence and its localization on the periphery of the cytoplasm.

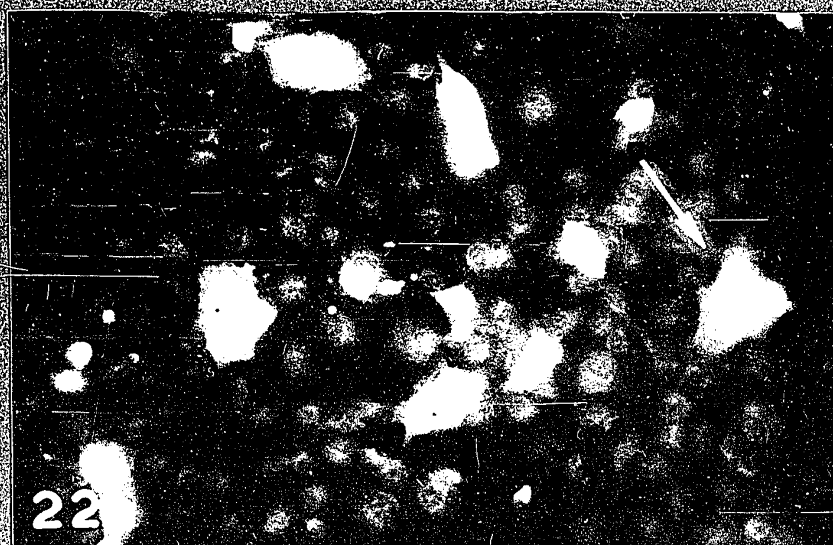
Rate of Virus Adsorption: Experiments were conducted to determine the rate of adsorption of influenza virus to GMK cells from a 0.3 ml. inoculum at 36°C.

GMK cell monolayers in slide chambers were inoculated with a dilution of the virus which was known to give countable IF cells. These were incubated at 36°C in a CO_2 incubator. Three chambers were removed at 60 minute intervals for a total period of five hours. The excess inoculum was removed and the cultures were placed back in the incubator after adding the overlay medium.

The excess inoculum from the five hour sample was placed in a fresh set of cultures to determine the presence of any residual virus left over in the inoculum even after five hours allowed for adsorption.

FIG. VS-22

INFLUENZA VIRUS-INFECTED GMK CELL MONO-
LAYER 13 HOURS AFTER VIRUS INOCULATION (500X)


22

The results obtained are presented in Fig. VS-23. As can be seen from the graph, virus adsorption reaches an equilibrium between two and three hours after virus inoculation. Not all the virus present in the inoculum is adsorbed even after five hours of incubation. This is evident from the presence of nearly 35% residual virus in the excess inoculum taken at the end of five hours.

Inability of all the infectious myxovirus particles in the inoculum to adsorb to susceptible cells has previously been demonstrated (Wheelock and Tamm, 1961a; Henle, 1949).

Under the test conditions, the system gives an adsorption efficiency of nearly 65%. In all subsequent experiments with this virus, an adsorption period of three hours at 36°C was used.

Relationship Between Virus Concentration and Number of IF Cells of Influenza Virus: A direct relationship between virus concentration and the number of IF cells of influenza virus was demonstrated as follows:

Two-fold dilutions (1/2-1/16) of the virus were inoculated into slide cultures of GMK cells, using at least three chambers for each dilution tested. Following a three hour incubation period at 36°C, excess inoculum was removed, overlay medium was added and the cultures were reincubated.

FIG. VS-23

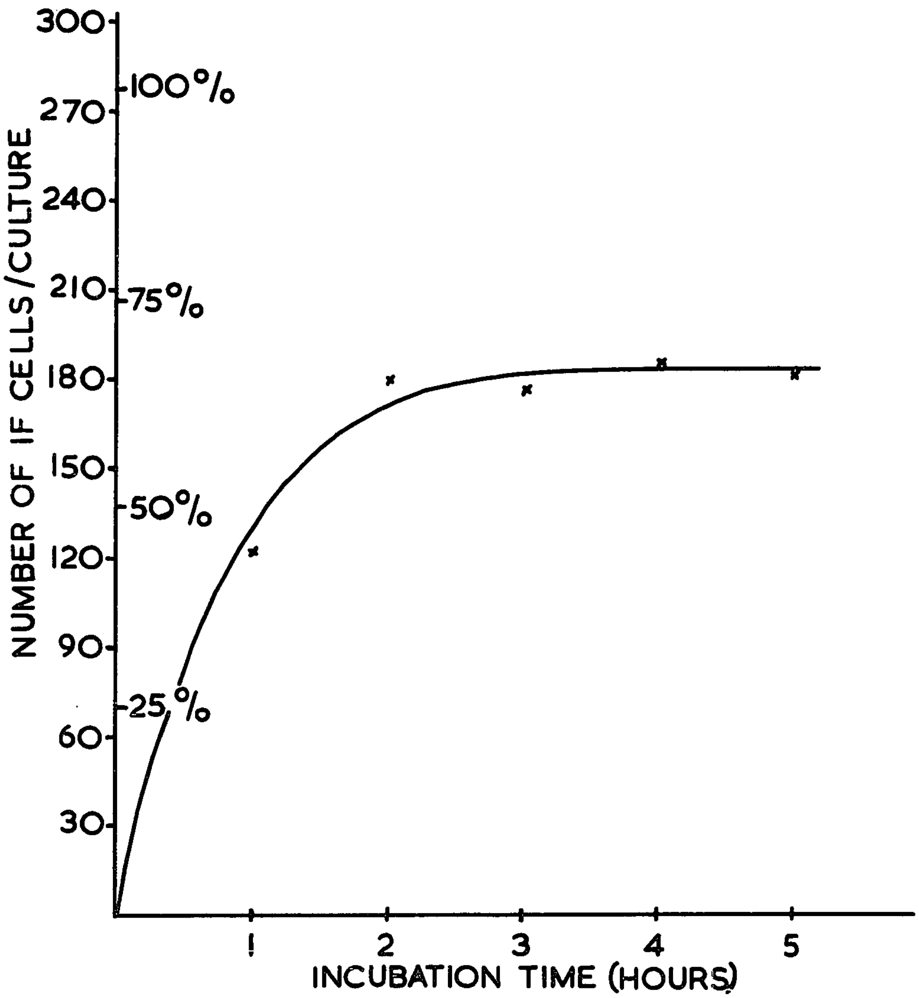
RATE OF ADSORPTION OF INFLUENZA
VIRUS TO GMK CELLS AT 36°C
(INOCULUM VOLUME 0.3 ML.)

TOTAL VIRUS IN THE INOCULUM
= 277 CIU (100%).

VIRUS ADSORBED AFTER FIVE
FIVE HOURS OF INCUBATION
= 180 CIU (65%).

