[CANCER RESEARCH 53, 5507–5511, November 15, 1993]

Macrophage Nitric Oxide Synthesis Delays Progression of Ultraviolet Light-induced Murine Skin Cancers[1]

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ABSTRACT

The role of macrophages in the host immune response against cancers remains uncertain. Since nitric oxide synthesis represents a significant macrophage antitumor mechanism *in vitro*, we evaluated whether NO was synthesized during the immune response to growing murine skin cancers. NO synthesis was readily detectable in enzymatically dissociated tumors (RD-995 and LR-298) and was inhibited by N^{ω}-monomethyl-L-arginine (MLA) and by macrophage depletion. Nitrosylation of iron-sulfur and heme complexes was observed in these tumors using electron paramagnetic resonance spectroscopy. NO production in the presence of increasing concentrations of MLA correlated inversely with tumor cell proliferation *in vitro*. To elucidate the role of NO during *in vivo* tumor progression, tumor-bearing mice were treated with continuous infusions of the nitric oxide synthase inhibitor MLA. MLA-treated mice demonstrated increased growth and delayed rejection of the highly antigenic UV radiation-induced regressor tumor LR-298. These experiments demonstrate that macrophage-derived NO synthesis can contribute to the antitumor immune response *in vivo*.

INTRODUCTION

Cancers growing in animals and humans are variably infiltrated by host T-cells (1–3) and macrophages (4–6). Infiltrating lymphocytes have the capacity to lyse tumor cells (7–10), but the role of TIM[4] has not been clarified. The degree of tumor infiltration by macrophages may correlate with a decrease in metastases or increased survival (5, 6, 11–13), but some reports have suggested that macrophage infiltration of tumors may correlate with adverse effects on host immune function or tumor growth (14–18). Attempts to evaluate the tumoricidal effector function of both mouse and human TIM have suggested that these cells are not in an activated state and require stimulation by cytokines for full antitumor activity (18, 19). Despite these observations, TIM frequently express major histocompatibility complex class II antigens, suggesting priming by cytokines such as IFNγ or macrophage-colony-stimulating factor (15). Pro-inflammatory cytokines, such as tumor necrosis factor α, IFNγ, IL-1, and IL-6 are also known to be secreted within tumors (20–22). TIM also have the potential to secrete inhibitors of cytotoxic lymphocyte function, such as transforming growth factor β and prostaglandins (reviewed in Ref. 14). Thus both antitumor and tumor-promoting activities of macrophages appear possible.

Recent investigations have established that macrophages acquire potent antitumor and antimicrobial activity following exposure to the cytokines IFNγ plus either IL-1 or tumor necrosis factor (recently reviewed in Refs. 23 and 24). In 1987, Hibbs *et al.* (25) reported that the induction of macrophage cytotoxicity was dependent on the amino acid L-arginine and that the effector molecule of L-arginine-dependent cytotoxicity was NO (26–28). Cytotoxic activated macrophages had been shown previously to have a variety of effects on tumor cells, including inhibition of mitochondrial respiration and DNA replication (29). Subsequent studies suggested that the intracellular targets of NO were intracellular iron-sulfur [4Fe-4S] prosthetic groups of Complex I and Complex II of the mitochondrial electron transport system and the citric acid cycle enzyme aconitase, as well as nonheme iron in ribonucleotide reductase (30–34). Iron-nitrosyl-sulfur complexes $[Fe(RS)_2(NO)_2]^-$ were later identified directly using EPR spectroscopy (35–37). The observation that MLA inhibits the cytokine-induced NOS provided another method to identify cell- mediated immune responses dependent on the L-arginine:NO pathway (25, 38).

Since cytokines that activate the inducible NOS may be present in the tumor microenvironment (14), we evaluated the activity of the inducible L-arginine:NO pathway in UV radiation-induced progressor and regressor murine skin cancers.

MATERIALS AND METHODS

Animals. Specific pathogen-free C3H/HeN mice (ages 6–8 weeks) were obtained from Harlan-Sprague Dawley (Indianapolis, IN) and housed in the Salt Lake City Veterans Administration Medical Center Animal Care Facility. Mice were maintained under guidelines established by the Salt Lake City Veterans Administration Medical Center Animal Care Committee, which also approved experimental protocols. Mice were age and sex matched at the onset of each experiment. All experiments described in this paper were performed at least twice with highly concordant results.

