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Biochemistry

Nitric oxide production in SJL mice bearing the RcsX lymphoma: A model for *in vivo* toxicological evaluation of NO·

(nitric oxide/macrophages/superantigen)

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ABSTRACT **SJL mice spontaneously develop pre-B-cell lymphoma that we hypothesized might stimulate macrophages to produce nitric oxide (NO·). Transplantation of an aggressive lymphoma (RcsX) was used to induce tumor formation. Urinary nitrate excretion was measured as an index of NO· production and was found to increase 50-fold by 13 days after tumor injection. NO· production was prevented by the addition of a nitric oxide synthase (NOS) inhibitor. The expression of inducible NOS (iNOS) in various tissues was estimated by Western blot analysis and localized by immunohistochemistry. The synthase was detected in the spleen, lymph nodes, and liver of treated but not control mice. To assess whether the iNOS-staining cells were macrophages, spleen sections from RcsX-bearing animals were costained with anti-iNOS antibody and the anti-macrophage antibody moma-2. Expression of iNOS was found to be limited to a subset of the macrophage population. The concentration of γ-interferon, a cytokine known to induce NO· production by macrophages, in the serum of tumor-bearing mice, was measured and found to be elevated 25-fold above untreated mice. The ability of RcsX-activated macrophages to inhibit splenocyte growth in primary culture was estimated and macrophage-derived NO· was found to inhibit cell division 10-fold. Our findings demonstrate that RcsX cells stimulate NO· production by macrophages in the spleen and lymph nodes of SJL mice, and we believe this experimental model will prove useful for study of the toxicological effects of NO· under physiological conditions.**

The free radical nitric oxide (NO·) has multiple functions in mammalian physiology. At physiological levels, it is associated with regulatory functions, such as long-term potentiation in the brain (1) and vasodilation (2). At higher levels, it has been associated with tissue damage (3) and tumoricidal (4) and bactericidal (5) effects. It also has been shown to be cytostatic to T cells and tumor cells *in vitro* (4) and has been reported to induce apoptosis (6). NO· mediates the oxidant injury caused by paraquat in isolated guinea pig lungs (7) and possesses tumoricidal activity against transplanted tumors in mice (8). Mice lacking the inducible isozyme of nitric oxide synthase (iNOS) are deficient in the ability to resist bacterial infection (9).

Long-term endogenous production of NO· has been suspected of causing adverse effects *in vivo*. 3-Nitrotyrosine, a biomarker of such production, has been detected in tissue proteins of patients with diseases thought to have autoimmune components, such as atherosclerosis (10) and rheumatoid arthritis (11). Evidence for involvement of NO· in autoimmune responses has also been produced in experimental animals, including MLR-lpr/lpr mice, in which development of spontaneous immune complex glomerulonephritis was prevented by administration of NOS inhibitors (12). In SJL mice, it was shown to play a role in the pathology of experimental autoimmune encephalomyelitis (13) and has been used as an experimental model for the disease (14).

SJL mice were derived from the Swiss–Webster strain (15) and exhibit multiple immunological disorders, paraproteinemia (16), and myositis (A. H. Weller, S. A. Magliato, K. P. Bell, and N. L. Rosenberg, unpublished data). They are suspected of having defective suppressor T-cell function (17), and during the first year of life, spontaneously develop lymphomas arising in germinal lymphoid centers (15, 18). The lymphomas are of B-cell lineage (19, 20) and present an mouse mammary tumor virus long terminal repeat-derived superantigen on their surface. This superantigen stimulates $Vb16^+$ T cells (21), which, in response, secrete cytokines required for growth of the lymphoma cells *in vivo* and *in vitro* (22, 23). The tumor may also require host macrophages for *in vivo* growth (24) and primary explant coculture of the tumor with SJL splenocytes is accompanied by production of γ-interferon (25).

