

Inhibition of Herpes Simplex Virus Multiplication by Activated Macrophages: A Role for Arginase?

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Proteose-peptone-activated mouse macrophages can prevent productive infection by herpes simplex virus in neighboring cells *in vitro* whether or not those cells belong to the same animal species. The effect does not require contact between the macrophages and the infected cells, may be prevented by adding extra arginine to the medium, and may be reversed when extra arginine is added 24 h after the macrophages. Arginase activity was found both intracellularly and released from the macrophages. The extracellular enzyme is quite stable; 64% activity was found after 48 h of incubation at 37°C in tissue culture medium. No evidence was found that the inefficiency of virus replication in macrophages was due to self-starvation by arginase. As might be predicted macrophages can, by the same mechanism, limit productive infection by vaccinia virus.

Much has been written on the role of macrophages as immunospecific and nonspecific mediators against virus infections (1, 18). Their crucial importance, for example, in herpes infection has been established (10, 12), and factors that modify their activity in one direction or another also influence the outcome of an infection (13, 17, 19, 29). Morahan et al. (19, 20) distinguish two sorts of resistance conferred by macrophages: intrinsic, where infectious virus is disposed of within macrophages acting either as phagocytes or as nonpermissive host cells (12, 26), and extrinsic, where macrophages retard or ablate virus multiplication in neighboring cells (9, 15). Although Morse and Morahan (21) have recently recognized that some factor must alter host-cell metabolism, no substantiated explanation of how extrinsic activity may work has been offered; the mechanism might equally well be positive, e.g., due to an interferon-like agent (15), or negative, e.g., due to depletion of essential molecules. We became interested in the extrinsic activity of peptone-activated macrophages as a result of the report of Hayashi and colleagues of restriction in plaque numbers (9), but we found the effect difficult to reproduce. However, more pronounced differences were observed when the medium was diluted. Plaque reduction was independent of the ratio of the number of macrophages to the number of infected cells, but did require high concentrations of macrophages. These preliminary findings suggested that the macrophages were acting by depleting the medium of an essential nutrient.

Kung and colleagues (14) discovered that activated macrophages contain arginase and ob-

tained evidence that the enzyme was responsible for macrophage-mediated suppression of mixed leucocyte reactions. Currie (6) found that mature macrophages release the enzyme, and he and his co-workers have drawn attention to arginase as a possible mechanism whereby macrophages suppress certain tumors. More recently Olds et al. (22) obtained evidence that arginase could be involved in the killing by macrophages of schistosomulae. Currie and colleagues (6, 7) have speculated that arginase activity may be important in restricting several arginine-requiring viruses. Since it is well known that arginine is required for the multiplication of herpes simplex virus (27), we have investigated the role of arginase as a mediator of extrinsic macrophage restriction of that virus and have also looked for evidence that vaccinia virus is similarly restricted.

MATERIALS AND METHODS

Macrophages. Adult BALB/c mice were injected intraperitoneally with 0.8 to 1.0 ml of 10% proteose peptone (Difco Laboratories, Detroit, Mich.). Peritoneal cells were harvested 3 days later by washing out with Eagle (Glasgow-modified) medium containing EDTA and 500 U of heparin (Evans Medical Ltd.) per ml, but no serum. The cells were centrifuged (100 rpm for 5 min), suspended in Eagle medium, counted, seeded into appropriate tissue culture vessels, and left at 37°C in a CO₂ incubator to adhere. The cultures were then washed vigorously with phosphate-buffered saline to remove nonadherent cells. For each experiment a sample culture was checked for phagocytosis by incubating with a loopful of Pelikan ink; virtually all of the adherent population phagocytosed avidly, and these cells we call macrophages.

