Analysis of Liver Lymphoid Cell Subsets Pre- and Post-in Vivo Administration of Human Recombinant Interleukin 2 in a C57BL/6 Murine System

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ABSTRACT

The systemic administration of high dose recombinant interleukin 2 (RIL-2) can mediate significant reductions in the number of hepatic metastases in a murine system. This effect is sensitive to host irradiation. Both large granular (LGLs) and small (SLs) lymphocytes have been implicated as the cells mediating the antitumor effect. Utilizing selective Percoll fractionation of liver nonparenchymal lymphoid cells, we have attempted to determine the cell types involved in tumor immunotherapy of murine liver metastases during RIL-2 administration. At a RIL-2 dose of 25,000 units given i.p. three times a day, the total number of lymphoid cells seen in murine livers reached a peak on day 6 after the onset of RIL-2 therapy, lasting up to 10 days and ranging from 25 to 29 times baseline values. Both LGLs and SLs were identified and SLs made up over one-half the cells present in murine livers. Phenotypic analysis of LGLs and SLs revealed that during exposure to RIL-2, bands 5 + 6 SLs expressed the Thy-1.2, Lyt-2, and Lyt-1 antigens to a greater degree than LGLs. LGLs exposed to RIL-2 demonstrated a decrease in the expression of the asialo Gm1 antigen during exposure to RIL-2; however, the 49H.8 antigen normally expressed on natural killer cells and not on circulating T-cells was found only on LGLs. The role of murine liver LGLs and SLs needs to be further characterized.

INTRODUCTION

In 1976 Morgan et al. (1) described the phenomenon whereby mitogen stimulated T-cells produced a factor in vitro that could mediate the selective expansion of T-cells obtained from bone marrow (2). This factor initially termed T-cell growth factor and subsequently renamed interleukin 2, is an important mediator of immune responses in vivo and in vitro in all animal systems (2-6).

The recent availability of large amounts of RIL-2,3 with known biological activity (7) has made it possible to study the in vivo effects of this molecule. The in vivo administration of interleukin 2 has been shown to have a wide variety of immunological effects in animals including the induction of specific T-helper cells, cytotoxic cells, and autoantibody production in nude mice (8-10), the restoration of in vivo immune functions in irradiated rats (11), in aged mice (12), or in mice treated with cyclophosphamide (13). In addition, IL-2 can specifically enhance alloimmune responses in either normal or primed mice in an antigen specific fashion (14, 15) and when given systemically to mice, has also been reported to induce a polyclonal IgM response (16).

It has been shown recently that high doses of recombinant interleukin 2 administered to mice can mediate the regression of established pulmonary (17, 18) and hepatic (19, 20) metastases from certain tumors, and that these cells appear to bear on their surface the Thy-1 antigen as demonstrated by Ettinhaus et al. (21). Recently however, Wiltrout et al. (22) reported on the presence of LGLs obtained from the parenchyma of murine livers that also bore on their cell surface both the Thy-1 and asialo Gm1 antigens.

In the present paper, we describe the nature of the lymphoid cells infiltrating murine livers. Specifically we report on the isolation, surface phenotype, growth characteristics, and cytotoxic activity of lymphoid cells obtained from livers of mice before and after treatment with high dose RIL-2.

MATERIALS AND METHODS

Animals. C57BL/6 mice, 12 weeks old or older, were obtained from the Animal Resources Center of the University of Calgary, Calgary, Alberta, Canada. These animals were immediately housed in a laminar flow caging system and tested for murine hepatitis virus on arrival and every month thereafter using standard enzyme linked immunosorbent assay methods. Results described in this paper are from mice that were and remained murine hepatitis virus negative. These animals were fed standard mouse chow and water ad libitum and were used in all in vivo experiments.

Tumor Cell Lines. Tumors used in these studies were (a) the YAC-1 lymphoma of A/Sn origin (American Type Culture Collection, ATCC TIB-160; Rockville, MD) maintained in continuous in vitro culture and harvested as necessary. This cell line has been shown to be sensitive to the cytotoxic activity of NK cells in mice (23); and (b) the MCA-102 sarcoma (a gift from Dr. S. A. Rosenberg, National Cancer Institute, NIH, Bethesda, MD) induced by Parker and Rosenberg in 1977 (24) which is resistant to lysis by NK cells in vitro (25).

