Inhibition of Tumor Cell Ribonucleotide Reductase by Macrophage-derived Nitric Oxide
By Nyoun Soo Kwon, Dennis J. Stuehr, and Carl F. Nathan

Summary
Macrophage-derived nitric oxide (NO) is cytostatic to tumor cells and microbial pathogens. We tested whether one molecular target for the cytostatic action of NO may be ribonucleotide reductase (RR), a rate-limiting enzyme in DNA synthesis. In a concentration-dependent manner, NO gas and lysates of activated macrophages that generated comparable amounts of NO led to the same degree of inhibition of partially purified RR from L1210 mouse lymphoma cells. Lysates from nonactivated macrophages, which do not produce NO, were noninhibitory. With lysates from activated macrophages, RR was protected by omitting l-arginine or by adding the NO synthase inhibitors diphenyleneiodonium, Nω-methyl-l-arginine, or Nω-amino-l-arginine. l-Arginine, but not D-arginine, abolished the protective effect of Nω-amino-l-arginine. The prototypic pharmacologic inhibitor of RR is hydroxyurea. Its structural resemblance to Nω-hydroxy-l-arginine, a reaction intermediate of NO synthase, prompted us to test if hydroxyurea can generate NO. In the presence of H2O2 and CuSO4, hydroxyurea produced NO2-/NO3-, aerobic reaction products of NO. Addition of morpholine blocked NO2-/NO3- generation from hydroxyurea and led to formation of nitrosomorpholine, as detected by gas chromatography/mass spectrometry. Thus, hydroxyurea can produce an NO-like, nitrosating reactant. L1210 cell DNA synthesis was inhibited completely by activated macrophages or by hydroxyurea, and was partially restored to the same degree in both settings by providing deoxyribonucleosides to bypass the block in RR. Thus, both NO gas and NO generated by activated macrophage lysates inhibit tumor cell RR. The RR inhibitor hydroxyurea can also generate an NO-like species. Similar, partial restoration of tumor cell DNA synthesis by deoxyribonucleosides in the presence of activated macrophages or hydroxyurea suggests that cytostasis by activated macrophages and by hydroxyurea has comparable mechanisms, including, but probably not limited to, inhibition of RR.

Activated macrophages can generate large amounts of nitric oxide (NO) from l-arginine (1−8). NO mediates many of the cytotoxic actions of macrophages toward tumor cells (2, 6, 8) and microbial pathogens (reviewed in reference 9). Inhibition of tumor cell respiration by activated macrophages (10, 11) and by NO (6, 8) is biochemically comparable. Before their mitochondria are damaged, however, tumor cells incubated with activated macrophages stop synthesizing DNA (2, 8, 12). This early cytostatic effect is also mediated by NO (6, 8), but its mechanism is unknown.

Authentic NO can react with iron (13), iron-sulfur centers (7), and thiols (14). NO-producing macrophages generate endogenous iron-nitrosyl complexes (15, 16), and inhibit iron-sulfur enzymes in tumor cells with which they are cultured (2, 6, 8, 11). Ribonucleotide reductase (RR), a rate-limiting enzyme in DNA synthesis, depends on thiols (17, 18) and on non-heme iron in its reaction center (17, 19), which are crucial for maintaining a key tyrosyl radical (20). Thus, RR is a candidate for inactivation by NO, and inactivation of RR might explain the cytostatic effect of NO-producing macrophages (8, 12).

In this report, we demonstrate that NO from activated macrophages can inactivate RR from tumor cells. We have assessed what proportion of tumor cell cytostasis could be ascribed to inhibition of RR, and compared the results to those obtained with NO gas and with the RR inhibitor hydroxyurea. Unexpectedly, hydroxyurea also proved capable of generating an NO-like species.