RESIDUAL VIRUS = 97 CIU (35%).

FIG. VS-23



NUMBER OF IF CELLS/CULTURE
300
270
240
210
180
150
120
90
60
30
100%
75%
50%
25%
1
2
3
4
5
INCUBATION TIME (HOURS)

After an additional nine hours of incubation, they were fixed, stained and examined to determine the number of IF cells per culture.

The results obtained are presented in Fig. VS-24. A direct relationship between virus concentration and the number of IF cells is evident.

Reproducibility of Results: To determine the reproducibility of influenza virus IF cell assay results, three titrations were carried out over a period of 25 days using the same virus pool.

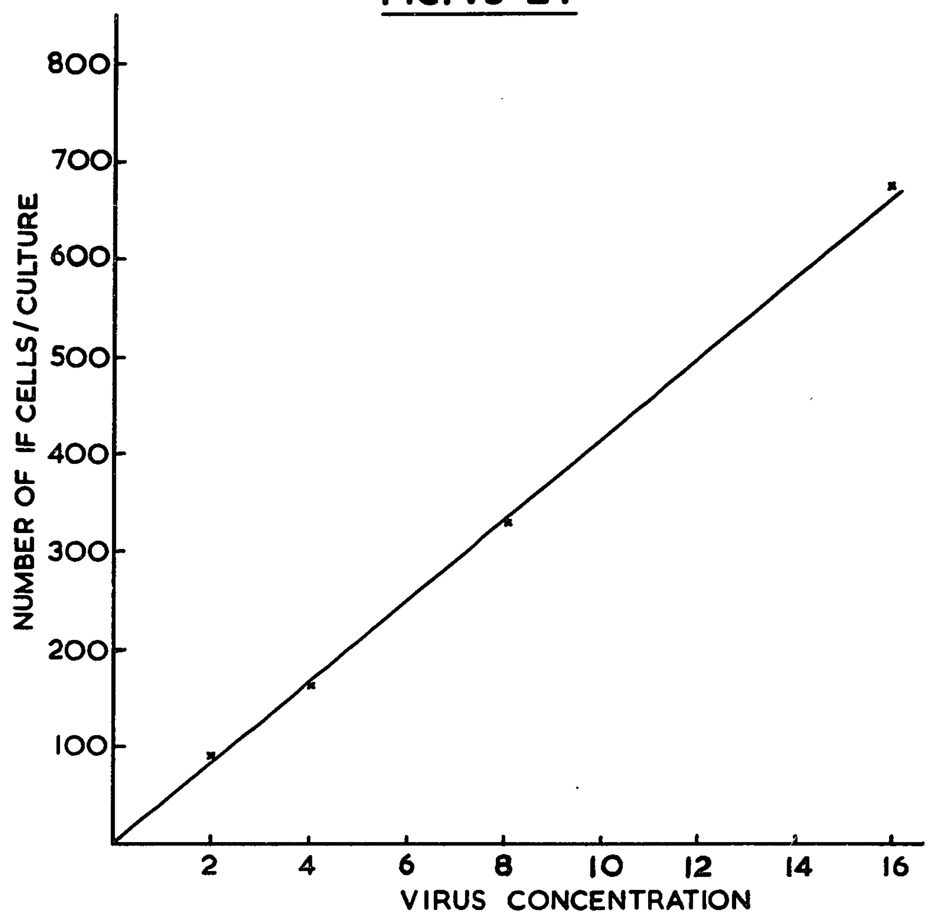
As can be seen from the first two columns of Table VS-VII, there was good agreement between the results obtained in these separate experiments.

Comparison of Influenza Virus IF Cell Assay with Quantitative Hemadsorption Technique: Hemadsorption can be demonstrated even in cells abortively infected with infuenza virus (White et al., 1965). Rozee et al. (1958) developed a method for titrating influenza virus by enumerating the cells showing hemadsorption in infected HeLa and monkey kidney cell cultures. They reported the technique to be sensitive and reproducible. Experiments were conducted to compare the IF cell assay with this quantitative hemadsorption test for titrating influenza virus.

FIG. VS-24

RELATIONSHIP BETWEEN CONCENTRATION AND NUMBER OF IF CELLS OF INFLUENZA VIRUS

FIG. VS-24



NUMBER OF IF CELLS / CULTURE
800
700
600
500
400
300
200
100
2
4
6
8
10
12
14
16
VIRUS CONCENTRATION

TABLE VS-VII

COMPARISON OF IMMUNOFLUORESCENT CELL ASSAY AND QUANTITATIVE HEMADSORPTION TECHNIQUE FOR TITRATING INFLUENZA (A PR8) VIRUS IN SECOND GENERATION GREEN MONKEY KIDNEY CELLS

Experiment Number	Immunofluorescent cell titer/0.3 ml.	Hemadsorbing cell titer/0.3 ml.
67426	3.0×10^7	9.0×10^6
67510	4.5×10^7	2.3×10^7
67521	2.5×10^7	9.0×10^6

The procedure used for the quantitative hemadsorption test was as follows:

Influenza virus-infected GMK cells in slide chambers were removed from the incubator between 12-13 hours after virus inoculation. Overlay medium was removed and the cultures were washed once with phosphate buffered saline (pH 7.2). One ml. of a 1% suspension of guinea pig red blood cells was added to each culture. They were allowed to sit at room temperature for ten minutes, and then were gently washed with buffered saline to remove unadsorbed red blood cells. They were fixed in methanol and stained with Giemsa's stain before counting the number of cells showing hemadsorption.

Table VS-VII shows the results obtained in three such comparative tests using the same virus pool. The IF cell assay titers were higher in every case than those obtained with the quantitative hemadsorption tests.

No attempt has been made to compare the IF cell assay with quantitative hemagglutination tests or infectivity titrations in embryonated eggs.

Effect of Multiplicity of Infection on the Time and Rate of Appearance of Specific Fluorescence in Influenza Virus-infected GMK Cell Cultures:

Qualitative Observations: Specific fluorescence was first detected as evenly distributed granules in the nuclei of the infected cells. These were soon transformed into a homogeneous mass of fluorescence occupying the whole nuclear area (Fig. VS-25). This was followed by the appearance of fluorescence in the perinuclear region of the cytoplasm, which appeared to progress towards the periphery of the cell. The number and size of these fluorescent granules increased till they covered the entire cytoplasm. At this stage these were bright and specific fluorescence both in the cytoplasm and nucleus of the infected cells (Fig. VS-26).

There was a noticeable increase in the size of the infected cells compared to the uninfected ones. These enlarged cells often showed cytoplasmic projections originating at their surfaces. Because of this enlargement in size and the presence of specific fluorescence both in the cytoplasm and nucleus, the infected could very easily be distinguished from the uninfected ones.

