Persistent, Noncytotoxic Viral Infection: Nonsynchrony of Viral and Cellular Multiplication

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ABSTRACT

WALKER, DUARD L. (University of Wisconsin Medical School, Madison), PING-PING CHANG, ROBERT L. NORTHROP, AND HARRY C. HINZE. Persistent, noncytotoxic viral infection: nonsynchrony of viral and cellular multiplication. *J. Bacteriol.* 92:983-989. 1966.—In cultures of human conjunctive cells persistently infected with mumps virus (C-M cultures), the degree to which viral multiplication is linked to cell multiplication was examined. First, cell multiplication was inhibited. This resulted in a 10-fold increase in virus-excreting cells in the culture and an 80-fold increase in virus in the medium as compared with vigorously growing control cultures. Second, by use of elevated incubation temperature, virus multiplication was inhibited without slowing multiplication of the cells, and uninfected cells that appeared in the culture were protected by virus antiserum. This resulted in accumulation of antigen-free cells and, in one experiment, in elimination of the virus. Evidence indicated that uninfected cells accumulated as a result of dilution of virus by repeated cell divisions, but not as a result of selection of uninfected cells. These data indicate that viral multiplication in the C-M system is not closely linked to cell multiplication and that each can proceed at a rate different from the other.

In previous reports (22, 23), we described a persistent infection of mumps virus in human conjunctiva cells in which the cultures continued to grow and to produce virus for unlimited periods of time. Evidence was presented indicating that the infected cells are capable of repeated division, and that the survival of cells in such cultures (C-M cultures) is dependent upon some mechanism other than selection of resistant cells, antiviral factors in the medium, or interference. Almost all of the cells in C-M cultures are infected with mumps virus and, under ordinary cultural conditions, they remain so. Cloning experiments (23) have indicated that at least 98 or 99% of cells from a typical culture are infected; yet, in actively growing cultures, only 1 to 5% of cells have sufficient virus at their surface to cause erythrocytes to adhere to the cell. We have interpreted this to indicate that only 1 to 5% of C-M cells are actively releasing virus at any given time, although practically all are infected.

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Viral infection in C-M cultures is transmitted from cell to daughter cell in a relationship that provides for continued infection of cells with release of virus by only an occasional cell and that uncommonly, if at all, results in cell destruction. Consideration of how such a persistent infection of cells could be maintained leads to two alternatives. One, multiplication of the viral genome may in some way be synchronized with multiplication of the cell genome; in an occasional cell, the virus may escape this control, may multiply independently and vigorously, and may cause active release of virus. Or two, the viral genome and the cell genome are not synchronized in multiplication and may either be completely independent of each other, or not synchronized but have some interplay between the genomes and at least some periodic repression of viral multiplication. The experiments reported here tested these two alternatives and provided evidence that, even though mumps virus in conjunctiva cells is not cytotoxic and allows cell multiplication to continue, there is no synchrony of viral and cellular multiplication.

MATERIALS AND METHODS

Cell cultures. The general methods by which C-M cultures were established and maintained after inoculation of human conjunctiva cells with mumps virus have been described (22). In the present study, the medium for growth of both C-M and uninfected control cell cultures was Eagle's minimal essential medium (4) supplemented with 20% horse serum. A low-serum medium, consisting of Eagle's medium and only 3% serum, was used to restrict cell growth with maintenance of cell viability.

Clones of cells were obtained by dispersing cultures of vigorously growing cells in a 10-min exposure to 0.05% trypsin at 37 C. Cell clumps were broken up by gentle pipetting, and the cell suspension was diluted 1:10 or 1:100 in warm growth medium that contained mumps virus antiserum. A hemocytometer was used to count the cells and to verify that they were dispersed as single cells. The concentration was adjusted to 100 cells per ml by further dilution in growth medium. Petri dishes (60-mm) were seeded with 50 cells in 5 ml of growth medium and were incubated at 37 C in an atmosphere of 5% CO₂. Clones were removed individually from the glass with trypsin, were planted in vials, and were grown into larger cultures.

