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Growth of Murine Cytomegalovirus in a Heterologous Cell System and Its Enhancement by 5-Iodo-2'-Deoxyuridine

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Mouse cytomegalovirus replicated in rabbit kidney cultures, a cell system of nonrodent origin. However, the sensitivity of these cultures, and the yields of virus therefrom, were lower than those of mouse cultures. Although a cytopathic effect developed in rabbit kidney cultures inoculated with sufficient amounts of the virus, such cultures were unsatisfactory for plaque assay. This was also true when rabbit fibroblast cultures were used, even though the murine cytomegalovirus replicated much better in mouse fibroblasts than in mouse kidney cultures, the latter of which contained extensive areas of epithelial cells. Viral growth in rabbit kidney cells was considerably enhanced when those cells had been initiated and grown in the presence of 5-iodo-2'-deoxyuridine; not only were the viral titers increased, but also the clarity and distinctness of the inclusion bodies.

The taxonomic delineation of the cytomegaloviruses from the other herpesviruses is of questionable validity. There has been a tendency to separate them as a distinct subgroup because of their slow replication, their attainment of low titers of infectivity in the fluid of infected cultures, and their specificity for the cells of their host species. But there is indication that herpesviruses with these properties form the end of a spectrum rather than a clearly distinct group, with viruses such as pseudorabies virus and the herpes simplex viruses at the other end of the spectrum. Also, it is by no means clear whether the viruses at one end of such a spectrum are phylogenetically closer to one another than they are to the viruses at the other end.

Specificity for the cells of the natural host is certainly becoming questionable as a distinguishing characteristic of the "cytomegaloviruses." Human cytomegalovirus can produce a cytopathic effect and viral antigen in guinea pig cells or in bovine cells, although viable viral progeny has not been produced (1, 10), and evidence is already available for the replication of mouse cytomegalovirus in cells of nonmurine origin (5, 7).

We have studied mouse cytomegalovirus—one of the "classical" cytomegaloviruses—in cell cultures from rabbits. We have noted considerable enhancement of viral growth by prior treatment of the cells with 5-iodo-2'-deoxyuridine (IUdR), a procedure which St. Jeor and Rapp (8, 9) found to increase the permissiveness of human cells to human cytomegalovirus. We do not mean to imply, however, that the present data indicate that IUdR enhancement is a distinguishing characteristic of "cytomegaloviruses"; subsequent work may show similar enhancement of other herpesviruses.

[1] Deceased.

MATERIALS AND METHODS

Viruses. The Smith strain of mouse cytomegalovirus was used in all the studies except where the French strain is specified. This latter virus (7) had been passaged extensively in laboratory mice (*Mus*) and in tissue cultures from such mice, although prior to such passage the original agent was isolated from the French field mouse, *Apodemus sylvaticus*; the virus had not been passaged in our laboratory, our stock having been grown directly from the material received from France. The actual origin of the French virus is somewhat open to question in view of its extensive passage in laboratory mice, but it at least represents a different isolate or strain of mouse cytomegalovirus even if its origin be laboratory mice.

Growth curves. Growth curves were done with the Smith strain; the inoculum had been grown, and passed 10 times, in rabbit kidney cultures. Primary kidney cultures were prepared in 60 by 15 mm Falcon plastic petri dishes from kidneys of newborn rabbits or newborn mice; Eagle medium with 10% fetal calf serum was employed. These cultures consisted of areas of epithelial cells interlaced with strands of fibroblasts. Mouse embryos were used for the preparation of cultures which appeared to be almost entirely

fibroblastic. The viral inoculum for the growth curves was either $10^{3.5}$ or $10^{4.5}$ plaque-forming units (PFU) per petri dish. After 1 h of absorption at 37 C, the cells were rinsed three times and then fed with 3 ml of Eagle medium containing 5% fetal bovine serum. At appropriate intervals, petri dishes were removed, wrapped in Parafilm (American Can Co.), and frozen at −70 C. Dishes were later thawed, the adhering cell sheet was scraped into the culture fluid, and the mixture was then homogenized in a Ten Broeck grinder. The homogenized cultures were then refrozen in tubes until titration. All plaque titrations were done in mouse embryo cultures with a 199-methocel overlay and read under the low power of the inverted microscope after 4 or 5 days.

