SUPPRESSION OF IN VITRO CYTOTOXIC RESPONSE BY MACROPHAGES DUE TO INDUCED ARGINASE*

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Excess numbers of macrophages are known to inhibit primary antibody production in vitro (1-3), mitogen-induced polyclonal B- or T-lymphocyte responses (4-6), and mixed lymphocyte reactions (7). The mechanism of this nonspecific immunosuppression is unclear although soluble suppressive factors produced by macrophages and lymphocytes have been reported by numerous investigators (6, 8-11). During an investigation of the role of medium depletion in macrophage-induced suppression we determined that arginine was not detectable in supernates of suppressed cultures and that arginase was induced in the macrophages during their in vitro culture.

Materials and Methods

Mice. Male C57BL/6 (H-2b), DBA/2 (H-2b), and B6D2F1, mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. BALB/c and CD1F1 mice were obtained from The American Medical Center in Denver, Colo. All mice used for experiments were between 2 and 4 mo of age.

Medium. In all experiments, except where specifically indicated, the medium consisted of Eagle's minimum essential medium (MEM) containing glucose, salts, vitamins, bicarbonate, and essential amino acids. To this was added Eagle's nonessential amino acids, 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U penicillin and 100 μg streptomycin/ml, and 10⁻⁴ M 2-mercaptoethanol (2-ME) (this was referred to as Med + 10% FCS). All media components, except 2-ME, were purchased from Grand Island Biological Co., Grand Island, N. Y. 2-ME was purchased from the Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.

Cell Suspensions. Spleens were removed aseptically and passed through stainless steel screens into Med to obtain single cell suspensions. Peritoneal cells were obtained by washing the peritoneal cavities of normal mice with Med. All cells were washed three times in Med and then resuspended in Med + 10% FCS before use.

Supplemental Feedings. 200 times stock solutions of arginine (100 mM), 100 times stock solutions of glucose (100 mg/ml), and 2-ME (10⁻² M) were made up in triple distilled water. A 100 times vitamin solution was used as purchased. 10 μl of each stock solution were added as described.

In Vitro Generation of Cytotoxic Cells. Either DBA/2 or B6D2F1 peritoneal cells served as stimulator cells while C57BL/6 spleen cells were used as responding cells. Mixtures of 5 x 10⁴-10⁶ stimulating cells and 2 x 10⁶ responding cells in 1 ml of Med + 10% FCS were cultured in Falcon

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Abbreviations used in this paper: FCS, fetal calf serum; GVH, graft vs. host; 2-ME, 2-mercaptoethanol; Med + 10% FCS, Eagle's minimum essential medium + 10% FCS; PC, peritoneal cells.
multiwell tissue culture plates (no. 3008; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). Cell cultures were maintained at 37°C in a gas mixture of 8.5% O₂, 79.5% N₂, and 12% CO₂ and rocked continuously on a Belco rocking platform (Belco Glass, Inc., Vineland, N. J.) for 5 days. At the end of this time, cells were harvested for cytotoxic assays.

**5₁²Cr Label of P-815 (H-2d) Target Cells.** P-815 mastocytoma cells were grown in vitro in Dulbecco's medium + 10% FCS. Occasionally, P-815 cells were passed in the peritoneal cavity of DBA/2 mice. 5 x 10⁶ P-815 cells were washed, suspended in 1 ml of serum-free Med, and 100 μCi Na₂¹⁵CrO₄ added. The mixture was incubated at 37°C for 1 h. The labeled P-815 target cells were then washed three times with Med + 10% FCS at 10⁶ cells/ml.