As the infection advanced, there was a progressive decrease in the nuclear fluorescence and eventually nuclei appeared as dark empty areas with fluorescence restricted

PHOTOGRAPHS SHOWING GMK CELLS STAINED WITH THE INDIRECT FLUORESCENT ANTIBODY STAINING TECHNIQUE

FIG. VS-25

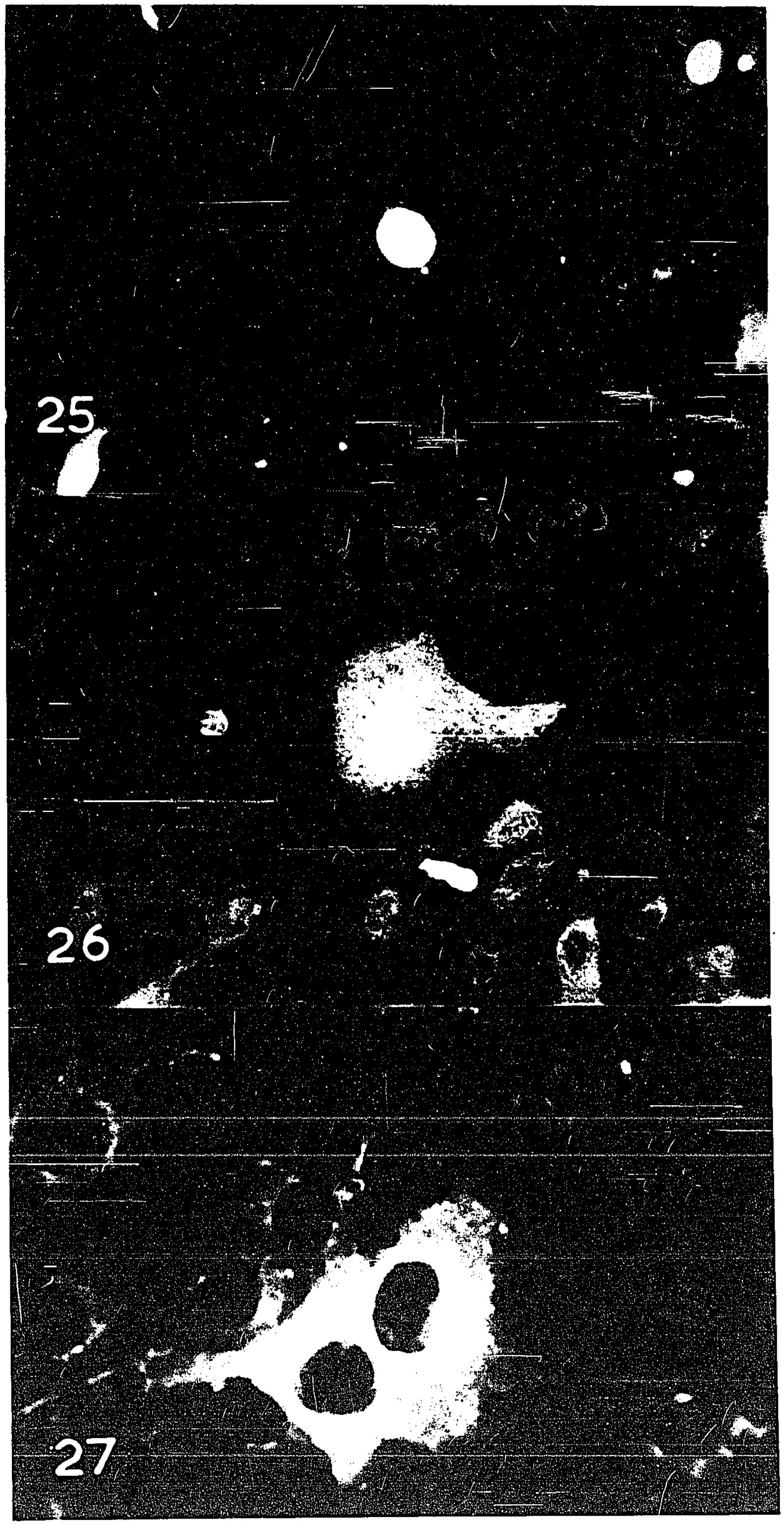
INFLUENZA VIRUS-INFECTED CELL SHOWING SPECIFIC FLUORESCENCE ONLY IN THE NUCLEUS

FIG. VS-26

INFLUENZA VIRUS-INFECTED CELL SHOWING SPECIFIC FLUORESCENCE BOTH IN THE CYTOPLASM AND NUCLEUS

FIG. VS-27

INFLUENZA VIRUS-INFECTED CELLS SHOWING SPECIFIC FLUORESCENCE ONLY IN THE CYTOPLASM


25
26
27


25
26
27

to the cytoplasm (Fig. VS-27). Cytoplasmic fluorescence also decreased gradually and became localized in the outer margins of the infected cells.

Since the strain of the virus used in this study produced only an abortive infection in the primarily infected cells, no spread of fluorescence to the neighbouring cells was found in these cultures even after 24 hours of incubation. The virus also failed to produce any detectable cytopathic effects in the infected cultures.

Examination of infected cultures often revealed the presence of "double" cells. These were seen as two nuclei surrounded by the same cytoplasmic boundary (Fig. VS-22, arrow). Whether this was due to stimulation of cell division as a result of virus infection or simply fusion of two neighbouring cells, could not be established.

The findings outlined here agree with the previously reported observations on the development of specific fluorescence in influenza virus-infected cultures (Traver et al., 1960; Fraser, 1967a and 1967b).

Quantitative Observations: Cultures representing the three different multiplicities of infection were examined to determine the percentage of cells showing specific fluorescence at different intervals after virus inoculation. The results obtained are presented in Table VS-VIII and Fig. VS-28.

At the highest multiplicity of infection (100:1) specific fluorescence could be detected in nearly 40% of the cells five hours after virus inoculation. At nine hours almost all the cells in these cultures showed specific fluorescence, whereas, the cultures which received a multiplicity of infection of 10:1 reached this stage eleven hours after virus inoculation. In the cultures with the lowest multiplicity of infection (1:1) the number of cells showing fluorescence reached its maximum between 11-13 hours. Since these cells were abortively infected, further increase in the number of fluorescent cells in these cultures was not possible.

