Effect of Recombinant Human Tumor Necrosis Factor on the Induction of Murine Macrophage Tumoricidal Activity

Kazuyoshi Hori, M. Jane Ehrke, Kenneth Mace, Darbie Maccubbin, Mary Jean Doyle, Yoshiki Otsuka, and Enrico Mihich

ABSTRACT

The ability of recombinant human tumor necrosis factor (rH-TNF) alone or in combination with lymphokines (LK) to induce the in vitro activation of murine macrophages was evaluated. The treatment of C57BL/6 mouse resident peritoneal exudate cells (PEC) with rH-TNF and LK was found to induce the activation of macrophages to a tumoricidal state against P815 mastocytes. Neither rH-TNF nor LK alone induced macrophage cytotoxic activity. Furthermore, the macrophage activation seen was not due to small amounts of contaminating lipopolysaccharide. The TNF plus LK-mediated macrophage activation could be totally ablated by rabbit antiserum to murine γ-interferon, thus suggesting a role for γ-interferon in this system. Since adherent cells (≥95% macrophages) only marginally responded to stimulation with rH-TNF plus LK and the addition of nonadherent PEC caused a marked augmentation of rH-TNF plus LK-mediated macrophage activation, the involvement of nonadherent PEC was suggested. In addition, using antibodies and complement to deplete subsets of cells from the nonadherent PEC, the requirement for cells bearing Thy 1.2 and asialo GMI surface markers was demonstrated. These results suggest that TNF may play an autocrine regulatory role in concert with lymphokines in macrophage-mediated host defense against malignant neoplasia.

INTRODUCTION

TNF was originally found by Carswell et al. in serum obtained from Bacillus Calmette-Guérin-infected mice after injection of endotoxin (1). Across species barriers, TNF causes hemorrhagic necrosis of some transplanted tumors and in vitro exhibits a cytotoxic or cytostatic effect against certain animal and human transformed cell lines, as well as a growth stimulating effect on some normal cell lines (1-6). TNF has been shown to be produced by cells of the monocyte/macrophage lineage and has been suggested to be one of the potent effector molecules for macrophage-mediated tumor cell killing (2, 7-9). The availability of pure human and murine recombinant TNF has permitted the re-investigation of this molecule in addition to its antitumor effect. These activities include suppression of the synthesis and activity of lipoprotein lipase (10), inhibition of adipocyte gene expression (11), stimulation of fibroblast growth (5), induction of β2-interferon in fibroblasts (12), and modulation of the hemostatic properties of endothelial cells (13). TNF has also been reported to enhance the functional activity of neutrophils and eosinophils (14-17).

Taken together this evidence is consistent with the possibility that TNF may have a role as a regulatory mediator of the immunological response in neoplasia and inflammation as well as other disease states. In this report, data are presented demonstrating that in vitro TNF is a stimulator of macrophage activation in concert with other cytokines.

MATERIALS AND METHODS

Animals. Specific pathogen-free female C57BL/6 mice were obtained from the Leo Goodwin Institute for Cancer Research. The mice were used between 8 and 12 weeks of age. The animals were maintained in conventional housing and fed mouse chow and water ad libitum.

Culture Conditions. The P815 mastocytoma cell line originating in DBA/2 mice was maintained as a continuous culture at 37°C in a humidified 5% CO2 atmosphere, and was passaged twice weekly. The cell line was maintained in Medium A which consisted of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT), 0.1 mg/ml gentamicin (Grand Island Biological Co., Grand Island, NY) and 25 mM HEPES buffer. Medium A was also used for washing the macrophage monolayers and for the macrophage-mediated cytotoxicity assay. The activation of macrophages was performed with Medium B, namely Medium A supplemented with 50 μM 2-mercaptoethanol. For preparation of lymphokines, Medium C (Medium B supplemented with 1 mM l-glutamine, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate) was used. All tissue culture media and sera were endotoxin free, defined as containing less than 0.05 ng/ml of endotoxin as quantified by a Limulus amebocyte lysate assay kit (M. A. Bioproducts, Walkersville, MD).

