L-Arginine-dependent Reactive Nitrogen Intermediates as Mediators of Tumor Cell Killing by Activated Macrophages

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ABSTRACT

The capacities of lymphokines and of various microbes to induce in a pure population of bone marrow-derived mononuclear phagocytes tumoricidal activity and/or the production of L-arginine-dependent reactive nitrogen intermediates, measured by the release of nitrite, were comparatively assessed. These parameters were found to be closely correlated in a variety of experimental situations, i.e., enhanced by a surplus of L-arginine and abrogated by N-monomethyl-L-arginine, a selective inhibitor of L-arginine-dependent effector mechanisms. In other macrophage/tumor cell combinations, such correlation was less obvious or not at all detectable, suggesting that, in these models, L-arginine-dependent reactive nitrogen intermediates are not or not alone responsible for the mediogenesis of tumoricidal activity by activated macrophages. Collectively, these observations suggest that the mechanism of tumor cell killing by activated macrophages may differ, depending on the tumor cell type and the pathway of macrophage activation. Among the various effector mechanisms considered to be involved in tumor cell killing by activated macrophages, L-arginine-dependent reactive nitrogen intermediates appear to hold a major role.

INTRODUCTION

Appropriately AMφ3 have the capacity to selectively and efficiently kill a wide array of neoplastic cells in a contact-dependent, nonphagocytic process (1-6). During their interaction, AMφ induce in tumor cells a characteristic pattern of metabolic alterations including inhibition of DNA synthesis (7, 8), of mitochondrial respiration (9), and of citric acid cycle enzyme aconitate (10), whereas other metabolic pathways, such as glycolysis, remain functional (9). However, the mechanisms which are critically involved in tumor cell killing have not as yet been clearly identified. Among the large number of agents secreted by macrophages (11), RNI have recently attracted particular attention (12-14). In the work reported here, the capacities of BMMφ, either resting or activated by a variety of agents, to release NO2− and to express tumoricidal activity were comparatively assessed under various experimental conditions.

MATERIALS AND METHODS

Amino acids, including l-arginine, d-arginine, and l-homoarginine, and sodium nitrite were obtained from Fluka AG, Buchs, SG, Switzerland; from Serva, Heidelberg, Federal Republic of Germany; or from Sigma, St. Louis, MO. Arginine from bovine liver (150 to 250 units per mg of protein), β-glucan, poly I:C, zymosan A, and IFN produced in rat kidney cell cultures by stimulation with poly I:C (IFN-α,β) were purchased from Sigma. NMMA was a generous gift from Dr. T. Ogawa (monoflavonivate) or was purchased from Bachem AG, Bubendorf, Switzerland (monoflavonivate) or from Calbiochem, Luzern, Switzerland [dil-hydroxyazobenzene-p′-sulfonate] salt. IMDM and its modifications (l-arginine free, glucose free) were freshly prepared. The medium was tested routinely for endotoxin contamination, and only batches containing endotoxin of less than 0.05 ng/ml (as assessed by the Limulus amebocyte lysate assay; E-toxate kit No. 120; Sigma) were used.

Bone Marrow-derived Mononuclear Phagocytes. Rat bone marrow cells were obtained and cultured as previously described (6, 15). Briefly, bone marrow was separated from femurs of adult male inbred DA rats by perfusion with ice-cold PBS, and the cells were suspended in IMDM supplemented with 10% FCS (Gibco, Grand Island, NY), 5% horse serum (Gibco), and antibiotics and conditioned with supernatant (final concentration, 10%) from strain L clone 929 cells (ATCC CCL 1). Ten ml of the cell suspension (4 × 108 bone marrow cells/ml) were cultured at 37°C in a humid atmosphere of 5% CO2 in bacteriological plastic Petri dishes (diameter, 100 mm; Greiner, Nütingen, Federal Republic of Germany). On Day 6, the cultures were repeatedly washed; the cells remaining adherent were removed with ice-cold Ca2+, Mg2+-free PBS, washed in PBS, and suspended in IMDM supplemented with 5% FCS and antibiotics.