**Assay of Cell-Mediated Cytotoxicity.** At the end of the 5-day culture period, cells from duplicate or triplicate cultures were pooled, centrifuged, and resuspended in one-third the original volume of Med + 10% FCS. 0.1-ml aliquots of cultured cells were pipetted into each of two wells of a Micro Test II Tissue Culture Plate (no. 3040; Falcon Plastics, Div. of BioQuest) and 0.1 ml (10⁵) of Na₂¹⁵Cr-labeled P-815 cells added to each well. The cell mixtures were incubated at 37°C in 8.5% O₂, 79.5% N₂, and 12% CO₂ for 4 h. At completion of this time 0.1-ml aliquots of each supernate was carefully removed for Na₂¹⁵Cr counting. Maximum release (100% lysis) was measured by mixing 0.1 ml of target cells and 0.9 ml of distilled water for 4 h at 37°C. The lysed cells were centrifuged and a 0.5-ml aliquot of the supernate counted. Spontaneous release (0% lysis) was measured by counting 0.1 ml of the supernate of a mixture of 0.1 ml target cells and 0.1 ml of Med + 10% FCS which had been incubated for 4 h.

**Calculation of Percent Lysis of Target Cells.** Cytotoxic activity was expressed as percent lysis of P-815 target cells.

\[
\% \text{ Lysis} = \frac{\text{cpm experimental release} - \text{cpm spontaneous release}}{\text{cpm maximum release} - \text{cpm spontaneous release}} \times 100.
\]

**Measurement of Arginase.** Cells were washed with 0.9% saline and lysed with 0.5 ml distilled water per 10⁶ cells for 20 min at room temperature. Arginase was activated by addition of 1.2 ml of 0.2 mM MnCl₂ in 0.04 M Tris-Cl and 0.9% saline (pH 7.2) to 0.5 ml lysate followed by incubation at 55°C for 1 h.

Aliquots of activated lysate diluted to a vol of 0.9 ml with distilled water were mixed with 0.1 ml of a pH 9.4 solution of 0.4 M arginine (Eastman Kodak Co., Rochester, N. Y.) containing 0.15 M glycine and incubated at 37°C for 15 min. The reaction was terminated by placing the tubes in a boiling water bath for 7 min.

A modification of the method of Geyer and Dabich (12) was used to measure urea content. The color reagent was a stock aqueous solution of 2.4 mM thiosemicarbazide and 41.1 mM 2,3-butanedione monoxime (both from Sigma Chemical Co., St. Louis, Mo.) and the acid reagent was a freshly prepared mixture of 0.1 ml of 0.12 M FeC₅ in 56.7% phosphoric acid and 100 ml of 20% H₂SO₄. Aliquots of the assay mixtures were made up to 1.0 ml with distilled water. 1.0 ml of the color reagent and 2.0 ml of the acid reagent were added, the tubes were mixed, capped, and placed in a water bath at 95°C for 23 min. The tubes were cooled and absorbancy at 520 nm in a Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) was determined. All results were corrected for blanks containing cells without arginine and arginine without cells. The latter produced significant color and reduced the overall sensitivity of the method to approximately 10 nm urea/min. At this level the amount of urea produced was proportional to cell number.

**Measurement of Amino Acids.** Amino acids were analyzed on a Beckman Model 120 automatic amino acid analyzer (courtesy of Virginia Sweeney and Dr. John Stewart, Department of Biochemistry, University of Colorado Medical Center, Denver, Colo.). 10% sulfosalicylic acid was added to Med + FCS to precipitate proteins, which were removed by centrifugation.

**Thioglycollate Suspension.** Powdered thioglycollate medium purchased from Baltimore Biological Laboratories, Baltimore, Md., was suspended in 33 parts of distilled water. 3 ml of this 3% suspension was injected intraperitoneally (i.p.) into each mouse.

**Results**

**Suppression by Stimulator or Responder Peritoneal Cells (PC).** Table I presents the results of two experiments showing that excess numbers of PC of either stimulator or responder strain cause complete suppression of the lytic
response of spleen cells. The PC were as effective when added on day 1 as when added on day 0. 300,000 PC caused marked suppression and one million PC produced complete suppression.

Amino Acid Concentration in Active and Suppressed Cultures. Multiple cultures were prepared containing $2 \times 10^6$ C57BL/6 spleen cells and either $5 \times 10^4$ or $5 \times 10^5$ B6D2F1 PC. After 5 days the original medium and supernates of the two types of cultures were examined in an amino acid analyzer. Cells from identical cultures were pooled and lysis of P-815 measured. Results are shown in Table I.

