The murine adenocarcinoma cell line TA 3 synthesized nitrite from L-arginine upon stimulation with γ-interferon (IFN-γ) associated with tumor necrosis factor (TNF), or bacterial lipopolysaccharide (LPS), but not with IFN-γ, TNF, or LPS added separately. Induction of the NO₂₇-generating activity caused an inhibition of DNA synthesis in TA 3 cells. This inhibition was prevented by the L-arginine analog N²-nitro-L-arginine, which inhibited under the same conditions nitrite production by TA 3 cells. The TA 3 M2 subclone, selected for enhanced ribonucleotide reductase activity, was found to be less sensitive than the wild phenotype TA 3 WT to the cytostatic activity mediated by the NO₂₇-generating system. Cytosolic preparations from TA 3 M2 cells treated for 24 or 48 h with IFN-γ, TNF, and LPS exhibited a reduced ribonucleotide reductase activity, compared to untreated control cells. No reduction in ribonucleotide reductase activity was observed when N²-nitro-L-arginine was added to treated cells. Addition of L-arginine, NADPH, and tetrahydrobiopterin into cytosolic extracts from 24-h treated TA 3 M2 cells triggered the synthesis of metabolic products from the NO₂₇-generating pathway. This resulted in a dramatic inhibition of the residual ribonucleotide reductase activity present in the extracts. The inhibition was reversed by N²-monomethyl-L-arginine, another specific inhibitor of the NO₂₇-generating activity. No L-arginine-dependent inhibition of ribonucleotide reductase activity was observed using extracts from untreated cells that did not express NO₂₇-generating activity. These results demonstrate that, in an acellular preparation, molecules derived from the NO₂₇-generating pathway exert an inhibitory effect on the ribonucleotide reductase enzyme. This negative action might explain the inhibition of DNA synthesis induced in adenocarcinoma cells by the NO₂₇-generating pathway.

The biosynthesis of the simple and highly reactive nitric oxide molecule NO has been recently demonstrated in cytotoxic macrophages (Hibbs et al., 1988; Marletta et al., 1988; Stuehr et al., 1989) and stimulated endothelial cells (Ignarro et al., 1987; Palmer et al., 1987). The nitrogen atom of NO is derived exclusively from one of the two guanidino nitrogen atoms of L-arginine (Marletta et al., 1988; Palmer et al., 1988), via an unusual metabolic route. Nitrite (NO₂⁻)/nitrate (NO₃⁻), the oxidation products of nitric oxide, and citrulline, are the stable end products of the pathway (Iyengar et al., 1987). In addition to macrophages and endothelial cells, neutrophils (Schmidt et al., 1989; McCall et al., 1989), Kupffer cells (Billiar et al., 1989), hepatocytes (Curran et al., 1989), and tumor cells (Amber et al., 1989; Lepoivre et al., 1989), cells from the adrenal gland (Palacios et al., 1989) and cerebellum (Gathwaite et al., 1988; Bredt and Snyder, 1989) can generate these L-arginine-derived molecules. The L-arginine:NO synthase activity responsible for this NO₂₇-generating pathway has been found in 100,000-150,000 x g cytosolic supernatants isolated from murine macrophages (Kwon et al., 1989; Tayed and Marletta, 1989), porcine endothelial cells (Maver et al., 1989; Palmer and Moncada, 1989), and bovine (Schmidt et al., 1989b) or rat (Knowles et al., 1989) brain homogenates. The activity is activated by Mg²⁺ or Ca²⁺. It requires L-arginine, NADPH, and, in macrophage preparations, a reduced bipterin such as BH₂⁻ (Kwon et al., 1989; Tayed and Marletta, 1989).

The NO₂₇-generating pathway has been invoked as an effect of the extracellular cytotoxic functions exerted by activated macrophages against tumor cells. A specific pattern of metabolic impairments induced in tumor cells by cytotoxic macrophages, including inhibition of the Krebs cycle enzyme aconitase (Draper and Hibbs, 1986), inhibition of complexes I and II of the mitochondrial electron transport chain (Granger and Lehninger, 1982), inhibition of DNA synthesis, and iron loss from tumor cells (Hibbs et al., 1984; Wharton et al., 1988), have been shown to be L-arginine requiring phenomena (Hibbs et al., 1987). Very similar metabolic alterations have also been observed in EMT-6 tumor cells endogenously generating L-arginine-derived nitrogen oxides, after stimulation by IFN-γ associated with bacterial lipopolysaccharide (LPS), tumor necrosis factor α (TNF), or interleukin 1 (IL-1) (Amber et al., 1988). Nitric oxide was proposed as one of the L-arginine-derived molecules exhibiting cytostatic properties, since authentic NO (Hibbs et al., 1988; Stuehr and Nathan, 1989), but not its oxidation products NO₂⁻/NO₃⁻ (Stuehr and Nathan, 1989), caused a profound inhibition of DNA synthesis in tumor cells. However, the biochemical basis for the antireplicative effect mediated by exogenously or endogenously formed L-arginine-derived molecules like NO is still unknown.
An L-Arginine-dependent Inhibition of Ribonucleotide Reductase Activity