The present study employed a line of C-M cells that were subjected to two successive clonal selections, in the manner just described, and then were grown into working cultures.

Virus assay. Confluent monolayers of HeLa cells in plastic bottles were washed with Earle's saline. Virus, diluted appropriately in Earle's saline, was added to each culture in a volume of 0.5 ml. The bottles were capped and rocked for 1 hr on a tilting platform at room temperature to provide even distribution of the inoculum in a thin layer over the cells. The inoculum was decanted, and a soft agar overlay, consisting of medium 199, 15% tryptose-phosphate broth, 0.3% purified agar (Difco), antibiotics (penicillin, 100 units; streptomycin, 100 µg/ml), and sodium bicarbonate to adjust the pH to 7.8, was added. The bottles were capped and left undisturbed at 35 C. On the 5th day, the overlay was decanted, and the cell sheet was washed with Earle's saline. A 1-ml amount of 0.5% chicken erythrocytes suspended in Earle's saline was added to each monolayer. After 30 min at 4 C, unadsorbed erythrocytes were washed from the monolayers with cold saline. The cell monolayers with foci of adherent erythrocytes were fixed with absolute methanol for 10 min and were stained with Kingsley stain for 30 to 50 sec. The excess stain was removed with water, and the foci of adsorbed erythrocytes were counted as in an ordinary plaque assay.

Hemadsorption. Tests for the capacity of cells in C-M cultures to adsorb erythrocytes to their surfaces were performed. Cells were cultivated on round (18-mm diameter) cover glasses in the bottom of shell vials (20-mm diameter). The cells on the cover glass were washed once with a balanced saline, and the cells were covered with 0.2 ml of a 0.25% suspension of chicken erythrocytes and were held for 20 min at

4 C. Unattached erythrocytes were removed by three gentle rinses in cold saline. The cells and adsorbed erythrocytes were dried at 4 C, were fixed with Bouin's fixative for 15 min, were stained with Harris' hematoxylin and eosin, and were mounted on slides. Random microscopic fields were examined, and at least 600 cells for each culture were counted. Cells were scored as hemadsorbing cells (i.e., having one or more adherent erythrocytes) or as nonhemadsorbing cells (i.e., having no adherent erythrocytes).

Detection of intracellular antigen with fluorescent antibody. The general methods used for staining C-M cells with fluorescent antibody have been described (22). Cells were stained with specific rabbit antibody-fluorescein conjugate by the direct method. In the present study, cells were classified as antigen-containing or antigen-free by dispersing cultures with trypsin and distributing scattered cells on 18-mm round cover glasses in the bottom of 20-mm shell vials (35,000 cells per vial). The cells were allowed to attach to the cover glass during 24 hr at 37 C in a growth medium containing antibody. They were then washed, were dried in air, were fixed in acetone, and were stained with fluorescent antibody. The cover glasses were mounted on slides; the slides were coded and were examined by ultraviolet microscopy by an observer who did not know the code. Cells were scored as antigen-containing if there were any recognizable grains of fluorescence in the cell and as antigen-free if none could be found. Where there was uncertainty, the cell was scored as antigen-containing. At least 500 cells from random fields were counted and scored for each culture.

Test of resistance of cultures to mumps virus. Cells from clones or cultures under test and stock conjunctiva cells were each established in tube cultures. When monolayers were confluent (ca. 200,000 cells per tube), the medium was removed. Ten-fold dilutions of a cytopathogenic line of mumps virus (0.1 ml per tube) were added; four tubes were used for each dilution. After 1 hr at room temperature to allow for virus adsorption, 0.9 ml of low-serum medium was added to each tube, and the tubes were incubated at 35 C. Those tubes showing cytopathic effect after 7 days were scored as infected. Under such conditions, in cultures of nonmultiplying cells, the cytopathogenic line of mumps virus (Dunai strain) caused a distinct cytopathogenic effect in 3 to 5 days (22).

