Immunotherapy of Murine Hepatic Metastases with Lymphokine-activated Killer Cells Expanded in Serum-free Media and Recombinant Interleukin 2

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ABSTRACT

It has been shown that the systemic administration of lymphokine-activated killer (LAK) cells with recombinant interleukin 2 (RIL-2) is effective in reducing the number of established pulmonary and hepatic metastases in murine models. Similarly, this modality of therapy has been proven effective against certain selected human tumors as well. In view of the rising concern with transmission of virally related communicable diseases such as hepatitis and AIDS, we have undertaken the evaluation of a serum-free medium (AIM V) for the generation and expansion of murine LAK cells for use in vivo tumor immunotherapy against murine hepatic metastases. Day 3 LAK cells generated in AIM V medium demonstrated a greater percentage of viable cells than cells generated in serum containing complete medium (CM) (mean percentage of yield, 59 versus 25%, AIM V medium versus CM, respectively, P < 0.001, N = 6 consecutive experiments). When day 3 LAK cells were transferred to new medium (CM to AIM V to AIM V), a highly reproducible expansion of these cells was demonstrated which was significantly better for cells expanded in AIM V medium versus cells expanded in CM (mean fold expansion on day 21 of culture; 201 versus 54, AIM V medium versus CM, respectively, P < 0.005, N = 4 consecutive experiments). When day 3 LAK cells, day 5 expanded LAK cells, and day 13 expanded LAK cells grown in CM or in AIM V medium were given in vivo with RIL-2 to mice harboring hepatic metastases, cells grown in AIM V medium demonstrated an increased antitumor activity compared to cells grown in CM. As an example in experiment 1, the mean number of metastases with day 5 expanded LAK cells grown in CM and given with RIL-2 was 47 while the mean number of metastases with day 5 expanded LAK cells grown in AIM V medium and given with RIL-2 was 5 (P < 0.002). These experiments demonstrate that AIM V medium can be utilized to generate greater numbers of murine LAK cells with enhanced in vivo antitumor activity compared to cells generated in CM. These findings could be applied to the expansion of cytotoxic cells for human antitumor therapy.

INTRODUCTION

The adoptive transfer of LAK3 cells in combination with RIL-2 to tumor-bearing hosts harboring tumor of various histologies and immunogenicities has recently been shown to be an effective approach in the therapy of established metastases (1–6).

LAK cells can easily be generated in large numbers by the incubation of murine splenocytes or human peripheral blood lymphocytes in medium containing RIL-2 (7–10). These cells have a high degree of antitumor reactivity as shown by their capacity in vitro to lyse a wide variety of fresh noncultured tumor cells, but not normal cells (11–12). Clinical trials of the therapy of human metastases in vivo have been established and objective regressions of metastatic cancers have been demonstrated by using therapy with LAK cells and RIL-2 (13).

In addition, it has been shown in experimental murine systems that LAK cells can be expanded to large numbers and that such cells can be given concomitantly with RIL-2 to tumor-bearing animals, leading to significant tumor regressions (14).

When day 3 LAK cells, murine splenocytes or human lymphocytes are cultured in medium containing 10% fetal calf serum for animal cell culture or 2% human AB serum for human cell culture. Of concern, particularly when one is dealing with human material, is the propagation to the patient of virally related diseases such as hepatitis B, hepatitis D, non-A, non-B hepatitis, and acquired immunodeficiency disease. The incidence of posttransfusion hepatitis has been reported to be of the order 10 to 20 cases per 100 patients transfused, and of these, non-A, non-B hepatitis accounts for approximately 90% of the cases (15). It is estimated that in the United States alone 12,000 people harbor transfusion-acquired human immunodeficiency virus infections (16).