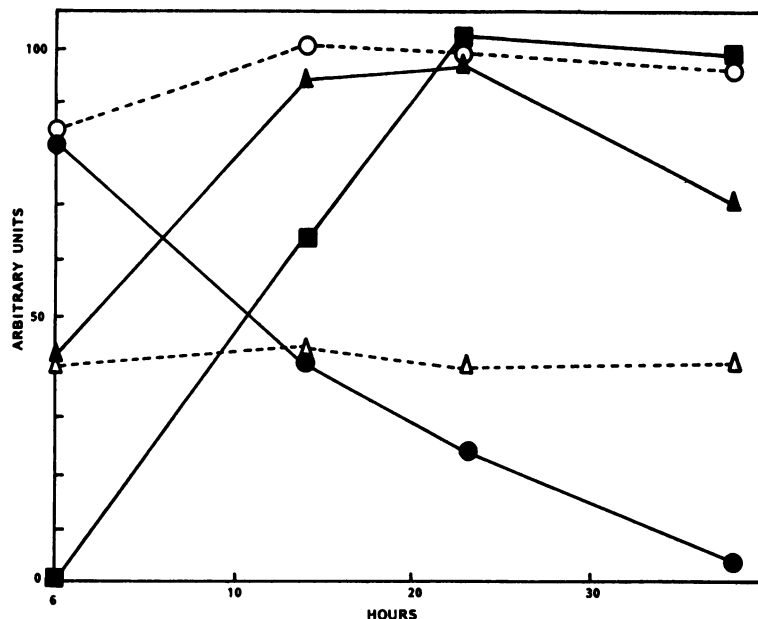


FIG. 1. Changes in amino acid concentration in medium incubated with macrophages. Volumes of 1 ml were incubated with 2×10^6 peptone-activated peritoneal macrophages (●, ■, ▲) or alone (○, △). Symbols: Arginine (●, ○); ornithine (■, □); glutamic acid (▲, △).

Although results within experiments with cell preparations made in this way are highly consistent, some inevitable variation in the performance of the various batches of macrophages occurred, possibly depending on the age of the mice used as donors, which was not standardized.

Measurement of amino acid depletion. Macrophage cultures were made in 24-well Linbro plates with 2×10^6 cells per well and 1.0 ml of Eagle medium containing 10% tryptose phosphate broth (Difco) and 10% fetal calf serum. Controls without macrophages were set up alongside. Cultures were incubated at 37°C; at intervals medium was withdrawn and centrifuged for 1 min in a microfuge, an equal volume of cold 10% trichloroacetic acid was added, and the precipitated protein was removed by centrifugation at 13,000 rpm. The supernatant fluid was extracted three times with ether. Samples were frozen at -70°C until they could be subjected to complete amino acid analysis with a Locarte amino acid analyzer.

Arginase estimations. Arginase was estimated by incubation with substrate at a high concentration under standardized conditions and subsequent direct determination of urea by means of thiosemicarbazide-diacetylmonoxime as previously described (23). The unit of activity used here is expressed in nanomoles of urea released per minute. Estimations of released activity were made directly on medium that had been centrifuged at 4,000 rpm for 10 min. Intracellular arginase activity was estimated on cell lysates made by adding 0.5 or 1.0 ml of a solution containing 9.5 ml of distilled water, 0.5 ml of aprotinin (Sigma Chemical Co., St. Louis, Mo.; A 6012), and 10 μl of Triton X-100. All estimates were made by using independent duplicate cultures.

Virus. A clone of the herpes simplex strain SC16 was used. It was titrated in BHK cells by the method of Russell (25).

Target cells. Most experiments were done in duplicate with either BHK 21 cells which were maintained in this department or BALB/c mouse embryo cells. The latter were cultured from roughly minced mid-term embryos. The fragments were washed with buffered saline, and cell suspensions were obtained by trypsinization. The cells were subcultured and used at the third passage. For most experiments 2×10^5 cells were seeded into the wells of Linbro trays in Eagle medium containing specified concentrations of arginine and 10% fetal calf serum which had been dialyzed for 24 h against 50 volumes of phosphate-buffered saline under sterile conditions.

To obtain standard target cells, cultures were incubated in arginine-free medium for 48 h before infection at low multiplicity.