Single cell suspensions of the MCA-102 sarcoma were prepared as described previously (26).

Complete Medium. Complete medium consisted of RPMI 1640 (Flow Laboratories) supplemented with 10% fetal calf serum (Gibco/BRL, Burlington, Ontario, Canada) and 0.03% fresh glutamine, 100 U/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 gentamycin (all from Flow Laboratories), and 5 × 10^-5 M of 2-mercaptopethanol (Aldrich, Milwaukee, WI).

RIL-2. Human recombinant interleukin 2 was kindly provided by the Cetus Corp. (Emeryville, CA) and its biological and biochemical properties have been characterized extensively (7). This material has been highly purified to homogeneity (27) and has a specific activity of 4 to 8 × 10^6 units/mg (28). In proliferative assays, approximately 1 unit of Cetus RIL-2 is equivalent to 2.3 units of the biological response modifiers program IL-2 standard (18).

Isolation of Effector Cells from Liver. Lymphocytes were isolated from murine livers by using the method of Richman (29) with some modifications. Mice were killed by cervical dislocation. The peritoneal cavity was then sterilized exposed and the portal vein was isolated and cut, the inferior vena cava was then exposed and cannulated with a 27-gauge needle, and 10 ml of RPMI 1640 with 5% fetal calf serum at room temperature were then injected to flush blood out of the liver in a retrograde fashion through the hepatic veins and out of the portal vein. When properly performed, the livers were completely blanched. This method was very effective in eliminating circulating RBC and did not affect the overall number or composition of the liver lymphoid cells

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3 The abbreviations used are: RIL-2, recombinant interleukin 2; LGLs, large granular lymphocytes; SLs, small lymphocytes; HBSS, Hanks' balanced salt solution; PBS, phosphate buffered saline; FITC, fluorescein isothiocyanate; FCA, flow cytometry analysis; IL-2, interleukin 2; NK, natural killer; TID, 3 times a day; IdUrd, 5-iodo-2'-deoxyuridine.

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when flushed and unflushed livers were analyzed for lymphoid cell content and phenotype in side by side experiments (data not shown). Any parts of the livers which were not completely blanched were discarded. The livers were then excised and their gall bladders were crushed; they were then weighed after being blotted dry with sterile gauze. Next, the livers were minced with scissors into pieces measuring 1 to 2 mm and then stirred in a triple enzyme solution for 3 h at room temperature, as described above for tumor digestion. Using this method, no undigested liver remained at 3 h. The liver digest was then filtered through NiteX (Lawshe Industrial Co., Bethesda, MD) and washed twice with HBSS at room temperature. The enzymatically digested livers were then resuspended in 50% metrizamide (Sigma) in HBSS at 4°C at a final ratio of 7 parts metrizamide to 5 parts of packed liver cells. Three ml of the mixture was then transferred into 15-ml conical tubes (Corning Glass Works, Corning, NY) and overlaid with 1.5 ml of PBS (Flow Laboratories). This metrizamide gradient was then centrifuged at 400 × g for 20 min at 4°C. The nonparenchymal cell layer was then carefully removed from the metrizamide-PBS interface with a 1-ml pipet, the cells were washed in HBSS, and the remaining erythrocytes were then lysed by incubating the cells for 1 min in a 8.5% buffered ammonium chloride solution at room temperature. The remaining cells were then washed twice more in HBSS at room temperature. The liver nonparenchymal cells were then separated into LGLs and SLs by a modification of a separation procedure previously described for human lymphocytes (30), but without passage on a nylon wool column as described by Richman et al., as side by side experiments with and without nylon wool columns had previously shown no differences in our results with cell number, cell phenotype, and cytotoxic activity (data not shown). Briefly, nonparenchymal cells at 5 × 10⁵ cells/gram were then fractionated at 300 × g for 1 h at room temperature on a 7-step discontinuous density gradient of Percoll (Pharmacia Canada Inc., Dorval, Quebec, Canada) (osmolality, <25 mosmol/kg of H₂O) at concentrations of 20, 40, 50, 60, 70, and 100%, LGLs were collected from fractions 2 and 3, representing the interphases of 20/40 and 40/50 concentrations of Percoll (uppermost low density fractions), and SLs were collected from fractions 4, 5, and 6, representing the interphases of 50/60, 60/70, and 70/100 concentrations of Percoll (high density bottom fractions). Cell preparations were evaluated for morphology by microscopic analysis of May-Grünwald stained cytocentrifuge preparations under blinded conditions by one of us (M.-C. P.), using standard criteria for classification of LGLs, macrophages, and T-cells; the composition of the nonparenchymal cells after Percoll fractionation was ascertained to be mostly lymphocytes. Less than 5% of all cells from each band was morphologically characterized as a macrophage of a Kupffer cell.

