

## The Altered Tumoricidal Capacity of Macrophages Isolated from Tumor-bearing Mice Is Related to Reduced Expression of the Inducible Nitric Oxide Synthase Gene

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### Summary

Nitric oxide (NO) is a major effector molecule in the destruction of tumor cells by activated macrophages. However, in many cases, developing neoplasms appear to be capable of impairing steps in the complex process leading to NO production as a means of avoiding immune destruction. After activation with lipopolysaccharide (LPS), peritoneal-elicited macrophages (PEM) from mice bearing mammary tumors display alterations in their ability to lyse tumor cells due to reduced production of NO. In contrast, when these same cells are stimulated with LPS in combination with interferon  $\gamma$  (IFN- $\gamma$ ), they are able to produce NO and lyse targets at normal levels. Since tumor-associated macrophages are intimately associated with the cells of the developing tumor, their ability to produce NO and lyse tumor targets is likely to be more relevant to controlling tumor growth. This population of macrophages exhibited a more profound inability to produce NO and lyse targets and, unlike the PEM, was not able to upregulate these functions even when treated with combinations of LPS and IFN- $\gamma$ . Northern and Western blots revealed that inducible nitric oxide synthase (iNOS) mRNA and protein levels correlated directly with the ability of each macrophage population to produce NO, and the levels of these macromolecules were altered sufficiently in tumor bearers' macrophages to account for the diminished NO production described. These results indicate that a spatial gradient of suppression of macrophage cytolytic activity and iNOS expression exists in mammary tumor-bearing mice, whereby macrophages from within the tumor exhibit a more pronounced suppression than the more distally located PEM. This suppression may be due to proximity of the macrophages to the developing tumor, macrophage maturational state, or both.

The activation of macrophages for tumoricidal activity is a complex process that can be accomplished with combinations of bacterial cell wall products, including LPS and cytokines, such as IFN- $\gamma$  (1–3). Once activated, macrophages are able to produce a variety of factors capable of lysing tumor cells. These factors include TNF- $\alpha$ , reactive oxygen intermediates, and nitric oxide (NO)<sup>1</sup> (1, 4, 5). Under normal conditions, the integrity of this complex process is maintained, but during tumor progression these mechanisms may become altered, resulting in the inability of the macrophages to lyse tumor cells. Several laboratories have demonstrated an inhibition of macrophage-mediated

cytotoxicity in the presence of developing tumors (6–9). Our previous studies revealed that, when compared with peritoneal-elicited macrophages (PEM) from normal mice (N-PEM), the lytic activity of PEM from tumor-bearing mice (TB-PEM) is altered in response to LPS activation; however, this defect is not due to impaired target binding or to decreased production of TNF- $\alpha$  or H<sub>2</sub>O<sub>2</sub> (8). In contrast, we have found that the production of NO by tumor bearers' PEM is inhibited in a manner that parallels their observed loss of cytotoxicity (8). Furthermore, when these same cells are stimulated with LPS in combination with IFN- $\gamma$ , they are able to recover the capacity to both produce NO and lyse tumor targets to near normal levels (8, 9).

Macrophage production of NO is regulated at the transcriptional level of the enzyme inducible nitric oxide synthase (iNOS) and is induced most efficiently in response to LPS and IFN- $\gamma$  (10, 11). Once produced, NO diffuses into adjacent cells and inactivates key enzymes involved in cell metabolism, eventually leading to cell stasis or death (12,

<sup>1</sup>Abbreviations used in this paper: iNOS, inducible nitric oxide synthase; NO, nitric oxide; N-PEM, PEM from normal mice; PEM, peritoneal-elicited macrophages; TAM, tumor-associated macrophages; TB-PEM, PEM from tumor-bearing mice.

13). However, the mechanisms underlying the effects of tumor growth on the production of NO by macrophages have not been elucidated.