Tumor Cell Lines. RD-995 and LR-358 skin tumors were induced in C3H/HeN mice by chronic UV light exposure as described previously (39). These tumors are spindle-cell variants of squamous cell carcinomas and stain with anti-cytokeratin monoclonal antibodies. RD-995 grows progressively in normal mice following s.c. implantation and is therefore termed a "progressor" tumor. In contrast, LR-298 is rejected by normal mice, will grow only in UV-irradiated or immunosuppressed mice, and has a "regressor" phenotype in normal animals (40). Tumor cells were maintained by serial passage in syngeneic mice or by culture in RPMI 1640 supplemented with 5% fetal calf serum (Hyclone Laboratories, Inc., Logan, UT), 100 units/ml penicillin G (Sigma Chemical Co., St. Louis, MO), 50 μg/ml streptomycin (Sigma), and 2 mM glutamine (working medium; Sigma).

Tumor cells were released from culture flasks by rinsing monolayers with isotonic phosphate-buffered saline (pH 7.4) followed by brief treatment (5–10 min) with a trypsin-EDTA solution [0.25% trypsin from hog pancreas, 490 USP units/mg (ICN Biomedicals, Cleveland, OH); 0.1 mM EDTA in Hanks' balanced salt solution without Ca^{2+}/Mg^{2+} (Flow Laboratories, McLean, VA)]. Cells were then extensively washed in serum-containing medium.

Mycoplasmal infection was excluded by surveillance cultures of supernatants derived from the tumor cell line grown in antibiotic-free media on pleuropneumonia-like organism agar (Baxter Health Care Corporation, McGaw Park, IL) supplemented with 10% horse serum (Hyclone), 25% yeast extract (Gibco BRL, Gaithersburg, MD), and 10 units penicillin G/ml.

Enzymatic Dissociation of Tumors Growing *in Vivo*. When tumors implanted into mice had grown to ~1.5 cm in diameter, tumors were excised using sterile technique and minced into 1-mm fragments. A single cell suspension was obtained by digesting tumor fragments with 0.1% collagenase

Received 7/2/93; accepted 9/9/93.

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[1] This work was supported by CATBRM Cooperative Agreement U01-CA58248 from the Biological Response Modifiers Program of the National Cancer Institute as well as medical research funds from the Department of Veterans Affairs. The University of Utah Cancer Center Core Flow Cytometry Facility is supported by Clinical Cancer Center Core Grant CA 42014 from the NIH.

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[4] The abbreviations used are: TIM, tumor-infiltrating macrophages; IFNγ, γ-interferon; IL, interleukin; EPR, electron paramagnetic resonance; MLA, N^{ω}-monomethyl-L-arginine; NOS, nitric oxide synthase; BCG, *Bacillus Calmette-Guérin*; dThd, thymidine.

(Sigma) and 0.1% dispase (neutral protease; Boehringer Mannheim, Indianapolis, IN) in Hanks' balanced salt solution (without Ca^{2+}/Mg^{2+}) at room temperature for 2–3 h. Cells were washed three times in RPMI 1640 (BioWhittaker, Walkersville, MD) containing 5% fetal calf serum (Hyclone), 1 mM L-glutamine (Sigma), 100 units/ml penicillin G (Sigma), and 50 μg/ml streptomycin (Sigma; working medium).

Assay for NO Generation in Cell Cultures. Nitrate production was assayed by a modification of the colorimetric Griess reaction (41). Briefly, 7.5×10^5 freshly dissociated or cultured tumor cells were placed into quadruplicate microtiter wells (in working medium with 5% fetal calf serum). In some experiments, varying concentrations (0–500 μM) of the NOS inhibitor MLA were added to cell cultures at the onset of the culture, maintaining a constant final volume of 0.2 ml/well. After 48 h, 50-μl samples of cell culture supernatant from each well were harvested into other (flat-bottomed) microtiter plates and incubated with 100 μl of a 1:1 mixture of 1% sulfanilamide in 30% acetic acid with 0.1% *N*-(1-napthyl)ethylenediamine dihydrochloride in 60% acetic acid. Once mixed, this reagent was used within 5 min. Following a 3-min incubation, sample absorbance at 570 nm was evaluated in a microtiter plate reader. Concentrations were determined from a linear standard curve generated from 6.25–100 μM sodium nitrite in medium. Results are presented as mean ± SD. RPMI 1640 with or without 5% fetal calf serum contained <0.5 μM nitrite.