Materials and Methods
Materials. L1210 mouse lymphoma and RAW 264.7 mouse macrophage cell lines were from the American Type Culture Collection (Rockville, MD). RPMI 1640, MEM (α-modification), peni-

Abbreviations used in this paper: FAD, flavin adenine dinucleotide; GC/MS, gas chromatography/mass spectrometry; m/z, mass-to-charge ratio; NO, nitric oxide; RR, ribonucleotide reductase.
cillin, streptomycin, l-glutamine, 2-deoxyoctytidine (for cell culture media), thymidine, 2-deoxyadenosine, 2'-deoxyguanosine were from JRH Biosciences. (Lenexa, KS) NADPH, l-arginine, d-arginine, Mn-superoxide dismutase (from Esherichia coli), hydroxyurea, snake venom (from Crotaulus atrox), ferredoxin (from Clostridium pasteurianum), sodium borate, LPS (from E. coli 0128:B12), FAD, magnesium acetate, Trisazma, dithioerythritol, 5'-adenylylimidodiphosphate, dCMP, CDP, cytidine, and deoxycytidine (for the RR assay) were from Sigma Chemical Co. (St. Louis, MO). Catalase (bovine liver) was from Calbiochem-Behring Corp. (La Jolla, CA). Dowex 1-X8 (200-400 mesh) was from Bio-Rad Laboratories (Richmond, CA). (6S)-erythro-5,6,7,8-tetrahydrobiopterin was from Dr. B. Schircks (Jona, Switzerland). [Methyl-3H]thymidine, [U-14C]CDP, and [5-3H]CDP were from New England Nuclear (Boston, MA). NO gas and N2 gas were from Matheson Gas Products (East Rutherford, NJ). N6-Methyl-l-arginine, N4- amino-l-arginine, and diphenyleneiodonium were gifts of Dr. Owen W. Griffith (Cornell University Medical College). Pure, recombinant mouse IFN-γ was a gift of Genentech (South San Francisco, CA).

**RR Preparations.** RR was partially purified from L1210 cells grown in humidified 5% CO2, 95% air at 37°C in 3-liter spinner bottles containing RPMI 1640 supplemented with bovine calf serum (10%), penicillin (50 U/ml), streptomycin (50 μg/ml), and l-glutamine (2 mM). When the cells reached a density of 0.5-0.7 × 10^6/ml, they were collected by centrifugation at 4°C, and resuspended in 0.9% NaCl, 25 mM glucose. Cell number was determined by a nuclear counting method (21) and viability by trypan blue exclusion. The cells were then pelleted and lysed by three cycles of freezing and thawing in cold distilled water containing protease inhibitors (0.1 mM PMSF, 5 μg/ml aprotonin, 1 μg/ml chymostatin, 5 μg/ml pepstatin A). Cell viability was >90% before and <5% after lysis. Lysate from 3-4 × 10^6 cells was centrifuged at 100,000 g for 2 h at 4°C. Solid ammonium sulfate (0.243 g/liter) was added with stirring at 4°C to the supernatant over 30 min (22). The supernatant was separated from cytidine (from CDP, the substrate) by chromatography on columns containing Dowex 1-borate. Dowex 1-borate was prepared as described with minor modifications (28). Dowex 1-X8 was washed with water four to five times to remove fines and then washed with saturated sodium borate. The resin (1 ml) was packed into a disposable plastic column (10-ml capacity; Bio-Rad Laboratories) just before use. The column was washed with 4 ml saturated sodium borate and then with 7 ml water before addition of the sample. Deoxycytidine was eluted with 6 ml water and radioactivity of the eluate measured. 1 U of RR activity was defined as 1 nmol of CDP converted from dCDP during the 30-min incubation under the conditions described above.

The assay is based on the selective reduction of nitroso-diol by Dowex-borate and hence should not be affected by the possible conversion of dCyt into dUrd by contaminating deaminases.

**Assay of Nitrogen Oxides from Hydroxyurea.** Hydroxyurea (0.3 or 1 mM) was mixed with CuSO4 (1-20 μM). Sodium phosphate (2.5 mM, pH 7.5) and H2O2 (1-2 mM)-mediated oxidation of hydroxyurea (0.3 mM) was performed in 100 mM Tris-HCl, pH 8.0, in the presence of 10 mM morpholine to trap NO (32). 5 ml of the reaction mixture was mixed with dichloroethane (2.5 ml) to extract nitrosomorpholine. The lower layer containing dichloroethane was concentrated to 50 μl under N2 at 25°C. Analysis of nitrosomorpholine was performed using a 5890 gas chromatograph and a 5970 mass selective detector (Hewlett-Packard, Avondale, PA). An HP-1 dimethylpolysiloxane column (length, 12 m; inner diameter, 2 × 10^-4 m; film thickness, 3 × 10^-2 m) was used for the gas chromatography. The column temperature was initially 60°C and then increased to 140°C at a rate of 20°C/min after 2 min. The carrier gas was helium at a flow rate of 1.05 ml/min.