In the studies reported herein, we examined the molecular events preceding the production of NO by PEM from both normal and tumor-bearing mice, and by tumor-associated macrophages (TAM). Our data indicate that the reduced capacity of tumor-bearers' macrophages to produce NO and lyse tumor targets results from diminished iNOS expression, and that this defect is quantitatively and qualitatively more profound in macrophages directly associated with the tumor.

## Materials and Methods

**Mice and Tumors.** Male and female 8–12-wk-old BALB/c mice, maintained by brother–sister matings in our facilities, were used in all experiments. The D1-DMBA-3 mammary adenocarcinoma was derived in a BALB/c mouse from a nonviral, carcinogen-induced, preneoplastic alveolar nodule treated with 7,12-dimethylbenzanthracene (14). This tumor is transplanted in BALB/c mice by s.c. injection of  $10^6$  tumor cells. The in vitro DA-3 cell line was derived from the in vivo D1-DMBA-3 mammary tumor (15).

**Reagents.** RPMI 1640 supplemented with 100 U of penicillin, 100  $\mu$ g/ml of streptomycin,  $5 \times 10^{-5}$  M 2-ME, 2 mM L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, and 10% FCS (all from GIBCO BRL, Gaithersburg, MD) was used as culture media in all experiments. *Escherichia coli* 055:B5 LPS (Difco Laboratories, Detroit, MI), mIFN- $\gamma$  (Genzyme Corp., Cambridge, MA), N<sup>G</sup>-monomethyl-L-arginine, and MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma Chemical Co., St. Louis, MO) were employed as described.

**Preparation of PEM.** Normal and 4-wk-old tumor-bearing mice were injected with 1.5 ml i.p. of 3% thioglycolate (Difco Laboratories). On day 4, the PEM were obtained by peritoneal lavage as previously described (16).

**Preparation of TAM.** TAM were isolated based on previously described protocols (17, 18). Briefly, tumors obtained 4 wk after transplantation were aseptically removed and minced into 1–2-mm<sup>3</sup> pieces of tissue. These pieces were then treated twice with a cocktail of 0.3% collagenase and 10% DNase (both from Boehringer Mannheim Corp., Indianapolis, IN) at 37°C for 20 min. The resultant supernatant containing the disaggregated cells was then spun through a Nycodenz density gradient (1.10 g/ml; GIBCO BRL) (19) at 4°C to remove dead cells and debris. The live cells were washed twice and the adherent population was obtained after a 2-h incubation on 10-cm plastic dishes at 37°C and 5% CO<sub>2</sub> (20). Using a hematoxylin and eosin (H&E) differential stain (Diff-Quick; Baxter Diagnostics, Inc., McGraw Park, IL), we have shown that >95% of this adherent population were cells of the monocyte/macrophage lineage, and >87% were nonspecific esterase positive (Sigma Chemical Co.). Contaminating cells consisted of, in order of frequency, lymphocytes ( $\leq 3\%$ ), blasts ( $\leq 2\%$ ), and unidentified cells ( $\leq 1\%$ ). Viability remained >95% by trypan blue exclusion. After adherence, TAM to be used in bioassays were placed on ice for 10 min in RPMI 1640, gently scraped free of the plate (20), and resuspended at the appropriate concentration.

**Nitrite Determination.** Peritoneal and tumor-associated macrophages were isolated and adhered in 96-well flat-bottom plates (Costar Corp., Cambridge, MA) at  $1.5 \times 10^5$  cells/well. Cells were

then cultured for 22–24 h 37°C and 5% CO<sub>2</sub> in plain media or media containing LPS alone or in combination with IFN- $\gamma$ . Nitrite concentrations in cell-free supernatants were measured by the Griess reaction according to the protocol of Stuehr and Nathan (12), and serve as a reflection of NO production.

**Tumor Target Cytotoxicity Assay.** Cytolytic capacity was measured after nitrite determination by the release of <sup>51</sup>Cr from labeled syngeneic DA-3 or allogeneic P-815 tumor targets as previously described (8).

