Inhibition of Tumor Cell Ribonucleotide Reductase by Macrophage-derived Nitric Oxide

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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, N^{ω}-methyl-L-arginine, or N^{ω}-amino-L-arginine. L-Arginine, but not D-arginine, abolished the protective effect of N^{ω}-amino-L-arginine. The prototypic pharmacologic inhibitor of RR is hydroxyurea. Its structural resemblance to N^{ω}-hydroxy-L-arginine, a reaction intermediate of NO synthase, prompted us to test if hydroxyurea can generate NO. In the presence of H_2O_2 and $CuSO_4$, hydroxyurea produced NO_2^-/NO_3^-, aerobic reaction products of NO. Addition of morpholine blocked NO_2^-/NO_3^- 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)[1] 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-

[1] *Abbreviations used in this paper:* FAD, flavin adenine dinucleotide; GC/MS, gas chromotography/mass spectrometry; *m/z*, mass- to -charge ratio; NO, nitric oxide; RR, ribonucleotide reductase.

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cillin, streptomycin, L-glutamine, 2'-deoxycytidine (for cell culture media), thymidine, 2'-deoxyadenosine, and 2'-deoxyguanosine were from JRH Biosciences. (Lenexa, KS) NADPH, L-arginine, D-arginine, Mn-superoxide dismutase (from *Escherichia coli*), hydroxyurea, snake venom (from *Crotalus atrox*), ferredoxin (from *Clostridium pasteurianum*), sodium borate, LPS (from *E. coli* 0128:B12), FAD, magnesium acetate, Trizma base, 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). (6R,S)-erythro-5,6,7,8-tetrahydrobiopterin was from Dr. B. Schircks (Jona, Switzerland). [Methyl-^{3}H]thymidine, [U-^{14}C]CDP, and [5-^{3}H]CDP were from New England Nuclear (Boston, MA). NO gas and N_2 gas were from Matheson Gas Products (East Rutherford, NJ). N^{ω}-Methyl-L-arginine, N^{ω}-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% CO_2, 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 aprotinin, 1 μg/ml chymostatin, 5 μg/ml pepstatin A). Cell viability was >90% before and <5% after lysis. Lysate from 3–4 × 10^9 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 solution was further stirred for 30 min and centrifuged at 22,000 *g* for 30 min. The precipitate was dissolved with 4 ml of 50 mM Tris-HCl, pH 7.6, dialyzed against 1 liter of 2.5 mM sodium phosphate, pH 7.5, for 15 h, and stored at −80°C.

Macrophage NO Synthase Preparations. The cytosolic fraction of RAW 264.7 cells was used as the source of NO synthase. Macrophages were cultured, harvested, and lysed as described previously (23). The cells were incubated for 12 h with (activated cells) or without (control cells) IFN-γ and LPS before harvest.

Protein Assay. The protein concentration of RR and NO synthase preparations was measured by the Bradford method (24) using Bio-Rad Laboratories protein assay solution with BSA as a standard.

Treatment of RR with Authentic NO or NO Synthase. N_2 was bubbled for 30 min through Tris-HCl buffer (10 mM, pH 8.0) in a test tube closed with a serum stopper to remove dissolved oxygen. Authentic NO gas was passed through 1 N KOH, and then bubbled into the deoxygenated buffer for 10 s. During this time, the head space of the test tube was ventilated with N_2. The initial concentration of NO was measured by exposing an aliquot of the NO solution to air, then measuring nitrite plus nitrate as described below. Another aliquot of NO solution was transferred to an RR preparation (5 mg protein/ml 10 mM Tris-HCl, pH 8.0) equilibrated with air. The mixture was incubated for 15 min at 25°C, and its RR activity measured as described below. Alternatively, partially purified RR (5 mg protein/ml) from L1210 was preincubated with macrophage lysate (4 mg protein/ml) in the presence of Mn-superoxide dismutase (200 U/ml), catalase (1,000 U/ml), 1 mM L-arginine, 0.25 mM NADPH, 4 μM 5,6,7,8-tetrahydrobiopterin (25), and 4 μM FAD (26) for 45 min in 10 mM Tris-HCl, pH 8.0, at 37°C. For assay of NO synthase activity under the same conditions, duplicate samples were prepared for the coincubation of macrophage lysate with L1210 cell RR as described above, except that radiolabeled CDP was omitted. Nitrite plus nitrate were measured as indicated below.