Since nitrite (NO_2^-) is further oxidized to nitrate (NO_3^-) in the presence of oxyhemoglobin, this metabolite can be used to evaluate NO synthesis *in vivo* (27, 42). The total nitrate plus nitrite concentration in urine samples was measured using a modification of techniques described previously (41). Briefly, 50 μl of urine samples were dispensed into triplicate wells of (flat-bottomed) microtiter plates and incubated for 90 min with 50 μl of a frozen suspension of *Escherichia coli* induced for the enzyme nitrate reductase (41). Following this incubation, plates were centrifuged and 50 μl of supernatant were transferred to a clean microtiter plate and analyzed for nitrite as described previously. Nitrate concentration in each sample was calculated from the total nitrite following bacterial reduction minus the concentration prior to reduction. Samples were always assayed in triplicate at each time point. Pooled urine from four animal groups was analyzed in triplicate for urinary nitrate excretion to establish the degree of NOS inhibition by MLA.

Immunomagnetic Bead Depletion of Macrophages from Dissociated Tumor. Discussed below is the effect of Mac-1^+ cell depletion on nitric oxide synthesis of freshly dissociated tumor. A single cell suspension was prepared by an enzymatic dissociation of RD-995 tumor. Cells were preincubated at 2×10^7 cells/ml in RPMI 1640 with saturating concentrations of anti-Mac-1 monoclonal antibody (affinity purified M1/70.15.11.5.HL; American Type Culture Collection, Rockville, MD) for 30 min at 4°C on a rotator. After two washings with RPMI 1640, cells were resuspended at 2×10^7 cells/ml and incubated with saturating concentrations of sheep anti-rat IgG-coated paramagnetic polystyrene beads (Dynabeads M-450; Dynal Inc., Great Neck, NY) for an additional 30 min at 4°C during slow rotation. The cell-bead mixture was placed into a magnetic particle concentrator (Dynal MPC-1; Dynal) and unbound cells (supernatant) was removed. The harvested unbound cells were washed twice and cultured at 7×10^5 cells/ml in 0.2 ml RPMI 1640/well in microtiter plates. After 48 h in culture (37°C; 5% CO_2), nitrite concentrations in the cell-free culture supernatants were measured as a reflection of NO synthesis. Freshly dissociated RD-995 tumor cells without depletion of Mac-1^+ cells served as a control.

Effect of NO on Tumor Cell Proliferation. Microtiter wells containing 7.5×10^5 freshly dissociated tumor cells were cultured for 30 h and then pulsed with tritiated thymidine (0.5 μCi/well [^{3}H]dThd, 2.0 Ci/mmol; DuPont, Boston, MA) for an additional 18 h (total 48 h), maintaining a constant final volume of 200 μl/well. Following harvesting onto glass fiber filters using a PhD harvester (Cambridge Technology, Cambridge, MA), samples were suspended in Optifluor scintillation fluid (Packard) and the incorporated [^{3}H]dThd was measured in a Packard Tri-Carb 1500 scintillation counter (Packard). Each assay was performed at least in triplicate and the results were presented as mean cpm ± SD.

EPR Spectroscopy. Cell suspensions (~2×10^8 cells/ml) derived from enzymatically dissociated s.c. RD-995 tumors or tumor cells grown in cell culture were placed into quartz EPR tubes and centrifuged for 5 min at 500 × *g*. The volume of the packed cells was controlled to maintain a final packed cell volume of 140 μl/tube (20-mm high). Samples were stored at −70°C until assayed. EPR spectra of the samples were recorded on a Bruker ER-200D spectrometer operating at a microwave frequency of 9.4 GHz at 77°K. A modulation frequency of 100 kHz and a modulation amplitude of 10 Gauss were used. The field was calibrated periodically by using a sample of reduced methyl viologen. The power was 1 mW and each tracing was corrected to an instrument gain of 2.5×10^5. Enzymatic dissociation with either trypsin-EDTA or collagenase-dispase had no demonstrable effect on EPR signals.[5]

