

NITRIC OXIDE

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

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Once activated by agents such as IFN- γ and bacterial LPS, macrophages (M ϕ)¹ can inhibit the growth of a wide variety of tumor and microbial targets (1, 2). Although M ϕ 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, M ϕ -mediated cytostasis and injury to the mitochondrial electron transport chain (METC) require a process associated with M ϕ 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 M ϕ have recently been shown to release a compound similar to or identical with the reactive radical nitric oxide (NO \cdot) during metabolism of L-arginine to NO₂⁻/NO₃⁻ (7). This report identifies NO \cdot (or a closely related product) as a mediator of M ϕ -induced cytostasis and mitochondrial respiratory inhibition in lymphoma cells.

Materials and Methods

Reagents. Cells were cultured in minimum Eagle's medium, α modification (α MEM) 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 μ g/ml). Catalase and N^G-monomethyl-L-arginine (NMA) were from Calbiochem-Behring Corp. La Jolla, CA. NO \cdot gas (99% pure) and N₂ gas (\leq 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 μ m, Millipore, Danvers, MA).

Collection and Culture of Mouse Peritoneal M ϕ and L1210 Cells. Peritoneal M ϕ 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

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¹ *Abbreviations used in this paper:* α glyPi, α -glycerophosphate; IC₅₀, dose producing 50% maximal inhibition; M ϕ , macrophages; METC, mitochondrial electron transport chain; NMA, N^G-monomethyl-L-arginine; NO \cdot , nitric oxide; NO₂, nitrogen dioxide; NO₂⁻, nitrite; 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 α MEM 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 α MEM, which in some cases contained 500 U/ml IFN- γ to activate the M ϕ . L1210 cells (American Type Culture Collection, Rockville, MD) were kept in continuous culture in RPMI 1640.

M ϕ -L1210 Cell Coculture. After M ϕ were cultured overnight in 96-well plates, the medium was aspirated and replaced with 50 μ l α MEM containing no added LPS (control M ϕ) or 3 μ g/ml LPS (activated M ϕ). L1210 cells (5×10^4 /well, 25 μ l) were then added along with solutions containing experimental agents and the volume of each well brought to 150 μ l with α MEM. After 6 h, 2.5 μ Ci [³H]TdR was added to each well for a period lasting 12–18 h. In some cases, 50 μ l volumes were removed from the cocultures at this point to measure NO₂⁻ production. Each experiment had control wells consisting of L1210 cells cultured without M ϕ under each experimental condition; L1210 [³H]TdR incorporation was unaffected by the additives in all cases. For experiments in Table II, 1.5×10^7 M ϕ were plated 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 \pm LPS or LPS and 250 μ M NMA. L1210 cells were added (2×10^6 /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 NO₂⁻ 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 morpholinoethane sulfonate, and various concentrations of NaNO₂ or NaNO₃. 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- μ m 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 \cdot -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 \cdot 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 \cdot] = 1.25 mM; reference 8). Various volumes were transferred with a gas-tight syringe into stoppered N₂-flushed tubes containing L1210 cells (4×10^7 to 4×10^8) 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 \cdot . 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 $\geq 80\%$ (trypan blue dye exclusion). For controls, the NO \cdot 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 \cdot from the solution and sparging with air converted residual NO \cdot to HNO₂ and NO₃⁻ (9). For [³H]TdR incorporation studies 4×10^6 cells were treated with NO \cdot as above, resuspended to 2 or 4×10^6 cells/ml in RPMI, plated at 5×10^4 /well, and pulsed with [³H]TdR. The NO \cdot -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 \cdot -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 5×10^7 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 10^8 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 μ l) were injected into a jacketed respiration

chamber, which was kept at 37°C and contained 1.4 ml Dulbecco's PBS (without Ca^{2+} or Mg^{2+}) plus 25 mM glucose. L1210 respiration was calculated as the rate of decrease in O_2 concentration following addition of cells, assuming an initial $[\text{O}_2] = 390 \text{ ng/ml}$ (11). Respiration of L1210 cells was dependent on glucose and 100% inhibitable by 3 mM KCN in all cases.

O_2 consumption by digitonin-permeabilized cells given mitochondrial substrates was measured as described previously (11). 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 (αglyPi), 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 (αglyPi) (11). 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 O_2 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 $\sim 15\%$ of O_2 consumption was not inhibitable. The cyanide-insensitive value for each run that used TMPD was subtracted.