Peritoneal Exudate Cells. Peritoneal exudate cells (PEC) were obtained from untreated C57BL/6 mice by lavage of the peritoneal cavity with 10 ml of cold Ca2+- and Mg2+-free Hank's balanced salt solution (Grand Island Biological Co., Grand Island, NY) with 25 mM HEPES buffer. The PEC were centrifuged at 350 x g for 4°C for 5 min and were resuspended in Medium B to a final density of 1.25 x 106 cells/ml. To obtain resident peritoneal macrophage monolayers, 0.1-ml aliquots of PEC suspensions were added to wells of 96-well flat-bottomed plates (No. 25860; Corning Glass Works, Corning, NY) and incubated for 2 h at 37°C in 5% CO2. After the incubation, the plates were washed three times with warm Medium B to remove nonadherent PEC. The resulting populations of cells were greater than 95% macrophages as determined by morphology and staining for nonspecific esterase (Sigma Diagnostic Kit; Sigma Chemical Company, St. Louis, MO) and the cell recoveries were usually between 50 and 55%. For preparation of nonadherent PEC, 3.4-ml aliquots of the PEC suspension (1.25 x 106 cells/ml) were incubated in wells of 6-well cluster dishes (No. 3406; Costar, Cambridge, MA) at 37°C in a CO2 incubator. After a 1.5-h incubation, the plates were washed three times with warm Medium B and incubated for an additional 24 h. The resulting populations of cells were greater than 95% macrophages as determined by morphology and staining for nonspecific esterase (Sigma Diagnostic Kit; Sigma Chemical Company, St. Louis, MO) and the cell recoveries were usually between 50 and 55%.

MATERIALS AND METHODS

Macrophage Activation and Tumor Cytotoxicity Assay. PEC (1.25 x 106 cells/well) were incubated in 0.15 ml of Medium B in 96-well flat-bottomed plates for 24 h at 37°C in 5% CO2 with or without the agents (TNF, LK, antibodies, etc.) to be tested. After the incubation, wells were washed three times with warm Medium B to remove nonadherent cells and test agents. In those experiments involving the recombination of separated PEC populations certain control wells received only nonadherent PEC. The purpose of this control was in order to evaluate the possible contribution of effector cells which may have become adherent during the 24-h activation period, therefore, these control wells also were washed before the addition of P815 target cells. The possibility that the nonadherent cells could develop lytic activity was
also evaluated, where appropriate, by adding P815 target cells to wells which had not been washed; the level of lytic activity observed was not significantly different from that of washed wells and therefore the data are not presented.

P815 target cells were labeling with Na251CrO4 (400 mCi/mgCr; Amersham Corporation, Arlington Heights, IL) for 30 min at 37°C and washed. The cells were resuspended in Medium A for 60 min at 37°C followed by gentle washing. Aliquots of 200 µl containing 109 51Cr-labeled cells were added to wells containing washed macrophage monolayers. After 18 h of incubation, the plates were centrifuged at 300 × g for 5 min, and the released label was determined in 0.1 ml of each culture supernatant. Radioactivity was assayed using an automatic γ counter (Model 119; Searle Analytic Inc., Nuclear Division, Des Plaines, IL). Supernatants from target cells incubated in media alone were used to determine the spontaneous release of label, and the total releasable radioactivity was determined after lysis of target cells with 1% (v/v) Triton X-100. Percentage of specific 51Cr release was calculated by the following formula:

\[
\text{Specific } 51\text{Cr release} (\%) = \frac{(\text{experimental release}) - (\text{spontaneous release})}{(\text{total release}) - (\text{spontaneous release})} \times 100 \tag{A}
\]

The rate of spontaneous release of radioactivity was 1–1.5% of total releasable radioactivity per hour.

Each test group was assayed in triplicate or quadruplicate and all experiments were repeated at least three times.

Interferon Assay. A microtiter assay for antiviral activity was used to assess murine IFN (18, 19). Briefly, the reduction of cytotoxic effect of vesicular stomatitis virus infected L-cells was evaluated following fixation and staining with methylene blue. The IFN titer is expressed as the reciprocal of the highest serial two-fold dilution of the sample showing 50% reduction of cytotoxic effect. A standard reference of mouse IFN-α/β (NIH standard G-002-904-511) was included in each assay. One unit of IFN in the assay corresponds to 1 U of the reference IFN.