Cells from cultures receiving $5 \times 10^4$ PC gave 47% lysis of $^{51}$Cr-P-815. Cells from cultures receiving $5 \times 10^6$ PC gave 4% lysis. The concentrations of alanine, glutamic acid, and glycine were elevated in both cultures compared to the original medium; glutamine was approximately 30% depleted in both cultures but arginine was undetectable in the suppressed culture. The level of ornithine plus lysine increased sharply. Other amino acids were unchanged or slightly elevated. In a second experiment a supplement of $1 \mu M$ (10 $\mu l$ of 100 mM) arginine was added to each culture increasing the initial concentration from 0.5 to 1.5 mM. Cultures containing $10^6$ PC were completely suppressed despite the extra arginine and arginine was not detectable in the medium at the end of these 5-day cultures.

Arginase Levels in Spleen Cells and in Adherent PC. Arginase was measured in fresh adherent PC and in adherent PC obtained 1-3 days after the i.p. injection of thioglycollate. In both cases the PC were cultured for 1 h in Med + FCS and the adherent cells washed twice with Med. The PC were then lysed

| Exp. | Stimulator PC $\times 10^5$ added | Responder PC $\times 10^5$ added | % Lysis $^{51}$Cr-labeled P-815 |
|------|----------------------------------|----------------------------------|----------------------------------|
| I    | 1                                | 1                                | 106, 95                          |
|      | 1                                | 10                               | 5, 1                             |
|      | 1                                | 10                               | 6, 1                             |
|      | 1                                | 10                               | 0, 0                             |
|      | 10                               | 1                                | 1, 0                             |
|      | 10                               | 10                               | 4, 2                             |
| II   | 0.5                              | 1                                | 77                               |
|      | 0.5                              | 10                               | 51                               |
|      | 0.5                              | 3                                | 11                               |
|      | 0.5                              | 10                               | 1                                |
|      | 10                               | 10                               | 5                                |

* $2 \times 10^6$ C57BL/6 spleen cells cultured 5 days from day 0.
† Stimulator PC were DBA/2 in exp. I and B6D2F1 in exp. II; responder PC were C57BL/6.
§ Set up in quadruplicate in exp. I and duplicate in exp. II; duplicates pooled for assay.
TABLE II

Amino Acid Concentration in Culture Medium

| Amino acid* | Original medium | Active† culture | Suppressed§ culture |
|-------------|-----------------|-----------------|--------------------|
| Alanine     | 30              | 53              | 96                 |
| Glutamic acid | 19           | 49              | 60                 |
| Glycine     | 20              | 30              | 34                 |
| Arginine    | 55              | 14              | 0                  |
| Glutamine   | 186             | 132             | 120                |
| Lysine + ornithine | 38 | 76 | 116 |

* The other amino acids were either unchanged or moderately elevated after culture. Results expressed as moles per liter × 10⁵.
† The active culture contained 2 × 10⁶ C57BL/6 spleen cells and 5 × 10⁴ B6D2F₁ peritoneal cells. After 5 days the cells produced 47% lysis of ⁵¹Cr-labeled P-815.
§ The suppressed culture contained 2 × 10⁶ C57BL/6 spleen cells and 5 × 10⁵ B6D2F₁ peritoneal cells. After 5 days the cells produced 4% lysis of ⁵¹Cr-labeled P-815.

with distilled water and the arginase levels determined as described above. Similarly arginase was measured in resident PC which had been incubated for 1 or 2 days in Med, in Med + FCS, and in arginine-free Med + FCS. In the latter case the FCS was first dialyzed against three daily changes of 40 volumes of balanced salt solution. Arginase levels were also assayed on spleen cells obtained from a normal CD1F₁ mouse and from CD1F₁ mice undergoing graft vs. host (GVH) reactions 1-3 wk after the injection of 10⁸ BALB/c spleen cells. Results are given in Table III. Fresh adherent resident PC, normal spleen cells, and PC cultured 2 days in Med all contained undetectable amounts of arginase (<10 nmol urea produced/min/million cells). Arginase levels were strikingly elevated in thioglycollate PC, in spleen cells of one F₁ mouse 3 wk after injection of parental spleen cells, and in PC cultured in Med + FCS with or without arginine. These results were confirmed in subsequent experiments with the exception of those on the spleen cells of mice undergoing a GVH reaction.

