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 (Mϕ)[1] 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 NO_2^-/NO_3^- (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 NO_2^-/NO_3^- (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^G-monomethyl-L-arginine (NMA) were from Calbiochem-Behring Corp. La Jolla, CA. NO· gas (99% pure) and N_2 gas ($\leq$5 ppm O_2) were from Matheson Gas Products, East Rutherford, NJ. [Methyl-^{3}H]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 $NaNO_2$, $NaNO_3$, $FeSO_4$) 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

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[1] *Abbreviations used in this paper:* αglyPi, α-glycerophosphate; IC_{50}, dose producing 50% maximal inhibition; Mϕ, macrophages; METC, mitochondrial electron transport chain; NMA, N^G-monomethyl-L-arginine; NO·, nitric oxide; NO_2, nitrogen dioxide; NO_2^-, nitrite; NO_3^-, 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% CO_2, 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 [^{3}H]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 NO_2^- production. Each experiment had control wells consisting of L1210 cells cultured without Mϕ under each experimental condition; L1210 [^{3}H]TdR incorporation was unaffected by the additives in all cases. For experiments in Table II, 1.5×10^7 Mϕ 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 ± LPS or LPS and 250 μM 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 [^{3}H]TdR incorporation studies.

Culture of L1210 Cells with NO_2^- at Reduced pH. L1210 cells (2×10^6/ml) were cultured without CO_2 in a 37°C incubator in bicarbonate-free DME containing 2% CS, 20 mM Hepes, 20 mM morpholinoethane sulfonate, and various concentrations of $NaNO_2$ or $NaNO_3$. 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. [^{3}H]TdR was added and its incorporation was measured over an 18-h period.

Treatment of L1210 Cells with NO·-saturated Solutions. For O_2 consumption studies solutions of saline (10 ml) containing 25 mM glucose were bubbled with N_2 for 45 min to remove dissolved O_2. Authentic NO· that had passed through 1 M KOH to remove nitrogen dioxide (NO_2) 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 N_2-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·. 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 N_2 for 15 min and then with air for 5 min before transfer to the cells. Sparging with N_2 removed most of the NO· from the solution and sparging with air converted residual NO· to HNO_2 and NO_3^- (9). For [^{3}H]TdR incorporation studies 4×10^6 cells were treated with NO· as above, resuspended to 2 or 4×10^6 cells/ml in RPMI, plated at 5×10^4/well, and pulsed with [^{3}H]TdR. The NO·-saturated saline/glucose solutions that were used to treat cells for the [^{3}H]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 K_2HPO_4, 10 mM $MgCl_2$, 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 O_2 respiration measurements.

O_2 Respiration Measurements. O_2 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 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 $[O_2]$ = 390 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 subtrate from the state 3 rate. Cyanide (3 mM) completely blocked respiration on all substrates except TMPD, where ~15% of O_2 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.

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 Mϕ NO_2^-/NO_3^- Synthesis but Is Not Due to NO_2^-/NO_3^- or their Metabolites. Initial experiments showed that induction of Mϕ NO_2^-/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-

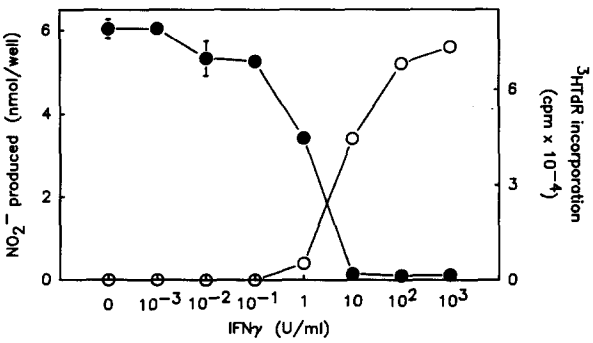

FIGURE 1. NO_2^- synthesis (○) and [³H]TdR uptake (●) by cocultures of C3H/HeJ macrophages and L1210 cells in the presence of 10 μg/ml LPS and various concentrations of IFN-γ. LPS and IFN-γ were added at the same time to cocultures, [³H]TdR was added 6 h later, and the incorporation period was 12 h.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

based inhibitor of Mϕ NO_2^- synthesis, NMA (not shown). This confirmed a previous report (6) that induction of the Mϕ NO_2^--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

	L1210 [^{3}H]TdR incorporation			
	Control Mϕ		Activated Mϕ + NMA	
Added NO_2^-/NO_3^-	NO_2^-	NO_3^-	NO_2^-	NO_3^-
mM	*cpm × 10^{-3}*			
0	89 ± 2	–	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 (10^5/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).