**Phagocytosis of Latex Beads.** Isolated peritoneal and tumor-associated macrophages were adhered at  $3 \times 10^5$  cells/well for 2 h to glass coverslips. The cells were then washed twice, and cultured for 1 h in supplemented RPMI 1640 containing  $\sim 10^7$  latex beads (Sigma Chemical Co.). Excess beads were washed free and the live macrophages were examined under an oil immersion objective for phagocytosis of the beads. 100 cells were counted from each sample, and data were expressed as the percentage of cells containing beads.

**Mitochondrial Dehydrogenase Activity.** Mitochondrial dehydrogenase activity was assessed by MTT assay as previously described (16).

**IL-6 ELISA.** Production of IL-6 by peritoneal and tumor-associated macrophages was determined by ELISA (Mouse IL-6 MiniKit; Endogen, Cambridge, MA) according to the manufacturer's lot-specific instruction.

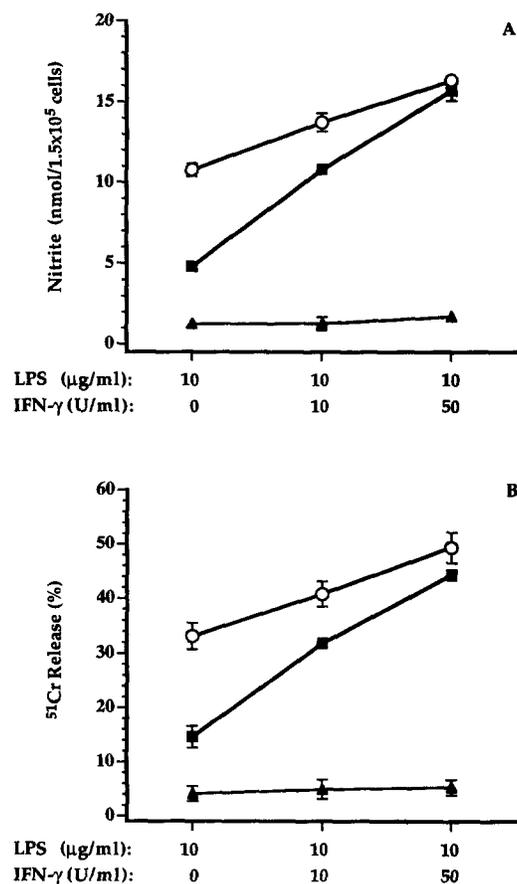
**Western Hybridizations.** Macrophages were plated on 10-cm plastic dishes and cultured for 24 h in media alone or media containing LPS or LPS and IFN- $\gamma$ . Total cellular protein was isolated as previously described (16). Protein concentration was normalized by comparison with BSA standards (Sigma Chemical Co.). The proteins were resolved on 5% SDS-PAGE and transferred onto 0.45- $\mu$ m nitrocellulose membranes (Costar Corp.). Membranes were probed with mouse IgG monoclonal anti-mouse iNOS (Transduction Laboratories, Lexington, KY; 1:1,000 dilution) at room temperature for 1 h. Protein was visualized by the electrochemiluminescence method (ECL; Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions.

**Northern Hybridizations.** Macrophages were plated and activated as described for Western hybridizations, and cells were lysed at the times indicated in the figure legends. Total RNA was isolated by Trizol reagent (GIBCO BRL) according to the manufacturer's instructions. RNA samples (15  $\mu$ g) were electrophoresed, transferred, and hybridized based on methods described by Fournier et al. (21). iNOS cDNA probes, a generous gift from Drs. Snyder and C.J. Lowenstein (Johns Hopkins University School of Medicine, Baltimore, MD), were radiolabeled with [<sup>32</sup>P]dCTP (3,000  $\mu$ Ci/mmol; New England Nuclear, Boston, MA) by random primer labeling (Prime-a-gene; Amersham Corp.). Blots were hybridized at 42°C overnight and then washed at 65°C for 1 h. Labeled blots were autoradiographed with Hyperfilm (Amersham Corp.) at –70°C for the times indicated in each experiment.  $\beta$ -actin levels were similarly examined, and served as controls to normalize RNA quantity.

