REGULATION OF MACROPHAGE FUNCTIONS
BY L-ARGININE

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It has been shown that the extracellular space of wounds contains an exceedingly low concentration of arginine (<0.05 mM), attributable to the activity of macrophage-derived arginase (1). In this regard, wounds are similar to tumors and other sites of inflammation with prominent macrophage infiltration (2, 3). A physiological role for macrophage arginase in inflammation and immunity has been proposed from results showing that arginine deprivation inhibits the replication of tumor cells, parasites, and viruses and supresses cellular immune functions in vitro (reviewed in reference 4). Because macrophages present in sites of inflammation must also function in conditions of reduced extracellular arginine availability, the impact of changes in environmental arginine concentration on macrophage physiology was investigated. Evidence to be presented will show that tumoricidal activity, superoxide production, phagocytosis, and protein synthesis by rat resident peritoneal macrophages are enhanced by culture in media containing <0.1 mM L-arginine. In contrast to resident macrophages, the tumoricidal activity of peritoneal macrophages collected from rats injected with Corynebacterium parvum was depressed by culture in L-arginine-deficient media. At the same time, however, C. parvum-elicited macrophages, like resident macrophages, exhibited increased superoxide production, phagocytosis and protein synthesis after incubation in L-arginine-deficient media. These results suggest that changes in the concentration of L-arginine at sites of inflammation can up- or downregulate macrophage functions depending on their prior state of activation.

Materials and Methods

Cell Culture. Male Fischer rats (150-175 g) (VAF-plus; Charles River Breeding Laboratories, Wilmington, MA) were used in all experiments. VAF-plus rats are certified free of common rat pathogens by the supplier and are housed in an isolation environment upon their arrival at the laboratory. Peritoneal cells from normal rats were obtained by sterile lavage using HBSS (Gibco Laboratories, Grand Island, NY) supplemented with 1% heat-inactivated FCS (HyClone Laboratories, Logan, UT), 10 mM 3-(N-morpholino)-propanesulfonic acid (MOPS) and antibiotics. Peritoneal cells were also harvested in identical fashion 5 d after the intraperitoneal injection of 2 mg Corynebacterium parvum (Wellcome Laboratories, Research Triangle Park, NC). The cells were washed, resuspended in culture media consisting of RPMI 1640 without L-arginine (Select Amine Kit, Gibco Laboratories), 10% FCS, 10 mM MOPS,
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5 x 10^{-5} M 2-ME, and antibiotics and plated (3 x 10^5 cells in 100 µl) in quadruplicate in flat-bottomed 96-well culture plates (No. 3598, Costar, Cambridge, MA). Where so indicated, dialyzed FCS containing undetectable free l-arginine was substituted for FCS. Nonadherent cells were removed by repeated washing after 2 h incubation at 37°C in 7% CO2 in air and culture media (200 µl) supplemented or not with l-arginine or other additives was added. Adherent cells comprised 76 ± 5% of the plated peritoneal cells and were >95% macrophages by Wright and nonspecific esterase staining or phagocytosis of opsonized SRBC. All functional assays were performed after overnight incubation. Viability after overnight incubation in media with or without l-arginine was determined by lactic dehydrogenase release (5) or trypan blue exclusion and exceeded 95%. All media and additives contained <3 pg/ml endotoxin, as assayed by a chromogenic assay (QCL 1000; Wittaker M. A. Bioproducts, Walkersville, MD). Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless indicated.

51Cr-release Assay. Macrophage tumor cytotoxicity was measured in a 51Cr-release assay as described previously (6). The macrophage-sensitive P815 mastocytoma used was obtained from Dr. Carl Nathan (The Rockefeller University, New York, NY) (7). For the determination of cytotoxicity, 100 µl of medium were removed from the wells and 5 x 10^5 51Cr-labeled P815 cells were added in 100 µl of culture medium. The plates were centrifuged and incubated for 6 h at 37°C. Supernatants (50 µl) were then harvested and counted. Cytotoxicity was calculated using the formula: Percent specific 51Cr release = 100 x [(experimental cpm - spontaneous cpm)/(total cpm - spontaneous cpm)]. Spontaneous 51Cr release averaged 8% of the total release (1% Triton X-100).

