ABSTRACT

We examined: (a) whether in vitro-generated lymphocyte-activated killer (LAK) cells from normal mice and splenical killer cells from tumorbearing mice subjected to interleukin-2 (IL-2) therapy alone or in combination with chronic indomethacin therapy have any detrimental effects on the spleen colony-forming units (CFU-S) of the normal bone marrow (BM); and (b) the effects of these immunotherapy protocols on BM cell numbers in host hematopoietic organs. Effects of in vitro-generated LAK cells (normal C3H/HeN mouse splenocytes cultured with 1000 units IL-2/10^6 cells for 72 h) on BM CFU-S were examined by incubating macrophage-depleted BM cells with LAK cells at 1:2.5 and 1:5 BM:LAK cell ratios or with LAK cell supernatant for 4 h. The cells were washed and subsequently injected into irradiated mice. Irradiated mice were also reconstituted with BM cells or LAK cells incubated alone. Spleen colonies were scored macroscopically and microscopically on day 7 after reconstitution of lethally irradiated mice with the various cell combinations. A comparison of colony numbers produced by LAK and BM cell mixture revealed that LAK cells at either dose had no suppressive effect on the colony-forming ability of BM at the macroscopic and microscopic levels of analysis. The supernatant of cultured LAK cells had a minor suppressive effect on colony formation at the macroscopic but not the microscopic level of analysis, indicating the presence of one or more suppressive factors capable of mediating a short-term inhibitory effect. In the immunotherapy experiment, C3H/HeN mice transplanted s.c. with 5 x 10^5 C3L5 mammary adenocarcinoma cells received either vehicle alone (controls), IL-2 (1.5 x 10^4 Cetus units i.p. every 8 h on days 10-14 and days 20-25), or chronic indomethacin therapy (10 µg/ml in drinking water from day 5 onwards) plus IL-2 as above. Animals were killed 24-25 days after tumor transplantation to examine: (a) the number of metastatic lung nodules; (b) the effects of co-incubating therapy-generated splenical effector cells with normal BM cells for 4 h on BM CFU-S, and (c) the CFU-S content of host BM and spleen. Results revealed a drop in spontaneous lung metastases from a mean of 50 in control mice to 18 with IL-2 therapy alone, and in 50% (macroscopic) and 75% (microscopic) of the controls, and a simultaneous rise in the splenic CFU-S content to 370% and 250%, respectively, indicating a CFU-S redistribution from the BM to the spleen and then expansion in the spleen. The computed total CFU-S number in the body rose by 30%. Immunotherapy augmented these findings, our laboratory devised an immunotherapy protocol including CIT initiated early during primary tumor development has been shown to cause regression of a number of primary tumors (16, 17) and prevent spontaneous metastasis of a highly metastatic mammary adenocarcinoma (16). However, this therapy alone is unable to cure advanced metastasis, most likely because of inadequate levels of endogenous IL-2 required for optimal activation of IL-2-dependent killer cells (18). Based on these findings, our laboratory devised an immunotherapy protocol combining CIT with multiple rounds of IL-2, using IL-2 dose levels similar to those reported by the Rosenberg group for tumor immunotherapy in mice (19). This protocol has been successful in curing advanced metastasis in various tumour-host models: experimental lung metastases of B16F10 melanoma in C57BL/6 mice (20) and KHT35-L1 fibrosarcoma in C3H/HeJ mice (21), experimental and spontaneous lung metastases of C3L5 mammary adenocarcinoma in C3H/HeJ mice (12), experimental lung (22), and multiorgan (23) metastases of human melanomas in BALB/c nude mice. In all these models, highly numbers was seen, despite a minor induction of CFU-S suppressor cells in the spleen of tumor-bearing hosts, which persisted during the immunotherapy.

