A CARRIER STATE OF MUMPS VIRUS IN HUMAN CONJUNCTIVA CELLS

II. Observations on Intercellular Transfer of Virus and Virus Release*

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We have described (1) a carrier culture of mumps virus in human conjunctiva cells (C-M cultures) characterized by lack of cytopathogenic effect on the cells, the continued release of small amounts of virus into the medium, the presence of antigen in the cytoplasm of 80 per cent or more cells, an unusual distribution of mumps virus antigen in the cells, and the continued growth of the cells at a rate similar to that of uninfected control cultures. The present report is concerned with the mechanisms by which the mumps virus is transferred from cell to cell, and with its release from cells. The data suggest that transfer through the medium is not necessary for continuation of the carrier state; that the virus can be passed to daughter cells through cell division; that, although practically all cells are infected and have the potential to produce infectious virus, in actively growing cultures less than 1 in 100 cells is releasing virus at any given time.

Materials and Methods

The way in which C-M cultures were established, as well as the methods for cultivation and transfer of C-M and control cell cultures, for assay of virus, and for fluorescence microscopy have been described (1).

Cell Clones.—Clones of C-M and control conjunctiva cells were obtained by the technique of Puck *et al.* (2). Rapidly growing cell cultures were dispersed by a 10 minute exposure to 0.05 per cent trypsin at 37°C. Cell clumps were broken up by gentle pipetting and the cell suspension was diluted 1:10 or 1:100 in growth medium warmed at 37°C. The cell suspension was counted in a hemocytometer and the concentration was adjusted to 100 cells per ml by further dilution in growth medium. Petri dishes (60 mm) were seeded with 100 cells in 5 ml of growth medium and incubated at 37°C in an atmosphere of 5 per cent CO_2 for 10 days. The clones were located either in the gross or microscopically, and either fixed and stained in the Petri dish or individually removed from the glass with trypsin, planted in tubes, and grown into larger cultures.

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Antiserum.—Antiserum was produced against the Dunai strain of mumps virus by using as antigen a chicken embryo amniotic sac line of the virus. Rabbits were given an intravenous injection of 1.0 ml of infected amniotic fluid followed by three additional injections of 1.0 ml given intraperitoneally at 1 week intervals. The rabbits were bled 1 week after the final antigen injection.

EXPERIMENTAL

In order to assess the importance of virus transfer through the culture medium in C-M cultures, experiments were performed in which C-M cells were serially cultivated in antibody.

C-M cell cultures were washed twice with balanced saline and dispersed with trypsin. Flasks (9 cm^2) were planted with 100,000 cells per flask in 1.5 ml of growth medium. One series of two flasks was started in medium containing 20 per cent horse serum and 10 per cent anti-mumps rabbit serum. A second series had in the medium 20 per cent horse serum and 10 per cent normal rabbit serum. The normal rabbit serum was from preimmunization bleedings of the same rabbit that provided the antiserum. The medium was changed twice weekly. At weekly intervals for 6 weeks the medium was removed from cultures and assayed for virus content, the cells were washed twice and dispersed with trypsin, the cells were counted, and new flasks were planted with 100,000 cells in the appropriate medium containing either immune or normal serum. At the end of the 6 week period the rabbit serum was omitted from the culture medium and the cultures were continued in standard growth medium.

The culture medium from the C-M cell cultures grown in antiserum was free of virus detectable by subculture throughout the test period, while medium from the cultures in normal rabbit serum regularly contained 10^4 to 10^5 $TCID_{50}$/ml. When cells from the antiserum series were planted in medium free of antiserum at the end of the experiment, the cells released sufficient virus to yield $10^{2.0}$ $TCID_{50}$/ml after 4 days and $10^{4.0}$ $TCID_{50}$/ml on the 7th day after antiserum removal.