Times at which 50% of the cells would show fluorescence at 100:1 and 10:1 ratios of virus to cells, were determined from Fig. VS-28 and were as follows:

Virus:Cell	Time (hours)
100:1	6
10:1	7 1/2
1:1	-

TABLE VS-VIII

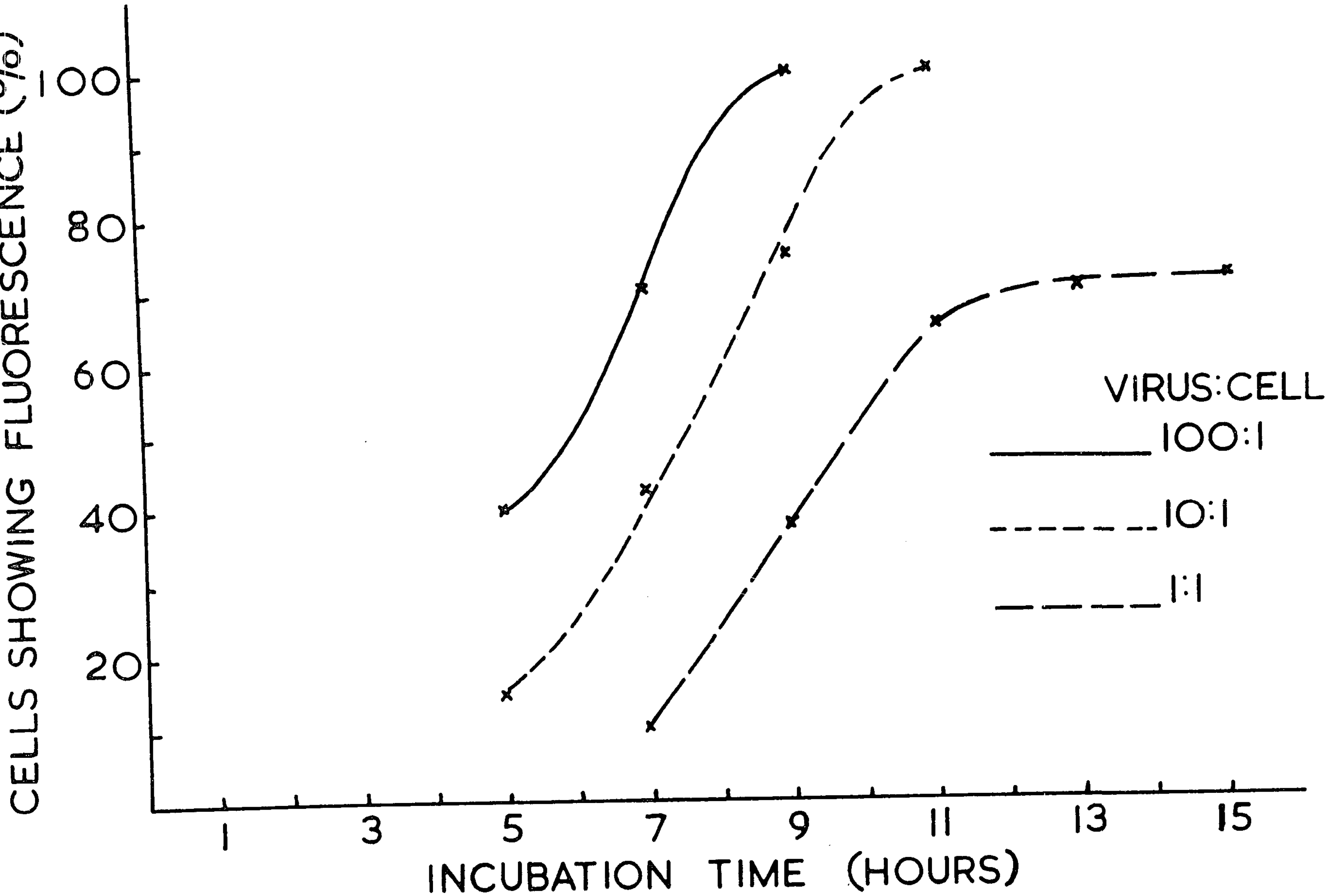
INFLUENZA (A PR8) VIRUS-INFECTED CELLS SHOWING SPECIFIC FLUORESCENCE AT DIFFERENT STAGES AFTER VIRUS INOCULATION

Time after virus inoculation (hours)	Cells showing specific fluorescence (%)		
	virus:cell		
	100:1	10:1	1:1
5	40	15	-
7	71	43	11
9	100	75	37
11	-	100	65
13	-	-	70
15	-	-	72

FIG. VS-28

RELATIONSHIP BETWEEN MULTIPLICITY OF INFECTION AND THE RATE OF APPEARANCE OF VIRUS SPECIFIC FLUORESCENCE IN INFLUENZA VIRUS-INFECTED GMK CELL CULTURES

FIG. VS-28



CELLS SHOWING FLUORESCENCE (%)
100
80
60
40
20
1
3
5
7
9
11
13
15
INCUBATION TIME (HOURS)
VIRUS:CELL
100:1
10:1
1:1

Discussion: Although influenza viruses have been shown to grow in a variety of cell cultures (Heath and Tyrrell, 1958; Lehmann-Grube, 1963; Lehmann-Grube and Fazekas de St. Groth, 1965), development of simple plaque assays for these viruses is often difficult. Because of their basic limitations, the techniques reported so far have not found general acceptance.

Many strains of influenza virus multiply in susceptible cultures without the production of visible cytopathic effects. Inability of certain cytopathogenic strains to produce visible plaques under agar overlay has also been reported (Lehmann-Grube, 1963).

An IF cell assay is described for influenza virus. It is based on the ability of virus particles to interact with primarily infected cells and to initiate formation of virus antigen, which can then be readily visualized by staining with fluorescent antibody. Since production of infective progeny virus was not a prerequisite for such a titrating system (unlike a plaque or IF focus assay), quantitation of influenza virus could be carried out even in abortively infected cells.

The technique has been shown to be rapid and reproducible. It was found to be more sensitive than the quantitative hemadsorption test. The experimental arrangement used for this titration technique was also suited to observations on the dynamics of appearance of virus specific fluorescence at the cellular level.

(4) Poliovirus I:

Development of a Macroplaque Assay for Poliovirus in using Slide Chambers: In the case of poliovirus, development of a titration technique based on immunofluorescence was not attempted because of time limitations. Instead, the virus was titrated by a macroplaque technique in slide chambers.

Second generation GMK cell monolayers in slide chambers were inoculated with 0.3 ml. of appropriate virus dilutions, using at least three cultures for each virus dilution tested. They were incubated for two hours in a CO_2 incubator at 36°C. At the end of the incubation period, excess inoculum was removed and 1.0 ml. of a semi-solid overlay (1% TMB + 1% calf serum + 0.6% agar in EBSS) medium was added to each culture. They were incubated for an additional 34 hours.

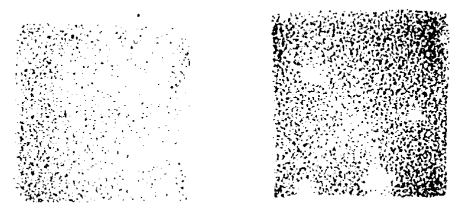
When ready for plaque counts, the cultures were fixed in 10% formalin and stained with Loeffler's methylene blue. As can be seen, from Fig. VS-29, these plaques could be counted with the naked eye.