Activation of BMMφ. To enhance their functional activity, Day 6 BMMφ were first incubated for various time intervals (standard, 24 h) with one of the macrophage-activating agents (activation step). MAF preparations were cell-free supernatants from 72-h rat spleen cells suspended in serum-free IMDM supplemented with Sepharose-bound concanavalin A (Pharmacia, Uppsala, Sweden) and 5 × 10−3 M 2-mercaptoethanol (16); for the activation of BMMφ, 10 or 20% MAF were used. Rat rIFN-γ (6), a generous gift from Dr. P. H. van der Meide, was used at 50 or 100 units. Corynebacterium parvum (ATCC 6916), Listeria monocytogenes, and Staphylococcus epidermidis organisms were selected from a single colony on solid brain-heart infusion (Difco Laboratories, Detroit, MI) and cultured for 3 to 4 days in liquid brain-heart infusion (Difco) before harvesting by centrifugation; after washing twice in PBS, the organisms were heat killed (240 min at 60°C), the humid preparations were stored at 4°C and used within 2 to 3 wk and 300 μg/well were used for in vitro activation. After incubation of BMMφ in medium (resting BMMφ) or in medium supplemented with one of the macrophage-activating agents for 24, 36, and/or 48 h, the release of nitrite (NO2−) was assayed in the culture supernatants, whereas tumoricidal activity was quantitated at the end of a 36-h interaction of BMMφ and prelabeled target cells in a 3H-thymidine release assay.

NO2− Release. The nitrite concentration was measured in supernatants from 105 BMMφ/ml cultured in IMDM supplemented with 2% FCS. One ml of culture supernatant was mixed with 100 μl of sulfosalicylic acid (70% in distilled water) and vortexed every 5 min for 30 min. After centrifugation (15 min, 7000 × g), the supernatant was mixed with 800 μl of NH4Cl buffer (5% aqueous NH4Cl buffer adjusted with sodium borate to pH 9.0) and 200 μl of 10% NaOH. The sample was mixed with 500 μl of modified Griess reagent (0.4% naphthylethylenediamine dihydrochloride in water/4% sulfanilamide in 5% H3PO4) and incubated for 10 min at 60°C and subsequently for at least 5 min at 4°C. The absorbance at 546 nm was determined in a Uvikon Model 800 spectrophotometer. Sodium nitrite (5, 10, 50, 100 μM in IMDM supplemented with 2% FCS) was used as a NO2− standard, and samples were processed as described for macrophage culture supernatants.

Macrophage-mediated Tumoricidal Activity. Tumoricidal activity was measured by the release of nitrite (NO2−) and nitric oxide (NO) and nitroscapic acid (NSA) (17). The capacities of BMMφ, either resting or activated by a variety of agents, to release NO2− and to express tumoricidal activity were comparatively assessed under various experimental conditions.

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2To whom requests for reprints should be addressed.

3The abbreviations used are: AMφ, activated macrophages; BMMφ, bone marrow-derived mononuclear phagocytes; FCS, fetal calf serum; IMDM, Iscove’s modified Dulbecco’s medium; IMDM-A, l-arginine-free IMDM, IFN, interferon; NMMA, N-monomethyl-L-arginine; PBS, phosphate-buffered saline; poly I:C, polyinosin-polycytidylic acid; RNI, reactive nitrogen intermediates; rIFN-γ, recombinant γ-interferon; ATCC, American Type Culture Collection; IL-1, interleukin 1; TNF-α, tumor necrosis factor α.