Continuous Infusion of MLA into Tumor-bearing Mice. Two groups of eight normal C3H/HeN mice were acclimated to a nitrate/nitrite-free diet (Ziegler Brothers, Gardners, PA) in metabolic cages (Nalgene, Rochester, NY) for 1 week. On days −1, 9, and 18 of the experiment, mice were implanted s.c. with Alzet continuous infusion pumps (Model 2001; Alza Corporation) containing 0.2 ml of 3.38 M MLA. This dose results in a calculated delivery of 18.52 mg MLA/mouse/day. In preliminary experiments, we demonstrated that this is sufficient to achieve 50–70% decrease in urinary nitrate excretion in mice on a nitrate-free diet and to prevent increases from suppressed levels due to cytokine stimulation triggered by BCG infection.[6] Control mice underwent anesthetic administration and s.c. tunnel formation but no pump implantation. On day 0, mice were implanted s.c. with 2.6×10^6 LR-298 tumor cells. Daily bidimensional tumor measurements were performed. Results are expressed as the mean tumor cross-sectional area ± SEM. This experiment was repeated twice with similar results.

RESULTS

Normal C3H/HeN mice were implanted s.c. with 3×10^6 syngeneic RD-995 (progressor) or LR-298 (regressor) tumor cells. When tumors reached 2–3 cm^2 in size, they were excised under sterile conditions and enzymatically dissociated into a single cell suspension. Dissociated cells were placed into 96-well flat-bottomed microtiter plates (final volume, 7.5×10^4 cells/well in 200 μl medium). After a 48-h culture (37°C; 5% CO_2), cell culture supernatants were evaluated for nitrite (NO_2^-) production, a metabolite of nitric oxide. Long-term cultures of RD-995 or LR-298 tumors, which were depleted of infiltrating host cells, served as controls. Supernatants of dissociated RD-995 and LR-298 tumors were found to contain significant nitrite (38 ± 3 and 70 ± 3 μM, respectively). In contrast, supernatants derived from the same tumor cells passaged *in vitro* 6–9 times produced little nitrite (RD-995, 0.5 ± 0.2 μM; LR-298, 0.6 ± 0.5 μM). This result suggested that NO synthesis was a product of cell-mediated immune responses to the tumor *in vivo*.

While macrophages represent a major source of nitric oxide, other cells such as endothelial cells and neural tissue can also secrete NO (43). Furthermore, Amber *et al.* (44, 45) have established that tumor cell lines are capable of synthesizing NO in response to cytokine stimulation. To test whether activated macrophages were a major source of NO within tumors growing *in vivo*, freshly dissociated tumor cells were depleted of macrophages by immunomagnetic separation. The effectiveness of the Mac-1 depletion of RD-995 tumor cell suspensions was confirmed by flow cytometry (unfractionated cells, 10.7% MAC-1^+; following MAC-1 depletion, <0.2%; >98% reduction). Cells were cultured in six replicate wells and assayed for NO_2^- synthesis. Macrophage depletion resulted in a 72% decrease in nitrite production (from 17 ± 0.58 to 4.75 ± 0.47 μM) compared to nondepleted tumor cell suspensions, establishing that TIM within progressively growing murine tumors accounted for a significant proportion of NO synthesis. Histological evaluation of tumors confirmed that lymphocytes and macrophages represented the only host-derived cells infiltrating the tumors. In addition, cytological examination of immunomagnetic bead-adherent cells revealed predominantly macrophages, excluding other MAC-1^+ cells (*e.g.*, neutrophils) as a significant source of NO (data not shown).

To further establish that NO was present within tumors in concentrations sufficient to produce cytostasis, we sought the typical EPR signals attributable to nitrosylation of cellular iron-sulfur complexes

[5] Unpublished data.
[6] Submitted for publication.

