ABSTRACT

The coculture of lymphoid cells from Fischer 344 rats with recombinant human interleukin 2 (rIL-2) resulted in the generation of lymphokine-activated killer (LAK) cells. Maximal LAK activity was obtained between 200 and 1000 units/ml rIL-2. Lymphoid cells from spleen, thymus, bone marrow, peripheral blood, and lymph nodes were able to generate LAK activity although the kinetics and magnitudes of the responses were appreciably different among these tissues. Thus, while spleen and blood lymphocytes responded quickly (by day 3) and gave the highest level of LAK activity in response to rIL-2, bone marrow and thymus cells responded only by 7 to 9 days in culture. LAK activity could be generated from a variety of rat strains regardless of whether there were high or low levels of endogenous splenic natural killer (NK) activity, but the early (day 3) response was lower in the strains with low levels of NK activity. Cells with LAK activity could lyse a variety of tumor targets including fresh ascites or fresh syngeneic solid tumor explants but could not lyse fresh normal cells including syngeneic fibroblasts, peripheral blood lymphocytes, bone marrow cells, thymocytes, or T,B blasts. The generation of LAK activity required a concomitant proliferative response and could be completely abrogated by mitomycin C, actinomycin D, or X-irradiation above 500 rads. These treatments, however, did not affect natural killer activity or short-term (4 h) IL-2-stimulated NK activity. LAK activity could be generated from spleen cells obtained from rats as early as 10 days of age but could not be generated from unfractonated neonatal spleen, neonatal liver, or peritoneal macrophages. The ontogeny of the development of splenic LAK activity correlated closely to the development of concurrent natural killer activity. When mixed with an NK-resistant mammary adenocarcinoma (MADB106) and adoptively transferred to normal syngeneic recipients in standard Winn-type assays, LAK cells were effective at inducing complete tumor inhibition.

INTRODUCTION

Recent advances in adoptive immunotherapy have used cytotoxic lymphocytes with broad antitumor reactivity. Studies have shown that coculture of normal lymphocytes with IL-2 + induces the generation of broadly cytotoxic antitumor killer cells commonly termed LAK cells (1-3) and these cells have proven useful in the control of metastatic tumors in animal models and humans (4-6).

Although studies on the basic description and therapeutic efficacy of LAK cells have used murine animal models (6-9), little has been done with other animal models (10) and no studies have been performed using rats. However, there are several advantages of using rat models for such studies: (a) rats are easily available from a single rat, compared to the limited numbers isolated from a mouse; (d) rats provide a means whereby surgical procedures and multiple anesthesias can be more readily performed, thus enabling more extensive in vivo analyses.

We have recently begun to investigate the generation of cells with LAK activity in the rat. This paper describes our initial observations regarding optimal conditions for their production, ontogeny, target spectrum, and general characteristics.

MATERIALS AND METHODS

Animals. Male Fischer 344 (F344) rats (75-100 g) were purchased from Taconic Farms (Germantown, NY) and were housed in a specific-pathogen-free animal facility at the Pittsburgh Cancer Institute. Other strains of rats (male, 75-100 g) were purchased from the Frederick Cancer Research Facility (Frederick, MD).

Tumor Cells. Routinely, the lysis of the NK-resistant mastocytoma, P815 (11), was used as an indicator of LAK activity. Other targets, however, included several NK-resistant rat tumor targets: CS8-NTD (a Gross virus-induced T-cell lymphoma from the W/F rat) (12); MADB106 (F344 mammary adenocarcinoma) (13); MT-13762A (F344 mammary adenocarcinoma) (14); and CRNK-16 (LGL leukemia) (15). Other targets included the mouse tumors: L5178Y (DBA/2 T-cell lymphoma); MDAY/D2 (DBA/2 lymphosarcoma); B-16/BL6 (C57BL/6 melanoma); 1.0/Anti-B (C57BL/6 fibrosarcoma); and the human tumor K562 (erythroleukemia) and Raji (B-cell lymphoma). All of these lines were grown in RPMI 1640, 10% FCS, and antibiotics. In several cases fresh tumor explants were used as targets including fresh ascites tumor of CRNK-16 leukemia and fresh solid tumor of MADB106 adenocarcinoma. The NK-sensitive Moloney virus-induced YAC-1 lymphoma (16) was used as the indicator of NK activity.