RESULTS

Macrophages deplete medium predominantly of arginine. Macrophage cultures were set up in Linbro wells (see above) and overlaid with medium. Wells without macrophages served as controls. At intervals amino acid estimations were made. Two changes were detected in the amino acid composition of the medium overlying the macrophages. These were (i) progressive depletion of arginine with concomitant accumulation of ornithine and (ii) the appearance of low levels of glutamic acid with a very small decrease in glutamine (Fig. 1). No other amino acid was

appreciably affected. These results, which are consistent with previous studies (6, 7), indicate considerable activity of arginase and also suggest that macrophages can deaminate glutamine. This latter effect can be discounted as important for two reasons. First, there was still abundant glutamine present at the end of the experiment; the addition of extra glutamine at this stage did not affect virus growth. Second, although ammonia (a product of deamination) is inhibitory to some viruses we have evidence that herpes simplex virus is rather resistant; replication is quite unaffected by concentrations as high as 1.0 mM.

Arginase activity persists in macrophage cultures. To interpret our findings, we needed to know what levels of arginase activity were to be found in our cultures of macrophages and how much variability there was. Samples of 3×10^6 to 4×10^6 macrophages were plated in each of several small petri dishes containing 2.0 ml of Eagle medium with 10% dialyzed fetal calf serum and 40 μg of arginine per ml. Within experiments, estimates of arginase activity in duplicate independent cultures gave satisfactory agreement to within 5% of the mean. There was, however, variation between experiments. For instance, in three separate experiments the values for intracellular enzyme activity were 12, 24,

and 45 U/ 10^6 freshly harvested macrophages. After incubation overnight the ratios of the levels of intracellular to extracellular enzyme were 23:5.3, 52:6.7, and 57:10.0 U/ 10^6 macrophages.

Levels of arginase were assayed in a set of replicate macrophage cultures incubated for various periods up to 67 h. The intracellular to extracellular enzyme level ratios for cultures harvested at 19, 28, 43, 52, and 67 h were, respectively, 57:10.0, 44:12.5, 44:12.8, 36:13.6, and 26:15.2 U/ 10^6 cells. Although some arginase activity persisted throughout the experiment, there was a slow progressive loss of intracellular enzyme activity accompanied by a slight increase in extracellular activity. The stability of released enzyme activity in tissue culture medium was also evaluated. Twenty-four-hour harvests of tissue culture medium with a starting concentration of 40 μg of arginine per ml and 4×10^7 macrophages were prepared. After centrifugation to remove cells, these were incubated at 37°C in an atmosphere of 5% CO₂, and samples were taken at intervals for arginase assay. The enzyme activity was evidently rather stable under these conditions; by 6 h 83% remained, by 12 h 77% remained, by 24 h 72% remained, and by 48 h 64% remained.