Reagents. rH-TNF (lot number L285811), produced in Escherichia coli (20) and purified (99.9%), was generously provided by Asahi Chemical Industry Co., Tokyo, Japan. The preparation had a specific activity of 2.3 × 106 units/mg protein as determined by its cytotoxic activity against mouse L fibroblast cells (21) and contained <0.1 ng of endotoxin/mg protein as measured by colorimetric limulus amebocyte lysate assay kit (Pyrodict; Seikagaku Kogyo Co., Tokyo, Japan). Dilutions of stock rH-TNF were prepared in assay medium (Medium B). Mouse monoclonal anti-rH-TNF antibody (IIH2F3, IgG1) was kindly provided by Asahi Chemical Industry Co. It was purified from murine ascites to 64.9 mg/ml and had a neutralization titer of more than 100 units/µg in the LM cell cytotoxicity assay (21, 22).

A rabbit antiserum to murine γ-interferon which was raised against the C-terminal peptide (93–133 amino acids) of the protein, was generously provided by Dr. Howard Johnson, University of Florida, Gainesville, FL. One ml of this preparation was capable of neutralizing 7 × 106 units of γ-interferon.

LPS (E. coli, 0111:B4) was purchased from Difco, Detroit, MI; polymyxin B was purchased from Sigma; Con A and Sephadex G-10 were obtained from Pharmacia (Uppsala, Sweden).

Lymphokines. Lymphokine-enriched supernatant from untreated mouse spleen cells was prepared by the modified method of Marino et al. (23). Briefly, spleen cells were cultured in 25 ml of Medium C (7.5 × 106 viable cells/ml) in 75-cm2 plastic culture flasks (No. 25116; Corning Glass Works, Corning, NY) at 37°C with Con A (5 µg/ml). After a 24-h incubation, the supernatants were harvested by centrifugation (450 × g for 10 min at 4°C) and were treated with Sephadex G-10 (10 mg/ml) at 37°C for 60 min to remove residual Con A from the lymphokine. Control supernatants (LK-control) were prepared by the addition of Con A at the time of harvesting cells that had been incubated without mitogen. The IFN titers of LK and LK-control were 10 and <1 U/ml, respectively. These LK preparations were stored at 4°C.

Antibody and Complement Treatments of Nonadherent PEC. For this treatment, RPMI 1640 medium containing 5% heat-inactivated fetal calf serum, 0.1 mg/ml gentamicin, 50 µM 2-mercaptoethanol, and 25 mM HEPES buffer was used. Thy 1.2 bearing nonadherent PEC were depleted by incubating the cells with monoclonal anti-Thy 1.2 (1:100 final dilution; New England Nuclear Co., Boston, MA), on ice for 45 min followed by centrifugation and resuspension in rabbit complement (1:15 final dilution; Low-Tox-M; Cederlane Laboratories, Ontario, Canada) followed by incubation at 37°C for 45 min. The cells were then washed twice with medium B and resuspended in the same medium at 106 cells/ml for further use.

For the elimination of ASGMi-bearing cells, the procedure was as described above, except, rabbit heterosera against ASGMi (Wako Pure Chemical Industries, Osaka, Japan) was used at a final dilution of 1:20.

Statistical Methods. Data are expressed as mean ± SD. P values were calculated by Student's t test.

RESULTS

TNF and LK-mediated Macrophage Activation. The data shown in Table 1 demonstrate the stimulatory effect of TNF on the induction of activated tumoricidal macrophages from resident PEC in the presence of LK. During the first 24 h of culture (the activation period), the whole PEC population was exposed to factors as indicated; then all unbound factors and nonadherent cells were removed by extensive washing and 51Cr labeled P815 target cells were added to the adherent PEC cells to determine tumoricidal activity. The treatment of the resident PEC with either TNF over the concentration range of 0.5 to 5000 units/ml (500 units/ml shown, Group B) or LK over the range of 1 to 17%:v/v (10% shown, Group C) alone caused little or no induction of tumoricidal activity. In contrast, the combination of these two factors (Group E) caused marked activation of adherent PEC to be tumoricidal against P815 target cells. No significant activation of adherent PEC was observed with the combination of TNF plus LK-control (Group F). Since TNF (10,000 units/ml) and/or LK (20%:v/v) did not have any direct tumoricidal effect by themselves on P815 cells (less than 2% specific lysis, data not shown), and these target cells are insensitive to natural killer cells (24), the observed tumoricidal activity is regarded to be mediated by activated macrophages.

