NITRIC OXIDE
A Macrophage Product Responsible for Cytostasis and Respiratory Inhibition in Tumor Target Cells

BY DENNIS J. STUEHR AND CARL F. NATHAN

From the Beatrice and Samuel A. Seaver Laboratory, Division of Hematology-Oncology, Department of Medicine, Cornell University Medical College, New York, New York 10021

Once activated by agents such as IFN-γ and bacterial LPS, macrophages (Mφ) can inhibit the growth of a wide variety of tumor and microbial targets (1, 2). Although Mφ products such as hydrogen peroxide, TNF-α, and IL-1 cause cytostasis and/or cytotoxicity (3–5), in many cases these mediators do not appear to be involved. With some targets, Mφ-mediated cytostasis and injury to the mitochondrial electron transport chain (METC) require a process associated with Mφ oxidation of the guanido nitrogens of l-arginine to NO2⁻/NO3⁻ (6). However, it is unknown if a metabolite of l-arginine causes these injuries, and if so, which metabolite.

Activated Mφ have recently been shown to release a compound similar to or identical with the reactive radical nitric oxide (NO) during metabolism of l-arginine to NO2⁻/NO3⁻ (7). This report identifies NO· (or a closely related product) as a mediator of Mφ-induced cytostasis and mitochondrial respiratory inhibition in lymphoma cells.

Materials and Methods

Reagents. Cells were cultured in minimum Eagle's medium, α modification (αMEM) or RPMI 1640 (RPMI; KC Biological Inc., Lenexa, KS), both supplemented with 8% bovine calf serum (CS; HyClone Systems, Logan, UT), l-glutamine (584 mg/liter), penicillin (50 U/ml), and streptomycin (50 μg/ml). Catalase and N°-monomethyl-l-arginine (NMA) were from Calbiochem-Behring Corp. La Jolla, CA. NO· gas (99% pure) and N2 gas (<5 ppm O2) were from Matheson Gas Products, East Rutherford, NJ. [Methyl-3H]Tdr (2 Ci/mmol) was from New England Nuclear, Boston, MA. Pure IFN-γ was generously provided by Genentech, South San Francisco, CA. LPS (Escherichia coli serotype 0127: B8) and all other reagents were from Sigma Chemical Co., St. Louis, MO. Concentrated stock solutions were prepared in culture medium (for myoglobin, ascorbate, catalase, NMA, LPS, and IFN-γ) or saline (for NaNO2, NaNO3, FeSO4) and sterile filtered (0.22 μm, Millipore, Danvers, MA).

Collection and Culture of Mouse Peritoneal Mφ and L1210 Cells. Peritoneal Mφ were obtained from C3H/HeJ (The Jackson Laboratories, Bar Harbor, ME), C3H/He, or CD-1 mice (Charles River Breeding Laboratories, Wilmington, MA) that had been injected 4 d previously with

This work was supported by National Institutes of Health grant CA-43610. D. J. Stuehr is a Fellow of the Leukemia Society of America. Address correspondence to C. Nathan, Box 57, Cornell University Medical College, 1300 York Ave., New York, NY 10021.

Abbreviations used in this paper: aglyPi, α-glycerophosphate; IC50, dose producing 50% maximal inhibition; Mφ, macrophages; METC, mitochondrial electron transport chain; NMA, N°-monomethyl-l-arginine; NO·, nitric oxide; NO2⁻, nitrogen dioxide; NO3⁻, nitrate; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates; TMPD, tetramethylphenylenediamine.
2 ml of 4% Brewer's thioglycollate broth (Difco Laboratories, Detroit, MI) by peritoneal lavage with PBS containing 25 mM glucose. The cells were pelleted at 4°C, resuspended in αMEM to 10^6/ml, and plated at 0.1 or 1 ml/well in 96- or 24-well plates, respectively. After culture for 2–3 h at 37°C in 5% CO₂, the medium was aspirated and replaced with an equal volume of αMEM, which in some cases contained 500 U/ml IFN-γ to activate the Mφ. L1210 cells (American Type Culture Collection, Rockville, MD) were kept in continuous culture in RPMI 1640.

*Mφ*-L1210 Cell Coculture. After Mφ were cultured overnight in 96-well plates, the medium was aspirated and replaced with 50 μL αMEM containing no added LPS (control Mφ) or 3 μg/ml LPS (activated Mφ). L1210 cells (5 × 10^4/well, 25 μl) were then added along with solutions containing experimental agents and the volume of each well brought to 150 μl with αMEM. After 6 h, 2.5 μCi [³H]TdR was added to each well for a period lasting 12-18 h. In some cases, 50 μl volumes were removed from the cocultures at this point to measure NO₂⁻ production. Each experiment had control wells consisting of L1210 cells cultured without Mφ under each experimental condition; L1210 [³H]TdR incorporation was unaffected by the additives in all cases. For experiments in Table II, 1.5 × 10^5 Mφ were kept in 100-mm culture dishes and in some cases activated overnight with IFN-γ as described for the 96-well experiments. The next day the medium was replaced with medium ± LPS or LPS and 250 μM NMA. L1210 cells were added (2 × 10^5/plate), cocultured for 24 h, and removed from the monolayers by rinsing with a pipette. The L1210 cells were counted and plated at 5 × 10^4/well for [³H]TdR incorporation studies.