The current study describes the generation and expansion of murine LAK cells in a serum-free medium named AIM V. We show that LAK cells can be generated and expanded to much greater numbers when grown in AIM V medium compared to CM containing fetal calf serum and that these LAK cells demonstrate improved in vitro cytotoxicity compared to day 3 LAK cells grown in CM. We also show that mice bearing hepatic metastases who are treated with LAK cells generated in AIM V medium in combination with RIL-2 demonstrate an increased reduction in the number of liver metastases compared to LAK cells generated in CM containing fetal calf serum. Findings outlined in this paper may be applicable to the treatment of human cancers by cytotoxic cells expanded in serum-free medium.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice, 12-weeks-old or older were utilized for all in vivo experiments and were obtained from the Animal Resources Centre of the University of Calgary, Calgary, Alberta, Canada. The animals were fed standard mouse chow and water ad libitum.

Tumors

MCA-38. MCA-38 (a gift from Dr. Steven Rosenberg, National Cancer Institute, NIH, Bethesda, MD) is a murine colon adenocarcinoma induced by Corbett et al. (17), through the s.c. injection of dimethylhydrazine in C57BL/6 mice. This tumor was passaged s.c. and its use was not restricted to early passage generations.

MCA-102. MCA-102 (a gift from Dr. Steven Rosenberg) is a non-immunogenic sarcoma syngeneic to the C57BL/6 strain which was induced by Rosenberg and Terry (18). After thawing from −70°C, this tumor was serially passaged and its use was not restricted to early passage generations. The MCA-102 sarcoma is resistant to lysis by NK cells in vitro (19).

YAC-1. YAC-1 (American Type Culture Collection, ATCC TIB 60, Rockville, MD) is a lymphoma of A/Sn origin which was maintained...
in continuous in vitro culture and harvested as necessary. The YAC-1 cell line is sensitive to the cytotoxic activity of NK cells in mice (20).

Single cell suspensions of fresh tumors were prepared as described previously (11). The YAC-1 lymphoma cells were harvested, washed three times in HBSS, and resuspended at the appropriate cell concentration in CM for cytotoxicity assays.

Complete Medium

CM consisted of RPMI 1640 (Flow Laboratories) with 10% fetal calf serum (Gibco/BRL, Burlington, Ontario, Canada), 0.03% fresh glutamine, 100 mg/ml of streptomycin, 100 units/ml of penicillin, 0.1 mM nonessential amino acids, 0.1 mM sodium pyruvate, 50 µg/ml of Fungizone, 50 µg/ml of gentamicin (all from Flow Laboratories), and 5 x 10⁻³ M 2-mercaptoethanol (Aldrich, Milwaukee, WI).

AIM V Medium

AIM V medium was kindly provided by the Gibco/BRL Life Technologies, Inc. and is a proprietary medium consisting of Dulbecco's modified Eagle's medium and Ham's F12 medium at a 1:1 ratio containing recombinant insulin and human transferrin plus human albumin obtained from donors screened for A and B hepatitis and human immunodeficiency virus at the time of donation. The transferrin and albumin come in a liquid pasteurized form and are added to the medium at the manufacturer's predetermined concentrations. For the purpose of our experiments, streptomycin, penicillin, Fungizone, and 2-mercaptoethanol were also added to the AIM V medium at the concentrations described under "Complete Medium." Recombinant Interleukin 2

The gene for interleukin 2 was isolated from a high producer Jurkat cell line (21) and was expressed at high levels in Escherichia coli; the resultant recombinant material was highly purified to homogeneity as described by Rosenberg et al. (9) and Wang et al. (22). Human IL-2 was kindly supplied by the Cetus Corp. (Emeryville, CA). Purified RIL-2 has a specific activity of 4 to 8 x 10⁶ units/mg (23). In proliferative assays, approximately 1 unit of Cetus RIL-2 is equivalent to 2.3 units of the biological response modifiers program interleukin 2 standard (24).

Generation of LAK Cells

Fresh splenocytes (3.2 x 10⁸), prepared as described previously (4), were placed into 175-cm² (750 ml) flasks (Falcon Labware, Fisher Scientific, Ottawa, Ontario, Canada), containing 175 ml of either CM or AIM V medium with 1000 units of RIL-2/ml. After 3 days of incubation at 37°C in a moist atmosphere containing 5% CO₂, the LAK cells were harvested, passed over Ficoll (Lympholyte-M, Cedarlane Laboratories, Hornsby, Ontario, Canada) to remove dead cells, and washed in HBSS before being resuspended in HBSS without calcium and magnesium for expansion or i.v. injection.

