A CARRIER STATE OF MUMPS VIRUS IN HUMAN CONJUNCTIVA CELLS

I. General Characteristics*

By DUARD L. WALKER, M.D., and HARRY C. HINZE, Ph.D.

(From the Department of Medical Microbiology, University of Wisconsin Medical School, Madison)

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Viral carrier states in cell cultures have been described by several investigators using a variety of virus-cell systems (reviewed in reference 1). Because of techniques applicable to cell cultures, such carrier states would seem to offer particularly good opportunities to gain understanding of interactions of virus and cell that may be important to those naturally occurring infections in which viruses invade and remain in hosts for relatively long periods without causing apparent harm. From the studies already reported, however, it is evident that there is considerable variation in the virus-cell relationships found in carrier systems *in vitro*. This is to be expected since, at least superficially, the examples of persistent, inapparent viral infections found in man and animals present a diversity of characteristics. Those carrier cultures so far described have, however, usually had several things in common in that they often have utilized cells relatively resistant to the virus; many have required antibody in the medium or have appeared to depend upon some form of interference for protection of the cells; many show evidence for infection of only a small proportion of the cells in the culture. In this and the following paper we shall describe a virus carrier system that is unusual in its combination of features that include (*a*) lack of any requirement for antibody, (*b*) indication that there is not a selection of resistant cells, (*c*) alteration in the cytopathogenicity of the persisting virus, (*d*) infection of almost all of the cells of the culture, and (*e*) indication that infected cells are capable of repeated division.

Materials and Methods

Viruses.—The Dunai strain of mumps virus was isolated from a patient with parotitis (2) by inoculation of throat washings into the amniotic sac of the chick embryo. After the initial amniotic passage, it was cultivated in human conjunctiva cells. Stock virus was prepared by

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inoculating conjunctiva cells in a maintenance medium and harvesting the fluid at the height of cytopathogenic effect, usually after 4 to 6 days. Stock virus was stored at −70°C. Sendai virus (parainfluenza 1) (2), the Victorian (3) and Roakin (4) strains of Newcastle disease virus, and vesicular stomatitis virus were cultivated in the allantoic sac of chick embryos and were used as suspensions of virus in allantoic fluid.

Cell Cultures.—Human conjunctiva cells (5) were cultivated in 200 ml flat-sided bottles at 37°C. Medium for the growth of cells was Eagle's basal medium (6) and 20 per cent horse serum. Cells were dispersed with 0.05 per cent trypsin 1:300 (Nutritional Biochemicals Corporation, Cleveland) and were distributed to tubes, bottles, or Petri dishes as needed for individual experiments. Horse serum was obtained from a local commercial source and was heated at 56°C for 30 minutes before use.

Virus Titrations.—Assays of virus from carrier cultures were performed by the end point dilution technique using human conjunctiva cells. Growth medium was removed from tubes containing cells in confluent sheets. Tenfold dilutions of the virus in maintenance medium were added to the tubes (0.1 ml/tube). The tubes were slanted so that the inoculum covered the cells in a thin layer and after a period of 1 hour at room temperature to allow adsorption of the virus to the cells, 0.9 ml of Eagle's medium containing 2 per cent horse serum was added as a maintenance medium. After 10 days of incubation at 37°C, the cultures were tested for hemadsorption (7). The medium was poured off and 0.2 ml of 5 per cent chicken erythrocytes was added. The tubes were slanted and held at 4°C for 30 minutes and were then examined microscopically and were scored as infected if there were distinct patches of erythrocytes adherent to the cell sheet. The titer was expressed as the dilution that resulted in infection of 50 per cent of inoculated tubes ($TCID_{50}$).

Fluorescence Microscopy.—Cells on coverslips were dried in air and were fixed in acetone at room temperature for 10 minutes. They were stained with a specific rabbit antibody-fluorescein conjugate by the direct method (8). Controls consisted of uninfected cells treated with specific conjugate, infected cells treated with normal serum, and infected cells pretreated with unlabeled antibody prior to staining with labeled antibody. Slides were examined using a 200 watt high pressure mercury vapor lamp as a ultraviolet light source with a BG 12 excitor filter and a Wratten K2 barrier filter.