RESULTS

To test the degree to which viral multiplication and cell multiplication were linked in C-M cultures, two kinds of experiments were used: one was designed to inhibit cell multiplication to allow viral multiplication to proceed and to out-distance cell division if it were able to do so; and the other was designed to inhibit viral multiplication with minimal effect on cell multiplication to allow the cells to dilute the number of viral genomes through repeated divisions and,

perhaps, to produce virus-free cells by segregation at cell division.

Effect of inhibition of cell multiplication. Vigorously growing cells of a cloned line of C-M cells were dispersed and redistributed into vials either as sparsely planted vials (100,000 cells) or as heavily planted vials (600,000 cells). At 24 hr after initial planting, two sets of conditions were established: (i) the medium in sparsely planted vials was replaced with fresh medium, and the cells were kept in vigorous multiplication in standard growth medium at 37 C; and (ii) cell multiplication in heavily planted vials was further inhibited by replacing the growth medium with one containing only 3% serum and by moving the vials to 35 C. After 72 hr under these conditions, the medium was removed for virus assay, and the proportion of hemadsorbing cells in each cultural condition was determined.

In crowded cultures in low-serum medium at 35 C, increase in cell numbers was completely stopped (Table 1). In the cultures with inhibited cell growth, there was a 10-fold increase in the number of cells having sufficient antigen at their surface to cause hemadsorption, and there was about an 80-fold increase in the level of infectious virus over the levels in cultures kept in vigorous growth. Experiments with other C-M cultures (cloned and uncloned lines) gave similar results, although clones varied considerably in the degree to which they could be shifted toward hemadsorption and virus excretion by these manipulations. Inhibition of cell multiplication by simple crowding in growth medium resulted in increased hemadsorption and virus release, but the change occurred more slowly than when low-serum medium and lower incubation temperature also were used. This observation, together with unpublished studies on the effects of metabolic inhibitors on C-M cultures, indicated that the lack of hemadsorption and virus release in growing cultures was not due to a virus-inhibiting effect of the higher serum content of the growth medium.

Effect of inhibition of viral multiplication. C-M cultures were routinely cultivated at 37 C. Simply increasing the incubation temperature to 38 C resulted in a moderate reduction in the amount of viral antigen demonstrable in the cell cytoplasm, and resulted in a distinct reduction in infectious virus released into the medium without any apparent effect on the rate of cell multiplication.

Cultures of a line of C-M cells that had been subjected to two clonal selections were dispersed and distributed into vials (50,000 cells per vial). Some of the vials were placed at 37 C, and others,

at 38 C. At each temperature, half of the vials were supplied with standard growth medium, and half were supplied with growth medium containing 2% of potent anti-mumps virus rabbit serum substituted for an equal quantity of horse serum. The antiserum was added to protect virus-free cells that might appear in the cultures. All cultures were divided and passed in series at regular intervals, usually weekly, as the vials became full.

Two experiments demonstrated the effect of incubation at 38 C on the virus-cell relationship. In one, after about three months, the proportion of cells in which viral antigen could be demonstrated with fluorescent antibody was clearly reduced in those cultures grown at 38 C under antibody. After 6 months, only occasional infected cells were demonstrable, and, after 7.5 months, cells containing antigen were no longer found in the cultures. The cultures were then moved to 37 C, antibody was removed from the medium, and cultivation was continued. During 2 months of cultivation at 37 C in standard medium, no infectious virus was recovered from the cultures, and viral antigen was not demonstrable in the cells by use of fluorescent antibody. Comparative tests of "cured" cultures and stock conjunctiva cells showed the "cured" cultures to be as sensitive as stock conjunctiva cells to infection with mumps virus. In this experiment, C-M cultures grown at either 37 or 38 C without antiserum in the medium did not develop any apparent reduction in number of antigen-containing cells, but cultures at 37 C under antibody had antigen-free cells estimated as comprising about 20% of the population after 7.5 months.

