Nitric oxide is an important mediator for tumoricidal activity *in vivo*

(murine ovarian teratocarcinoma cells/NIH:OVCAR-3 cells/cancer)

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ABSTRACT   When cultured *in vitro*, peritoneal macrophages, obtained from mice previously inoculated with bacillus Calmette-Guérin, release nitric oxide, which is cytostatic and/or cytolytic for tumor cells. However, it is not known whether nitric oxide has antitumor effects *in vivo*. Here we demonstrate that nitric oxide is an important mediator of host resistance to syngeneic and xenogeneic ovarian tumor grafts in C3HeB/FeJ mice. A murine ovarian teratocarcinoma cell line, utilized to study the mechanism of bacillus Calmette-Guérin-induced host resistance to a syngeneic ovarian tumor, proliferated when transplanted intraperitoneally. Marked tumoricidal activity was observed, however, when these murine ovarian teratocarcinoma cells were transplanted 8 days after intraperitoneal bacillus Calmette-Guérin inoculation. In studies related to xenogeneic ovarian tumor grafts, tumoricidal activity was observed after intraperitoneal transplantation of a human epithelial ovarian cancer cell line, NIH:OVCAR-3. This cell line proliferates only in athymic nude (immunologically incompetent) mice. In both sets of experiments, tumoricidal activity was reduced by inhibition of nitric oxide synthesis. These results demonstrate the tumoricidal action of nitric oxide *in vivo*.

The precise mechanism by which the host defense to malignant tumors is mediated *in vivo* is not known. Cancer cells could trigger an immune response, which in turn could limit the development of the tumor. If this mechanism is not operative, it is possible to induce nonspecific resistance to cancer cells therapeutically. Nitric oxide (NO)-mediated mechanisms of cytotoxicity have mainly been studied *in vitro* (1–3). When cultured *in vitro*, peritoneal macrophages obtained from mice previously inoculated with bacillus Calmette-Guérin (BCG) release NO, which is cytostatic and/or cytolytic for tumor cells (4, 5). This *in vitro* cytostatic and/or cytolytic activity is also observed after activation of the macrophages by cytokines (6, 7). NO produced by the activated macrophages is responsible for this cytostatic and/or cytolytic activity (1–3). Interferon γ (IFN-γ) is important for the priming of macrophages, and tumor necrosis factor α (TNF-α) or some other cytokine or bacterial lipopolysaccharide (LPS) is necessary for full induction of activated macrophage cytotoxicity (6–9). However, the role of NO in mediating tumoricidal activity *in vivo* is not known. We therefore evaluated the *in vivo* role of NO in mediating both the host resistance to a syngeneic ovarian tumor in BCG-inoculated mice as well as the host resistance to a xenogeneic ovarian tumor graft and the role of IFN-γ and TNF-α in this process.

We utilized two different ovarian cancer cell lines for our study. The murine ovarian teratocarcinoma (MOT) cell line, utilized to study the mechanism of BCG-induced host resis-

tance to a syngeneic ovarian tumor, spreads throughout the peritoneal cavity and produces ascites in C3HeB/FeJ mice akin to human ovarian cancer (10, 11). i.p. inoculation of *Corynebacterium parvum*, which increases nonspecific resistance to MOT tumor cells, can cure mice bearing these tumor cells (12). In studies related to xenogeneic ovarian tumor grafts, we utilized a human epithelial ovarian cancer cell line, NIH:OVCAR-3. This cell line grows and develops as a lethal tumor only in athymic nude (immunologically incompetent) mice (13). We therefore transplanted these two cancer cell lines i.p. into female C3HeB/FeJ mice, which served as our model system.