Poorly understood. Ribonucleotide-diphosphate reductase (EC 1.17.4.1), a non-heme iron-containing molecule and a key enzyme for DNA synthesis, has been proposed as a potential target for cytostasis-inducing molecules derived from the NO$	extsubscript{2}$-generating pathway (Hibbs et al., 1988; Stuehr and Nath, 1989). Ribonucleotide reductase consists of two nonidentical homodimeric subunits denoted protein M1 and M2 in mammals. Protein M1 contains nucleotide binding sites and supported the complex allosteric regulation of the enzyme. Protein M2 contains a hydroxyurea-sensitive tyrosyl residue. Protein M2, but not protein M1, is cell cycle-dependent (Eriksson et al., 1984; Engström et al., 1986), and its availability within the cell regulates and limits the enzyme activity during the cell cycle. This activity is enhanced in hydroxyurea-resistant, M2-overexpressing cell lines (Akerblom et al., 1981; Eriksson et al., 1984).

In this report, we investigated the relationships existing between induction of the L-arginine-requiring pathway, inhibition of DNA synthesis, and variations in the ribonucleotide reductase activity. The experiments were performed with the TA 3 M2 clone, in which both overexpressing M2 subunits of the ribonucleotide reductase enzyme and responds to appropriate stimulations by endogenous induction of the NO$	extsubscript{2}$-generating system. This is the first report to show an inhibitory effect of metabolites from the NO$	extsubscript{2}$-generating pathway on the ribonucleotide reductase activity.

**Materials and Methods**

**Cell Lines and Cultures**—The TA 3 cell line is a mammary adenocarcinoma from a mouse (Klein, 1951). The hydroxyurea-resistant, M2-overproducing TA 3 M2 clone was obtained from cells of the wild-type phenotype (called TA 3 WT in this paper) as previously described (Eriksson et al., 1984).

TA 3 cell cultures were maintained in RPMI 1640 medium (GIBCO BRL SARL, Cergy-Pontoise, France) plus 5% heat-inactivated fetal calf serum (GIBCO BRL SARL), antibiotics, and 25 mM HEPES, pH 7.4. The TA 3 M2 clone was routinely propagated in a culture medium containing 2 mM hydroxyurea, except when cells were grown for experiments.

**Chemicals and Cytokines**—N$	extsubscript{6}$-Monomethyl-L-arginine, acetate salt from Calbiochem (La Jolla, CA); L-arginine hydrochloride; L-2,3-diaminopimelic acid; L-biopterin; 2HCl was from Serva (Heidelberg, Federal Republic of Germany). L-Arginine HCl and HEPES were purchased from Merck (Darmstadt, FRG). Arginine from bovine liver, CDP, ATP, dithioerythritol, N$	extsuperscript{6}$-nitro-L-arginine, hydroxyurea, NADPH, and Crotalus adamanteus venom were from Sigma. Lipopolysaccharide from Salmonella enteritidis was purchased from Difco (Detroit, MI).