In the second experiment, this process was examined in more detail. After 15 subcultures, it appeared that about half of the cells cultivated at 38 C in medium containing antibody no longer contained antigen. Cells of the 16th and 17th subcultures were then distributed as scattered cells on cover glasses, and were fixed and stained with fluorescent antibody. Under ultraviolet microscopy, the cells on coded slides were examined individually and were scored as antigen-containing or antigen-free cells (Table 2). Although there were differences between the counts obtained from the 16th and 17th subcultures, in both instances, there was a marked increase in the proportion of cells that were antigen-free in those cultures that had been cultivated under antibody at 38 C. In the cultures under antibody at 37 C, the proportion of cells that scored as antigen-free was considerably smaller, but was, nonetheless, greater than in cultures not containing antibody.

TABLE 1. *Effect of inhibition of cell multiplication on virus excretion by cells in C-M cultures*

Cultural conditions	Initial cell count (cells/vial)	Final cell count ^a (cells/vial)	Virus in medium ^a (PFU/cell)	Hemadsorbing cells in culture	
				HAd+cells ^b /no. counted	Per cent HAd+ cells
Growth medium at 37 C.....	72,000	384,000	0.0034	82/1408	5.8
Low-serum medium at 35 C.....	644,000	605,000	0.27	986/1773	55.6

^a After 72 hr under experimental conditions.^b Hemadsorption-positive cells.TABLE 2. *Effect of cultivation at elevated temperature and in antibody on viral infection of C-M cells*

Incubation temp	Medium	Sub-culture no.	Antigen-free cells	
			Ag-free cells/no. examined	Per cent of Ag-free cells
37	No antibody	16	28/530	5.3
		17	5/577	0.9
	Antibody	16	71/506	14.0
		17	104/666	15.6
38	No antibody	16	43/563	7.6
		17	23/530	4.3
	Antibody	16	245/522	46.9
		17	288/678	42.5

In such an experiment, it is difficult to rule out the possibility that the antigen-free cells arose not from infected cells but from outgrowth of a small minority of uninfected cells possibly existing in the original culture. The use of a twice-cloned line of C-M cells in the experiment reduced the likelihood that the accumulation of antigen-free cells was due to the latter possibility, but cloning by distribution of dispersed cells in dishes involved some uncertainty that every colony of cells developed from a single cell. The experiment was, therefore, amplified to provide more information on the mechanism of accumulation of antigen-free cells.

If antigen-free cells were accumulated by selection of cells that had never been infected, it seemed probable that examination of individual clones of cells from those C-M cultures that had been under antibody at 38 C for 17 subcultures would reveal two kinds of clones, infected and uninfected, and the clones would be isolated with a frequency proportional to antigen-free and antigen-containing cells already demonstrated in the cultures (i.e., approximately 45% uninfected and 55% infected clones). On the other hand, if a process of gradual dilution of viral components and viral genomes were involved, it seemed probable that, when 45% of cells appeared to be

antigen-free, some cells would, in fact, be completely free from virus and no longer infected, whereas others, still containing one or more viral genomes, would have insufficient antigen to be recognized. These latter cells would have the potential to produce virus if conditions were changed to favor viral multiplication. Moreover, if some cells contained only one or two viral genomes, there would be a good possibility of production of mixed clones containing both infected and uninfected cells, because of segregation and of production of virus-free daughter cells in the cell divisions required for building up clonal populations from single cells.

Clones from the cultures carried for 17 subcultures in standard medium at 37 C and those carried under antibody at 38 C were isolated and grown into cultures of 10⁶ or more cells. The cloning efficiency was 39 and 34%, respectively. Cloning was done under a medium containing antibody, and incubation was at 37 C for cells of both cultures. Cells in confluent monolayers were next held for 72 hr at 35 C in low-serum medium; then the cell sheet was washed, fixed, stained with fluorescent antibody, and examined for mumps virus antigen.