Preparation of Lymphoid Cells. Spleens, cervical lymph nodes, and thymus were aseptically removed and single cell suspensions prepared in RPMI 1640-10% FCS. When spleen cells were used, mononuclear cells were obtained after centrifugation on Ficoll-Hypaque gradients (density, 1.077) at 300 x g for 20 min. Peripheral blood was obtained by cardiac puncture into heparinized syringes. Mononuclear cells were then obtained after centrifugation on Ficoll-Hypaque gradients (density, 1.077) at 400 x g for 30 min. Bone marrow was harvested from femurs using a syringe and needle and a single cell suspension was prepared by pressing the marrow through fine nylon mesh. Peritoneal cells were obtained from the peritoneal cavity after lavaging with 50 ml of cold phosphate-buffered saline. All lymphoid cells were washed twice in RPMI 1640-10% FCS, and counted prior to use. Viabilities were routinely >97% by trypan blue exclusion.

Interleukin 2. Human IL-2 was kindly provided by the Cetus Corporation (Emoryville, CA) and contained 1.25 x 10⁶ units of IL-2/mg protein (as defined by [3H]thymidine incorporation into CTLL-2 cells).

Generation of LAK Cells. LAK cells were produced from F344 rats because IL-2. Optimal medium conditions for the generation of LAK activity included the following: RPMI 1640 (GIBCO) supplemented with 10% FCS (GIBCO), 2 mM glutamine, 5 x 10⁻⁵ M 2-mercaptoethanol, antibiotics (streptomycin/penicillin), and 10⁵ units/ml IL-2 (hereafter referred to as LAK medium). The lymphoid cells were cultured in T-75 flasks (Corning) lying flat at an optimal density
of \(2 \times 10^6\) viable cells/ml in LAK medium for up to 5 days in 5% CO\(_2/\)
95% air at 37°C.

Cytotoxicity Assay. Cytotoxicity was measured by a standard 4-h
\(\text{^{51}Cr}\) release microcytotoxicity assay using 96-well, round-bottomed
microplates (Costar, Cambridge, MA). Target cells were labeled with 100
\(\mu\text{Ci}\) of Na\(^{51}\text{CrO}_4\), washed, and seeded into 96-well microplates at \(5 \times 10^3\) cells/well in 50 \(\mu\text{l}\). Suspensions of effector cells were added to
triplicate wells to give various E:T ratios in a final volume of 200 \(\mu\text{l}\). After
incubation at 37°C for 4 h, 100 \(\mu\text{l}\) of supernatant were removed
from each well and counted in a gamma counter to determine experi-
mental release (ER). Spontaneous release (SR) was obtained from wells
receiving target cells and medium only, and total release (TR) was
obtained from wells receiving 1% Triton X-100. The percentage of
cytotoxicity was calculated as

\[
\% \text{ of cytotoxicity} = \frac{(ER) - (SR)}{(TR) - (SR)} \times 100
\]

Proliferation Assay. Proliferation was measured by the incorporation of
\(\text{[3H]}\text{dThd}\) into DNA. Cells (5 \(\times\) 10\(^4\)) were cultured in 200 \(\mu\text{l}\) in 96-
well flat-bottomed microplates (Costar) and pulsed with 1 \(\mu\text{Ci}\) \(\text{[3H]}\)-
dThd for 12 h. The DNA was collected onto glass fiber filters using a
MASH-II harvester, and \(\text{[3H]}\text{dThd}\) was counted in a scintillation coun-
ter (LKB).

Statistics. Statistical analysis was performed using Student’s \(t\) test.