MATERIALS AND METHODS

Reagents and Media. N^G-Methyl-L-arginine acetate (L-NMA), L- and D-arginine hydrochloride, LPS (phenol-extracted *Escherichia coli*, serotype 0.128:B12), minimum essential medium (MEM) without phenol red, sodium nitrate, FAD, NADPH, ammonium chloride, sulfanilic acid, N-(1-naphthyl)ethylenediamine, and *Aspergillus* nitrate reductase were obtained from Sigma. Glacial acetic acid and sodium nitrite were obtained from Fisher Scientific. Fetal calf serum, L-glutamine, penicillin, streptomycin, and amphotericin B were obtained from Irvine Scientific. BCG (Thera-cys strain) was obtained from Connaught Laboratories. Recombinant murine IFN-γ (Mu-rIFN-γ) and recombinant murine TNF-α (Mu-rTNF-α) were obtained from International Biosource (Camarillo, CA). Recombinant human IFN-γ and recombinant human TNF-α were obtained from Collaborative Biomedical Products (Bedford, MA). Monoclonal antibody to Mu-rIFN-γ (IFNγAB), Mu-rTNF-α (TNF-αAB), and irrelevant murine immunoglobulin (IgG immunoglobulin isotype standard) were obtained from PharMingen.

Cancer Cell Lines. MOT cell line was obtained from Robert Bast (Duke University, Durham, NC). The MOT cell line is a murine germ-cell tumor, which arose spontaneously in a C3HeB/FeJ mouse (10, 11). These MOT cells are syngeneic for C3HeB/FeJ mice and were grown in this strain. The human epithelial ovarian cancer cell line NIH:OVCAR-3 (hereafter referred to as OVCAR) was obtained from American Type Culture Collection.

Animals. Six- to 10-week-old female C3HeB/FeJ mice (The Jackson Laboratory) weighing 17–28 g were utilized. These animals were housed five per cage in standard facilities.

In Vivo **Studies.** *Studies related to the mechanism of BCG-induced host resistance to a syngeneic ovarian tumor*

graft. MOT cells (10^5) were transplanted i.p. into each animal. Animals were divided into nine groups (Gp): GP I ($n = 14$) served as the control group and was transplanted with MOT cells. Gp II ($n = 21$) were transplanted with MOT cells 8 days after i.p. BCG (10^6 colony-forming units) to allow for maximal increase in macrophage activation (14). Gp III ($n = 10$) was similar to Gp II except that BCG was administered s.c. Gp IV ($n = 16$) was similar to Gp II and, in addition, received i.p. L-NMA (250 mg/kg of body weight) daily to inhibit NO synthesis (3, 15, 16), starting 2 hr prior to MOT cell transplantation. Gp V ($n = 8$) was similar to Gp II and, in addition, received i.p. IFN-γ_{AB} (200 μg per animal) (14), administered 2 hr prior to MOT cell transplantation and again 7 days later. Gp VI ($n = 5$) was similar to Gp V but received TNF-α_{AB} (200 μg per animal) (14) instead of IFN-γ_{AB}. Gp VII ($n = 5$) was similar to Gp V but received i.p. irrelevant murine immunoglobulin (200 μg per animal) (14) instead of IFN-γ_{AB}. Gp VIII ($n = 12$) and Gp IX ($n = 3$) were similar to Gp IV and, in addition, received i.p. either L-arginine or D-arginine (750 mg/kg of body weight), respectively, daily along with L-NMA. These two groups were included to assess whether only L-arginine, the precursor of NO (1, 17–19), but not D-arginine could stereoselectively reverse the effects of L-NMA. Experiments were terminated 10 days after MOT cell transplantation, ensuring 100% survival of the animals prior to the termination of the experiment. This time period was selected on the basis of preliminary experiments in which increased mortality (40%) of animals was observed 15 days after transplantation of MOT cells.