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iated by resting and activated BMMφ, derived from the same pool as used for the measurements of NO2− secretion, was determined in a [14C]thymidine release assay (6). DA rat dimethylbenz(a)anthracene (D-12; 17) and methylcholanthrene-induced (M-1, M-3) tumor cell lines derived from continuous in vivo passage in ascites form, BDX rat AS variant of BSP73 (18; a gift from Dr. S. Matzku, Heidelberg, Federal Republic of Germany), P-815 murine mastocytoma, YAC-1 murine lymphoma, WEHI-164 murine fibrosarcoma clone 13 cells (19; a gift from Dr. J. Tschopp, Lausanne, Switzerland), and the NCTC clone L-929 murine cell strain (ATCC CCL 1) were cultured in vitro in IMDM supplemented with 5% FCS and antibiotics. Tumor cell cultures were regularly checked to ensure the absence of Mycoplasma by direct fluorescent DNA staining, by chemiluminescence, and by assessing the growth on PPLO agar as described (20). Tumor cells, seeded at an initial density of 2.5 × 105/ml in 10 ml of IMDM supplemented with 10−3 m uridine and 5% FCS, were labeled with 0.02 μCi of [14C]thymidine/ml (methyl-14C; 40 to 50 mCi/mm; New England Nuclear, Boston, MA). After 20 to 24 h, tumor cells were washed twice and resuspended in IMDM supplemented with 10−4 m cold thymidine and 5% FCS. Labeled tumor cells (5 × 105) were interacted for 36 h at 37°C in a volume of 1 ml in 16-mm wells (tissue culture plates; Petrac Plastic, Chur, Switzerland) with increments of BMMφ to result in initial effector/target cell ratios of 1:1, 2.5:1, and 5:1. Thereafter, radioactivity in the supernatants was measured, and the following was calculated.

% of specific cytotoxicity

\[
\text{cpm in test wells - cpm in medium control wells} = \times \frac{100}{\text{maximal releasable cpm - cpm in medium control wells}}
\]

For all cell lines, spontaneous medium release within 36 h was in the range of 2 to 10%. Statistical significance was calculated by the Mann-Whitney U test.

RESULTS

The present study addresses the question of whether the abilities of macrophages to produce RNI, measured by the release of NO2−, and to mediate tumoricidal activity, measured by the release of [14C]thymidine, are related phenomena. These capacities were assessed in parallel in BMMφ derived from the same pool, either after incubation in medium (resting BMMφ, control), or after incubation with agents exhibiting different macrophage-activating potential. To discriminate between activation and effector steps, BMMφ were first incubated for 24 h with one of the activating agents (activation step); the medium was then replaced by fresh medium supplemented with the same activating agent and prelabeled target cells, and isotopic release was measured after a further 36 h (effector step). Under these standard conditions, the ability of the agents used to induce in BMMφ tumoricidal activity differed considerably; MAF, C. parvum, L. monocytogenes, and S. epidermidis consistently triggered marked tumoricidal activity whereas the potential of zymosan, and in particular of IFN-γ, poly I:C, and β-glucan, was more variable and often clearly weaker; IFN-α,β was inactive under these conditions. The results in Fig. 1, obtained with the particularly sensitive P-815 murine mastocytoma cells, illustrate these differences.

Comparative Ability of Macrophage-activating Agents to Trigger BMMφ Tumoricidal Activity versus NO2− Production. As NO2− production by BMMφ steadily decreased on continuing incubation without medium change, it was necessary to replace the medium (including the corresponding activator) every 24 h; under these conditions, the decrease in NO2− production during the time interval required for the assessment of tumoricidal activity (24-h activation, 36-h effector step) was small. In resting BMMφ that do not express cytoidal activity against the majority of tumor cell lines, the generation of NO2− was low, and these parameters were not noticeably affected by IFN-α,β (Fig. 1) or the presence of tumor cells. Among the agents utilized to trigger in BMMφ tumoricidal activity, C. parvum, S. epidermidis, zymosan, β-glucan, and rIFN-γ also considerably enhanced NO2− production; incubation with MAF, consistently eliciting marked tumoricidal activity, resulted in variable NO2− secretion (25 to 75 μM/106 BMMφ/24 h); and induction of macrophage cytolytic activity by L. monocytogenes was associated with no or an only minor increase in NO2− release. The values in Fig. 1 are derived from 24-h supernatants; in the 36-h and 48-h supernatants, NO2− concentration was somewhat lower, but the differences between the various macrophage-activating agents remained within the same range as after 24 h.