Culture of L1210 Cells with NO⁻ at Reduced pH. L1210 cells (2 × 10^6/ml) were cultured without CO₂ in a 37°C incubator in bicarbonate-free DME containing 2% CS, 20 mM Hepes, 20 mM morpholineethanesulfonate, and various concentrations of NaN₃ or NaN₃. Solutions were prepared fresh for each experiment, the pH adjusted to range from 6.2 to 7.2 with 1 M NaOH, and were sterile filtered through a 0.22-μm membrane. After culture for various times, the cells were pelleted and resuspended in conventional RPMI (pH 7.2) at 2 × 10^6/ml and plated at 5 × 10^4/well. Viabilities at this point ranged between 77 and 90% by trypan blue exclusion. [³H]TdR was added and its incorporation was measured over an 18-h period.

Treatment of L1210 Cells with NO⁻-saturated Solutions. For O₂ consumption studies solutions of saline (10 ml) containing 25 mM glucose were bubbled with N₂ for 45 min to remove dissolved O₂. Authentic NO⁻ that had passed through 1 M KOH to remove nitrogen dioxide (NO₂) was then bubbled in for 15–20 min to form saturated solutions ([NO⁻] = 1.25 mM; reference 8). Various volumes were transferred with a gas-tight syringe into stoppered N₂-flushed tubes containing L1210 cells (4 × 10⁷ to 4 × 10⁸) suspended in 0.1 ml CS and the contents were mixed. After 5 min on ice, 2 ml of cold, aerated RPMI was added to destroy the remaining NO⁻. The cells were centrifuged and resuspended to 5 × 10^⁸/ml in the respiration medium used for intact cells (see below). Cell viabilities at this point were ≥80% (trypan blue dye exclusion). For controls, the NO⁻ solutions were first sparged with N₂ for 15 min and then with air for 5 min before transfer to the cells. Sparging with N₂ removed most of the NO⁻ from the solution and sparging with air converted residual NO⁻ to HNO₂ and NO₃⁻ (9). For [³H]TdR incorporation studies 4 × 10⁸ cells were treated with NO⁻ as above, resuspended to 2 or 4 × 10⁹ cells/ml in RPMI, plated at 5 × 10⁴/well, and pulsed with [³H]TdR. The NO⁻-saturated saline/glucose solutions that were used to treat cells for the [³H]TdR studies were buffered with 25 mM succinate to prevent the pH from falling below 4.5.

Digitonin Permeabilization of L1210 Cells. This was done as described previously (10). NO⁻-treated or control cells were washed twice by centrifugation in 30 ml cold respiration buffer used for permeabilized cells (250 mM sucrose, 20 mM Hepes, 2 mM K₂HPO₄, 10 mM MgCl₂, 1 mM EGTA, and 0.7% BSA, pH 7.2), resuspended to 5 × 10⁸ cells/ml, and treated with digitonin (0.01%) for 10 min on ice. Tests with trypan blue dye exclusion showed each cell preparation was <15% permeable before and >95% permeable after digitonin treatment. The cells were centrifuged at 200 g for 8 min at 4°C, washed once in 35 ml respiration buffer, and resuspended to 10⁶ cells/ml for O₂ respiration measurements.

O₂ Respiration Measurements. O₂ consumption was measured using a Clark electrode (Yellow Springs Instrument Co., Yellow Springs, OH). For respiration measurements with intact cells, L1210 cell suspensions (50 or 100 μl) were injected into a jacketed respiration
chamber, which was kept at 37°C and contained 1.4 ml Dulbecco's PBS (without Ca²⁺ or Mg²⁺) plus 25 mM glucose. L1210 respiration was calculated as the rate of decrease in O₂ concentration following addition of cells, assuming an initial [O₂] = 390 ng/ml (II). Respiration of L1210 cells was dependent on glucose and 100% inhibitable by 3 mM KCN in all cases.

O₂ consumption by digitonin-permeabilized cells given mitochondrial substrates was measured as described previously (II). 50 or 100 µl of cell suspensions were injected into a respiration chamber containing 1.4 ml of the respiration buffer used for permeabilized cells (composition detailed above). After 3–5 min, a mitochondrial substrate was added in 10 µl to give a final concentration of 5 mM for malate, succinate, or α-glycerophosphate (aglyPi), or 200 µM for tetramethylphenylenediamine (TMPD). Rotenone (100 nM) inhibits electron flow from complex 1 into the METC and was added in order to measure respiration on substrates that donate electrons into the METC through complex 2 (succinate) or Coenzyme Q (aglyPi) (II). For the same reason, antimycin A (40 nM) was added to block electron flow from Coenzyme Q into the METC so that respiration on TMPD, which donates electrons to cytochrome c, could be measured. State 3 respiration was initiated by adding 10 µl ADP (giving 1 mM) and the rate of O₂ consumption was calculated by subtracting the rate observed without substrate from the state 3 rate. Cyanide (3 mM) completely blocked respiration on all substrates except TMPD, where ~15% of O₂ consumption was not inhibitable. The cyanide-insensitive value for each run that used TMPD was subtracted.