**Densitometric Quantification of Blots.** Images were scanned on a Scanmaster 3+ scanner (Howtek, Inc. Hudson, NH). Band intensity was assessed on a Sun Sparcstation 5 computer (Sun Microsystems Computer Corp., Mountain View, CA) with BioImage Whole Band Analyzer software (BioImage Systems Corp., Ann Arbor, MI). In Northern analysis, band intensity was normalized to control  $\beta$ -actin signals to account for any differences in total RNA content for each sample. Band intensity was reported in relative densitometric units. The units were calculated as a fraction relative to the maximum sample density for each blot, which was provided an arbitrary value of one.

## Results

In response to activation with LPS (10  $\mu\text{g/ml}$ ), TB-PEM had a diminished capacity to produce NO and lyse syngeneic DA-3 tumor targets when compared with N-PEM. In contrast, when TB-PEM were stimulated with LPS (10  $\mu\text{g/ml}$ ) in combination with IFN- $\gamma$  (10 or 50 U/ml), they were able to produce NO and lyse targets at near normal levels (Fig. 1). IFN- $\gamma$  alone did not induce NO production or cytolytic activity in these cells. Moreover, N<sup>G</sup>-monomethyl-L-arginine (an inhibitor of NO production) blocked cytolytic activity, indicating that NO is the major mediator of tumoricidal function (data not shown). Because of their proximity to the developing tumor, we evaluated the ability of TAM to perform these functions. Upon stimulation with LPS, TAM were unable to produce NO or lyse DA-3 targets above basal levels and, further, TAM failed to exert these functions even after the addition of IFN- $\gamma$  (Fig. 1). A similar reduction in lytic capacity was exhibited toward allogeneic P815 murine mastocytoma targets by TAM (data not shown).



**Figure 1.** NO production and cytolytic capacity are altered in tumor bearers' macrophages. N-PEM (○), TB-PEM (■), and TAM (▲) were cultured at  $1.5 \times 10^5$  cells/well for 24 h in media containing LPS (10  $\mu\text{g/ml}$ ) alone or in combination with varying concentrations of IFN- $\gamma$  (10 or 50 U/ml). NO production (A) was evaluated in cell supernatants as nitrite by the Griess reaction. Cytotoxic activity (B) was measured as <sup>51</sup>Cr-release by syngeneic DA-3 targets. Values represent mean  $\pm$  SD of triplicate samples from a representative experiment.

Further analyses revealed that the enzymatic process employed to isolate TAM from the tumor tissue is unlikely to have contributed to the unresponsiveness of these macrophages as this procedure had no effect on NO production or cytolytic activity by either N-PEM or TB-PEM (data not shown). Additionally, the TAM were morphologically and functionally similar to PEM from normal and tumor-bearing mice (Table 1). Although the TAM exhibited a diminished capacity to produce NO and lyse targets for up to at least 72 h in culture, they retained other complex functions such as adherence to plastic, phagocytosis of latex beads, mitochondrial dehydrogenase activity, and the ability to exclude trypan blue during this same time period. The TAM were also able to produce IL-6, as measured by ELISA, at comparable levels to those produced by similarly treated N-PEM and TB-PEM (Table 2). These data indicate that the defects noted in the TAM were specific.

Western blots were performed to evaluate the expression of iNOS protein in PEM and TAM. As seen in Fig. 2, iNOS protein could not be detected in unstimulated PEM or TAM. However, when stimulated with LPS (10  $\mu\text{g/ml}$ ), N-PEM produced substantial quantities of the enzyme, whereas levels of this protein remained undetectable in TB-PEM and TAM. After stimulation with LPS (10  $\mu\text{g/ml}$ ) and IFN- $\gamma$  (50 U/ml) in combination, both populations of PEM produced approximately equal and high levels of the enzyme whereas iNOS protein levels remained undetectable in TAM. Furthermore, treatment with 50 U/ml of IFN- $\gamma$  alone did not produce detectable quantities of iNOS protein in any population (data not shown).