Is L-Arginine Critical for the Manifestation of Tumoricidal Activity by Macrophages? Recent work has suggested that the guanidino nitrogen atom(s) of L-arginine is involved in the formation of RNI by macrophages (13, 21). Therefore, various approaches were made in the present study to assess the role of L-arginine-dependent effector mechanisms in these processes. In cultures in which complete medium was supplemented with arginine (2 and/or 4 units/ml), or in IMDM-A, a spontaneous release of [14C]thymidine from targets was in most effector/target cell combinations too high to allow interpretation of the data. However, L-homoarginine, but not D-arginine, could effectively substitute for L-arginine (data not shown). When IMDM-A was supplemented with a surplus of L-arginine (9 × 10−4 m instead of 5 × 10−4 m in IMDM), tumoricidal activity was often clearly higher than under the ordinary experimental conditions (Fig. 2). The presence of NMMA (10−4 m), a specific substrate-analogue inhibitor of L-arginine-dependent effector mechanisms (13), during the activation step in no case affected subsequent manifestation of tumoricidal activity triggered in BMMφ by macrophage-activating agents (not shown). When present during the effector phase, NMMA affected tumoricidal activity by BMMφ differently, depending on the target cell type. Tumoricidal activity manifesting against P-815 (Table 1), M-1,
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Fig. 2. Expression of tumoricidal activity by BMMφ can be enhanced in the presence of a surplus of L-arginine. BMMφ were first cultured for 24 h in complete medium (IMDM, control) or in IMDM supplemented with macrophage-activating agent. The medium was then replaced by IMDM or IMDM-A supplemented with \(9 \times 10^{-4}\) M L-arginine, either alone (control) or in the presence of the corresponding macrophage-activating agent, and interacted for 36 h with prelabeled target cells. The initial effector:target cell ratio was 1:1 for P-815 and 2.5:1 for BSp73AS cells. The effects on target cell viability are expressed as the net percentage of \[^{14}C\]thymidine release. Columns, mean of eight experiments, each performed in triplicate; bars, SD. Concentrations of macrophage-activating agents were specified as in Fig. 1. P-815 cells in IMDM; D-12, P-815 cells in IMDM-A supplemented with \(9 \times 10^{-4}\) M L-arginine; BSp73AS cells in IMDM; YAC-1 BSp73AS cells in IMDM-A supplemented with \(9 \times 10^{-4}\) M L-arginine. According to the Mann-Whitney U test, the values for cultures supplemented with \(9 \times 10^{-4}\) M L-arginine were statistically significantly higher than the corresponding values in normal IMDM (*), LM, L. monocytogenes; ZYMO, zymosan.

Table 1 Dose-dependent inhibition by NMMA of tumoricidal activity triggered in BMMφ by MAF and/or C. parvum and manifested against P-815 mastocytoma cells

<table>
<thead>
<tr>
<th>Concentration of NMMA present during effector step (m)</th>
<th>Activating agent present*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (medium only)</td>
<td>MAF</td>
</tr>
<tr>
<td>10^{-5}</td>
<td>None</td>
</tr>
<tr>
<td>10^{-5}</td>
<td>MAF</td>
</tr>
<tr>
<td>5 \times 10^{-5}</td>
<td>C. parvum</td>
</tr>
<tr>
<td>4 \times 10^{-4}</td>
<td>None</td>
</tr>
<tr>
<td>4 \times 10^{-4}</td>
<td>MAF</td>
</tr>
<tr>
<td>2 \times 10^{-4}</td>
<td>C. parvum</td>
</tr>
</tbody>
</table>

* BMMφ were first cultured for 24 h in medium alone (control) or in medium supplemented with MAF (20%) or C. parvum (300 µg/well). The medium was then replaced for 36 h by medium plus prelabeled P-815 mastocytoma cells (controls) or by medium supplemented with MAF and/or C. parvum and prelabeled P-815 cells before the net percentage of \[^{14}C\]thymidine release was determined. In the experiments indicated, NMMA was additionally present during the effector phase.