RESULTS

General Characteristics of LAK Cells from F344 Rats. Initial
experiments were designed to establish the optimal culture
conditions to generate cells with LAK activity from F344 rat
spleen cells. Although complete LAK medium yielded optimal
activity, by selective elimination of each medium component,
we found the presence of 2-mercaptoethanol to be a most
critical factor (data not shown). Variations in LAK activity were
also noted with different lots of fetal calf serum. Fig. 1 shows
that the concentrations of rIL-2 required to generate LAK
activity ranged from 100 units/ml to 10,000 units/ml with 200-
1000 units/ml sufficient for maximal activity. Levels of rIL-2
between 10 and 100 units/ml also generated weak but consistent
levels of cytotoxic activity but mainly against the YAC-1 target.

Fig. 2 shows the kinetics of generation of LAK activity from
various lymphoid organs in F344 rats. Clearly spleen, periph-
eral lymph nodes, and peripheral blood generated high levels
of cytotoxic activity by 3 to 5 days in culture with 10\(^3\) units/ml
rIL-2. Thymus and bone marrow cells did not begin to generate
LAK activity until approximately day 5 in culture and peaked
by 7 to 9 days. LAK activity persisted in all cultures (without
refeeding) through 15 days. We have not investigated longer
culture periods. Peritoneal exudate cells (>80% macrophages
as judged morphologically in Giemsa-stained cytospin prepa-
rations) did not generate cytotoxic activity even after extended
culture times and varying amounts of rIL-2.

In addition to the development of LAK activity, lymphoid
cells from each organ were analyzed for their level of prolifera-
tion by measuring \(\text{[3H]}\text{dThd}\) incorporation into DNA during
each day in culture. As shown in Fig. 3, rIL-2 induced a strong
proliferative response in all lymphoid organs tested. This pro-
liferative response occurred concomitant with the development
of LAK activity. Proliferation by spleen cells was apparent as
early as day 2 and peaked by day 7. Bone marrow cells did not
initiate a proliferative response until day 5 and peaked at day
7. Although thymus cells showed a biphasic response, peak
proliferative activity was also noted on days 7 and 8. Peritoneal
cells showed no detectable proliferative response to rIL-2.

The correlation between the kinetics of proliferation and the
generation of LAK activity raised the question of whether cell
proliferation was required for the development of LAK activity.
Spleen cells were treated with varying doses of X-irradiation or
with agents which irreversibly blocked DNA (mitomycin C) or
RNA synthesis (actinomycin D). After treatment, the cells were tested for proliferation as well as their ability to generate cytotoxicity. The results of these experiments are shown in Table 1. Clearly, the generation of LAK activity as well as the rIL-2-induced proliferation at 3, 4, or 5 days was completely suppressed above 500 rads. Although the data are shown only for day 4, essentially identical results were noted on days 3 and 5. Treatment with mitomycin C or actinomycin D also inhibited proliferation and generation of the LAK response. However, none of these treatments had any significant effect on NK activity or short-term (4h) rIL-2 booster NK activity.

Specificity of Rat LAK Cells. Although we used the NK-resistant murine mastocytoma P815 as a routine indicator of LAK activity, the cytolytic activity of LAK cells generated from F344 spleen or peripheral blood was tested extensively against a panel of targets. These data are shown in Table 2. Clearly, very high levels of cytolytic activity were detected on tumor cells from a variety of sources including in vitro-grown tumors of various histological origins or tumors derived from mice, or humans. In addition, LAK cells could easily lyse fresh syngeneic ascites tumors (CRNK-16, a syngeneic LGL leukemia) or fresh solid tumor explants (MADB106, a syngeneic mammary adenocarcinoma). Extensive testing also showed that F344 LAK cells were unable to lyse fresh normal targets including syngeneic fibroblasts, thymocytes, bone marrow cells, LGL, granulocytes, or concanavalin A-induced T-cell or lipopolysaccharide-induced B-cell blasts, even when tested at high E:T ratios (100:1). Using cold target competition we found that the failure of these LAK cells to lyse normal cells was due to an inability of the LAK cells to recognize the normal cells. The data in Fig. 4 show that normal syngeneic bone marrow or