Assay for Cell-mediated Cytotoxicity Determined by Chromium Release. Cells obtained from the MCA-102 sarcoma or the YAC-1 lymphoma were labeled with ⁵¹Cr (NEN Dupont, Lachine, Quebec, Canada) as described previously (26). Spontaneous release of ⁵¹Cr was measured in complete medium and maximum release in 0.1 N HCl (Sigma). The percentage of cytotoxicity was determined by the following formula:

\[
\text{Experimental cpm} - \text{spontaneous cpm} \times 100
\]

\[
\text{Maximal cpm} - \text{spontaneous cpm}
\]

Immunofluorescence Experiments. Cell surface phenotyping of LGLs and SLs was carried out through indirect immunofluorescence, using a 50-H cytofluorograph interfaced to a Model 2150 computer system (Ortho Diagnostics Systems, Inc., Westwood, MA). Single cell suspensions of LGLs and SLs obtained from bands 2 and 3, and bands 4, 5, and 6, respectively, were incubated for 30 min at 4°C with 10 to 50 µl (depending on the source and concentration) of the following antibodies, either directly conjugated with (a) FITC: anti-Thy-1.2, anti-Lyt-2, anti-Lyt-1 (Becton Dickinson, Mountain View, CA); or with (b) phycoerythrin: anti-L3T4 (Becton Dickinson), or with the following purified unconjugated antibodies: anti-Ly-6.2, anti-Ly-21,2, anti-Ly-15.2, anti-Qa2 (Australian Monoclonal Development, New South Wales, Australia), anti-Ia (clone 25-9-17, IgG2a), a gift of Dr. James Yang, National Cancer Institute, NIH, Bethesda, MD) and anti-49H.8 (IgM, a gift of Dr. M. Longenecker, University of Alberta, Edmonton, Alberta, Canada). Unconjugated bound antibodies were detected by incubation with an appropriately labeled FITC labeled IgG FC fragment, γ chain specific goat monoclonal antibody to mouse proteins (Cappel, Malvern, PA), for 30 min at 4°C. Anti-asialo GM₁, rabbit heteroserum (Wako Chemicals, Dallas, TX) and a corresponding secondary reagent, FITC labeled sheep anti-rabbit F(ab')₂ fragments (Biosystems, Compiègne, France) were also appropriately titered and incubated with samples of LGLs and SLs for 30 min each at 4°C. The anti-Ly-6.2 antibody identifies activated 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-Qa2 antibody is linked to the murine H2 system and the Qa antigens are found on T-lymphocytes (cytotoxic and helper). The anti-Ly-15.2 antibody defines a genetic polymorphism of the lymphocyte function associated antigen 1 molecule in the mouse. This molecule is intimately involved in cytotoxic T-cell functions and this antibody binds to all T- and B-cells. The anti-49H.8 antibody identifies an antigen present on NK cells and unlike the asialo GM₁ antigen, it is not present on peripheral T-cells (31).

Statistics. Two sided P values were calculated by using the Student’s t test for paired and unpaired samples (32).