Superoxide Production. Superoxide production was measured by the method of Pick and Mizel (8). Briefly, after removal of the culture supernatants, 100 µl of a 160 µM solution of ferricytochrome c in phenol red-free HBSS were added to the wells. Superoxide production was stimulated with 200 nM PMA (L. C. Services, Co., Woburn, MA). Additional control wells also received 30 U of superoxide dismutase. The plates were incubated at 37°C for 60 min and the absorbance at 550 nm measured in a Multiskan MC plate reader (Flow Laboratories, McLean, VA). The quantity of superoxide produced was calculated using the equation \( \Delta A_{550\text{nm}} = 2.1 \times 10^4 \text{M}^{-1} \times \text{cm}^{-1} \).

Phagocytosis. Phagocytosis of fluorescent latex beads (2 µm, Fluoresbrite, Polysciences Inc., Warrington, PA) was measured as described by Oda and Maeda (9). The beads were opsonized with normal rat serum and added to the cells at a ratio of 50 particles per macrophage. The plates were centrifuged at 300 g for 3 min and incubated at 37°C for 30 min. Noningested beads were removed by repeated washing, the cells were lysed with 1% Triton X-100, and the fluorescence of the supernatant was measured in a fluorescence spectrophotometer. Nonspecific binding of beads by macrophages incubated at 4°C was subtracted. The number of phagocytized particles was calculated from the relationship between fluorescence and number of particles in a standard particle dispersion and the number of plated cells.

Protein Synthesis. Protein synthesis was calculated from the incorporation of U-[14C]phenylalanine (0.1 µCi/well; Dupont-NEN, Wilmington, DE) into TCA-precipitable material over 6 h and the specific activity of phenylalanine in the culture media.

Wound Fluid and Serum. Sterile circular polyvinyl alcohol (PVA) sponges (Unipoint Industries, Inc., Highpoint, NC) measuring ~1 cm in diameter and 0.4 cm in thickness were inserted subcutaneously in the dorsum of animals anesthetized with 50 mg/kg pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL). Each animal received 10 sponges. The implants were removed under sterile conditions after 7 d. The fluid contained in the sponges was obtained by centrifugation and the cell free supernatants were pooled, 0.45 µm filtered, and stored at −80°C. This fluid contained no detectable arginine or endotoxin. Blood was obtained by cardiac puncture of anesthetized, normal animals and serum was 0.45 µm filtered and stored at −80°C.

Arginine Determination. Arginine was measured using a Durrum D500 amino acid analyzer (Dionex Corp., Sunnyvale, CA) with lithium eluents and norleucine as an internal standard (1).

Data Presentation. Data reported are means ± one standard deviation from a representative experiment. When not shown in the figures, the standard deviation was <5% of the value.
of the mean. All macrophage functions were measured in at least three independent experiments.

Results and Discussion

L-Arginine Inhibits Activation-associated Functions in Resident Macrophages. Normal rat resident peritoneal macrophages were cultured in media containing either 1.2 mM L-arginine (RPMI 1640 plus 10% FCS) or 6 μM L-arginine (identical media prepared using arginine-free RPMI 1640, 6 μM L-arginine was provided by the serum). Marked morphological differences were apparent after overnight incubation (Fig. 1). Macrophages incubated in media containing 1.2 mM L-arginine presented a generally rounded appearance and little evidence of spreading. In contrast, macrophages incubated in media containing 6 μM L-arginine exhibited pronounced spreading and elongation, properties associated with macrophage activation (10).

In line with the changes in morphology, all activation-associated functions of macrophages examined were enhanced after overnight culture in L-arginine-deficient media. It can be seen first in Fig. 2 A that macrophage cytotoxicity against P815 tumor cells in a short-term assay (6 h) increased as the L-arginine concentration of the media was reduced below that of plasma (~0.1 mM). Macrophages were required for cytotoxicity since culture supernatants were devoid of tumoricidal activity (data not shown). Fig. 2, B, C, and D, show that superoxide production, phagocytosis of opsonized latex beads, and protein synthesis were also enhanced when the L-arginine concentration in the media was reduced. Thus, culture of resident macrophages in L-arginine concentrations similar to those found in sites of inflammation (1) results in a generalized increase in activation-associated functions.