These experiments show that arginase activity

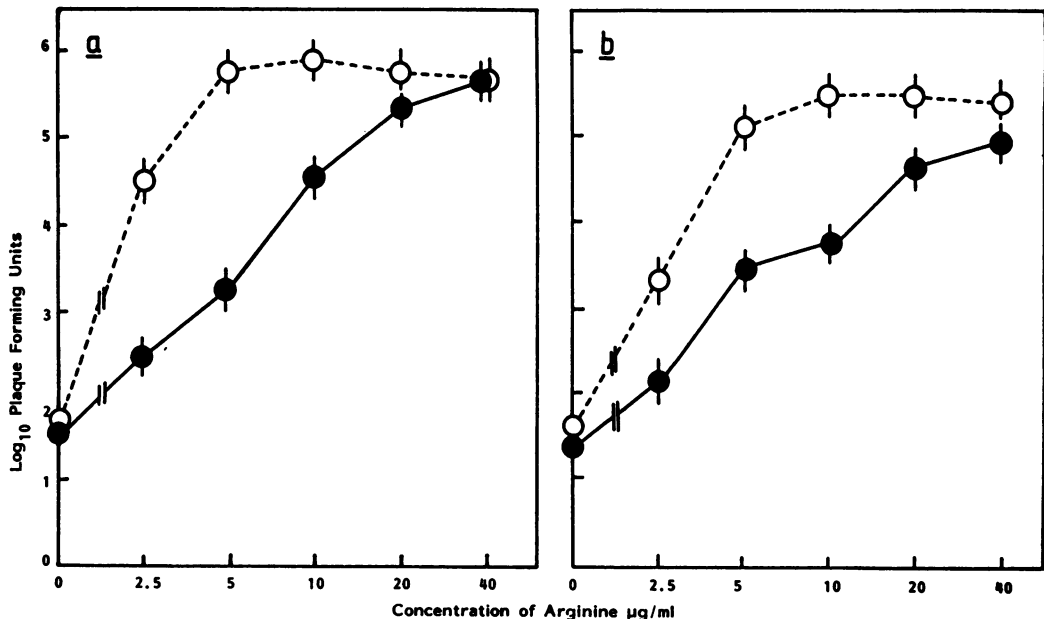


FIG. 2. Suppression of virus multiplication by macrophages. (a) Virus multiplying in BHK cells; (b) virus multiplying in mouse embryo cells. Samples of 2×10^5 cells were infected with 10^4 PFU of virus and overlaid with 0.5 ml of medium containing the indicated concentrations of arginine with (●) or without (○) 2×10^6 macrophages. Each point is the geometric mean titer of yields taken from two independent cultures at 24 h. The bar indicates range. Total harvests were also taken at 4 h; all gave values below 10^2 PFU.

persists well in macrophage cultures and that although reproducible values are obtained within experiments we must expect some variation between experiments.

Suppression by macrophages of virus multiplication in the presence of various concentrations of arginine. BHK cells or mouse embryo cells were dispensed in Linbro wells to give 2×10^5 cells per cell. They were allowed to form monolayers in arginine-free medium. Two days later they were infected with 10^4 PFU of the SC16 strain of herpes simplex virus (type 1) in 0.2-ml volumes. After 1 h at 37°C to allow adsorption, the cells were washed twice with phosphate-buffered saline and overlaid either with freshly harvested macrophages in arginine-free medium or with the medium alone. One hour later, the wells were again washed twice with phosphate-buffered saline to remove nonadherent cells. The medium was then replaced by 0.5-ml volumes of medium containing various concentrations of arginine. Total virus harvests were taken at 4 and 24 h by removing the supernatant medium, lysing the cells in distilled water, pooling the

lysate with the medium, sonicating, and freezing at -70°C until the infectivity could be assayed. The results clearly show (Fig. 2) that within the physiological range of arginine concentrations (2.5 to $20 \mu\text{g/ml}$) macrophages effectively suppress the multiplication of virus.

Macrophage suppression of virus multiplication can be reversed by the addition of extra arginine. Monolayers of 2×10^5 mouse embryo cells were prepared, infected with 10^4 PFU of virus, washed, and overlaid with macrophages exactly as in the previous experiment. They were covered with Eagle medium containing 10% dialyzed fetal calf serum and either $10 \mu\text{g}$ of arginine per ml or no arginine. At 24 h some of the wells had arginine added to bring the concentration to $40 \mu\text{g/ml}$. Total harvests were made at 4, 24, and 48 h, and the infectivity titers were determined. Once again the suppressive effect of the macrophages was obvious and even more pronounced than in the previous experiment (Fig. 3). The virus titers at 24 and 48 h in the presence of macrophages resembled those of cultures which had no arginine. When arginine