Studies related to the mechanism of host resistance to a xenogeneic ovarian tumor graft. OVCAR cells (5.0×10^7) were transplanted i.p. into each animal. Animals were divided into seven groups: Gp I ($n = 5$) served as the control group and was transplanted with OVCAR cells. Gp II ($n = 5$) was similar to Gp I and, in addition, received i.p. L-NMA (250 mg/kg of body weight) daily starting 2 hr prior to OVCAR cell transplantation. Gp III ($n = 5$) was similar to Gp I and, in addition, received i.p. IFN-γ_{AB} (200 μg per animal) (14), administered 2 hr prior to OVCAR cell transplantation and again 7 days later. Gp IV ($n = 5$) was similar to Gp III, but the animals received i.p. TNF-α_{AB} (200 μg per animal) (14) instead of IFN-γ_{AB}. Gp V ($n = 5$) was similar to Gp III but received i.p. irrelevant murine immunoglobulin (200 μg per animal) instead of IFN-γ_{AB}. Gp VI ($n = 3$) and Gp VII ($n = 3$) were similar to Gp II and, in addition, received i.p. either L-arginine or D-arginine (750 mg/kg of body weight), respectively, daily along with L-NMA. OVCAR cells proliferate slowly and only in athymic nude mice (13). Experiments were therefore terminated 14 days after OVCAR cell transplantation to ensure adequate time for significant differences in tumor cell numbers to be observed, should this occur among the treatment groups.

Assessment of cancer cell growth in vivo. Animals were weighed initially at the time of tumor cell transplantation and then again just prior to sacrifice. Euthanasia was performed

by intracardiac injection of pentobarbital sodium (100 mg/kg). Through a midline incision, ascitic fluid was collected, followed by four sequential lavages of the peritoneal cavity. The lavage effluent was centrifuged at $250 \times g$ at 8°C for 10 min. The cell pellets were resuspended in Hepes buffer with 0.3% bovine serum albumin, the cells were counted in a hemacytometer, and viability was measured by trypan blue exclusion (20, 21). Cytopreps were stained with Wright–Giemsa and cell differential counts were then performed. The number of viable MOT or OVCAR cells in each group was calculated from the total viable cell count and the differential count.

Studies *in Vitro*. *Culture and* in vitro *stimulation of MOT cells, OVCAR cells, and rat pulmonary alveolar macrophage cell line* (NR8383 *cells*). We assessed whether selected cytokines and LPS were capable of affecting viability as well as stimulating NO formation by these cell lines utilizing the method similar to that reported by Sherman *et al.* (20). NR8383 macrophages were utilized as our positive control as these cells can be stimulated by cytokines *in vitro* to produce NO (20). NO production was indirectly assessed by estimation of nitrite and nitrate concentration in the culture supernatant. Viability of cells was measured by trypan blue exclusion (21). If viability exceeded 97%, cells were suspended in MEM with 2% fetal bovine serum, 2 mM glutamine, and antibiotics at a density of 5×10^5 cells per ml, and 1 ml was placed into 8 wells of 24-well dishes (Costar). MOT or OVCAR cells were placed in the other 16 wells. A combination of LPS and cytokines (25 μl) was incubated with these cell lines for 22 hr at 37°C in 5% CO_2. In some wells, LPS and cytokines were not added and these wells served as our negative controls for each cell type. The combinations of cytokines and LPS were as follows: (*i*) IFN-γ (1000 units/ml per well) and LPS (100 ng/ml per well) or (*ii*) IFN-γ (1000 units/ml per well), LPS (100 ng/ml per well), and TNF-α (1000 units/ml per well). Species-specific recombinant IFN-γ and TNF-α were utilized in these experiments.

Assessment of cytokine-induced cytotoxicity in cancer cells studied in vitro. The cytotoxic effect of cytokines on cultured MOT and OVCAR cells was assessed by determining viability using trypan blue exclusion (21).

Nitrite determinations. Nitrite was measured by the modified Griess reaction (20, 22). Standard curves were constructed using sodium nitrite concentrations ranging from 0 to 50 μM in MEM without phenol red (20, 22).