To test the effectiveness of IUdR, growth curves were done in rabbit kidney cultures as described above, but in cells initiated and grown on medium that contained 100 μg of IUdR per ml; from the same pair of kidneys, normal cultures (no IUdR) were prepared for the control growth curve. When these cultures were confluent, at 6 or 7 days, they were washed three times in Eagle medium, and parallel growth curves were commenced in the IUdR-treated and nontreated cultures. No IUdR was, of course, present in the medium during the viral growth.

RESULTS

Growth in rabbit cells. Inoculation of mouse cytomegalovirus in relatively small amounts (i.e., less than about 10^3 PFU, as measured in mouse fibroblast cultures) into tube or petri-dish cultures of rabbit kidney produced no cytopathic effect (CPE). If larger amounts were inoculated, CPE developed after a period of about 7 days; this CPE could be transmitted from culture to culture provided the inoculum was not diluted or was diluted only slightly. The French isolate behaved the same as the Smith strain in these respects.

Comparative growth curves were therefore done in rabbit kidney cultures, mouse kidney cultures, and mouse embryo cultures. Virus which had been passed 10 times in rabbit kidney cultures was used in each case as the virus inoculum. As can be seen in Fig. 1, viral replication in the rabbit cells is markedly less than in the mouse cultures.

The mouse embryo cultures, which appeared to consist entirely of fibroblasts, supported viral growth better than did the mouse kidney cultures, which consisted of areas of epithelial cells extensively interlaced with strands of fibroblasts. Figures 2A and B demonstrate the preferential development of CPE in the fibroblasts of a mouse kidney culture rather than in the epithelial cells. Figure 2C shows a rabbit kidney culture at a similar number of days after infection; even though there are what seem to be extensive areas of fibroblastic cells, no CPE is apparent.

The mouse cytomegalovirus could not be successfully plaque-assayed in rabbit kidney cultures, because not only did CPE develop much more slowly than in mouse cells but the "plaques" varied widely in size and shape; it was impossible to identify and count them accurately, even under the low power of the microscope. In contrast, the plaques which developed in mouse cultures could be readily discerned from one another and achieved a diameter of about 0.4 mm by 4 days post-inoculation.

The attempts to titrate the virus in rabbit cultures gave the marked impression that a 10-fold dilution of the inoculum resulted in more than a 10-fold decrease in the area of cell sheet involved in the CPE and in the number of "plaques." No precise dose-response curve could be plotted because of the difficulty in counting the foci of CPE.

