In allogeneic tumor or skin transplantation, the rejection process that destroys the allogeneic cells leaves syngeneic cells intact by discrimination between self and nonself. Here, we examined whether the cells infiltrating into the allografts could be cytotoxic against syngeneic immortal cells in vitro and in vivo. The leukocytes (i.e., macrophages (Mφ); 55–65% of bulk infiltrates), granulocytes (20–25%), and lymphocytes (15–20%) infiltrating into allografts, but not into autografts, in C57BL/6 mice were cytotoxic against syngeneic tumor cells and cell lines, whereas the cytotoxic activity was hardly induced in allografted, IFN-γ−/− C57BL/6 mice. Among the leukocytes, Mφ were the major population of cytotoxic cells; and the cytotoxic activity appeared to be cell-to-cell contact dependent. When syngeneic tumor cells were s.c. injected into normal C57BL/6 mice simultaneously with the Mφ-rich population or allogeneic, but not syngeneic, fibroblastic cells, tumor growth was suppressed in a cell number-dependent manner, and tumor cells were rejected either with a Mφ:tumor ratio of about 30 or with an allograft:tumor ratio of ~200. In the case of IFN-γ−/− C57BL/6 mice, however, the s.c. injection of the allograft simultaneously with tumor cells had no effect on the tumor growth. These results suggest that allograft or allograft-induced Mφ may be applicable for use in cancer treatment and that IFN-γ induction by the allograft may be crucial for the treatment. The Journal of Immunology, 1999, 163: 148–154.

Materials and Methods

Reagents

Materials and Methods

Reagents

Na2SO3 (10.5 GBq/mg) was purchased from New England Nuclear (Boston, MA). RPMI 1640 medium was obtained from Nissui Seiyaku (Tokyo, Japan). FCS was obtained from ICN Biomedicals (Costa Mesa, CA) and was used after heat inactivation. Casein sodium was purchased from Wako Pure Chemicals (Osaka, Japan). d-[α-methyl]Mannoside and Con A were obtained from Sigma (St. Louis, MO). LPS (Escherichia coli O55:B5) prepared by the Westphal method and Brewer thiglycolate medium were products of Difco (Detroit, MI). IFN-γ, penicillin, streptomycin, and L-glutamine were obtained from Life Technologies (Gaithersburg, MD). Anti-TNF-α Ab was a product of Genzyme (Cambridge, MA). Anti-Thy-1.2 (53-2.1) Ab, anti-NK-1.1 (PK136 or 3A4) Ab, biotin-labeled anti-IL-2R (7D4) Ab, and PE-conjugated streptavidin were purchased from PharMingen (San Diego, CA). N3-(monomethyl-t-arginine (N-MMA) was obtained from Calbiochem (La Jolla, CA). All other chemicals were of reagent grade.

Animals

Male specific pathogen-free C57BL/6 (B6) (H-2b) mice, 7 wk old, were purchased from Japan SLC (Hamamatsu, Japan). IFN-γ−/− B6 mice were donated by Dr. Y. Iwakura (Institute of Medical Science, University of Tokyo, Tokyo, Japan) (7). In Con A blasts from IFN-γ−/− B6 mice, IFN-γ mRNA was not detected by RT-PCR analyses, whereas IL-2, IL-4, and TNF-α mRNAs were present at levels of expression similar to those in normal (IFN-γ+/+) B6 mice (8). After an s.c. inoculation of tumor cells into normal or IFN-γ−/− B6 mice, the mice were maintained for several weeks.
weeks in our animal facility under specific pathogen-free conditions in an air-conditioned room (25 ± 2°C; −50% humidity).

Tumor cells and cell lines

3-Methylcholanthrene-induced ascites-type fibrosarcoma (Meth A; H-2d) cells were provided by Dr. S. Muramatsu (Department of Zoology, Kyoto University Faculty of Science, Kyoto, Japan), and were maintained by routine i.p. injection of 3 × 10⁶ cells into a syngeneic mouse strain (BALB/c; H-2b), EL-4 (lymphoma; H-2b), 3LL (lung carcinoma; H-2b), B16 (melanoma; H-2b), and CMT-93 (rectum carcinoma; H-2b) tumor cells, and NOR10 (H-2b), NCTC4093 (H-2b), and BALB/3T3 (H-2b) fibroblastic cell lines were purchased from American Type Culture Collection (Manassas, VA).