Nitrate determinations. An enzymatic assay that utilizes *Aspergillus* nitrate reductase to reduce NO_3^- to NO_2^- was modified to measure nitrate in tissue culture supernatants by monitoring NADPH oxidation (23). Absorbance was monitored at 340 nm for 10 min at 25°C using a Beckman DU-70 spectrophotometer. A standard curve was constructed using sodium nitrate dissolved in MEM at concentrations ranging from 0 to 50 μM. For each spectrophotometric run, a blank containing MEM alone was included to establish a back-

Table 1. Weights and ascitic fluid volume (mean $\pm$ SEM) in animals inoculated with MOT cells

Group	n	Initial body weight, g	Weight at sacrifice, g	% change in weight	Ascitic fluid volume, ml
Control (MOT only)	14	26.9 ± 0.8	32.6 ± 0.5	22.3 ± 3.2	6.2 ± 0.8
BCG + MOT i.p.	21	28.6 ± 0.4	26.1 ± 0.5	$-8.9 \pm 1.0^*$	0
BCG + MOT s.c.	10	28.6 ± 0.9	32.3 ± 0.6	13.5 ± 3.3	6.5 ± 0.2
BCG + MOT + L-NMA, i.p.	16	26.9 ± 0.8	33.0 ± 0.6	20.7 ± 2.5	5.1 ± 0.6
BCG + MOT + IFN-γ_{AB}, i.p.	8	29.3 ± 0.5	33.0 ± 0.6	12.6 ± 0.9	5.0 ± 0.2
BCG + MOT + TNF-α_{AB}, i.p.	5	19.7 ± 0.6	18.3 ± 0.4	$-7.1 \pm 1.1^*$	0

The body weight of the animals at the time of i.p. transplantation of MOT cells, body weight at the time of euthanasia, percent change in body weight, and ascitic fluid volume in different groups are shown.
*Significant difference in the percent (%) change in weight as compared to the control group.
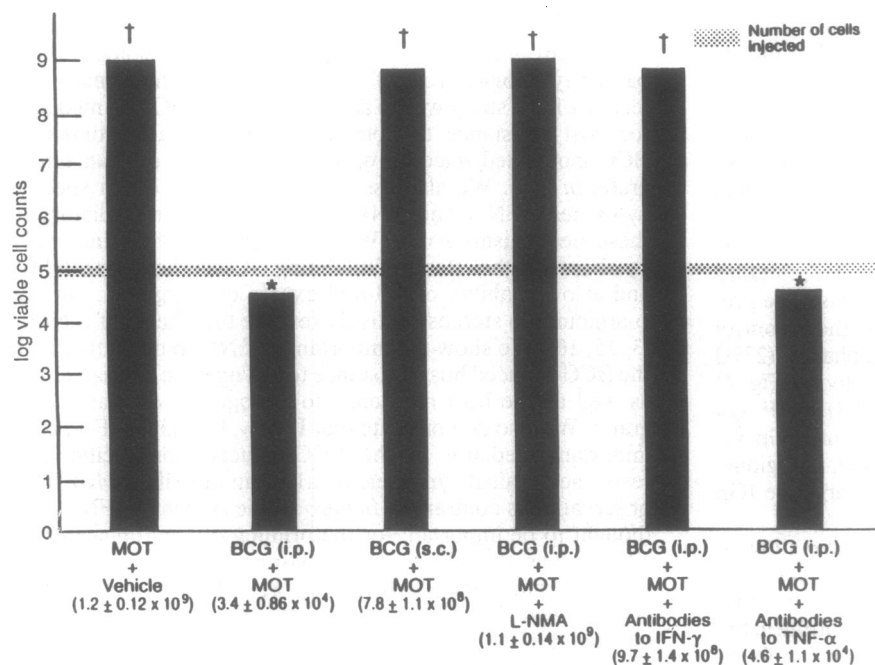
FIG. 1. Role of NO in the BCG-induced host resistance to a syngeneic ovarian tumor. Each column represents (in logarithmic scale) the number of viable MOT cells (mean ± SEM) of 5–21 animals in each group. The hatched horizontal bar represents the total number of MOT cells transplanted i.p. in each animal. †, Significant difference in the total viable MOT cell count as compared to the counts obtained in BCG (i.p.)-preinoculated animals subsequently transplanted with MOT cells. *, Significant difference between the number of MOT cells transplanted and the cell counts in the peritoneal cavity.

ground for calculation of data. The blank was subtracted from each rate determination made in the presence of enzyme.