RESULTS

Characteristics of Lymphoid Cells Isolated from Murine Livers Prior to RIL-2 Administration. Base-line data prior to RIL-2 administration are shown in Tables 1 and 2. Several experiments involving 5 to 25 mice are documented. The mean weight of murine livers before RIL-2 administration was 1230 ± 103 mg (n = 10) (mean ± SEM, n = number of consecutive experiments performed, 5 animals per experiment). The mean number of lymphocytes isolated per liver was 10.5 ± 1 × 10⁸ (n = 8) (n = number of consecutive experiments performed). The mean number of lymphocytes isolated per mg of liver substance was 11 ± 1 × 10⁷ (n = 8). This was higher than reported previously by Wiltout et al. (22, 33) and is probably due to the more intense 3-h liver digestion done with stirring that we use in our laboratory, as opposed to injection of the animals because the animals used in these experiments were always pathogen free. Although Table 1 is the summary of 8 consecutive experiments, this method of liver lymphoid cell isolation has been used in over 50 experiments without any change in the efficiency or reproducibility of the procedure. The mean weight of mouse livers obtained from 6 consecutive experiments each containing 25 animals is shown in Table 2. Several experiments involving 5 to 25 mice are documented. The mean weight of mouse livers obtained from 25 livers was 1230 ± 103 mg (n = 10) (mean ± SEM, n = number of consecutive experiments performed, 5 animals per experiment). The mean number of lymphocytes isolated per liver was 10.5 ± 1 × 10⁸ (n = 8) (n = number of consecutive experiments performed). The mean number of lymphocytes isolated per mg of liver substance was 11 ± 1 × 10⁷ (n = 8). This was higher than reported previously by Wiltout et al. (22, 33) and is probably due to the more intense 3-h liver digestion done with stirring that we use in our laboratory, as opposed to injection of the animals because the animals used in these experiments were always pathogen free. Although Table 1 is the summary of 8 consecutive experiments, this method of liver lymphoid cell isolation has been used for over 50 experiments without any change in the efficiency or reproducibility of the procedure.
Utilized by one of us (A. R. A.) over 150 times with similar results (data not shown).

Lymphocytes collected from the metrizamide-PBS gradients were also separated on a 7-step discontinuous Percoll gradient as described in “Materials and Methods.” Following separation, the total cell population was divided into cells that histologically were classified as LGLs (bands 2 + 3) and SLs (bands 4, 5, and 6). Less than 5% of all cells from each band were classified as macrophages.

Cell counts and percentages of LGLs and SLs obtained are shown in Table 2. From a series of 6 consecutive experiments isolating LGLs and SLs from the substance of normal murine liver parenchyma, it was determined that LGLs outnumbered SLs by a 2.5:1 ratio, 71% of all cells recoverable from the liver being LGLs and 29% being SLs. The mean number of LGLs recovered per liver in these experiments was 7.9 x 10⁶, while the mean number of SLs was 3.2 x 10⁶ (each experiment consisted of 25 mice). These results thus demonstrate a predominance of LGLs versus SLs in base-line murine livers.

RIL-2 Administration in Vivo Leads to Presence of Large Numbers of Liver Lymphoid Cells within Murine Livers. A characteristic experiment to study the in vivo effects of RIL-2 on the lymphocyte subpopulations in murine livers is shown in Table 3. RIL-2 was injected i.p. 3 times a day at a dose of 25,000 units/injection from days 0 through 6 and mice were sacrificed every other day for 18 days to measure the lymphoid cell contents in their livers. Significant increases in the number of lymphoid cells obtained per liver were seen as early as 2 days after the onset of RIL-2 administration compared to control mice (34 x 10⁶ versus 11 x 10⁶, day 2 versus day 0, respectively; Table 3). This expansion of lymphoid cells reached a plateau within 2 days of RIL-2 administration, this ratio changed to 55 versus 45% (P < 0.5 compared to control). This change, seen early during RIL-2 administration (days 2 to 4), was due to a doubling in the number of SLs (SLs x 10⁶: 2, 13, and 14 on days 0, 2, and 4, respectively). Subsequently the maximal number of LGLs on day 6 was 14.5 times that of control livers (116 x 10⁶ cells versus 8 x 10⁶), and the maximal number of SLs was 54 times that of control livers (109 x 10⁶ cells versus 2 x 10⁶ cells). However, for SLs, this was seen to occur on day 10 instead of on day 6 for LGLs. The LGLs:SLs ratio on day 10 was also reversed to 39% for LGLs and 61% for SLs. While the number of SLs was still elevated on day 14 of the experiment (24 x 10⁶), the number of LGLs had now returned close to base-line levels (17 x 10⁶). By day 18, SLs had also returned to base-line levels returning the LGLs:SLs ratio to a preexperimental value of 78:22. Thus despite an initial increase in LGLs, it appears that a large proportion of the lymphoid infiltration seen in murine livers during RIL-2 administration is due to a significant contribution by SLs (54 times base-line values versus 14.5 for LGLs, P < 0.001).