Fig. 3. Comparative abilities of NMMA (NM) and dexamethasone (DX) to affect the manifestation of tumoricidal activity by and the release of NO\(_2^-\) from BMMφ activated by various agents. BMMφ were first incubated for 24 h with a macrophage-activating agent in the concentrations given in Fig. 1; the medium was then replaced by medium supplemented with the same activating agent alone (control, C) or, in addition, NMMA (10^{-6} M) and/or dexamethasone (10^{-4} M). BMMφ derived from the same pool were used for the assessment of tumoricidal activity (36-h \[^{14}C\]thymidine release assay) and/or NO\(_2^-\) release (after 24, 36, and 48 h); here the 24-h NO\(_2^-\) values were presented. Columns, mean of 8 to 12 experiments, each performed in triplicate; bars, SD. LM, L. monocytogenes; CP, C. parvum; SE, S. epidermidis; ZYMO, zymosan.

Table 2 Tumor cell lines and their abilities to be killed by macrophages

<table>
<thead>
<tr>
<th>Tumor cell line</th>
<th>Culture conditions during effector phase</th>
<th>Activating agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>L. monocytogenes (300 µg/well)</td>
<td>rIFN-γ (50 IU/well)</td>
</tr>
<tr>
<td>D-12 IMDM</td>
<td>10 ± 6*</td>
<td>33 ± 5*</td>
</tr>
<tr>
<td>D-12 IMDM + NMMA</td>
<td>12 ± 5</td>
<td>16 ± 4</td>
</tr>
<tr>
<td>D-12 IMDM + NMMA</td>
<td>12 ± 5</td>
<td>33 ± 6*</td>
</tr>
<tr>
<td>BSp73 AS IMDM</td>
<td>0 ± 2</td>
<td>31 ± 6*</td>
</tr>
<tr>
<td>BSp73 AS IMDM + NMMA</td>
<td>1 ± 3</td>
<td>36 ± 7*</td>
</tr>
<tr>
<td>WEHI-164/13 IMDM</td>
<td>30 ± 8</td>
<td>66 ± 19*</td>
</tr>
<tr>
<td>WEHI-164/13 IMDM</td>
<td>40 ± 9</td>
<td>76 ± 22*</td>
</tr>
<tr>
<td>YAC-1 IMDM</td>
<td>7 ± 3</td>
<td>32 ± 5*</td>
</tr>
<tr>
<td>YAC-1 IMDM + NMMA</td>
<td>7 ± 4</td>
<td>38 ± 8*</td>
</tr>
</tbody>
</table>

* Mean ± SD of 8 to 12 experiments, each performed in triplicate.

DISCUSSION

Although the extraordinary efficiency of AMφ to cope with a large array of neoplastic cells has been established almost 20 yr ago, the mechanisms of tumor cell killing still remain largely unknown. Among the large number of biologically relevant molecules secreted by mononuclear phagocytes, IL-1 (22, 23), TNF-α (24–27), proteases (28, 29), reactive oxygen (30), and reactive nitrogen intermediates (12–14) have been considered to be critically involved in tumor cell killing by activated macrophages. Various findings suggest, however, that, on their own,
some of these agents, in particular TNF-α (Ref. 31; Footnote 4), IL-1 (32), and reactive oxygen intermediates (2, 33, 34), are operative only in a limited number of selected model systems. To assess the possible role of RNI as mediators of tumor cell killing, the capacities of BMMφ to secrete RNI and to manifest tumoricidal activity were comparatively assessed in the present study.

The capacity of mammalian cells, such as vascular endothelium and mononuclear phagocytes, to generate RNI has been discovered only recently (13, 21, 35–38). It has in particular been shown that various stimuli, including Bacillus Calmette-Guérin infection, lymphokines, and bacterial lipopolysaccharides, induce in macrophages the generation of NO2⁻ and NO3⁻ (35, 36). Other work suggested that RNI, generated during the conversion of L-arginine to NO2⁻ and NO3⁻, are critically involved in macrophage-mediated tumor cell killing (12–14). Our knowledge on the synthesis and regulation of RNI and on their possible role as regulatory molecules is, however, still rather limited (39). The present study addressed two basic questions: (a) whether a parallelism can be established between the potential of microbes and lymphokines to induce in macrophages tumoricidal activity and/or the generation of RNI; and (b) whether inhibitors of the metabolism of L-arginine, such as the L-arginine analogue NMMA (13), do affect tumoricidal activity and NO2⁻ production in a similar manner. Comparison of tumoricidal activity and secretion of RNI in a population of resting BMMφ, which were homogeneous with respect to the cell lineage (6, 15, 40), has shown that these parameters were at a low level (Fig. 1) and were not affected by their interaction with tumor cells. On incubation of BMMφ with macrophage-activating agents, the increase in tumoricidal activity expressed against P-815 mastocytoma cells and in the production of NO2⁻ was in most models in a similar range (Fig. 1). Some discrepancy in the induction of these abilities was observed on incubation of BMMφ with L. monocytogenes where expression of high tumoricidal activity was associated with no or only a minor increase in NO2⁻ secretion (Fig. 1).