Statistical analysis. Differences in mean between groups were tested utilizing analysis of variance (ANOVA). Multiple comparisons were performed using Dunnett's procedure for comparing treatment groups with a single control. For each group, a one-sample *t* test was used to compare baseline injected values to absolute total viable cancer cells and to compare differences in percent change in weight between groups. All tests were two-tailed. Significance was defined as $P \leq 0.05$.

RESULTS

In Vivo Studies. In vivo *studies related to the mechanism of BCG-induced host resistance to a syngeneic ovarian tumor graft.* In preliminary experiments, increased mortality (40%) of animals was observed 15 days following transplantation of MOT cells as well as in the group which received s.c. BCG followed by MOT cell transplantation 8 days later (60%). No mortality was observed in animals which received i.p. BCG followed by MOT cell transplantation 8 days later. By contrast, increased mortality (40%) was observed in animals which received i.p. BCG and were transplanted with MOT cells 8 days later and which also received daily i.p. L-NMA. It is for this reason that the subsequent experiments were terminated 10 days following MOT cell transplantation and there was no mortality of the animals in any of the groups. The results of these experiments are summarized in Table 1 and Fig. 1. Significant increase in the number of MOT cells and an increase in body weight accompanied by ascites were observed in control (non-BCG treated) animals (Gp I) and in those preinoculated with s.c. BCG (Gp III). Tumoricidal activity in animals preinoculated with i.p. BCG (Gp II) was evident by a reduction in the number of viable intraperitoneal MOT cells and absence of ascites. In this group (Gp II), the predominant cell types in the peritoneal washings were macrophages (54%) and lymphocytes (23%) (Fig. 2). By contrast, daily i.p. administration (250 mg/kg) of L-NMA, an inhibitor of NO synthesis (3, 15, 16), prevented the tumoricidal activity in animals preinoculated with i.p. BCG and subsequently transplanted with MOT cells (Gp IV) (Fig. 2). The MOT cells in this group of animals proliferated to the same extent as that of control animals which received MOT cells alone (Gp I). In animals in Gp VIII, the effect of L-NMA

was not observed when L-arginine (750 mg/kg), the precursor of NO synthesis (1, 17–19), was injected simultaneously with L-NMA (data not shown). D-Arginine (Gp IX) did not reverse the effects of L-NMA (data not shown). These results indicated that the BCG-induced host defense mechanism to MOT cells *in vivo* was due to stimulation of the L-arginine-NO pathway. IFN-γ_{AB} (200 μg per animal) (14), administered i.p. 2 hr before transplantation of MOT cells, and again 7 days
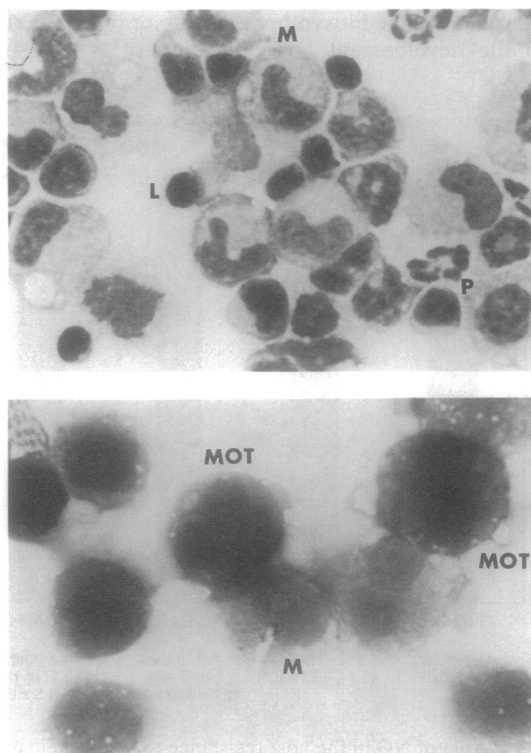