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at 150°C. Head pressure of the column was 10 psi. Injector and transfer line temperature was 225°C. Electron impact ionization was achieved at 70 eV and an ion source temperature was 220°C. Abundances of fragmented ions between m/z 50 and m/z 140 were analyzed by an attached computer.

Effects of Exogenous Deoxyribonucleosides on the Cytostatic Activity of Macrophages or Hydroxyurea. Thioglycollate broth-elicited peritoneal macrophages were obtained from CD1 female mice and activated in vitro with IFN-γ (2.5–50 U/ml) and LPS (0.5 μg/ml) in 96-well plates (1.5 × 10⁶ cells/well) as described (8). MEM, α-modification, with bovine calf serum (8%), penicillin (50 U/ml), streptomycin (50 μg/ml), and L-glutamine (2 mM) was used. The medium (100 μl) was changed twice after a 12-h incubation in 5% CO₂ at 37°C. L1210 cells (70 μl, 0.5 × 10⁶ cells/well) were then added. 10 μl [methyl-3H]thymidine (0.5 μCi/well, sp act 2 Ci/mmol) was added after a 1-h incubation. After 0.5 h, 0.4 mM deoxyadenosine and 0.4 mM deoxyguanosine (in 20 μl) were added for a further 3.5 h. Nitrite was measured in 50 μl of the medium, and thymidine incorporation was measured in the rest of the culture using a PHD cell harvester (Cambridge Technology Inc., Watertown, MA).

Results

Inactivation of RR by NO Gas. In a concentration-dependent manner, NO gas inhibited RR partially purified from L1210 lymphoma cells (Fig. 1). Addition of 0.23 mM NO reduced RR activity by 37% within 15 min at 25°C (Fig. 1). As a control, the buffer in which NO was dissolved was aerated before its addition to the RR preparation. This markedly diminished its inhibitory effect, consistent with the rapid oxidation of NO to nitrite/nitrate in oxygenated, aqueous media (8). Incomplete inhibition of RR may likewise have resulted from rapid oxidation of NO by O₂ in the RR preparation during the co-incubation, and/or from reaction of NO with the iron and thiol needed to demonstrate RR activity.

Inactivation of RR by NO Generated by Lysates of Activated Macrophages. Partially purified L1210 cell RR was incubated with the NO-generating cytosolic fraction of activated RAW 264.7 murine macrophages in the presence of NADPH, tetrahydrobiopterin, and FAD. RR activity was not detected in the macrophage cytosol itself at the concentration used. Upon addition of 1 mM L-arginine to the co-incubate, RR activity was reduced 36% (Fig. 2), coincident with the accumulation of 0.14 mM nitrite/nitrate. Incubation beyond 45 min at 37°C, RR activity was measured. Cross-hatched bars indicate RR activities; solid bars indicate the concentration of nitrate plus nitrate generated. Bars and brackets indicate the mean and SD of triplicates.
verse correlation between NO production (reflected in the accumulation of nitrite/nitrate) and RR activity (Fig. 2).

**Dependence of Inhibition of RR on Amount of Macrophage Cytosol, and on Macrophage Activation.** When RR was exposed to increasing amounts of activated macrophage cytosol, NO generation increased and RR activity diminished in parallel (Fig. 3). Accumulation of as little as 35 μM nitrite/nitrate was associated with a 38% reduction in RR activity. Additional production of NO was associated with progressively less inhibition of RR. When macrophages had not been exposed to IFN-γ and LPS, their lysates were unable to produce NO or to inhibit RR over the entire range of amounts of lysate tested (Fig. 3, inset).

**Oxidation of Hydroxyurea to Nitrite/Nitrate.** Hydroxyurea resembles the N-hydroxyguanidine group of N^o-arginine, an intermediate in NO production by macrophage NO synthase (35). This prompted us to consider that hydroxyurea might generate NO within cells. As a model for possible oxidative metabolism of hydroxyurea, we exposed hydroxyurea (1 mM) to H₂O₂ (1 mM) in the presence of CuSO₄ (1-20 μM). This led to a time-dependent accumulation of nitrite/nitrate (Fig. 4). Nitrite/nitrate generation was optimal at 5 μM Cu(II), but was not supported by Fe(II) or Fe(III). No nitrogen oxides were detected if H₂O₂ was omitted, or if catalase (500 U/ml) or EDTA (1 mM) were added (data not shown).