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

NO_2^- and NO_3^- Determination. NO_2^- 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% H_3PO_4) 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). NO_2^- was determined using NaNO_2 as a standard and double-distilled H_2O as a blank. Background NO_2^- values of buffers or media were determined in each case and subtracted from the experimental values. In certain cases the NO_2^- and NO_3^- concentrations were measured by an automated method described elsewhere (12).

Results

Cytostasis Depends on a Process Associated with *M ϕ* $\text{NO}_2^-/\text{NO}_3^-$ Synthesis but Is Not Due to $\text{NO}_2^-/\text{NO}_3^-$ or their Metabolites. Initial experiments showed that induction of *M ϕ* $\text{NO}_2^-/\text{NO}_3^-$ 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-

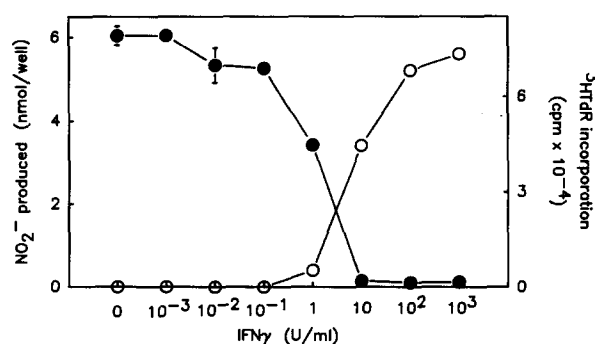


FIGURE 1. NO_2^- synthesis (O) and $[\text{^3H}]\text{TdR}$ uptake (\bullet) by cocultures of C3H/HeJ macrophages and L1210 cells in the presence of 10 $\mu\text{g/ml}$ LPS and various concentrations of IFN- γ . LPS and IFN- γ were added at the same time to cocultures, $[\text{^3H}]\text{TdR}$ was added 6 h later, and the incorporation period was 12 h. The points represent the mean \pm SD of four wells.

based inhibitor of M ϕ NO₂⁻ synthesis, NMA (not shown). This confirmed a previous report (6) that induction of the M ϕ NO₂⁻-producing pathway correlates with and is required for cytoxicity in this system. Since NO₂⁻ can be growth inhibitory (13-16), we tested if exogenous NaNO₂ could cause cytoxicity in the cocultures. NaNO₃ served as a control. As shown in Table I, neither NaNO₂ nor NaNO₃ inhibited [³H]TdR incorporation in cultures containing L1210 cells and either nonactivated or activated M ϕ (NMA was added to cocultures containing activated M ϕ to block conversion of L-arginine to NO₂⁻/NO₃⁻). NO₂⁻ and NO₃⁻ were ineffective even when added at concentrations 50-fold higher than those typically achieved under coculture conditions (125 μ M). Thus, activated M ϕ did not convert added NO₂⁻ or NO₃⁻ into cytoxic agents.

Generation of Cytoxic Reactive Nitrogen Intermediates (RNI) from Acidification of NO₂⁻. In bacterial systems (13-16), the cytoxic action of NO₂⁻ increases upon mild acidification through formation of nitrous acid (HNO₂, *pK_a* 3.4) and its dismutation, which generates other RNI, including NO \cdot and NO₂ (9, 17). Thus, we tested if acidified NO₂⁻ solutions would inhibit replication of L1210 in the absence of macrophages. Fig. 2 shows that L1210 [³H]TdR incorporation was inhibited after culture with NO₂⁻ under mildly acidic conditions. The degree of growth inhibition was directly proportional to the time of exposure, the acidity, and the concentration of NO₂⁻, consistent with a requirement for formation of HNO₂. At pH 6.2, a 10-h exposure to 250 μ M NO₂⁻ (a concentration twice that typically achieved in activated M ϕ cultures) caused >50% cytoxicity. Cytoxicity did not occur when L1210 cells were cultured at these pH values in the absence of NO₂⁻ (Fig. 2), nor when NO₃⁻ replaced NO₂⁻ (not shown).