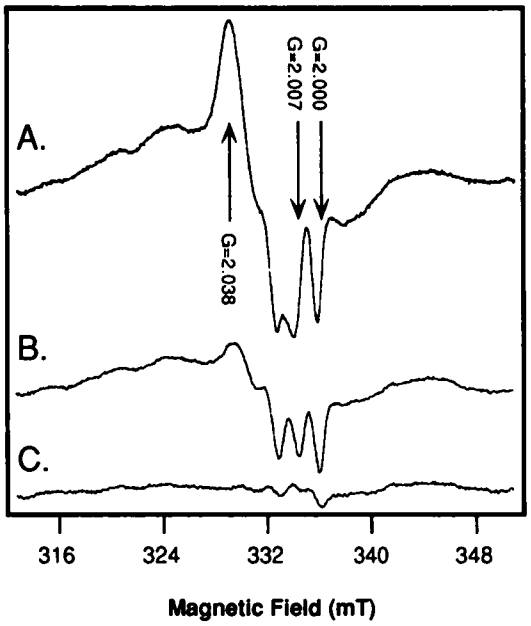

Fig. 1. Effect of MLA on EPR signal induction in freshly dissociated tumors. Cell suspensions derived from enzymatically dissociated s.c. RD-995 tumors were placed into cell culture at 7.5×10^5 cells/ml in RPMI 1640 with 5% fetal calf serum without (*A*) or with (*B*) 500 μM MLA in 75-cm^2 culture flasks (in a final volume of 20 ml/flask). After 48 h in culture (37°C; 5% CO_2), cells were harvested using a cell scraper and washed twice in serum-free medium. Thick cell suspensions were dispensed into quartz EPR tubes and centrifuged for 5 min at 500 × *g*. The volume of the packed cells was controlled to maintain a final packed cell volume of 140 μl/tube (20 mm high). Samples were stored at −70°C until assayed. EPR spectra were recorded on a Bruker ER-200D spectrometer operating at a microwave frequency of 9.4 GHz at 77°K. A modulation frequency of 100 kHz and a modulation amplitude of 10 Gauss were used. The field was calibrated periodically by using a sample of reduced methyl viologen. The power was 1 mW and each tracing is corrected to an instrument gain of 2.5×10^5. *Tracings A* and *B* contain both $Fe(RS)_2(NO)_2$ signals (upward deflection at G = 2.038) and nitrosyl heme signals (a downward signal centered at G = 2.007). The magnitude of both signals was decreased by MLA exposure (*B*). Long-term *in vitro* RD-995 cultures served as a control (*C*). These cells exhibited a semiquinone signal at only G = 2.000.

in tumors growing *in vivo*. Freshly dissociated RD-995 tumor cells cultured for 48 h without added MLA demonstrated prominent $Fe(RS)_2(NO)_2$ signals as well as nitrosyl heme signals (Fig. 1*A*). Similar signals were also seen if tumors were assayed by EPR immediately following mechanical or enzymatic dissociation. The addition of MLA to dissociated tumor cells resulted in a marked decrease in both nitrite production (82% decrease) and EPR signals derived from $Fe(RS)_2(NO)_2$ complex formation (73% decrease; Fig. 1*B*). In contrast, the same tumor cell line cultured *in vitro* for 5–9 passages exhibited only a small (G = 2.000) signal attributable to semiquinones but no NO-derived signals (Fig. 1*C*). These results establish that NO generation is required to produce detectable $Fe(RS)_2(NO)_2$ and nitrosyl-heme complex formation in tumors *in vivo*.

MLA was used to establish that nitrite detected in cell cultures was a product of the cytokine-induced L-arginine:NO pathway. Cells from freshly dissociated RD-995 tumor were placed into cell culture with varying concentrations (0–500 μM) MLA. MLA inhibited nitrite production in a dose-dependent manner (Fig. 2). In contrast, MLA had no effect on the trace amounts of nitrite produced by serially cultured tumor cells (data not shown). Experiments performed in the presence of 10 μg/ml polymyxin B to exclude inadvertent endotoxin contamination gave similar results. Bacterial lipopolysaccharide was undetectable in the culture medium, fetal calf serum, and the collagenase/dispase solution by *Limulus* lysate assay (sensitivity, 100 pg/ml).

One of the major effects of NO on tumor growth is inhibition of ribonucleotide reductase, a rate-limiting step in DNA synthesis (34, 46). To determine if NO synthesis within tumors inhibited tumor cell proliferation, we measured thymidine incorporation into DNA in the presence of increasing concentrations of MLA. Thymidine incorporation by freshly dissociated tumor cells was increased in the presence of MLA, suggesting that NO synthesis within the tumor had significant antiproliferative effects. The enhancement of proliferation in the presence of MLA was reciprocally related to nitrite levels (Fig. 2). In contrast, tumor cells in long-term cultures *in vitro* demonstrated high levels of [^{3}H]dThd incorporation which was unaffected by MLA (data not shown).