FIG. 2. Cytopreps of cell pellets obtained from peritoneal washings of MOT-transplanted animals and stained with Wright–Giemsa. (*Upper*) i.p. BCG-preinoculated animals. Macrophages (M), lymphocytes (L), and polymorphonuclear leukocytes (P) are present. (*Lower*) i.p. BCG- and L-NMA-treated animals. Pleomorphic, hyperchromatic MOT cells with multiple and multilobular nuclei are seen. (×300.)

later also inhibited the tumoricidal activity of BCG-treated animals receiving MOT cells (Gp V). TNF-α_{AB} (200 μg per animal) (14) (Gp VI) or irrelevant murine immunoglobulin (200 μg per animal) (14) (Gp VII) were without effect.

In vivo *studies related to the mechanism of host resistance to a xenogeneic ovarian tumor graft.* The results of these experiments are summarized in Fig. 3. Marked tumoricidal activity was observed in animals (Gp I) transplanted i.p. with OVCAR cells, as evidenced by a significant reduction in the number of tumor cells. No ascites was observed. No mortality was observed in any OVCAR-transplanted animals. The predominant cell types in the peritoneal washings in these control animals were lymphocytes (70%) and macrophages (27%) (Fig. 4). L-NMA (Gp II) reduced tumoricidal activity (Fig. 4) and resulted in ascites formation. IFN-γ_{AB} (Gp III), TNF-α_{AB} (Gp IV) (Fig. 3) or irrelevant murine immunoglobulin (Gp V), did not affect tumoricidal activity (data not shown). L-Arginine reversed the effects of L-NMA (Gp VI), but D-arginine (Gp VII) was without effect (data not shown).

In Vitro **Studies.** The results of culture and *in vitro* stimulation of MOT cells, OVCAR cells, and NR8383 cells are summarized in Table 2. MOT cells, OVCAR cells, and NR8383 cells did not produce detectable nitrite or nitrate in the absence of LPS and cytokines. IFN-γ and LPS, in combination or along with TNF-α, activated the macrophages to produce increased nitrite and nitrate. Only very small quantities of nitrite and nitrate were produced by MOT cells and OVCAR cells after stimulation with a combination of LPS and cytokines and the viability of these cells was also not affected.

DISCUSSION

Studies *in vitro* indicate that certain cytokines are capable of activating macrophages and inducing synthesis of NO, thereby exerting cytostatic and/or cytolytic activity (6, 7). Evidence that L-arginine is the biosynthetic precursor of NO (1, 17–19) led to the development and utilization of N^G-substituted

analogs of L-arginine such as L-NMA to inhibit NO synthesis (3, 15, 16) and thereby the study of the physiological and pathophysiological role of NO (14–16, 24). The primary objective of this study was to assess the role of NO as a mediator for host resistance to both a syngeneic ovarian tumor in BCG-inoculated mice as well as xenogeneic ovarian tumor grafts *in vivo*. We also assessed the role of certain specific cytokines, IFN-γ and TNF-α, as effectors or mediators in these mechanisms *in vivo*. We studied the role of NO indirectly by observing the effects of inhibiting NO synthesis by L-NMA and also the ability of a 3-fold excess of L-arginine, but not D-arginine, to stereoselectively reverse the effects of L-NMA (3, 15, 16). We show the importance of NO in mediating both the BCG-induced host resistance to a syngeneic ovarian tumor as well as the host resistance to xenogeneic ovarian tumor grafts. We also demonstrate that IFN-γ, but not TNF-α, is an important mediator of the BCG-induced nonspecific host resistance against syngeneic ovarian tumor cells *in vivo*. This observation is contrary to *in vitro* studies in which IFN-γ was thought to be important for the priming of macrophages and TNF-α was thought to be necessary for full induction of activated macrophage cytotoxicity (8, 9). Furthermore, others (14) showed that neutralization of either IFN-γ or TNF-α blocked the BCG-induced *in vivo* synthesis of nitrogen oxides from L-arginine as well as the protection against *Francisella tularensis*. Our observation that only IFN-γ_{AB}, but not TNF-α_{AB}, neutralized BCG-induced host resistance to the syngeneic tumor is therefore surprising. Further studies are necessary to explain this possible discrepancy.