Assay of RR Activity. After the preincubation described above, RR activity was assayed by the conversion of radioactive CDP to dCDP, measured as described, with minor modifications (27–29). The reaction mixture was incubated with 47 μM $FeCl_3$, 3.8 mM magnesium acetate, 5.6 mM dithioerythritol, 1.9 mM 5'-adenylylimidodiphosphate, and 20 mM sodium phosphate, pH 6.5, for 30 min at 37°C in the presence of 25 μM radioactive substrate CDP, either [U-^{14}C]CDP (sp act, 6.5 mCi/mmol) or [5-^{3}H]CDP (131 mCi/mmol). At the end of the assay incubation, the reaction mixture (160 μl) was boiled for 3 min, then treated with snake venom for 2 h at 37°C to convert nucleotides to nucleosides (30). The treatment was performed by adding 50 μl snake venom (40 mg/ml) and 50 μl dCMP-magnesium acetate solution (5 mM dCMP and 20 mM magnesium acetate in 100 mM Tris-HCl, pH 9.0). The snake venom–treated sample was boiled again for 3 min. Cold cytidine and deoxycytidine (8 μg each) were added. The mixture was centrifuged at 10,000 *g* for 15 min. Radioactive deoxycytidine (derived from dCDP, the product of the RR-catalyzed reaction) in 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 retention of *cis*-diols by Dowex-borate and hence should not be affected by the possible conversion of dCyt into dUrd by contaminating deaminases.

Assay of Nitrogen Oxides. Nitrite plus nitrate, the accumulating oxidation products of NO, were measured by the colorimetric Griess reaction after reducing nitrate to nitrite on a copper-cadmium column, as described (31).

Generation of Nitrogen Oxides from Hydroxyurea. Hydroxyurea (0.3 or 1 mM) was mixed with $CuSO_4$ (1–20 μM). Sodium phosphate (2.5 mM, pH 7.5) and H_2O_2 (1–2 mM) were added to start the reaction. After 60 min at 37°C, the reaction was stopped by addition of catalase (1,000 U/ml) and EDTA (0.1 mM), and the concentration of nitrite, or nitrite plus nitrate, was measured.

Gas Chromatography/Mass Spectrometry (GC/MS). The Cu(II)/H_2O_2 (10 μM/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 N_2 at 25°C. Analysis of nitrosomorpholine in the concentrated sample (2 μl) 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^{-7} 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 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 × 10^5 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% CO_2 at 37°C. L1210 cells (70 μl, 0.5 × 10^5 cells/well) were then added. 10 μl [methyl-^{3}H]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 O_2 in the RR preparation during the co-incubation, and/or from reaction of NO with the iron and thiol needed to demonstrate RR activity.