Since this system was found to give rapid and reproducible results, all poliovirus titrations for this study were carried out with this technique.

FIG. VS-29

PHOTOGRAPH SHOWING THE APPEARANCE OF POLIOVIRUS PLAQUES IN SLIDE CULTURES OF GMK CELLS 36 HOURS AFTER VIRUS INOCULATION

(CULTURES FIXED IN 10% FORMALIN AND STAINED WITH LOEFFLER'S METHYLENE BLUE)



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Effect of Multiplicity of Infection on the Time and Rate of Appearance of Specific Fluorescence in Poliovirus-infected GMK Cell Cultures:

Qualitative Observations: In the early stages of poliovirus infection, specific fluorescence could be seen as a bright halo around the nuclei of the infected cells. There was a gradual increase in the intensity of cytoplasmic fluorescence, and soon became organized as a well defined cytoplasmic inclusion. An increase in the size of this inclusion resulted in the obliteration and shrinkage of the nucleus. In the advanced stages of the infection, there was an abundance of brightly fluorescent rounded cells in which the inclusion occupied the whole cytoplasmic area and the nuclei were reduced to a crescent shape (Fig. VS-30, VS-31 and VS-32).

Quantitative Observations: Cultures representing the three multiplicities of infection were further examined to determine the percentage of cells showing specific fluorescence at different intervals after virus inoculation. The data obtained are presented in Table VS-IX and Fig. VS-33.

At the highest multiplicity of infection (100:1) nearly 12% of the cells showed specific fluorescence only two hours after virus inoculation. At twelve hours after virus inoculation, almost all the cells showed fluorescence even

PHOTOGRAPHS OF GMK CELLS STAINED WITH THE INDIRECT FLUORESCENT ANTIBODY STAINING TECHNIQUE

FIG. VS-30

POLIOVIRUS-INFECTED GMK CELL MONOLAYER IN EARLY STAGES OF VIRUS INFECTION (500X)

FIG. VS-31

POLIOVIRUS-INFECTED GMK CELL MONOLAYER IN TERMINAL STAGES OF VIRUS INFECTION (500X)

FIG. VS-32

POLIOVIRUS-INFECTED GMK CELLS SHOWING ADVANCED CYTOPATHIC EFFECTS WITH SPECIFIC FLUORESCENCE IN THE CYTOPLASM (2,000X)

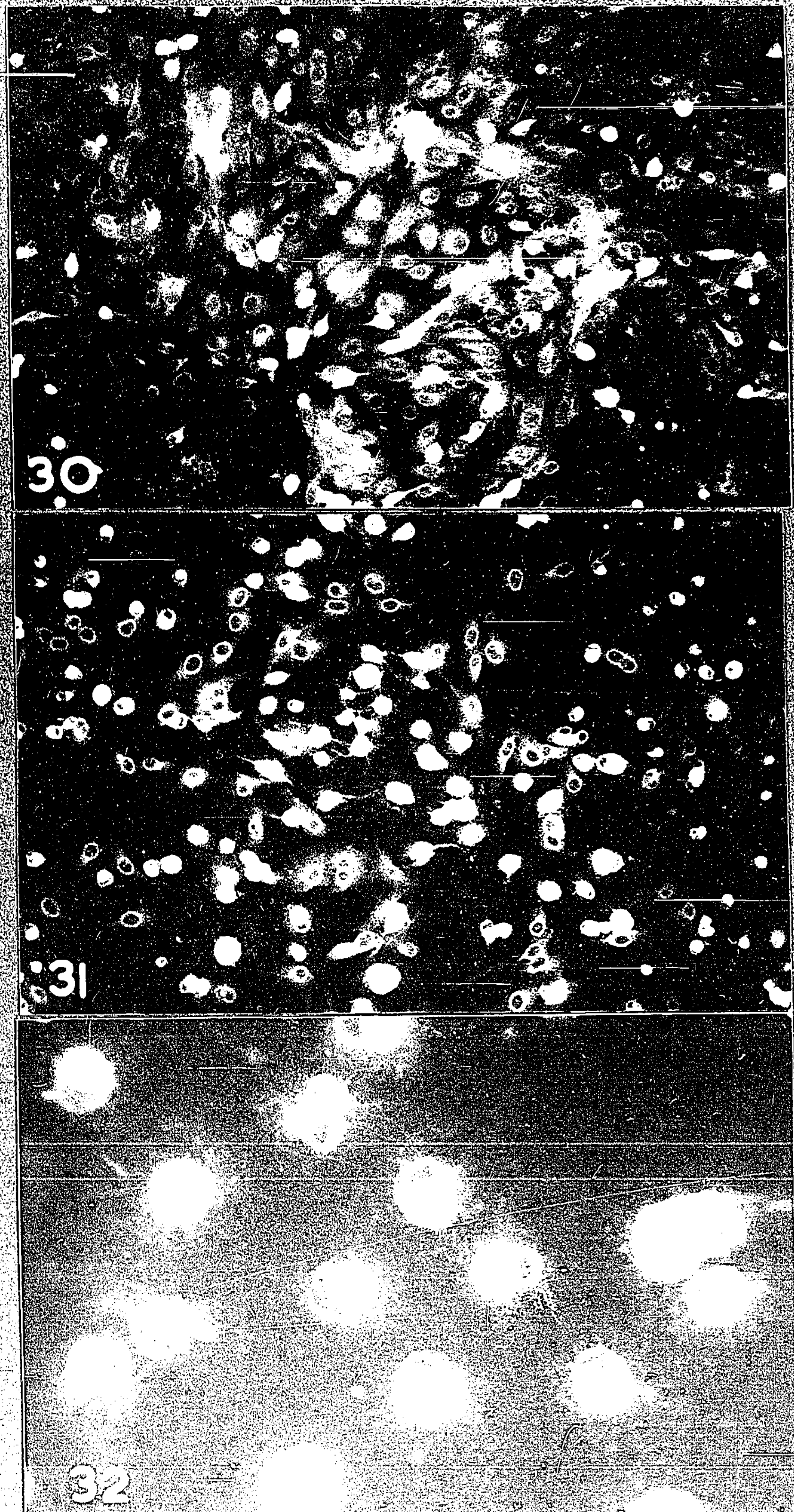

30
31
32

TABLE VS-IX

POLIOVIRUS-INFECTED CELLS SHOWING SPECIFIC FLUORESCENCE AT DIFFERENT STAGES AFTER VIRUS INOCULATION

Time after virus inoculation (hours)	Cells showing specific fluorescence (%)		
	virus:cell		
	100:1	10:1	1:1
2	12	-	-
4	50	21	10
6	85	58	33
8	100	90	65
10	-	100	88
12	-	-	100

in cultures with the lowest (1:1) multiplicity of infection.