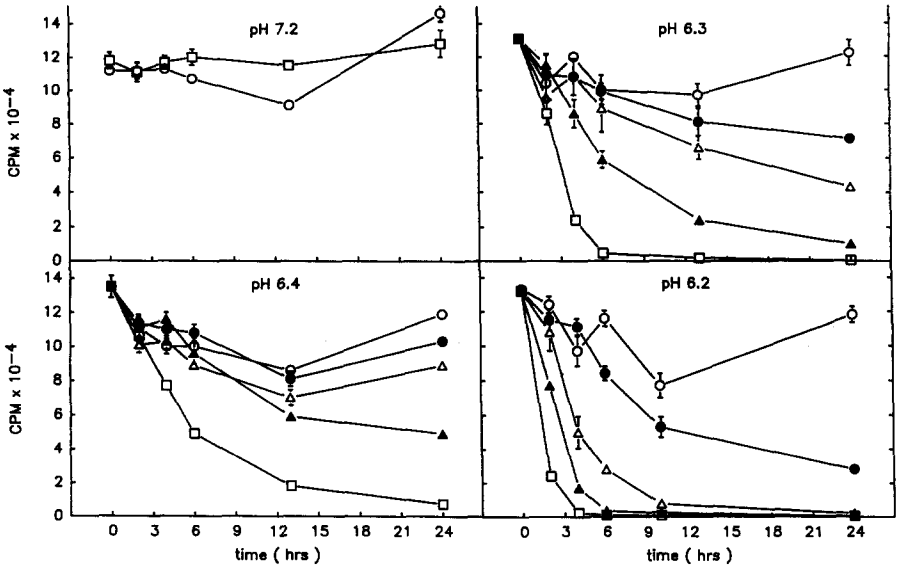

FIGURE 2. [^{3}H]TdR incorporation by L1210 cells after culture for various times in NO_2^--containing medium at various pH. NO_2^- concentrations were 0 (○), 0.25 (●), 0.5 (△), 1.0 (▲), and 2.5 mM (□). The experiment is representative of three and the values are the mean cpm ± SD for four cultures over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

[^{3}H]TdR incorporation in a dose-dependent manner during a subsequent 3-h labeling period, with an IC_{50} of ~20 nmol NO·/10^6 cells (Fig. 3). Solutions that had been rid of NO· by N_2 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 NO_2^-/NO_3^-. 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 [^{3}H]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

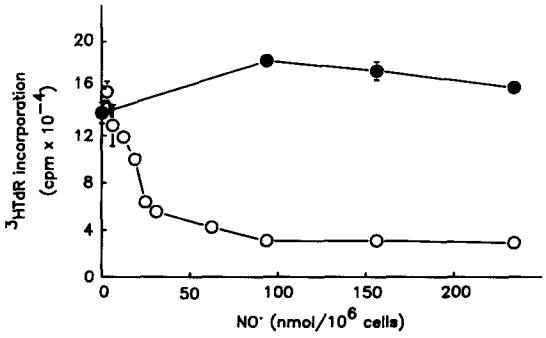

FIGURE 3. Dose-response curve for NO· inhibition of L1210 [^{3}H]TdR incorporation. Cells were treated for 5 min with NO· solution (○) or NO· solution that had been sparged with N_2/air (●). [^{3}H]TdR incorporation was measured over the first 3 h period after NO· treatment. The experiment shown represents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

TABLE II

Timecourse of L1210 Recovery from Mϕ- or NO·-induced Cytostasis

Pulse period	L1210 [^{3}H]TdR incorporation after exposure to:				
	NO·	Aerated NO·	Control	Activated Mϕ	Control Mϕ
h			*cpm* × 10^{-3}		
0-3	3 ± 0	17 ± 1	14 ± 1	3 ± 1	27 ± 2
3-6	25 ± 1	34 ± 2	29 ± 2	3 ± 0	42 ± 2
6-9	34 ± 3	26 ± 11	30 ± 3	4 ± 0	51 ± 0

L1210 cells that were cocultured 24 h with CD-1 Mϕ or treated for 5 min with NO· solution (625 nmol), N_2-sparged/aerated NO· solution, or no solution (control), were plated at 5 × 10^4/well, and pulsed with [^{3}H]TdR for the indicated periods. For L1210 cells harvested from Mϕ plates, the resuspension and pulsing medium contained 250 μM NMA to prevent RNI synthesis by activated Mϕ that might be carried over in the washing step. The experiment is representative of four and the values are the mean cpm ± SD of four cultures. The NO· solution had a pH of 5.4 and a NO_2^- concentration of 4.1 mM. Mϕ 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· 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 [^{3}H]TdR incorporation to the level of controls for Mϕ-injured cells was not seen until 30 h (not shown). Thus, a pulse of NO· caused cytostasis of shorter duration than that caused by cocultivation with activated Mϕ for 24 h.

NO·-mediated Respiratory Inhibition. Like cytostasis, inhibition of target cell respiration by activated Mϕ is dependent on metabolism of L-arginine to NO_2^-/NO_3^- (6). We therefore determined if NO· could inhibit respiration of L1210 cells in the absence of Mϕ. Fig. 4 shows that NO· 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· specific, since NO· solutions that had been sparged with N_2 and aerated were inactive.