Histological sections obtained during RIL-2 administration revealed that most of the lymphoid cell infiltration was periportal in location and was distributed between the cords of liver cells and the sinusoidal spaces. Both large and small lymphoid cells could be seen at high power with a preponderance of small cells appearing late during RIL-2 administration (Fig. 2).
Fig. 1. Female C57BL/6 mice were given injections of 25,000 units of RIL-2 i.p. TID for 6 days. On day 10, livers were harvested, fixed in 10% formalin, and sent for histological analysis. Shown are the central vein (dark arrow) and the hepatocyte necrosis seen with high dose RIL-2 (open-ended arrow). Notice that the liver sinusoids are filled with small dark nucleated cells.

Table 4 Contribution of LGLs and SLs to overall infiltration of murine livers during RIL-2 administration

<table>
<thead>
<tr>
<th>Day of data collection</th>
<th>Bands 2 + 3 LGLs/liver (mean × 10^6)</th>
<th>Bands 4 + 5 + 6 SLs/liver (mean × 10^6)</th>
<th>% of all cells recovered</th>
<th>Total cells/liver (mean × 10^6)</th>
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<tbody>
<tr>
<td></td>
<td>LGLs</td>
<td>SLs</td>
<td>LGLs</td>
<td>SLs</td>
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<tr>
<td>2</td>
<td>15</td>
<td>13</td>
<td>55</td>
<td>45</td>
</tr>
<tr>
<td>4</td>
<td>26</td>
<td>14</td>
<td>66</td>
<td>34</td>
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<tr>
<td>6</td>
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<td>84</td>
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<td>18</td>
<td>7</td>
<td>2</td>
<td>78</td>
<td>22</td>
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* Female C57BL/6 mice were given RIL-2 i.p. TID from days 0 through 6, livers were then harvested every other day from days 0 through 18, and processed as described in "Materials and Methods."

* Liver nonparenchymal lymphoid cells were separated on a 7-step discontinuous Percoll gradient for 1 h. Bands 2 + 3 cells representing LGLs were harvested and counted, each value representing the mean number of cells per liver obtained from each group of animals.

* After Percoll separation SLs were harvested from bands 4, 5, and 6 and counted. Each value represents the mean number of SLs obtained per mouse liver. Each group contained 5 animals.

* Mean number of liver lymphoid cells recovered on each day of harvest; each group consisted of 5 animals.

In Vivo Administration of RIL-2 Leads to Phenotypic Changes within Lymphoid Cell Subpopulations of Liver. Animals receiving RIL-2 at a dose of 25,000 units i.p. TID had their livers harvested pre-RIL-2 and then every other day during and post-RIL-2 administration. Their liver lymphoid cells were analyzed phenotypically as described in "Materials and Methods." Tables 6, 7, and 8 describe one characteristic experiment wherein each value is the mean of a triplicate measurement. In Table 6, results of the effects of RIL-2 on LGLs are documented. Of note is the increase in Thy-1.2 expression from 18% on day 0 to 63% on day 6 with no increase in the Lyt-2 and Lyt-1 antigens and a decrease in the L3T4 and asialo GM1 antigens. The Ly-21.2, Ly-15.2, and Qa2 antigens all increased, while the Ly-6.2 differentiation antigen remained the same. The 49H.8 antigen defining NK cells similarly increased from 12 to 75% on day 8.

Bands 4, 5, and 6 SLs shown in Tables 7 and 8 demonstrated a marked increase in the Thy-1.2 antigen, but only bands 5 and 6 SLs had an increase in the Lyt-2 antigen. The Lyt-1 antigen increased in both bands, perhaps representing activation by RIL-2, and the asialo GM1 antigen also increased in bands 5 and 6 SLs, while the band 4 SLs demonstrated a picture which was more closely associated with that seen with bands 2 and 3 LGLs. The differentiation antigens Ly-21.2, Ly-15.2, Ly-6.2, and Qa2, and the NK antigen 49H.8 were expressed to a similar extent on bands 4, 5, and 6 SLs. These findings led us to believe that perhaps band 4 SLs were early precursors to NK or T-cells or could possibly represent a population of cells distinct from both.

