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Effect of Arginine Deprivation on Murine Cytomegalovirus Replication

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Murine cytomegalovirus (CMV) has often been used as a model for human CMV because of its many similarities to human CMV (13).

Multiplication of murine CMV in fibroblastic cells has been found to depend on the S-phase of the host cell cycle (11). It has been reported that the cell growth can be controlled by deprivation of a single amino acid of the medium (1). These facts led us to study infection of murine CMV under single amino acid deprivation. The Smith strain of murine CMV, provided by Dr. T. Nakao, Sapporo Medical College, was used throughout the experiments. The virus stock was prepared from infected mouse embryo fibroblasts (MEF) by freeze-thawing followed by low speed centrifugation, and dialyzed against Hanks' balanced salt solution (BSS) for 4 hr with one change. Cells derived from osteosarcoma of the hairless mouse (hr/hr), designated as HOT, at the 40th to 45th passages and MEF at the 3rd passage were used for replication of the virus. The virus was plaque-titrated with MEF at the 3rd to 5th passages, under a methylcellulose overlay (9). Unless otherwise stated cells were grown in Eagle's minimum essential medium (MEM) (3) supplemented with 5% calf serum. To determine the amino acid requirements of murine CMV, the infected cells were maintained either with MEM or with MEM lacking each of 13 amino acids. Except for the experiment shown in Fig. 1, those media were supplemented with 2.5% calf serum which was dialyzed against Hanks' BSS for 3 days at 4 C with six changes. Supplementing with the dialyzed serum prevented the cells from detaching during incubation without altering the arginine requirement for murine CMV replication.

The amino acid requirement for multiplication of murine CMV was determined in HOT cells and MEF as follows. Prior to inoculation cells were prestarved for 13 individual amino acids by incubating them in MEM lacking each amino acid for 24 hr at 37 C and then inoculated with murine CMV at a multiplicity of infection (MOI) of 1. After 1-hr adsorption at room temperature, the infected cells were again maintained in the amino acid-deficient media for 38.5 hr at 37 C and the virus yield was determined. All 13 amino acids were found to be necessary for maximum production of murine CMV in HOT (10^6) and in MEF (10^5). Under deprivation of a single amino acid the virus yield decreased, and the decrease was generally more marked in HOT cells (10^3–10^5) than in MEF (10^1–10^3). Relevant-

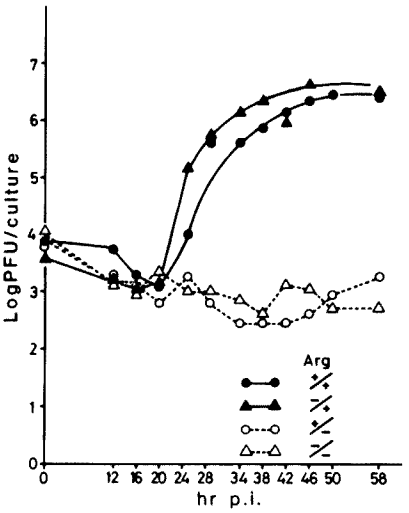

Fig. 1. Growth curve of murine CMV in HOT cells. HOT cells were maintained in complete medium (+) or in arginine-deficient medium (−) for 24 hr at 37 C prior to inoculation with murine CMV at an MOI of 1. The infected cells were maintained either in complete medium or in arginine-deficient medium at 37 C. All the media used were serum-free. The virus yield is shown in $\log_{10}$ PFU per culture.