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**Table 1 Importance of proliferation for development of LAK activity**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 0</th>
<th>Day 4</th>
<th>Proliferation on Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>39</td>
<td>1</td>
<td>1,055</td>
</tr>
<tr>
<td>Control + IL-2</td>
<td>51</td>
<td>62</td>
<td>489,485</td>
</tr>
<tr>
<td>250 rads</td>
<td>37</td>
<td>53</td>
<td>42,21</td>
</tr>
<tr>
<td>500 rads</td>
<td>39</td>
<td>52</td>
<td>18,18</td>
</tr>
<tr>
<td>1000 rads</td>
<td>37</td>
<td>51</td>
<td>42</td>
</tr>
<tr>
<td>2000 rads</td>
<td>35</td>
<td>49</td>
<td>1</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>34</td>
<td>49</td>
<td>0</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>37</td>
<td>50</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 2 Killing of tumor cells but not normal cells**

<table>
<thead>
<tr>
<th>Species</th>
<th>Histological type</th>
<th>40:1</th>
<th>20:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRNK-16</td>
<td>LGL leukemia</td>
<td>66</td>
<td>38</td>
</tr>
<tr>
<td>MADB106</td>
<td>Mammary adenocarcinoma</td>
<td>81</td>
<td>55</td>
</tr>
<tr>
<td>MT13762</td>
<td>Mammary adenocarcinoma</td>
<td>72</td>
<td>51</td>
</tr>
<tr>
<td>NTD</td>
<td>T-cell lymphoma</td>
<td>80</td>
<td>60</td>
</tr>
<tr>
<td>GITC</td>
<td>T-cell lymphoma</td>
<td>88</td>
<td>66</td>
</tr>
<tr>
<td>YAC-1</td>
<td>T-cell lymphoma</td>
<td>92</td>
<td>78</td>
</tr>
<tr>
<td>P815</td>
<td>Mastocytoma</td>
<td>65</td>
<td>45</td>
</tr>
<tr>
<td>L5178Y</td>
<td>T-cell lymphoma</td>
<td>56</td>
<td>39</td>
</tr>
<tr>
<td>MDAY/D2</td>
<td>Lymphosarcoma</td>
<td>50</td>
<td>27</td>
</tr>
<tr>
<td>B-16</td>
<td>Melanoma</td>
<td>48</td>
<td>29</td>
</tr>
<tr>
<td>L0/anti-B</td>
<td>Fibrosarcoma</td>
<td>61</td>
<td>32</td>
</tr>
<tr>
<td>K562</td>
<td>Erythroleukemia</td>
<td>70</td>
<td>56</td>
</tr>
<tr>
<td>Raji</td>
<td>B-cell lymphoma</td>
<td>62</td>
<td>48</td>
</tr>
</tbody>
</table>

* CRNK-16 and MADB106 were obtained from fresh ascites tumor or fresh solid tumor explant, respectively.

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**Fig. 4.** Normal cells fail to cold target compete for the lysis of tumor cells by rat LAK cells. On day 5, F344 LAK cells were tested for cytotoxicity against MADB106, P815, CRNK-16, syngeneic peripheral blood lymphocytes or unfraccionated bone marrow target cells at various E:T ratios (left). Right, lysis of MADB106 fresh tumor targets in the presence of various cold targets: (P) P815; (C) MADB106; (x) CRNK-16; (y) F344 PBL; (w) unfraccionated F344 bone marrow.
peripheral blood lymphocytes did not cold target compete for the lysis of fresh MADB106 tumor cells. Similar data were observed when P815 or CRNK-16 were used as LAK targets.