A second similar experiment was continued with weekly subcultures in antiserum for 5½ months. At the end of that time the cells promptly produced virus upon removal of antiserum-containing medium. When cells grown for 5½ months in antiserum were examined for the presence of intracellular antigen by use of fluorescent antibody, masses of cytoplasmic antigen could be recognized in about 40 per cent of cells.

Isolation of Clones of C-M Cells.—Isolation of clones of cells from C-M cultures could provide two kinds of information. If performed in such a manner that transfer through the culture medium was prevented, it would provide additional and probably more sensitive measurement of the proportion of cells in C-M cultures infected by virus. Also, the development of colonies of infected cells from single cells under a medium prohibiting extracellular virus would provide additional strong evidence that virus could be transmitted from cell to daughter cell through cell division.

With these aims in mind, C-M cultures were dispersed with trypsin and dis-

tributed as scattered single cells in Petri dishes in a growth medium containing 10 per cent antiserum. This concentration of antiserum was shown to be sufficient to keep the medium clear of infective virus even when the cells were confluent. The single cells were either allowed to grow into colonies which were then removed to tubes and grown until the cultures were large enough to test for intracellular antigen, or the colonies were fixed in the Petri dishes when they reached 50 to 100 cells per colony, and examined for intracellular antigen after staining with fluorescent antibody.

Carrier cultures were cloned under antibody, grown into clonal lines of carrier cells, and recloned with cloning efficiencies equal to those of uninfected control cells. Table I summarizes several experiments with the original line of C-M cells and the clonal lines derived from the original C-M cultures. The carrier cultures varied in their cloning efficiency from experiment to experiment, but the usual efficiency was in the range of 40 to 100 per cent. Although

TABLE I

Isolation of Clones from Infected and Control Cultures

Cell culture	Clonal plating efficiency of culture	No. of clones examined	No. of clones containing antigen
	per cent		
Stock carrier culture	60	51	46
Carrier clone 2	43	76	76
Carrier clone 4	100	89	89
Carrier clone 8	46	53	52
Control clone 2	100	50	0

Table I happens to include an experiment in which the uninfected control cells had a cloning efficiency of 100 per cent, they too varied from 40 to 100 per cent. Clones examined by use of fluorescent antibody appeared to have antigen in all the cells of the clone or in none. There was notable variation from clone to clone in the amount of antigen in each cell, although within each clone this was fairly uniform from cell to cell. Of 269 C-M cell clones examined, 262 (97.4 per cent) clearly contained antigen, while the cells of only seven clones appeared not to have cytoplasmic antigen.

Sensitivity of Virus-free Clones to Mumps Virus.—Uninfected cell colonies presented no identifying characteristics while in antibody-containing medium, and since they were in such minority, few uninfected clones could be isolated and grown into workable cultures. Two apparently uninfected clones were obtained, however, and were grown into large cultures. These clones repeatedly were found free of evidence of infection when tested by hemadsorption or fluorescent antibody. Since one of the possible mechanisms by which a carrier state of the C-M cultures might have been established was by destruction of

susceptible cells in the original cultures with survival of relatively resistant cells, the two virus-free clones were tested for resistance to the cytopathogenic line of Dunai strain of mumps virus.

In their resistance to cytopathogenic mumps virus the virus-free clones were compared with control conjunctiva cell cultures, two carrier cell clones, and the original line of C-M cells. This was done by performing parallel titrations of mumps virus in tube cultures of the several cell lines and scoring the tubes for the presence or absence of CPE. The cultures of the virus-free clones developed typical mumps CPE indistinguishable from that seen in control cell cultures and, since the titer of the challenge virus in the virus-free clones was not significantly different from that in control cells (Table II), it was evident that the virus-free clones were not more resistant even to small inocula of mumps virus than were control conjunctiva cell cultures. The carrier clones and C-M cells were refractory to the cytopathogenic effect of the challenge

TABLE II

Titration of Cytopathogenic Mumps Virus in Virus-Free Cell Clones, Carrier Cultures, and in Control Cell Cultures

Cell cultures	Virus titer by CPE endpoint
Virus-free clone 1	$10^{-5.5}$
Virus-free clone 2	$10^{-5.9}$
Control cells	$10^{-5.75}$
Carrier clone 4	$<10^{-1.0}$
Carrier clone 5	$<10^{-1.0}$
C-M cells	$<10^{-1.0}$

virus. In addition to their lack of resistance to the cytopathogenic line of mumps virus, it was shown that the virus-free clones could readily be infected with C-M virus and typical carrier cultures established.