Human recombinant TNF-α (Genentech Inc., San Francisco, CA) had a specific activity of 7.6 × 10$^6$ units/mg (1 unit of TNF activity is defined as that amount producing 50% lysis of actinomycin D-treated L929 cells in our standard assay (Lepoivre et al., 1988)). Human recombinant IL-1α (2.1 × 10$^6$ LAF units/mg) was generously provided by Dr. F. Lomedico (Hoffman-La Roche Inc., Nutley, NJ). Mouse recombinant TNF-α interferon with a specific activity of 1.9 × 10$^6$ units/mg was kindly given by Dr. G. R. Adolf (Ernst-Boehringer Institut für Arzneimittel Forschung, Vienna, Austria). Diluted stock solutions of TNF (1 × 10$^6$ units/ml) or IFN-γ (1 × 10$^6$ units/ml) in RPMI 1640 medium plus 10% heat inactivated fetal calf serum were stored as small aliquots at -20 °C, thawed, used once, and discarded. Determination of PH/Thymidine Incorporation—Cells plated in 24-well, flat-bottomed microtiter plates (Nunclon, Roskilde, Denmark) were incubated for 24 h in a 100 μl volume of culture medium, at 37 °C in a humidified 6% CO$	extsubscript{2}$ incubator. Then, 100 μl of fresh medium, or 100 μl of IFN-γ, LPS, TNF, and/or IL-1 dilutions in fresh medium, were added into the well. Cultures were incubated for 16 h or 48 h and then pulsed for an additional 3 h with 1 μCi [3H]thymidine (1.5 mCi/μmol) (CEA, Saclay, France; specific activity, 37 Bq/mmol). Cells were lysed with 20 μl of 1 N NaOH, and DNA was harvested onto glass fiber filters with a Titertek cell harvester (Skatron, Lierneby, Norway). The radioactivity present on the filter was counted in 2 ml of a Lipofluor scintillation mixture (J. T. Baker, NJ) and stored as small aliquots at -20 °C, thawed, used once, and discarded. Identical cultures were grown for measurement of nitrite production by TA 3 M2 cells, except that no thymidine was added. At the end of incubation, supernatants of triplicate wells were collected, pooled, centrifuged, stored at -30 °C, and assayed for nitrite content.

**Determination of Nitrite**—Nitrite concentration in cell-free culture supernatants was determined with the Griess reagent, as previously described (Lepoivre et al., 1989). The calibration curve obtained with NO$	extsubscript{2}$-linear from 3 to 100 μM was used.

**Preparation of Cell Extracts**—Exponentially growing cell cultures in 14-cm dishes (Nunclon) were used. At zero time, culture medium was replaced by 20 ml of fresh medium or 20 ml of medium with indicated additives. Cells were incubated for 24 or 48 h, after which 2 ml of the culture supernatants were collected for nitrite assay. Cells were harvested by trypsinization, washed once in 20 ml of phosphate-buffered saline, pH 7.4, and washed again in 20 ml of ice-cold HEPES buffer (100 mM HEPES, pH 7.6, 15 mM magnesium acetate, and 10 mM DTE). After centrifugation, the cell pellet containing between 20 and 70 × 10$^6$ cells was resuspended in 150 to 500 μl of HEPES buffer (see above) and transferred to a glass homogenizer. The homogenate was centrifuged for 20 min at 4 °C in a Beckman TL-100.2 rotor at 13,000 × g. The supernatant, containing 4–10 mg of protein per ml, was rapidly frozen in liquid nitrogen and stored at -30 °C.

**Protein Determination**—Protein content in cell extracts was determined with the Bio-Rad protein assay kit (Bio-Rad SA, Paris, France), using ovalbumin as a standard.

**Assay of Ribonucleotide Reductase Activity**—Assays were performed with [3H]CDP as substrate, ATP as effector and DTE as reducing agent. The following reagents were incubated at 30 °C for 30 min in a final volume of 10 μl: 100 mM HEPES, pH 7.8, 15 mM magnesium acetate, 10 mM DTE, 5 mM ATP, 200 μM CDP, 74 kBq of [5-3H]CDP (Amersham France SA, specific activity: 680 GBq/mmol), and variable amounts of cell extracts. In some experiments, 150 μM NADPH, 500 μM CTP, 2.2 mM L-NMA, arginase (4.7 units/ml), and/or indicated concentrations of L-arginine were also added at the beginning of the assay. The reaction was stopped by heating at 90 °C for 2 min. Further steps were as described by Darling et al. (1987). Nucleotides were converted to their corresponding nucleosides by treatment for 2 h at 37 °C with 10 μl of C. adamanteus venom (200 mg/ml in 15 mM MgCl$	extsubscript{2}$). Precipitates formed after heating at 90 °C for 2 min were pelleted by centrifugation for 5 min in a Beckman Microfuge B. Supernatants were removed and analyzed by HPLC after addition of 29 μl of a 2 mM solution of unlabeled deoxycytidine. Ten microliters of the mixture were loaded on a 5-μm Zorbax ODS C18 column (250 × 4.6 mm, Société Française Chromato Colonne, Neuilly-Paisance, France) connected to a Waters model 481 absorbance detector set at 254 nm, and a Berthold LB 506 C-1 flow-through radioactivity monitor. An isocratic elution of [3H]cytidine and [3H]deoxycytidine was performed at 1 ml/min with a 10 mM sodium acetate buffer, pH 5.4, supplemented with 2% of the ion-pairing reagent pentane sulfonic acid (Waters, Low UV Picket). Data from UV and H detectors were stored in an Epon PC AX computer running under a “Berthold HPLC” program version 9.6. Typical retention times of cytidine and deoxycytidine were 13.7 and 21.7 min, respectively.