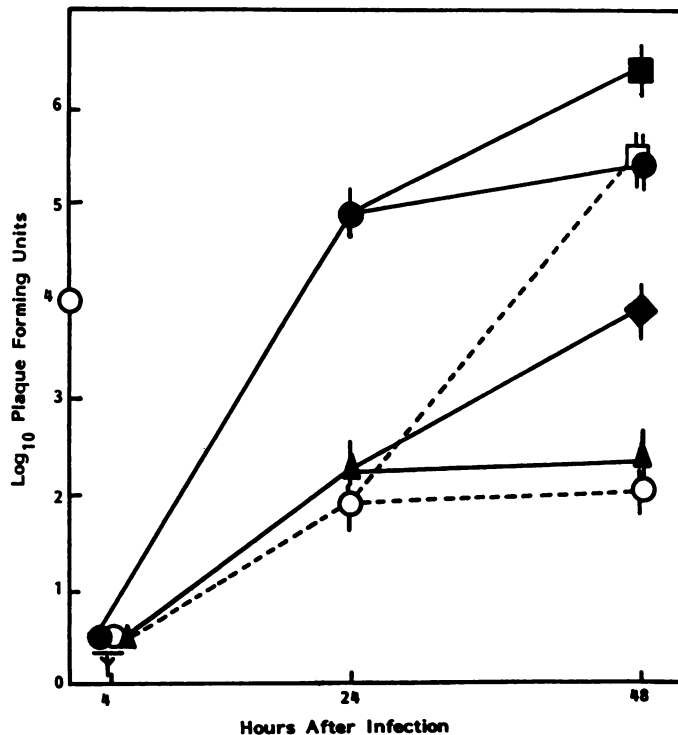


FIG. 3. Reversal of macrophage suppression by added arginine. Samples of 2×10^5 mouse embryo cells were infected with 10^4 PFU of virus and overlaid with medium containing no arginine (○), $10 \mu\text{g}$ of arginine per ml (●), or $10 \mu\text{g}$ of arginine per ml plus 2×10^5 macrophages (▲). Further arginine was added to some cultures at 24 h to bring the concentration to $40 \mu\text{g/ml}$ (□, ■, ◆). Total harvests were taken at 4, 24 and 48 h. All cultures were set up in duplicate, and titers were determined independently. Points are geometric means; range is indicated by the bars. The open circle on the ordinate indicates the inoculum.

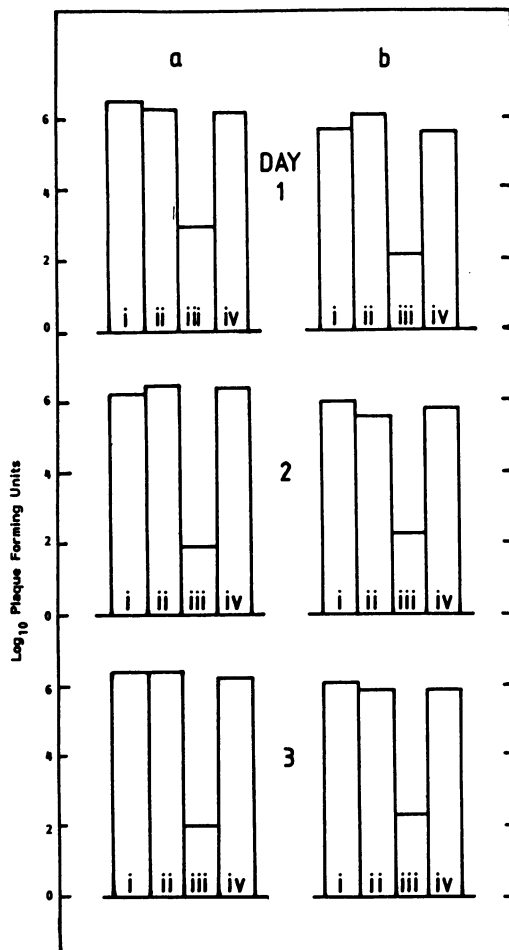


FIG. 4. Macrophages can deplete medium of arginine sufficiently to abort virus multiplication in BHK cells (a) or mouse embryo cells (b). Eagle medium containing 10 μg of arginine per ml was incubated for 24 h either alone or with macrophages (see text). The same macrophages were used on three consecutive days (1, 2, 3). These media were used to cover infected cells either alone or with extra arginine added to give 40 $\mu\text{g}/\text{ml}$. Bars: (i) control medium, (ii) control medium plus added arginine, (iii) macrophage-conditioned medium, and (iv) macrophage-conditioned medium plus added arginine.

was added at 24 h to the control cultures containing no arginine, suppression of virus production was reversed dramatically. With the macrophage-suppressed cultures the reversal was less marked but appreciable; it seems likely that this reversal was less striking owing to the accumulation of arginase in the medium at 24 h.

