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) 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 N⁴-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...
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 x 10^4/well, 25 μl) were then added along with solutions containing experimental agents and the volume of each 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 x 10^6 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 x 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 x 10^4/well for [3H]TdR incorporation studies.

**Culture of L1210 Cells with NO2 at Reduced pH.** L1210 cells (2 x 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 NaN02 or NaN03. Solutions were prepared fresh for each experiment, the pH adjusted from 6.2 to 7.2 with 1M 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 x 10^6/ml and plated at 5 x 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.

**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 (BioTek Instruments, Inc., Burlington, VT). NO₂⁻ was determined using NaN₂O₃ 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 NaN$_2^-$ could cause cytostasis in the cocultures. NaN$_3$ served as a control. As shown in Table I, neither NaN$_2^-$ nor NaN$_3^-$ inhibited [³H]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$_3^-$(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 [³H]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

<table>
<thead>
<tr>
<th>Added NO$_2^-$/NO$_3^-$ (mM)</th>
<th>NO$_2^-$</th>
<th>NO$_3^-$</th>
<th>Control Mφ</th>
<th>Activated Mφ + NMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>89 ± 2</td>
<td>79 ± 0</td>
<td>93 ± 8</td>
<td>62 ± 3 78 ± 6</td>
</tr>
<tr>
<td>5</td>
<td>96 ± 2</td>
<td>76 ± 2</td>
<td>96 ± 3</td>
<td>76 ± 7</td>
</tr>
<tr>
<td>10</td>
<td>99 ± 11</td>
<td>88 ± 5</td>
<td>84 ± 13</td>
<td>78 ± 4</td>
</tr>
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</table>

C3H/He Mφ (10³/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. [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 ± SD of four wells. [³H]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 [³H]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
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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 Mo 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 Mo-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 Mo for 24 h.

**NO\textsuperscript{-}-mediated Respiratory Inhibition.** Like cytostasis, inhibition of target cell respiration by activated Mo is dependent on metabolism of \(L\)-arginine to NO\textsubscript{2}/NO\textsubscript{3} (6). We therefore determined if NO\textsuperscript{-} could inhibit respiration of L1210 cells in the absence of Mo. 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.** Mo-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

![Figure 4](https://example.com/figure4.png)

*Figure 4.* Dose-response curve for NO\textsuperscript{-} inhibition of L1210 respiration on glucose. Cells were treated for 5 min with various amounts of NO\textsuperscript{-} solution (●) or NO\textsuperscript{-} solution that had been N\textsubscript{2} air-sparged (○) to remove NO\textsuperscript{-}. The experiment shown represents one of seven. The points are the mean ± SD of three determinations.

<table>
<thead>
<tr>
<th>Pulse period (h)</th>
<th>NO\textsuperscript{-}</th>
<th>Aerated NO\textsuperscript{-}</th>
<th>Control</th>
<th>Activated Mo</th>
<th>Control Mo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm × 10\textsuperscript{3}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-3</td>
<td>3 ± 0</td>
<td>17 ± 1</td>
<td>14 ± 1</td>
<td>3 ± 1</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>3-6</td>
<td>25 ± 1</td>
<td>34 ± 2</td>
<td>29 ± 2</td>
<td>3 ± 0</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>6-9</td>
<td>34 ± 3</td>
<td>26 ± 11</td>
<td>30 ± 3</td>
<td>4 ± 0</td>
<td>51 ± 0</td>
</tr>
</tbody>
</table>

L1210 cells that were cocultured 24 h with CD-1 Mo or treated for 5 min with NO\textsuperscript{-} solution (625 nmol), N\textsubscript{2}-sparged/aerated NO\textsuperscript{-} solution, or no solution (control), were plated at 5 × 10\textsuperscript{4}/well, and pulsed with \[^{3}H\]TdR for the indicated periods. For L1210 cells harvested from Mo plates, the resuspension and pulsing medium contained 250 μM NMA to prevent RNI synthesis by activated Mo 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\textsuperscript{-} solution had a pH of 5.4 and a NO\textsubscript{2}\textsuperscript{-} concentration of 4.1 mM. Mo NO\textsubscript{2}\textsuperscript{-} production over the coculture period was 0.0 ± 0.4 and 11.7 ± 0.4 nmol/well for control and activated cultures, respectively.