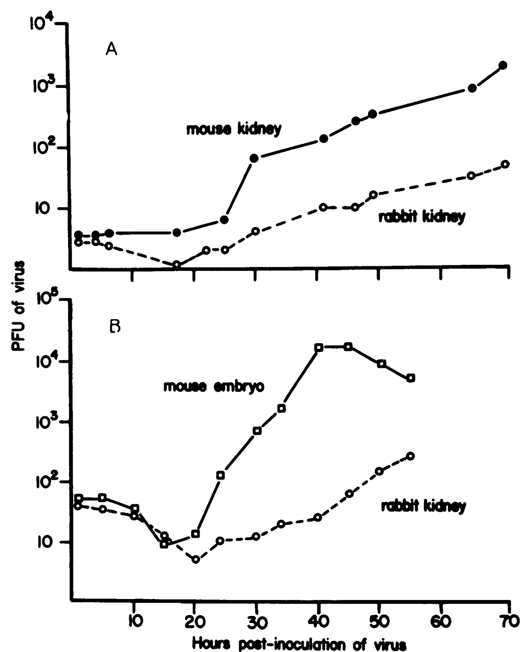
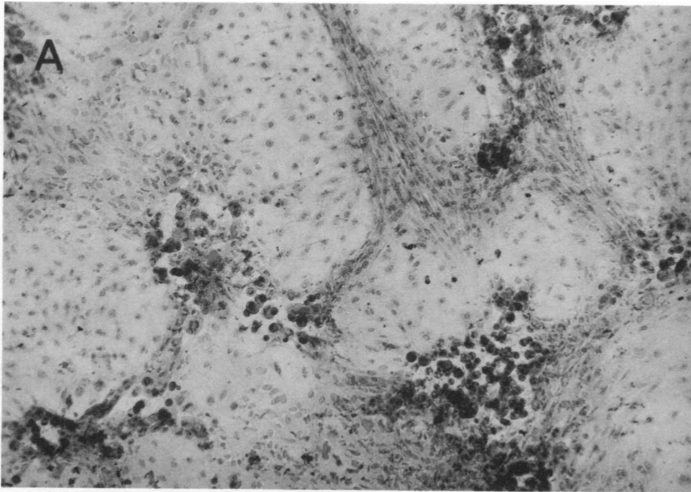
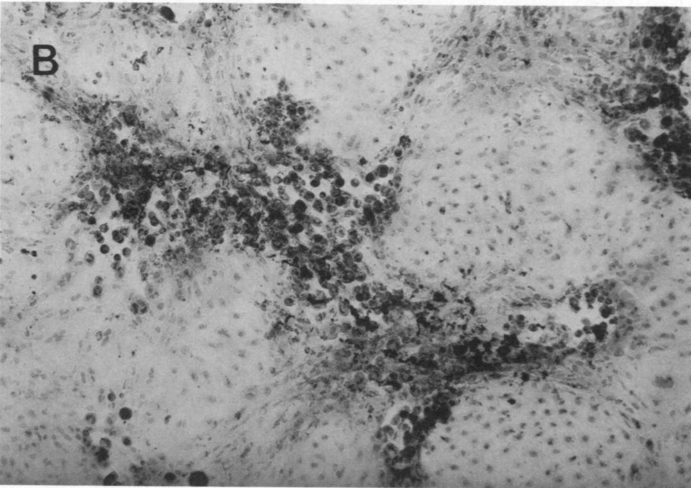


FIG. 1. *(A) Growth curves of mouse cytomegalovirus in primary cultures of mouse kidney and rabbit kidney. The viral inoculum was $10^{3.5}$ PFU per petri-dish culture. (B) Growth curves in mouse fibroblasts (i.e., the mouse embryo cultures) and rabbit kidney. The viral inoculum was $10^{4.5}$ PFU per petri-dish culture. All of these growth curves represent combined virus from the culture fluid and the homogenized cells. The titers plotted are per 0.2 ml of the total 3 ml of homogenate.*





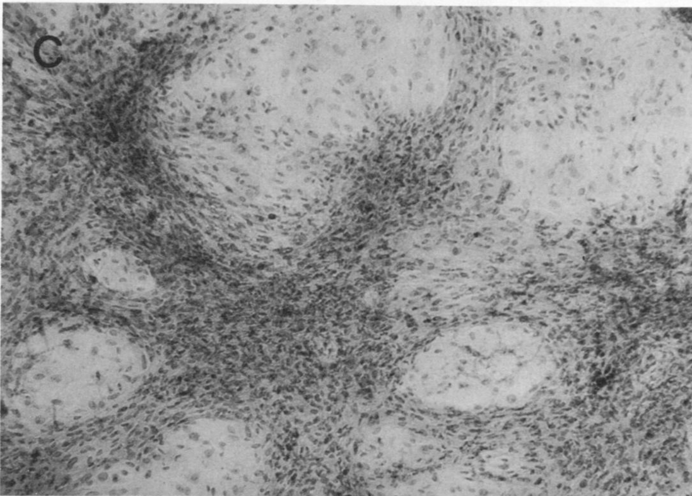


FIG. 2. *(A,B) Mouse kidney cultures stained with hematoxylin and eosin 4 days after receiving a low inoculum of mouse cytomegalovirus. The CPE is largely confined to the fibroblasts. (C) Rabbit cultures similarly infected and stained show no CPE after 4 days even though extensive regions of fibroblasts are present in the culture.*

In view of the apparent preference by mouse cytomegalovirus for mouse fibroblasts and by human cytomegalovirus for human fibroblasts, rather than for epithelial cells, an attempt was made to assay the mouse virus in rabbit fibroblast cultures prepared from lungs or skin of very young animals. The results were no different from those in rabbit kidney cultures. Once again, the areas of CPE were slow to develop, were irregular in shape, and seemed to be of a lesser number than was obtained in mouse cultures receiving a similar virus inoculum.

