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MACROPHAGE ARGININE METABOLISM AND THE INHIBITION OR STIMULATION OF CANCER[1]

CHARLES D. MILLS,[2]* JEFFRY SHEARER,* ROBERT EVANS,† AND MICHAEL D. CALDWELL*

*From the *Department of Surgery, University of Minnesota, Minneapolis, MN 55455; and †From the Jackson Laboratory, Bar Harbor, ME 04609*

The potential of the immune system to inhibit or stimulate tumor growth is a vivid example of the "two-edged sword" nature of immune responses. Our results provide evidence that this dual capacity can be attributed, in part, to the dual pathways of arginine metabolism exhibited by intratumor macrophages. Specifically, i.p. tumor rejection in P815-preimmunized mice is accompanied by an upshift in intratumor macrophage arginine metabolism to the nitric oxide (NO) synthase pathway that yields citrulline and NO. A rapid and marked local increase in IFN-γ (both mRNA and protein) in preimmunized mice during tumor rejection suggests that this cytokine plays a role in up-regulating nitric oxide production in vivo. Unlike tumor rejection, progressive i.p. P815 tumor growth in naive mice is associated with a marked decline in the production of citrulline/NO by intratumor macrophages. Examination of macrophage arginine metabolism via arginase revealed a pattern opposite that of NO synthase. The local production of ornithine/urea markedly increases during progressive tumor growth whereas arginase activity decreases during tumor rejection. Inasmuch as nitric oxide inhibits tumor cell replication whereas ornithine is the precursor of polyamines required for cell replication, these results are consistent with the conclusion that the pathway macrophages use to metabolize arginine can influence the type of host immune responses against cancer and other conditions.

Despite intensive and prolonged research efforts in tumor immunology dating back to the turn of the century when Ehrlich (1) and others (2) first proposed an anticancer role for the immune system, there are still no consistently effective protocols for the immunotherapy of human cancer. Clearly the immune system has the potential to inhibit cancer. Indeed, several different potent antitumor mechanisms have been identified in animal models (3, 4, reviewed in 5–7). At the same time, although anticancer potential can be demonstrated experimentally it is not clear that cancer is routinely eliminated by "immunosurveillance" as originally envisaged by Ehrlich (1) and developed by Burnet (8). That is, a picture of the immune system as serving a purely inhibitory role in cancer had to be rethought when it was discovered during the 1970's that immunodeficient hosts did not exhibit an increased incidence of cancer (with the minor exception of lymphoid malignancies) (5, 9, 10). Also during this period, Prehn and co-workers (11, 12) advanced the theory that the immune system could actively stimulate cancer. Although the "immunostimulation" theory has received little attention by most investigators in their pursuit of anticancer defenses, sufficient evidence has accumulated to conclude that cancer growth can be stimulated as well as inhibited by the immune system (13–15). In particular, macrophages have been demonstrated to exhibit this dual capacity under different circumstances (16–21).

Recent results in this laboratory (22–24) and others (25–28) have demonstrated that the pathway macrophages use to metabolize arginine can promote or inhibit their functions, and that of cells with which they communicate. This knowledge combined with the aforementioned background in tumor immunology led us to postulate that macrophage arginine metabolism at the site of a tumor could influence whether tumor growth is inhibited or stimulated. It was proposed that macrophage arginine metabolism in the tumor bed via the NO[3] synthase pathway would favor tumor inhibition because NO is tumoristatic/tumoricidal (26). If instead, NO production was down- regulated, tumor growth might be allowed or even stimulated because macrophages are a primary source of growth factors that promote proliferation of a variety of cells such as those involved in wound healing and regeneration (29, 30). Further, if the predominant pathway of arginine metabolism by intratumor macrophages was via arginase (yielding ornithine and urea) (31) rather than NO synthase then tumor growth might be further promoted because ornithine is the precursor of polyamines that are required for cell replication (32, 33). This report examines these hypotheses. A model was used in which tumor growth or rejection occurs in the peritoneum. This model facilitates analyses of the intratumor cellular infiltrate and local extracellular environment (peritoneal fluid). It will be shown that tumor rejection or growth is associated with local up regulation of the NO synthase or arginase pathway, respectively. These and other data suggest that intratumor macro-

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[2] Address correspondence and reprint requests to Charles D. Mills, Department of Surgery, University of Minnesota, Box 120 University of Minnesota Hospitals and Clinics, Minneapolis, MN 55455.