Measurement of [³H]TdR Incorporation by L1210 Cells. At the end of each incorporation period, the 96-well culture plates were frozen and stored at −80°C. [³H]TdR incorporation was measured by liquid scintillation counting after processing the plates with an automatic cell harvester (Dynatech, Wesbart, UK). Incorporation by cultures of Mφ without L1210 was determined for each experiment (typically 400–1,400 cpm) and subtracted from the coculture values to obtain L1210-specific [³H]TdR uptake.

NO₂⁻ and NO₃⁻ Determination. NO₂⁻ concentrations were determined by a microplate assay that will be described in detail elsewhere (Stuehr, D. J., manuscript in preparation). Briefly, 50- or 100-µl sample aliquots, diluted if needed, were mixed with an equal volume of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2% H₃PO₄) and incubated at room temperature for 10 min. The absorbance at 550 nm was measured in a microplate reader (Bio-Tek Instruments, Inc., Burlington, VT). NO₂⁻ was determined using NaN₃O₂ as a standard and double-distilled H₂O as a blank. Background NO₂⁻ values of buffers or media were determined in each case and subtracted from the experimental values. In certain cases the NO₂⁻ and NO₃⁻ concentrations were measured by an automated method described elsewhere (12).

Results

Cytostasis Depends on a Process Associated with Mφ NO₂⁻/NO₃⁻ Synthesis but Is Not Due to NO₂⁻/NO₃⁻ or their Metabolites. Initial experiments showed that induction of Mφ NO₂⁻/NO₃⁻ synthesis by IFN-γ and LPS correlated closely with L1210 cytostasis in coculture (Fig. 1) and that the cytostasis could be reversed 85% by a substrate-
based inhibitor of Mφ NO$_2^-$ synthesis, NMA (not shown). This confirmed a previous report (6) that induction of the Mφ NO$_3^-$-producing pathway correlates with and is required for cytostasis in this system. Since NO$_2^-$ can be growth inhibitory (13-16), we tested if exogenous NaNO$_2$ could cause cytostasis in the cocultures. NaNO$_3$ served as a control. As shown in Table I, neither NaNO$_2$ nor NaNO$_3$ inhibited $[^3]$H]TdR incorporation in cultures containing L1210 cells and either nonactivated or activated Mφ (NMA was added to cocultures containing activated Mφ to block conversion of L-arginine to NO$_2^-$/NO$_3^-$). NO$_2^-$ and NO$_3^-$ were ineffective even when added at concentrations 50-fold higher than those typically achieved under coculture conditions (125 μM). Thus, activated Mφ did not convert added NO$_2^-$ or NO$_3^-$ into cytostatic agents.

*Generation of Cytostatic Reactive Nitrogen Intermediates (RNI) from Acidification of NO$_2^-$.* In bacterial systems (13-16), the cytostatic action of NO$_2^-$ increases upon mild acidification through formation of nitrous acid (HNO$_2$, $pK_a$ 3.4) and its dismutation, which generates other RNI, including NO$^-$ and NO$_2^-$ (9,17). Thus, we tested if acidified NO$_2^-$ solutions would inhibit replication of L1210 in the absence of macrophages. Fig. 2 shows that L1210 $[^3]$H]TdR incorporation was inhibited after culture with NO$_2^-$ under mildly acidic conditions. The degree of growth inhibition was directly proportional to the time of exposure, the acidity, and the concentration of NO$_2^-$, consistent with a requirement for formation of HNO$_2$. At pH 6.2, a 10-h exposure to 250 μM NO$_2^-$ (a concentration twice that typically achieved in activated Mφ cultures) caused >50% cytostasis. Cytostasis did not occur when L1210 cells were cultured at these pH values in the absence of NO$_2^-$ (Fig. 2), nor when NO$_3^-$ replaced NO$_2^-$ (not shown).

*Cytostasis by NO$^-$.* Since RNI generated from acidified NO$_2^-$ were cytostatic, we tested the effect of authentic NO$^-$ Exposure to NO$^-$ for 5 min inhibited L1210

### Table I

Inability of Added NO$_2^-$ and NO$_3^-$ to Inhibit L1210 Growth in Mφ Coculture

| Added NO$_2^-$/NO$_3^-$ | Control Mφ | Activated Mφ + NMA |
|---------------------------|-------------|---------------------|
| mM                        | $[^3]$H]TdR incorporation | $[^3]$H]TdR incorporation |
|                           | NO$_2^-$ | NO$_3^-$ | NO$_2^-$ | NO$_3^-$ |
| 0                         | 89 ± 2   | 79 ± 0   | 79 ± 0   | 79 ± 0   |
| 1                         | 93 ± 8   | 96 ± 2   | 62 ± 3   | 78 ± 6   |
| 5                         | 96 ± 2   | 96 ± 3   | 76 ± 2   | 76 ± 7   |
| 10                        | 99 ± 11  | 84 ± 13  | 88 ± 5   | 78 ± 4   |