PEC cultured in medium before TNF and LK treatment rapidly lost their capacity to respond to these two factors in a time-dependent manner and incubation over 4 h in medium resulted in irreversible ABL of PEC responsiveness to TNF plus LK (Fig. 1). This loss of responsiveness by cultured PEC was not due to cell death and could be prevented by the addition of LK but not of TNF before 4 h (data not shown). Based on these findings, in subsequent experiments, treatments of TNF and LK were always completed within 3.5 h of PEC plating.

Table 1 Effect of TNF, LK, and LK control on the induction of tumoricidal macrophages from resident mouse PEC

<table>
<thead>
<tr>
<th>Group</th>
<th>Supplements</th>
<th>% Specific 51Cr release</th>
<th>P values&lt;br&gt;*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None</td>
<td>4.0 ± 1.5</td>
<td>NS</td>
</tr>
<tr>
<td>B</td>
<td>TNF</td>
<td>3.0 ± 1.0</td>
<td>NS</td>
</tr>
<tr>
<td>C</td>
<td>LK</td>
<td>6.0 ± 1.7</td>
<td>NS</td>
</tr>
<tr>
<td>D</td>
<td>LK-control</td>
<td>2.0 ± 2.2</td>
<td>NS</td>
</tr>
<tr>
<td>E</td>
<td>TNF + LK</td>
<td>51.4 ± 2.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>F</td>
<td>TNF + LK-control</td>
<td>2.0 ± 1.3</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Concentrations were as follows: TNF, 500 units/ml; LK, 10% (v/v), LK-control, 10% (v/v).

\* Mean ± SD of triplicate wells.

\* NS, not significant.
Dependence of Activation upon Concentration of TNF and LK. The induction of tumoricidal macrophages from resident PEC was completely dependent on both TNF and LK concentrations (Fig. 2). Under the experimental conditions employed when LK was kept constant, significant macrophage activation by TNF was observed at 5 units/ml and full activation was obtained at concentrations above 500 units/ml (Fig. 2A). Similarly, when TNF was kept constant, increasing macrophage activation was observed with increasing LK concentrations over the range of 5 to 17% (v/v) (Fig. 2B). TNF plus LK-mediated macrophage activation could be observed using not only normal resident PEC but also thioglycollate-elicited PEC, and there was no significant difference between TNF and LK dependencies of the activation could be observed using not only normal resident PEC but also thioglycollate-elicited PEC, and there was no significant difference between TNF and LK dependencies of the activation of these two PEC populations (data not shown).

Evaluation of Possible Role for LPS in the TNF-induced Effects. Bacterial LPS stimulates macrophages in combination with LK (23, 25). In fact under experimental conditions similar to those described in "Materials and Methods," LPS at concentrations above 1 ng/ml with LK (23, 25) had no effect on macrophage activation. These results suggested that LPS contamination did not contribute to macrophage activation by TNF plus LK.

Effect of Length of Activation Period on Induction of Tumoricidal Macrophages. PEC were treated with TNF and LK for several time periods (Fig. 3), in order to determine the period of exposure to these factors required for tumoricidal macrophage activation. TNF plus LK-mediated macrophage activation was time dependent, increasing over 24 h at TNF concentrations of 5–500 units/ml. The onset of detectable tumoricidal activity was, however, delayed, and significant increases in macrophage activation could be observed only after 12–18 h incubation of PEC with TNF plus LK.

Inhibition of TNF plus LK-mediated Macrophage Activation by Antimurine IFN-γ Antiserum. It is now established that IFN-γ is a highly potent macrophage activating factor for the induction of tumoricidal macrophages in the presence of LPS. Therefore, the involvement of IFN-γ in TNF plus LK-mediated

Table 2. Effect of heat, monoclonal anti-TNF antibody or polymyxin B on TNF plus LK- and on LPS plus LK-mediated activation of macrophages