[3] Abbreviations used in this paper: NO, nitric oxide; PEC, peritoneal exudate cells.

phage arginine metabolism plays a role in determining what course cancer takes.

MATERIALS AND METHODS

Animals. C57Bl/6 × DBA/2 (B6D2F1) female mice 8-wk-old were purchased from Taconic Farms, Germantown, NY. Mice were used in experiments when they were 10- to 14-wk-old. They were free of common murine pathogens as assessed by routine testing by the supplier and by the University of Minnesota animal care facility. Mice were killed with carbon dioxide gas.

Tumors. The P815 mastocytoma employed has been described in detail previously (3, 34, 35). Briefly, the tumor was passaged weekly in vivo in irradiated (750R) B6D2F1 mice. Mice were specifically immunized against P815 by axillary intradermal injection of 2.5×10^6 tumor cells mixed with 50 μg *Corynebacterium parvum* (Burroughs Wellcome Ltd., London, England). By 21 days postimmunization complete tumor regression has occurrred. At this time, immunized or age-matched naive mice were challenged with 5×10^5 P815 tumor cells in the peritoneum in 0.5 ml of PBS. For in vitro use, P815, L5178Y, and YAC tumor cells were grown in RPMI 1640 supplemented with 10% FCS and antibiotics (penicillin/streptomycin). New stocks of tumors were used in vivo and in vitro every few months from cells kept frozen in liquid nitrogen.

PEC and peritoneal fluid. PEC and peritoneal fluid were collected simultaneously by the injection of 0.5 ml PBS containing antibiotics followed by immediate recovery of available fluid/cells. Injecting a relatively small volume reduces the dilution of peritoneal fluid and yet allows more than half of the PEC to be recovered. Recovery of fluid was quite consistent averaging 0.42 ± 0.04 ml in normal mice. The volume of the peritoneal fluid was determined using radioisotope dilution. Mice received injections in the peritoneum with 0.02 μCi [^{51}Cr] in 0.5 ml PBS and then peritoneal fluid was immediately recovered. A comparison of the radioactive counts injected and recovered allowed the peritoneal fluid volume to be calculated.

Cell culture. Details of cell culture have been previously reported (24). PEC were washed and resuspended in complete culture media consisting of RPMI 1640 supplemented with 10% FCS (Sterile Systems, Logan, UT), 10 mM morpholinopropane sulfonic acid, 5×10^{-5} M 2-ME, and antibiotics. Unless indicated, these and other tissue culture reagents were purchased from GIBCO/BRL, Gaithersburg, MD. Cells were cultured in 96-well flat-bottomed plates at 1.5×10^6/ml in a volume of 0.2 ml for 20 h at 37°C in 7% CO_2 in air. Cell supernatants for NO_2 or amino acid analysis were collected and stored at -80°C. Macrophage-rich cultures were obtained by a 2-h incubation of 3×10^5 PEC in 0.1 ml followed by repeated washing (22). The PEC that did not adhere to the wells were placed in other wells and referred to as nonadherent.

[^{51}Cr] release assay. The assay was the same as that used previously (34). Briefly, P815 or other tumor cells were harvested during log-phase growth and 10^6 cells were labeled in 0.3 ml of media with 100 μCi of $Na_2[^{51}Cr]O_4$ (CJS.11, Amersham Corp, Arlington Heights, IL) for 1 h at 37°C. PEC and [^{51}Cr]-labeled tumor target cells (5×10^3) were plated in round-bottomed 96-well plates in quadruplicate in a total volume of 0.2 ml and the plate was centrifuged to initiate the assay. After 4 h at 37°C, 0.05 ml of medium was removed and counted (LKB Instruments, Inc., Gaithersburg, MD). "Total" [^{51}Cr] release was that released by 0.5% Triton-X. Total release was >97% and spontaneous release was 6 to 10% of the total depending on the tumor. The percentage of specific [^{51}Cr] release was calculated as follows: (experimental cpm - spontaneous cpm/total cpm - spontaneous cpm) × 100.