Cell preparations

Meth A cell-induced peritoneal exudate cells (PEC) were obtained by peritoneal lavage on day 7 (or at various intervals) after an i.p. transplantation of Meth A tumor cells (3 × 10⁶ cells/mouse) to normal or IFN-γ−/− B6 mice as previously described (8–18). The Mφ-rich (−95% purity and −5% T lymphoblast contamination) population was isolated from PEC by FACS (FACStar, Becton Dickinson, Mountain View, CA) with the gate set in the forward scattering/side scattering mode, as described previously (9–18). Skin grafting and preparation of the cells infiltrating into the skin graftraft bed border were performed as described previously (19). The grafts and the surrounding tissues of recipients were removed en bloc and cut into small blocks with scissors. The blocks were digested with 0.15% protease/0.075% collagenase/0.001% DNase. All digested cells were centrifuged, and a granulocyte-, lymphocyte-, or Mφ-rich population was sorted with the gate set in the forward scattering/side scattering mode by FACS. The morphological characteristics of the cells in each fraction were assessed by May-Giemsa stain. The cell number in all the digested cells was determined with a hemocytometer. Positive control was examined by FACS.

In vivo measurement of tumor growth

EL-4 (H-2b) or 3LL (H-2b) tumor cells (10⁴ cells/mouse) were s.c. injected simultaneously with an Mφ (H-2b)-rich population, BALB/3T3 (H-2b) cells, or NOR10 (H-2b) fibroblastic cells into normal or IFN-γ−/− B6 (H-2b) mice. At various intervals after the treatment, the growth of these syngeneic tumor cells was determined by measuring two diameters (perpendicular to each other) of the tumor with Vernier calipers (22). The surface area of the tumor was previously shown to correlate precisely with the tumor weight (23).

Results

Cytotoxic activities of Mφ against various kinds of syngeneic tumor cells and cell lines

Bulk cells (Mφ, 55–65%; granulocytes, 20–25%; lymphocytes, 15–20%) (11) infiltrating into allografts of Meth A tumor cells in B6 mice were cytotoxic against various kinds of syngeneic tumor (e.g., 3LL, EL-4, B16, and CMT-93) cells and cell lines (e.g., NOR10 and NCTC4093) with a 45–65% cytotoxic activity in an 18-h incubation (Fig. 1). Most of the activity was retained after T cell elimination (Fig. 1A); and the cytotoxic activity was not suppressed (3LL (~103% of the cytotoxic activity with control serum), B16 (~133%), NCTC4093 (~103%), and NOR10 (~123%)) by the addition of anti-CD3 Ab to the culture medium. Furthermore, bulk allospecific CTLs that had been induced in mixed lymphocyte cultures (B6 anti-BALB/c) were virtually inactive toward syngeneic tumor cells (e.g., 0.5 ± 3.1 and 3.4 ± 2.1% (mean ± SD; n = 4) specific lysis for EL-4 and 3LL cells, respectively and cell lines (e.g., 5.2 ± 3.0% (mean ± SD; n = 4) and 3.2 ± 2.0% (mean ± SD; n = 4) specific lysis for NCTC4093 and NOR10 cells, respectively), indicating that T cells are not involved in the cytotoxic activity of bulk PEC against syngeneic tumor cells and cell lines. The granulocyte-rich population had no cytotoxic activities against 3LL cells (0.2 ± 1.3% (mean ± SD; n = 4) specific lysis) and NCTC4093 cells (0.5 ± 2.1% (mean ± SD; n = 4) specific lysis). In addition, the cytotoxic cells were phenotypically IL-2R− (Fig. 2/NK-1.1− (10)/Mac-1− (19) by FCM, and neither the cytotoxic activity nor the cell number changed significantly after magnetic removal of cells attached to anti-NK-1.1 Ab-coated beads from PEC or after the in vitro specific elimination of NK or NKT cells from PEC, PEC were incubated with anti-NK-1.1 Ab and complement.