C3H/He Mφ (10$^3$/well) were activated overnight with IFN-γ (500 U/ml). Medium was replaced the next day with an equal volume containing no LPS (control) or 2 μg/ml LPS plus 250 μM NMA (activated). L1210 cells and 10 μl of NaNO$_2$ or NaNO$_3$ solutions were added. $[^3]$H]TdR was added 6 h later for an 18-h period. The experiment is representative of three and the values are the mean cpm ± SD of four wells. $[^3]$H]TdR incorporation in activated Mφ/L1210 cocultures not receiving NMA was 1,208 ± 706 cpm (98% cytostasis). NO$_2^-$ production (nmol/well) by cultures that did not receive $[^3]$H]TdR was 0.0 ± 0.0, control Mφ; 0.7 ± 0.1, activated Mφ + NMA; and 4.6 ± 0.1, activated Mφ without NMA ($n = 4$).
[3H]TdR incorporation in a dose-dependent manner during a subsequent 3-h labeling period, with an IC50 of ~20 nmol NO−/10⁶ cells (Fig. 3). Solutions that had been rid of NO− by N2 sparging and aeration were incapable of causing cytostasis. This indicated that the active principle was NO− and not its nonvolatile or oxygen-resistant reaction products, such as NO2−/NO3−. Maximal inhibition required NO− exposures as short as 30 s; inhibition remained NO− specific through at least 10 min of exposure (not shown). Thus, 5-min exposures were used routinely.

Recovery from NO−-induced Cytostasis. Activated Mφ cause target cell cytostasis within 8 h of coculture and it characteristically lasts ≥24 h (18, 19). Table II compares [3H]TdR incorporation by L1210 cells rendered cytostatic either by treatment with NO− or by 24-h coculture with activated Mφ. DNA synthesis by L1210 cells in the

![Figure 2](image1.png)

**Figure 2.** [3H]TdR incorporation by L1210 cells after culture for various times in NO2−-containing medium at various pH. NO2− concentrations were 0 (○), 0.25 (●), 0.5 (△), 1.0 (▲), and 2.5 mM (□). The experiment is representative of three and the values are the mean cpm ± SD for four cultures over an 18-h period.

![Figure 3](image2.png)

**Figure 3.** Dose-response curve for NO− inhibition of L1210 [3H]TdR incorporation. Cells were treated for 5 min with NO− solution (○) or NO− solution that had been sparged with N2/air (●). [3H]TdR incorporation was measured over the first 3 h period after NO− treatment. The experiment shown represents one of five. The points are the mean cpm ± SD for four cultures.
first, second, and third 3-h periods after exposure to NO\textsuperscript{-} was 22, 73, and 128\% of controls, respectively. In contrast, DNA synthesis by L1210 cells that had been cocultured with activated Mφ within the same three time periods was 12, 8, and 9\% of controls. Recovery of \[^3H\]TdR incorporation to the level of controls for Mφ-injured cells was not seen until 30 h (not shown). Thus, a pulse of NO\textsuperscript{-} caused cytostasis of shorter duration than that caused by cocultivation with activated Mφ for 24 h.

**NO\textsuperscript{-}-mediated Respiratory Inhibition.** Like cytostasis, inhibition of target cell respiration by activated Mφ is dependent on metabolism of L-arginine to NO\textsuperscript{-}/NO\textsubscript{3}\textsuperscript{-} (6). We therefore determined if NO\textsuperscript{-} could inhibit respiration of L1210 cells in the absence of Mφ. Fig. 4 shows that NO\textsuperscript{-} treatment inhibited cyanide-sensitive oxygen uptake by L1210 cells in a dose-dependent manner, with an IC\textsubscript{50} of 66 nmol/10\textsuperscript{7} cells. The effect was NO\textsuperscript{-} specific, since NO\textsuperscript{-} solutions that had been sparged with N\textsubscript{2} and aerated were inactive.

**Sites of NO\textsuperscript{-} Injury within the METC.** Mφ-mediated respiratory inhibition results from specific injury within complex 1 (NADPH:ubiquinone oxidoreductase) and complex 2 (succinate:ubiquinone oxidoreductase) of the METC (11). To determine if NO\textsuperscript{-} exhibited similar specificity, NO\textsuperscript{-}-treated cells were permeabilized with digitonin.
and mitochondrial substrates were used to measure electron flow through complex 1 (malate), complex 2 (succinate), coenzyme Q (α-GlycerPi), and cytochrome c (TMPD). NO· (40 nmol/10⁷ cells) decreased L1210 cell respiration on malate or succinate to 16 and 44%, while respiration on α-glycerophosphate or TMPD was 122 and 105% of controls, respectively (Table III). Thus, NO· specifically injured complex 1 and 2.

Scavenging of MΦ-generated NO· in Coculture. The above results indicated that NO·, a MΦ product, was capable of causing target cell cytostasis and respiratory inhibition. To test if NO· mediated these effects in MΦ-L1210 cell cocultures, we added agents that scavenge NO· and monitored their effect on MΦ-mediated cytostasis. Superoxide reacts rapidly with NO· to produce the inactive product NO₃⁻ (20). Fig. 5 depicts the effect of a superoxide-generating system (FeSO₄/ascorbate; 21) on MΦ-mediated, L-arginine-dependent cytostasis. Catalase (1,000 U/ml) was added to prevent HOOH-mediated cytotoxicity to L1210 cells (22) that otherwise occurred when ascorbate (1 mM) was present. MΦ-induced cytostasis was partially prevented by the superoxide-generating system. The antagonism was dependent on added Fe²⁺ in a concentration-dependent manner. Ascorbate alone was inactive, but greatly enhanced the ability of Fe²⁺ to prevent cytostasis at all Fe²⁺ concentrations, presumably by providing electrons for Fe⁴⁺-catalyzed superoxide production (21). At 100