Antibody treatment. T lymphocyte subsets were depleted as previously described (35). PEC (2×10^7/ml) were incubated for 30 min at 4°C in a 1/5 dilution of a hybridoma culture supernatant. Anti-L3T4 (GK1.5) and anti Lyt-2 (TIB-150) hybridomas were obtained from the American Type Culture Collection (Rockville, MD). Low-tox rabbit complement (Accurate Chemical and Scientific Co., Westbury, NY) was added and cells were incubated for an additional 60 min at 37°C.

Nitrite determination. Nitrite in cell-free culture supernatants was measured as described by Ding et al. (36). Briefly, 0.05 ml of test fluid was mixed with 0.05 ml of Griess reagent in quadruplicate and absorbance read at 550 nm using an automatic plate reader. Nitrite concentration was calculated from a $NaNO_2$ standard curve.

Amino acid analysis. Amino acids were measured as previously described (22) using a Dionex BioLC Amino Acid Analyzer System (Dionex Corp., Sunnyvale, CA) with lithium eluents and norleucine as an internal standard. Variation between replicate HPLC runs routinely averaged less than 5%.

IFN-γ. A solid phase, two-site "sandwich immunoassay" (Amgen, Thousand Oaks, CA) was used to measure IFN-γ in cell culture supernatants and peritoneal fluids.

Northern hybridization. RNA was extracted from cells by a modification of the guanidine hydrochloride technique described fully elsewhere (37, 38). The IFN-γ cDNA probe was obtained from DNAX, (Palo Alto, CA). All probes were labeled with [^{32}P] dCTP by the method of Feinberg and Vogelstein (39) to a sp. act. of ≈10^9 dpm/μg cDNA. In all experiments, RNA was blotted onto ZetaProbe nylon membranes (Bio-Rad Laboratories, Richmond, CA) and hybridized with ≈10^6 cpm/ml hybridization mix. Blots were exposed to XAR-5 x-ray film (Kodak) between 2 Du Pont Cronex Lightening Plus (Du Pont, Wilmington, DE) screens for variable times at -70°C. Densitometry was carried out on the autoradiograms using the Bio-Rad model 620 Video Densitometer (Bio-Rad). Data were normalized relative to the β-actin signal.

RESULTS AND DISCUSSION

Properties of tumor model used to determine intratumor arginine metabolism. Mice exhibiting specific immunity to the P815 were produced as in preceding publications (3, 34, 35). Specifically, B6D2F1 mice receiving intradermal injections with P815 tumor cells mixed with *Corynebacterium parvum* reject the tumor in 12 to 15 days and then exhibit long-lived memory immunity. For our experiments, immunized (21 days postimmunization) or naive mice were challenged with 5×10^5 P815 tumor cells in the peritoneum and the intratumor cellular infiltrate (PEC) and the local extracellular environment (peritoneal fluid) were analyzed during tumor growth or rejection. It can be seen first in Figure 1*A* that exponential growth of tumor occurs in naive mice such that by 9 days postimplantation >10^8 P815 tumor cells are present in the peritoneal cavity (*line graphs*). In contrast, in preimmunized mice there are less than 10^5 tumor cells present by 5 days after tumor implantation. As previously observed at other anatomical sites (34), tumor rejection in the peritoneum of preimmunized mice is accompanied by the appearance of robust cytolytic activity (Fig. 1*A*, *histobars*) mediated by tumor-specific T lymphocytes of the Lyt-2^+ (CD8), L3T4$^-$ (CD4) phenotype (Fig. 1*B*). As shown previously, T lymphocytes are known to be required for tumor rejection and immunity is tumor specific (6, 35). In contrast, no significant cytolytic activity was detectable during progressive P815 tumor growth in naive mice.