EXPERIMENTAL

Introduction of Dunai strain mumps virus into cultures of human conjunctiva cells in a maintenance medium resulted in destruction of the cell culture in 4 to 6 days. Fluids from such cultures contained 10^6 to 10^7 $TCID_{50}$ and when transferred at the height of cell destruction, the cytopathogenic effect (CPE) could readily be passed in series. When, however, the conjunctiva cells were kept in a medium rich enough in horse serum (10 per cent or more) to promote cell multiplication, the effects of both large and small inocula of mumps virus were minor and transient and the cultures continued to grow in a manner similar to uninoculated cultures. In fluids from such cultures the virus was present even after the cells were dispersed with trypsin and subcultured by the usual procedures employed for serially cultured cells. One such line of persistently infected cells has now been passed through 100 subcultures over a period of 3 years. All passages have been in Eagle's medium with 20 per cent horse serum, and specific antibody in the medium has not been required and has not been included at any time.

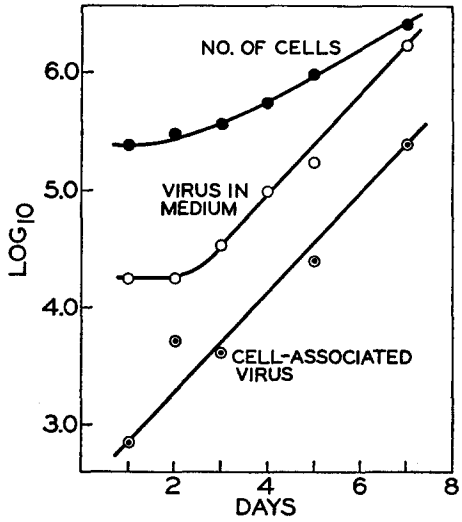
Change in Cytopathogenic Effect of the Virus.—Initial experimentation showed that carrier cultures could regularly be produced by inoculating the original Dunai strain of mumps virus into cultures of actively growing human conjunctiva cells, and that virus from carrier cultures would readily establish a carrier relationship when transferred to new cultures of conjunctiva cells. Study was then concentrated on the original carrier cultures, designated C-M cultures.

When study of the virus-cell relationships in the C-M cultures was begun several months and 30 subcultures after initial inoculation, it was found that the virus had changed to the extent that it did not produce cytopathogenic effect in cultures of several cell lines in which the original virus did have cytopathogenic effect. Inocula of supernatant fluids, unwashed cells, washed cells, or pellets from high-speed centrifugation of supernatant fluid from C-M cultures did not result in easily recognizable CPE in cultures of human conjunctiva, HeLa, human tonsil, human liver, or monkey kidney cells. In such cultures, however, there was evidence for multiplication of the virus in the absence of appreciable CPE in the fact that the addition of chicken erythrocytes 7 or 8 days after inoculation resulted in their adsorption to the cultured cells (hemadsorption).

The change in the cytopathogenic characteristics of the virus was interesting as it related to processes leading to development of the carrier cultures, but it created problems in virus assay. The virus from the C-M cultures was not adapted to chicken embryos and while it would grow in the amniotic sac if inoculated in low dilutions, this was not a sensitive or satisfactory method for routine assay of the virus. A search was made for an "indicator" cell line in which the virus would cause sufficient CPE to provide a reliable virus assay. This was unsuccessful, however, even though many cell types, including primary cultures of swine kidney cells, chick kidney cells, and chick embryo lung cells, were tested. Various methods of altering the sensitivity of human conjunctiva, HeLa, tonsil, and liver cells were tried. These included maintenance of inoculated cultures at temperatures above and below the usual 37°C, and use of cultures of giant cells produced by x-irradiation. In such cultures the virus was not sufficiently cytopathogenic, however, to provide an assay system with CPE as the means of recognizing infected cultures. Study of the C-M cultures was eventually undertaken using the hemadsorption technique to recognize infection in tubes of human conjunctiva cells inoculated with virus from the carrier cultures and held in maintenance medium for 10 days or longer. This provided an assay method that allowed study of the carrier system, although its sensitivity in measurement of the virus remained uncertain.