To test the role of NO in inhibiting tumor growth *in vivo*, mice were first acclimated to metabolic cages and a low-nitrate diet for 1 week; urinary nitrate excretion was measured in sequential 24-h urine collections. Since dietary nitrate intake and endogenous NO synthesis are the major sources of urinary nitrate excretion (47), this protocol allows assessment of NOS activity within intact animals. One day prior to s.c. tumor implantation (day −1), normal C3H/HeN mice were

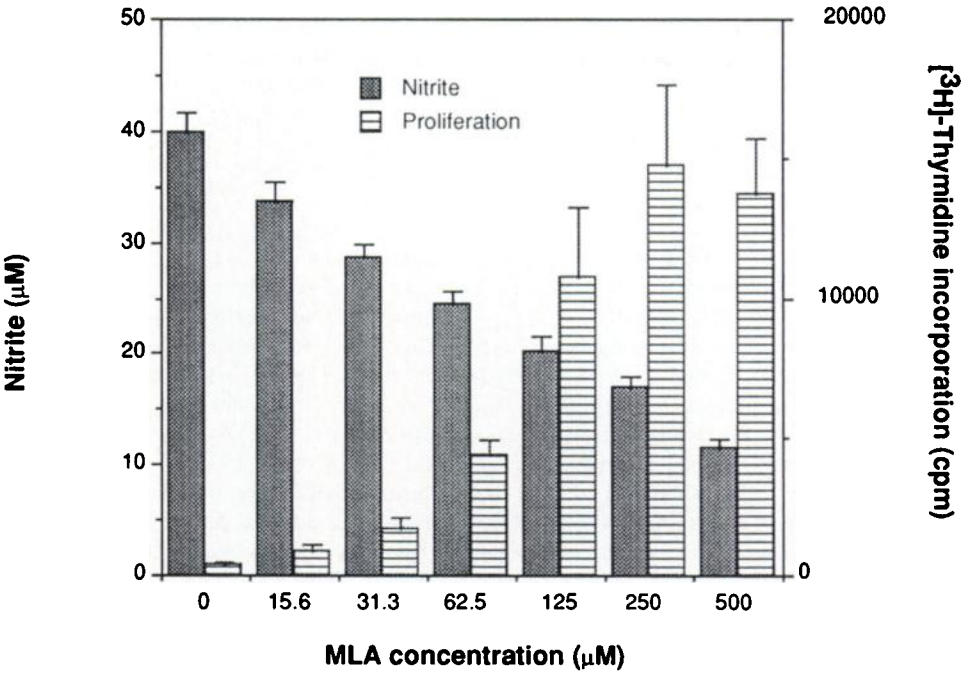

Fig. 2. Effect of MLA on NO synthesis and proliferation of freshly dissociated murine skin cancers. RD-995 implanted s.c. fibrosarcomas (growing in C3H/HeN mice) were excised, enzymatically dissociated, and then washed extensively in serum-containing medium. Endogenous NO production within tumors was assessed by culturing 7.5×10^5 cells/well in microtiter plates (in RPMI 1640 with 5% fetal calf serum) with varying concentrations (0–500 μM) of MLA, maintaining a constant final volume of 0.2 ml/well. After a 48-h culture (37°C; 5% CO_2), nitrite (NO_2^-, a stable degradation product of NO) was measured in 50-μl culture supernatant by a modification of the colorimetric reaction using the Griess reagent. Parallel wells containing freshly dissociated tumor cells were cultured for 30 h and then pulsed with tritiated thymidine (0.5 μCi/well [^{3}H]dThd, 2.0 Ci/mmol) for an additional 18 h (total 48 h). Following harvesting onto glass fiber filters, these cells were analyzed for thymidine incorporation into DNA by scintillation counting. Results are mean ± SD (*bars*) of quadruplicate wells. Parental RD-995 cell line carried *in vitro* contained 3.0 ± 1.0 μM nitrate/well and exhibited a high proliferative rate (150,000 ± 10,000 cpm/7.5×10^4 cells). These values were unaffected by MLA addition to cultures (data not shown). RPMI 1640 with or without 5% fetal calf serum contained less than 1 μM nitrite.