We demonstrated (26) overproduction of NO· in spontaneous myositis concurrently involving macrophage infiltration into skeletal muscle tissue in SJL mice. These findings, together with the spontaneous development of autoimmune disease, stimulation of the host immune system by transplantation of the lymphoma, and the production of γ-interferon by tumor cells in primary culture suggested the possibility that transplantation of the lymphoma into SJL mice might induce NO· production by host macrophages. The RcsX tumor cell line (22) was derived by successive transplantation of an SJL lymphoma into syngeneic host animals (20, 23). Intraperitoneal injection of RcsX cells into SJL mice leads to rapid growth of tumor as well as host T cells in the lymph nodes, spleen, and liver, resulting in morbidity 15 days later. We report herein results supporting the conclusion that large amounts of NO· are indeed produced by macrophages in the spleen and lymph nodes of SJL mice bearing the RcsX tumor. This experimental model will be useful for further characterization of the toxicology of NO· *in vivo*.

MATERIALS AND METHODS

Animal Experiments. Female SJL mice, 14 weeks old (The Jackson Laboratory), were fed a low nitrate control diet (AYN-76A, Bio-Serve, Frenchtown, NJ) for 4 weeks to minimize the background rate of nitrate excretion. The mice were then weighed and placed individually in metabolic cages, and their drinking water was replaced with 30 mM solution of the nitric oxide inhibitor N^G-methyl-L-arginine acetate (NMA) (Chem Biochem Research, Salt Lake City, UT) or 30 mM ammonium acetate (Sigma). Two days later, six mice receiving NMA and six mice receiving ammonium acetate were each

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Abbreviations: iNOS, inducible nitric oxide synthase; NMA, N^G-methyl-L-arginine acetate.

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injected intraperitoneally with 0.2 ml of PBS containing 10^7 cells of the RcsX line (provided by N. Ponzio, University of New Jersey Medical Center, Newark, NJ), isolated from lymph nodes of mice bearing the actively growing tumor. Urine was collected on alternate days into tubes containing 0.5 ml of 0.5 M NaOH to inhibit bacterial growth. Total urinary nitrate concentration was determined as described (27) and normalized to body weight. All animals were weighed and killed by CO_2 asphyxiation 14 days after injection of cells. Spleen, liver, peripheral lymph nodes, kidneys, thigh skeletal muscle, heart, and serum were removed, and portions of each tissue were fixed in formalin and embedded in paraffin; the remainder was frozen in liquid nitrogen for further analysis.

Western Blot Analysis. iNOS was isolated from tissues as described by Oguchi *et al.* (28) with minor modifications. Tissues were homogenized in buffer A [50 mM Tris·HCl, pH 7.4/0.5 mM EDTA/0.5 mM EGTA/1 mM dithiothreitol/1 μM leupeptin/0.1 mM phenylmethylsulfonyl fluoride (all from Sigma)] and centrifuged at 100,000 × *g* for 60 min at 4°C. Proteins of the cytosolic fraction (500 mg) was incubated with 2′,5′-ADP agarose, 50:1 (vol/vol), for 30 min at 4°C. The agarose was washed three times with buffer A and iNOS was eluted with buffer A containing 10 mM NADPH. The eluted protein was concentrated on a Rotovap (Savant), loaded onto an 8% SDS/PAGE gel, and transferred to a nitrocellulose filter (Schleicher & Schuell) overnight. The membrane was blocked for 2 hr with 3% bovine serum albumin (Biowhittaker) in PBS at room temperature and then incubated with rabbit anti-mouse NOS (5 μg/ml, a gift from M. Marletta, University of Michigan, Ann Arbor, MI) in blocking buffer for 2 hr. The membrane was washed then incubated with mouse anti-rabbit alkaline phosphatase at 1 μg/ml and developed using a substrate kit (Bio-Rad) using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and *p*-nitroblue tetrazolium chloride (NBT) according to manufacturer's instructions.

Immunohistochemistry. Immunohistochemical staining for iNOS was performed on sections prepared from formalin-fixed paraffin-embedded tissues. Sections were deparaffinized in xylene and then rehydrated by passage through decreasing concentrations of ethanol in water. Sections were incubated successively with avidin and biotin (Dako) for 10 min to block reagent binding to endogenous biotin and with CAS block (Zymed) for 15 min to block nonspecific binding. Sections were incubated with polyclonal rabbit anti-mouse iNOS antiserum (10 mg/ml) in PBS for 2 hr at room temperature. Thereafter goat anti-rabbit IgG conjugated to biotin (Dako) was incubated with the tissues for 10 min, followed by incubation with alkaline phosphatase-conjugated streptavidin (Dako) for 10 min. Color was developed using fast red as the chromogen, resulting in a bright red precipitate at antigen sites. Sections were counterstained with Mayer's hematoxylin.