Reversal of Macrophage-Induced Suppression by Arginine. Repeated attempts to reverse macrophage-induced suppression by direct daily addition of arginine to cultures were unsuccessful. Partial reversal occurred if the added daily cocktail included glucose and 2-ME. Results of such an experiment are given in Table IV.

The results shown in Table IV suggested that arginine feeding produced either secondary nutritional depletion or the accumulation of toxic products. Consequently, an experiment was performed in which the nonadherent cells of some cultures were transferred to new wells with their culture medium followed by the addition of various nutrients. Results are shown in Table V. Complete reversal of suppression occurred when arginine and glucose were added to the nonadherent cells after their transfer at 62 h. Nearly complete reversal was seen after the addition of arginine alone.

Discussion

The data presented show that the presence of excessive numbers of peritoneal macrophages of either donor or responder type block the immune response of
Arginase Levels in PC and Spleen Cells

| Source of cells                        | First experiment | Subsequent experiment |
|---------------------------------------|------------------|-----------------------|
|                                       | nmoles urea/min/10^6 cells* |                       |
| Fresh adherent PC                     | <10              | <10                   |
| Adherent PC cultured 1 day in Med + FCS | 109              | 73                    |
| Adherent PC cultured 2 days in Med + FCS | 65               | 52-109                |
| Adherent PC cultured 1 day in Med-Arg + FCS‡ | 119              |                       |
| Adherent PC cultured 2 days in Med    | <10              | <10                   |
| Adherent PC from thioglycollate injection Removed after 1 day | 44               |                       |
|                                        | 2 days           | 219                   |
|                                        | 3 days           | 70                    |
| Normal spleen cells                   | <10              | <10                   |
| Spleen cells from GVH reaction        | 164‡             | <10                   |

* PC were counted before removal of nonadherent cells. Thus, the PC values are low by a factor of approximately 2.
† FCS in this case only was dialyzed against three daily changes of a balanced salt solution.
§ The spleen of this F1 mouse taken 3 wk after injection of parental spleen cells was unusually large. Subsequent experiments with three spleens taken from 1 to 3 wk after injection failed to show a detectable level of arginase.

Failure to Reverse Macrophage-Mediated Suppression by Direct Daily Addition of Arginine

| Cells added x 10^-5 | DBA/2 PC C57BL/6 spleen cells | Daily supplement* | % Lysis |
|---------------------|--------------------------------|-------------------|--------|
|                     | C57BL/6 PC                     |                   |        |
| 0.5                 | 20                             | Arg, Glu, 2-ME    | 69     |
| 0.5                 | 20                             | Arg, Glu, 2-ME    | 67     |
| 0.5                 | 20                             | Arg + Glu         | 11     |
| 0.5                 | 20                             | Arg + Glu + 2-ME  | 19     |

* 10 μl of each indicated supplement were added on days 2, 3, and 4. Arg was 200 × arginine (0.1 M), Glu was 100 × glucose (100 mg/ml), and 2-ME was 10^-2 M.

spleen cells in culture through a depletion of arginine. The mechanism of this depletion was found in the marked increase in the enzyme arginase in adherent macrophages. The level of arginase in resident peritoneal macrophages was initially low but rapidly increased within 24 h of cultures in Med + 10% FCS. Increased levels of enzyme were also found in thioglycollate-activated peritoneal cells and in one spleen activated by a GVH reaction. Thus, the synthesis of the enzyme by macrophages may be a result and a sign of their activation, but since
TABLE V