Negative immunomagnetic selection of PEC

Sterile magnetic polymer beads (Dynabeads M-450, Dynal, Oslo, Norway) were supplied coated with covalently bound afffinity-purified sheep anti-mouse IgG. Anti-NK-1.1 Ab-coated immunomagnetic beads were prepared as described previously (20). Washed mAb-coated beads (10⁶ beads/2 ml) were added to bulk PEC (5 × 10⁶ cells). The mixture of cells and beads was incubated 5 min at 4°C, and beads with any attached cells were removed magnetically. The supernatant was centrifuged, the pellet was resuspended in the culture medium, and then the cell number and the cytotoxic activity were determined.

FACS analysis

The expression of the IL-2R Ag on Mφ with Con A blasts used as a positive control was examined by FACS.

Cell number and viability

The cell number in the suspensions was determined with a hemocytometer after dilution of the cells in Turk’s solution. The viability of cells was determined by the trypan blue exclusion method.

Cytotoxicity assay

Target cells (10⁶ cells) in 25 μl of culture medium were labeled with 25 μl (925 kBq) of Na₂⁵¹CrO₄ at 37°C for 2 h, and effector cells (3 × 10⁵ to 5 × 10⁶ cells) in 200 μl of culture medium were mixed with 20 μl of ⁵¹Cr-labeled targets (10⁵ cells). After an 18-h incubation, an aliquot of supernatant was removed from each well and assayed for released ⁵¹Cr in a Hewlett-Packard (Meriden, CT) gamma counter. Lysis of ⁵¹Cr-labeled targets in each well was quantified as the percent specific lysis, as described previously (21).
and autografts (2.0 ± 1.1% (mean ± SD; n = 8) specific lysis) as previously described (19).

Distribution of cytotoxic activities against syngeneic tumor cells and cell lines in i.p. Meth A cell-treated or BALB/c skin-grafted B6 mice

When the cytotoxic activities against 3LL cells of lymphoid cells in a variety of locations in i.p. Meth A cell-treated and untreated mice were quantified, the cytotoxic activities were associated exclusively with PEC of Meth A cell-treated mice and not with peritoneal cells of control mice (Fig. 3, left). Not only the cells in all lymphoid organs tested to date but also peripheral mononuclear leukocytes of Meth A cell-treated mice had little, if any, cytotoxic activity toward the targets. Furthermore, casein- or thioglycolate-elicited inflammatory Mφ were totally inactive toward all these target cells (e.g., 0.2 ± 1.5 or 0.0 ± 1.2% (mean ± SD; n = 8) specific lysis for 3LL cells). Similarly, the cytotoxic activities against 3LL cells of cells in a variety of locations in BALB/c

<table>
<thead>
<tr>
<th>Target</th>
<th>Condition</th>
<th>Relative and Specific Lysis</th>
<th>Relative Cell Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCTC4093</td>
<td>Complement (C) alone</td>
<td>100 (50.9 ± 4.1)%</td>
<td>100 (10^6 cells)</td>
</tr>
<tr>
<td></td>
<td>Anti-NK-1.1 (PK136) + C</td>
<td>92.9 ± 17.5</td>
<td>94.2 ± 9.6</td>
</tr>
<tr>
<td></td>
<td>Anti-NK-1.1 (3A4) + C</td>
<td>87.2 ± 15.9</td>
<td>89.6 ± 13.5</td>
</tr>
<tr>
<td>3LL</td>
<td>Complement (C) alone</td>
<td>100 (29.2 ± 3.5)</td>
<td>100 (3 × 10^6 cells)</td>
</tr>
<tr>
<td></td>
<td>Anti-NK-1.1 (PK136) + C</td>
<td>86.6 ± 16.9</td>
<td>101.5 ± 25.3</td>
</tr>
<tr>
<td></td>
<td>Anti-NK-1.1 (3A4) + C</td>
<td>93.2 ± 19.2</td>
<td></td>
</tr>
<tr>
<td>NCTC4093</td>
<td>Bulk PEC</td>
<td>100 (44.2 ± 2.7)</td>
<td>100 (3 × 10^6 cells)</td>
</tr>
<tr>
<td></td>
<td>Bulk PEC – NK-1.1+ cells</td>
<td>91.9 ± 18.1</td>
<td>101.5 ± 25.3</td>
</tr>
<tr>
<td>3LL</td>
<td>Bulk PEC</td>
<td>100 (26.9 ± 2.3)</td>
<td>100 (3 × 10^6 cells)</td>
</tr>
<tr>
<td></td>
<td>Bulk PEC – NK-1.1+ cells</td>
<td>90.6 ± 16.7</td>
<td></td>
</tr>
</tbody>
</table>

* Relative to the percent specific lysis of target cells exposed to control effector cells (E:T = 50).