Double staining with the macrophage-specific antibody moma-2 and anti-iNOS was performed on frozen sections. Tissue sections were fixed in ice-cold methanol (4°C) for 15 min and incubated with rat anti-mouse moma-2 antibody (1:2.5; Serotec) for 1 hr and then in goat $F(ab)_2$ anti-rat IgG conjugated to fluorescein isothiocyanate (FITC) (Caltag, South San Francisco, CA) for 10 min. The slides were dipped in formalin, rinsed for 5 min in running water and incubated with iNOS and goat anti-rabbit IgG as described above, then with streptavidin Cy-3 (Caltag) at 2 μg/ml for 10 min.

γ-Interferon Assay. Blood (1 ml) was collected by heart puncture from six tumor-bearing and six control mice at the time they were killed and allowed to coagulate, and serum was then separated by centrifugation. Serum levels of γ-interferon were measured by ELISA (R & D Systems) according to manufacturer instructions.

Cell Culture Experiment. Lymph nodes and spleen were aseptically isolated from mice 14 days after the injection of RcsX cells. A single cell suspension was prepared by mincing and repeated pipetting in ice-cold PBS. Cells were pelleted and washed three times and stained with trypan blue to assess viability. Only preparations containing more than 95% dye-excluding cells were used. Cells were cultured in 96-well plates (Falcon), at a density of 40,000 cells per well in 0.2 ml of medium containing Isocove's modified minimal essential medium [2 mM L-glutamine/penicillin (0.2 unit/ml)/streptomycin (0.2 unit/ml)/1 mM sodium pyruvate/1 mM oxaloacetic acid/15% fetal calf serum (BioWhittaker)/insulin (0.2 unit/ml)/50 μM 2-mercaptoethanol (Sigma)]. After 4 hr nonadherent cells were transferred to another well and NMA was added to a final concentration of 1 mM. [^{3}H]Thymidine (DuPont/NEN) was added at a level of 1 μCi per well and allowed to remain in the medium for a period of 24 hr. Cells were then harvested with an automated cell harvester (Sketron, Sterling, VA) and incorporated radioactivity was measured by scintillation counting (LBK).

RESULTS

RcsX-Bearing SJL Mice Excrete Elevated Amounts of Urinary Nitrate; Excretion Is Inhibited by the iNOS Inhibitor NMA. To test the hypothesis that NO· would be overproduced in tumor-bearing SJL mice, animals were each injected with RcsX tumor cells, and total NO· production was monitored by measuring urinary excretion of nitrate, its ultimate metabolic product in mammals (29). Treated mice showed a time-dependent increase in urinary nitrate excretion ($P < 0.05$ by *t* test on day 6, Fig. 1), beginning 4 days after cells were injected and culminating in a 50-fold increase on the 14th day of the experiment. Treated mice also receiving NMA showed no elevation in nitrate excretion until the last 2 days of the experiment. Only minor variations in nitrate excretion occurred in untreated and NMA-treated control animals throughout the experiment. These results demonstrated that growth of RcsX cells was temporally associated with significant elevation of NO· production in SJL mice.

Administration of NMA alone did not affect body weights of the animals, and no differences were observed in the weights or gross appearance of organs from mice receiving NMA and those from mice receiving ammonium acetate in the drinking water. Spleens from RcsX-injected mice receiving ammonium acetate weighed 1.43 ± 0.34 g and those from treated mice