INTRODUCTION

Despite the presence of various effector cells with potential tumoricidal function in the host, many tumors develop, grow, and eventually metastasize to distant sites. This is because host defense can be circumvented by the tumor in a variety of ways. One mechanism has recently been recognized as a general phenomenon for many host-tumor systems: an inactivation of the host killer cell function by tumor-derived or host-derived PGE2 (1). With progressive tumor growth, host macrophages are triggered to produce elevated amounts of PGE2 (1-3) which, in turn, prevents activation of NK cells (2, 4), LAK cells (2), cytotoxic T cells (2), and tumoricidal macrophages (2). The subpopulation of PGE2-producing macrophages in the normal host is I-A negative (5), and tumor bearing leads to a preponderance of this subset in host lymphoid organs as well as the tumor site (6). Several mechanisms of PGE2 action have been identified for the effector cell inactivation: inhibition of IL-2 production (7-9) and down-regulation of receptors for IL-2 (9), transferrin (8), and interferon-γ (10) on the effector cell surface. Treatment with indomethacin, a prostaglandin synthetase inhibitor, is capable of relieving these suppressive effects both in vitro (2, 4, 11) and in vivo in hosts with developing tumors (12). High levels of tumor-derived or host-derived PGE2 can also promote metastasis by its action on tumor cells, either by promoting tumor cell migration or dissemination (13, 14) or by increasing NK cell resistance of tumor cells (15).

CIT initiated early during primary tumor development has been shown to cause regression of a number of primary tumors (16, 17) and prevent spontaneous metastasis of a highly metastatic mammary adenocarcinoma (16). However, this therapy alone is unable to cure advanced metastasis, most likely because of inadequate levels of endogenous IL-2 required for optimal activation of IL-2-dependent killer cells (18). Based on these findings, our laboratory devised an immunotherapy protocol combining CIT with multiple rounds of IL-2, using IL-2 dose levels similar to those reported by the Rosenberg group for tumor immunotherapy in mice (19). This protocol has been successful in curing advanced metastasis in various tumour-host models: experimental lung metastases of B16F10 melanoma in C57BL/6 mice (20) and KHT35-L1 fibrosarcoma in C3H/HeJ mice (21), experimental and spontaneous lung metastases of C3L5 mammary adenocarcinoma in C3H/HeJ mice (12), experimental lung (22), and multiorgan (23) metastases of human melanomas in BALB/c nude mice. In all these models, highly

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2 To whom requests for reprints should be addressed.
active NK cells, LAK cells, and killer macrophages were generated in situ. In vivo depletion of such killer cells led to a complete annulment of the therapeutic effects (20).

Encouraged by these findings in mice, and the simplicity of the protocol, a phase 2 human clinical trial with CIT combined with venous infusions of IL-2 have recently been conducted locally in patients with advanced melanoma (24, 25) and renal cell carcinoma4 (26) with promising results. It remains unknown whether IL-2 or CIT combined with IL-2 therapy has adverse effects on hemopoietic stem cells as has been documented for chemotherapy and radiotherapy. Immunocompetent cells, in particular, NK and LAK cells, have been shown to exert positive (27–31), negative (27–29, 31–36), or no (27–30) effects on hemopoietic cells. The objective of this present study was, therefore, to evaluate the effects of the above protocols of tumor immunotherapy in mice on hemopoietic stem cells. We examined the effects of in vitro-generated LAK cells and therapy-generated killer cells on hemopoietic stem cells of the BM as well as the effects of these therapeutic protocols on the stem cell content of host hemopoietic organs by using the CFU-S assay.

MATERIALS AND METHODS

Mice. Eight- to 10-week-old female C3H/HeN mice (Charles River Laboratories, Wilmington, MA) were used as hosts for tumor transplantation, as sources of BM and spleen cells, and as irradiated recipients of hemopoietic cells for spleen colony assays.

Preparation of BM Cells. Epiphyseal ends of femurs were cut, and the marrow was flushed with RPMI 1640 (Flow Laboratories, Mississauga, Ontario, Canada). A single cell suspension was made by repeated aspirations of cells with a Pasteur pipette. Cells were washed, plated onto plastic Petri dishes, and incubated for 45 min with RPMI 1640 supplemented with 10% fetal calf serum (Flow) in a 37°C, 5% CO2-filled incubator to remove adherent macrophages (5). This was done to exclude the possibility of introducing inhibitory effects of macrophase-derived PGE2 on NK (16) or LAK (2) cell function or hemopoietic colony formation by BM cells (37) during the 4-h co-incubation as will be described later. Nonadherent cells were carefully collected, washed, and counted with a Coulter model B particle counter (Coulter Electronics, Inc., Hialeah, FL). Suspensions were kept on ice until use. Immediately prior to their use, the cells were washed once more in warmed RPMI 1640, counted, and tested for viability using the trypan blue exclusion test.