Northern hybridizations were performed to evaluate iNOS mRNA accumulation in PEM and TAM. After activation with LPS (10  $\mu\text{g/ml}$ ), detectable levels of iNOS

**Table 1.** Characteristics of Normal and Tumor Bearers' Peritoneal Macrophages and TAM

Properties	Percent		
	N-PEM	TB-PEM	TAM
Viability by trypan blue exclusion	95	97	96
Adherence to plastic	100	100	100
Macrophages/monocytes by			
H&E staining	94	97	95
Nonspecific esterase positive	93	94	87
Phagocytosis of latex beads	96	95	97
Activity of mitochondrial dehydrogenases*	100	101	99

N-PEM, TB-PEM, and TAM were isolated as described in Materials and Methods, and then adhered to plastic for 2 h before washing to remove nonadherent cells. The adherent cells were then cultured in complete media overnight before morphologic and functional assessment. These results represent data obtained from a representative experiment.

\*Expressed as a percentage of N-PEM formazan absorbance.

**Table 2.** *IL-6 Production by Normal and Tumor Bearers' Peritoneal Macrophages and TAM*

Population	IL-6 (pg/ml) $\pm$ SD	
	Unstimulated	LPS (10 $\mu$ g/ml)
N-PEM	42.7 $\pm$ 3.9	7,587.4 $\pm$ 163.9
TB-PEM	35.4 $\pm$ 0.7	7,077.5 $\pm$ 237.9
TAM-1	44.4 $\pm$ 5.1	5,664.2 $\pm$ 195.7
TAM-2	41.1 $\pm$ 2.6	9,154.0 $\pm$ 197.3

N-PEM, TB-PEM, and two separate populations of TAM were isolated as described in Materials and Methods. The N-PEM and TB-PEM were then subjected to the same enzymatic protocol employed to obtain the TAM. Cells were plated in 96-well microtiter plates at  $1.5 \times 10^5$  cells/well, and cultured in plain media or media containing LPS (10  $\mu$ g/ml) for 18 h. IL-6 levels were evaluated by ELISA, and results represent mean  $\pm$  SD of triplicate samples from a representative experiment.

message appeared in PEM from both types of mice by 4 h and reached maximum levels by 8 h (Fig. 3 A). After this time, the levels of iNOS mRNA began to decline. Although the pattern of iNOS mRNA accumulation in TB-PEM resembles that seen in N-PEM, the quantities of this message consistently remained depressed in the TB-PEM and became nearly undetectable by 24 h. At similar autoradiograph times, iNOS mRNA could not be observed in TAM at any time point after stimulation with LPS.

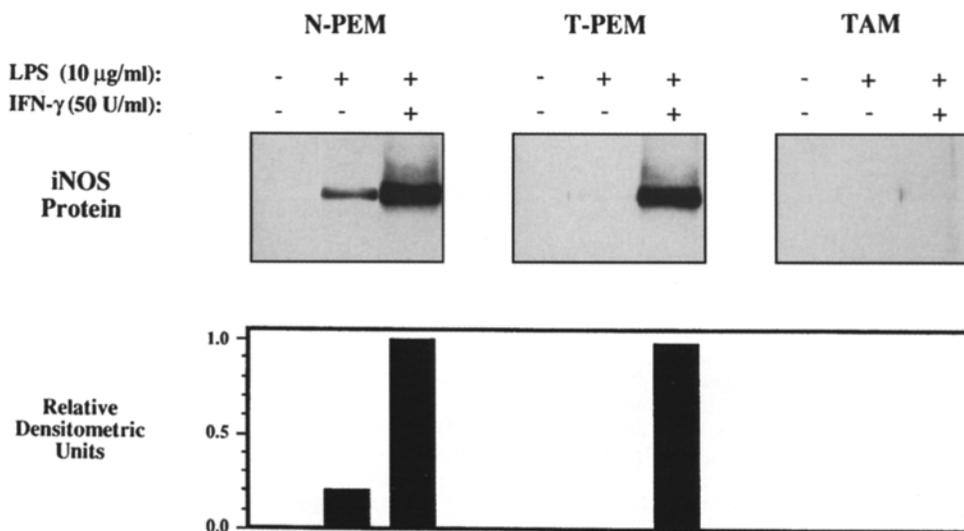
Similar experiments were performed after stimulation of PEM and TAM with LPS (10  $\mu$ g/ml) in combination with IFN- $\gamma$  (50 U/ml) (Fig. 3 B). iNOS mRNA levels increased more rapidly and remained elevated longer in PEM from normal and tumor-bearing mice when compared with the induction observed subsequent to stimulation with LPS alone. More importantly, the TB-PEM were able to recover the accumulation of iNOS mRNA to near normal levels after receiving the stronger signal provided by the