PEC (1.25 x 10⁶ cells/well) were treated with the indicated supplements for 24 h. After the incubation, each well was washed and macrophage-mediated tumor cytotoxicity against ⁵¹Cr-labeled P815 cells was determined in an 18 h assay as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Group</th>
<th>Supplements*</th>
<th>% Specific ⁵¹Cr release</th>
<th>P values^</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNF</td>
<td>LK</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>None</td>
<td>1.8 ± 1.5</td>
<td>NS</td>
</tr>
<tr>
<td>B</td>
<td>TNF</td>
<td>2.3 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>C</td>
<td>LK</td>
<td>1.5 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>D</td>
<td>TNF + LK</td>
<td>52.8 ± 4.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>E</td>
<td>Heated TNF + LK</td>
<td>0.7 ± 1.2</td>
<td>NS &lt;0.001</td>
</tr>
<tr>
<td>F</td>
<td>TNF + LK + anti-TNF</td>
<td>0.7 ± 1.1</td>
<td>NS &lt;0.001</td>
</tr>
<tr>
<td>G</td>
<td>TNF + LK + polymyxin B</td>
<td>50.3 ± 2.3</td>
<td>&lt;0.001 NS</td>
</tr>
<tr>
<td>H</td>
<td>LPS</td>
<td>1.5 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>I</td>
<td>LPS + LK</td>
<td>56.8 ± 3.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>J</td>
<td>LPS + LK + anti-TNF</td>
<td>55.6 ± 1.9</td>
<td>&lt;0.001 NS</td>
</tr>
<tr>
<td>K</td>
<td>LPS + LK + polymyxin B</td>
<td>3.1 ± 1.4</td>
<td>NS &lt;0.001</td>
</tr>
<tr>
<td>L</td>
<td>LK + anti-TNF</td>
<td>1.0 ± 1.1</td>
<td>NS</td>
</tr>
<tr>
<td>M</td>
<td>LK + polymyxin B</td>
<td>0.1 ± 0.6</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Concentrations were as follows: TNF, 500 units/ml; LK, 10% (v/v); anti-TNF, 8.3 µg/ml; polymyxin B, 10 µg/ml; LPS, 1 ng/ml.
^ Mean ± SD of quadruplicate wells.
# A, compared with group A; B, compared with group D; C, compared with group I.
^ NS, not significant.
* TNF heated at 100°C for 5 min.
macrophage activation was examined by using a rabbit antisera against murine IFN-γ. As shown in Fig. 4 the presence of the anti-IFN-γ antisera inhibits macrophage activation induced by TNF plus LK as well as that induced by LPS plus LK.

Requirement of Nonadherent PEC for TNF plus LK-mediated Macrophage Activation. Studies were performed to determine whether TNF plus LK acted directly on macrophages to induce tumoricidal activity as reported for LK plus LPS- or IFN-γ plus LPS-stimulation (23, 25, 26). As shown in Table 3, separation of PEC into adherent and nonadherent populations markedly abrogated the effect of TNF plus LK. The treatment of adherent PEC with TNF (500 units/ml) plus LK (10%).v/v) caused only a marginal activation (less than 10% specific lysis), and the incubation of nonadherent PEC with those factors could not produce any adherent tumoricidal effector cells. In contrast, when adherent PEC were recombined with purified nonadherent PEC, macrophage activation comparable to that observed with unseparated PEC populations could be obtained. Under the same conditions, both whole PEC and adherent PEC populations stimulated with LK (10%.v/v) plus LPS (1 ng/ml) exhibited similar levels of tumor cytotoxic activity (42.6 ± 1.8% and 42.4 ± 1.6% of specific lysis, respectively).

Evaluation of the Nonadherent PEC Populations Contributing to TNF plus LK-mediated Macrophage Activation. Further evidence for the contribution of nonadherent PEC to the TNF plus LK-mediated activation of macrophage is illustrated in Fig. 5. The activation of resident macrophages in the presence

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**Table 3** Involvement of nonadherent PEC in the TNF plus LK-mediated macrophage activation

<table>
<thead>
<tr>
<th>Group</th>
<th>Cells</th>
<th>% Specific $^{51}$Cr release</th>
<th>$P$ values</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>PEC</td>
<td>40.7 ± 6.2</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Adher PEC</td>
<td>1.9 ± 3.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C</td>
<td>Nonadher PEC</td>
<td>3.9 ± 1.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>D</td>
<td>Adher PEC + nonadher PEC</td>
<td>35.0 ± 0.4</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Adherent PEC and nonadherent PEC were prepared as described in "Materials and Methods."