Because of the change in the cytopathogenic character of the virus as compared to that originally introduced into the cultures, tests were made to be certain that the virus in the C-M cultures was a derivative of the Dunai mumps line and not a virus contaminating the cells or the culture medium. Several

findings indicated that the virus in the C-M cultures was mumps virus and that it was a variant of the original inoculum. First, control bottles of uninoculated conjunctiva cells cultivated in parallel with the C-M cultures and exposed to all media and manipulations, except the mumps virus inoculum, remained free of any evidence of viral infection. Second, virus from the C-M cultures multiplied in the amniotic sac of the chicken embryo and produced hemagglutinating virus at a rate characteristic of mumps virus. Third, antiserum produced in rabbits against the original Dunai line of mumps virus, and



TEXT-FIG. 1. Comparison of the number of cells in the culture, the extracellular infectious virus, and the cell-associated virus during 7 days of growth of C-M cultures. The cells are represented as the total number of cells per culture and the virus as the total $TCID_{50}$ per culture.

also antiserum against other strains of mumps virus, neutralized the virus from the C-M cultures.

Relationship between Cell Number and Infectious Virus Units.—Continued observations and virus assays showed that infectious mumps virus was regularly detectable in the medium from carrier cultures. Virus was usually present only in low concentration, however, and was rarely measurable by the hemagglutination technique. An experiment was designed to measure the rate of intracellular production of infectious virus units, to measure the rate of virus release from the cells, and to relate virus production to the rate of cell multiplication.

Replicate cultures of C-M cells were prepared, each initially consisting of 5×10^5 cells in 6 ml of medium. The cultures were incubated at 37°C, and at 24 hour intervals, beginning 1 day after the cells were planted, two cultures were removed for cell counts and virus assay.

The fluid medium was withdrawn from the cultures, loose cells were removed by centrifugation, and the fluid was assayed for virus. The cell sheet was washed 2 times with 5.0 ml of saline, the cells were dispersed with trypsin, and an aliquot was removed for cell count. The cells were sedimented by centrifugation and resuspended in a measured volume of growth medium. The cells were frozen and thawed once and were further disrupted by mechanical grinding. Cell debris was removed by centrifugation and the supernatant fluid was assayed for virus.

Text-fig. 1 shows the curves representing cell growth, cell-associated virus, and extracellular infectious virus, during 7 days of culture. In the first 24 hours after trypsinization and transfer, the virus in the cells and fluid was relatively

TABLE I

Incidence of Infected Cells in C-M Culture during One Cycle of Culture Growth

Days of culture growth	Cells containing cytoplasmic inclusions*	Cells containing specific antigen‡
	per cent	*per cent*
1	49	88
2	44	89
3	49	79
5	54	91
6	53	95
7	42	87
10	55	84
15	58	85

* 500 cells examined.

‡ 100 cells examined.

low, but thereafter it increased in a linear fashion. After the first 48 hours, the virus in the fluid bore a quite constant relationship to that associated with the cells and was always about 0.9 log higher. During growth of the cultures the number of infectious units of virus produced progressed from $\frac{1}{13}$ $TCID_{50}$ per cell per 24 hours on the 1st day to $\frac{2}{3}$ $TCID_{50}$ per cell per 24 hours on the 7th day.

Intracellular Antigen and Inclusions.—Study of monolayers of C-M cells fixed and stained with hematoxylin and eosin showed that many cells contained cytoplasmic inclusion bodies. An experiment was designed to provide an estimate of the number of cells containing cytoplasmic inclusions and the number containing mumps virus antigen identified by fluorescent antibody.

New cultures of C-M cells were started in Leighton tubes containing coverglasses. The growth medium was renewed every 3rd day. At regular intervals cultures were fixed and stained with hematoxylin and eosin or with fluorescent antibody. These coverglass preparations were examined systematically and counts were made of the proportion of cells exhibiting recognizable inclusion bodies and intracellular antigen.

The results of this study are presented in Table I. Although cultures of C-M

cells were examined over an extensive period from first planting until cultures were complete monolayers, the proportion of cells containing recognizable inclusion bodies or specific antigen remained almost constant throughout the experiment. Approximately half the cells in the cultures could be seen to contain one or more inclusion bodies. From 84 to 95 per cent of cells contained sufficient antigen to result in fixation of visible quantities of fluorescent antibody.