Preparation of Single Cell Suspension of Spleen Cells. Spleens from normal, tumor-bearing control, and tumor-bearing mice subjected to immunotherapy were removed and gently dispersed using a glass homogenizer. The cells were then washed, resuspended in RPMI 1640, counted, and tested for viability. In experiments using normal splenocytes as a source of NK cells or for generating LAK cells in vitro, red blood cells and dead cells were removed by density gradient centrifugation with modified Ficoll-Paque (1.5 g Ficoll 400/100 ml Ficoll-Paque; Pharmacia, Dorval, Quebec, Canada).

Generation of LAK Cells. Ficoll-Paque separated spleen cells were cultured with a mixture (1:1 ratio) of AIM-V medium (Grand Island Biological Co./Bethesda Research Laboratories, Burlington, Ontario, Canada) and RPMI 1640 complete medium with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml Fungizone (Flow) for 72 h at 37°C, in a 5% CO2-filled humidified incubator. Human recombinant IL-2 (lot LSP-805 with endotoxin content <8 x 10−3 ng/ml, kindly supplied by Cetus Corp., Emeryville, CA) was added at a concentration of 103 Cetus units (6 x 103 IU)/ml x/106 cells. After incubation, the cells were washed, resuspended, and tested for viability for use in spleen colony assay.

51Cr Release Assay to Measure Killer Cell Activity. Fresh and in vitro or in vivo IL-2-activated spleen cells were incubated for 4 h at various effector:target ratios with 51Cr-labeled NK-sensitive YAC-1 lymphoma cells (American Type Culture Collection, Rockville, MD) and NK-resistant but LAK-sensitive 8911 lymphoma target cells (2.5 x 105 target cells/well) as described elsewhere (38). In addition, NK-resistant C3L5 mammary adenocarcinoma cells were also used as targets for in vivo IL-2-activated splenocytes.

Tumor Transplantation. C3 is a cloned mammary adenocarcinoma line, derived in our laboratory from a spontaneous C3H/HeJ mammary tumor, which initially showed a strong ability for spontaneous metastasis to the lungs when transplanted s.c. With in vitro passage for a number of years, the metastatic ability of this line declined (16). A highly metastatic line, C3L5, was produced by 5 cycles of in vivo selection of C3 cell micrometastases to the lungs following s.c. transplantation (12). This line maintained its metastatic phenotype both in C3H/HeJ and C3H/HeN strain mice.

C3L5 mammary adenocarcinoma cells (5 x 104) were injected s.c. in the mammary line near the axilla into approximately 90 C3H/HeN female mice (day 0 of tumor transplantation).

Protocols for Immunotherapy (Fig. 1). Tumor-transplanted mice were randomly separated into 3 groups. The first group of mice (controls) received ethanol (0.2%), the vehicle used for dissolving indomethacin, as a placebo in their drinking water, which was changed every second day. The second group of mice received human recombinant IL-2 (Cetus IL-2, as specified earlier) on days 10–14 and 20–24 (or 25) of tumor growth at a dose of 1.5 x 104 Cetus units (equivalent to 9 x 104 IU) i.p. every 8 h. The third group received IL-2 as above in combination with CIT. Indomethacin (Sigma Chemical Co., St. Louis, Missouri) was added to the drinking water at a concentration of 10 μg/ml drinking water started on day 5 and IL-2 as in (B).

Preparation of BM Cells. Epiphyseal ends of femurs were cut, and the marrow was flushed with RPMI 1640 (Flow Laboratories, Mississauga, Ontario, Canada). A single cell suspension was made by repeated aspirations of cells with a Pasteur pipette. Cells were washed, plated onto plastic Petri dishes, and incubated for 45 min with RPMI 1640 supplemented with 10% fetal calf serum (Flow) in a 37°C, 5% CO2-filled incubator to remove adherent macrophages (5). This was done to exclude the possibility of introducing inhibitory effects of macrophase-derived PGE2 on NK (16) or LAK (2) cell function or hemopoietic colony formation by BM cells (37) during the 4-h co-incubation as will be described later. Nonadherent cells were carefully collected, washed, and counted with a Coulter model B particle counter (Coulter Electronics, Inc., Hialeah, FL). Suspensions were kept on ice until use. Immediately prior to their use, the cells were washed once more in warmed RPMI 1640, counted, and tested for viability using the trypan blue exclusion test.

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MO) was administered on days 5-24 (or 25) at 10 µg/ml in drinking water changed every second day with fresh solutions. Normal age-matched healthy control mice were also housed 24 to 25 days for their use as donors of hemopoietic cells in CFU-S assays when such assays were performed with experimental mice as listed above.