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**Table 4** Effects of anti-Thy 1.2 or anti-Asialo GM1 and complement treatment on the helper activity of nonadherent PEC

<table>
<thead>
<tr>
<th>Group</th>
<th>Cells</th>
<th>% Specific $^{51}$Cr release</th>
<th>$P$ values</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>PEC</td>
<td>39.0 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Adher PEC</td>
<td>5.4 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Nonadher PEC</td>
<td>4.8 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Adher PEC + Nonadher PEC</td>
<td>29.8 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>Adher PEC + Nonadher PEC (C)</td>
<td>28.0 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>F</td>
<td>Adher PEC + Nonadher PEC (anti-Thy 1.2)</td>
<td>28.0 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>Adher PEC + Nonadher PEC (anti-Thy 1.2 + C)</td>
<td>17.2 ± 0.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>H</td>
<td>Adher PEC + Nonadher PEC (anti-Asialo GM1)</td>
<td>28.0 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>I</td>
<td>Adher PEC + Nonadher PEC (anti-Asialo GM1 + C)</td>
<td>16.9 ± 0.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>J</td>
<td>Adher PEC + Nonadher PEC (anti-Thy 1.2 + anti-Asialo GM1)</td>
<td>28.7 ± 1.1</td>
<td>NS</td>
</tr>
<tr>
<td>K</td>
<td>Adher PEC + Nonadher PEC (anti-Thy 1.2 + anti-Asialo GM1)</td>
<td>7.0 ± 0.6</td>
<td>&lt;0.001</td>
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</table>

*Adherent PEC and nonadherent PEC were prepared as described in "Materials and Methods."

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![Fig. 4](image) Inhibition of TNF plus LK- or LPS plus LK-mediated macrophage activation by anti-IFN-γ antisera. PEC (1.25 × 10⁶ cells/well) were incubated with 10% (v/v) LK, 500 U/ml TNF or 1 ng/ml LPS alone or with the indicated combination of these reagents, either in the presence or in the absence of 1/360 dilution of normal rabbit serum or of rabbit anti-IFN-γ antisera. After the 24 h incubation, each well was washed and macrophage-mediated tumor cytotoxicity was determined as described in "Materials and Methods." Bars, mean of quadruplicate values ± SD.

![Fig. 5](image) Effect of nonadherent PEC on the TNF plus LK-mediated macrophage activation. PEC, adherent PEC (macrophages) and nonadherent PEC were obtained as described in "Materials and Methods." The indicated numbers of nonadherent PEC were added to each well containing adherent cells (macrophages) derived from 1.25 × 10⁶ whole PEC/well, and treated with 0 U/ml (B) or 500 U/ml (C) of TNF in the presence of 10% (v/v) LK for 24 h. Nonadherent PEC alone were treated with 0 U/ml (A) or 500 U/ml (D) of TNF and 10% (v/v) LK in the same manner. After the treatment, each well was washed, and tumor cytotoxicity was determined as described in "Materials and Methods." Solid horizontal line, level of cytolytic activity obtained with whole (unseparated) PEC activated with TNF (500 U/ml) plus LK (10% v/v); dashed lines, ± SD.
although these treatments caused complete inhibition of allospecific killer T-lymphocytes and natural killer cell activities, respectively (data not shown). Treatment of nonadherent PEC with both anti-Thy 1.2 and anti-ASGM1 plus complement completely abrogated their ability to induce macrophage activation (Group K).

These findings suggested that nonadherent PEC populations contributing to TNF plus LK-mediated macrophage activation express Thy 1.2 and/or ASGM1 cell surface markers.

DISCUSSION

The participation of activated macrophages in host defense against microorganisms and malignant neoplasia is well established (27, 28). It is also known that a variety of stimuli including microbial components and lymphokines have been reported to activate macrophages or monocytes to a tumoricidal state (29-31). Recently, IFN-γ, granulocyte-macrophage colony-stimulating factor and interleukin-1 have been identified as cytokines that can prime or activate macrophages/monocytes (26, 32-34).

The data presented in this report demonstrate that human TNF, which is a monokine released from macrophages/monocytes, can also serve as a signal in vitro to induce tumoricidal macrophages from murine resident PEC in combination with LK. TNF alone, in the concentration range tested (0.5–5000 units/ml), could not stimulate PEC, however, the combined treatment of TNF (~5 units/ml) plus IFN-γ containing LK exerted a marked stimulation of tumoricidal macrophage activity. While the mechanism by which the macrophage activation is induced has not been clarified, the recent reports (35, 36) that TNF induces differentiation of human myeloid cell lines to monocytes and, in synergy with IFN-γ, can induce terminaly differentiated myelomonocytic cells are consistent with its proposed role as a regulatory monokine.