**Inhibition of Nitrogen Oxide Generation from Hydroxyurea by Ferredoxin and Morpholine.** Oxidation of hydroxyurea might form nitrate/nitrate directly, or could generate a more reactive oxide of nitrogen as an immediate product. To help distinguish between these possibilities, we added ferredoxin from *Clostridium pasteurianum* (7) or morpholine (32), which are both known to react with NO or certain of its oxidation products (such as N₂O₃ or N₂O₄; reference 36), but not with nitrite or nitrate under the same conditions. In the control, 58 μM nitrite was produced after 1 h from the oxidation of 300 μM hydroxyurea with 1 mM H₂O₂ and 10 μM CuSO₄. Ferredoxin (0.1 mg/ml) inhibited nitrite production by 76%. Inhibition was not overcome by increasing the concentration of Cu(II) (Fig. 5 A). As a control, albumin was tested in place of ferredoxin. Albumin was also inhibitory, but in this case, inhibition was readily reversed by increasing the concentration of Cu(II) (Fig. 5 B), suggesting that albumin acted as a chelator of Cu(II). Like ferredoxin, morpholine effectively blocked nitrite/nitrate generation from hydroxyurea, with an IC₅₀ of 40 μM (data not shown).

**Detection of Nitrosomorpholine by GC/MS.** When the oxidation of hydroxyurea (0.3 mM) with Cu(II) (10 μM) and H₂O₂ (2 mM) was carried out, 0.1 mM nitrite accumulated. However, in the presence of 10 mM morpholine, nitrite could no longer be detected. GC/MS analysis of this reaction mixture contained a product eluting at 3.8 min that displayed a molecular ion of m/z 116 and a base fragment ion of m/z 56 (Fig. 6). The mass spectrum was identical with that of nitrosomorpholine generated by reaction with NO gas. Neither nitrite nor nitrate (0.1 mM) led to the formation of nitrosomorpholine from morpholine in the presence of the Cu(II)/H₂O₂.

**Effects of Exogenous Deoxyribonucleosides on the Cytostatic Effects of Intact, Activated Macrophages and Hydroxyurea.** Ex-
ogenous deoxyribonucleosides can be taken up by cells and phosphorylated to deoxyribonucleotides (37-39). If cytostasis by activated macrophages were due mainly to inhibition of tumor cell RR, exogenous deoxyribonucleosides might partially reverse the cytostatic effect by bypassing RR. We therefore added deoxyribonucleosides to tumor cells whose DNA synthesis was blocked either by the RR inhibitor hydroxyurea (22), or by NO-generating activated macrophages. A wide range of concentrations of each of the four deoxyribonucleosides was tested, alone and in all possible combinations (data not shown). The optimal regimen to reverse partially the cytostasis caused by hydroxyurea was the addition of deoxyadenosine and deoxyguanosine at 0.4 mM each. Under these conditions, [3H]thymidine incorporation was restored to 38-63% of that by L1210 cells not receiving hydroxyurea (Fig. 7, inset). When activated macrophages were used in place of hydroxyurea, cytostasis was equally profound, and was reversed to a similar degree by deoxyadenosine and deoxyguanosine (Fig. 7, main figure). The deoxyribonucleosides did not affect nitrite production by the macrophages (data not shown).

Figure 5. Inhibition of nitrite production from hydroxyurea by ferredoxin but not by albumin. The ability of CuSO₄ at the indicated concentrations and H₂O₂ (1 mM) to generate nitrogen oxides from hydroxyurea (0.3 mM) was examined without further additions (O) or in the presence of Clostridium pasteurianum ferredoxin (A) or BSA (B) at concentrations of 0.1 mg/ml (△), 0.01 mg/ml (▽), or 0.001 mg/ml (●). After a 60-min incubation at 37°C, the reaction was stopped by adding EDTA (0.1 mM) and catalase (1,000 U/ml), and the concentration of nitrite was measured. Data are the means of duplicates.