Parameters Measuring Effects of Immunotherapy. Since the time required to analyze parameters exceeded the course of one day, the animals were sacrificed on days 24 and 25. The parameters investigated included: measurement of primary tumor size, metastatic lung colony counts, effects of therapy-generated splenic killer cells on normal BM CFU-S, and effects of therapy on the distribution of CFU-S numbers in host hemopoietic organs (BM and spleen).

Tumor growth s.c. was measured with calipers as the average diameter of the tumor throughout days 15-24. Isolated lungs were fixed with Bouin's fixative, and the number of lung metastases was scored using a dissecting microscope.

The spleens removed on day 24 were used to determine the effects of therapy-generated splenic killer cells on the spleen colony-forming ability of normal BM cells. Spleens and femurs from mice killed on day 25 were used to examine the CFU-S numbers in these organs. The total number of nucleated cells in the spleen and per pair of femurs was determined using a particle counter. Spleens removed on day 25 were also weighed.

CFU-S Assay. The CFU-S assay designed by Till and McCulloch (39) was used. Seven-week-old C3H/HeN mice were subjected to 500-rad γ-radiation from a 60Co source (Atomic Energy of Canada, Ltd., Chalk River, Ontario, Canada) and 12 h later received injections of various hemopoietic cell populations as specified for individual experiments. Seven days after inoculation, colonies in the spleens of irradiated mice were scored, at both macroscopic and microscopic levels. Briefly, microscopic colonies were counted after spleens were processed for light microscopy with 6-µm-thick sections at 72-µm intervals and stained (40). A 7-day, rather than conventional 10-day, CFU-S analysis was utilized to permit a microscopic analysis of the colony phenotypes.

The various phenotypic colonies included CFU-E, CFU-GM, CFU-ML, CFU-MEG, CFU-MEGE, and CFU-MIX.

Effects of in Vitro-generated LAK Cells on CFU-S Content of Normal BM. In determining the effects of in vitro-generated LAK cells on CFU-S, irradiated mice were reconstituted i.v. with various cell populations that were preincubated at 37°C (in a humidified 5% CO₂-containing incubator) for 4 h and washed: BM cells alone (10⁵ cells); fresh splenocytes alone (10⁶ cells); IL-2-activated splenocytes alone (2.5 × 10⁵ or 5 × 10⁵ cells); BM cells (10⁵ cells) plus fresh splenocytes (10⁶ cells); BM cells (10⁵ cells) plus IL-2-activated splenocytes (2.5 × 10⁵ or 5 × 10⁵ cells). BM cells were also incubated with the LAK cell culture supernatant for 4 h, after which cells were washed and injected into irradiated recipients.

Effects of in Vitro Immunotherapy-generated Killer Cells in Tumor-bearing Mice on Normal BM CFU-S (Fig. 1, flow chart). This was tested by noting whether a preincubation of therapy-generated splenic killer cells with normal BM cells had any effect on CFU-S numbers in the BM. The following cell populations were incubated in vitro for 4 h at 37°C in a humidified, 5% CO₂ incubator prior to their injection into irradiated mice: normal BM cells from 7-week-old mice; spleen cells from 3 groups of tumor-bearing mice (vehicle-treated, IL-2-treated, and CIT + IL-2-treated as described earlier) and one normal healthy age-matched control group; and a combination of normal BM and spleen cells from the above groups co-incubated at a 1:2 ratio of cell numbers. A total of 9 groups of mice with 10-15 mice/group were given injections of 5 × 10⁶ BM cells/mouse for BM alone, 10⁶ cells/mouse for splenocytes alone, and a combination of the two when injected together.

CFU-S Content in the Femurs and the Splenums of Tumor-bearing Mice Subjected to Therapy (Fig. 1, flow chart). BM and spleen cells isolated from the experimental groups mentioned above were injected immediately after their isolation, without any incubation, into irradiated mice. A total of 8 groups of mice (10-15 mice/group) were given injections of either 10⁵ BM or 10⁵ spleen cells. The absolute numbers of CFU-S (macroscopic and microscopic) in the femurs and the spleens were computed from CFU-S incidence in the inocula and the total cellularity of these organs.

Statistical Evaluation. The difference in CFU-S numbers between various groups and the difference in primary tumor size from untreated and treated mice were compared by using the Student’s t test. The values obtained from the metastatic lung colony counts did not conform to a normal distribution. In this case, the nonparametric Wilcoxon rank sum test (41) was used to measure the significance of difference between control and experimental groups.