| Substrate | Acceptor     | Control            | NO· treated     |
|-----------|--------------|--------------------|-----------------|
| Malate    | Complex 1    | 0.353 ± 0.028      | 0.058 ± 0.028 (16.4%) |
| Succinate | Complex 2    | 0.694 ± 0.059      | 0.306 ± 0.006 (44.1%)  |
| α-GlyPi   | Coenzyme Q   | 0.398 ± 0.039      | 0.486 ± 0.030 (122%) |
| TMPD      | Cytochrome C | 0.387 ± 0.041      | 0.619 ± 0.029 (105%) |

L1210 cells were treated for 5 min with NO· solution (48 nmol NO·/10⁷ cells) or an equivalent volume of N₂-sparged/aerated NO· solution (pH 2.9, [NO₂⁻] = 12 mM) and permeablized as described in Materials and Methods. The experiment is representative of four and the values are the mean ± SD of three measurements. Values in parentheses are percents of the controls.

![Figure 5](https://example.com/figure5.png)
Inhibition of activated Mφ-induced cytostasis by myoglobin. L1210 cells were cultured with activated Mφ and various concentrations of myoglobin alone (△), myoglobin plus 1 mM ascorbate and 1,000 U/ml catalase (○), or myoglobin plus ascorbate/catalase and 500 U/ml SOD (●). Results for L1210 cultured with control Mφ, ascorbate/catalase, and varying amounts of myoglobin (□) are also shown. The experiment represents one of six. The points are the mean cpm ± SD for four wells. For comparison, [3H]Tdr incorporation by L1210 cells in cocultures containing activated Mφ and 250 μM NMA was 75,297 ± 2,683 cpm.

μM FeSO₄ (plus ascorbate), L1210 DNA synthesis returned to 44% that of control cocultures (L1210 cells and activated Mφ given NMA). Inclusion of 500 U/ml superoxide dismutase (SOD) eliminated the protective effect of Fe²⁺/ascorbate and fully restored Mφ-mediated cytostasis, while boiled SOD was inactive. Thus, Fe²⁺/ascorbate prevented Mφ cytostasis primarily through generation of superoxide.

Ferroheme complexes such as ferrous myoglobin bind NO· with high affinity (23, 24) and have been used to scavenge NO· generated by endothelial cells (8, 25, 26) and Mφ (7). The effect of a myoglobin NO·-scavenging system on Mφ-mediated inhibition of L1210 DNA synthesis is shown in Fig. 6. Myoglobin, when kept in the ferrous state by inclusion of 1 mM ascorbate, blocked Mφ-mediated cytostasis of cocultured L1210 cells in a dose-dependent manner. At 3 mg/ml myoglobin (plus ascorbate), L1210 [3H]Tdr incorporation recovered to 97% that of control (NMA-treated) cocultures. Myoglobin was inactive in the absence of the reductant ascorbate; ascorbate alone was inactive. Inclusion of 500 U/ml SOD did not abrogate scavenging by ferrous myoglobin, consistent with a mechanism independent of superoxide production.

Discussion
These results identify NO· (or NO₂, formed via reaction of NO· with O₂) as an l-arginine-derived Mφ metabolite responsible for inhibition of DNA synthesis and mitochondrial respiration in L1210 cells. Initial experiments (6), extended here, showed that although metabolism of l-arginine was required for these effector functions, the observed endproducts (NO₂− and NO₃−) were inactive. However, NO₂− became cytostatic at acidic pH, a condition under which NO₂− is chemically converted into more reactive species, including HNO₂, NO−, and NO (9, 17). During their metabolism of l-arginine to NO₂−/NO₃−, Mφ produce a compound with biological, biochemical, and physical properties of NO· or NO₂ (7). Authentic NO· inhibited L1210 DNA synthesis and mitochondrial respiration in a dose-dependent manner. The pattern of respiratory injury was strikingly similar to that reported for activated Mφ (11). Finally, systems that scavenge NO· (superoxide or ferrous myoglobin) partially blocked Mφ-mediated cytostasis in a coculture system.
NO−-induced lesions within the METC were restricted to complex 1 and 2. Complex 1 and 2 contain several FeS clusters that may be susceptible to destruction by both authentic and Mφ-derived NO− and NO2− (7, 27). In cell-free systems, NO− reacts with certain FeS proteins, forming paramagnetic complexes similar to Fe(NO)2(cysteine)2 (28). This suggests a molecular mechanism by which Mφ may cause mitochondrial iron loss and respiratory inhibition (29).