Different pathways of intratumor leukocyte arginine metabolism predominate during tumor rejection or growth. Examination of arginine metabolism at the site of tumor rejection or progressive tumor growth revealed fundamental differences. First, it can be seen that leukocytes recovered from preimmunized mice 1 day after tumor implantation demonstrate increased metabolism of arginine to citrulline and NO (measured as sodium nitrite) (Fig. 2, *A* and *B*). Also, the up-regulation of the NO synthase pathway is maintained for at least 1 wk after tumor implantation. In contrast, during progressive tumor growth in naive mice, local leukocyte arginine metabolism to citrulline and NO declines below that observed with resident normal peritoneal exudate leukocytes. Second, precisely the opposite pattern is observed when arginine metabolism via the arginase pathway is evaluated. That is, it can be seen that leukocyte arginine metabolism to ornithine and urea markedly increases during progressive tumor growth, whereas during tumor rejection the arginase pathway in leukocytes is decreased below normal (Fig. 2, *C* and *D*).

Intratumor shifts in NO synthase and arginase are primarily attributable to macrophages. To confirm that the changes in leukocyte arginine metabolism observed

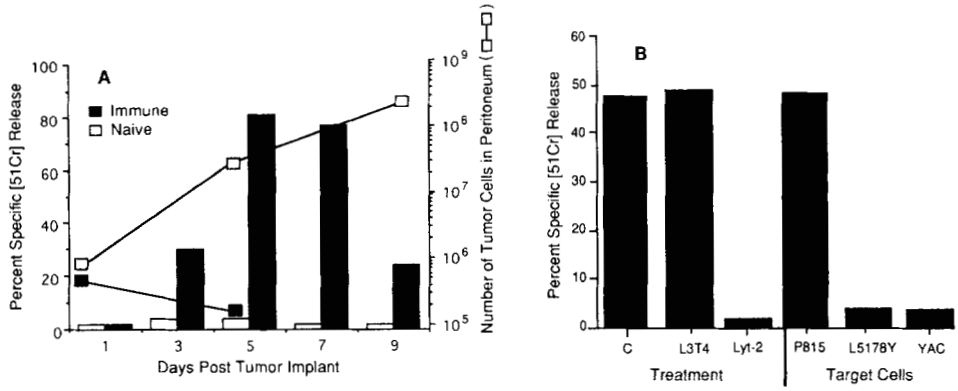

Figure 1. Properties of tumor model used to analyze intratumor arginine metabolism. *A*, Kinetics of tumor rejection or progressive tumor growth (*line graphs* in background) in immune or naive mice. Cytolytic activity (*histobars* in foreground) of PEC from immunized or naive mice on the days indicated following implantation of 5×10^5 P815 tumor cells in the peritoneum. Unfractionated PEC pooled from five mice tested at a 50:1 E:T ratio in quadruplicate in a 4-h[^{51}Cr] release assay. *B*, Phenotype and specificity of cytolytic cells from immunized mice 4 days after P815 tumor implantation (10:1 E:T ratio). Cytolytic phenotype (Lyt-2$^+$ L3T4$^-$) determined by treating PEC with the indicated mAb or a control culture supernatant and C before assay. The specificity of the PEC for the P815 tumor was determined by incubating PEC with [^{51}Cr]- labeled L5178Y (another immunogenic tumor of DBA/2 origin) or [^{51}Cr]-labeled YAC-1, a tumor sensitive to lysis by NK cells. Representative data from three experiments.