combination of LPS and IFN- $\gamma$ . Additionally, a significant amount of this message remained in PEM from normal and tumor-bearing mice at 24 h after stimulation. Again, TAM failed to accumulate detectable levels of iNOS mRNA at all time points.

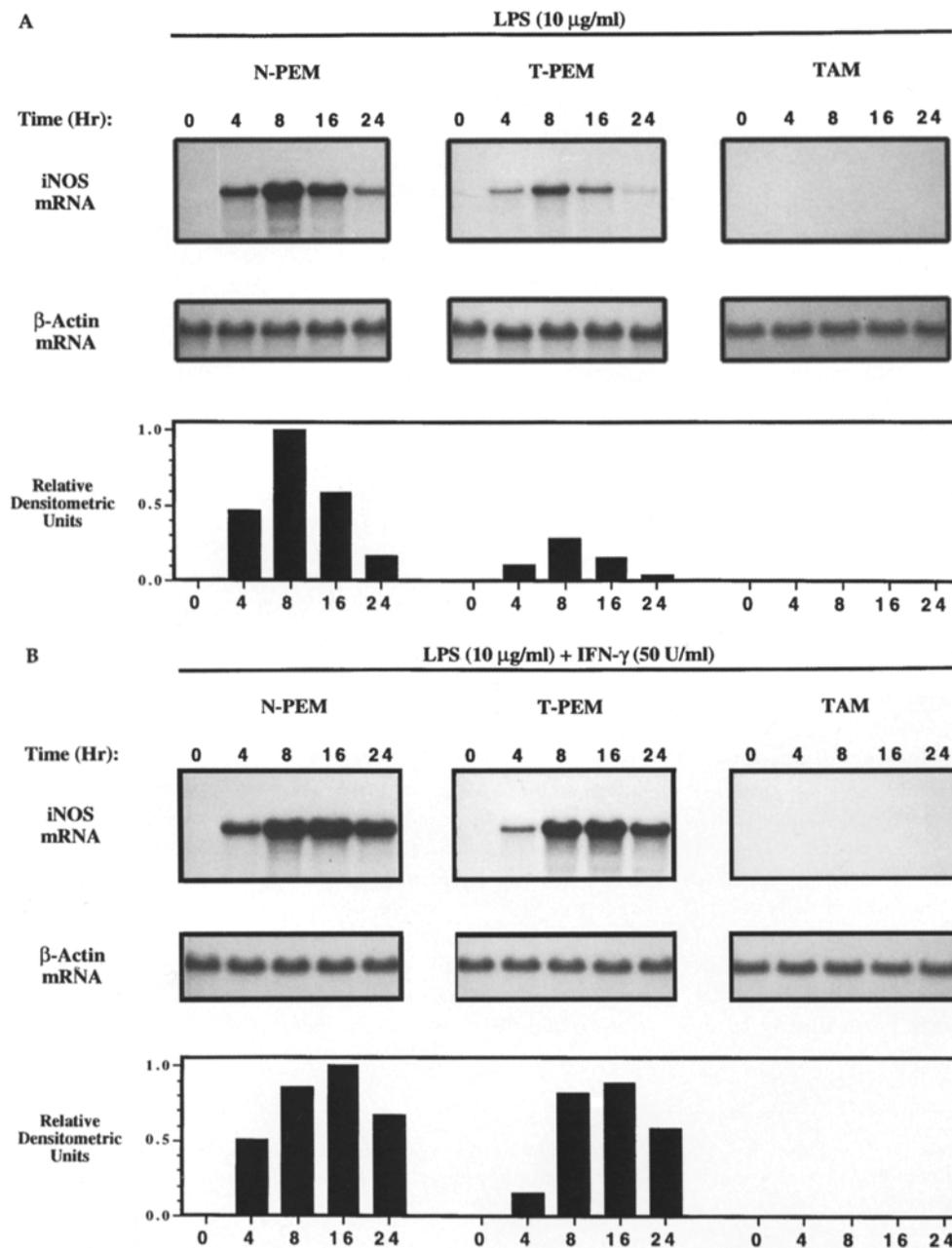
## Discussion

Activated macrophages play an important role in the destruction of a variety of tumor cells (1–5, 11–13); however, the integrity of the pathways for macrophage activation must be preserved for NO production and cytolytic activity to be maintained. In many cases, developing neoplasms appear to be capable of impairing steps in this process as a means of avoiding immune destruction (6–9).

Here we present evidence that a progressively growing mammary tumor can suppress host macrophage cytolytic activity through alterations in NO production as a consequence of reduced iNOS expression. Alterations in NO production and cytolytic activity became more profound in PEM with progression of the tumor (data not shown) and were most severe in macrophages isolated directly from the tumor. TAM were unable to produce significant amounts of NO or lyse targets regardless of stimulation employed. Our data suggest that the enzymatic process employed to obtain the TAM was not responsible for these alterations, and further experiments with resident peritoneal macrophages suggest that the elicitation process also was not involved in the functional differences noted between the PEM and TAM (data not shown). Despite the fact that TB-PEM and TAM exhibited alterations in their ability to produce NO and lyse targets, these cells remained fully capable of performing other complex functions, including the production and release of IL-6. These data indicate that the alterations leading to the inhibition in NO production and cytolytic activity were not occurring pervasively within the tumor bearers' macrophages, but rather were affecting specific functions.



