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# Mouse Macrophage Arginase (40777)[1]

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We reported recently the isolation of a macromolecular factor in macrophage culture supernatant which was able to inhibit the proliferation of mouse lymphoma cells and various immune reactions of mouse spleen cells *in vitro* (1). Shortly afterward Kung *et al.* (2) presented evidence that macrophages inhibited *in vitro* immune responses by depleting arginine from the culture medium, and arginase activity was demonstrated in macrophage lysate. However, in the report arginase activity in the culture medium was only slightly increased above background, and the products of arginine degradation were not identified. Hence there was uncertainty over whether this or another enzyme was responsible for arginine depletion. Arginine deiminase has been shown to cause similar growth inhibition in other culture systems (3). Furthermore, the macrophage arginase was not characterized; its mode of action and its relation to liver arginase, which have been extensively studied, were not shown. In the present paper these points have been investigated. Our inhibitor proved to be arginase (EC 3.5.3.1.), since it converted the arginine in the medium to urea and ornithine. The amount of enzyme activity found in the supernatants is quantitatively sufficient to account for the depletion of arginine to below a growth permissive level for lymphoma cells. The enzyme has a relatively low affinity for its substrate ($K_m$ 1.0 $\times$ $10^{-2}$ $M$) and has properties very similar to those of bovine liver arginase as reported by previous workers.

*Materials and methods. Mouse macrophage culture supernatant (MCS).* This was prepared as in the previous report (1). Briefly, peritoneal cells were collected from DBA/2 mice 4 days after ip injection of 2 ml thioglycollate medium. The cells, comprising more than 80% macrophages as judged by latex particle ingestion, were washed and cultured in Dulbecco's medium without serum for 48 hr at 1 $\times$ $10^7$ cells/ml. The viability at 48 hr culture was 80–92%. The culture fluid was then collected, centrifuged at 20,000 rpm for 20 min to remove cells, and (unless specified) dialyzed against Dulbecco's medium. The fluid was then filtered and stored at −20°C.

*Mouse lymphoma cells.* L1210A was maintained in culture in Dulbecco's medium and 10% fetal calf serum (FCS) as described previously (1). For inhibition assay, 2 $\times$ $10^5$ cells were inoculated in 1 ml of test fluid, cultured for 48 hr, and the viable cells were counted by trypan blue exclusion.

*Reagents.* [*guanido*-$^{14}$C]- or [U-$^{14}$C]arginine HCl and [$^{14}$C]urea, all used at 50 mCi/mmole, were purchased from Amersham Corporation. Ingredients for culture medium were obtained from GIBCO, chromatographic reagents and salts were from Fisher, and other chemicals were from Sigma.

*Identification and measurement of products of arginine degradation in media treated with macrophage supernatant.* One microcurie of C$^{14}$ [*guanido*-$^{14}$C]- or [U-$^{14}$C]arginine and unlabeled arginine at a final concentration of 30 mg/liter were added to 0.1 ml of MCS dialyzed previously against arginine-free medium (made in the laboratory). The material was then incubated at 37° in a $CO_2$ incubator for 18 hr and deproteinized by passage through the Amicon Centriflo CF-25 membrane cone, and an aliquot was subjected to high-voltage paper electrophoresis (Savant Instruments) for 45 min at 2500 V(30 V/cm) using

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pyridine–acetate buffer at pH 6.5. After drying, the paper was run in the vertical direction for descending liquid chromatography, using *n*-butanol–acetic acid–water as solvent. The paper was dried and placed in contact with an X-ray film overnight. The spots on the developed autoradiograph were identified with known radiolabeled compounds run in identical manner. Since only urea (neutral) and arginine (basic) were detected on the autoradiograph using [*guanido*-$^{14}$C]arginine (see Results), the amount of residual arginine in the incubation mixture could be measured by the radioactivity after electrophoretic separation. The spots corresponding to the neutral and arginine regions were cut and transferred to vials containing 10 ml of scintillation fluid and the activity was counted. The concentration of residual arginine was calculated as original arginine (30 mg/liter) × activity of arginine/activity of (arginine + urea).