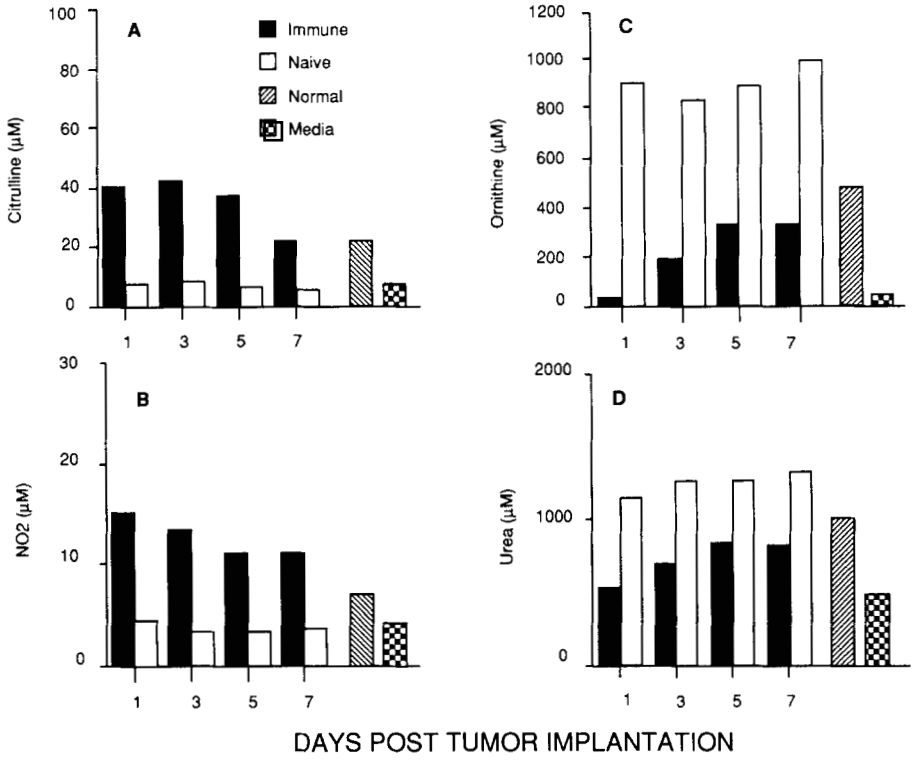

Figure 2. Changes in intratumor leukocyte arginine metabolism during tumor rejection or progressive tumor growth. PEC were collected from groups of five immunized or naive mice after P815 tumor implantation on the days indicated. PEC were cultured at 1.5×10^6/ml (0.2 ml) in quadruplicate in 96-well microtiter plates for 20 h. Cell-free supernatant was collected and stored at -80°C until analyzed. "Normal" = resident peritoneal exudate leukocytes of normal mice. "Media" = cell free media cultured for 20 h. Citrulline, ornithine, and urea were analyzed by HPLC; nitrite (NO_2) determined using the Griess reagent. The same pattern of intratumor leukocyte arginine metabolism was observed in three similar experiments.

in the preceding section could be attributed to macrophages, PEC were fractionated. Peritoneal exudate leukocytes were incubated for 2 h at 37°C; leukocytes remaining adherent after thorough washing are "adherent" and the rest are "nonadherent." Adherent cells comprised on average 54% of immune PEC and 45% of naive PEC. More than 93% of adherent immune or naive PEC were macrophages as assessed by morphology or nonspecific esterase staining. The majority of remaining cells exhibited lymphocyte-like morphology. It can be seen first in Figure 3*A* that, again, local NO production by peritoneal exudate leukocytes from immunized mice is elevated 5 days after tumor implantation. More importantly, Figure 3*A* shows that virtually all of the NO (measured as NO_2) produced can be attributed to the adherent cell fraction. In Figure 3*B* it can be seen that PEC recovered instead from naive mice 5 days after tumor implantation demonstrated elevated ornithine production as compared with resident PEC from normal mice. Again, the majority of leukocyte ornithine production from naive tumor bearers or normal mice is found in the adherent fraction. Although a percentage (approximately 50%) of the PEC in naive mice at this time are tumor cells, P815 tumor cells are nonadherent and produce little ornithine. Specifically, a culture supernatant of P815 tumor cells contained 36 μM ornithine after 20 h incubation at 1.5×10^6/ml (the maximum cell density if all PEC were tumor cells). Together, these data indicate that macrophages are primarily responsible for the increased local citrulline/NO or ornithine/urea production during tumor rejection or progressive tumor growth, respectively.