**Determination of Urea and Citrulline in Cytosolic Extracts**—Metabolic products from L-[guanido-14C]arginine were analyzed with the Anion-HPLC device described above. Reaction mixture in a final volume of 90 μl was exactly as described for the ribonucleotide reductase assay, including cold CDP, ATP, and DTE, except that 130 μM NADPH, 500 μM BH$	extsubscript{4}$, and 8 kBq of [L-14C]arginine (CEA, Saclay, specific activity: 1.88 GBq/mmol), and indicated concentrations of L-arginine were added immediately after filtering the mixture through a brown filter. Incubations at a final concentration of 2.2 mM. Samples were incubated for 50 min at 30 °C and then heated at 90 °C for 2 min. Precipitates were pelleted and 200 μl of 20 mM sodium citrate, pH 2.5, was added to the supernatant, which was filtered through a YM-10 ultrafiltration membrane (M, cut-off 10,000, Amicon, Danvers, MA). A 50-μl aliquot of this solution was analyzed as a standard, using a scintillation counter (Zorbax 300 SCX, 250 × 4.6 mm, Société Française Chromato Colonne). Radiolabeled L-arginine metabolites were eluted at a flow rate of 1 ml/min under the following conditions: 0–7 min, isocratic, 20 mM sodium citrate, pH 2.2; 7–17 min, linear gradient to 100 mM
was investigated by the measurement of nitrite production in the culture medium. As expected, unstimulated cells were not stimulated. Curve fittings were obtained with a Cricket Graph program, version 1.2.1 (Cricket Software, Malvern, PA).

**RESULTS**

**Induction of Nitrite Production in TA 3 M2 Cells**—Induction of an L-arginine:NO synthase activity in TA 3 M2 cells was investigated by the measurement of nitrite production in the culture medium. As expected, unstimulated cells were not able to produce nitrite spontaneously. In another murine adenocarcinoma cell line, EMT6, nitrite synthesis was induced by treatment with IFN-γ associated with LPS, TNF, or IL-1 (Amber et al., 1988; Lepoivre et al., 1989). These factors were also tested on TA 3 M2 cell cultures (Table I). When added separately, none of the above-mentioned stimulants could induce a significant production of nitrite by TA 3 M2 cells. However, this activity was induced when IFN-γ was associated with IL-1 (not shown), TNF, and/or LPS. The most potent binary association was IFN-γ + LPS and maximal production was observed with either a ternary association of IFN-γ, LPS, and TNF or a combination of the four stimuli IFN-γ, LPS, TNF, and IL-1 (not shown). Nitrite synthesis was sustained over a 72-h period, but in most cases the rate of NO3 production was markedly reduced during the 3rd day. Results presented in Table I were limited to the first 48 h.

**Growth Inhibition of TA 3 M2 Cells**—Interferon, TNF, and LPS were tested for their ability to inhibit DNA synthesis by TA 3 M2 cell cultures. Their antiproliferative effect was compared to the amounts of nitrite produced under the same conditions. Interferon, TNF, and LPS tested separately exhibited a very moderate cytostatic effect, even after a 48-h treatment (Table I). The growth inhibition was higher but still moderate when TNF and LPS were added together. In no case did such treatments induce a significant production of nitrite in the culture medium. Combinations of LPS, TNF, or LPS + TNF with IFN-γ exerted a strong cytostatic effect on TA 3 M2 cells, which accompanied the induction of nitrite synthesis. The extent of DNA synthesis was inversely linked to the amounts of nitrite produced by TA 3 M2 cells. This observation suggested that the NO3-generating pathway induced in TA 3 M2 cells might exert a strong cytostatic effect on the cell line. In agreement with this hypothesis, addition of a potent inhibitor of the NO3-generating activity, N4-nitro-L-arginine, inhibited nitrite synthesis by TA 3 M2 cells and correspondingly inhibited the cytostatic effect induced by IFN-γ, LPS, and TNF (Table I). For instance, a 24-h treatment with the inhibitor completely blocked nitrite synthesis by TA 3 M2 cells and inhibited the antiproliferative action of the three combined stimuli IFN-γ, LPS, and TNF, which became similar to the moderate, L-arginine-independent cytostatic activity displayed by LPS + TNF alone (%[^3H]dThd uptake = 0.45% ± 0.8 versus 0.65% ± 5.3, respectively).