Table II

**Timecourse of L1210 Recovery from Mo- or NO\textsuperscript{-}-induced Cytostasis**

<table>
<thead>
<tr>
<th>Pulse period (h)</th>
<th>NO\textsuperscript{-}</th>
<th>Aerated NO\textsuperscript{-}</th>
<th>Control</th>
<th>Activated Mo</th>
<th>Control Mo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm × 10\textsuperscript{3}</td>
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<td></td>
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<tr>
<td>0-3</td>
<td>3 ± 0</td>
<td>17 ± 1</td>
<td>14 ± 1</td>
<td>3 ± 1</td>
<td>27 ± 2</td>
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<tr>
<td>3-6</td>
<td>25 ± 1</td>
<td>34 ± 2</td>
<td>29 ± 2</td>
<td>3 ± 0</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>6-9</td>
<td>34 ± 3</td>
<td>26 ± 11</td>
<td>30 ± 3</td>
<td>4 ± 0</td>
<td>51 ± 0</td>
</tr>
</tbody>
</table>
and mitochondrial substrates were used to measure electron flow through complex 1 (malate), complex 2 (succinate), coenzyme Q (α-glyPi), 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 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 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^{4+}-catalyzed superoxide production (21). At 100

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Acceptor</th>
<th>Control</th>
<th>NO- treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate</td>
<td>Complex 1</td>
<td>0.353 ± 0.028</td>
<td>0.058 ± 0.028 (16.4%)</td>
</tr>
<tr>
<td>Succinate</td>
<td>Complex 2</td>
<td>0.694 ± 0.059</td>
<td>0.306 ± 0.006 (44.1%)</td>
</tr>
<tr>
<td>α-GlyPi</td>
<td>Coenzyme Q</td>
<td>0.398 ± 0.039</td>
<td>0.486 ± 0.030 (122%)</td>
</tr>
<tr>
<td>TMPD</td>
<td>Cytochrome C</td>
<td>0.387 ± 0.041</td>
<td>0.619 ± 0.029 (105%)</td>
</tr>
</tbody>
</table>

L1210 cells were treated for 5 min with NO- solution (48 nmol NO- /10^7 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.

![Figure 5](https://example.com/figure5.png)
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**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, \[^3\text{H}\]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 \[^3\text{H}\]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 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 NO₂ (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\textsuperscript{-}-induced lesions within the METC were restricted to complex 1 and 2. Complex 1 and 2 contain several Fe\textsubscript{55} clusters that may be susceptible to destruction by both authentic and Mϕ-derived NO\textsuperscript{-} and NO\textsubscript{2\textsuperscript{-}} (7, 27). In cell-free systems, NO reacts with certain Fe\textsubscript{55} proteins, forming paramagnetic complexes similar to Fe(\textsuperscript{2+}NO\textsubscript{2})(cysteine)\textsubscript{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\textsuperscript{-} 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\textsubscript{2\textsuperscript{-}}. Perhaps a sustained exposure to moderate amounts of NO\textsuperscript{-}, as occurs during coculture with activated Mϕ or during acidification of NO\textsubscript{2\textsuperscript{-}}, has a more lasting effect than a brief exposure to larger concentrations of NO\textsuperscript{-}. Alternatively, other RNI (such as HNO\textsubscript{2}), may contribute. The molecular target(s) involved in Mϕ- or NO\textsuperscript{-}-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\textsuperscript{-}-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\textsuperscript{-} and have been used to prevent its biological effects (8, 20, 24, 25). In addition to scavenging NO\textsuperscript{-}, FeSO\textsubscript{4} and myoglobin may have helped injured cells to recover faster from cytostasis by furnishing Fe to replenish intracellular pools and rebuild Fe\textsubscript{55} clusters (10, 31). However, our findings that SOD reversed the Fe\textsuperscript{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\textsuperscript{-} or acidified NO\textsubscript{2\textsuperscript{-}} 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\textsuperscript{-} may play a role in host resistance against a wide range of microbial pathogens.

The factors regulating cytotoxicity by Mϕ-derived NO\textsuperscript{-} are not yet well understood. For example, the flux of NO\textsuperscript{-} 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\textsuperscript{-}, such as superoxide dismutase (39). NO\textsuperscript{-} bound to certain transition metal complexes, as in nitroprusside, is sufficiently stable to be used as a source of slowly released NO\textsuperscript{-} (37, 38, 40). Similarly, while thiols can scavenge NO\textsuperscript{-}, 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\textsuperscript{-}, 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


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