Enhancement by IUdR. As can be seen in Fig. 3, the mouse cytomegalovirus multiplied considerably better in rabbit kidney cultures which had been initiated and grown in the presence of 100 μg of IUdR per ml prior to the inoculation of the virus. Similar results were obtained in each of three such experiments. Even though the IUdR-treated cultures, as well as the nontreated cultures, appeared to be confluent at the time of virus inoculation, the cell counts in the treated cultures were between 38 and 46% those of the untreated cultures. If one wished to compare the virus yields per given cell population, the IUdR virus titers would need to be increased 0.4 $\log_{10}$, although the results shown in Fig. 3 have not been so adjusted.

Prior treatment of the rabbit kidney cells with IUdR appeared also to enhance the clarity of the intranuclear and cytoplasmic inclusion bodies. In the untreated rabbit cultures, inclusions were difficult to discern, even though the cells were involved in CPE and many of the nuclei were elongated and curved in the characteristic fashion. However, in the IUdR-treated cells, inclusions were easily seen when stained with hematoxylin and eosin and were remarkably similar to those caused by human cytomegalovirus in human cells; Fig. 4B and C show mouse cytomegalovirus inclusions in IUdR-treated rabbit cells. For comparison, inclusion bodies formed in mouse fibroblasts are shown in Fig. 4A.

DISCUSSION

The growth of the mouse cytomegalovirus in rabbit cell cultures, even though somewhat tardy, means that cell specificity cannot be used as a delineating characteristic of the "cytomegaloviruses"; this is emphasized by the apparent specificity for cells of their natural hosts of the dog and cat herpesviruses, two viruses which replicate rapidly and which on that basis would not be labeled cytomegaloviruses. But when an attempt is made to correlate the deoxyribonucleic acid (DNA) densities of the herpesviruses with the number of different species of tissue culture cells in which each herpesvirus will readily multiply, then a correlation becomes tentatively evident (Fig. 5). A greater proportion of viruses with DNA of a high density seems to replicate easily in a wide range of cell species than of viruses with a low DNA density. We wish to emphasize the very speculative nature of Fig. 5 and of the correlation which it is meant to convey. Although there is little doubt that viruses such as the herpes simplex viruses, pseudorabies virus, infectious bovine rhinotracheitis virus, and equine herpesvirus type 1 grow readily in a broad range of tissue culture species without prior adaptation, there are a number of viruses for which we know the DNA density but for which we have poor knowledge of their behavior in tissue cultures; thus, the cottontail-rabbit herpesvirus is reported to be specific for the cells of rabbits (4), although we have evidence that it will grow in hamster cells but not in monkey cells. We are unable to define at this stage precisely what should be meant by "ready growth in a broad range of tissue culture species," but we feel, nevertheless, that the possible correlation with DNA density should be pointed out. Figure 5 also begs the question of whether the herpes-