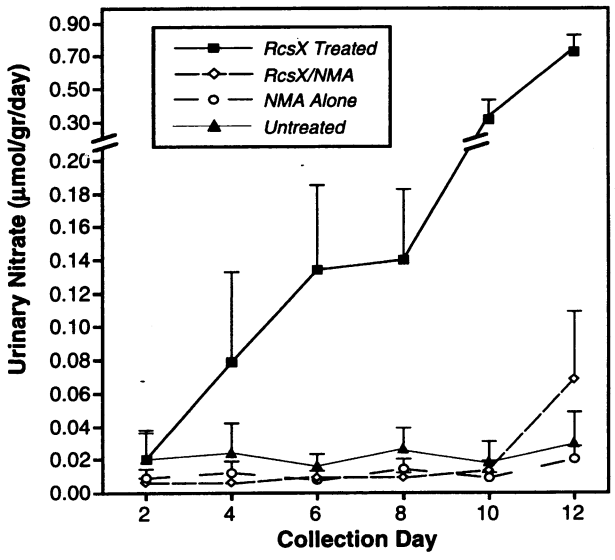

FIG. 1. Nitrate excretion by RcsX-bearing SJL mice and controls. Twelve SJL mice were injected i.p. on day 0 with 10^7 RcsX cells. Six RcsX-bearing and six control mice received 30 mM NMA in drinking water starting on day 2. All tumor-bearing mice were killed on day 13.

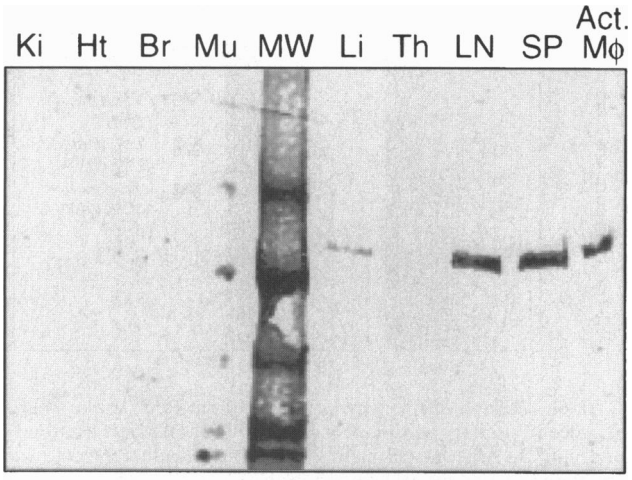

FIG. 2. Western blot analysis of iNOS expression. Tissues from an RcsX tumor-bearing SJL mouse were isolated 14 days after injection of 10^7 RcsX cells and Western blot analysis was performed. Activated macrophages were used as positive control. Control mice had no detectable iNOS expression in any of the tissues analyzed. Ki, kidney; Ht, heart; Br, brain; Mu, muscle; MW, molecular weight; Li, liver; Th, thymus; LN, lymph node; SP, spleen; Act. Mϕ, activated macrophage.

receiving NMA weighed 1.33 ± 0.31 g; peripheral lymph nodes from animals receiving the same treatments weighed 0.77 ± 0.25 g and 0.81 ± 0.21 g, respectively.

iNOS Is Expressed in Lymphoid Tissue and Liver of Tumor-Bearing Mice. To localize the NOS isozyme serving as the ultimate source of urinary nitrate observed in the above animals, presence of the endothelial, brain, and inducible isoforms of the enzyme was determined by Western blot analysis of liver, spleen, kidney, lymph node, heart, brain, and muscle. Detectable levels of iNOS were present in spleen, lymph nodes, and liver of RcsX-bearing animals, but not in control mice (Fig. 2). Neither brain NOS nor endothelial NOS was detectable in any tissues of the tumor-bearing or control animals. These results support the conclusion that NO· was produced by the inducible isoform of NOS in tumor-bearing animals.

iNOS Protein Is Localized to Small Subpopulations of Tumor Cells in Lymph Nodes and Spleen, as well as in Metastatic Cells in Liver. Immunohistochemical staining for iNOS was performed to determine the cellular localization of NO· production. The iNOS protein was detected in cells scattered throughout the lymph nodes and spleen in RcsX-treated (Fig. 3*A*) but not control (Fig. 3*B*) mice. Staining was distinctly localized in individual cells estimated to represent collectively less then 5% of the total cell population. In the spleen, staining was concentrated in, but not limited to, follicular regions (Fig. 3*A*). Cells staining for iNOS typically were larger than surrounding cells and contained a relatively large amount of cytoplasm and vacuoles (Fig. 3 *C* and *D*), an appearance consistent with the morphology of macrophages. In the liver, staining was limited to islands of metastatic cells located around central veins (Fig. 3*E*). Some tumor cells infiltrating into the kidney were also stained (Fig. 3*F*), but in both liver and kidney very few cells contained detectable levels of iNOS, and those that were present were morphologically similar to iNOS-positive cells in the spleen and lymph nodes. Liver parenchyma was devoid of iNOS-containing cells and the kidney parenchyma stained only weakly. These results indicated that iNOS-expressing cells were localized primarily within lymphoid tissues of RcsX tumor-bearing mice and further suggested that a small subpopulation of cells present in the tumor mass was responsible for the observed elevation of NO· production.