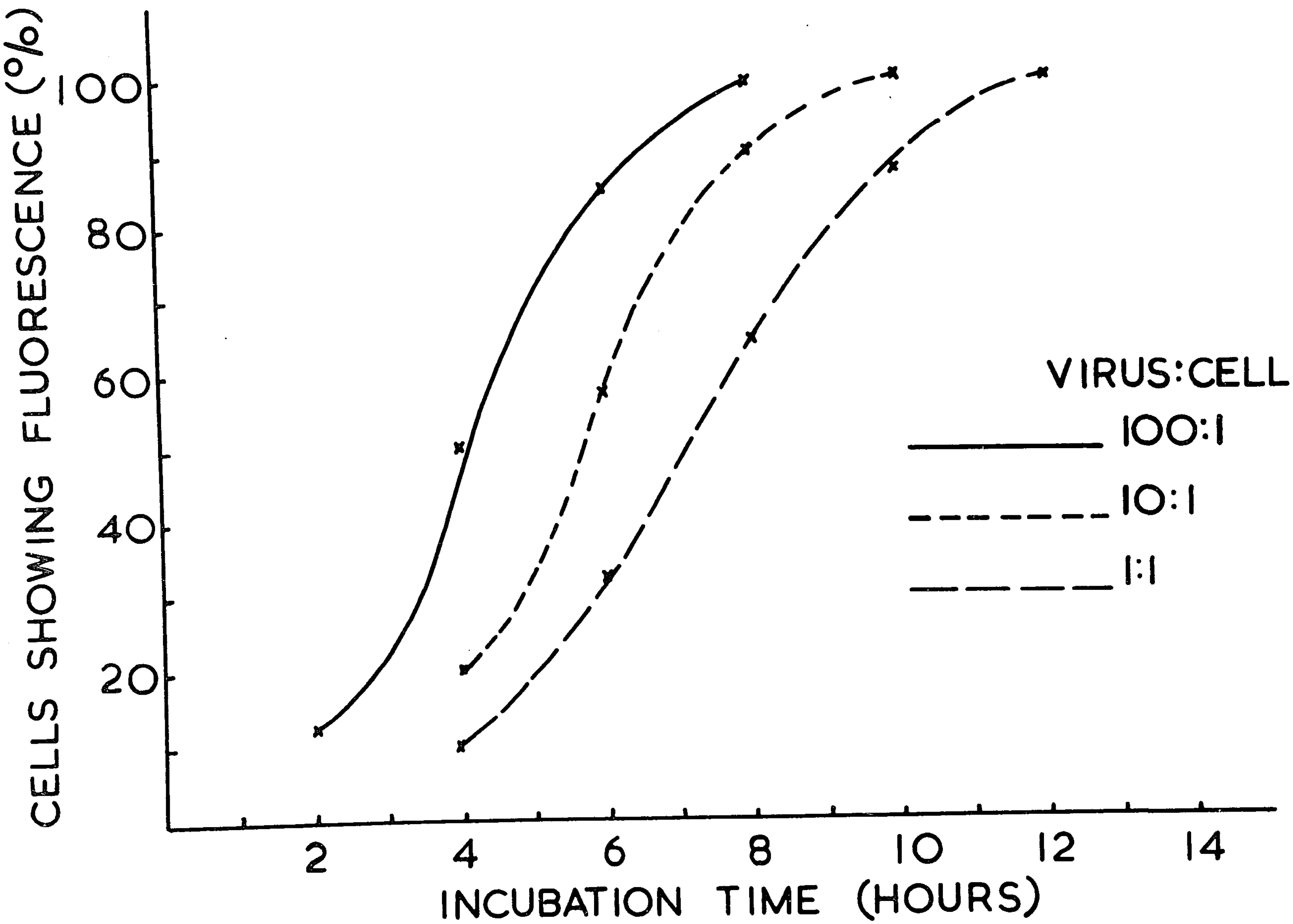
Times at which 50% of the cells would show specific fluorescence were determined from Fig. VS-33 and were as follows:

virus:cell	Time (hours)
100:1	4
10:1	5 1/2
1:1	7

FIG. VS-33

RELATIONSHIP BETWEEN MULTIPLICITY OF INFECTION AND THE RATE OF APPEARANCE OF SPECIFIC FLUORESCENCE IN POLIOVIRUS-INFECTED GMK CELL CULTURES

FIG. VS-33



CELLS SHOWING FLUORESCENCE (%)
100
80
60
40
20
2
4
6
8
10
12
14
INCUBATION TIME (HOURS)
VIRUS:CELL
100:1
10:1
1:1

<u>Discussion</u>: Though technically feasible, an IF cell or IF focus assay could not be developed for poliovirus because of time limitations. In preliminary experiments carried out in this connection, it was found that the time for harvesting the cultures for IF cell assay was most critical. Poliovirus infected cells are easily detached from the glass during the washing and staining procedures. However, fifteen hours after virus inoculation IF foci could be demonstrated in cultures overlaid with agar.

A sensitive macroplaque assay is described for poliovirus using second generation GMK cells cultivated on microscope slides. The experimental arrangement used for this titration procedure was also suited to studies of the dynamics of appearance of specific fluorescence in infected cultures.

PART VI

FLUORESCEIN-LABELLED GOAT MILK ANTIBODIES: USE IN THE DETECTION OF MUMPS VIRUS IN TISSUE CULTURE

SUMMARY

Specific antibodies are found in the globulin fraction of the milk following instillation of mumps virus in the mammary gland of the goat. These antibodies, conjugated with fluorescein isothiocyanate, have been successfully employed in the direct fluorescent antibody staining of mumps virus in infected BS-C-1 cells.

INTRODUCTION

One possible approach in solving the problem of non-specific staining lies perhaps in the use of antibodies from sources other than animal sera; Larson (1962) has found fluorescein-labelled Coxsackie A14 globulin from immune mouse ascitic fluid to be free from non-specific staining reactions. Levinthal et al. (1962) have successfully used the mouse ascitic fluid antibodies in the immunofluorescence studies of polyoma viruses. Recently, Sartorelli et al. (1966) have obtained specific antibodies from mouse ascitic fluid to several different antigens including arboviruses.

Mitchell et al. (1958) demonstrated that large quantities of highly specific milk antibodies could be obtained following instillation of viruses in the mammary gland of the goat. These antibodies were shown (Mitchell et al., 1967; Pasieka et al., 1967) to possess complement fixing, hemagglutination-inhibiting and virus neutralizing activities. With these observations in view, it was of interest to investigate their possible application in immunofluorescence. The present section reports certain preliminary observations on the use of goat milk antibodies in the immunofluorescence studies of mumps virus in tissue culture (Sattar et al., 1967).