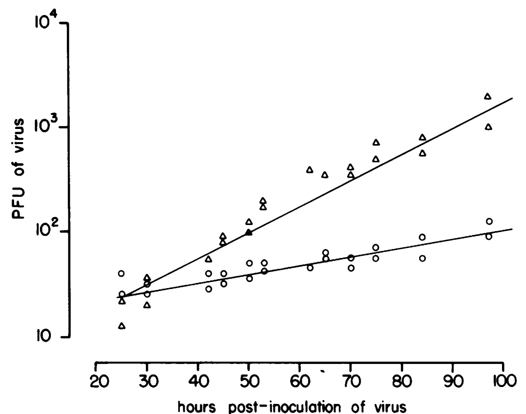
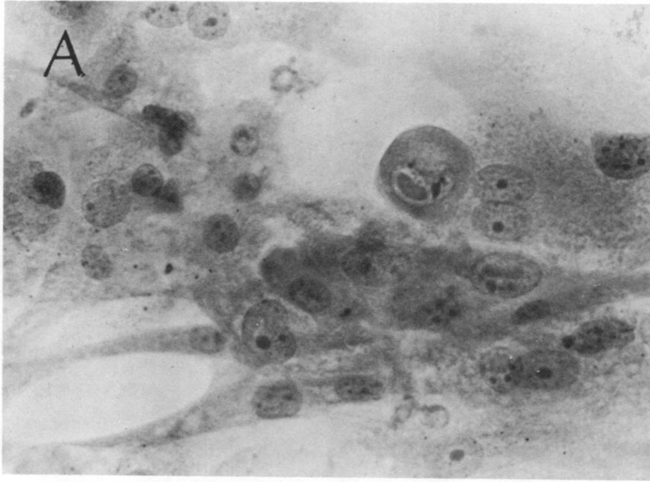
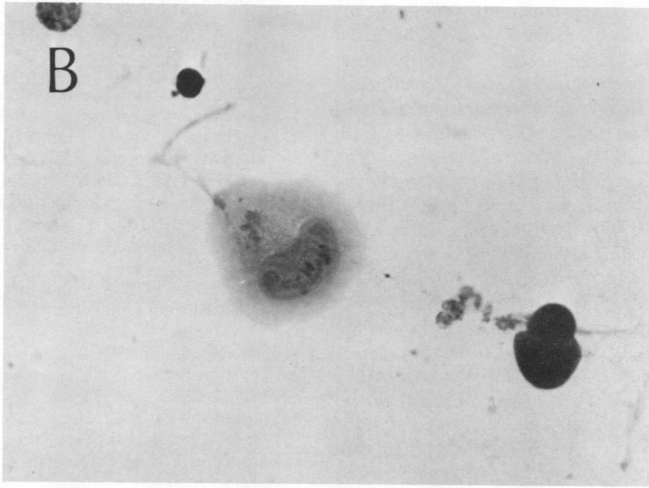


FIG. 3. *Growth curves of mouse cytomegalovirus in rabbit kidney cultures pretreated with IUdR* (Δ) *and untreated* (O). *The viral inoculum was* $10^{4.5}$ *per petri-dish culture. Each point represents combined virus from culture fluid and homogenized cells. The two IUdR points and two control points shown at most of the sampling times represent the titers of duplicate IUdR plates and duplicate control plates harvested at those times. The IUdR-treated cultures contained 2.5-fold less cells than did the control cultures. To make the results comparable per given cell population the IUdR points would need to be increased by 0.4* $\log_{10}$, *but this has not been done in the above graph.*





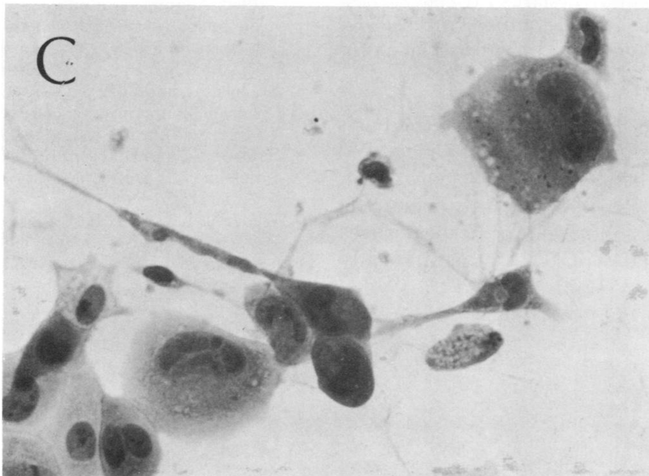


FIG. 4. (*A*) *Inclusions in mouse fibroblast cultures which were not pretreated with IUdR*). (*B, C*) *Inclusions in rabbit kidney cultures which were pretreated with IUdR.*