We were not able to inhibit the host resistance to the xenogeneic ovarian tumor graft by utilizing either IFN-γ_{AB} or TNF-α_{AB}. One possible explanation for the inability of IFN-γ_{AB} to inhibit the host resistance to the xenogeneic ovarian
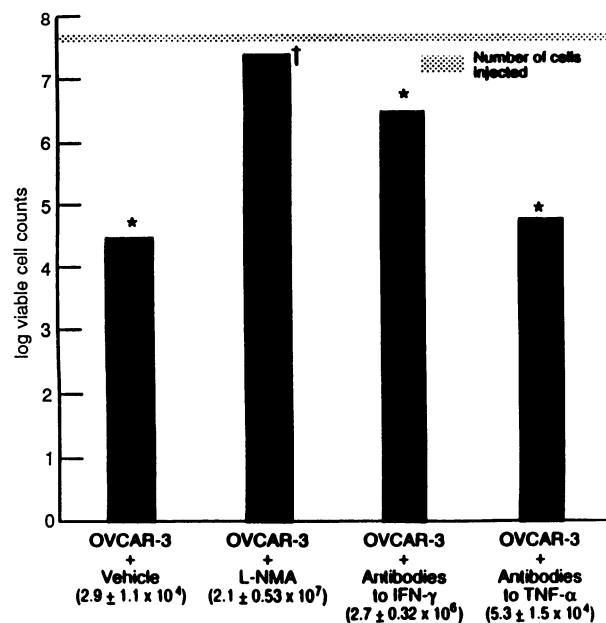

FIG. 3. Role of NO in the host resistance to xenogeneic ovarian tumor grafts. Each column represents (in logarithmic scale) the number of total viable OVCAR cells (mean ± SEM) of five animals in each group. The hatched horizontal bar represents the total number of cells transplanted i.p. in each animal. *, Significant difference between number of OVCAR cells transplanted and the counts in the peritoneal cavity. †Significant difference in the OVCAR cell count in L-NMA-treated animals as compared to the counts obtained in the OVCAR and vehicle-treated group.
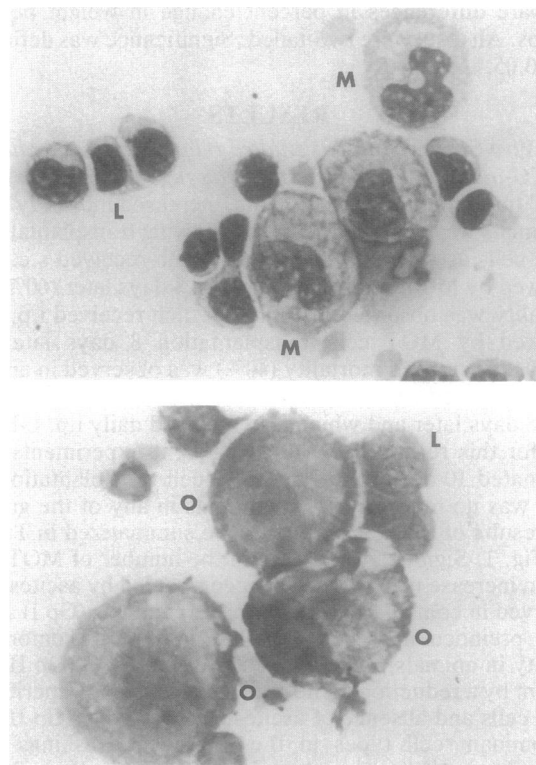

FIG. 4. Cytopreps of cell pellets obtained from the peritoneal washings of OVCAR (O)-transplanted animals and stained with Wright–Giemsa. (*Upper*) Animals transplanted i.p. with OVCAR cells. Macrophages (M) and lymphocytes (L) are present. (*Lower*) OVCAR-transplanted and L-NMA-treated animals. Large malignant epithelial cells (O) with prominent multiple nucleoli and pleomorphic nuclei are seen. (×300.)