iNOS Expression Colocalizes with the Macrophage-Specific moma-2 Antigen. To determine whether cells expressing iNOS were macrophages, spleen sections isolated from tumor-bearing mice were stained with the rat anti-mouse macrophage antibody moma-2 (30) as well as with anti-iNOS. Expression of iNOS was limited to a subpopulation of moma-2-positive cells (Fig. 4), consistent with the conclusion that macrophages were indeed the major source of NO· in these animals.

Serum Levels of γ-Interferon Are Elevated in Tumor-Bearing Mice. Because γ-interferon has been implicated in the induction of iNOS expression in macrophages *in vitro* and *in vivo*, levels of this cytokine in serum from RcsX bearing SJL mice were measured and found to be elevated by 25-fold compared with those in controls (Fig. 5). NMA treatment had no detectable effect in either tumor-bearing or control mice. This response provides further supportive evidence concern-

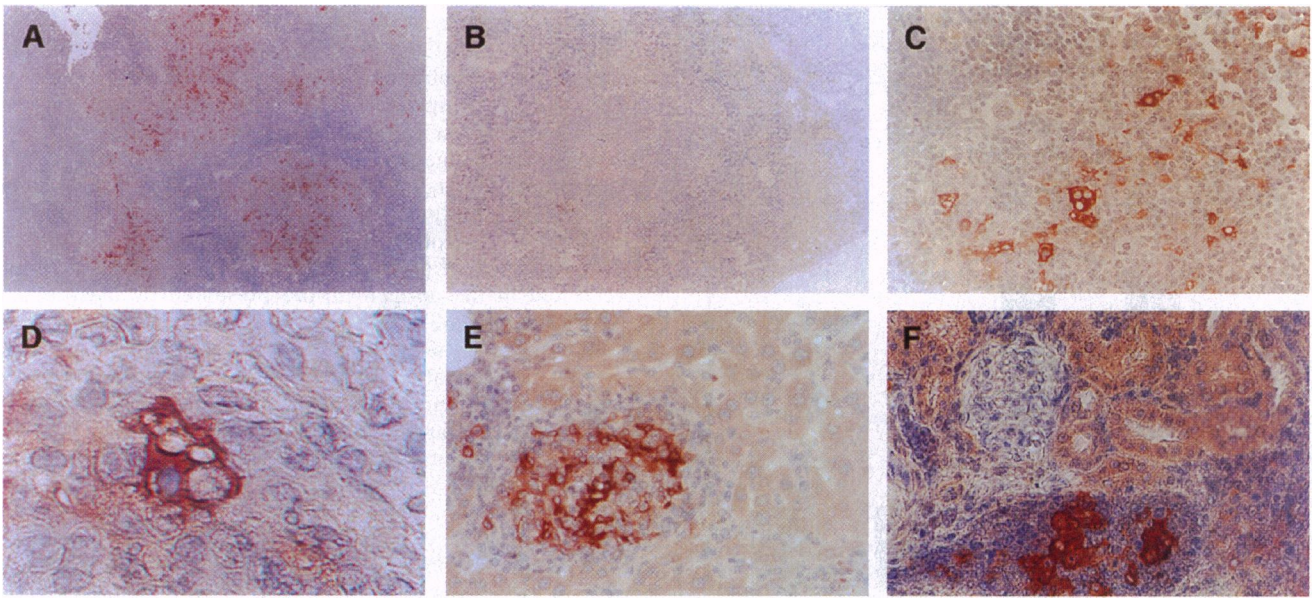

FIG. 3. Immunohistochemical analysis of iNOS expression in tissues of RcsX tumor-bearing SJL mice. Sections were prepared and stained. (*A*) Spleen of RcsX-bearing mouse. (×100.) (*B*) Spleen of control mouse. (×100.) (*C*) Spleen of RcsX-bearing mouse. (×400.) (*D*) Spleen of RcsX-bearing mouse. (×1000.) (*E*) Liver of RcsX-bearing mouse. (×400.) (*F*) Kidney of RcsX-bearing mouse. (×400.)