L-Arginine Is Necessary and Sufficient to Inhibit Resident Macrophage Tumoricidal Activity. To determine the specificity of the inhibitory activity of L-arginine, the effects of arginine analogues, products of arginine catabolism, or other basic amino acids on tumoricidal activity were measured. Overnight culture with 1 mM L-arginine or homo-arginine, but not with D-arginine nor any of the other substrates tested, resulted in decreased macrophage tumor cytotoxicity (Fig. 3). L-arginine also suppressed tumoricidal activity by macrophages cultured in serum-supplemented, amino acid–free RPMI 1640 (Fig. 3). Thus, other amino acids are not required for inhibitory activity in concentrations higher than those provided by 10% FCS contained in the culture media. Since the inclusion of 10% FCS in culture media results in an L-arginine concentration of 6 μM, experiments were conducted using 10% dialyzed-FCS in order to more precisely determine the effect of low L-arginine concentrations on tumoricidal activity. It can be seen in Fig. 4 that little tumoricidal activity was expressed until the L-arginine concentration in the media containing dialyzed-FCS was increased to 4-10 μM. It can also be seen that cytotoxicity never reached that observed using media containing FCS as the L-arginine concentration was varied, indicating that other low molecular weight species in serum promote tumoricidal activity.

L-Arginine Promotes Tumoricidal Activity while Inhibiting other Activation-associated Functions of C. parvum–elicited Macrophages. In marked contrast with resident macrophages, the cytotoxicity of peritoneal macrophages collected 5 d after intraperitoneal C. parvum injection was increased by culture in media supplemented with L-arginine (Table I). Thus, C. parvum–elicited macrophages would be classified as activated for tumori-
cidal activity as compared with resident macrophages after incubation in standard tissue culture media. However, precisely the opposite pattern is observed after incubation in L-arginine-deficient media, indicating that the assessment of macrophage activation can be markedly influenced by culture conditions.
Figure 2. Evidence that arginine-deficient media promotes activation-associated functions of macrophages. Tumoricidal activity (A), superoxide production (B), phagocytosis (C), and protein synthesis (D) by resident macrophages after overnight culture in media containing 6 μM to 1.2 mM arginine. The abscissa shows L-arginine added over the 6 μM L-arginine provided by 10% FCS contained in the culture media.

The L-arginine dependent cytotoxicity of C. parvum–elicited macrophages is consistent with reports that activated murine macrophages require L-arginine to induce metabolic inhibition and cytostasis in tumor cells (11, 12). It has been proposed that these effects are mediated by toxic intermediates generated during the catabolism of arginine via a novel pathway involving arginine deiminase-like activity (11–14). This mechanism may be responsible for the presently reported findings, since it has been shown that rat peritoneal macrophages metabolize L-arginine primarily through

Figure 3. Specificity of the inhibitory effect of L-arginine on macrophage cytotoxicity. Tumoricidal activity of resident macrophages after overnight culture in media supplemented with 1 mM L-arginine, D-arginine, homo-arginine, ornithine, citrulline, urea, lysine, or histidine. Macrophages were also cultured in medium formulated with RPMI 1640 without amino acids (−AA) or this same medium containing 1 mM L-arginine (−AA + Arg).
this pathway (1). However, it can also be seen in Table I that culture with 1.2 mM L-arginine markedly inhibited superoxide production, phagocytosis, and protein synthesis in C. parvum-elicited macrophages as in resident macrophages. As for an explanation for the up- and downregulation of different functions in C. parvum-elicited macrophages by L-arginine, the catabolism of L-arginine by activated murine macrophages through the arginine deiminase–like pathway is known to result in a similar pattern of metabolic inhibition in these cells as that observed in tumor cells undergoing cytostasis (15). It may then be proposed that, although L-arginine catabolism through this pathway can promote cytotoxicity by certain macrophage populations, the concomitant metabolic inhibition results in generalized suppression of other macrophage functions as observed here.