Release of Infective Virus by C-M Cells.—From our experiments on isolation of cell clones under antibody, it appeared that cells containing antigen could divide and could do so repeatedly with continued production of antigen. But the presence of antigen in the cloned cells did not necessarily indicate that such cells were capable of producing and releasing complete, infectious virus. It was found, however, that of eight clones isolated under antibody, grown into larger cultures, and found to have cytoplasmic antigen, after a short period of culture in medium free of antibody all eight showed positive hemadsorption and released infective virus. Since the cells that released the virus could only have been progeny of antigen-containing cells, this suggested that the large number of antigen-containing cells in the C-M cultures carried the potential for production and release of complete, infectious virus particles as well as the capacity to produce intracellular viral protein.

Further work was directed toward several questions: (*a*) are all antigen-containing cells in the culture producing and releasing virus at a relatively steady but very slow rate, (*b*) is virus production and release an intermittent activity that does not seriously damage the cells and is followed by non-releasing periods, or (*c*) does virus production and release occur in only an occasional cell followed by destruction of the cell? Complete answers to these questions were not achieved, but some pertinent information was obtained. Cultivation of single cells in droplets under oil with periodic removal of medium for virus assay was attempted. C-M cells were quite fragile in such droplets, however, and the few that survived more than a few hours were thought not to be a reliable sample of the cell population. The problem was, therefore, approached in a less direct way.

C-M cells were planted on coverglasses in flat-sided tubes (Leighton tubes) and at regular intervals (daily for 7 days and then every 2nd day) during 15 days of growth cultures were removed and the coverglasses were washed with balanced saline, then immersed in a 10 per cent suspension of chicken erythrocytes, washed again in saline, and examined by phase contrast microscopy. In random microscopic fields cells with and without adsorbed erythrocytes were counted.

In such cell preparations hemadsorption to individual cells was readily recognizable and was usually seen as 4 to 12 erythrocytes adherent to a single conjunctiva cell. Cells exhibiting hemadsorption were found in all C-M cultures and varied from 0.1 to 1.0 per cent of the cell population, but at no time in healthy, growing cultures did these exceed 1.0 per cent of cells. On the other hand, if C-M cells were allowed to become crowded and the medium to become depleted, or if the serum content of the medium was reduced to less than 3 per cent, then most of the cells in the cultures (50 to 90 per cent) adsorbed erythrocytes. Accompanying this increase in hemadsorption there was an increase, usually about tenfold, in the level of infective virus in the medium. Treatment of C-M cultures with specific antiserum prior to the addition of erythrocytes completely blocked hemadsorption. These observations suggested a close relationship between hemadsorption and release of infective virus, and suggested that in actively growing cultures virus release was probably limited to less than 1 per cent of cells, although most of the other cells contained large quantities of viral antigen and were capable of releasing virus under certain conditions.

Close examination of cells exhibiting hemadsorption in healthy, growing cultures revealed that they were usually quite normal in appearance. This impression was reinforced by study of coverglass preparations on which the cells and their adsorbed erythrocytes were fixed and stained by Macchiavello's method. In such preparations it could be seen that cells to which erythrocytes were adsorbed frequently contained cytoplasmic inclusion bodies, but did not otherwise appear abnormal. Sometimes cells were found in mitosis with clear-cut erythrocyte adsorption (Figs. 1 and 2) suggesting that the cells were under-

going mitosis and were excreting virus at the same time. It was not possible in the present study to locate such cells in living cultures and to follow them through mitosis to be certain that virus-excreting cells actually completed mitosis.