**Figure 2.** iNOS protein levels are altered in tumor bearers' macrophages. N-PEM, TB-PEM, and TAM were cultured for 24 h in plain media or media containing LPS (10  $\mu$ g/ml) alone or in combination with IFN- $\gamma$  (50 U/ml). Total cellular protein was isolated and the levels of iNOS protein were assessed by Western analysis. Blots were exposed for 90 s to allow optimum visualization of bands. Levels of iNOS protein are depicted in the accompanying histogram as densitometric units relative to the maximum iNOS signal obtained for this blot.



**Figure 3.** Accumulation of iNOS mRNA in PEM and TAM in response to LPS alone or in combination with IFN- $\gamma$ . N-PEM, TB-PEM, and TAM were cultured in media containing LPS (10  $\mu$ g/ml) alone (A) or in combination with IFN- $\gamma$  (50 U/ml) (B). Total cellular RNA was isolated at the times indicated, and the levels of iNOS mRNA were assessed by Northern analysis. Blots from cells stimulated with LPS (A) were autoradiographed for 8 h, whereas those from cells stimulated with LPS and IFN- $\gamma$  (B) were autoradiographed for 2 h to prevent overexposure. After densitometric analysis, iNOS levels were normalized to their respective  $\beta$ -actin controls and presented in the accompanying histograms as densitometric units relative to the maximum iNOS signal for each blot.

Because of the intimate association of TAM with the cells of the developing tumor, TAM are believed to play a crucial role in regulating tumor growth (22–24). In this system, the almost complete loss of the capacity of TAM to produce NO and lyse tumor targets may explain the progressive nature of this mammary tumor. In support of the significance of NO production by infiltrating macrophages in controlling tumor growth, Yim et al. (25) have demonstrated that the production of NO by TAM leads to the in vivo regression of ultraviolet-induced murine skin cancers. Similarly, Umansky et al. (26) have recently reported that a transient arrest in T lymphoma metastasis was accompanied by an increase in NO production by liver Kupffer cells and

splenic macrophages, whereas metastatic expansion correlated with a reduction in NO production.