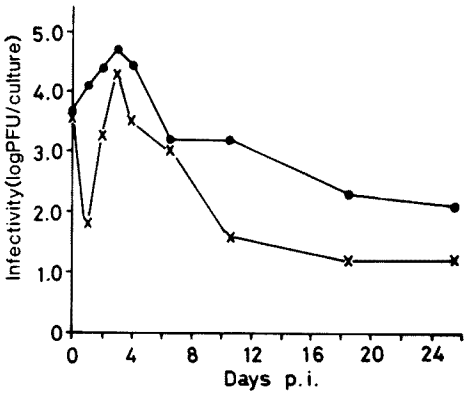

Fig. 2. Persistence of murine CMV genomes in HOT cells under arginine deprivation. HOT cells in tubes, prestarved for arginine for 24 hr, were inoculated with murine CMV at an MOI of 0.7. After adsorption, the infected cells were maintained at 37 C in arginine-deficient medium supplemented with 2.5% dialyzed calf serum. The medium was changed every 3 days. On the indicated days the cells in two tubes were harvested, trypsinized and suspended in 1 ml of MEM. Samples (0.1 ml) of appropriate dilutions of the cell suspensions were inoculated onto monolayers of MEF to determine the number of infectious centers (●). In parallel, the cell suspensions were sonicated to disrupt the cells and the intracellular (cell-associated) virus was plaque-titrated (×).

ly, it has been reported that the effect of arginine deprivation on the replication of herpes simplex virus is observed in cell lines but not in primary cells (6). Cystein and glutamine deficiencies were strongly cytotoxic, especially to HOT cells; there-

fore the requirement of these amino acids for virus multiplication could not be evaluated. Arginine deficiency markedly reduced the virus yield in HOT cells without a toxic effect on the host cells. The arginine dependence of virus production has also been reported for human and simian CMV (5, 8).

The infectious process under arginine deprivation was further investigated.

First, the experiment on growth of murine CMV in HOT cells with or without arginine prestarvation revealed that the arginine deprivation inhibited multiplication of murine CMV even without prestarvation (Fig. 1).

Second, the survival of the virus genome under arginine deprivation was examined as follows. Murine CMV-infected cells were fed with arginine-deficient medium for 20–26 days. The numbers of infectious centers and cell-associated viruses were determined at appropriate intervals. A representative result is shown in Fig. 2. The number of infectious centers and the virus titer increased until 3 days after infection and then decreased. After 10 days, the infective virus titer remained less than one-tenth of the number of infectious centers up to 26 days. Similar results were obtained repeatedly. Hamatoxylin-eosin staining of the infected cultures revealed that the cells contained small, restricted inclusion bodies within 1 day after infection and that only a small percentage of the cells had fully developed, intracellular inclusion bodies 4–6 days after infection. This may account for leaky production of infective viruses in some infected cells under arginine deprivation.

Third, a lysate of infected or mock-infected cells with extracellular fluid was layered on a 10–50% potassium tartrate density gradient and centrifuged for 3 hr at 25,000 rpm as described by Kim et al (7). The gradients were examined for the presence of virions of murine CMV by plaque titration and by electron microscopy. No virions were detected in the infected cells maintained in arginine-deficient medium.

Fourth, the inhibitory effect of arginine deprivation on virus production was apparently reversible, and infective viruses were recovered after arginine was supplied. However, after addition of arginine, infective viruses were produced more rapidly, with a shorter latent period (5–12 hr), in infected cells starved for arginine for 48 hr prior to the addition of arginine than in the infected cells without arginine starvation after infection (20 hr). The shorter latent period in the infected cells with arginine starvation after infection cannot be explained only by omission of the adsorption and penetration process. A preferable explanation is that the synthesis of DNA, RNA and protein might partially proceed but not be completed under arginine deprivation.

Fifth, the effect of arginine deprivation on the synthesis of viral macromolecules was determined in the following experiments. HOT cells prestarved for arginine for 24 hr were infected with murine CMV at an MOI of 1. The infected cells were maintained in arginine-deficient medium for 48 hr and then fed for an additional 48 hr with arginine-containing medium (complete medium) or complete medium containing inhibitors of protein synthesis (cycloheximide, 1.0 μg/ml; canavanine, 24 mM), an inhibitor of RNA synthesis (actionmycin D, 0.2 μg/ml),