The findings showing that, in a variety of experimental models, the cytolytic activity of BMMφ was markedly enhanced by a surplus of L-arginine suggest that L-arginine and its metabolites may play an important role in the mediation of tumor cytotoxicity by AMφ (Fig. 2). Such a concept is further supported by the demonstration that NMMA, a specific substrate-analogue inhibitor of L-arginine, suppressed cytoidal activity by AMφ in a variety of situations in a dose-dependent manner (Tables 1 and 2; Fig. 3). When present during the effector step, NMMA abrogated tumoricidal activity manifested against P-815, M-1, and YAC-1 target cells and also markedly diminished spontaneous cytolytic activity of resting BMMφ (Fig. 2). These findings suggest that, in these models, L-arginine-dependent RNI are critically involved in the mediation of cytoidal activity by BMMφ. As tumoricidal activity triggered in BMMφ by L. monocytogenes was enhanced by a surplus of L-arginine (Fig. 2) and abrogated by NMMA (Fig. 3), it appears likely that RNI are also critically involved in this model, although their induction was not associated with increased NO2⁻ secretion (Fig. 1). The causes responsible for this discrepancy remain to be elucidated, but measurements of nitrite may not always truly reflect the formation of RNI. One possibility to be considered is that the usual stoichiometry of about 60% nitrite and 40% nitrate could be shifted to a preponderance of nitrate. Recent work, published after the completion of the present study, has arrived at the conclusion that highly reactive nitric oxide is the effector molecule involved in macrophage-mediated, L-arginine-dependent tumor cytostasis (41–44). The present study focused on tumor cell lysis and nitrite formation but did not examine cytostasis or the production of nitric oxide and/or nitrate. Other recent work has shown that some non-macrophage tumor cell lines produce RNI in response to soluble products from activated macrophages, resulting in cytostasis and the release of iron (45, 46). It remains to be elucidated whether similar mechanisms are operative in the present model systems.

Unlike the situation encountered with P-815, M-1, and YAC-1 tumor cells, the killing of other targets by activated BMMφ was either not at all or only under certain defined conditions affected by NMMA, suggesting that in these models, metabolites of L-arginine are not or not alone responsible for the mediation of macrophage cytolytic activity. Recent work has shown that targets that are particularly sensitive to TNF-α, such as L-929 and WEHI-164/13 cells, differ from other targets in that they are susceptible to killing by resting BMMφ. This spontaneous cytolytic activity of resting BMMφ was fully neutralized by sheep anti-mouse TNF-α antiserum, indicating that TNF-α is crucially involved in the killing of these targets by macrophages.* The mechanisms underlying the killing of D-12 cells by IFN-γ-activated BMMφ and BSp73AS tumor cells by L. monocytogenes-activated BMMφ (Table 2), that appears as well to be L-arginine independent, remain to be elucidated. Collectively, the present and earlier findings (Ref. 6; Footnote 4) suggest that macrophage tumoricidal activity can be induced via various pathways and be manifested via various mechanisms. Which and whether a single or a combination of several mechanisms is becoming operative and whether it will be efficacious appear to depend on variables such as the quality and/or extent of macrophage tumoricidal activity, its maintenance, and the properties (e.g., reactivity, susceptibility) of the target cell type. Among the effector mechanisms hitherto identified, L-arginine-dependent RNI appear to represent valuable candidates, but their exact nature and their precise role remain to be established.

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