Our results concur with those of Mills et al. (27) and Murata et al. (28), who have recently demonstrated a suppression of NO production in intratumor macrophages and cerebral endothelial cells, respectively, associated with the growth of tumor cells. In addition, evidence that NO production by macrophages within tumors may be reduced was suggested as early as 1977, when Spitalny and North (29) noted that tumors act as privileged sites for the growth of *Listeria monocytogenes*, a bacteria that is now known to be susceptible to killing by macrophage-produced NO (30). However, the molecular events leading to the reduced pro-

duction of NO in response to tumor growth have not been previously evaluated.

In these studies, Northern and Western blots from tumor bearers' macrophages revealed that iNOS mRNA and protein levels correlated directly with the ability of each macrophage population to both produce NO and lyse tumor targets. Furthermore, the addition of exogenous L-arginine had no effect on NO production (data not shown), ruling out the possibility that diminished substrate concentrations were contributing to the observations. However, effects on the availability of cofactors cannot be dismissed.

Using immunohistochemistry, Thomsen et al. (31) have recently demonstrated an increase in iNOS protein in macrophages within sections of human breast cancer. Although our data contrast with these results, we have found that, after prolonged exposure of both Western and Northern hybridizations, trace quantities of iNOS protein and mRNA, respectively, are detectable in TAM from our system. In addition, where detectable, these molecules appear to be of identical size to those from N-PEM, suggesting that the basis for the altered levels of iNOS protein is more likely to be reduced synthesis rather than increased degradation of the protein, and that truncation or incorrect splicing of the mRNA has not occurred and is therefore not likely to have contributed to the reduced iNOS protein levels.

We have demonstrated that qualitative and quantitative differences exist in the alterations noted in TAM and TB-PEM. There appears to be a spatial gradient of suppression of macrophage cytolytic activity and iNOS expression, whereby macrophages isolated directly from the tumor exhibit a more profound suppression than the more distally located PEM. In the tumor microenvironment, TAM may be affected by higher concentrations of tumor-derived or tumor-induced factors, or by cells present within the tumor. Alternatively, TAM dysfunction may be secondary to the

maturation stage of these cells. TAM are believed to arise from blood monocytes that are recruited into the tumor by tumor factors (23). The fact that the TAM do not recover their ability to produce NO or lyse targets even after several days in culture suggests that cells or factors within the tumor may have induced the infiltrating monocytes to have remained in or to have differentiated into a state less capable of responding to LPS and/or IFN- $\gamma$ . In support of this idea, Moore and McBride (32) have hypothesized that within the tumor microenvironment, infiltrating monocytes may be unable to fully mature into tissue macrophages capable of lysing targets. Moreover, it has been demonstrated that the less mature WEHI-3 macrophage cell line similarly does not respond to IFN- $\gamma$  for the induction of cytolytic activity (33), and that genes containing IFN-stimulated response element or gamma-activation site binding motifs within their promoter region are induced only in the more mature RAW 264.7 macrophage cell line (33, 34). TAM isolated from a murine B16 melanoma display a similar, less mature response to IFN- $\gamma$  as observed in the WEHI-3 cells; Paulnock (35) suggests that macrophages recruited during tumor growth may be under regulatory constraints which limit their activation to the tumoricidal state. Lastly, conditions of hypoxia and nutrient depletion within the tumor may further reduce the effectiveness of the macrophages to lyse cancerous cells, allowing the tumor to grow progressively despite the presence of TAM (24). Our results indicate that the reduced tumoricidal capacity of macrophages from mammary tumor-bearing mice results from diminished iNOS expression, and that this defect is more pronounced in macrophages directly isolated from the tumor. This inability of tumor-infiltrating macrophages to produce NO and lyse tumor cells may contribute to the continued development of the tumor and the eventual subversion of the host.

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