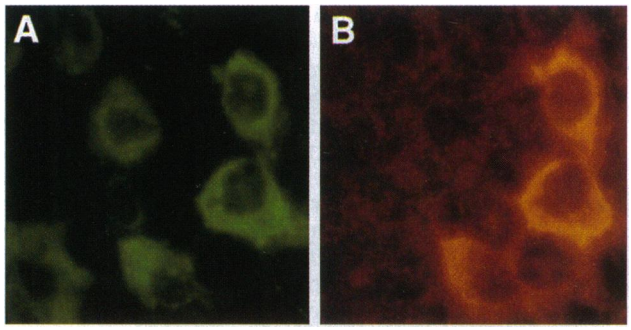

FIG. 4. iNOS and moma-2 expression in spleen of RcsX tumor-bearing SJL mice. The section was stained with moma-2 (fluorescein isothiocyanate, green) and iNOS (Cy3, red). (×1000.)

ing a possible mechanism of iNOS induction in macrophages of RcsX tumor-bearing mice.

Adherent Cells Isolated from RcsX Tumor Produce Nitric Oxide in Primary Culture. Nitric oxide has been shown to inhibit growth of spleen cells *in vitro* (31, 32), and we utilized this property to provide additional evidence concerning the source of NO· in this experimental system. Primary cultures of splenocytes from RcsX-treated mice showed no detectable [^{3}H]thymidine incorporation over a 48-hr period (Fig. 6). However, addition of NMA to the culture medium resulted in a substantial increase in cell division (Fig. 6). Suspensions of spleen cells were cultured in microwell plates for 4 hr, during which time macrophages were expected to become adherent. Nonadherent cells (estimated to represent 80–90% of the total) were then transferred into a well devoid of adherent cells. Separation of cells in this way (i.e., culture of splenocytes in the absence of macrophages) led to increased thymidine incorporation by nonadherent cells, which was further enhanced by incorporation of NMA in the medium (Fig. 6). These results indicate that adherent cells (primarily macrophages) were responsible for inhibiting the growth of nonadherent cells, putatively through NO· production, thus providing additional evidence that macrophages were the source of NO· in this experimental model.

DISCUSSION

The objective of this work was to characterize an animal model in which NO· production can be physiologically induced over time periods and at levels sufficient for investigation of damage to cellular macromolecules. Excretion of nitrate, the final metabolite of NO· *in vivo*, was measured in urine of RcsX tumor-bearing animals, and results (Fig. 1) demonstrated a

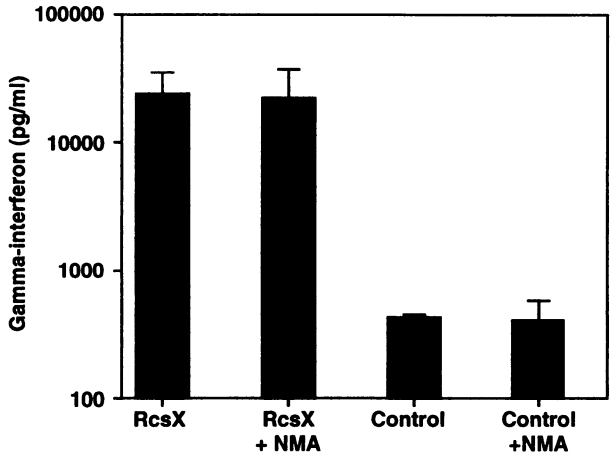

FIG. 5. Serum levels of γ-interferon in tumor-bearing SJL mice.