**Sensitivity of Two TA 3 Clones to the L-Arginine-Dependent Cytostatic Potential**—The wild-type TA 3 WT and the M2-overproducing TA 3 M2 cell lines were examined for their sensitivity to the L-arginine-requiring cytostatic action induced by a combination of IFN-γ, LPS, and TNF. The NO3-producing activity of the TA 3 WT cell line was very similar to that of the M2-overproducing clone, respectively, 9.5 versus 10.0 nmol of NO3/24 h/105 cells were produced by TA 3 WT (n = 6) and TA 3 M2 (n = 12) cells, when treated with IFN-γ, LPS, and TNF. Nitrite production and DNA synthesis by TA 3 M2 cells were measured under the same conditions, in response to a 24-h treatment with IFN-γ, LPS, and TNF (Fig. 1). In order to produce increasing nitrite concentrations in cell cultures, cells were plated at increasing densities. As already described for the results presented in Table I, a logarithmic correlation was found between the amounts of nitrite produced and the residual DNA synthesis exhibited by treated TA 3 cells. Surprisingly, the two clones were not equally sensitive to the cytostatic consequences of the NO3-generating metabolism, TA 3 M2 cells being less affected than the wild type phenotype. Since the TA 3 M2 cell line overexpressed the M2 subunit of the ribonucleotide reductase, a crucial and unique enzyme for DNA synthesis, it was suggested that the L-arginine-dependent cytostatic action of the NO3-generating pathway might be related to an inhibition of ribonucleotide reductase.

**Ribonucleotide Reductase Activity in TA 3 M2 Cell Extracts**—Ribonucleotide reductase activity was measured in cytosolic extracts from TA 3 M2 cells cultured for 24 or 48 h with IFN-γ, LPS, and TNF, and was compared to the amounts of nitrite that had accumulated during these periods in the culture medium (Table II). Cytosolic extracts from

### Table I

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Additive*</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[^3H]dThd uptake</td>
<td>n</td>
<td>Nitrite</td>
</tr>
<tr>
<td>% control</td>
<td>µM</td>
<td></td>
<td>µM</td>
</tr>
<tr>
<td>LPS</td>
<td>80.8 ± 17.9</td>
<td>4</td>
<td>&lt;3</td>
</tr>
<tr>
<td>TNF</td>
<td>91.9 ± 10.0</td>
<td>4</td>
<td>&lt;3</td>
</tr>
<tr>
<td>IFN</td>
<td>92.0 ± 16.8</td>
<td>4</td>
<td>&lt;3</td>
</tr>
<tr>
<td>LPS + TNF</td>
<td>73.6 ± 23.0</td>
<td>4</td>
<td>&lt;3</td>
</tr>
<tr>
<td>TNF + IFN</td>
<td>38.0 ± 7.5</td>
<td>4</td>
<td>8.5 ± 0.4</td>
</tr>
<tr>
<td>LPS + IFN</td>
<td>30.9 ± 11.4</td>
<td>4</td>
<td>7.3 ± 1.2</td>
</tr>
<tr>
<td>TNF + LPS + IFN</td>
<td>14.3 ± 3.7</td>
<td>4</td>
<td>9.5 ± 0.8</td>
</tr>
<tr>
<td>LPS + TNF</td>
<td>109.8 ± 15.2</td>
<td>3</td>
<td>&lt;3</td>
</tr>
<tr>
<td>TNF + LPS + IFN</td>
<td>64.5 ± 5.8</td>
<td>3</td>
<td>&lt;3</td>
</tr>
</tbody>
</table>

*An initial number of 1 × 10⁶ cells/200 µl was plated in 96-well plates. Cells were treated for 24 or 48 h with IFN-γ (10 units/ml), LPS (1 µg/ml), and TNF (380 units/ml).