The results (Table 3) supported the view that the antigen-free cells arose by a process of virus dilution. Of 38 clones isolated from the culture carried under antibody at 38 C, 4 (10.5%) appeared to be virus-free, and 6 (15.8%) were mixed cultures comprised of both antigen-free and antigen-containing cells in various proportions. All of the 52 clones from the culture carried at 37 C in standard medium were infected.

The four antigen-free clones obtained from cultures at 38 C remained antigen-free and released no detectable virus even after 2 months of cultivation in antibody-free medium at 37 C; they did not produce recognizable quantities of antigen even after 72 hr in confluent monolayers at 35 C in low-serum medium. They were tested for resistance to infection by mumps virus from C-M cultures and to a cytopathogenic line of mumps virus. Growing cultures derived from each of the four clones could be converted back to typical C-M cultures simply by reintroducing

TABLE 3. *Characteristics of clones of cells isolated from C-M cultures after 17 serial subcultures at 38 C in mumps virus antiserum*

Culture	No. of clones tested	Infected clones	Mixed clones	Uninfected clones
38 C, in antibody.	38	28	6	4
37 C, in standard medium.....	52	52	0	0

10 to 100 PFU of virus from C-M cultures. When compared with stock conjunctiva cells, nongrowing cultures of the four clones showed no significant increase in resistance to challenge by various quantities of a cytopathogenic line of mumps virus and exhibited characteristic cytopathic effect at the expected time.

DISCUSSION

In the C-M system, mumps virus is able to maintain a persistent infection that is passed from cell to daughter cell for generation after cell generation. The data presented here, however, appeared to exclude the possibility that this is because viral multiplication is synchronized with cell multiplication. The demonstration that conditions inhibiting cell multiplication caused increased hemadsorption and virus release suggested that, under these conditions, viral multiplication continued and virus accumulated until it reached levels resulting in the appearance of virus at the cell surface and in virus release by most of the cells of the culture. The increased hemadsorption and virus release could have been due, however, to a disruption of synchrony brought about by the metabolic changes that occurred in cells as their multiplication was inhibited. But the opposite maneuver, inhibition of viral multiplication with continued multiplication of the cells, provided additional and more convincing indication that viral and cellular multiplication were not tied together. Cultivation at 38 C resulted in the accumulation of uninfected cells in the culture. If such virus-free cells did not accumulate from selective multiplication of uninfected cells in an originally mixed culture, they must have arisen from previously infected cells. Evidence indicated that the cells did not come from outgrowth of uninfected cells: (i) a twice-cloned line of C-M cells was used, (ii) examination of clones from cultures at a time when about 45% of cells appeared to be antigen-free revealed a spectrum of virus-free, mixed, and infected clones, and (iii) virus-free clones were not resistant to virus from C-M cultures or to stock mumps virus and, therefore, did not come from genetically resistant cells. Thus, it appears

most likely that virus-free cells arose from previously infected cells by a process of virus dilution.

These data indicate that multiplication of virus and cell were not closely linked in C-M cells and that each could proceed at a rate different from the other. The simplest explanation of the continued cell-virus association would be that they were completely independent, each multiplying at its own rate, and competing for nutrients and synthetic sites. In such a system, continued long-term infection of cells without either cell destruction or loss of virus would depend upon a balance of viral and cellular multiplication rates. The presently available data do not, however, rule out the possibility that, even though virus and cell are not synchronized, they do have some interplay and that viral multiplication may be, at least part of the time, under some specific control.