Generation of Expanded LAK Cells

Day 3 LAK cells generated as above were cultured in 850-cm² roller bottles (Corning No. 25140; Corning Glass Works, Corning, NY) as described previously (11), either in CM or in AIM V medium containing 1000 units of RIL-2/ml. Bottles were sampled daily from days 1 through 24 for expansion and cells were harvested on days 5 and 13 of expansion for immunotherapy and on days 5 and 8 of expansion for cytotoxicity.

Adoptive Immunotherapy Model

To induce hepatic metastases, a single cell tumor suspension prepared as described above was injected directly into the spleen of anesthetized animals, as described previously (1). Three days after tumor induction, 1 x 10⁸ day 3 LAK cells or day 5 or 13 expanded LAK cells grown either in CM or in AIM V medium were injected into the tail veins of the animals. HBSS or RIL-2 at a dose of 25,000 units per injection was administered i.p. every 8 h from days 3 through 7. Six mice were included in each treatment group. On day 14 after tumor induction, all mice were ear-tagged, randomized, killed by cervical dislocation, and their livers were harvested and the number of metastatic deposits was enumerated as described previously (1). Livers with metastases too numerous to count were assigned an arbitrary value of 250 since we were only able to count reliably numbers of metastases approaching 250 per organ. After all data were recorded, the codes were broken and the data were analyzed.

LAK Cell Cytotoxicity Determined by Chromium Release

Cells from the MCA-102 sarcoma or the YAC-1 lymphoma obtained as described above were labeled with 51Cr (NEN-DuPont, Lachine, Quebec, Canada) for 1 h and were subjected to a standard 4-h chromium release assay as described previously (11). Cytotoxicity is also expressed in this paper in terms of lytic units per 10⁶ cells with 1 LU defined as the number of cells causing 30% lysis of 10⁴ target cells.

Fluorescence-activated Cell Sorter Analysis

The analysis of cell surface phenotype was carried out by indirect immunofluorescence with a 50-H cytotofluorograph interfaced to a Model 2150 computer system (Ortho Diagnostic Systems, Inc., Westwood, MA). Single cell suspensions (1 x 10⁶) of day 3 LAK cells or day 5 or 8 expanded LAK cells cultured either in CM or in AIM V medium were incubated for 30 min at 4°C with 10 to 50 µl (depending on the source and concentration) of the directly conjugated antibodies. (a) Anti-Thy-1.2, anti-Lyt-2, anti-Lyt-1 (Becton Dickinson, Mountain View, CA; fluorescein isothiocyanate); (b) anti-L3T4 (Becton Dickinson; phycoerythrin), or with purified unconjugated antibodies anti-Ly-6.2, anti-Ly-21.2, anti-Qa-2, anti-Ly-15.2 (Australian Monoclonal Developments, New South Wales, Australia), and anti-Ia² (clone 25-9-17 IgG2a, a gift of Dr. Jim Yang, National Cancer Institute, NIH, Bethesda, MD). Unconjugated bound antibodies were detected by incubation with an appropriately titered fluorescein isothiocyanate-labeled goat monoclonal antibody to mouse IgG fragments γ chain specific (Cappel, Malvern, PA) for 30 min at 4°C. Anti-asialo-GM₁ monoganglioside rabbit heteroserum (Wako Chemical, Dallas, TX) and the corresponding secondary reagent fluorescein isothiocyanate-labeled sheep anti-rabbit F(ab)² fragments (Biosystems, Compiègne, France) were also appropriately titered and incubated with cells for 30 min each at 4°C. The anti-Ly-6.2 antibody identifies effector T-cells, the anti-Ly-21.2 antibody identifies an antigen present on T- and B-cells, and detects a new leukocyte-differentiation antigen. The anti-Qa-2 antibody is linked to the murine H₂ system and Qa antigens are found on T-lymphocytes (cytotoxic and helper T-cells) and are also known to be found on NK cells. The anti-Ly-15.2 antibody defines a genetic polymorphism of the LFA-1 (lymphocyte-function-associated antigen-1) molecule in the mouse. This molecule is intimately involved in cytotoxic T-cell functions. This antibody binds to all T-cells and B-cells.