Reversal of Macrophage-Induced Suppression by Addition of Arginine After Transfer of Nonadherent Cells

| DBA/2 PC added × 10^5* | Transfer of nonadherent cells† | Additions at time of transfer§ | % Lysis |
|------------------------|-------------------------------|-------------------------------|--------|
| 0.5                    | No                            | None                          | 82     |
| 0.5                    | Yes                           | None                          | 83     |
| 7                      | No                            | None                          | 2      |
| 7                      | Yes                           | None                          | 2      |
| 7                      | Yes                           | Glu                           | 3      |
| 7                      | Yes                           | Arg                           | 66     |
| 7                      | Yes                           | Arg + Vit Mix                 | 61     |
| 7                      | Yes                           | Arg + Glu                     | 85     |
| 7                      | Yes                           | Arg + 2-ME                    | 63     |
| None                   | Yes                           | None                          | 14     |

* 2 × 10^5 C57BL/6 spleen cells were incubated for a total of 132 h with indicated numbers of DBA/2 PC.
† Nonadherent cells with their medium were transferred to a clean well at 62 h. Adherent cells were discarded.
§ 10 μl of each supplement were added to cultures at 62 h as indicated except that 10 μl of arginine (0.1 M) were added at 62 and 86 h.

activation appears to occur spontaneously in vitro, macrophages of both donor and recipient strains have a similar effect.

Arginase has been reported in FCS (13) and may account for part of the drop in arginine levels in active cultures (Table II). Arginase levels increased slightly (approximately twofold) in the supernates of 5-day cultures containing 10^6 PC/ml (data not shown). The significance of these findings has not been determined because of unresolved background problems associated with measuring arginase in medium containing relatively high concentrations of protein and urea. This problem was not encountered in measuring arginase in washed cells.

Arginine is not an essential amino acid for adult animals since it is resynthesized in the urea cycle, but arginine has been found to be essential for most in vitro cell cultures (14). The failure to readily reverse the effect of arginine depletion by daily addition of the amino acid may be an indication of the rapidity of arginine breakdown. It may also be due to secondary effects of this breakdown or other effects of actively metabolizing cells. In any case the first and most striking defect in the medium produced by macrophages was depletion of arginine since the response of spleen cells could be almost completely restored by the addition of arginine alone if the nonadherent cells and medium were removed from the adherent cells after 2.5 days of culture.

There have been a number of reports of soluble suppressive factors produced by macrophages or by cultures containing macrophages (6, 8-11). One low molecular weight factor which inhibits labeled thymidine uptake appears to be thymidine (8). It is conceivable that the high molecular weight factors observed in some experiments may have been arginase. Ham has noted that numerous suppressors of cell growth in vitro have been later identified as arginase (15).

We are not aware of previous reports of high concentrations of arginase in macrophages. The enzyme is normally present in the liver where it plays an
essential role in urea formation. Its function in macrophages is obscure but may be to provide a source of ornithine.

The findings of arginase in activated macrophages may be no more than an interesting problem of in vitro culture. It may also lead to a simple method of studying macrophage activation. However, we cannot dismiss the possibility that arginase may play a role in vivo in immunosuppression by activated macrophages. The failure to reproduce the finding of a striking elevation of arginase in one enlarged spleen is puzzling. Macrophages have been found in large numbers in the spleens of animals undergoing GVH reactions (16) but local accumulations of high arginase levels in vivo may be unstable because of feedback suppression (17–19). Eccles and Alexander have noted a correlation between the number of macrophages in a tumor and the degree of accompanying immunosuppression (20). The injection of another enzyme, asparaginase, has been shown to produce immunosuppression in vivo (21, 22).

Summary

Arginine was found to be completely depleted from cell-free supernates of mixed leukocyte cultures suppressed by the addition of excess macrophages. Partial reversal of macrophage-mediated suppression was accomplished by daily addition of a cocktail containing arginine and nonamino acid nutrients. Complete reversal of the suppression was accomplished by the addition of arginine and glucose to the medium and the nonadherent cells after their separation from the adherent macrophages. A marked increase in the enzyme arginase was found in macrophages that had been cultured 24 h in vitro in Eagle's minimum essential medium plus 10% fetal calf serum, in peritoneal cells activated by prior injection of thioglycollate, and in one spleen activated by a graft vs. host reaction.

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