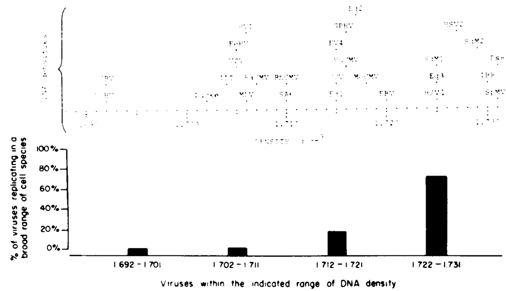


FIG. 5. *The DNA densities of the various herpesviruses are compared with the percentage of viruses in a given DNA density range which will readily grow in a wide variety of tissue culture species. The viral initials indicate the following viruses (in some instances, when confusion may arise as to the identity of the virus concerned, the provisional label recommended by the Herpesvirus Study Group [3] is used alone or is given in parentheses): CCV, channel catfish virus (catfish herpesvirus 1); CRV, cottontail-rabbit herpesvirus; DoHV, dog herpesvirus; EBV, Epstein-Barr virus; Eq1, equine herpesvirus 1; Eq2, equine herpesvirus 2; Eq3, coital exanthema virus (equine herpesvirus 3); FeHV, feline rhinotracheitis virus (feline herpesvirus 1); FV4, frog virus 4 (ranid herpesvirus 2); GPHV, an isolate of guinea pig herpesvirus; HSV1 herpes simplex virus type 1; HSV2, herpes simplex virus type 2; HuCMV, human cytomegalovirus; HVT, herpesvirus of turkeys; IBR, infectious bovine rhinotracheitis virus; ILT, infectious laryngotracheitis virus of chickens; Lucké, ranid herpesvirus 1; MDV, Marek's disease virus; MoCMV, mouse cytomegalovirus; PsR, pseudorabies virus; RaCMV, rat cytomegalovirus; RhCMV, an isolate of cytomegalovirus from the rhesus monkey; SA6, the South African isolate of Cercopithecid herpesvirus 2; SpMV, a herpesviral isolate from the spider monkey; SqMV1, squirrel monkey herpesvirus 1 (Cebid herpesvirus 1); SqM2, herpesvirus saimiri of the squirrel monkey (Cebid herpesvirus 2); VZV, varicella-zoster virus.*

viruses fall into four natural groups on the basis of DNA density; Nahmias (6) has also suggested the possible subdivision of the herpesviruses on the basis of DNA density. But only when the DNA densities of many more herpesviruses have been measured will it be unequivocally clear if the four clusters remain discrete from one another or if they merge together.

The enhancement of viral replication in rabbit cells by pretreatment of the cells with IUdR is in harmony with the observations of St. Jeor and Rapp (8, 9) on the increased permissiveness of human cells to human cytomegalovirus, when those cells were pretreated with IUdR. They suggested that the IUdR prevented, or reduced, the formation of viral inhibitors by the cells; we have no better hypothesis to offer.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Fioretti, A., T. Furukawa, D. Santoli, and S. A. Plotkin. 1973. Nonproductive infection of guinea pig cells with human cytomegalovirus. J. Virol. **11**:998-1003.
2. Goodheart, C. R., G. Plummer, and J. L. Waner. 1969. Density difference of DNA of human herpes simplex viruses, type 1 and 2. Virology **35**:473-475.
3. Herpesvirus Study Group, International Committee for the Nomenclature of Viruses (Chairman, B. Roizman). 1973. Provisional labels for herpesviruses. J. Gen. Virol. **20**:417-419.
4. Hinze, H. G. 1971. New member of the herpesvirus group isolated from wild cottontail rabbits. Infect. Immunity **3**:350-354.
5. Kim, K. S., and R. I. Carp. 1971. Growth of murine cytomegalovirus in various cell lines. J. Virol. **7**:720-725.
6. Nahmias, A. J. 1972. Herpesviruses from fish to man—a search for pathobiologic unity, p. 153-182. *In* H. L. Ioachim (ed.), Pathobiology annual. Appleton-Century-Crofts, New York.
7. Raynaud, J., P. Atanasiu, C. Barreau, and M. Jahkola. 1969. Adaptation d'un virus cytomegalique provenant du mulot (*Apodemus sylvaticus*) sur differentes cellules heterologues, y compris les cellules humaines. C. R. Acad. Sci. Ser. D **269**:104-106.
8. St. Jeor, S., and F. Rapp. 1973. Cytomegalovirus: conversion of nonpermissive cells to a permissive state for viral replication. Science **181**:1060-1061.
9. St. Jeor, S., and F. Rapp. 1973. Cytomegalovirus replication in cells pretreated with 5-iodo-2'-deoxyuridine. J. Virol. **11**:986-990.
10. Waner, J. L., and T. H. Weller. 1974. Behavior of human cytomegaloviruses in cell cultures of bovine and of simian origin. Proc. Soc. Exp. Biol. Med. **145**:379-384.