Statistics

Two-sided P values are calculated by using the Wilcoxon rank sum test (25) for analysis of the hepatic metastases data. No mice were excluded from the statistical evaluation. All other statistical data were determined by the Student’s t test for paired and unpaired samples (26).

RESULTS

The Generation of Day 3 LAK Cells in AIM V Medium Leads to a Significantly Greater Number of Viable Cells Compared to CM. In order to determine if AIM V medium could generate LAK cells as effectively as CM, we performed the next series of experiments. Fresh splenocytes were put in culture to generate day 3 LAK cells as described in "Materials and Methods." The percentage of yield of viable cells was determined by using the total number of cells put into culture on day 0 as the denominator. Table 1 summarizes the results of 6 consecutive experiments. For each experiment, the percentage of yield of day 3 LAK cells obtained with AIM V medium was higher than the percentage of yield seen with CM. The mean percentage of
Table 1  Incubation of splenocytes in AIM V medium with RIL-2 and generation of a greater number of day 3 LAK cells compared to complete medium with RIL-2

Fresh C57BL/6 splenocytes were incubated either in CM or in AIM V medium with 1000 units of RIL-2/ml at a final cell number of $3.2 \times 10^6$ in 175 ml of medium for 3 days. The number of viable cells on day 3 of culture was enumerated and represents the percentage of yield compared to the cell number on day 0 of culture.

<table>
<thead>
<tr>
<th>Media utilized</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Experiment 4</th>
<th>Experiment 5</th>
<th>Experiment 6</th>
<th>Mean*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM</td>
<td>21</td>
<td>23</td>
<td>18</td>
<td>27</td>
<td>26</td>
<td>33</td>
<td>25</td>
</tr>
<tr>
<td>AIM V</td>
<td>57</td>
<td>52</td>
<td>60</td>
<td>45</td>
<td>75</td>
<td>67</td>
<td>59</td>
</tr>
</tbody>
</table>

* Preparation of the media were described in "Materials and Methods," each medium contained 1000 units of RIL-2/ml.

yield for all experiments done with CM was 25 versus 59% for experiments done with AIM V medium ($P < 0.001$). Thus the mean percentage of yield of day 3 LAK cells obtained through the use of AIM V medium was over twice that seen with CM.

Optimization of Expansion of Day 3 LAK Cells with Use of AIM V Medium. In order to generate large numbers of cytotoxic LAK cells that could be utilized for the immunotherapy of tumors, we compared standard techniques of expanded LAK cell generation in CM containing RIL-2 (14) with similar techniques using AIM V medium in side by side experiments. Day 3 LAK cells grown either in CM or AIM V medium were harvested sterilely and were resuspended in roller bottles in medium similar to the one they had been generated in. The mean fold expansion for days 5, 8, 13, and 18 expanded cells is shown in Table 2. Day 3 LAK cells generated in AIM V medium and left in AIM V medium demonstrated the greatest expansion compared to day 3 LAK cells generated in CM and left in CM (mean fold expansion in 4 consecutive experiments on day 18 of expansion: 201 versus 55 for AIM V versus CM, respectively; $P < 0.001$). Thus the expansion of LAK cells was significantly more productive with AIM V medium compared to CM.