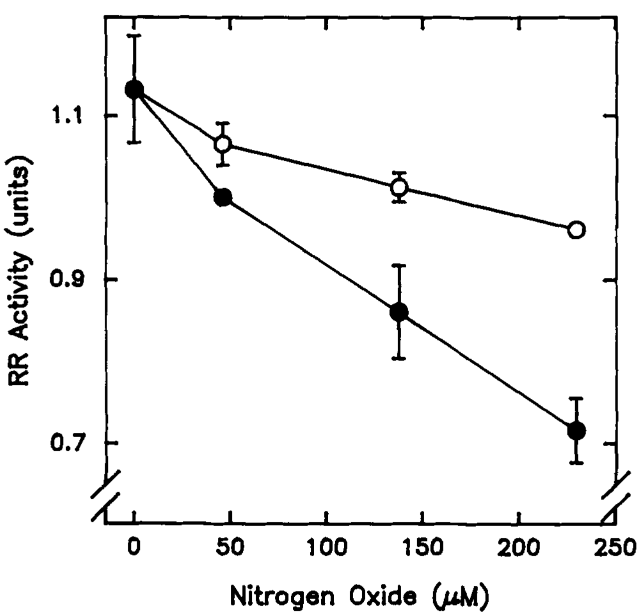

Figure 1. Inactivation of RR activity by authentic NO. NO gas was dissolved in deoxygenated 10 mM Tris-HCl, pH 8.0. Varying volumes of the NO solution (●) were added to an aerated preparation of RR for 15 min at 25°C, after which RR activity was measured under aerobic conditions over an additional 30 min. As a control (O), aliquots of NO solution were aerated to oxidize NO before its addition to the RR preparation. 1 U of RR was defined as the activity converting 1 nmol of CDP to dCDP during the 30-min assay. NO was measured as described in Materials and Methods. Data are means ± SD for triplicates. Where error bars are not visible, they are smaller than the symbol representing the mean.

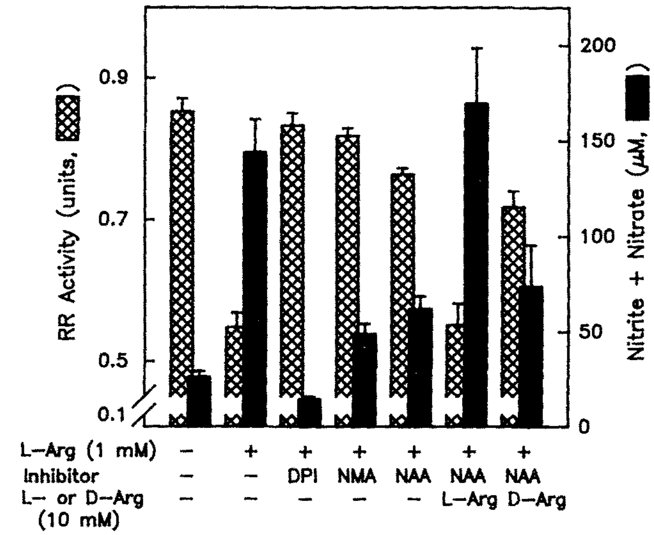

Figure 2. Inactivation of RR by NO synthase in lysates of activated macrophages. Partially purified RR (5 mg protein/ml) was incubated with NO synthase from activated macrophages (4 mg protein/ml) in the presence of Mn-superoxide dismutase (200 U/ml), catalase (1,000 U/ml), 0.25 mM NADPH, 4 μM tetrahydrobiopterin, and 4 μM FAD. L-Arginine (1 mM), diphenyleneiodonium (DPI, 10 μM), N^{ω}-methyl-L-arginine (NMA, 0.5 mM), N^{ω}-amino-L-arginine (NAA, 0.5 mM), and excess L- or D-arginine (10 mM) were further added as indicated. After incubation for 45 min at 37°C, RR activity was measured. Cross-hatched bars indicate RR activities; solid bars indicate the concentration of nitrite plus nitrate generated. Bars and brackets indicate the mean and SD of triplicates.

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 led to an increase in nitrite/nitrate production, but did not cause additional inactivation of RR (not shown). RR activity was protected when NO generation was inhibited by agents from both of the known classes of inhibitors of macrophage NO synthase: diphenyleneiodonium, an irreversible inhibitor that appears to interact with NO synthase via its flavin- and/or NADPH-binding sites (33), and the competitive substrate analog inhibitors N^{ω}-methyl-L-arginine and N^{ω}-amino-L-arginine (34). The protective effect of N^{ω}-amino-L-arginine was abolished by excess L-arginine, but not by D-arginine. Under all these conditions, there was an in-