Study of C-M cultures stained with fluorescent antibody further revealed that the antigen in these cells was distributed differently than that usually seen in mumps-infected cells. A comparison was made with conjunctiva cells infected with the cytopathogenic line of mumps virus. The distribution of the antigen of the latter virus during the full cycle of multiplication has been

TABLE II

Location of Cell-Associated Virus in C-M Cultures

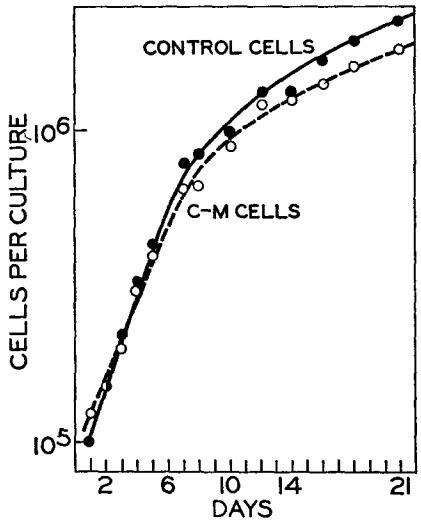
Aliquot No.	Time of cell disruption	Serum added	Virus titer
			$TCID_{50}/ml$
1	After serum treatment	Normal	$10^{-3.24}$
2	After serum treatment	Immune	$10^{-1.74}$
3	Before serum treatment	Normal	$10^{-3.50}$
4	Before serum treatment	Immune	0

described previously (9). The major point to be emphasized here is that in the cytopathogenic, acute infection the antigen was widely dispersed throughout the cytoplasm, first in fine granules, and later in dozens of granules and larger clumps (Fig. 1). In the C-M cells, the antigen was confined to one or a very few discrete, sharply outlined masses (Fig. 2). These masses were often much larger than those in cytopathogenic infections and, except for the few discrete masses, the C-M cell cytoplasm was usually clear of recognizable antigen.

Location of Cell-Associated Virus.—It is evident in Text-fig. 1 that although most of the virus in the carrier cultures was free in the culture fluid, there was a significant quantity of virus associated with the washed cells. For several of the myxoviruses in the ordinary multiplication cycle this fraction has been shown to lie in a position neutralizable by antibody, presumably at the cell surface. In order to determine whether the virus in the C-M cultures accumulated in infectious form at a position in the cell not accessible to antibody, or whether its release conformed to the usual pattern for myxoviruses, the following experiment was performed.

The medium was removed from a bottle of carrier cells and the cell sheet was washed twice with saline. The cells were dispersed with trypsin, sedimented by centrifugation, and resuspended in saline. They were then divided into four aliquots and treated as follows: to

aliquot 1 normal rabbit serum was added to a final concentration of 10 per cent, and to aliquot 2 mumps-immune rabbit serum was added to a final concentration of 10 per cent. Both aliquots were held for 1 hour at 4°C with frequent agitation. The cells of each aliquot were then removed from the serum by centrifugation, washed 3 times in saline, and resuspended in saline. The cells were disrupted by grinding, the cell debris was removed by centrifugation, and the supernate was assayed for infectious virus. The cells of aliquots 3 and 4 were disrupted first, and this was then followed by addition of normal rabbit serum to aliquot 3 and immune serum to aliquot 4 to a concentration of 10 per cent. The homogenate-serum mixtures were held for 1 hour at 4°C, followed by removal of cell debris by centrifugation and assay for virus in the supernatant fluid.



TEXT-FIG. 2. Comparison of the multiplication of C-M and control cells during 21 days of cultivation.

The data in Table II show that although some virus escaped inactivation by antibody when whole cells were treated with immune serum, this fraction was only a small part of the total and about 95 per cent of cell-associated virus was neutralized. This suggests that almost all of the cell-associated virus was at the cell surface and that there was little intracellular accumulation of infectious units in this system of carrier cells.

Multiplication of Infected Cells as Compared to Control Cells.—In day to day observation of infected and control cells it appeared that the infected cells multiplied at a rate similar to that of control cultures. This was determined more accurately in an experiment comparing the growth rates of infected and control cell cultures.

Stock cultures of infected and control cell cultures were dispersed with trypsin, sedimented by centrifugation, resuspended in growth medium, and adjusted to a concentration of 10^5

cells/ml. A group of tubes for each cell line were planted with 1.0 ml of cell suspension per tube. The tubes were incubated at 37°C and at chosen intervals 3 to 4 tubes of each cell line were removed for counting. Fluids were removed from tubes to be counted, the cell sheet was washed with 2.0 ml of saline, and 0.05 per cent trypsin was applied to disperse the cells. After incubation in trypsin at 37°C for 10 minutes trypan blue was added to stain non-viable cells. Two counts were immediately made from each tube, counting separately cells stained and cells not stained by trypan blue. Counts from the 3 to 4 tubes of each cell line at each interval were averaged to provide the count for each time period. During the growth period medium was renewed at 2-day intervals.