MATERIALS AND METHODS

Monolayers of BS-C-1 cells (Hopps et al.,1963) were infected with mumps virus (Ender's strain) in allantoic fluid; control cultures received an equivalent amount of uninfected allantoic fluid. Following an incubation of 24 hours at 36°C, the cultures were thoroughly washed in buffered saline (pH 7.2) and fixed in acetone for ten minutes at room temperature. To ascertain the presence of the virus, one lot of unfixed cultures from the same batch was subjected to hemadsorption and hemadsorption-inhibition tests using chicken red blood cells and guinea pig serum (Microbiological Associates) against mumps virus.

To obtain the lactoglobulin fractions from normal and mumps virus-infected goat milks, skim milk was treated with renin to remove casein, and the milk serum fractionated by precipitation with saturated ammonnium sulphate. Protein determinations on the lactoglobulin fractions were made using a Beckman DB spectrophotometer at a wavelength of 280 mu with Lab-trol (LT23YE, Dade) as the standard. Conjugation of the lactoglobulins with fluorescein isothiocyanate (Baltimore Biologicals) was carried out by the method of Riggs et al.,(1958); for every 80 mg of protein, one mg of the dye was used. Unconjugated dye was removed from the reaction mixture by passing it through a column of 'Sephadex' (G-25, medium). The conjugates were absorbed twice with mouse liver powder. They were passed through a clarifying filter prior to use in staining.

EXPERIMENTAL AND RESULTS

The direct fluorescent antibody technique was used in this study. The pattern followed for the staining of cultures and the results obtained are presented in Table MA-I. On the basis of these observations the specificity of the staining reaction is firmly established.

Mumps virus-infected cells, stained with the conjugated milk antibody showed brightly fluorescent granules dispersed throughout the cytoplasm (Fig. MA-1). A large proportion of these granules was located in close proximity to the nuclear membrane. No specific fluorescence could be detected in the nuclei of the infected cells. These findings are in essential agreement with the previous observations concerning the immunofluorescent localization of mumps virus in infected cells (Watson, 1952; Traver *et al*., 1962).

DISCUSSION

The lack of non-specific fluorescence in these preparations was particularly striking. Whereas, this may be partly due to the use of a purified antibody fraction from the milk, similar attempts at the elimination of non-specific staining from rabbit antisera have met little success (Goldstein *et al*.,1961; Myers *et al*., 1965)

The present study, though limited in data, indicates the usefulness of goat lactoglobulins in the fluorescent antibody studies of viruses. Apart from being free of non-

TABLE MA-I

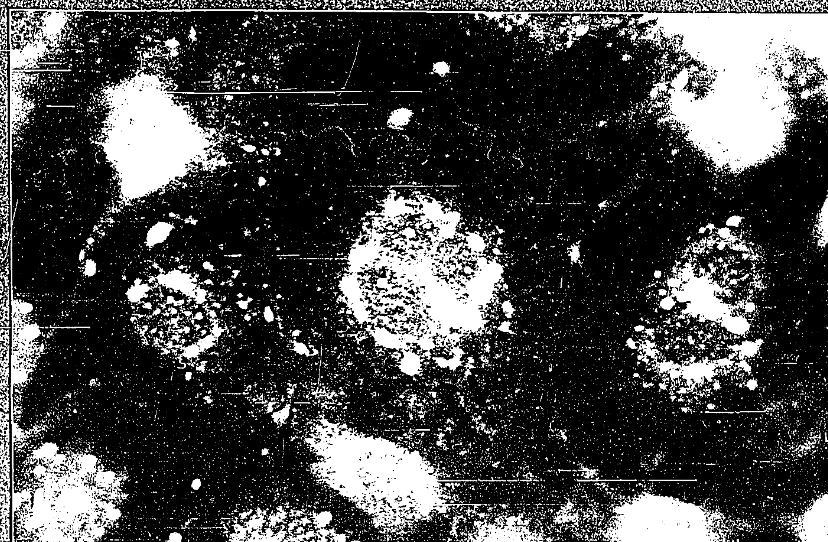
STAINING PATTERN USED FOR ESTABLISHING THE SPECIFICITY OF THE FLUORESCENCE

Culture	Staining Steps*		Specific Fluorescence
	First	Second	
Uninfected	Conjugated mumps milk antibody	-	-
Infected	1. Conjugated normal milk globulin	-	-
	2. Unconjugated mumps milk antibody	Conjugated mumps milk antibody	-
	3. Unconjugated normal milk globulin	Conjugated mumps milk antibody	++++
	4. Conjugated mumps milk antibody	-	++++

*Staining done at room temperature with 20 minutes at each step.

FIG. MA-1

MUMPS VIRUS-INFECTED BS-C-1 CELLS
TREATED WITH FLUORESCEIN-LABELED
GOAT MILK ANTIBODY TO MUMPS VIRUS
(2,000 X)



specific staining reactions, they can be obtained in large quantities relatively easily. High titered goat milk antibody preparations have also been obtained for a variety of other viruses, including influenza A (PR8), influenza B (Great Lakes), adenovirus 3 and herpes simplex virus. It is suggested that the afore- mentioned studies be extended to these viruses. The use of milk antibodies in the indirect fluorescent antibody staining technique should also be attempted.

GENERAL COMMENTS AND CONCLUSIONS

The present investigation was aimed at the initiation of experimental studies to provide a solid foundation for the application of immunofluorescence in the laboratory diagnosis of virus diseases. The experimental approach was divided into three main phases:

(1) Since staining and identification of viruses was to be carried out in infected cell cultures, a tissue culture system was first developed.

(2) The staining technique was standerdized to demonstrate that immunofluorescence could be used in the detection and identification of many different viruses with least possible modifications in the stainig procedure.

(3) Finally, the technique was tested for its sensitivity and rapidity in detecting specific fluorescence when inocula with varying virus concentrations were employed.

Bacteria, protozoa and fungi, because of their large size, can be visualized and identified in smears prepared directly from certain clinical specimens. Although it has been reported that specific fluorescence could be demonstrated in smears of leukocytes (Baratawidjaja et al., 1963) and nasal mucosa (Tateno et al., 1965a), direct demonstration of viruses in clinical specimens with immunofluorescence is often impossible. Inoculation of clinical specimens in tissue culture provides a possible 'amplification' of the virus present, thus increasing the chances of its detection and identification. This combination of tissue culture with the fluorescent antibody staining technique has been successfully used previously (Kalter et al.,1959; Hatch,1963).

The tissue culture system reported here has been shown to be suitable for virus diagnostic work, since it combines versatility with simplicity of operation.

Cultivation of cells on microscope slides using plastic chambers overcomes the disadvantages inherent in working with coverslip cultures. Cultivation of cells in this fashion offers another possible advantage. In preliminary experiments it has been shown that virus-inoculated slide cultures could be subjected to centrifugation (upto 500 xg), thereby significantly reducing the time required for virus adsorption and increasing the efficiency of adsorption.