implanted with osmotic continuous infusion pumps designed to deliver 18.52 mg MLA/mouse/day. The dose of MLA required for these experiments was defined in preliminary experiments, and continuous infusion of this dose resulted in 50–70% depression in urinary nitrate excretion in control mice for a period of 8–9 days. This dose also decreased nitrate excretion 83% in mice infected with BCG[6]. Control mice underwent sham pump implantation. One day following pump implantation, mice received injections s.c. of 2.6×10^6 LR-298 tumor cells. This UV-induced regressor tumor was chosen since it elicits a strong immune response that results in rejection beginning 6–8 days postimplantation. To determine if this tumor would be rejected more slowly in MLA-treated mice, bidimensional tumor size and urinary nitrate excretion were measured daily during the course of the experiment. The results of two experiments demonstrated that MLA treatment resulted in increased tumor growth. A longer time to peak tumor growth and rejection was also observed. All tumors were eventually rejected (Fig. 3*A*). In order to confirm the effectiveness of NOS inhibition, pooled daily urines were analyzed for urinary nitrate excretion (Fig. 3*B*). Approximately 60% suppression of nitrate excretion was maintained in the experimental mice for the 26-day duration of the experiment, which required three successive pump implantations.

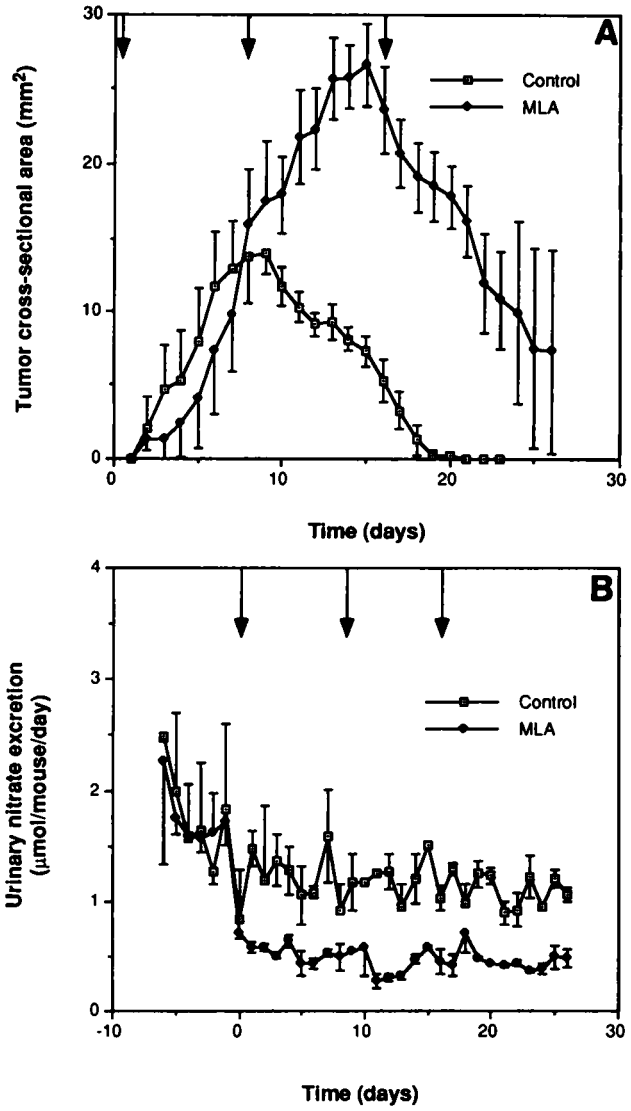

Fig. 3. Evaluation of the role of NO synthesis in the host defense against murine tumors. In *A*, two groups of eight normal C3H/HeN mice were acclimated to a nitrate-free diet in metabolic cages for 1 week. On days −1, 9, and 18 of the experiment, mice were implanted s.c. with Alzet continuous infusion pumps (Model 2001), containing 0.2 ml of 3.38 M MLA (*arrows*). This results in a calculated delivery of 18.52 mg MLA/mouse/day. In preliminary experiments, we demonstrated that this is sufficient to achieve 50–70% decrease in urinary nitrate excretion in mice on a nitrate-free diet and to prevent increases from suppressed levels due to cytokine stimulation triggered by BCG infection. Control mice underwent sham pump implantation. On day 0, mice were implanted s.c. with 2.6×10^6 LR-298 tumor cells. Daily bidimensional tumor measurements were performed (mean tumor cross-sectional area ± SEM). In *B*, pooled urine from four animal groups were analyzed in triplicate for urinary nitrate excretion to establish the degree of NOS inhibition by MLA [mean ± SD (*bars*)]. This experiment was repeated twice with similar results.

DISCUSSION

Our experiments have demonstrated that NO is produced during the cellular immune response to highly antigenic skin cancers implanted in mice. Inhibition of the NOS enzyme by MLA markedly reduced the formation of nitrite, the major metabolite in aqueous solution. A significant proportion of NO synthesis appeared to be macrophage derived as demonstrated by immunomagnetic depletion of macrophages within tumors. Since macrophage depletion could not completely reverse NO production, cytokine-induced NO synthesis within tumor cells may also have contributed to the observed results. The induction of endogenous NOS activity by cytokines has been reported in a wide variety of murine cells, including some cancers (44, 45).