The growth curves for the infected and control cultures are plotted in Text-fig. 2. Both the infected and control cultures grew at a logarithmic rate for about 6 days with a generation time of approximately 50 hours. After 6 days

TABLE III

Titration of Viruses in C-M and Control Cell Cultures

Virus	Virus titer	
	Control cultures	C-M cultures
	$TCID_{50}/ml$	$TCID_{50}/ml$
Sendai	$10^{-6.7}$	$10^{-5.7}$
NDV—Victoria strain	$10^{-6.5}$	$10^{-5.8}$
NDV—Roakin strain	$10^{-8.3}$	$10^{-7.5}$
VSV	$10^{-5.9}$	$10^{-5.8}$
Mumps	$10^{-6.0}$	$<10^{-1.0}$

the rate of growth for both cultures gradually decreased, with the infected cultures falling off in growth rate somewhat more quickly than control cultures.

Sensitivity of Cultures to Cytopathogenic Viruses.—Experiments were carried out comparing the sensitivity of carrier and control cultures to the cytopathogenic effect of several viruses. These were performed as parallel titrations of the challenge virus in tube cultures of carrier cells and control cells. Sendai virus and NDV, biologically related to mumps virus, were included in these tests, as well as a cytopathogenic line of mumps virus. Because of the previous finding by Henle *et al.* (11) of resistance to vesicular stomatitis virus (VSV) in a line of cells persistently infected with mumps virus, VSV was included in the tests. In Table III one such experiment is presented and it can be seen that carrier cells were resistant to more than $10^{5.0}$ $TCID_{50}$ of cytopathogenic mumps virus. This represented about 1 infectious unit per cell and was the maximum available. There was suggestion of a very low degree of resistance to Sendai virus and equivocal resistance to NDV. There was no indication of resistance to VSV.

DISCUSSION

In the carrier cultures of mumps virus in human conjunctiva cells described in this report the selection of a variant virus of reduced cytopathogenicity is a

prominent feature, and would seem very likely to be a major factor both in the establishment of the carrier infection and in its stability. Antiserum was not needed in the cultures, and data presented in the accompanying report (10) suggest that selection of resistant cells was not a factor in this system. We have not made an extensive study of interferon in the C-M cultures, but several observations suggest that it does not have a major role. First, in two carrier systems where interferon or autointerference appeared to be important (12, 13), the use of large initial inocula of virus resulted in less CPE than did small inocula. This was not observed in the present study. The largest available inocula of Dunai strain mumps virus caused more CPE than small inocula although even the largest caused relatively little CPE in cultures in growth medium. Second, while Glasgow and Hable (14) could upset the equilibrium and bring about destruction of their cultures of mouse embryo cells infected with vaccinia virus by frequent medium changes or trypsinizations, our C-M cultures were not adversely affected by such manipulations. Third, in cultures persistently infected with mumps virus Henle *et al.* (11) found a quite high degree of resistance to VSV, which they attributed to an effect of interferon produced in the cultures. In our C-M cultures there was no detectable resistance to VSV.

The C-M carrier system was notably different from other carrier systems of myxoviruses in another respect. Puck and Cieciura studying NDV in HeLa cells (15), Henle *et al.* studying mumps virus and NDV in MCN and Lung-To cells (11), and Mason and Kaufman studying NDV in L and U_{12} cells (16), found most of the virus associated with the cells. In the C-M cultures on the other hand, although the virus levels were low, most of the virus was in the medium. In this regard the relationship between virus and cell in the C-M cultures is quite like that found in the cytocidal infection of cells by several of the myxoviruses (reviewed in reference 17) in that the virus is released from the cell very soon after it is formed.

The distribution of antigen in C-M cells is strikingly different from that of the cytocidal mumps infection. The limitation of cytoplasmic antigen in C-M cells to sharply circumscribed, discrete masses rather than spreading throughout the cytoplasm may be of some importance in determining the effect of the virus on the cell. Its possible bearing on processes in the carrier cultures will be discussed further in the succeeding report.