* Relative to the cell number of control effector cells.

* Data in parentheses represent the mean ± SD of the observed percent specific lysis (n = 8) in an 18-h assay.
Mechanisms of MΦ-mediated cytotoxicity against syngeneic tumor cells and cell lines

When MΦ isolated from tumor- or skin-allografted B6 mice were cocultured with 3LL or NCTC4093 cells, the MΦ exhibited high cytotoxic activities against these targets, but they were totally inert against 3LL and NCTC4093 cells in a Transwell consisting of two upper and lower chambers with a cell-impermeable membrane (Table II). Although N-MMA, a NO synthase inhibitor, completely inhibited NO release from the MΦ (58.3 ± 6.4 μM/18 h without inhibitor to 2.7 ± 1.3 μM/18 h with inhibitor), the inhibitor had no effect on the MΦ-mediated cytotoxic activity against 3LL and NCTC4093 cells. By contrast, LPS/IFN-γ-activated casein MΦ were highly cytotoxic (40.3 ± 4.2% specific lysis in an 18-h assay) against NO-sensitive P815 cells even in a Transwell, and the cytotoxic activity was completely suppressed by the addition of N-MMA (0.0 ± 2.3%). Furthermore, in contrast to LPS/IFN-γ-activated casein-MΦ, the allograft-induced MΦ (AIM) did not release TNF-α (16), and the addition of anti-TNF-α Ab did not affect the AIM-mediated cytotoxic activities against 3LL and NCTC4093 cells. LPS/IFN-γ-activated casein-MΦ, however, were cytotoxic (38.5 ± 2.8% specific lysis in an 18-h assay) against TNF-α-sensitive L929 cells even in a Transwell, and anti-TNF-α Ab largely (∼61%) inhibited the activity. These results indicated that in contrast to other activated MΦ, AIM exhibited cytotoxic activity against syngeneic tumor cells and cell lines in a cell-to-cell contact-dependent, soluble factor-independent manner.

The cytotoxic activities of PEC against various kinds of syngeneic tumor cells and cell lines were time dependently detected in the transplantation site of allogeneic Meth A tumor cells with a peak around day 8 after transplantation in normal (IFN-γ−/−) B6 mice (Fig. 4A). Thereafter, the cytotoxic activities decreased, and almost no cytotoxicity remained against syngeneic tumor cells and cell lines on day 17. By contrast, very low cytotoxic activities of PEC against these syngeneic tumor cells and cell lines were induced in Meth A cell-treated IFN-γ−/− B6 mice (Fig. 4B), suggesting IFN-γ-dependent cytotoxicity.

### Table II. Cell-to-cell contact-dependent cytotoxicity of PEC against 3LL or NCTC4093 cells

<table>
<thead>
<tr>
<th>Target</th>
<th>Culture Condition</th>
<th>Inhibitor</th>
<th>% Specific Lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCTC4093</td>
<td>Coculture</td>
<td>N-MMA</td>
<td>34.6 ± 3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-TNF-α</td>
<td>31.2 ± 5.1</td>
</tr>
<tr>
<td></td>
<td>Transwell</td>
<td></td>
<td>0.0 ± 0.9</td>
</tr>
<tr>
<td>3LL</td>
<td>Coculture</td>
<td>N-MMA</td>
<td>26.6 ± 1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-TNF-α</td>
<td>28.4 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>Transwell</td>
<td></td>
<td>0.0 ± 0.8</td>
</tr>
</tbody>
</table>

* Allografted Meth A tumor cell-induced PEC in B6 mice were cocultured with target cells or cultured separately in a Transwell at an E:T ratio of 30 in the presence or absence of N-MMA (0.5 mM) or anti-TNF-α Ab (2 μg/ml).