DISCUSSION

Two experiments reported here suggest that, although C-M cultures regularly contain infective virus in the medium, transfer through the medium is not necessary for maintenance of the carrier infection. The first was the demonstration that infection could persist through 5½ months of weekly subcultures with continued vigorous cell growth, and with the medium kept clear of virus by antibody. The second was the demonstration that colonies of C-M cells could be grown from single cells in an antibody-containing medium with cloning efficiency comparable to control cultures. 262 of 269 C-M cell clones contained viral antigen and, presumably, possessed the potantial to produce virus. These experiments, particularly the second, also provide evidence for an alternative means of intercellular transmission, that of passage to daughter cells through cell division. These experiments, plus the fact that cultures in which as many as 95 per cent of cells contain mumps virus antigen can still multiply at a rate close to that of control cultures (1), provide strong evidence that such cells can divide repeatedly and that the antigen, or the antigen-producing potential, can be passed from cell to daughter cell. We did not determine in every instance that the potential to produce infectious virus was also transferred, but in eight clones of eight tested, the capacity to release infective virus was found in these clones along with the presence of intracellular antigen.

The isolation from C-M cultures of cells that were apparently uninfected and yet did not show increased resistance to mumps virus was noteworthy. That there could be any uninfected susceptible cells after several months of cultivation in the presence of virus and of a majority of infected cells, suggests that uninfected cells may continuously be arising in the cultures. The localized, sharply circumscribed character of the intracellular antigen of C-M cells may make it possible for the intracellular focus of infection to go to one daughter cell during division and to leave the other free of infection. However, the fact that such an uninfected daughter can be isolated from the infected environment of the C-M cultures suggests that something must be acting to provide some temporary protection against infection. We have not found in C-M cultures any appreciable resistance to heterologous viruses (1) to indicate an extensive interferon effect, but there may be enough interferon or inactivated virus in the cultures to provide temporary protection to uninfected cell through the mechanism of interference.

Although most of the cells of the C-M cultures contained mumps virus antigen, the evidence obtained from testing of the cells' capacity to adsorb erythrocytes suggests that at any given time only a small portion of the cells

are excreting virus under the ordinary conditions of growing cultures. The possibility is not excluded that some cells are producing and excreting virus at a low rate not detectable by hemadsorption, but Marcus has recently provided (3) quantitative evidence of a close relationship between virus release and erythrocyte-adsorbing capacity in cells infected with Newcastle disease virus, and his studies indicated that hemadsorption was quite sensitive in signaling virus release. The fate of virus-excreting cells was not demonstrated in this study. The observation that cells in mitosis could be excreting virus, as indicated by their capacity to adsorb erythrocytes, suggests that mumps virus excretion is not a seriously damaging process or one that necessarily leads to cell death, but since it is not certain that these cells completed mitosis and continued to survive, other experiments will have to be devised to clarify this point.

SUMMARY

Mumps virus in a carrier culture of human conjunctiva cells (C-M cultures) persisted through 5½ months of cultivation in a medium containing sufficient specific antiserum to keep the fluid free of infectious virus. Cells from the C-M cultures were cloned under antiserum with an efficiency equal to control, uninfected cultures (40 to 100 per cent). Of 269 C-M cell clones examined, the cells of 262 contained antigen. Eight infected clones were grown into cultures sufficiently large to demonstrate that they released infectious virus and adsorbed erythrocytes in a manner similar to the original C-M cultures. Two uninfected clones were as susceptible to the effects of a cytopathogenic line of mumps virus as were uninfected control cells.

In healthy, growing cultures of C-M cells 0.1 to 1.0 per cent of cells adsorbed erythrocytes to their surfaces, suggesting that these cells were releasing virus. Reduction of serum content of the medium to less than 3 per cent, depletion of the medium, or crowding of cultures resulted in hemadsorption by 50 to 90 per cent of cells and an increase of virus in the medium. In growing cultures hemadsorbing cells did not appear damaged. It was observed that cells could simultaneously exhibit hemadsorption and mitosis.