Table 2.   Action of selective cytokines and LPS on cancer cell lines

Treatment	NO_3^-, μM	NO_2^-, μM	$NO_2^- + NO_3^-$, μM	Cell viability, %
NR8383 cells alone	0.0	0.0	0.0	80
OVCAR cells alone	0.0	0.0	0.0	83
MOT cells alone	0.0	0.0	0.0	85
NR8383 + IFN-γ + LPS	32.7 ± 0.6	23.2 ± 0.4	55.9 ± 1.0	80
OVCAR + IFN-γ + LPS	0.0	0.0	0.0	93
MOT + IFN-γ + LPS	0.0	0.33	0.33	81
NR8383 + IFN-γ + LPS + TNF-α	38.1 ± 1.9	22.6 ± 2.1	60.7 ± 0.2	84
OVCAR + IFN-γ + LPS + TNF-α	0.14	0.40	0.54	93
MOT + IFN-γ + LPS + TNF-α	0.0	0.24	0.24	90

Each value represents the mean concentration of nitrite and nitrate (μM) and cell viability after treatment with the following reagents: IFN-γ (1000 units/ml); TNF-α (1000 units/ml); LPS (100 ng/ml). The concentrations of cells per well were as follows: NR8383, 10^6 cells per ml per well; OVCAR, 10^6 cells per ml per well; MOT, 0.5×10^6 cells per ml per well. No significant production of NO by tumor cells was detected. The cytokines and LPS also did not affect cell viability.

tumor graft, unlike that observed with syngeneic ovarian tumor, may have been due to an overwhelming release of IFN-γ, which may not have been completely neutralized by the amount of IFN-γ_{AB} utilized in our studies. A similar explanation may also hold true with regard to our observation that TNF-α_{AB} had no effect on host resistance to the ovarian xenogeneic tumor graft. However, other potential mechanisms of tumoricidal activity independent of IFN-γ and TNF-α may also have been involved in host resistance to xenogeneic ovarian tumor grafts *in vivo*.

The source of NO responsible for the cytolytic activity *in vivo* was most likely from activated macrophages (1–3, 25). We did not observe any cytolytic activity nor were we able to stimulate significant NO production by the tumor cells *in vitro* by incubating these cells with selected cytokines and LPS. However, the cytokines and LPS were able to stimulate NO production by macrophages. It is possible that a much longer exposure time of tumor cells to cytokines and LPS may be necessary to observe increased NO synthesis *in vitro*. It is also possible that stimulation of our tumor cell lines to produce NO *in vivo* requires cytokines other than those utilized in our *in vitro* studies. Therefore, other potential sources of NO in addition to macrophages, such as the tumor cells, cannot be completely ruled out. The ability of human ovarian malignant tumors (26) to produce NO has recently been demonstrated.

Results from our studies may have potential clinical significance. Our observation that only i.p. inoculation of BCG, and not s.c. administration, was capable of stimulating the host immune response to i.p. transplantation of syngeneic tumor cells by an NO-mediated mechanism *in vivo* may explain why local instillation of BCG into the bladder is effective in the treatment of superficial mucosal bladder cancers in humans (27, 28). Our results may also explain why s.c. and i.v. administration of BCG to women was not effective for treatment of human ovarian cancer (29), whereas i.p. instillation of *C. parvum*, which, like BCG, stimulates the host defense mechanism (30), was at least partially effective in the treatment of minimal residual ovarian cancer in women (31). It would, therefore, be interesting to evaluate whether the tumoricidal action in humans is also mediated by NO.

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