NO synthase or arginase pathway predominates in local extracellular tumor environment during rejection or growth, respectively. Direct examination of arginine metabolism at the site of tumor rejection or progressive

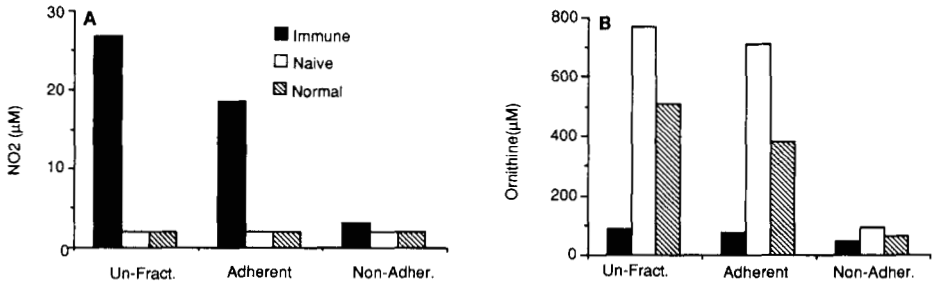

Figure 3. Evidence that macrophages are primarily responsible for changes in intratumor leukocyte arginine metabolism. PEC were collected from groups of five immune or five naive mice 5 days after P815 tumor implantation. Unfractionated PEC, adherent PEC, or nonadherent PEC were cultured overnight as in Figure 2.

tumor growth (peritoneal fluid) revealed the same characteristic differences observed with intratumor leukocytes. That is, the local concentration of citrulline is elevated in peritoneal fluid in immunized mice within 1 day of tumor implantation and remains somewhat elevated during tumor rejection (Fig. 4*A*). Inasmuch as citrulline and NO are produced from arginine in equimolar quantites by NO synthase (25), the concentration of this amino acid should reflect NO synthase activity. In marked contrast to these findings, it can be seen that during progressive tumor growth, the intratumor citrulline concentration declines to levels significantly below that found in peritoneal fluid from normal mice. Examination of arginine metabolism via arginase revealed precisely the opposite pattern to that of NO synthase. Specifically, there was a progressive rise in the local concentration of ornithine in peritoneal fluid during progressive tumor growth, but not during tumor rejection (Fig. 4*B*). In contrast, the local concentration of ornithine is below normal during specific tumor rejection.

Regulation of local arginine metabolism during tumor rejection or growth. Our results indicate that tumor rejection and progressive tumor growth are associated with characteristic and opposite shifts in macrophage arginine metabolism. As for the signals that regulate these changes in arginine metabolism, IFN-γ has been reported (36) to up-regulate the NO synthase pathway. Evidence consistent with the ability of IFN-γ IFN to up-regulate this pathway is shown in Figure 5*A*. It can be seen that the local extracellular concentration (peritoneal fluid) of IFN-γ is markedly increased in immunized mice, but not naive mice, after tumor implantation. Evidence that intratumor leukocytes are responsible for the local increase in IFN-γ is shown in Figure 5*B* where it can be seen that leukocytes recovered from the site of tumor rejection, but not from the site of tumor growth, secreted markedly elevated levels of IFN-γ. Leukocyte secretion of IFN-γ and its concentration in peritoneal fluid were both observed to be maximal 1 day after tumor implantation and to disappear by day 5. In support of these findings, Figure 5*C* shows that leukocyte mRNA coding for IFN-γ is abundant early after tumor challenge and rapidly declines indicating a close correspondence between gene transcription and translation/secretion. Finally, because local production of citrulline/NO remains elevated during tumor rejection for at least 1 wk, the data suggest that if IFN-γ is responsible for increased NO production in vivo, then it is either required only transiently or that other signals play a role as well.

In contrast to our knowledge of factors such as IFN-γ that "activate" macrophages, less is known concerning the signals that down-regulate local macrophage arginine metabolism to citrulline/NO and increase the production of ornithine/urea during progressive tumor growth. However, some informed speculation may be useful.