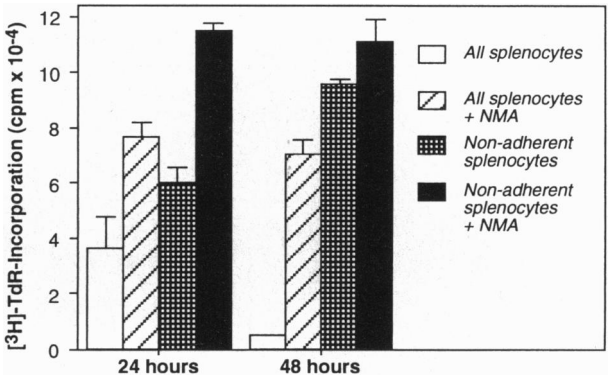

FIG. 6. Growth of splenocytes isolated from RcsX tumor-bearing SJL mice in primary culture. Cells, 40,000 per well, were cultured in quadruplicate in a 96-well plate. After 4 hr nonadherent cells were transferred to another well and 1 mM NMA was added. For [^{3}H]thymidine incorporation, [^{3}H]thymidine was added 24 hr before harvest to quantify growth.

50-fold elevation in endogenous production of the radical. In the context of sites of potential NO·-induced damage, cells of the spleen and lymph nodes would be exposed to higher doses than those of other tissues, inasmuch as production was localized in those tissues. Further, localized production in a small subpopulation of cells would suggest that gradients of NO· concentration likely existed within those tissues, in which exposure of individual target cells would depend on their position in relation to generating cells.

Although administration of NMA in drinking water effectively prevented the production of NO· through most of the experiment, relatively small amounts were produced during the final 2 days, possibly due to induction of iNOS. We believe the time-dependent increase in amount of NO· produced was related to increased mass of the spleen and lymph nodes not to differential expression of iNOS during the experiment, on the basis of invariate intensity of staining in Western blots and immunohistochemical analyses of tissues isolated at different stages of the experiment (data not shown). It should be noted that nitrate excretion in control mice was also decreased by NMA. While this may have been due to inhibition of background levels of endothelial or brain NOS, it was more likely associated with the spontaneous development of myositis as we previously reported (26).

Induction of iNOS expression in lymphoid tissue was limited to a small subpopulation of splenocytes and lymph node cells (Fig. 3). In the spleen, iNOS-expressing cells were concentrated in follicular regions, the zones implicated in the presentation of antigens (33, 34). This localization could potentially be related to the action of the superantigen, which mimics antigen presentation to T cells, or to differential regulation of macrophages in follicular and nonfollicular regions. Differential regulation of macrophages in different compartments is reflected in the antigen profile presented by macrophages. While the moma-2 antibody recognized iNOS-expressing cells (Fig. 4), two other anti-macrophage antibodies, mac-1 and F4/80, did not (data not shown). In the liver, iNOS expression was observed in macrophages forming metastatic islands but not in resident Kupffer cells.

We have observed a substantial increase in the serum concentration of γ-interferon in RcsX-bearing SJL mice (Fig. 5), but this was unaffected by inhibition of NO· production with NMA. Since this cytokine is known to induce iNOS expression, this result provides additional support to the interpretation that macrophages were the source of NO· in this model. In contrast, increases did not occur in serum tumor necrosis factor α and interleukin 1β, two additional cytokines reported to be involved in iNOS induction (35–37) (data not shown).

The observation that adherent cells inhibited growth of tumor cells in primary culture (Fig. 6) is also consistent with the interpretation that macrophages were the source of NO·. However, since the same cells did not appear to prevent tumor cell growth *in vivo*, it could be argued that cell inhibition by NO· *in vitro* may be a cell culture artifact.

Mechanisms underlying the induction of NO· production by macrophages in this model are largely uncharacterized. It has previously been reported that two endogenous signals are required under different conditions, one of which is usually γ-interferon (4), while the identity of the second varies, in some instances involving direct interaction of macrophages with a membranal protein on T cells (38). We conjecture that in the experimental model described herein, Vβ16-T cells induced by the superantigen-bearing tumor cells may have directly interacted with macrophages. This hypothesis would be consistent with both the localized concentration of iNOS in the follicular regions of the spleen and the lack of iNOS staining by the resident Kupffer cells of the liver.

Collectively, these results define an experimental model in which target tissues can be chronically exposed to a persistent flux of NO· generated by host macrophages intermingled with tumor and host T cells. This model should be useful in further elucidating toxicological effects of NO· under *in vivo* conditions.

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