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EXPLANATION OF PLATE

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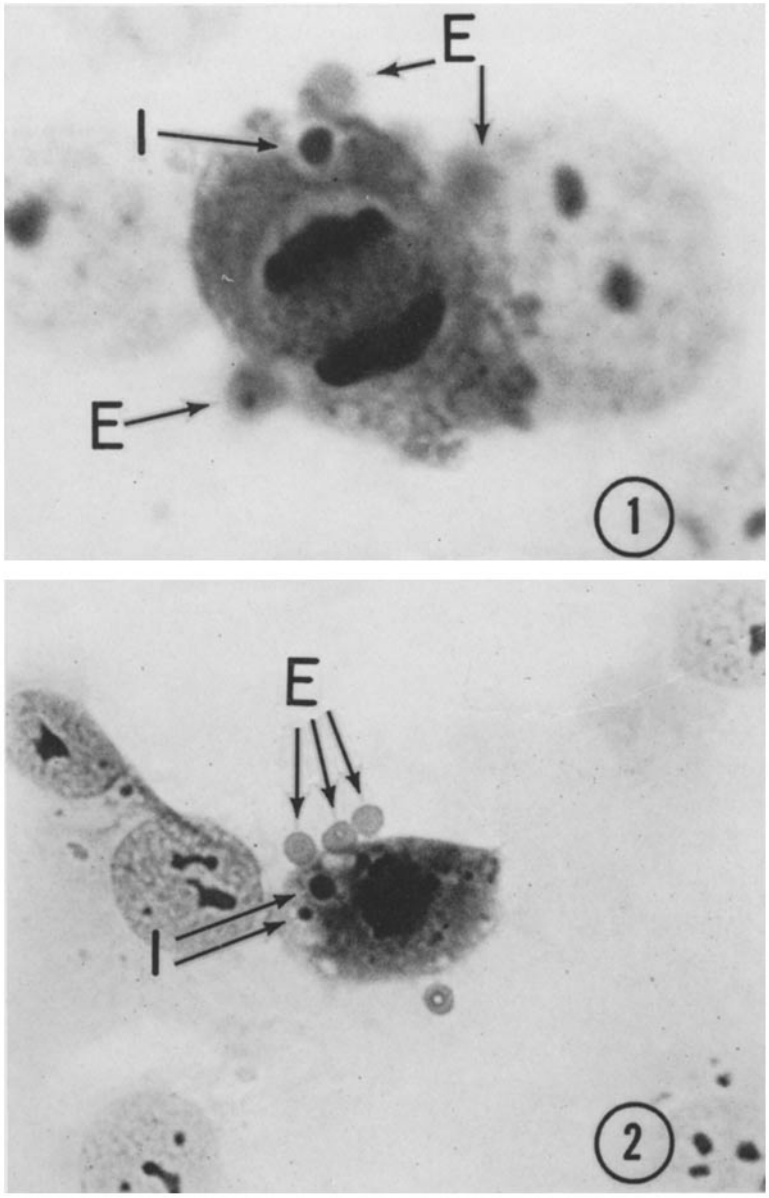
FIG. 1. A cell from C-M carrier cultures in mitosis. The arrow labeled I points to a cytoplasmic inclusion body characteristic of the inclusion bodies containing mumps virus antigen found in cells of the C-M cultures. The arrows labeled *E* point to chicken erythrocytes adsorbed to the cell surface. The erythrocytes are not in sharp focus since the nucleus, the inclusion body, and the erythrocytes are in different planes. Stained with a modified Macchiavello's stain. × 1,000.

FIG. 2. A cell from C-M cultures in mitosis. The arrows labeled I point to two cytoplasmic inclusion bodies; those labeled *E* indicate erythrocytes adsorbed to the cell surface. Stained with a modified Macchiavello's stain. × 500.



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