Cytoxicity by NO \cdot . Since RNI generated from acidified NO₂⁻ were cytoxic, we tested the effect of authentic NO \cdot . Exposure to NO \cdot for 5 min inhibited L1210

TABLE I
Inability of Added NO₂⁻ and NO₃⁻ to Inhibit
L1210 Growth in M ϕ Coculture

Added NO ₂ ⁻ /NO ₃ ⁻ <i>mM</i>	L1210 [³ H]TdR incorporation			
	Control M ϕ		Activated M ϕ + NMA	
	NO ₂ ⁻	NO ₃ ⁻	NO ₂ ⁻	NO ₃ ⁻
0	89 \pm 2	—	79 \pm 0	—
1	93 \pm 8	96 \pm 2	62 \pm 3	78 \pm 6
5	96 \pm 2	96 \pm 3	76 \pm 2	76 \pm 7
10	99 \pm 11	84 \pm 13	88 \pm 5	78 \pm 4

C3H/He M ϕ (10⁵/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 μ g/ml LPS plus 250 μ M NMA (activated). L1210 cells and 10 μ l of NaNO₂ or NaNO₃ solutions were added. [³H]TdR was added 6 h later for an 18-h period. The experiment is representative of three and the values are the mean cpm \pm SD of four wells. [³H]TdR incorporation in activated M ϕ /L1210 cocultures not receiving NMA was 1,208 \pm 706 cpm (98% cytoxicity). NO₂⁻ production (nmol/well) by cultures that did not receive [³H]TdR was 0.0 \pm 0.0, control M ϕ ; 0.7 \pm 0.1, activated M ϕ + NMA; and 4.6 \pm 0.1, activated M ϕ without NMA (*n* = 4).

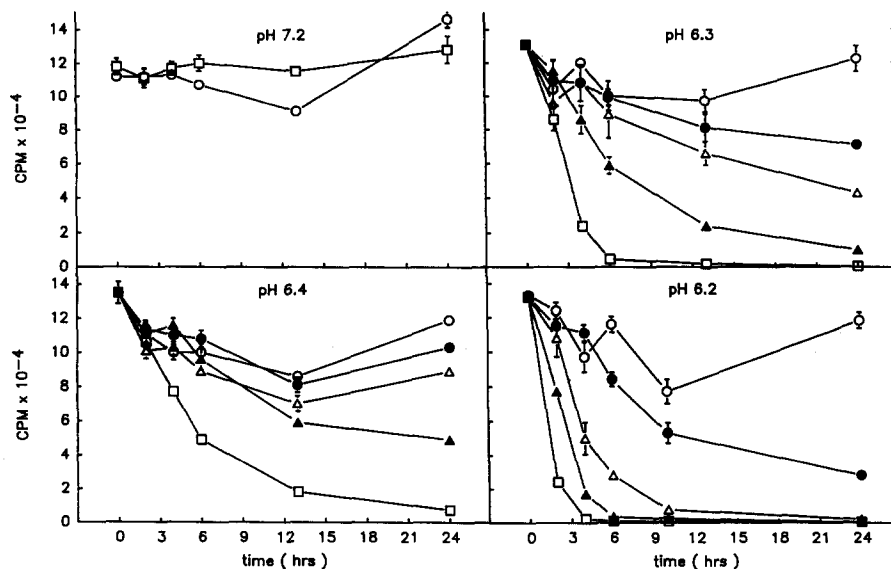


FIGURE 2. $[^3\text{H}]\text{TdR}$ incorporation by L1210 cells after culture for various times in NO_2^- -containing medium at various pH. NO_2^- concentrations were 0 (○), 0.25 (●), 0.5 (△), 1.0 (▲), and 2.5 mM (□). The experiment is representative of three and the values are the mean $\text{cpm} \pm \text{SD}$ for four cultures over an 18-h period.

$[^3\text{H}]\text{TdR}$ incorporation in a dose-dependent manner during a subsequent 3-h labeling period, with an IC_{50} of $\sim 20 \text{ nmol NO}\cdot/10^6$ cells (Fig. 3). Solutions that had been rid of $\text{NO}\cdot$ by N_2 sparging and aeration were incapable of causing cytostasis. This indicated that the active principle was $\text{NO}\cdot$ and not its nonvolatile or oxygen-resistant reaction products, such as $\text{NO}_2^-/\text{NO}_3^-$. Maximal inhibition required $\text{NO}\cdot$ exposures as short as 30 s; inhibition remained $\text{NO}\cdot$ specific through at least 10 min of exposure (not shown). Thus, 5-min exposures were used routinely.