* The cytotoxic activities were determined after an 18-h incubation. Each value represents the mean ± SE of six cultures from two different experiments.
after tumor transplantation. However, the growth of 3LL cells was suppressed by a simultaneous inoculation of AIM in a cell number-dependent manner, and in the presence of 2 × 10⁶ cells in an AIM-rich population the tumor cells were rejected without forming any tumor mass in the transplantation site. Table IV shows similar inhibitory effects of cells in the AIM-rich population on the growth of EL-4 lymphoma cells. The growth was completely abrogated by the simultaneous inoculation of AIM at an AIM:tumor ratio of 50.

Effects of AIM on growth of 3LL cells s.c. transplanted into B6 mice

<table>
<thead>
<tr>
<th>Days</th>
<th>3LL Alone</th>
<th>+ AIM (cells/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10³</td>
<td>2 × 10³</td>
</tr>
<tr>
<td>14</td>
<td>10.3 ± 1.3³</td>
<td>9.8 ± 1.9</td>
</tr>
<tr>
<td>19</td>
<td>15.9 ± 3.6</td>
<td>14.8 ± 2.2</td>
</tr>
<tr>
<td>26</td>
<td>23.9 ± 2.6</td>
<td>23.4 ± 4.7</td>
</tr>
<tr>
<td>29</td>
<td>28.0 ± 3.9</td>
<td>25.1 ± 4.9</td>
</tr>
<tr>
<td>34</td>
<td>32³</td>
<td>29.0 ± 0.5</td>
</tr>
</tbody>
</table>

* Days after s.c. injection of 3LL tumor cells (2 × 10⁵ cells/mouse) into B6 mice.
* Diameter of tumor mass, mm; mean ± SD (n = 4).
* Value for one mouse only, as the other three mice died.

Rejection of 3LL tumor cells by the simultaneous inoculation of allogeneic, but not syngeneic, fibroblastic cells

As it may sometimes be difficult to isolate AIM from tumor-bearing patients, we next tried to induce AIM in the transplantation site of syngeneic tumor cells (Fig. 5). 3LL cells (2 × 10⁵ cells/mouse) s.c. injected into B6 mice grew time dependently, and the tumor mass reached 16 mm diameter on day 24 after transplantation. However, a simultaneous inoculation of allogeneic (BALB/3T3; H-2b), but not syngeneic (NOR10; H-2b), fibroblastic cells suppressed tumor growth in a cell-number dependent manner and resulted in rejection without forming any tumor mass in the transplantation site at an allograft:tumor ratio of 200. In contrast, sonicated BALB/3T3 fibroblastic cells totally lost their inhibitory effect; and the simultaneous inoculation of allogeneic BALB/3T3 fibroblastic cells with 3LL cells into IFN-γ-/- B6 mice had virtually no effect on tumor growth (Fig. 5).

Discussion

Mφ recognize, phagocytose, and dispose of senescent RBC, damaged cells, and foreign invaders (24). These continuous functions are constitutive and therefore do not require external regulation. In contrast, sporadic functions, such as participation in host defense against chronic infections and cancer, require activation signals to initiate the recruitment and activation of Mφ to perform a specific task. Once this task is completed, the cells can revert to the non-activated state (25). AIM fulfill these demanding properties. 1) The cytotoxic activity against syngeneic tumor cells was found only in the rejection site of allografted tumor or skin (Fig. 3). 2) Resident (Fig. 3) and casein- or thioglycolate-elicited inflammatory (Table II) Mφ were inactive toward syngeneic tumor cells or cell lines. 3) AIM are a type of activated Mφ (9, 10, 16, 17). 4) When allografts were rejected, AIM reverted to the nonactivated state, and the number of AIM was rapidly reduced in the rejection site (9–12, 19) (Fig. 4).