Although a pulse of authentic NO− inhibited target cell DNA synthesis in a rapid and dose-dependent manner, its effect was short-lived compared with the cytostasis caused by activated Mφ or acidified NO2−. Perhaps a sustained exposure to moderate amounts of NO−, as occurs during coculture with activated Mφ or during acidification of NO2−, has a more lasting effect than a brief exposure to larger concentrations of NO−. Alternatively, other RNI (such as HNO2), may contribute. The molecular target(s) involved in Mφ- or NO−-mediated cytostasis are unknown; thus, it is not yet possible to compare the treatments at the target level. An enzyme catalyzing the rate-limiting step in DNA synthesis, ribonucleotide reductase, contains catalytically essential non-heme iron that is easily removed (30). We are investigating whether NO−-mediated inhibition of this enzyme is involved in Mφ-induced cytostasis.

Mφ cytostasis was blocked 97% in the presence of reduced myoglobin and 44% in the presence of an Fe-catalyzed superoxide generating system. These systems scavenge NO− and have been used to prevent its biological effects (8, 20, 24, 25). In addition to scavenging NO−, FeSO4 and myoglobin may have helped injured cells to recover faster from cytostasis by furnishing Fe to replenish intracellular pools and rebuild FeS clusters (10, 31). However, our findings that SOD reversed the Fe2+/ascorbate effect and that myoglobin was inactive unless reduced by ascorbate suggest that increased availability of Fe was not the mechanism by which cytostasis was blocked.

NO− or acidified NO2− have long been known to inhibit growth, respiration, and active transport in fungi, bacteria, and bacteriophages (13–16). Molecular targets include ferredoxins (7, 32), hydrogenases (33, 34), and glycolytic enzymes that contain essential sulfhydryl groups (35, 36). The ubiquitous distribution of these enzyme systems suggests that Mφ-derived NO− may play a role in host resistance against a wide range of microbial pathogens.

The factors regulating cytotoxicity by Mφ-derived NO− are not yet well understood. For example, the flux of NO− reaching a target will depend on the concentrations of species that can scavenge it, such as oxygen (9), superoxide (20), reduced hemes (23, 24), transition metals (37), and thiols (38), as well as on the activity of species that can protect NO−, such as superoxide dismutase (39). NO− bound to certain transition metal complexes, as in nitroprusside, is sufficiently stable to be used as a source of slowly released NO− (37, 38, 40). Similarly, while thiols can scavenge NO−, resulting S-nitrosothiols can release it (38, 40).

The interrelationships between RNI and reactive oxygen intermediates (ROI) are also likely to be complex. Cytokines and bacterial products that induce production of RNI in Mφ and those that enhance the capacity of Mφ for release of ROI comprise overlapping but distinct sets (41). RNI release proceeds over ~36 h after exposure of Mφ to activating signals alone (42–44). The respiratory burst is less dependent on activating signals, but more dependent on additional triggering stimuli, following which the release of ROI usually lasts <3 h (45). Although respiratory burst products may inactivate NO−, they can also deplete species that otherwise would
scavenge or protect against NO·, such as glutathione. Moreover, ROI may synergize with RNI in mediating injury, particularly to FeS proteins involved in electron transport (46). Also unknown is the subcellular location of the NO· synthetase (our preliminary work suggests it is cytoplasmic) and whether there can be a directional component to NO· release.

In biological systems, the distance over which NO· travels is probably limited by its reaction with dissolved O2. This may explain why Mφ-mediated cytotoxicity often requires proximity between Mφ and target cells (47). NO2− and NO3− (formed via decomposition of Mφ-derived NO· and NO2 in aqueous, oxygenated environments) are relatively stable and could diffuse from the site of NO· production. Although NO2− that entered the circulation would be oxidized to NO3− by oxyhemoglobin (48), any portion entering acidic microenvironments, such as phagolysosomes, tumors, sites of infection, or exercising muscle, could reconvert to cytotoxic RNI through an acid-catalyzed reaction. This provides a mechanism by which RNI-related injury could occur at sites other than the point of origin. In pathologic states with sustained production of the appropriate cytokines (41), such a process might contribute to cachexia (49).

**Summary**

A metabolic pathway of activated macrophages (Mφ) involving oxidation of the guanido nitrogens of L-arginine is required for inhibition of growth and respiration of some target cells. The goal of this study was to identify the Mφ metabolite(s) that induce these injuries. The stable products of the L-arginine pathway, NO2− and NO3−, were incapable of causing cytostasis under coculture conditions. However, NO2− became cytostatic upon mild acidification, which favors its transformation into nitrogen oxides of greater reactivity. This suggested that NO· (and/or NO2), recently identified as an Mφ metabolite of L-arginine, could be a mediator. Authentic NO· caused cytostasis and respiratory inhibition in L1210 cells in a dose-dependent manner. The mitochondrial lesions caused by NO· were confined to complex 1 and 2, a pattern of injury identical to that seen after coculture with activated Mφ. Inclusion of NO· scavenger systems prevented cytostasis from developing in Mφ-L1210 cocultures. Thus, Mφ-generated NO· can account for L-arginine-dependent cytostasis and respiratory inhibition.

We thank Dr. Jack Peisach of Albert Einstein Medical College for helpful discussion, and Claudia Morris and Bonnie Thiel for expert assistance. IFN-γ was a generous gift of Genentech.

Received for publication 21 December 1988.

Note added in proof: Evidence that Mφ produce NO· was recently reported by two additional laboratories (50, 51).