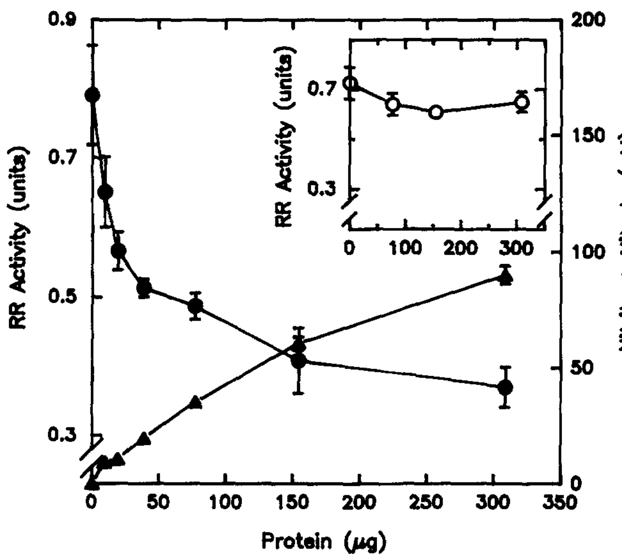

Figure 3. Treatment of RR with varying amounts of NO synthase. The RR preparation (0.5 mg protein) was preincubated with different amounts of lysates from activated macrophages (*main figure*) or nonactivated macrophages (*inset*) in the presence of Mn-superoxide dismutase, catalase, L-arginine, NADPH, tetrahydrobiopterin, and FAD at 37°C. The reaction volume was 100 μl. After the incubation, RR activity (●, ○) and production of nitrite plus nitrate (▲) were measured. Data are means ± SD of triplicates.

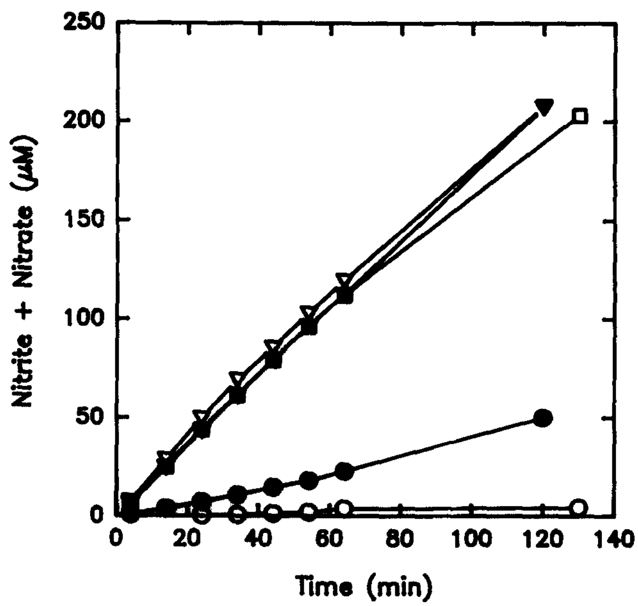

Figure 4. Oxidation of hydroxyurea to nitrite/nitrate. Hydroxyurea (1 mM) was incubated with varying concentrations of $CuSO_4$ (○, no addition; ●, 1 μM; ▽, 5 μM; ▼, 10 μM; □, 20 μM) in the presence of 1 mM H_2O_2 in 2.5 mM sodium phosphate, pH 7.5, at 37°C. After the indicated periods of incubation, samples were analyzed for nitrite plus nitrate.

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^{ω}-hydroxy-L-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 H_2O_2 (1 mM) in the presence of $CuSO_4$ (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) nor Fe(III). No nitrogen oxides were detected if H_2O_2 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 N_2O_3 or N_2O_4; 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 H_2O_2 and 10 μM $CuSO_4$. 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 IC_{50} 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 H_2O_2 (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 $Cu(II)/H_2O_2$.

Effects of Exogenous Deoxyribonucleosides on the Cytostatic Effects of Intact, Activated Macrophages and Hydroxyurea. Ex-

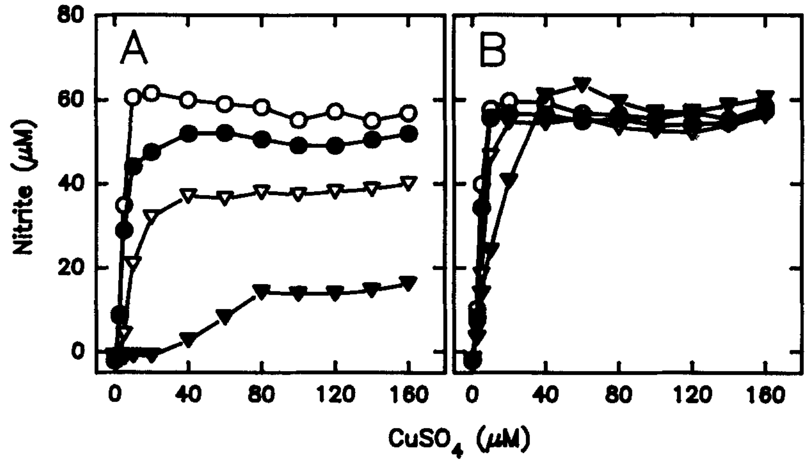

Figure 5. Inhibition of nitrite production from hydroxyurea by ferredoxin but not by albumin. The ability of $CuSO_4$ at the indicated concentrations and H_2O_2 (1 mM) to generate nitrogen oxides from hydroxyurea (0.3 mM) was examined without further additions (○) or in the presence of *Clostridium pasteurianum* 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.