Figure 6. Mass spectrum of nitrosomorpholine generated from the oxidation of hydroxyurea in the presence of morpholine. Oxidation of hydroxyurea (0.3 mM) with 10 μM CuSO₄ and 2 mM H₂O₂ was carried out in the presence of 10 mM morpholine to trap generated NO. The reaction mixture was extracted with dichloroethane, concentrated under N₂, and analyzed by GC/MS. A peak eluting at 3.8 min showed a molecular ion of m/z 116 and base fragment ion of m/z 56.

Figure 7. Effects of exogenous deoxyribonucleosides on the cytostatic effects of intact, activated macrophages or hydroxyurea. Thioglycollate broth-elicited peritoneal macrophages were activated with LPS (0.5 μg/ml) and IFN-γ (2.5-50 U/ml, as indicated) for 12 h, and cocultured with L1210 cells in the presence (O) or absence (O) of 0.4 mM deoxyadenosine and 0.4 mM deoxyguanosine (main figure). As a positive control, hydroxyurea (0.1-1 mM) was used in place of activated macrophages (inset). Incorporation of [3H]thymidine was measured, and expressed as a percent of control. The controls were incubated with nonactivated macrophages (main figure) or without hydroxyurea (inset) in the presence of the same concentrations of deoxyribonucleosides. Deoxyribonucleosides alone caused ~50% inhibition of thymidine incorporation. Data are means ± SD of triplicates.
Discussion

Understanding cell-mediated cytotoxicity requires identifying not only the toxins released by effector cells, but also the critical molecules with which they interact in tumor cells and microbes. Known targets for macrophage-derived NO include cis-aconitase (11), NADH/succinate oxidoreductase (8), and NADH/ubiquinone oxidoreductase (8). Inactivation of these enzymes appears to account for inhibition of tumor cell respiration by activated macrophages. Suppression of tumor cell proliferation can now be ascribed, in part, to inhibition of RR.

RR was susceptible to inhibition at a very low level of NO generation, but inactivation of RR in vitro was far from complete. This probably reflects limitations imposed by the assay conditions, which require oxygen and relatively large concentrations of exogenous iron and thiols. These reactants can deplete NO and restore RR (40, 41). The major catalytic components of RR are ferric ions coupled through μ-oxo bridges, tyrosyl radicals, and reduced thiols (17–20, 22). Each is a possible site for NO-mediated inactivation.

While this work was in progress, Lepoivre et al. (42) reported the partial sensitivity of RR in a hydroxyurea-resistant, RR-overproducing murine adenocarcinoma cell line that was stimulated to generate endogenous NO by treatment with cytokines and LPS. The present report extends those results by using tumor cells that were not selected for overexpression of RR; by comparing the effects of biosynthetic NO with those of reagent NO and hydroxyurea; by studying interactions between macrophages and tumor cells; by assessing the degree to which tumor cell cytostasis by intact macrophages could be ascribed to inhibition of RR; and by demonstrating that hydroxyurea can generate an NO-like compound.

Generation of NO by hydroxyurea was unexpected. Hydroxyurea is thought to inhibit RR by quenching the active site tyrosine radical through a one-electron transfer (22). The present study suggests that formation of an NO-like reagent from hydroxyurea may be involved in actions of the drug in those cells that can oxidize it. If so, then this pharmacologic agent, and perhaps other structurally related chemotherapeutics (43, 44), may imitate a component of cell-mediated cytotoxicity.

Exogenous purine deoxyribonucleosides partially protected tumor cells against inhibition of DNA synthesis by intact, activated macrophages and by hydroxyurea. While far from complete, protection was comparable for macrophages and hydroxyurea. Incomplete protection may reflect the known methodologic limitations of bypassing a block in RR with products that compete with each other for transport (39) and phosphorylation (38) and can themselves be cytostatic (37, 38). Alternatively, both hydroxyurea and macrophage-derived NO may have additional targets besides RR, whose inactivation contributes to cytostasis. This is to be expected for NO, which is broadly reactive with redox-sensitive molecules, and for hydroxyurea, if it generates NO.

We have only studied RR from tumor cells. However, it is likely that RR in microbes is also susceptible to macrophage-derived NO. If so, inhibition of RR may contribute to the ability of activated macrophages to inhibit the proliferation of bacteria, fungi, protozoa, and helminths (9).

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