Ontogeny of the LAK Progenitors. To determine the ontogeny of the progenitors of cells with LAK activity, F344 rats of various ages were obtained and spleen cells were prepared. These cells were then tested for NK activity and then placed in culture with 1000 units/ml rIL-2 and tested for the development of LAK activity over a 13-day period. As can be seen in Fig. 5, LAK activity could be generated from F344 spleen cells from 10 days of age to greater than 300 days of age. The kinetics of the generation of LAK activity was generally slower (5–6 days) for spleen cells obtained from rats less than 30 days old (data not shown). Neither neonatal spleen cells nor neonatal liver cells could generate LAK activity even after 7 days of culture. When compared to the generation of NK activity, we found a close correlation between the ontogeny of NK activity in the spleen and the development of LAK activity.

Development of LAK Activity in Various Rat Strains. Various strains of rats (all male and 3 months of age) were obtained and spleen cells were prepared. These cells were tested for NK activity, short term (4-h) rIL-2 boosted NK activity, and the generation of LAK activity 1, 3, or 5 days after culture in 1000 units/ml rIL-2. These data are summarized in Table 3. All strains tested generated substantial levels of LAK activity after 5 days in culture with rIL-2. However, at earlier time points (days 1–3), those strains having low endogenous NK activity (BN, Lou, Buffalo) showed generally lower levels of LAK activity than strains with high NK activity (DA, F344, nu/nu).

LAK Cells Have Potent Antitumor Activity in Standard Adoptive Transfer (Winn-Type) Assays. In order to obtain preliminary data on the in vivo effectiveness of the LAK cells generated in these cultures, we tested their ability to inhibit the growth of an NK-resistant syngeneic adenocarcinoma, MADB106. Tumor cells were mixed with syngeneic F344 LAK cells (5-day rIL-2-cultured spleen cells) or normal spleen cells at various E:T ratios and injected s.c. into the backs of normal F344 recipients. The results of these experiments are shown in Table 4. While all control animals grew tumors and died within 2 months, animals that received tumor cells mixed with LAK cells at ratios of 100:1 and 50:1 did not grow tumors and remained alive and healthy. Animals receiving tumor cells mixed with normal spleen cells also grew tumors and died within 2 months. Animals that received the LAK plus tumor cell mixtures and that did not grow tumors were rechallenged with 5 × 10^4 viable tumor cells after 2 months. All such rechallenged animals grew tumors and died.

**DISCUSSION**

Previous studies in humans and mice have demonstrated that the incubation of lymphocytes in IL-2 induces the generation of cytotoxic lymphocytes with broad antitumor cytotoxicity. Generation of cells with such activity required high levels of rIL-2 (>1000 units/ml) and 3–5 days of culture. Lower levels of rIL-2 (~250 units/ml) generated only low levels of cytotoxicity and required 5–7 days of culture (16, 17). The present studies are the first to demonstrate the successful generation of cells with broad antitumor cytotoxicity (LAK activity) in rats. LAK activity could be generated rapidly (2–3 days) using lower doses of rIL-2 (250 units/ml) resulting in the induction of killer cells with very high levels of cytolytic activity. The optimal culture conditions for the generation of cells with LAK activity in rats were carefully analyzed and we found that the presence of 2-mercaptoethanol and carefully screened lots of fetal calf serum which optimally supported this activity to be the key factors.

These studies demonstrate that rIL-2 not only induces the rapid expression of broad antitumor cytotoxic activity but also induces a strong proliferative response resulting in the expansion of large numbers of highly active cytolytic cells. Thus, in rats as in humans (18, 19) and mice (20), rIL-2 appears to be capable of directly activating both a proliferative as well as a LAK cytolytic response. Further, the magnitude and kinetics of the proliferative responses closely paralleled the generation of LAK activity for a given lymphoid organ. While spleen cells...
LYMPHOKINE-ACTIVATED KILLER CELLS IN RATS

responded quickly to rIL-2, cells from bone marrow or thymus were delayed (days 7–8) in their response. Our studies suggest that proliferation is a requisite event in the development of LAK activity. Treatment of spleen cells with agents which prevent proliferation (mitomycin C, irradiation above 500 rads) abolished the generation of cells with LAK activity. These treatments, however, did not affect endogenous NK activity or short-term (4-h) rIL-2-boosted NK activity, indicating that these treatments were not nonspecifically toxic to the cells. These results confirm the results of Itoh et al. (21, 22) who also noted depression in the generation of LAK activity from mitomycin C-treated or irradiated human peripheral blood lymphocytes.