**Results and Computerized Curve Fitting**—Statistical analysis of the data was performed with the Student's t test. Computerized curve fittings were obtained with a Cricket Graph program, version 1.2.1. Statistics and Computerized Curve Fitting—Statistical analysis of the data was performed with the Student's t test. Computerized curve fittings were obtained with a Cricket Graph program, version 1.2.1 (Cricket Software, Malvern, PA).
treated cells exhibited a reduced ribonucleotide reductase activity, as compared to untreated controls. The decrease in ribonucleotide reductase activity was moderate after a 24-h treatment (approximately 50% of control samples) and became severe after 48 h (more than 90% of the control activity was lost at this time). This reduction was related to the NO2-generating activity since addition of N°-nitro-l-arginine to TA 3 M2 cultures treated with IFN-γ, LPS, and TNF prevented the loss of ribonucleotide reductase activity in cell extracts. There was a qualitative rather than a quantitative parallelism between the decrease in cytosolic ribonucleotide reductase activity and the production of nitrite in TA 3 M2 supernatants. Approximately 80% of the nitrite production occurred during the first 24 h, whereas the ribonucleotide reductase activity regularly decreased over 48 h. In the presence of N°-nitro-l-arginine, TA 3 M2 cells still produced significant amounts of nitrite over 48 h (35% of the nitrite levels produced by treated cells cultured without the inhibitor) but ribonucleotide reductase activity measured at 48 h in the corresponding cell extracts had fully recovered to a normal level (95% of the activity of cytosolic preparations from untreated cells).

**Inhibition of Ribonucleotide Reductase Activity after Triggering of the L-Arginine: NO Synthase Activity.**—These experiments were performed with cytosolic extracts of treated or untreated cells cultured for 24 h (Table III). Ribonucleotide reductase activity was measured as described above. The L-arginine metabolism in cytosolic preparations was investigated by studying L-[guanidino-14C]arginine degradation through the L-arginine:NO synthase and arginase pathway, leading, respectively, to radiolabeled citrulline and urea. Arginase was constitutively expressed in TA 3 M2 cells. Urea was thus synthesized by untreated as well as treated cells. Synthesis of peak X, an unidentified metabolite of L-arginine, was found to be remarkably constant in all cytosolic preparations from treated and untreated cells. As expected from an inducible activity, synthesis of citrulline was not observed in cell extracts from untreated cells and had to be induced in living TA 3 M2 cells by IFN-γ, TNF, and LPS. Citrulline synthesis in cytosolic extracts from such treated cells was greatly enhanced by addition of NADPH and BH4, two co-factors of the L-arginine:NO synthase activity. It was inhibited by L-NMA, a potent and selective inhibitor of the NO2-generating pathway. Presence of N°-nitro-L-arginine, NADPH, and/or L-NMA did not affect urea and peak X synthesis. To examine the effects of metabolic products from the NO2-generating pathway on ribonucleotide reductase activity, unlabeled L-arginine, NADPH, and BH4 were added to cytosolic extracts from treated cells, at the beginning of the ribonucleotide reductase assay. Under these conditions, reactive intermediates and stable products of the L-arginine:NO synthase activity were continuously generated while ribonucleotide reductase was converting [3H]CDP into [3H]dCDP. The additives L-arginine, NADPH, and BH4 caused a profound inhibition

![Graph](image)

**Table II**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Additives</th>
<th>Ribonucleotide Reductase Activity (24 h)</th>
<th>Ribonucleotide Reductase Activity (48 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS + TNF + IFN</td>
<td>NO2-ARG ND</td>
<td>125.1 ± 43.4 (64.4)</td>
<td>159.1 ± 84.6 (99.2)</td>
</tr>
<tr>
<td>LPS + TNF + IFN</td>
<td>NO2-ARG ND</td>
<td>149.5 ± 65.3 (95.8)</td>
<td>27.6 ± 3.8</td>
</tr>
</tbody>
</table>

*Exponentially growing cell cultures were treated for 24 or 48 h with INF-γ (40 units/ml), LPS (10 μg/ml), and TNF (150 units/ml) in a final volume of 20 ml.

*Final concentration of NO2-ARG added at zero time was 760 μM.

*Ribonucleotide reductase activity in cellular extracts and concentration of nitrite in culture supernatants were determined as reported under "Materials and Methods." Mean ± S.E. of n independent experiments.

*Significantly different from the untreated control, (p < 0.01).

*ND, not done.