In Vitro Incubation of Liver Lymphoid Cell Subpopulations with High Dose RIL-2 Induces Expression of T-Cell Antigens on Band 2 + 3 LGLs. In a separate series of experiments, Percoll enriched LGLs and SLs were then combined into LGLs classified cytologically as LGLs. Similarly, bands 5 and 6 represent SLs cytologically that also bear the same phenotypic antigens. However, despite being SLs cytologically, the band 4 lymphoid subpopulation appears to be distinct phenotypically from bands 5 and 6 lymphocytes, and may represent a different subpopulation of small lymphoid cells.
Fig. 2. Female C57BL/6 mice were given injections of RIL-2 at a dose of 25,000 units i.p. TID for 6 days and their livers were then harvested and fixed in 10% formalin and sent for histological analysis. Two central veins can be seen (dark arrows). Note that the liver sinusoids are filled with small nucleated cells (open-ended arrow).

Table 5 Nonparenchymal lymphoid cells obtained through Percoll separation express differential cell surface phenotypes

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<thead>
<tr>
<th>Antigen</th>
<th>% of cells expressing antigen</th>
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<td>Thy-1.2</td>
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<tr>
<td>Ina</td>
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<tr>
<td>49H8</td>
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Table 6 Modulation of phenotypic antigenic expression of livers band 2 + 3 LGLs by RIL-2 administered in vivo

<table>
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<tr>
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<th>Day 6</th>
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Table 7 Modulation of phenotypic antigenic expression of livers band 4 small lymphocytes by RIL-2 administered in vivo

<table>
<thead>
<tr>
<th>Antigen</th>
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<td>Lyt-2</td>
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<td>L3T4</td>
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<td>Lyt-1</td>
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<td>Ly-21.2</td>
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Table 8 Modulation of phenotypic antigenic expression of livers bands 5 + 6 SLs by RIL-2 administered in vivo

<table>
<thead>
<tr>
<th>Antigen</th>
<th>% of bands 5 + 6 SLs expressing antigens</th>
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<tr>
<td>Thy-1.2</td>
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<td>Lyt-2</td>
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Results of a typical representative experiment are shown in Table 9. Most of the major phenotypic changes occurred by day 8 of culture. Of interest is that the LGL subpopulation began expressing high levels of the Thy-1.2 antigen compared to day 0 LGLs (77 versus 28%, P < 0.001, for day 8 and day 0 LGLs, respectively). In addition the expression of the Lyt-2
and the asialo G_{M1} antigens increased to a maximum of 43 and 82%, respectively, on day 8 of culture. Of even greater interest is that bands 4 and 5 + 6 SLs although expressing the Thy-1.2 antigen on 97% of all cells on day 8 of culture, also expressed the asialo G_{M1} antigen on 80 and 70% of all cells for band 4, and bands 5 and 6 SLs, respectively, while the Lyt-2 marker was expressed on 68 and 74% of all cells for band 4, and bands 5 and 6 SLs, respectively. The Lyt-1 antigen, a T-cell antigen associated with helper functions, was mostly expressed on day 3 for band 4 and bands 5 + 6 SLs (65 and 75%, respectively) while remaining relatively stable in bands 2 and 3 LGLs (21 and 34% on days 3 and 8, respectively). Of interest on day 8 of culture were the findings that the Ly-21.2 differentiation antigen and the Ly-15.2 cytotoxic T-cell antigen were mostly expressed on cells obtained from bands 2, 3, and 4 compared to cells from bands 5 and 6. Ly-21.2 (85 and 87% for bands 2 + 3 and 4 versus 65% for bands 5 + 6), Ly-15.2 (80 and 82% for bands 2 + 3 and 4 versus 63% for bands 5 + 6). The Ly-6.2 antigen expressed on activated lymphoid cells was identified on 97% of all cells on day 8 of culture, also expressed the Thy-1.2 antigen on 97% of all cells on day 8 of culture, also expressed the asialo G_{M1} antigen on 80 and 70% of all cells for band 4, and bands 5 and 6 SLs, respectively.

During administration of RIL-2, on day 3 one could see a minimal elevation of cytotoxicity against the MCA-102 target in bands 5 and 6 with similar findings against the YAC-1 target. However, no cytotoxicity was seen other than background NK lysis for band 4 lymphocytes and bands 2 and 3 lymphocytes. On day 8 of the experiment, the band 2 and 3 LGLs expressed the highest cytotoxicity against the YAC-1 target with 60% lysis at the 100:1 effector:target ratio, while little lysis was seen against the MCA-102 target. Band 4 SLs demonstrated little lysis against both targets while bands 5 and 6 SLs demonstrated 50% lysis against the MCA-102 target and 70% lysis against the YAC-1 target. Thereafter on days 13 and 18, the cytotoxicity disappeared rapidly, presupposing that RIL-2 was necessary for the induction of cytotoxicity seen in murine livers.