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

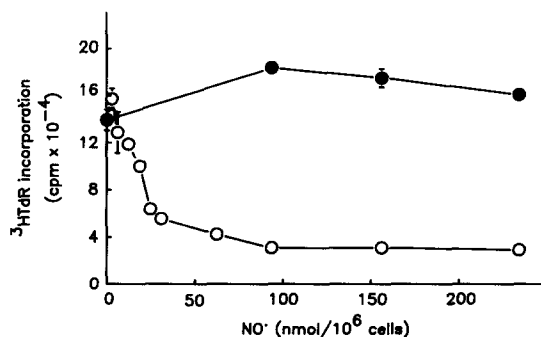


FIGURE 3. Dose-response curve for $\text{NO}\cdot$ inhibition of L1210 $[^3\text{H}]\text{TdR}$ incorporation. Cells were treated for 5 min with $\text{NO}\cdot$ solution (○) or $\text{NO}\cdot$ solution that had been sparged with N_2/air (●). $[^3\text{H}]\text{TdR}$ incorporation was measured over the first 3 h period after $\text{NO}\cdot$ treatment. The experiment shown represents one of five. The points are the mean $\text{cpm} \pm \text{SD}$ for four cultures.

TABLE II
Timecourse of L1210 Recovery from M ϕ - or NO \cdot -induced Cytostasis

Pulse period <i>h</i>	L1210 [^3H]TdR incorporation after exposure to:				
	NO \cdot	Aerated NO \cdot	Control	Activated M ϕ	Control M ϕ
	<i>cpm</i> $\times 10^{-3}$				
0-3	3 \pm 0	17 \pm 1	14 \pm 1	3 \pm 1	27 \pm 2
3-6	25 \pm 1	34 \pm 2	29 \pm 2	3 \pm 0	42 \pm 2
6-9	34 \pm 3	26 \pm 11	30 \pm 3	4 \pm 0	51 \pm 0

L1210 cells that were cocultured 24 h with CD-1 M ϕ or treated for 5 min with NO \cdot solution (625 nmol), N $_2$ -sparged/aerated NO \cdot solution, or no solution (control), were plated at 5×10^4 /well, and pulsed with [^3H]TdR for the indicated periods. For L1210 cells harvested from M ϕ plates, the resuspension and pulsing medium contained 250 μM NMA to prevent RNI synthesis by activated M ϕ that might be carried over in the washing step. The experiment is representative of four and the values are the mean $\text{cpm} \pm \text{SD}$ of four cultures. The NO \cdot solution had a pH of 5.4 and a NO $_2^-$ concentration of 4.1 mM. M ϕ NO $_2^-$ production over the coculture period was 0.0 ± 0.4 and 11.7 ± 0.4 nmol/well for control and activated cultures, respectively.

first, second, and third 3-h periods after exposure to NO \cdot was 22, 73, and 128% of controls, respectively. In contrast, DNA synthesis by L1210 cells that had been cocultured with activated M ϕ within the same three time periods was 12, 8, and 9% of controls. Recovery of [^3H]TdR incorporation to the level of controls for M ϕ -injured cells was not seen until 30 h (not shown). Thus, a pulse of NO \cdot caused cytostasis of shorter duration than that caused by cocultivation with activated M ϕ for 24 h.

NO \cdot -mediated Respiratory Inhibition. Like cytostasis, inhibition of target cell respiration by activated M ϕ is dependent on metabolism of L-arginine to NO $_2^-$ /NO $_3^-$ (6). We therefore determined if NO \cdot could inhibit respiration of L1210 cells in the absence of M ϕ . Fig. 4 shows that NO \cdot treatment inhibited cyanide-sensitive oxygen uptake by L1210 cells in a dose-dependent manner, with an IC $_{50}$ of 66 nmol/10 7 cells. The effect was NO \cdot specific, since NO \cdot solutions that had been sparged with N $_2$ and aerated were inactive.

Sites of NO \cdot Injury within the METC. M ϕ -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 \cdot exhibited similar specificity, NO \cdot -treated cells were permeabilized with digitonin

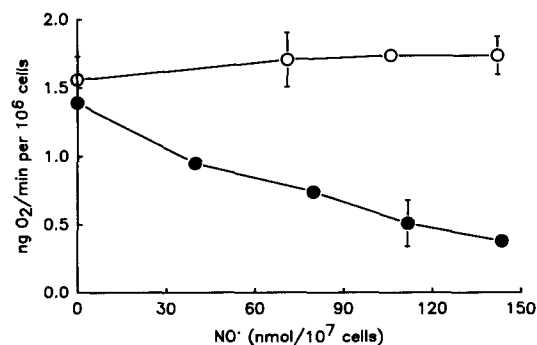


FIGURE 4. Dose-response curve for NO \cdot inhibition of L1210 respiration on glucose. Cells were treated for 5 min with various amounts of NO \cdot solution (●) or NO \cdot solution that had been N $_2$ /air-sparged (○) to remove NO \cdot . The experiment shown represents one of seven. The points are the mean \pm SD of three determinations.