While most studies in humans or mice have analyzed the generation of LAK activity from spleen or peripheral blood lymphocytes, our studies demonstrate that this activity can be generated from a variety of lymphoid organs. However, the kinetics of development of LAK activity and the magnitude of the cytolytic response from the various organs were noticeably different. In the presence of high levels of rIL-2 (1000 units/ml), spleen cells were the quickest to respond (2–3 days) while bone marrow cells and thymocytes required longer culture periods (7–9 days). Nevertheless, the levels of cytotoxicity generated in these latter cultures were not significantly different from those obtained with spleen cells. We believe the delay in the induction of cells with LAK activity from these organs is due to the low frequency of direct LAK precursors rather than active suppressor mechanisms. While the phenotypes of the cells from spleen and blood responding to rIL-2 to generate LAK activity are the subject of the following paper, the phenotypes of the responding cells from the marrow and thymus are currently under study.

The specificity of cells with LAK activity generated by rIL-2 was extensively studied. While we used the mouse mastocytoma, P815, as a routine LAK target, cells with LAK activity from F344 rats were fully capable of efficiently lysing a panel of syngeneic and allogeneic tumor lines, as well as syngeneic fresh tumor explants. However, we did not observe lysis of syngeneic fresh normal lymphoid cells including proliferating cells such as unfractonated bone marrow cells or T-cell or B-cell blasts. The failure of lysis of these normal lymphoid cells appeared to be due to the inability of the effector cells to recognize/bind these targets since normal bone marrow, thymus, or peripheral blood lymphocytes could not cold target compete for the lysis of cultured tumor cells or syngeneic fresh tumor explants.

The ontogeny of the development of LAK activity was also studied. Spleen cells from F344 rats older than 30 days of age generated good levels of LAK activity. Further, as noted by Mule et al. (23) using mice, this response in rats did not appear to subside with age. However, cells obtained from neonatal spleens or livers or adult peritoneal cells were unable to generate LAK activity. Additional testing revealed that this failure may be due to suppressor mechanisms rather than the absence of precursor cells since neonatal spleen cells as well as adult peritoneal cells were fully capable of suppressing the generation of LAK activity from normal adult spleen cells.5

Previous studies have shown that the adoptive transfer of cells with LAK activity (24) or cytotoxic T-lymphocytes (25) could inhibit the growth of syngeneic tumor cells in Winn-type assays in the absence of additional IL-2. Our adoptive transfer experiments using Winn-type assays demonstrated unequivocally that the effector cells generated in these cultures were highly effective at eliminating NK-resistant tumor cells. All animals receiving either tumor cells alone or tumor cells mixed with normal spleen cells developed tumors and died within 2 months. In contrast, none of the animals receiving tumor cells mixed with LAK cells developed tumors and all animals have remained alive and healthy. Evidence that this effect was not due to the development of specific immunity was shown by rechallenging these animals with fresh tumor cells. When this was done, all animals developed tumors and subsequently died. We are currently studying the therapeutic efficacy of these LAK cells in rats bearing experimental metastases.

In summary, this study establishes the parameters for investigating cells with broad antitumor (LAK) activity in rats. This will allow for additional analysis in animal models for the general applicability of LAK therapy in the treatment of cancer. The rat model also provides an efficient model for studying the phenotype of rIL-2-activated killer cells since large quantities of highly purified LGL or T-cells can be obtained from rat peripheral blood or spleens. The following paper (26) describes the phenotypic and morphological characteristics of these cells in rats.

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LYMPHOKINE-ACTIVATED KILLER CELLS IN RATS


Lymphokine-activated Killer Cells in Rats: Analysis of Tissue and Strain Distribution, Ontogeny, and Target Specificity

Nikola L. Vujanovic, Ronald B. Herberman and John C. Hiserodt


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