*Significantly different from the treated sample without the inhibitor (p < 0.01).
An L-Arginine-dependent Inhibition of Ribonucleotide Reductase Activity

**Table III**

Inhibition of ribonucleotide reductase activity after triggering of the NO\(_2\)-generating pathway in TA 3 M2 cell extracts

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Additives*</th>
<th>L-[guanidino-(^{14})C]Arginine metabolism(^d)</th>
<th>Ribonucleotide reductase(^n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Citrulline</td>
<td>Urea</td>
</tr>
<tr>
<td>L-Arg (1.1 mM) + NADPH + BH(_4)</td>
<td>0</td>
<td>2.9</td>
<td>16.9</td>
</tr>
<tr>
<td>L-Arg (1.1 mM) + NADPH + BH(_4) + L-NMA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L-Arg (1.1 mM) + NADPH + BH(_4) + L-NMA</td>
<td>4.3</td>
<td>2.8</td>
<td>16.8</td>
</tr>
<tr>
<td>L-Arg (250 (\mu)M) + NADPH + BH(_4)</td>
<td>27.5</td>
<td>1.3</td>
<td>18.4</td>
</tr>
<tr>
<td>L-Arg (250 (\mu)M) + NADPH + BH(_4) + L-NMA</td>
<td>9.8</td>
<td>1.0</td>
<td>20.7</td>
</tr>
<tr>
<td>Arginase + NADPH + BH(_4)</td>
<td>39.4</td>
<td>0.5</td>
<td>7.2</td>
</tr>
<tr>
<td>Arginase + NADPH + BH(_4) + L-NMA</td>
<td>3.9</td>
<td>0.0</td>
<td>7.6</td>
</tr>
<tr>
<td>Arginase + NADPH + BH(_4)</td>
<td>80.1 ± 2.7</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

*Cells were treated as described in Table II.

*Final concentrations of NADPH, BH\(_4\), L-NMA, and arginase were, respectively, 130 \(\mu\)M, 500 \(\mu\)M, 2.2 \(\mu\)M, and 4.7 units/ml.

*Experiments with cold CDP and radiolabeled arginine. Metabolites from L-arginine were determined as described under "Materials and Methods." Mean of two separate experiments, with SE. <10% of the mean. ND, not done.

*Experiments with cold CDP (and cold arginine, if required). Ribonucleotide reductase activity was 211.2 ± 9.9 and 177.6 ± 75.1 pmol/min/mg of protein for untreated and treated cells, respectively. Mean ± S.E. of \(n\) independent experiments.

The biochemical mechanisms whereby the NO\(_2\)-generating pathway induces an L-arginine-dependent inhibition of DNA replication in tumor cells are still speculative. The metabolic alterations accompanying the inhibition of DNA synthesis seem to be centered around perturbations of iron metabolism or iron-containing enzymes (see introduction to the text). For this reason, an iron-containing protein essential for DNA replication has been supposed to be dramatically affected by the induction of the NO\(_2\)-generating activity in tumor cells. Ribonucleotide reductase might be such a protein. Its activity is correlated to the extent of DNA synthesis (Thelander and Reichard, 1979), and the \(\beta_2\) subunit of the \(\alpha_2\beta_2\) molecule contains two binuclear ferric iron centers (Sahlin et al., 1989).