Cytotoxicity of Murine Liver Lymphoid Subpopulations Activated in Vitro with RIL-2. It is known that peripheral blood lymphocytes or splenocytes can be incubated in vitro with RIL-2 and will generate cells capable of lysing NK resistant targets (34). In vitro assays of cytotoxicity were similarly performed with cells isolated from Percoll fractionated murine liver lymphocyte subsets. Percoll fractions of liver nonparenchymal lymphoid cells enriched for LGLs (bands 2 + 3) and SLs (bands 4 and bands 5 + 6) were treated with various monoclonal and polyclonal antibodies on day 0 of harvest and after 3, 8, and 70 days of in vitro culture in complete medium containing RIL-2 at a final concentration of 1,000 units/ml as described in "Materials and Methods."
The antitumor effects of high dose RIL-2 have been shown to be totally abrogated when RIL-2 is given to sublethally irradiated mice (17–19), and this decrease in antitumor activity in irradiated mice was subsequently shown by Ettinghausen et al. (21) to be secondary to a lack of proliferation of endogenous lymphoid elements in the irradiated animals. This suggested that IL-2 was not mediating tumor regression directly but rather was acting through a host component. Ettinghausen et al. (21) also went on to show in mice that the systemic administration of high doses of RIL-2 led to the generation of lymphoid cells that bore the Thy-1 antigenic marker and that these cells could also express cytotoxicity against fresh tumor targets in a fashion similar to what had been shown previously for LAK cells (34).

Earlier studies of the in vivo antitumor effectiveness of RIL-2 have shown that at higher doses, this lymphokine is capable of significantly reducing the number of artificially induced pulmonary and hepatic micrometastases, as well as s.c. implants from a variety of tumors (17, 19, 20). More recently, clinical trials have shown that the administration of high dose RIL-2 in humans can result in significant regressions of certain systemic tumors as well (35–37).
population, raising a question as to the adequacy of measuring induced proliferation with $[^{3}H]$IdUrd incorporation, or that (b) the cells that infiltrate the liver actually come from other lymphoid compartments as recently determined by Wiltrout et al. in mice receiving MVE-2 (33). Similar studies as done by Wiltrout with two color labeling with propidium iodide will be part of a future communication on bands 2 + 3 LGLs.

In an attempt to identify and compare the cellular components possibly involved in the reduction of hepatic metastasis by RIL-2, the liver lymphoid populations were separated on Percoll gradients every other day from days 0 through 18, while RIL-2 was administered i.p. TID at a dose of 25,000 units for 6 days. In these experiments, an early increase of both SLs and LGLs could be seen within 4 days of RIL-2 administration (Table 4) (SLs day 0, $2 \times 10^4$, day 2, $13 \times 10^4$, day 4, $14 \times 10^4$, respectively; LGLs days 0, $8 \times 10^4$, day 2, $15 \times 10^4$, day 4, $26 \times 10^4$). Although the average LGLs:SLs ratio in control animals was 71:29, this ratio quickly changed and was reversed on day 8 of the experiments, achieving a maximal reversal on day 10, with 39% of the cells being LGLs while 61% were SLs (Table 4).

The increase in the number of lymphoid cells was documented to be secondary to an increase in the number of SLs which demonstrated an increase of 54 times over control on day 10 versus 14.5 times over control for the LGLs on day 6. These results demonstrated that the presence of maximal numbers of SLs occurred late during RIL-2 administration (days 8 to 10), while the presence of LGLs occurred a few days earlier (days 6–8).

When cells from each band were characterized phenotypically through the use of monoclonal and polyclonal antibodies directed against murine lymphoid antigens, it was found that LGLs from bands 2 and 3 represented similar cells with a low Thy-1.2 antigenic expression. Of these LGLs, 75% expressed the asialo GMI marker and 55% expressed the L3T4 antigen (Table 5). These phenotypic analysis results are not single occurrence findings. Results of over 150 liver harvest experiments have confirmed the same L3T4 positivity on cells of bands 2 + 3. These cells were classified blindly by one of us (M-C. P.) as LGLs morphologically. However, the L3T4 antigen also occurs on some myeloid cells including monocytes, as reviewed recently by Lanier et al. (40) and CD4, the human equivalent of L3T4, is also reportedly expressed on bone marrow derived macrophage precursors. In view of these findings, we are continuing our work with phenotyping of bands 2 + 3 LGLs with antimonocyte antibodies, in particular the F4/80 monoclonal antibody. It is known that macrophage precursors and LGLs are indistinct morphologically and that both can lyse the YAC-1 target and both can demonstrate an increase in cell numbers and cytotoxic potential with the administration of biological response modifiers in mice (41). So it is possible that the changes reported by Wiltrout et al. (22, 33) with the administration of biological response modifiers, may not be secondary to LGLs but could be a combination of LGLs and macrophage precursors; this is presently being studied in our laboratory.