**References**

1. Adams, D. O., and T. A. Hamilton. 1984. The cell biology of macrophage activation. *Annu. Rev. Immunol.* 2:283.

2. Nathan, C. F. 1986. Interferon-gamma and macrophage activation in cell-mediated immunity. In *Mechanisms of Host Resistance to Infectious Agents, Tumors, and Allografts.*
R. M. Steinman and R. J. North, editors. The Rockefeller University Press, New York. 165-184.

3. Nathan, C. F. 1982. Secretion of oxygen intermediates: role in effector functions of activated macrophages. *Fed. Proc.* 41:2206.

4. Urban, J. L., H. M. Shepard, J. L. Rothstein, B. J. Sugarman, and H. Schreiber. 1986. Tumor necrosis factor: a potent effector molecule for tumor cell killing by activated macrophages. *Proc. Natl. Acad. Sci. USA.* 83:5233.

5. Onozaki, K., K. Matsushima, E. S. Kleinerman, T. Saito, and J. J. Oppenheim. 1985. Role of interleukin 1 in promoting human monocyte-mediated tumor cytotoxicity. *J. Immunol.* 135:314.

6. Hibbs, J. B. Jr., R. R. Taintor, and Z. Vavrin. 1987. Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science (Wash. DC).* 235:473.

7. Stuehr, D. J., S. S. Gross, I. Sakuma, R. Levi, and C. F. Nathan. 1989. Activated murine macrophages secrete a metabolite of arginine with the bioactivity of endothelium-derived relaxing factor and the chemical reactivity of nitric oxide. *J. Exp. Med.* 169:1011.

8. Ignarro, L. J., R. E. Byrns, G. M. Buga, and K. S. Wood. 1987. Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. *Circ. Res.* 61:866.

9. Cotton, F. A., and G. Wilkinson. 1980. Advanced Inorganic Chemistry, a Comprehensive Text. 4th Ed. Wiley-Interscience, New York. 422-430.

10. Drapier, J. C., and J. B. Hibbs, Jr. 1986. Murine cytotoxic activated macrophages inhibit aconitase in tumor cells. *J. Clin. Invest.* 78:790.

11. Granger, D. L., and A. L. Lehringer. 1982. Sites of inhibition of mitochondrial electron transport in macrophage-injured neoplastic cells. *J. Cell Biol.* 95:527.

12. Green, L. C., K. Ruiz de-Luzuriaga, D. A. Wanger, W. Rand, N. Istan, V. R. Young, and S. R. Tannenbaum. 1981. Nitrate biosynthesis in man. *Proc. Natl. Acad. Sci. USA.* 78:7764.

13. Tarr, H. L. A. 1941. Bacteriostatic action of nitrates. *Nature (Lond.).* 147:417.

14. Castellani, A. G., and C. F. Niven, Jr. 1955. Factors affecting the bacteriostatic action of sodium nitrite. *Appl. Microbiol.* 3:154.

15. Shank, J. L., J. H. Silliker, and R. H. Harper. 1962. The effect of nitric oxide on bacteria. *Appl. Microbiol.* 10:185.

16. Lytle, C. D., and W. Ginoza. 1970. Inactivation of bacteriophage φX174 by sublethal nitrous acid-produced lesions. *Biochem. Biophys. Res. Commun.* 39:809.

17. Taylor, T. W. J., E. W. Wignall, and J. F. Cowley. 1927. The decomposition of nitrous acid in aqueous solution. *J. Chem. Soc.* 11:1923.

18. Krahenbuhl, J. L., L. H. Lambert Jr., and J. S. Remington. 1976. The effects of activated macrophages on tumor target cells: escape from cytostasis. *Cell. Immunol.* 25:279.

19. Granger, D. L., R. R. Taintor, J. L. Cook, and J. B. Hibbs, Jr. 1980. Injury of neoplastic cells by murine macrophages leads to inhibition of mitochondrial respiration. *J. Clin. Invest.* 65:357.

20. Blough, N. V., and O. C. Zafiriou. 1985. Reaction of superoxide with nitric oxide to form peroxonitrite in alkaline aqueous solution. *Inorg. Chem.* 24:3502.

21. Winterbourn, C. 1979. Comparison of superoxide with other reducing agents in the biological production of hydroxyl radicals. *Biochem. J.* 182:623.

22. O'Donnell-Tormey, J., C. J. DeBoer, and C. F. Nathan. 1985. Resistance of human tumor cells in vitro to oxidative cytolysis. *J. Clin. Invest.* 76:80-86.

23. Yonetani, T., H. Yamamoto, J. E. Erman, J. S. Leigh, Jr., and G. H. Reed. 1972. Electromagnetic properties of hemoproteins. *J. Biol. Chem.* 247:2447.

24. Goretski, J., and T. C. Hollocher. 1988. Trapping of nitric oxide produced during denitrification by extracellular hemoglobin. *J. Biol. Chem.* 263:2316.
MEDIATION OF CYTOTOXICITY BY NITRIC OXIDE

25. Martin, W., G. M. Villani, D. Jothianandan, and R. F. Furchgott. 1985. Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. Pharmacol. Exp. Ther. 232:708.

26. Palmer, R. M. J., A. G. Ferrige, and S. Moncada. 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature (Lond.). 327:524.