LAK Cells Grown in AIM V Medium Demonstrate Increased Early and Late Cytotoxicity against Fresh and Cultured Tumor Targets Compared to LAK Cells Grown in CM. In order to document if day 3 LAK cells and days 5 and 8 expanded LAK cells grown in AIM V medium demonstrated in vitro antitumor activity, we performed a series of experiments wherein cells were assayed for cytotoxicity using a $^{51}$Cr cytotoxicity release assay against a fresh NK-insensitive tumor, the MCA-102 sarcoma, and against a NK-sensitive cultured cell line, the YAC-1 lymphoma. Fig. 1 documents the findings of one characteristic experiment. As can be seen, day 3 LAK cells grown in AIM V medium demonstrated a greater lysis of the fresh and cultured targets compared to day 3 LAK cells grown in CM (70 versus 48% and 60 versus 25% AIM V medium versus CM for MCA-102 and YAC-1 targets, respectively). After 8 days in culture (day 5 expanded LAK cells) both groups of cells demonstrated similar cytotoxicity. As documented previously, however (14), day 8 expanded LAK cells (11 days in culture) grown in CM lost much of their cytotoxicity against a fresh tumor target (24%, 20:1 effectortarget ratio against the MCA-102 sarcoma). However day 8 expanded LAK cells grown in AIM V medium maintained a higher level of cytotoxicity (60% using the same target at the same effectortarget ratio). Of interest was the fact that lysis of the cultured cell line was maintained by both groups of cells without significant differences.

The cells grown in AIM V medium also expressed a greater number of lytic units/$10^6$ cells on days 3 and 11 of culture, especially against the fresh sarcoma target. Results of one characteristic experiment are shown in Table 3. As shown, LAK cells grown for 3 days (day 3 LAK cells), 8 days (day 5 expanded LAK cells), or 11 days (day 8 expanded LAK cells), were assayed in a $^{51}$Cr release assay against the fresh and cultured targets described above. Day 3 LAK cells grown in AIM V medium demonstrated more lytic units than cells grown in CM (145 versus 40 LU against the MCA-102 target, 140 versus 25 LU against the YAC-1 target, AIM V medium versus CM, respectively). On day 5 of expansion, both groups of cells demonstrated the same number of lytic units against both targets (120 versus 128 LU against the MCA-102 target and 200 versus 200 LU against the YAC-1 target, AIM V medium versus CM, respectively). Again on day 11 of culture (day 8 expanded LAK cells), there was a loss of lytic units against the MCA-102 sarcoma for cells grown in CM compared to cells grown in AIM V medium (10 versus 35 LU, CM versus AIM V medium, respectively). There was however little difference in the number of lytic units between CM and AIM V medium cells when tested against the YAC-1 lymphoma target (72 versus 100 LU, CM versus AIM V medium, respectively).

In accordance with the greater expansion of LAK cells seen with the AIM V medium, there were also more total lytic units for LAK cells grown in AIM V medium compared to CM. For example, day 3 LAK cells against MCA-102, total lytic units, 27,840 versus 2320, day 8 expanded LAK cells against MCA-102, total lytic units, 183,050 versus 16,880 (AIM V medium versus CM, respectively).

Phenotypic Analysis of LAK Cells Grown in AIM V Medium Demonstrates More Thy-1.2- and Lyt-2-positive Cells during Early Stages of Culture. In an attempt to identify whether the culture of splenocytes in AIM V medium would alter cell surface antigenic expression of LAK cells, we analyzed the cell surface antigens through the use of various monoclonal antibodies.

Table 2  Maximal Expansion of Day 3 LAK Cells in AIM V medium compared to complete medium

Day 3 LAK cells grown either in CM or in AIM V medium were plated in roller bottles at a cell concentration of $1 \times 10^6$ cells/ml in a medium similar to the one they were harvested from. Each medium contained RIL-2 at a final concentration of 1000 units/ml. Bottles were sampled on days 5, 8, 13, and 18 of expansion, and cell numbers were compared to numbers of day 3 LAK cells put into culture on day 0 of expansion.

<table>
<thead>
<tr>
<th>Media utilized</th>
<th>Day 5 expanded LAK cells</th>
<th>Day 8 expanded LAK cells</th>
<th>Day 13 expanded LAK cells</th>
<th>Day 18 expanded LAK cells</th>
<th>N*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM</td>
<td>5.7  ± 1.7 (A)</td>
<td>24.2 ± 2.3 (C)</td>
<td>36.36 ± 4.2 (E)</td>
<td>54.54 ± 6.1 (G)</td>
<td>4</td>
</tr>
<tr>
<td>AIM V</td>
<td>6.33 ± 1.6 (B)</td>
<td>28.6 ± 3.2 (D)</td>
<td>77.25 ± 3.6 (F)</td>
<td>200.85 ± 4.5 (H)</td>
<td>4</td>
</tr>
</tbody>
</table>

* Statistical significance of differences A versus B, $P = 0.058$; C versus D, $P < 0.002$; E versus F, $P < 0.001$; G versus H, $P < 0.0005$.