Macrophages suppress virus multiplication at a distance by means of arginine deprivation. Two plastic petri dishes were set up, each containing 5.0 ml of medium and 2×10^7 macrophages.

After 1 h of incubation, nonadherent cells were washed away, and 5.0 ml of Eagle medium with 10% dialyzed fetal calf serum and 10 μg of arginine per ml was added. Two control dishes with no cells were set up alongside. The dishes were incubated at 37°C for 3 days in a CO₂ incubator. At 24, 48, and 72 h the media were harvested and centrifuged at 4,000 rpm for 10 min to remove any detached cells, and the samples were frozen at -70°C until required. The cells were overlaid with fresh medium at each time point. Monolayers of BHK and mouse embryo cells were prepared in Linbro wells. They were infected with 10^4 PFU of virus and after washing were overlaid with 0.5 ml of macrophage-conditioned medium or control medium each with or without extra arginine added to bring the concentration to 40 $\mu\text{g}/\text{ml}$. The results (Fig. 4) demonstrate that at this concentration macrophages deplete medium of arginine sufficiently to prevent virus multiplication. There is no requirement for the cells to make contact. Clearly the macrophages remained active over a period of 3 days.

DISCUSSION

More than one mechanism must underlie the extrinsic resistance conferred by macrophages; for instance, macrophages have been shown to produce interferon (8), and this could obviously act upon virus-producing cells. However, Morse and Morahan (21) have evidence of another mechanism acting against herpes simplex virus, though its nature remains undefined. Our experiments unambiguously show that arginase produced by activated macrophages can so deplete medium of arginine that the replication of herpes simplex virus in neighboring cells can be aborted. Not surprisingly, the host species of the cells is unimportant, and the restriction works at a distance. This is possibly the same phenomenon as that of Morse and Morahan (21), although they found that cell contact was needed for optimal activity.

The arginine requirement of growing herpes simplex virus is well known (3-5, 11, 16, 27). Virus appears to be able to undergo the early steps in multiplication, and even virus DNA may be synthesized in the absence of arginine. Some virus-specified proteins are made, but the necessary movement of certain of these from the cytoplasm to the nucleus seems to be impeded so that replication is arrested at this stage. When arginine is supplied, the block is quickly reversed, even when cells have been deprived for some days.

The most important question is whether this extrinsic effect, so easily demonstrated *in vitro*, has any relevance *in vivo*. We are at present examining this. It seems unlikely, as Currie (6)

argues, that macrophages could deplete systemic arginine levels sufficiently to affect replicating virus. However, it is much easier to imagine effective local depletion in a focus of inflammation. This is analogous to the suppression of certain tumors by macrophages (7).

If macrophage-produced arginase turns out to be important in herpes simplex virus infections *in vivo*, we may be sure that other viruses will be affected in the same way. Arginine is essential for the production of poxviruses (2), adenoviruses (24), and papovaviruses (28). We have evidence that vaccinia virus is indeed restricted in culture by macrophages; an experiment done in the same way as that illustrated in Fig. 2 gave the following results at 24 h: mouse embryo cells infected in the presence of 10 μ g of arginine per ml yielded 2.7×10^6 PFU in the absence of macrophages and 1.7×10^5 PFU when they were present. In the presence of 40 μ g of arginine per ml the respective yields were 3.2×10^6 and 3.7×10^6 PFU.

Finally, we may speculate whether the intrinsic restriction of virus growth within macrophages could arise from self-starvation of arginine. At present the evidence is against this. In a series of experiments macrophages were infected with herpes simplex virus at many input multiplicities of infection and were assayed after 4 h for infective centers (after trypsinization), at 7 h for the proportion of antigen-positive cells (by immunofluorescence), and at 24 h for virus yields. The results indicated very inefficient antigen production, even less efficient infective center formation, and miniscule yields. All three determinations varied strictly with input multiplicity; however, all three were quite unaffected by concentrations of arginine in the medium (from 0 to 1,000 μ g/ml).

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