Two separate subpopulations of SLs were identified, band 4 SLs expressing little Thy-1.2 antigen (32%) and Lyt-2 antigen (11%) on their cell surface, and bands 5 + 6 SLs with a higher expression of both antigens (68 and 34%) for Thy-1.2 and Lyt-2 antigens, respectively. Thus a subpopulation of T-lymphocytes appears to be present in the SL subpopulation of nonparenchymal liver lymphoid cells and the role of this subpopulation is unknown at this time.

Etinghousen et al. (21) described that lymphoid cells from the liver demonstrate the Thy-1 antigen by indirect immunofluorescence. Wiltrout et al. (22) also identified the Thy-1 antigen on the LGL subpopulation of liver lymphoid cells. It was thus important to evaluate in vivo and in vitro the phenotypic characteristics of each subpopulation of liver lymphoid cells during exposure to RIL-2. As shown in Tables 6–8 the bands 2 + 3 LGLs expressed the Lyt-2 marker to a limited degree during RIL-2 administration; however, the NK antigen 49H.8 was highly enhanced and this increase in the expression of the 49H.8 antigen correlated well with the increasing YAC-1 lysis seen in Fig. 3 for bands 2 + 3 LGLs. Despite the fact that the Thy-1.2 antigen increased, there were no increase in the expression of the Lyt-2 or L3T4 antigens and very little cytotoxicity if any was seen against the fresh tumor target for bands 2 + 3 LGLs. An interesting find was the loss of the asialo GMI marker on the LGLs during the RIL-2 administration in vivo, perhaps reflecting a change in the bands 2 + 3 cells phenotypically rather than morphologically. Band 4 lymphoid cells had no increase in Lyt-2, L3T4, asialo GMI, or 49H.8 antigenic expression and demonstrated little cytotoxicity against the cultured and fresh targets. Bands 5 + 6 SLs had a marked increase in the Lyt-2 and asialo GMI antigen expression, perhaps reflecting their activation by RIL-2 into LAK cells which could lyse both the cultured and fresh tumor targets. There were no increase of the 49H.8 antigen on these cells with RIL-2 administration. During in vitro cultures, results were different with all cells from all bands acquiring similar overall phenotypic labels, perhaps reflecting secondary changes from a very high dose of RIL-2 present in the culture media selecting out certain cellular subtypes. Despite these findings, cells from bands 4, 5, and 6 SLs demonstrated the greatest expression of Lyt-2 and asialo GMI, antigen on day 8, which was also reflected as shown in Fig. 4 by an increase in lysis of both tumor targets.

The in vivo phenotype and cytotoxic findings are probably more representative of the in vitro effects of RIL-2 and do demonstrate a possible role for both bands 2 and 3 LGLs and bands 5 and 6 SLs in tumor immunotherapy with RIL-2 in murine livers.

The experimental results reported herein attempted to define the role of LGLs and SLs in the antitumor therapy of liver metastases induced by RIL-2. There appears to be a role for both LGLs and SLs, and two subpopulations of SLs exist, although bands 5 + 6 SLs are probably more relevant than band 4 SLs in the overall picture seen with in vivo RIL-2. The fact that most of the liver lymphoid cells seen with RIL-2 result from an increase in the number of SLs possibly indicates a very important role of the SLs subpopulation in tumor eradication. Further studies are needed to determine the role of the LGLs and SLs subpopulations in actual in vivo tumor experiments with high dose RIL-2.

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* Dr. Larry Gilbert, University of Alberta, personal communication.


Analysis of Liver Lymphoid Cell Subsets Pre- and Post-in Vivo Administration of Human Recombinant Interleukin 2 in a C57BL/6 Murine System

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


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