* N, number of consecutive experiments.

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and polyclonal antibodies. Cells grown either in AIM V medium or CM for 3, 8, or 11 days were labeled with various antibodies as described in "Materials and Methods," and the percentage of cells expressing particular antigens was evaluated through the use of a flow cytometer (Ortho Diagnostic Systems 50-H).

The surface phenotype of cells grown in the two media, assayed at various days of culture, is shown in Table 4. LAK cells grown in AIM V medium for 3 days expressed more of the Thy-1.2, Lyt-1, and Lyt-2 antigens compared to LAK cells grown in complete medium. There were no differences between the percentage of cells expressing L3T4 and asialo-dv1 monoganglioside antigens as described in "Materials and Methods."

Thus, a greater percentage of cells grown in AIM V medium expressed the Thy-1.2 and the Lyt-2 antigens on day 3 of culture. There were no differences between the percentage of expression of L3T4 and asialo-GM1 monoganglioside antigens for both groups of cells and similar results were found with the 49H.8 monoclonal antibody which is thought to be highly specific for NK cells (27) (data not shown).

Day 3 LAK Cells, Day 5 Expanded LAK Cells, and Day 13 Expanded LAK Cells Grown in CM or in AIM V Medium and Enhanced in Vivo Antitumor Activity against Established Liver Micrometastases When Given with RIL-2. Fresh splenocytes were cultured either in CM or in AIM V medium containing RIL-2 at a final concentration of 1000 units/ml were harvested after 3, 8, and 11 days in culture, washed in HBSS, and resuspended at a final cell concentration of 1 × 10⁶/ml for staining with monoclonal antibodies.

Table 3 Fresh splenocytes cultured and expanded in AIM V medium and greater cytotoxicity against tumor targets compared to cells grown in complete medium.

Fresh splenocytes were plated at a final cell number of 3.2 × 10⁶ in 175 ml of either CM or AIM V medium, each containing RIL-2 at a final concentration of 1000 units/ml. Cells were grown for 3 days (day 3 LAK cells), 8 days (day 5 expanded LAK cells), or 11 days (day 8 expanded LAK cells), and then were harvested and tested in a chromium release assay against a fresh NK-insensitive sarcoma, the MCA-102 tumor, and an NK-sensitive cell line, the YAC-1 lymphoma.

Table 4 Phenotype of LAK cells grown in complete medium versus LAK cells grown in AIM V medium.

<table>
<thead>
<tr>
<th>Antigens</th>
<th>3 days in culture</th>
<th>8 days in culture</th>
<th>11 days in culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CM</td>
<td>AIM V</td>
<td>CM</td>
</tr>
<tr>
<td>Thy-1.2</td>
<td>57</td>
<td>70</td>
<td>90</td>
</tr>
<tr>
<td>Lyt-1</td>
<td>48</td>
<td>59</td>
<td>28</td>
</tr>
<tr>
<td>Lyt-2</td>
<td>36</td>
<td>49</td>
<td>76</td>
</tr>
<tr>
<td>Lyt-21.2</td>
<td>73</td>
<td>82</td>
<td>88</td>
</tr>
<tr>
<td>Lyt-15.2</td>
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<td>96</td>
</tr>
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<td>Ly-6.2</td>
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<td>97</td>
</tr>
<tr>
<td>Qa-2</td>
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<tr>
<td>Pid</td>
<td>41</td>
<td>43</td>
<td>24</td>
</tr>
</tbody>
</table>

*Cells labeled with selected antibodies were analyzed for cell surface antigen expression in an Ortho 50-H flow cytometer at a rate of 10,000 cells/min. The percentage of cells expressing a particular antigen compared to the total population of cell analyzed is shown here (coefficient of variation, 4%). Italicized values delineate at least a 10% difference in antigenic expression between cells cultured in AIM V and CM.*

*Cells from each group were labeled with monoclonal and polyclonal antibodies as described in "Materials and Methods."