*Assay of arginase activity by colorimetric method.* The method of Kung *et al.* (2) was used with some modifications. The sample, 0.1 ml, was mixed with 0.05 ml of 5 m*M* $MnCl_2$ in 0.04 *M* Tris–HCl, pH 7.2, and 0.05 ml of 5% bovine serum albumin (BSA) in saline, and incubated for 15 min at 55° to activate the arginase. The addition of BSA was found necessary in all the colorimetric assays; in its absence only very low arginase activity could be demonstrated in MCS. The use of BSA was first reported by Loeb and Stuhlman (4) to stabilize dilute arginase solution. The mixture was then incubated with 0.1 ml of 0.84 *M* arginine–HCl (pH 9.5) for 15 min at 37°. The urea generated was measured from its reaction with thiosemicarbazide and butadiene monoxime in acid reagents (2). For measuring the pH optimum of the arginase 0.84 *M* arginine solution was adjusted to various pH with concentrated HCl and added to the reaction mixture. For the study of metal ion requirement, 5 m*M* $FeSO_4$, $ZnSO_4$, Co-acetate, or Ni-acetate was used instead of $MnCl_2$. For measuring the $K_m$ value, various concentrations of arginine in 0.3 *M* glycine with final pH adjusted to 9.5 were used.

*Gel filtration for arginase activity.* Five milliliters of MCS was loaded on a column of Sephadex G-150 and eluted with 1 m*M* sodium phosphate buffer in saline, pH 7.2, as before (1). The individual fractions were tested for the arginase activity by urea production. Human IgG (MW 150,000) and bovine serum albumin (67,000) were used to calibrate the molecular weight of macrophage arginase.

*Results. Inhibition of lymphoma cells by macrophage culture supernatant (MCS) and its reversal by arginine or citrulline.* Evidence that the lymphoma inhibitory factor in MCS was arginase was first obtained after finding that arginine supplementation was protective. Thus, when L1210 lymphoma cells were cultured in medium containing 50% of dialyzed MCS, their proliferation was inhibited and the cell number actually declined to less than the starting number when examined at 48 hr of culture, as shown in Table I. The inhibition was readily reversed by adding arginine or citrulline at a concentration of $1.4 \times 10^{-3}$ *M*, but not by adding their metabolic precursors, carbamyl phosphate or ornithine up to 0.1 *M*. The concentration of arginine in medium is $2 \times 10^{-4}$ *M* normally. To elucidate the nature of the arginine-depleting factor, [*guanido*-$^{14}$C]-labeled and nonlabeled arginine were incubated with MCS for 18 hr and the mixture was analyzed by two-dimensional electrophoresis–liquid chromatography as described. On the autoradiograph only two spots were found. These were identified as urea and arginine by comparison with radiolabeled standards run in an identical manner, and by the known $R_f$ values of the compounds (urea 0.5, neutral; arginine, 0.15, basic) (5). No citrulline was present (0.18, neutral). Using [U-$^{14}$C]arginine as substrate, ornithine (0.12, basic but with different mobility) was identified as a reaction product. These suggested that arginine in the medium was decomposed by arginase (arginine amidinohydrolase, EC 3.5.3.1.).

*Depletion of arginine.* The arginase activity in the dialyzed MCS was further studied by following the decomposition of arginine during 16 hr of incubation. For quantitative measurements of arginine, [*guanido*-$^{14}$C]arginine was used and elec-

TABLE I. INHIBITION AND REVERSAL OF LYMPHOMA CELL PROLIFERATION IN CULTURE BY VARIOUS REAGENTS

| Reagents | Viable cell count after 48 hr ($\times 10^5$/ml) |
|---|---|
| Control[a] | 10.1[c] |
| DM-MCS[b] | 0.5 |
| DM-MCS + arginine (250 mg/liter) | 9.1 |
| DM-MCS + citrulline (250 mg/liter) | 9.4 |
| DM-MCS + carbamyl phosphate and/or ornithine ($10^{-5}$–$10^{-1}$ *M*) | 1.0 |

[a] L1210A Mouse lymphoma cells, $2 \times 10^5$, were cultured in 1 ml of Dulbecco's medium with 10% FCS.
[b] Containing 50% of Dulbecco's medium-dialyzed culture supernatant of $10 \times 10^6$/ml mouse peritoneal exudate cells.
[c] Mean of triplicate. Variation all within ±20%.

trophoresis was employed to separate urea from arginine. As shown in Fig. 1, MCS from $10^7$ cells/ml hydrolyzed arginine in the medium following nearly first-order kinetics. At 16 hr less than 3 mg/liter of arginine ($0.17 \times 10^{-4}$ *M*) remained, from an initial concentration of 30 mg/liter ($1.7 \times 10^{-4}$ *M*). Supernatants from macrophage cultures of lower densities showed proportionally less activity.