One of the most important deterrents to the more widespread acceptance of immunofluorescence as a diagnostic tool is the diversity of techniques involved in its application. However, in the present study it has been demonstrated that the technique could be used in uniform steps, with least possible modifications, for the detection and identification of a number of different viruses in infected cell cultures.

The technique has been shown to be just as sensitive as (if not more sensitive than) the conventional methods of virus quantitation. It not only has the advantage of being rapid, because of its immunological specificity it identifies the infecting agent at the same time.

It has been shown that the time of appearance of detectable specific fluorescence in infected cultures is related to the amount of virus present in the inoculum. There was a corresponding decrease in the time at which specific fluorescence became detectable with an increase in the dose of the virus inoculated. The data obtained for the viruses studied are summarized in Table GC-I.

Amount of infectious virus present in clinical specimens varies greatly depending on the stage of the disease and methods of specimen collection and preservation. Smith and Melnick (1962) have shown that the amount of herpes simplex virus in human vesicular fluid varies from 3×10^{9} to 7×10^{10} particles/ml. However, in many other infections the amounts of virus present may not be as high, but presence of even a few infectious particles in a clinical specimen may be of diagnostic significance, when considered together with the clinical symptoms of the disease.

In the present study it has been demonstrated that the fluorescent antibody staining technique, when combined with tissue culture, is capable of rapidly detecting and identifying small amounts of infectious virus present in the inoculum.

The true significance of the key aspects of this basic work can be demonstrated only by their application in the laboratory diagnosis of actual clinical material. Although

TABLE GC-I

ESTIMATED TIMES AT WHICH 50% OF THE CELLS IN AN INFECTED CULTURE* WOULD SHOW VIRUS-SPECIFIC FLUORESCENCE

Virus	Time in Hours		
	Virus:Cell		
	100:1	10:1	1:1
Herpes simplex virus	7	9	11
Vaccinia virus	2	4	5 1/2
Influenza (A PR8) virus	6	7 1/2	-
Poliovirus (Type I)	4	5 1/2	7

*Second generation green monkey kidney cells in slide cultures.

such field exploration was beyond the scope of the present investigation, limited expeirments on the laboratory diagnosis of suspected herpetic infections have proved encouraging.

APPENDIX

PREPARATION OF MOUSE LIVER POWDER

(Nairn,1964)

(1) Remove livers from adult mice and wash them in four changes of physiological saline.

(2) Homogenize the livers with acetone in a Waring blender. Filter the homogenate through a coarse grade filter paper.

(3) Wash the deposit on the paper several times with acetone to complete dehydration.

(4) Dry the deposit overnight at room temperature. Grind it to a powder and store in a specimen jar.

For absorption, use 100 mg of the powder for every ml of original serum.

CONJUGATION OF ANTIBODY WITH FLUORESCEIN ISOTHIOCYANATE

(Riggs et al.,1958)

(1) Mix the following in a beaker:

Saline(0.85%)----10.0 ml

Carbonate-bicarbonate
buffer(pH 9.0)---3.0 ml

Acetone----------2.0 ml

Chill the mixture in liquid nitrogen (or alcohol-dry ice) till ice crystals appear.

(2) Add 10.0 ml of cold globulin solution (with known protein concentration) to the above mixture with stirring.

(3) Dissolve the required amount of fluorescein

isothiocyanate (1.0 mg of the dye to every 80-100 mg of protein is suitable) in 2.0 ml of acetone. Add this solution to the reaction mixture drop by drop with constant stirring.

(4) Transfer the beaker to a refrigerator (4°C) and allow the reaction to continue for at least 16 hours.

(5) Remove the unconjugated dye by passing the reaction mixture through a column of 'Sephadex' (G-25,medium).

(6) Absorb the conjugate twice with mouse liver powder. Clarify by filtration and store at -20°c.

CARBONATE-BICARBONATE BUFFER

(pH 9.0, 0.5 M)

$NaHCO_3$------------3.7 g

Na_2CO_3------------0.6 g

Distilled water---------100 ml

Store the buffer in a well-stoppered bottle to prevent absorption of carbon dioxide from the atmosphere.

PREPARATION OF TRYPTIC MEAT (HARTLEY'S) BROTH

(Mackie and McCartney, 1948)

(A) Cole and Onslow's Pancreatic Extract

(1) Place fresh pig pancreas (fat-free) in a Waring blender with some water and run it till all the tissue is in a fine suspension.

(2) For each gram of pancreas add 3.0 ml of water and 1.0 ml of absolute alcohol. Shake the mixture thoroughly in a stoppered bottle and allow it to stand for three days at room temperature, the shaking being repeated occasionally.

(3) Strain through muslin, and filter through Chardin paper.

(4) Measure the filtrate and add concentrated hydrochloric acid in the proportion of 0.1%. This causes a cloudy precipitate which settles in 2-3 days, and can be filtered off. (Hydrochloric acid is added to retard the deterioration of trypsin in the extract.)

(5) The extract keeps for two months in stoppered bottles in the cold (4°C).

(B) Meat Broth

(1) Mix together 1500 gms of ox heart or lean beef (fat-free) and 2500 ml of deionized water.

(2) Heat to a temperature of 80°C and then add 2500 ml of a 0.8% solution of sodium bicarbonate (anhydrous).

(3) Cool to 45°C and add 50 ml of the pancreatic

extract. Incubate the mixture at 45°C for three hours with frequent stirring.

(4) Filter while hot and adjust the reaction to pH 7.6.

(5) Filter sterilize and store frozen (-20°C).

TRYPSIN-VERSENE SOLUTION

Trypsin (1-250)---------0.125gm

Versene (EDTA)----------0.05gm

Calcium and Magnesium free
phosphate buffered saline------100.0 ml

Adjust the pH to 7.4. Sterilize by filtration.

PREPARATION OF 'SEPHADEX' COLUMNS

(1) Weigh out 6.0 grams of Sephadex(G-25, medium)

(2) Suspend in about 100 ml of water in a flask and decant the supernate containing the lighter material when the heavier Sephadex has settled to the bottom.

(3) Place about 20.0 ml of distilled water in the column and pour the 6.0 grams of Sephadex (suspended in less than 50.0 of water) into the column. Open the stopcock and rinse the Sephadex adhering to the upper part of the column.

(4) When the Sephadex has settled , draw off the water above the column and run through about 50.0 ml of phosphate buffer (pH 7.2, M/15).

(5) Add the sample carefully to the top of the column immediately after the last of the buffer has entered the column.

The unconjugated dye remains in a bright band at the top of the column, whereas the conjugated material passes through rapidly. With a 6.0 gram column and about 11.0 ml of conjugate, roughly 3-5 ml of conjugate will pass out of the column before the last has entered. The remainder is eluted by the addition of about 10.0 of buffer.

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