In this report, we showed that a TA 3 ahelone expressing an enhanced ribonucleotide reductase activity was less sensitive than the normal parent to the cytostatic action of an endogenous NO\(_2\)-generating system, induced by the combination of IFN-\(\gamma\) with LPS and TNF. This observation suggested that the observed L-arginine-dependent inhibition of DNA synthesis in TA 3 cells was effectively supported by an inactivation of the ribonucleotide reductase enzyme. Alternatively, the mutant clone might have also been modified in cellular functions other than nucleotide reduction. These modified functions (e.g. accelerated iron uptake, overexpression of antioxidant systems . . .) might have protected the TA 3 M2 clone against the cytostatic injury of the NO\(_2\)-generating pathway. In fact, there was no clear kinetic correlation between the decrease in ribonucleotide reductase activity in cellular extracts and the amount of nitrite produced by TA 3 M2 cells stimulated with IFN-\(\gamma\), LPS, and TNF. The ribonucleotide reductase activity is controlled during the cell cycle by the amounts of the M2 protein, which is present at high levels during the S and G2 phases and greatly reduced during the G1 phase (Eriksson et al., 1984). Therefore, if the stimulation of TA 3 M2 cells with IFN-\(\gamma\), LPS, and TNF had resulted in a blockage of the cell cycle in G1 phase, the ribonucleotide reductase activity of the treated cells would have been much lower than that of untreated control cells, in the absence of any inhibitory effect of the NO\(_2\)-generating pathway on the enzyme. On the contrary, the NO\(_2\)-generating pathway might have exerted a reversible inhibitory action on the ribonucleotide reductase, induced by a sustained production of L-arginine-derived molecules. In cytosolic extracts prepared for the ribonucleotide reductase assay, and in the absence of any additives, the NO\(_2\)-generating activity is in-
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terrupted, enabling a possible reactivation of the ribonucleotide reductase (inhibition of ribonucleotide reductase by hydroxyurea can thus be reversed by removing the inhibitor and adding iron and a reductant (Eriksson et al., 1984)). Cell cycle blockage of treated cells in G1 phase and reactivation of a reversibly inhibited enzyme during preparation of cytosolic extracts are hypothetical events with opposite effects on the ribonucleotide reductase activity. Therefore, the results reported in Table II could not be clearly explained and have required additional experiments. Those reported in Table III, although performed with acellular preparations, exhibited some analogy with a physiological situation since the ribonucleotide reductase activity was measured while the NO2-generating system was functioning (after addition of L-arginine, NADPH, and BH4 to extracts of TA 3 M2 cells stimulated with IFN-γ, LPS, and TNF). This experiment was possible since assay conditions for both activities were compatible (see “Materials and Methods” and Tayed and Marletta, 1989). It resulted in a profound inhibition of the ribonucleotide reductase activity, specifically caused by metabolic products of the NO2-generating pathway. Even in the presence of L-arginine, NADPH, and BH4, extracts from untreated, L-arginine:NO synthase-deficient cells exhibited a normal ribonucleotide reductase activity, indicating that the additives did not cause, per se, an inhibition of the ribonucleotide reductase enzyme, and that the presence of the NO2-generating activity was required for the inhibition to occur. The inhibitory effect was L-arginine-dependent since it was not observed in the presence of arginine and it was specific of the NO2-generating pathway because L-NMA, a specific inhibitor of this pathway in macrophages (Hibbs et al., 1987) and in murine adenocarcinoma cells (Lepoivre et al., 1989 and Table III), prevented the inhibition.

The inhibitory effect of the NO2-generating system on the ribonucleotide reductase enzyme might be directly mediated by metabolites derived from L-arginine oxidation or might involve an intermediary step. Such a step should be related to the well known activation by L-arginine-derived nitric oxide of the soluble guanylate cyclase enzyme (Marletta, 1989, for review). Although phosphorylation of the M2 subunit by a cyclic AMP-dependent protein kinase has been reported to inhibit the ribonucleotide reductase enzyme (Albert and Nodenzski, 1989), so far no relationship has been described between the enzyme and cGMP-dependent activities. We think that a direct inhibition of ribonucleotide reductase by metabolic products from the NO2-generating pathway seems a more relevant hypothesis. The tyrosyl radical and the nonheme iron of the M2 subunit of the ribonucleotide reductase are essential structures for the active enzyme. They are also potential targets for metabolites synthesized via the NO2-generating pathway. One of them, nitric oxide, has been described to complex with the binuclear iron site of heme-thrin, a prototype for proteins with ω-hydroxyl or ω-oxo-bridged binuclear iron structure, such as the M2 protein (Noceti et al., 1988). Furthermore, under anaerobic conditions, authentic nitric oxide has been shown to inhibit DNA replication of murine and guinea pig tumor cells (Hibbs et al., 1988; Stuehr and Nathan, 1989). Hydroxy-2-guanidine is a potent inhibitor of ribonucleotide reductase (Tai et al., 1983), acting like hydroxyurea by scavenging the tyrosyl radical of the M2 subunit of the enzyme. There is an evident structural homology between N4-hydroxyguanidine and N4-hydroxy-L-arginine, the first product of L-arginine oxidation by the L-arginine:NO synthase system, as proposed by Marletta (1989).

We have demonstrated that the L-arginine:NO synthase activity can exert an inhibitory effect on the ribonucleotide reductase activity in acellular preparations. Since the relative resistance of the TA 3 M2 clone to the cytostatic effect of the NO2-generating pathway seems to be due to overexpression of the M2 subunit of ribonucleotide reductase, it is tempting to speculate that the function of the M2 protein might be specifically altered in intact cells by an L-arginine-dependent mechanism. Nitric oxide and N4-hydroxyarginine are two L-arginine-derived molecules with a potential reactivity on crucial structures of the M2 protein. They might be the cytostatic effectors of the L-arginine:NO synthase activity.

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REFERENCES

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