SUMMARY

Mumps virus produced a carrier state in human conjunctiva cells that was maintained for more than 100 subcultures over a period of 3 years. Antiserum in the medium was not required. The virus had little apparent effect on the cells which grew at a rate similar to uninfected control cells. Mumps virus was regularly found in the culture medium at levels about 0.9 log higher than the cell-associated virus. When first tested after 30 subcultures, the virus was found

to have lost its cytopathogenicity for cells ordinarily susceptible to mumps virus, but was identifiable as mumps virus by neutralization with specific antiserum. Use of fluorescein-labeled antiserum revealed that 80 to 95 per cent of cells in the carrier cultures contained mumps virus antigen. The antigen was concentrated in a few sharply circumscribed, discrete masses in the cell cytoplasm rather than in many granules throughout the cytoplasm as is characteristic of cell infection by cytopathogenic mumps virus. The carrier cultures were resistant to the destructive effect of a cytopathogenic line of mumps virus, but showed little resistance to the cytopathogenic effect of vesicular stomatitis, Sendai, or Newcastle disease viruses.

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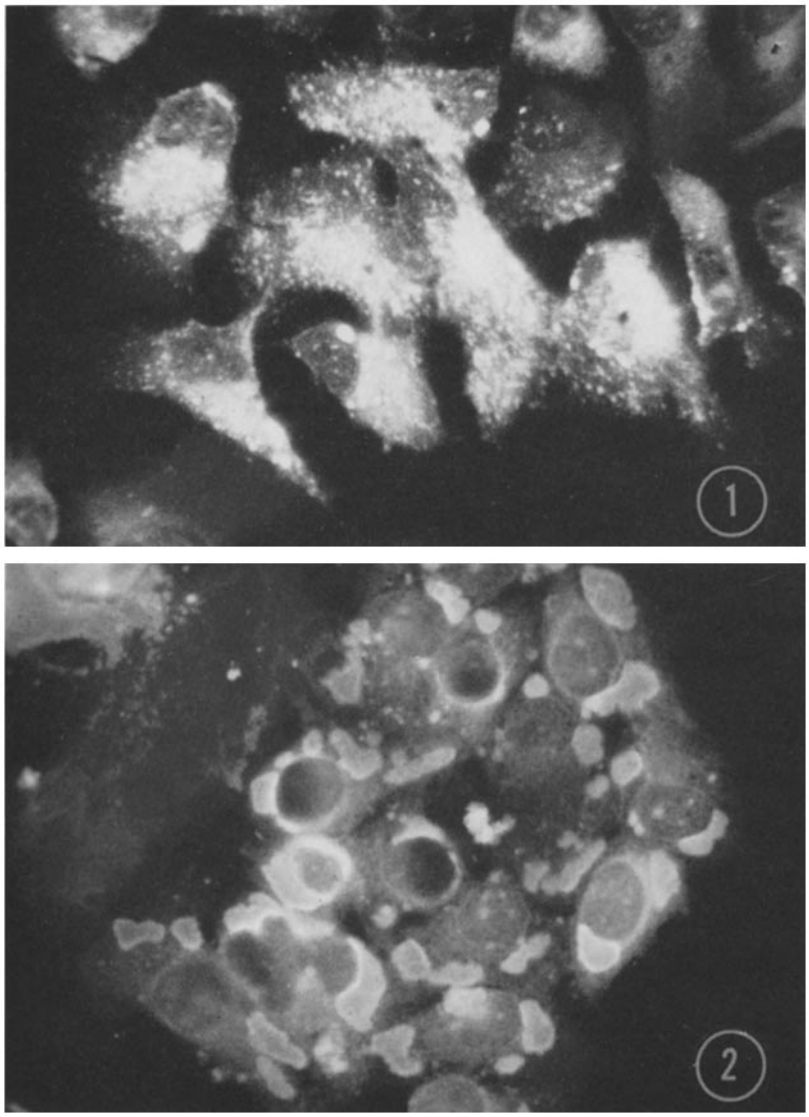
EXPLANATION OF PLATE

PLATE 94

FIG. 1. Infection of human conjunctiva cells with a cytopathogenic line of Dunai strain mumps virus. Stained with fluorescent antibody 30 hours after inoculation. Mumps virus antigen is distributed throughout the cytoplasm in small and large granules. × 500.

FIG. 2. Carrier cultures of mumps virus in human conjunctiva cells (C-M cultures). Stained with fluorescent antibody. Mumps virus antigen is confined mainly to large, discrete cytoplasmic masses. × 500.



(Walker and Hinze: Carrier state of mumps virus. I)