TABLE III
Sites of NO· Injury Within the Mitochondrial Electron Transport Chain

Substrate	Acceptor	Mitochondrial O ₂ consumption by permeabilized L1210 cells	
		Control	NO· treated
<i>ng O₂/min/10⁶ cells</i>			
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	0.587 ± 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 permeabilized 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.

and mitochondrial substrates were used to measure electron flow through complex 1 (malate), complex 2 (succinate), coenzyme Q (αglyPi), 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 cytoxicity 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 cytoxicity. 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 cytoxicity. 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 cytoxicity 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 cytoxicity at all Fe²⁺ concentrations, presumably by providing electrons for Fe²⁺-catalyzed superoxide production (21). At 100

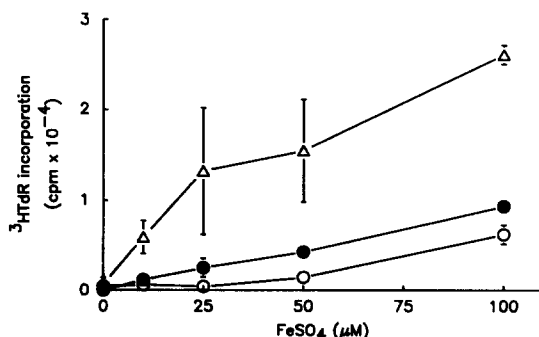


FIGURE 5. Inhibition of activated Mφ-induced cytoxicity by a superoxide-generating system. [³H]TdR incorporation by L1210 cells cocultured with activated Mφ in the presence of various concentrations of FeSO₄ alone (●), with 1 mM ascorbate and 1,000 U/ml catalase (Δ), or with ascorbate, catalase, and 500 U/ml SOD (○) was measured over an 18-h period. The experiment represents one of three. 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 60,018 ± 1,678 cpm.

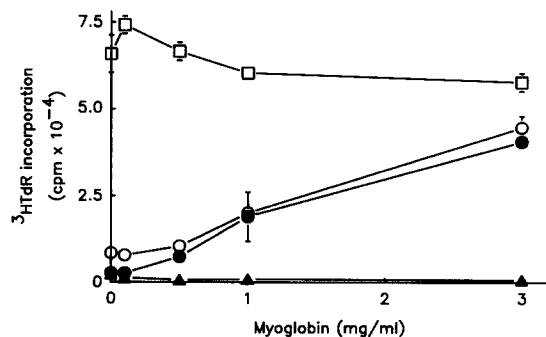


FIGURE 6. Inhibition of activated M ϕ -induced cytostasis by myoglobin. L1210 cells were cultured with activated M ϕ and various concentrations of myoglobin alone (\blacktriangle), myoglobin plus 1 mM ascorbate and 1,000 U/ml catalase (\circ), or myoglobin plus ascorbate/catalase and 500 U/ml SOD (\bullet). Results for L1210 cultured with control M ϕ , ascorbate/catalase, and varying amounts of myoglobin (\square) are also shown. The experiment represents one of six. The points are the mean cpm \pm SD for four wells. For comparison, [^3H]TdR incorporation by L1210 cells in cocultures containing activated M ϕ and 250 μM NMA was $75,297 \pm 2,683$ cpm.

μM FeSO_4 (plus ascorbate), L1210 DNA synthesis returned to 44% that of control cocultures (L1210 cells and activated M ϕ given NMA). Inclusion of 500 U/ml superoxide dismutase (SOD) eliminated the protective effect of Fe^{2+} /ascorbate and fully restored M ϕ -mediated cytostasis, while boiled SOD was inactive. Thus, Fe^{2+} /ascorbate prevented M ϕ cytostasis primarily through generation of superoxide.

Ferroheme complexes such as ferrous myoglobin bind $\text{NO}\cdot$ with high affinity (23, 24) and have been used to scavenge $\text{NO}\cdot$ generated by endothelial cells (8, 25, 26) and M ϕ (7). The effect of a myoglobin $\text{NO}\cdot$ -scavenging system on M ϕ -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 M ϕ -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 $\text{NO}\cdot$ (or NO_2 , formed via reaction of $\text{NO}\cdot$ with O_2) as an L-arginine-derived M ϕ 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 (NO_2^- and NO_3^-) were inactive. However, NO_2^- became cytostatic at acidic pH, a condition under which NO_2^- is chemically converted into more reactive species, including HNO_2 , $\text{NO}\cdot$, and NO_2 (9, 17). During their metabolism of L-arginine to $\text{NO}_2^-/\text{NO}_3^-$, M ϕ produce a compound with biological, biochemical, and physical properties of $\text{NO}\cdot$ or NO_2 (7). Authentic $\text{NO}\cdot$ 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 M ϕ (11). Finally, systems that scavenge $\text{NO}\cdot$ (superoxide or ferrous myoglobin) partially blocked M ϕ -mediated cytostasis in a coculture system.