**Arginine-deficient Wound Fluid Promotes Resident Macrophage Cytotoxicity.** As for the potential relevance of the foregoing results to macrophage function in sites of inflammation, the tumoricidal activity of resident macrophages was enhanced by overnight culture in L-arginine– and serum-free media supplemented with arginine-free, sterile wound fluid, as compared with normal rat serum (Fig. 5). The addition of L-arginine

### Table I

**Effect of L-Arginine on Resident and C. parvum-elicited Peritoneal Macrophage Functions**

| Media L-arginine concentration | Resident macrophages | C. parvum-elicited macrophages |
|------------------------------|----------------------|--------------------------------|
|                              | 6 μM                 | 1.2 mM                         | 6 μM                         | 1.2 mM |
| Cytotoxicity                 | 32.9 ± 3.0           | 14.8 ± 3.1                     | 17.9 ± 3.2                   | 29.4 ± 1.4 |
| (percent specific 51Cr release) | 2.0 ± 0.1           | 0.6 ± 0.1                      | 2.2 ± 0.3                    | <0.1 |
| Superoxide production (nmol/h) | 7.5 ± 1            | 3.2 ± 0.4                      | 15.2 ± 1                     | 6.7 ± 1 |
| Phagocytosis (beads ingested/cell) | 18.6 ± 0.2        | 5.2 ± 0.2                      | 18.9 ± 0.3                   | 3.3 ± 0.1 |
| Protein synthesis (pmol Phe incorporated/h) | 1.2 | 1 | 1.2 | 1 | 1.2 | 1 |

Before assay, resident and C. parvum-elicited macrophages were cultured overnight in arginine-deficient culture media or in media supplemented with 1.2 mM L-arginine.
to either media again markedly suppressed cytotoxicity. Therefore, other conditions present at a site of inflammation (cytokines, hormones, etc.) allow the promoting effect of low arginine concentration on tumoricidal activity to be expressed.

The present results have implications for the understanding of macrophage physiology in vitro and in vivo. For example, commonly used culture media contain L-arginine in concentrations shown here to be inhibitory for all activation-associated functions measured with the exception of tumoricidal activity by C. parvum–elicited macrophages (RPMI 1640 = 1.2 mM L-arginine, Ham's F10 = 1 mM L-arginine, Eagle's MEM = 0.6 mM L-arginine, and DMEM = 0.4 mM L-arginine). It follows that standard culture conditions are not appropriate for the functional assessment of macrophages in different states of activation. More importantly, because extracellular arginine concentrations become markedly reduced in sites of inflammation, it is postulated that a similar L-arginine–dependent modulation of macrophage functions occurs in vivo. In particular, the results in this study suggest that a decrease in arginine availability may contribute to the activation of unprimed macrophages migrating into inflammatory foci. Since macrophages themselves are primarily responsible for reducing extracellular arginine in sites of inflammation, it is concluded that activation-associated functions in these cells can be autoregulated through the control of the extracellular arginine concentration.

Summary

Sites of inflammation with prominent macrophage infiltration, such as wounds and certain tumors, are uniquely deficient in free arginine. The effects of arginine availability on macrophage physiology were investigated. When cultured in media containing <0.1 mM L-arginine, rat resident peritoneal macrophages exhibited enhanced spreading, tumor cytotoxicity, superoxide production, phagocytosis, and protein synthesis. Thus, arginine concentrations similar to those found in sites of inflammation can augment macrophage functions, while those found in plasma (~0.1 mM) and in commonly used culture media (0.4 to 1.2 mM) are inhibitory. Culture in homo-arginine, but not D-arginine, ornithine, citrulline, urea, histidine, or lysine also inhibited macrophage tumor cytotoxicity, indicating the specificity of the effect. In
contrast to resident macrophages, the tumor cytotoxicity of peritoneal macrophages obtained after C. parvum injection was suppressed by culture in arginine-deficient media. However, L-arginine-deficient media enhanced all other activation-associated functions in C. parvum-elicited macrophages as in resident cells. Arginine-free wound fluid promoted resident macrophage tumoricidal activity when compared with rat serum, and again, the addition of L-arginine was inhibitory. The marked effects of L-arginine availability on macrophage functions, together with the knowledge that these cells modify the extracellular arginine concentration in sites of inflammation through arginase, provide evidence for an autoregulatory mechanism of macrophage activation.

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