EPR spectroscopy of cancers growing *in vivo* demonstrated both $Fe(RS)_2(NO)_2$ and nitrosyl-heme signals as evidence of local NO synthesis within the tumor microenvironment. Evidence of similar heme-nitrosylation products can be detected within some human tumors as well.[5] While the $Fe(RS)_2(NO)_2$ signals are believed to be due to nitrosylation of iron-sulfur-containing prosthetic groups of enzymes such as aconitase (Krebs cycle) and Complexes I and II (electron transport chain), the origin of nitrosyl-heme signals is as yet uncertain. While nitrosylation of contaminating hemoglobin or myoglobin in samples could account for this signal, this signal could alternatively be the result of nitrosylation of heme prosthetic groups of enzymes in tumor cells. Possible candidates include cytochromes, catalase, and peroxidases. NO is thought to trigger intracellular signals via nitrosylation of a heme prosthetic group of guanylate cyclase (48). Further studies are in progress to evaluate the origin of these signals. Induction of NO synthesis appeared to be confined to the cancer because other tissues of tumor-bearing mice (*e.g.*, adjacent abdominal muscle and lungs) did not contain EPR nitrosylation signals.[6] Urinary nitrate excretion, a reflection of total body NO synthesis, also appeared to remain stable during progressor tumor growth in mice.

The present study clearly demonstrates that endogenous NO secretion during the immune response to tumors can depress the ability of tumor cells to proliferate. Inhibition of NOS in freshly dissociated tumors by increasing concentrations of MLA resulted in increased tumor cell proliferation *in vitro*. To further examine the role of NO in delaying tumor progression, we developed a murine model that allowed us to chronically inhibit the NO synthesis using continuous infusions of MLA. While only partial (~60%) inhibition of NO production was achieved *in vivo*, MLA infusions resulted in increased tumor growth, delayed immune recognition, and delayed rejection in mice bearing a highly antigenic UV regressor tumor. Intake of food and water and the weight of mice receiving MLA infusions did not differ significantly from control mice (data not shown).

Macrophage-derived NO production may contribute to the development of cell- mediated immune responses in a number of ways. These include induction of localized vasodilation and increasing cell localization to the tumor via the bloodstream. Cytokine-activated macrophages are known to increase secretion of IL-1, tumor necrosis factor, and IL-6, which may also contribute to antitumor responses by priming natural killer or T-cells for activation via IL-2 (49–52). These cytokines may also directly induce NOS activity within tumor cells, resulting in direct antitumor effects (44, 45). Our results demonstrate that macrophages are important participants in cell-mediated immune responses against antigenic tumors *in vivo* and serve to decrease the

rate of tumor progression. It should be noted, however, that other investigators have demonstrated that NO can depress T-cell-mediated immune responses (53, 54). The interrelationship of immunostimulatory and immunosuppressive roles of NO is likely to be complex and will require further evaluation. The role of the L-arginine:NO pathway in less antigenic or spontaneous tumors also remains to be clarified.

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