NO \cdot -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 M ϕ -derived NO \cdot and NO $_2^-$ (7, 27). In cell-free systems, NO \cdot reacts with certain FeS proteins, forming paramagnetic complexes similar to Fe(NO) $_2$ (cysteine) $_2$ (28). This suggests a molecular mechanism by which M ϕ may cause mitochondrial iron loss and respiratory inhibition (29).

Although a pulse of authentic NO \cdot inhibited target cell DNA synthesis in a rapid and dose-dependent manner, its effect was shortlived compared with the cytostasis caused by activated M ϕ or acidified NO $_2^-$. Perhaps a sustained exposure to moderate amounts of NO \cdot , as occurs during coculture with activated M ϕ or during acidification of NO $_2^-$, has a more lasting effect than a brief exposure to larger concentrations of NO \cdot . Alternatively, other RNI (such as HNO $_2$), may contribute. The molecular target(s) involved in M ϕ - or NO \cdot -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 \cdot -mediated inhibition of this enzyme is involved in M ϕ -induced cytostasis.

M ϕ 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 \cdot and have been used to prevent its biological effects (8, 20, 24, 25). In addition to scavenging NO \cdot , FeSO $_4$ 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 Fe $^{2+}$ /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 \cdot or acidified NO $_2^-$ 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 M ϕ -derived NO \cdot may play a role in host resistance against a wide range of microbial pathogens.

The factors regulating cytotoxicity by M ϕ -derived NO \cdot are not yet well understood. For example, the flux of NO \cdot 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 \cdot , such as superoxide dismutase (39). NO \cdot bound to certain transition metal complexes, as in nitroprusside, is sufficiently stable to be used as a source of slowly released NO \cdot (37, 38, 40). Similarly, while thiols can scavenge NO \cdot , 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 M ϕ and those that enhance the capacity of M ϕ for release of ROI comprise overlapping but distinct sets (41). RNI release proceeds over \sim 36 h after exposure of M ϕ 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 \cdot , they can also deplete species that otherwise would

scavenge or protect against $\text{NO}\cdot$, 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 $\text{NO}\cdot$ synthetase (our preliminary work suggests it is cytoplasmic) and whether there can be a directional component to $\text{NO}\cdot$ release.

In biological systems, the distance over which $\text{NO}\cdot$ travels is probably limited by its reaction with dissolved O_2 . This may explain why $\text{M}\phi$ -mediated cytotoxicity often requires proximity between $\text{M}\phi$ and target cells (47). NO_2^- and NO_3^- (formed via decomposition of $\text{M}\phi$ -derived $\text{NO}\cdot$ and NO_2 in aqueous, oxygenated environments) are relatively stable and could diffuse from the site of $\text{NO}\cdot$ production. Although NO_2^- that entered the circulation would be oxidized to NO_3^- 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 ($\text{M}\phi$) 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 $\text{M}\phi$ metabolite(s) that induce these injuries. The stable products of the L-arginine pathway, NO_2^- and NO_3^- , were incapable of causing cytostasis under coculture conditions. However, NO_2^- became cytostatic upon mild acidification, which favors its transformation into nitrogen oxides of greater reactivity. This suggested that $\text{NO}\cdot$ (and/or NO_2), recently identified as an $\text{M}\phi$ metabolite of L-arginine, could be a mediator. Authentic $\text{NO}\cdot$ caused cytostasis and respiratory inhibition in L1210 cells in a dose-dependent manner. The mitochondrial lesions caused by $\text{NO}\cdot$ were confined to complex 1 and 2, a pattern of injury identical to that seen after coculture with activated $\text{M}\phi$. Inclusion of $\text{NO}\cdot$ scavenger systems prevented cytostasis from developing in $\text{M}\phi$ -L1210 cocultures. Thus, $\text{M}\phi$ -generated $\text{NO}\cdot$ can account for L-arginine-dependent cytostasis and respiratory inhibition.

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Note added in proof: Evidence that $\text{M}\phi$ produce $\text{NO}\cdot$ was recently reported by two additional laboratories (50, 51).

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