*Arginine requirement of L1210 cells.* The concentration of arginine required to sustain the mouse L1210 lymphoma cells in culture was examined by inoculating $2 \times 10^5$ cells/ml initially in arginine-free Dulbecco's medium in which different amounts of arginine and 10% saline-dialyzed FCS were added. As shown in Fig. 2, the cells did not grow with less than 15 mg/liter of arginine, and below 3 mg/liter the cells were actually killed. The arginase in MCS would, therefore, be sufficient in itself to inhibit lymphoma cells *in vitro* after a few hours of incubation.

*Gel filtration of MCS.* MCS was passed through a column of Sephadex G-150 and the emerging fractions were tested for arginase activity. As shown in Fig. 3, activity was found in the region corresponding to a molecular weight of approximately 110,000. This was the same as that of the inhibitor previously reported (1), and was close to that reported for rat liver arginase (118,000) (6) but different from that of mouse liver

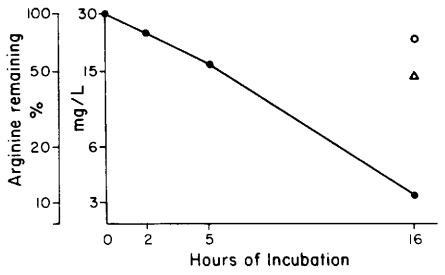


FIG. 1. Conversion of arginine by arginase in mouse macrophage culture supernatant. Cell-free supernatants from cultures of $10 \times 10^6$ (●), $5 \times 10^6$ (△), and $1 \times 10^6$ (○) macrophages were dialyzed against arginine-free medium and incubated with 1 μCi of [$^{14}$C]arginine and 30 mg/liter of unlabeled arginine. Urea and arginine were separated by electrophoresis (see text) and the amount of arginine remaining (in mg/liter and % of original concentration) was determined.

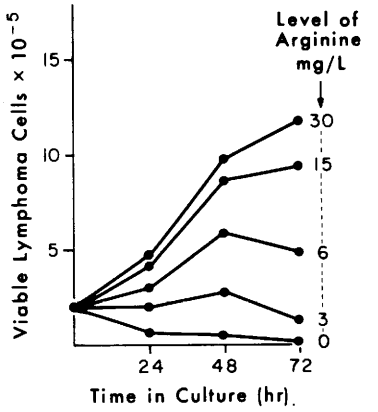


FIG. 2. Effect of arginine level on proliferation of L1210A lymphoma cells in culture. Cells, $2 \times 10^5$, were inoculated in 1 ml of Dulbecco's medium containing different amounts of arginine (in mg/liter) and 10% saline-dialyzed FCS. The viable cells were counted at various hours of incubation.

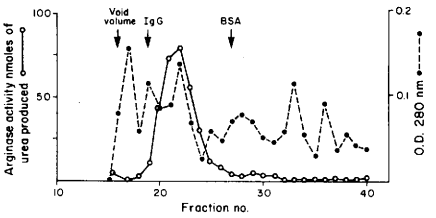


FIG. 3. Sephadex G-150 elution profile of macrophage culture supernatant assayed for arginase activity. Five milliliters of MCS was passed through the Sephadex column and eluted with 1 m*M* phosphate-buffered saline, pH 7.2. The individual fractions were tested for the arginase activity by urea production (see under Materials and methods). Human IgG (MW 150,000) and bovine serum albumin (BSA 68,000) were used to calibrate the column.

arginase (137,000) (7). Mouse peritoneal cells, $1 \times 10^7$, yielded 2.5 IU of arginase.