A tumor could regulate intratumor macrophage arginine metabolism directly or indirectly through other leukocytes or another organ system. Regarding direct regulation, tumors have been reported to produce a variety of products that could affect macrophage arginine metabolism. In particular, Nathan and co-workers (40, 41) have isolated a protein from several murine tumors that down-regulates the NO synthase pathway in macrophages. One tumor that secretes this protein (macrophage deactivating factor) is the P815 mastocytoma used in our study. Thus, it seems reasonable to postulate that such a factor plays a role in down-regulating the NO synthase pathway during progressive P815 tumor growth. Another factor that seems likely to play a regulatory role in intratumor macrophage arginine metabolism is TGF-β. This cytokine is produced by some tumors and has been shown to down-regulate NO production in macrophages in vitro (41). CSF including granulocyte-macrophage CSF are also produced by certain tumors (42) and these cytokines have been demonstrated to inhibit activation-associated functions of macrophages (43). Although CSF have not been

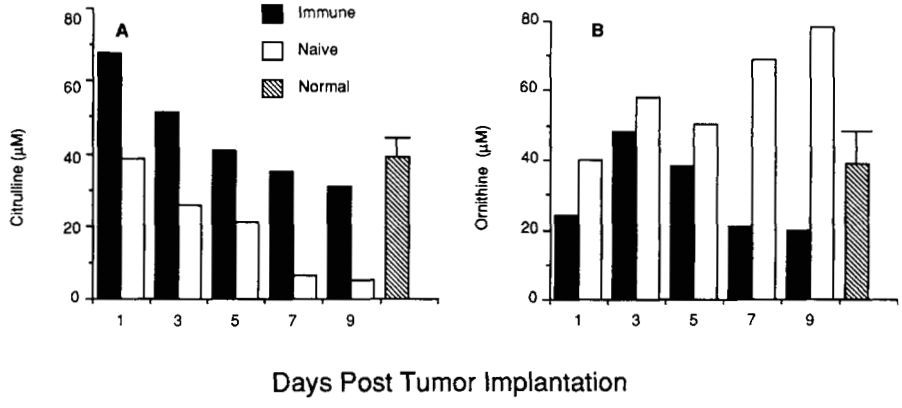

Figure 4. Changes in arginine metabolism in the local extracellular environment during tumor regression or progressive tumor growth. Peritoneal fluid was collected and pooled from groups of five immunized or naive mice on the indicated days after P815 tumor implantation. Citrulline and ornithine concentrations were determined using HPLC. A virtually identical pattern of changes in citrulline and ornithine in peritoneal fluid was observed in another experiment of the same design. Normal peritoneal fluid represents the mean ± SD of five mice.