Sites of NO· 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· exhibited similar specificity, NO·-treated cells were permeabilized with digitonin

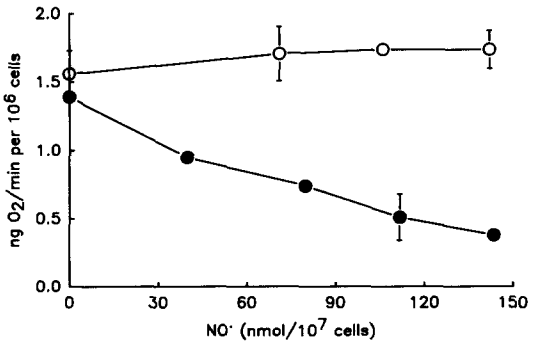

FIGURE 4. Dose-response curve for NO· inhibition of L1210 respiration on glucose. Cells were treated for 5 min with various amounts of NO· solution (●) or NO· solution that had been N_2/air-sparged (○) to remove NO·. The experiment shown represents one of seven. The points are the mean ± SD of three determinations.

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

Substrate	Acceptor	Mitochondrial O_2 consumption by permeablized L1210 cells	
		Control	NO· treated
		ng O_2/min/10^6 cells	
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·/10^7 cells) or an equivalent volume of N_2-sparged/aerated NO· solution (pH 2.9, [NO_2^-] = 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.

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/10^7 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 NO_3^- (20). Fig. 5 depicts the effect of a superoxide-generating system ($FeSO_4$/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 Fe^{2+} in a concentration-dependent manner. Ascorbate alone was inactive, but greatly enhanced the ability of Fe^{2+} to prevent cytostasis at all Fe^{2+} concentrations, presumably by providing electrons for Fe^{2+}-catalyzed superoxide production (21). At 100

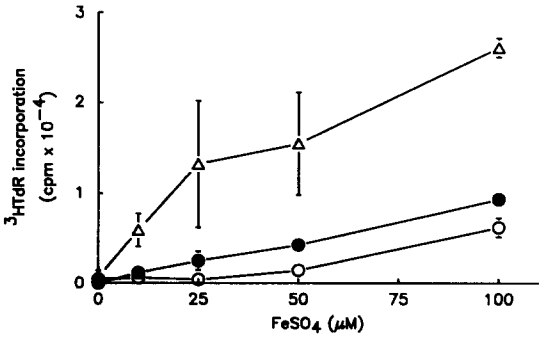

FIGURE 5. Inhibition of activated Mϕ-induced cytostasis by a superoxide-generating system. [^{3}H]TdR incorporation by L1210 cells cocultured with activated Mϕ in the presence of various concentrations of $FeSO_4$ alone (●), with 1 mM ascorbate and 1,000 U/ml catalase (Δ), or with ascorbate, catalase, 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, [^{3}H]TdR incorporation by L1210 cells in cocultures containing activated Mϕ and 250 μM NMA was 60,018 ± 1,678 cpm.

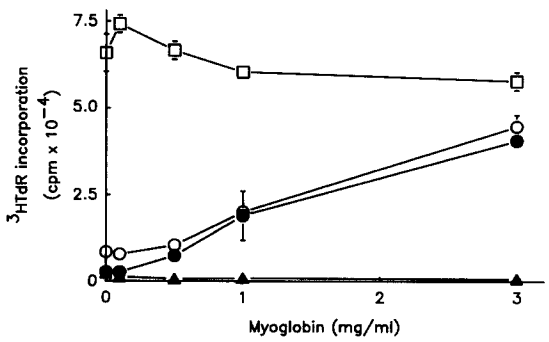

FIGURE 6. Inhibition of activated Mϕ-induced cytostasis by myoglobin. L1210 cells were cultured with activated Mϕ and various concentrations of myoglobin alone (▲), myoglobin plus 1 mM ascorbate and 1,000 U/ml catalase (○), or myoglobin plus ascorbate/catalase and 500 U/ml SOD (●). Results for L1210 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, [^{3}H]TdR incorporation by L1210 cells in cocultures containing activated Mϕ and 250 μM NMA was 75,297 ± 2,683 cpm.

μM $FeSO_4$ (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 Fe^{2+}/ascorbate and fully restored Mϕ-mediated cytostasis, while boiled SOD was inactive. Thus, Fe^{2+}/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 [^{3}H]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 NO_2, formed via reaction of NO· with O_2) 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 (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, NO·, and NO_2 (9, 17). During their metabolism of L-arginine to NO_2^-/NO_3^-, Mϕ produce a compound with biological, biochemical, and physical properties of NO· or NO_2 (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 NO_2 (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 shortlived compared with the cytostasis caused by activated Mϕ or acidified NO_2^-. Perhaps a sustained exposure to moderate amounts of NO·, as occurs during coculture with activated Mϕ or during acidification of NO_2^-, has a more lasting effect than a brief exposure to larger concentrations of NO·. Alternatively, other RNI (such as HNO_2), 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·, $FeSO_4$ 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· 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 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 O_2. This may explain why Mϕ-mediated cytotoxicity often requires proximity between Mϕ and target cells (47). NO_2^- and NO_3^- (formed via decomposition of Mϕ-derived NO· and NO_2 in aqueous, oxygenated environments) are relatively stable and could diffuse from the site of NO· 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 (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, 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 NO· (and/or NO_2), 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.

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

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