A number of persistent infections of cells in culture that appear to be similar to the C-M system have been described. These include infections by measles virus (17), Sendai virus (10, 11, 25, 26), hemadsorption 1 virus (3), hemadsorption 2 virus (7, 15, 16), simian virus 5 (2), and rabies virus (5). Although complete information is not available for some of these systems, and although there is some degree of variation in characteristics among the various cell-virus combinations, they have several important features in common and make up a group of persistent infections distinctly different from the usual carrier cultures (*see* 21) and, in fact, different from other types of cell-virus complexes. In this group of cultures, the survival of cells does not depend upon heritable cell resistance, upon antiviral factors in the medium, or upon interference or interferon. All, or most, of the cells in the cultures are infected. The infected cells are not destroyed and can divide and transmit the infection to daughter cells. Infected cells have been found resistant to superinfection by homologous virus and may show some resistance to closely related viruses but, in general, display little or no increase in resistance to heterologous viruses. In regard to this latter point, the rabies-rabbit endothelial cell system of Fernandes et al. (5) is something of an exception; i.e., the cultures were found quite resistant to challenge with vesicular stomatitis virus. However, evaluation of the significance of the heterologous resistance must await clarification of the influence of lymphocytic choriomeningitis virus that was later found present in the cultures in addition to rabies virus (24). None of these persistent infections, other than the C-M system, has been examined closely in regard to cell-virus synchrony; however, it is noteworthy that when the conjunctiva cells in-

fected with HA1 virus described by Cole and Hetrick (3) were cultivated in an antibody-containing medium, there was a gradual decrease in the number of antigen-containing cells in the culture until, after 67 subcultures, only an occasional cell appeared to be infected. This observation suggests that in the HA1-conjunctiva cell system, also, the infection is nonsynchronized and cytoplasmic and can be diluted out. It seems likely that this will be a general characteristic of this type of cell-virus complex, although the ease or difficulty of separating virus and cell can be expected to vary greatly among different cell-virus combinations.

All of the viruses so far demonstrated to enter into this type of cell-virus relationship are myxoviruses or, as in the case of rabies virus, have a myxovirus-like structure. Other viruses of the membrane type, namely, rubella virus (8, 13) and some of the arboviruses (1, 12, 14), can sometimes establish infections in which there is a fairly high proportion of infected cells without overt cytopathic effect, and in which there is some indication of continued division of infected cells. Analysis of the cell-virus relationship in rubella virus and arbovirus infections has been complicated, however, by interfering factors that, under the usual methods of testing, make the cultures refractory to heterologous, as well as to homologous, viruses. Whether the cell-virus relationship in rubella virus and certain arbovirus systems has any basic similarity to that in the C-M system is not yet clear.

Infections of the C-M type represent a cell-virus relationship quite different from the cytocidal infection characteristic of viruses such as poliovirus, mengovirus and vaccinia viruses. Clearly, in the C-M system, cellular deoxyribonucleic acid (DNA) and ribonucleic acid synthesis cannot be seriously inhibited, and viral multiplication can proceed, although at a fairly slow rate, without appreciable cell damage for an indefinite time. The infection also seems quite different from the noncytotoxic infections of the DNA, intranuclear viruses, such as polyoma virus, simian virus 40, and adenoviruses, in transformed cells. Closest to the C-M system in general characteristics are the cell-virus complexes established by Rous sarcoma virus (RSV) and the other viruses of the membrane type comprising the avian leukosis complex. RSV establishes infections in which there usually is continued production of virus without cell destruction but frequently, also, cell conversion. Infected chicken cells can produce viral infectious units at a rate that exceeds that of cellular multiplication; on the other hand, Macpherson (9) has reported isolation of clones of reverted cells from popu-

lations of RSV-infected converted hamster cells. Nonetheless, the cell-virus complex is usually very stable and is one that can cause cell transformation not dependent upon viral multiplication (6, 18). The production of virus and the cell transformation may involve a DNA intermediate (provirus) in the cell (19, 20), and, although some parts of the virus cycle and of the virus-cell interaction are not yet clear, it does appear that the RSV system represents a further step in complexity beyond that seen in infections of the C-M type.

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