Table 1. Multiplication of murine CMV after arginine restoration

Medium		Virus yield
Complete		++
Complete with addition of		
Actinomycin D	(0.2 μg/ml)	−
Canavanine	(24 mM)	−
Cycloheximide	(1.0 μg/ml)	−
5-Bromodeoxyuridine	(100 μg/ml)	+
5-Fluorodeoxyuridine	(50 μg/ml)	−
Arabinosylcytosine	(50 μg/ml)	−
Phosphonoacetic acid	(200 μg/ml)	−
2-Deoxy-D-glucose	(0.1%)	−
Arginine-deficient		−

Virus yield (PFU/culture) is expressed as: $-(<10^3)$, $+(10^5$–$10^6)$, and $++(10^6$–$10^7)$.

inhibitors of DNA synthesis (arabinosylcytosine, 50 μg/ml; 5-bromodeoxyuridine, 100 μg/ml; 5-fluorodeoxyuridine, 50 μg/ml; phosphonoacetic acid, 200 μg/ml) or an inhibitor of glycosilation (2-deoxy-D-glucose, 0.1%). If synthesis of viral DNA, RNA, and protein or glycosilation of the protein was completed under arginine deprivation, those inhibitors should no longer be effective and subsequently the infectious viruses should be produced in the presence of the inhibitor upon arginine restoration. However, every inhibitor except BUDR was still effective after the arginine restoration (Table 1). The results suggest that under arginine deprivation, synthesis of DNA, RNA and protein essential for virus multiplication was at least not completed and must have been initiated or completed after arginine was supplied. The finding that BUDR inhibited virus replication only moderately may reflect the fact that thymidine kinase is not necessary for multiplication of murine CMV (4, 5).

Sixth, attempts were made to examine the viral DNA and RNA synthesis under arginine deprivation by incorporation of radiolabeled precursors of DNA ([^{3}H]thymidine and [^{3}H]deoxycytidine) and RNA ([^{14}C]uridine). Cultures prestarved for arginine for 24 hr were infected with murine CMV at an MOI of 1–4 or mock-infected. Duplicate cultures were labeled with 100 μCi of [^{3}H]-thymidine and 1 μCi of [^{14}C]uridine or 20 μCi of [^{3}H]deoxycytidine 1.5–36 hr after infection at 37 C. The cultures were digested with Sarkosyl and pronase as described by Crouch and Rapp (2). DNA was determined as alkaline-stable, acid-precipitable material, and RNA as alkaline-unstable, acid-precipitable material. Incorporation of those isotopes into DNA and RNA in mock-infected cells was not affected by arginine deprivation, but incorporation of [^{3}H]thymidine and [^{3}H]-deoxycytidine into DNA was reduced by murine CMV infection to one-fifth and one-half, respectively, regardless of arginine starvation. At the same time, the incorporation of [^{14}C]uridine into RNA was not much affected by the virus infection. Analysis by CsCl centrifugation, described by Moon et al (10), revealed that the labeled DNA synthesized under such conditions was only host cell DNA, with no peak of viral DNA even in the presence of arginine as well as in the absence of arginine.

These results suggest that murine CMV infection also suppresses the cellular enzyme(s) involved in phosphorylation of deoxycytidine and the virus does not code for the enzyme(s), as observed for thymidine kinase (4, 12). Thus, the viral DNA was unable to be labeled for reasons *per se* even in the presence of arginine. Yet, the infectious progeny viruses were sufficiently produced, if arginine was present. Therefore, it is not clear whether viral DNA is synthesized in the absence of arginine. It cannot be ruled out that the viral DNA is synthesized to a certain extent under arginine deprivation. In fact, the host cell DNA was synthesized even in the absence of arginine as shown above, and it is known that replication of murine CMV depends on the events associated with the S-phase of the host cell cycle (11). On the other hand, it was shown that synthesis of viral DNA was not completed, if it occurred at all, in the absence of arginine.

In summary, murine CMV requires arginine for replication, especially in HOT cells. It was suggested that, in the absence of arginine, synthesis of viral DNA, RNA and protein was not completed. The viral genome persisted under such a condition for 26 days, with leaky production of infective viruses in certain cells.

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