*The properties of arginase in MCS.* The arginase in the peak fraction from Sephadex filtration of MCS had a $K_m$ of $1.0 \times 10^{-2}$ *M*, as shown in Fig. 4. Additional representative experiments examining the properties of the macrophage arginase are shown in Table II and summarized in Table III. The enzyme has an absolute requirement for metal ions, and $Mn^{2+}$ is the most active. The pH optimum was 9.5 for $Mn^{2+}$-activated enzyme. Ornithine was a potent inhibitor, while canavanine was a substrate but was hydrolyzed at only one-sixth the rate of arginine. Argininic acid and γ-guanidinobutyric acid were not hydrolyzed. These properties are similar to those of the extensively studied bovine liver arginase (6).

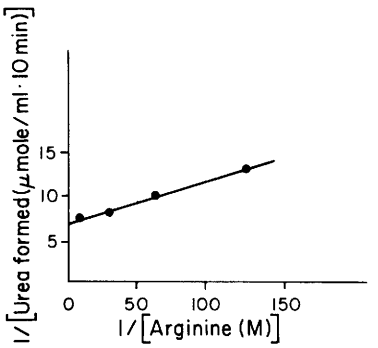


FIG. 4. The Lineweaver–Burk plots of arginase activity from mouse MCS. The arginase fraction was preactivated at 55° with 1 m*M* $Mn^{2+}$ and 1% BSA in 0.04 *M* Tris buffer, pH 7.2, for 15 min. Aliquots were then mixed with arginine and 0.3 *M* glycine–NaOH, pH 9.5, and reacted for 10 min.

*Arginase activity in mouse peritoneal fluid.* To test whether arginase was also release under *in vivo* conditions, the peritoneal cavities of several mice were washed with 3 ml of saline 4 days after injection of thioglycollate medium or saline. The washing fluid was withdrawn and immediately centrifuged at 2000 rpm for 20 min, and the supernatant was tested for anginase activity. As shown in Table IV, the thioglycollate medium-treated mice, containing more macrophages, showed 0.12–0.23 unit/ml of activity in the fluid, while the control mice had no measurable activity. Taken together with the *in vitro* data, it is possible that macrophages can release arginase *in vivo*.

*Discussion.* From the data which have been presented, it is clear that the inhibition of the growth of lymphoma cells by macrophage supernatants (1) can be fully explained by their containing arginase. Medium is depleted of arginine to levels which are demonstrably insufficient for the growth of these cells. Ornithine, the product of arginase action, cannot be utilized by these cells for growth. Arginine depletion has been shown to inhibit other lymphoma cells *in vitro* (8) as a result of the action of deiminase, an enzyme product in mycoplasma and other sources. Citrulline, which is product of this enzyme, can be utilized efficiently by some but not by other lymphoma cells (8) and can replace arginine. In the present experiment arginine was hydrolyzed to urea and ornithine only, as expected from the action of arginase. The deiminase pathway, hence contamination of mycoplasma, as the cause of arginine depletion can be excluded.

The properties of macrophage arginase as shown in Table III are similar to those of liver arginase found in numerous species. The molecular weight of 110,000 estimated by gel filtration here is near those reported for liver arginase (110,000–138,000) (7). The $K_m$ value of $1.0 \times 10^{-2}$ *M* is also within the reported range ($6 \times 10^{-3}$–$2 \times 10^{-2}$ *M*) (6). The concentration of arginine in the

TABLE II. FACTORS AFFECTING MACROPHAGE ARGINASE ACTIVITY

| | Effect of metal ions[a] | | | | | |
|---|---|---|---|---|---|---|
| | $M^{2+}$ | $Co^{2+}$ | $Ni^{2+}$ | $Zn^{2+}$ | $Fe^{2+}$ | Na,K only |
| Arginase activity[b] | 0.65 | 0.22 | 0.18 | 0.11 | 0.09 | 0.09 |
| | Effect of pH[c] | | | | | |
| | 7.5 | 8.5 | 9.0 | 9.5 | 10.0 | 10.7 |
| Arginase activity[b] | 0.16 | 0.33 | 0.38 | 0.66 | 0.46 | 0.38 |

| | Specificity and inhibitor[d] | | | | |
|---|---|---|---|---|---|
| | Arginine | Arginine + Ornithine | Canavaline | Argininic acid | γ-Guanidinobutyric acid |
| Arginase activity[b] | 0.70 | 0.05 | 0.12 | <0.01 | <0.01 |

[a] The arginase fractions, 0.1 ml, from gel filtrate of MCS were activated with 1.25 m*M* of various salts in 0.01 *M* Tris buffer, pH 7.0, and 1.25% BSA, then incubated with 0.285 *M* arginine, pH 9.5. See text for detail.