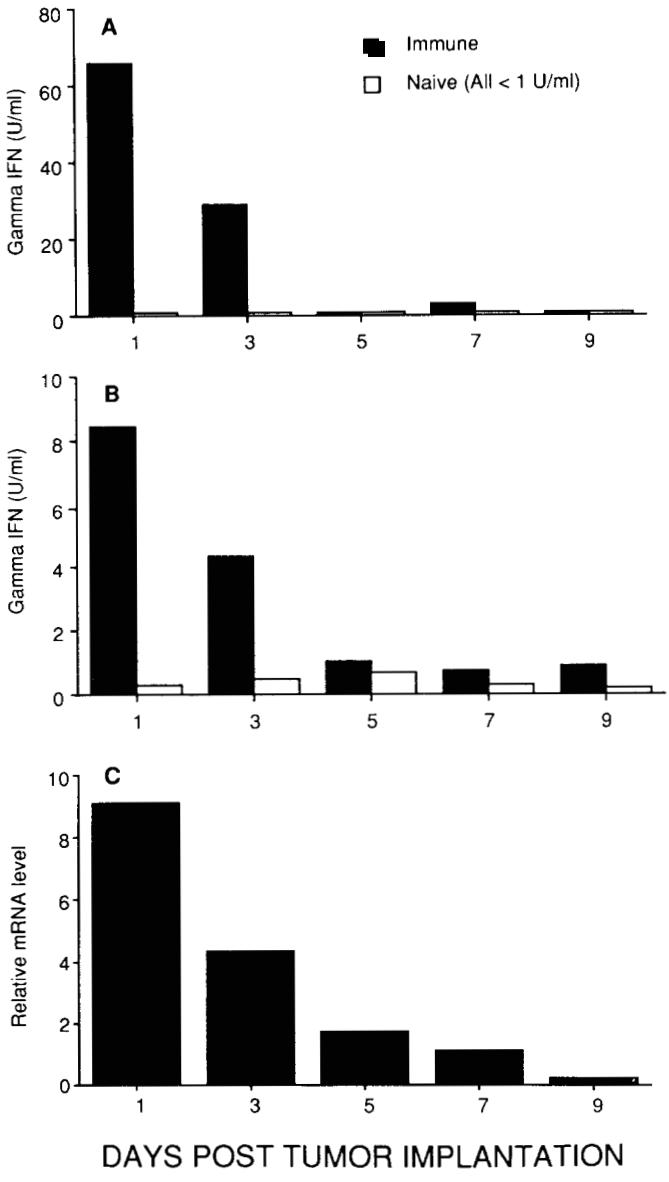

Figure 5. Local elevation in IFN-γ during tumor rejection. Peritoneal fluid and PEC were collected from immunized or naive mice after tumor implantation as in preceding figures. *A*, IFN-γ concentration in peritoneal fluid. *B*, Leukocyte secretion of IFN-α into supernatant after overnight culture as in Figure 2. *C*, Level of mRNA determined by densitometry tracing of Northern blots. IFN-γ mRNA normalized for B-actin mRNA content of each sample. No detectable IFN-γ or IFN-γ mRNA was present in normal mice.

shown to inhibit NO production, per se, it seems reasonable to postulate that they might. In particular, CSF can promote macrophage proliferation (44) which seems unlikely during precocious NO production because macrophages themselves are susceptible to the toxic effects of their NO (22, 23, 27).

It is likely that tumors also regulate macrophage arginine metabolism indirectly. In particular, the immune system itself is a likely source of indirect regulation by tumor cells because lymphocytes produce TGF-β and CSF (45, 46). In this connection, the type of indirect regulation that macrophages receive may depend on the type of T lymphocyte activated. For example, Th1 lymphocytes produce IFN-γ, and thus could increase NO production (47). However, Th2 lymphocytes produce IL-4 and IL-10 both of which have been shown to block the NO production of macrophages stimulated by IFN-γ (48, 49). Finally, other organ systems could contribute to indirect regulation of arginine metabolism in macrophages. In particular, participation by the pituitary-adrenal axis seems likely because circulating steroids are sometimes elevated in cancer (50) and steroids have been shown (51) to down-regulate NO synthase activity.

Regarding the regulation of macrophage arginine metabolism, the relationship between NO production and ornithine/urea production via the arginase pathway is largely unknown. It is known (52) that both enzyme activities can increase simultaneously in macrophages because activated murine macrophages produce more citrulline/NO and ornithine/urea than resident macrophages. In the tumor model used in this study, citrulline/NO production and ornithine/urea production by macrophages were inversely related. Relatedly, it is known that blocking the NO synthase pathway with N-G-monomethyl-L-arginine increases ornithine production by murine macrophages (52). An increase in ornithine production as a consequence of inhibition of NO production would be expected because the K_m of arginase is higher than that of NO synthase (51). However, whether down-regulation of NO production necessarily increases ornithine production is not known. Ongoing experiments are designed to more clearly delineate how these two pathways of arginine metabolism are regulated in macrophages.

Our results provide evidence that intratumor macrophage arginine metabolism is a molecular explanation for the dual ability of the immune system to inhibit or stimulate tumor growth. It is proposed that arginine metabolism in the tumor bed yielding citrulline and NO favors tumor rejection, whereas production of ornithine and urea could promote tumor growth. Inasmuch as macrophages are the primary intratumor source of NO synthase, their presence in a tumor could be considered a favorable prognosis. However, if intratumor macrophages are expressing the arginase pathway then their presence could be unfavorable. It should be added that although ornithine-derived polyamines can stimulate tumor growth, we do not have direct evidence of stimulation of the P815 tumor cells used in this study. Although it is a cruel paradox that macrophages can promote as well as inhibit tumor growth, this dual capacity is in keeping with the multiple roles that these cells play in physiology. In conclusion, our results suggest that the ability to modulate immune responses against cancer and other conditions can depend, in part, on stimulating the appropriate pathway of arginine metabolism in macrophages.

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