Fig. 1: LAK cells grown either in CM (O) or AIM V (D) medium containing 1000 units of RIL-2/ml were sampled on days 3 (day 3 LAK cells), 8 (day 5 expanded LAK cells), and day 11 (day 8 expanded LAK cells) of in vitro culture and were then assayed in a chromium release assay against a fresh NK-insensitive sarcoma, the MCA-102 tumor, and an NK-sensitive cell line, the YAC-1 lymphoma.
only one injection of the LAK cells on day 3 posttumor induction. In four consecutive experiments shown in Table 5, cells grown in AIM V medium were more effective than cells grown in CM in reducing the number of established liver metastasis.

The antitumor effectiveness of LAK cells grown in AIM V medium was higher whether the cells had been grown for 3 days (day 3 LAK cells), 8 days (day 5 expanded LAK cells), or 16 days (day 13 expanded LAK cells). For example, in Table 5, Experiment 2, while 25,000 units of RIL-2 reduced the number of metastases to 155 from 250, the addition of $1 \times 10^6$ day 3 LAK cells grown in CM reduced the number of deposits to 29, while the addition of $1 \times 10^6$ day 3 LAK cells grown in AIM V medium reduced the number of deposits to 13 ($P < 0.05$). In the same experiment, day 5 expanded LAK cells grown in CM or in AIM V medium reduced the number of metastatic deposits to 30 and 14, respectively ($P < 0.002$).

Thus, there was a significant enhancement of antitumor activity seen with LAK cells grown in AIM V medium compared to CM when these cells were given RIL-2.

**DISCUSSION**

Previous studies have demonstrated that the in vivo administration of LAK cells plus recombinant interleukin 2 could lead to marked decreases in the number of established pulmonary and hepatic metastases induced from various tumors (1, 2, 4, 5). Similar results have been documented in human studies with selected tumors (13). In humans, LAK cells have been generated in medium containing 2% human AB serum (13).

Serum is a well-known carrier for several viruses, some leading to infections, at times fatal, such as hepatitis or AIDS (16). Thus, any investigations into serum-free medium for growth of cytotoxic cells might have long-term benefits for patients who have been selected to receive adoptive immunotherapy with cultured lymphocytes.

In this paper, we have analyzed the potential usefulness of a new serum-free medium (AIM V medium; Gibco) for the generation and expansion of murine LAK cells.

As shown in Table 1, in a series of 6 experiments, the mean percentage of yield on day 3 for LAK cells cultured in CM was of the order of 25%. However, when cells were grown in AIM V medium, the mean percentage of yield increased to 59%, double the viability expected from cells grown in CM.

When day 3 LAK cells cultured either in CM or in AIM V medium were put back into similar media in roller bottles, optimal expansion of LAK cells was seen with cells grown in serum-free medium (Table 2). The mean fold expansion for cells grown in AIM V medium was 201 compared to 55 for cells grown in CM ($P < 0.002$) on day 21 of culture.

When the cells generated and expanded in both media were evaluated for in vitro cytotoxicity in a $^{32}P$ release assay, it was noticed that LAK cells generated in AIM V medium were more cytotoxic than cells grown in CM, at least for the cell lines tested (Fig. 1). This finding was true both for a fresh NK-insensitive sarcoma target and a NK-sensitive lymphoma target.

Day 3 LAK cells generated in CM and expanded in similar medium demonstrated increasing cytotoxicity against a fresh target up to 8 days in culture, at which time they would then lose their in vitro cytotoxicity as described previously (14). Interestingly, cells generated in AIM V medium and expanded in the same medium maintained their cytotoxicity against a fresh target. Against an NK-sensitive target, both groups of cells demonstrated equal cytotoxicity. Thus, not only did the AIM V medium lead to a greater number of cells, it also led to cells which demonstrated greater lysis against a fresh target early and late during LAK cell generation and expansion compared to cells grown in CM. This information presented in terms of lytic units/10⁶ cells and in total lytic units is shown in Table 3. The increase in cytotoxicity seen with day 3 LAK cells grown in AIM V medium could partially be explained by the fact that a greater percentage of cells grown in AIM V medium expressed the Lyt-2 antigen compared to cells grown in CM (49 versus 36%; Table 4). However, since both groups had an equal percentage of cells expressing the same antigens on day 8 of expansion (11 days in culture), we cannot explain why the expanded LAK cells harvested from the AIM V medium maintained a higher cytotoxicity against a fresh tumor target compared to cells grown in CM.