Among bulk infiltrates in allografts, T cells were found to be the major producers of IFN-γ (8). Therefore, it is reasonable that, as was shown in Fig. 1, the IFN-γ-dependent cytotoxicity of cells in an AIM-rich population against syngeneic tumor cells and cell lines was significantly reduced after T cell elimination. Although IFN-γ was essential for the induction of AIM-mediated cytotoxicity against syngeneic tumor cells and cell lines (Figs. 4 and 5), IFN-γ alone was insufficient to induce significant Mφ-mediated antitumor activity even in vitro, because resident or thioglycolate-activated Mφ activated by IFN-γ in vitro had very low cytotoxic activity against tumor cells (data not shown). Also in vivo, an s.c. injection of IFN-γ (0.05–5 μg in 0.2 ml of PBS) simultaneously with 3LL tumor cells was ineffective on the tumor growth (unpublished data). The nature of the additional factor(s) essential for the induction of AIM-mediated cytotoxicity against syngeneic tumor cells and cell lines remains to be elucidated. Of particular interest, Hayashi (26) reported recently that the inducibility of IFN-γ in cancer patients is a good marker for the effectiveness of immunotherapy with bacillus Calmette-Guérin cell wall skeleton.

It has been generally believed that CTLs recognize allografts as nonself through the difference in the histocompatibility Ags and destroy the allografts (27). Recent studies using β₂-microglobulin, CD4, or CD8 knockout mice, however, revealed that the main

Table IV. Effects of AIM on growth of EL-4 cells s.c. transplanted into B6 mice

<table>
<thead>
<tr>
<th>Days</th>
<th>EL-4 Alone</th>
<th>+ AIM (cells/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10³</td>
<td>7.5 × 10³</td>
</tr>
<tr>
<td>13</td>
<td>16.5 ± 2.1³</td>
<td>5.0 ± 7.1</td>
</tr>
<tr>
<td>17</td>
<td>20.3 ± 3.2</td>
<td>8.0 ± 11.3</td>
</tr>
<tr>
<td>25</td>
<td>35.0 ± 1.4</td>
<td>16.5 ± 23.3</td>
</tr>
<tr>
<td>34</td>
<td>35.0 ± 1.4</td>
<td>23.7 ± 16.8³</td>
</tr>
</tbody>
</table>

* Days after s.c. injection of EL-4 tumor cells (2 × 10⁵ cells/mouse) into B6 mice.
* Diameter of tumor mass, mm; mean ± SD (n = 3).
* All mice died.

One mouse died.
A novel type of activated Mϕ is involved in rejection of both i.p. and s.c. allografted tumor cells in B6 mice. These Mϕs are cytotoxic against syngeneic tumor cells or cell lines as well as with allografted Meth A tumor cells, which were cultured in a series of cold target inhibition assays. The cytotoxic activity of Mϕ against $^{31}$Cr-labeled allograft Meth A cells was inhibited by the addition of unlabeled H-2d, but not H-2k or H-2b, Con A blasts (11), whereas the cytotoxic activities of Mϕ against $^{31}$Cr-labeled 3LL and NCTC4093 cells were not affected by the addition of cold H-2d, H-2k, or H-2b Con A blasts (our unpublished data).

The application of clinical allogeneic bone marrow transplantation has expanded as a treatment modality for hematologic malignancies (32), and it has been suggested that graft-vs-host disease is associated with an antileukemic effect (33, 34). Some patients without graft-vs-host disease, however, have remained free of leukemia 2–8 yr after bone marrow transplantation, and recurrent leukemia has developed in other patients with severe graft-vs-host disease (35). Furthermore, Chaplin et al. (36) demonstrated that bone marrow transplantation using donor marrow depleted of CD8 lymphocytes decreased the incidence of a graft-vs-host disease without abrogating the graft-vs-leukemia effect. In the present study we revealed that host-derived AIM were cytotoxic not only against parenchymal tumor (e.g., 3LL, CMT-93, and B16) cells and cell lines (e.g., NCTC4093 and NOR10) but also against lymphoid tumor cells, including EL-4 lymphoma cells. Therefore, it is plausible that the cytotoxicity of recipient-derived Mϕ against the lymphoid tumor cells is a mechanism responsible for the antileukemic effect following allogeneic bone marrow transplantation and that it may be possible to make a therapeutic application of AIM (or the inducer) to leukemia.

Acknowledgments

We thank J. Sato and Y. Miyamoto for skillful technical assistance and K. Hirata, C. Yao, Y. Nakadeguchi, and H. Yoshii for excellent secretarial assistance.

References