Recently it has been shown in human and murine systems that TNF synergizes with IFN-γ (a component of LK) to be directly cytotoxic for certain tumor cell lines in vitro (6, 37). Therefore, it could be argued that residual TNF plus IFN-γ on macrophages may be responsible for the cytotoxicity of TNF plus LK-treated PEC. This possibility is unlikely, however, because the cytotoxicity of TNF plus LK-treated PEC could not be diminished by extensive washing. In addition, P815 mastocytoma used as target cells are resistant to direct cytotoxicity of TNF and/or LK. Therefore, the tumoricidal effect of TNF plus LK-treated PEC is regarded to be macrophage mediated.

Although LPS is also a potent stimulator of PEC in the presence of LK (23, 25), the stimulation of PEC by TNF plus LK is not due to LPS contamination since: (a) the concentration of LPS in the TNF preparation is very low; (b) polymyxin B abolished the LPS-induced effect, but not that of TNF; and (c) the addition of anti-TNF monoclonal antibody could inhibit TNF- but not LPS-induced tumoricidal activity.

While TNF plus LK induced high levels of tumoricidal activity when the whole PEC population was present during activation, they appeared to provide only a weak activation stimulus for adherence purified macrophages. Thus, the treatment of adherent PEC (more than 95% of which were macrophages) with a high concentration of TNF (5000 U/ml) plus LK (10%) caused significant but marginal tumor cytotoxicity (less than 20% specific lysis). The TNF plus LK-mediated macrophage activation could be augmented markedly by the presence of nonadherent PEC during the activation period of the assay. Nonadherent PEC bearing Thy 1.2 and/or ASGM1 surface markers appeared to be involved in the augmentation of the TNF plus LK-mediated macrophage activation. These results, however, are in conflict with data published very recently describing a direct stimulation of human macrophages by TNF alone (38). There are a number of differences between the two studies including species (human versus mouse), adherent cell preparations (removed from plastic and further cultured under non-adhering conditions versus those left undisturbed), cytotoxic assays (8 h assay against an actinomycin D-treated WEHI-164 target versus 18 h assay against untreated P815 targets) and target sensitivity to TNF (sensitive versus insensitive). Another recent report provides a possible alternative explanation: Kornbluth and Edgington found that contamination with as little as 3 pg/ml of LPS is capable of inducing human monocytes to become cytotoxic for actinomycin D-treated WEHI 164 cells through a TNF-mediated mechanism (39). Further experimentation will be required to determining if the difference in responsiveness to TNF alone is species related or a consequence of the variations in techniques used.

The mechanism of TNF plus LK-mediated macrophage activation is not clear at present, but the inhibitory effects of anti-IFN-γ antiserum suggest the involvement of IFN-γ in the macrophage activation. Several studies have demonstrated that IFN-γ alone cannot activate resident macrophages to be tumoricidal, and that it acts to prime the macrophages, making them more sensitive to triggering signals such as LPS (26, 32). Furthermore, a T-cell-derived lymphokine distinct from IFN-γ (macrophage cytotoxicity-inducing factor-2) has been reported to synergize with IFN-γ in stimulating murine resident macrophages (40-42). Thus, it is tempting to speculate that macrophages primed with TNF and/or LK require the additional signals of cytokines derived from nonadherent PEC before becoming fully activated. Preliminary experiments have indicated that culture supernatant from LK-stimulated nonadherent PEC can directly activate resident macrophages to be tumoricidal in the presence of TNF plus LK. Since, as shown by the data in this report, the resident adherent cells were not activated by TNF plus LK alone even though the LK has demonstrable IFN-γ activity, it is possible that the nonadherent PEC are producing such additional signals. These results are consistent with the hypothesis described above and tend not to support the alternative possibility that direct cell-to-cell contact between macrophage and nonadherent PEC are required.

Collectively, these in vitro results suggest that TNF may play an autocrine regulatory role in concert with lymphokines in macrophage-mediated host defense against tumor cells.

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