[b] Micromoles of urea produced in 10 min.

[c] Sample was activated with 1.25 m*M* $MnCl_2$ as above and incubated with 0.285 *M* arginine adjusted to various pH with HCl.

[d] The $Mn^{2+}$-activated arginase fractions were incubated with 0.2 *M* concentrations of each substrate at pH 9.5.

medium ($4 \times 10^{-4}$ *M*) is much lower than the $K_m$ of arginase, which theoretically would result in a first-order kinetics for arginine decomposition, as shown in Fig. 1. Other properties such as pH optimum, requirement for $Mn^{2+}$ ions, and inhibitor and substrate specificities resemble those of bovine liver arginase (9). Although arginase was released by macrophages, the conditions in the culture were far from optimal for its action. The medium was maintained at neutral pH, the concentration of arginine was low, no exogenous $Mn^{2+}$ was added, and various amino acids present were probably inhibitors of arginase (10).

As suggested by Kung *et al.* (2), the synthesis of arginase might be a sign of macrophage activation, although the role of this enzyme in the function of macrophage is still unclear. An immunosuppressive activity could be postulated. The finding that arginase was present in the washing fluid of mouse peritoneal cavity after induction of macrophages by thioglycollate medium injection suggested that arginase is released *in vivo*. The detection of arginase activity in the washing fluid was facilitated by our modified assay method, in which albumin was added to activate and stabilize arginase in the dilute solution. One possible function of arginase is to suppress the growth of extracellular or intracellular (but intralysosomal) microorganisms by arginine deprivation. Conversely, some pathological effect of inflammation might be due to the local accumulation of arginase.

Since macrophage can be easily cultivated *in vitro*, the regulation of arginase synthesis and its release can now be studied.

TABLE III. SOME PROPERTIES OF MOUSE MACROPHAGE ARGINASE

| | |
|---|---|
| 1. | MW 110,000 (by gel filtration) |
| 2. | $K_m$ $1.0 \times 10^{-2}$ *M* (at pH 9.5 with $Mn^{2+}$) |
| 3. | Activation by cations<br>$Mn^{2+} >> Co^{2+} > Ni^{2+} > Zn^{2+} > Fe^{2+} > Na^+$ or $K^+$ |
| 4. | pH optimum 9.5 (with $Mn^{2+}$) |
| 5. | Inhibitor—ornithine |
| 6. | Substrate specificity: canavanine—one-sixth the rate of arginine;<br>Arginic acid and γ-guanidinobutyric acid—no activity |

TABLE IV. ARGINASE ACTIVITY IN PERITONEAL WASHING FLUID

| Mice injected ip | Peritoneal cells/mouse | Arginase activity[a] (unit/ml of washing fluid) |
|---|---|---|
| Thioglycollate medium | $6 \times 10^6$, over 90% activated macrophages | 0.12–0.23 |
| Saline | $1.5 \times 10^6$, 40% nonactivated macrophages | <0.01 |

[a] The peritoneal cavities of six mice in each group were washed with 3 ml of saline. The fluid was withdrawn and immediately centrifuged to remove the cells.

*Summary.* Mouse peritoneal macrophages in culture released a macromolecular factor capable of inhibiting mouse L1210 lymphoma cells *in vitro*. The factor was proved to be arginase by several criteria. The inhibition was reversed by adding arginine or citrulline to culture, the arginine in the culture medium was consumed, and the guanido group of arginine was hydrolyzed exclusively to urea as demonstrated by electrophoresis and chromatography. The factor has a molecular weight of 110,000 and a $K_m$ value of $1.0 \times 10^{-2}$ *M*. These and other parameters, such as a requirement for $Mn^{2+}$, alkaline pH optimum, substrate specificities, and inhibition by ornithine, were similar to those of liver arginases. The arginine in the medium was decomposed by first-order kinetics, and the arginase activity in the supernatant of $10 \times 10^6$ peritoneal exudate cells/ml was enough to deplete arginine in the medium to below the level required for L1210 cells to grow in culture.

Arginase activity was also detected in the peritoneal washing fluid 4 days after thioglycollate medium stimulation, suggesting *in vivo* release of the enzyme.

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