27. Salerno, J. C., and T. Ohnishi. 1976. Tetranuclear and binuclear iron-sulfur clusters in succinate dehydrogenase: a method of iron quantitation by formation of paramagnetic complexes. Biochem. Biophys. Res. Commun. 73:833.

28. McDonald, C. C., W. D. Phillips, and H. F. Mower. 1965. An electron spin resonance study of some complexes of iron, nitric oxide and anionic ligands. J. Am. Chem. Soc. 87:3319.

29. Wharton, M., D. L. Granger, and D. T. Durack. 1988. Mitochondrial iron loss from leukemia cells injured by macrophages. A possible mechanism for electron transport chain defects. J. Immunol. 141:1311.

30. Graslund, A., M. Sahlin, and B. M. Sjoberg. 1985. The tyrosyl free radical in ribonucleotide reductase. Environ. Health Perspect. 64:139.

31. Weinberg, J. B., and J. B. Hibbs, Jr. 1977. Endocytosis of red blood cells or haemoglobin by activated macrophages inhibits their tumoricidal effect. Nature (Lond.). 269:245.

32. Woods, L. F. J., J. M. Wood, and P. A. Gibbs. 1981. The involvement of nitric oxide in the inhibition of the phosphoroclastic system in Clostridium sponges by sodium nitrite. J. Gen. Microbiol. 125:399.

33. Tibelius, K. H., and R. Knowles. 1984. Hydrogenase activity in Azospirillum brasilense is inhibited by nitrite, nitric oxide, carbon monoxide, and acetylene. J. Bacteriol. 160:103.

34. Krasna, A. I., and D. Rittenberg. 1954. The inhibition of hydrogenase by nitric oxide. Proc. Natl. Acad. Sci. USA. 40:225.

35. O'Leary V., and M. Solberg. 1976. Effect of sodium nitrite inhibition on intracellular thiol groups and on the activity of certain glycolytic enzymes in Clostridium perfringens. Appl. Environ. Microbiol. 31:208.

36. Yarbrough, J. M., J. B. Rake, and R. G. Eagon. 1980. Bacterial inhibitory effects of nitrite: inhibition of active transport, but not of group translocation, and of intracellular enzymes. Appl. Environ. Microbiol. 39:831.

37. McCleverty, J. A. 1979. Reactions of nitric oxide coordinated to transition metals. Chem. Rev. 79:53.

38. Ignarro, L. J., H. Lippton, J. C. Edwards, W. H. Baricos, A. L. Hyman, P. J. Kadowitz, and C. A. Gruetter. 1981. Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrates, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates. J. Pharmacol. Exp. Ther. 218:739.

39. Gryglewski, R. J., R. M. J. Palmer, and S. Moncada. 1986. Superoxide anion is involved in the breakdown of endothelium-derived relaxing factor. Nature (Lond.). 320:454.

40. Feilisch, M., and E. A. Noak. 1987. Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. Eur. J. Pharmacol. 139:19.

41. Ding, A., C. F. Nathan, and D. J. Stuehr. 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. J. Immunol. 141:2407.

42. Stuehr, D. J., and M. A. Marletta. 1985. Mammalian nitrite biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide. Proc. Natl. Acad. Sci. USA. 82:7738.

43. Stuehr, D. J., and M. A. Marletta. 1987. Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-γ. J. Immunol. 139:518.

44. Iyengar, R., D. J. Stuehr, and M. A. Marletta. 1987. Macrophage synthesis of nitrite, nitrate and N-nitrosamines: precursors and role of the respiratory burst. Proc. Natl. Acad. Sci. USA. 84:8369.
45. Nathan, C. F., and R. K. Root. 1977. Hydrogen peroxide release from mouse peritoneal macrophages. Dependence on sequential activation and triggering. J. Exp. Med. 146:1648.
46. Rosen, H., and S. J. Klebanoff. 1985. Oxidation of microbial iron-sulfur centers by the myeloperoxidase-H2O2-halide antimicrobial system. Infect. Immun. 47:613.
47. Lu, C. Y., M. J. Lombardi, C. M. Shea, and L. B. Dustin. 1988. High strength binding of P815 mastocytoma cells is not necessary for their lysis by macrophages which have been primed and triggered in vitro. J. Immunol. 141:1083.
48. Kosaka, H., K. Imaizumi, K. Imai, and I. Tyuma. 1979. Stoichiometry of the reaction of oxyhemoglobin with nitrite. Biochim. Biophys. Acta. 581:184.
49. Billiar, T. R., R. D. Curran, D. J. Stuehr, M. A. West, B. G. Bentz, and R. L. Simmons. 1989. An L-arginine-dependent mechanism mediates Kupffer cell inhibition of hepatocyte protein synthesis in vitro. J. Exp. Med. 169:1467.
50. Marletta, M. A., P. S. Yoon, R. Iyengar, C. D. Leaf, and J. S. Wishnok. 1988. Macrophage oxidation or L-arginine to nitrite and nitrate: nitric oxide is an intermediate. Biochemistry. 27:8706.
51. Hibbs, J. B., Jr., R. R. Taintor, Z. Vavrin, and E. M. Rachlin. 1988. Nitric oxide: a cytotoxic activated macrophage effector molecule. Biochem. Biophys. Res. Commun. 157:87.