To demonstrate the antitumor effectiveness of LAK cells generated in AIM V medium we infused them along with RIL-2 to animals harboring hepatic metastases and compared their antitumor effectiveness to LAK cells grown in CM. As can be seen from Table 5, the antitumor therapy seen with cells grown in AIM V medium on days 3, 8, and 16 of culture was significantly better than that seen with LAK cells grown in CM.

Results reported herein confirm that the use of a serum-free medium can lead to the generation and expansion of LAK cells. Such expansion is far superior in terms of numbers of cells generated and in terms of in vitro cytotoxicity compared to LAK cells grown in CM; but even more important is that these cells can maintain an in vivo cytotoxic activity against established hepatic metastases which is superior to that seen with

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**Table 5**

**Effectiveness of day 3 LAK cells and days 5 and 13 expanded LAK cells grown in various media and given with RIL-2 against established day 3 MCA-105 liver metastases**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>(A) HBSS</th>
<th>(B) RIL-2</th>
<th>Day 3 LAK cells grown in</th>
<th>Day 5 expanded LAK cells grown in</th>
<th>Day 13 expanded LAK cells grown in</th>
</tr>
</thead>
<tbody>
<tr>
<td>(C) CM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(D) AIM V</td>
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<td></td>
</tr>
<tr>
<td>(E) CM</td>
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</tr>
<tr>
<td>(F) AIM V</td>
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*a On day 0, 3 x 10⁶ MCA-38 tumor cells were injected intrasplenically. The number of mice per group was 6. Livers were removed 14 days after tumor induction.

*b Statistical significance of differences: Experiment 1: A versus B, P < 0.05; C, P < 0.001; D, P < 0.001; E, P < 0.001; F, P < 0.002; G, P < 0.002; H, P < 0.002; C versus D, P < 0.001; F versus P, P < 0.005; G versus H, P < 0.002; Experiment 2: A versus B, P < 0.01; C, P < 0.002; D, P < 0.001; E, P < 0.001; F, P < 0.004; G, P < 0.001; H, P < 0.005; C versus D, P < 0.001; F versus P, P < 0.002; G versus H, P < 0.002; H, P < 0.001; E, P < 0.002; G versus H, P < 0.002; Experiment 3: A versus B, not significant; C, P < 0.003; D, P < 0.002; E, P < 0.002; F, P < 0.004; G, P < 0.002; H, P < 0.002; C versus D, P < 0.001; F versus P, P < 0.003; E, P < 0.002; F, P < 0.002; G versus H, P < 0.002; H, P < 0.001; E, P < 0.002; G versus H, P < 0.002; H, P < 0.001.

*c HBSS or RIL-2 at a dose of 25,000 units i.p. 3 times/day was given from days 3 through 7 after tumor induction in experiments 1 through 4.

d Day 3 LAK cells (1 x 10⁶), day 5 expanded LAK cells, or day 13 expanded LAK cells prepared as described in “Materials and Methods” were given on day 3 after tumor induction in Experiments 1 through 4.

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LAK cells grown in medium containing serum. These results may play an important role in improving current methods of human cytotoxic cell culture utilizing medium containing serum, thus leading to a safer environment both for the workers generating these cells and for the patient receiving them.

ACKNOWLEDGMENTS

The authors wish to thank Rejeanne Barolet for her careful preparation of this manuscript.

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Immunotherapy of Murine Hepatic Metastases with Lymphokine-activated Killer Cells Expanded in Serum-free Media and Recombinant Interleukin 2

Rene Lafreniere, Knut Borkenhagen, Laurette D. Bryant, et al.


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