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, [^{3}H]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).

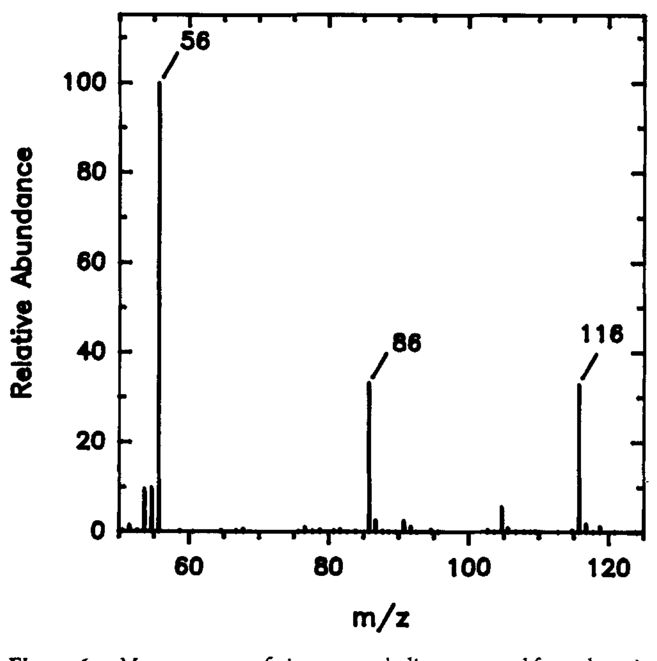

Figure 6. Mass spectrum of nitrosomorpholine generated from the oxidation of hydroxyurea in the presence of morpholine. Oxidation of hydroxyurea (0.3 mM) with 10 μM $CuSO_4$ and 2 mM H_2O_2 was carried out in the presence of 10 mM morpholine to trap generated NO. The reaction mixture was extracted with dichloroethane, concentrated under N_2, 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.

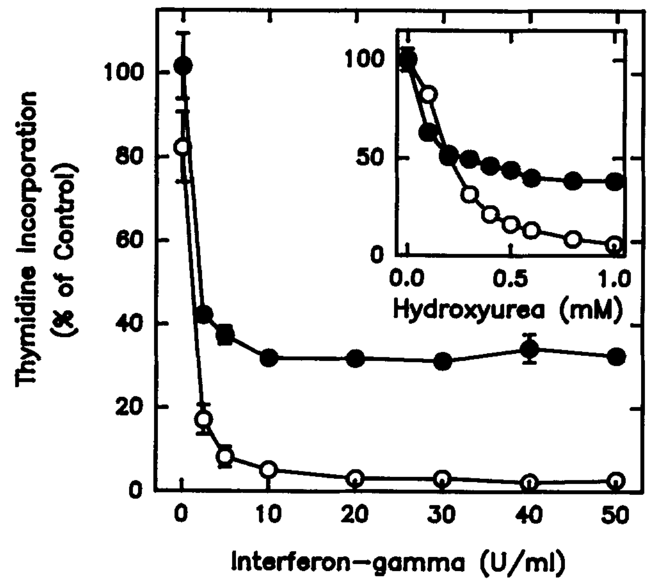

Figure 7. Effects of exogenous deoxyribonucleosides on the cytostatic effects of intact, activated macrophages or hydroxyurea. Thioglycollate broth–elicited peritoneal macrophages were activated with LPS (0.5 μg/ml) and IFN-γ (2.5–50 U/ml, as indicated) for 12 h, and cocultured with L1210 cells in the presence (●) or absence (○) 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 [^{3}H]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 reactant 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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