Since metastatic lung colonies were counted after removal of lungs on days 24 and 25, the Kruskal-Wallis test (41) was used to ascertain the similarity of colony numbers in lungs harvested from similarly treated mice on 2 consecutive days.

RESULTS

In Vitro-generated LAK Cells and BM CFU-S

Antitumor Cytotoxicity of Fresh and IL-2-activated Splenocytes

This was tested at effector:target ratios of 25:1, 50:1, and 100:1 (data not shown). Fresh splenocytes exhibited moderate cytotoxic activity (54%) towards the NK-sensitive YAC-1 cells at the 100:1 effector:target cell ratio but little cytotoxicity towards the NK-resistant, LAK-sensitive 8911 lymphoma cells (8.6%). Spleen cells, cultured in the presence of IL-2, showed a high degree of cytotoxicity at the same ratio towards both YAC-1 lymphoma cells (70.9%) and 8911 cells (78.2%), indicating the presence of LAK activity in this cell population.

Aliquots from the same populations of fresh and IL-2-activated splenocytes were used to test their effects on the CFU-S content of normal BM, when co-incubated in vitro.
Effects of in Vitro-generated LAK Cells on BM CFU-S

Macroscopic Analysis of CFU-S Numbers. Fig. 2 shows the number of macroscopic CFU-S colonies produced when fresh and IL-2-activated spleen cell populations were incubated for 4 h alone or in combination with BM cells and injected into irradiated recipients. In animals receiving no cell, very few CFU-S colonies appeared after 7 days (Fig. 2A). Irradiated mice given injections of fresh splenocytes (Fig. 2I) produced confluent colony numbers. When $2.5 \times 10^5$ (Fig. 2F) or $5 \times 10^5$ (Fig. 2G) spleen cells with LAK activity were injected, a low number (0–2) of colonies was observed.

Injection of BM cells co-incubated with IL-2-activated splenocytes at both 1:2.5 (Fig. 2C) and 1:5 (Fig. 2D) ratios resulted in colony numbers similar to the computed sum of the values produced by BM and LAK cell populations injected alone. BM cells incubated with fresh spleen cells at 1:10 ratio (Fig. 2H), and subsequently injected, produced confluent colonies. The effects of soluble factors (remaining within the cell-free supernatant from IL-2-activated spleen cells) on CFU-S numbers was determined by incubating the supernatant with BM cells for 4 h. The resulting colony formation (Fig. 2E) was significantly reduced when compared to normal BM values.

Microscopic Analysis of CFU-S Numbers. A microscopic analysis of colony numbers with phenotypes, although extremely laborious, was performed for 2 reasons: (a) to allow scoring of all CFU-S colonies, irrespective of size and location; and (b) to examine whether LAK cells or (LAK cell-derived factors) had any effect on a particular colony phenotype. The proportions of the different types of histologically identifiable CFU-S colonies produced in the various inocula groups and their sum totals are listed in Table 1.

Analysis of the number of microscopic colonies revealed that when fresh splenocytes were injected alone, or in combination with BM cells at a 1:10 ratio, confluent microscopic colony numbers were obtained (not presented in Table 1). As a result, the effect of NK cells on hemopoietic cells could not be determined in this series of experiments. The IL-2-activated spleen cell populations ($2.5 \times 10^5$ and $5 \times 10^5$ cell inocula) produced a small number of colonies. To examine the effects of LAK cells on the microscopic colony-forming ability of hemopoietic cells in the BM, the experimental and computed colony numbers resulting from injection of BM and LAK cells were compared. This comparison revealed that LAK cells at the 1:2.5 BM:LAK cell ratio had no effect on colony formation by the BM cells. However, at the 1:5 BM:LAK cell ratio, there was a small but significant augmentation of colony numbers ($P = 0.04$). When BM cells were incubated with the supernatant of IL-2-activated spleen cells and subsequently injected, there was no significant change in colony number when compared to normal control values.

The frequency of appearance of colony types resulting from reconstitution with BM cells was as follows (in descending order): CFU-E, CFU-BL or CFU-MEG, CFU-GM, CFU-MEGE, and CFU-MIX. Erythroid colonies accounted for half of the total colonies. When BM cells were preincubated with LAK cells (experimental), the frequency of colony phenotypes did not deviate significantly from the computed data derived from separate BM and LAK cell injections. Similarly, preincubation of BM cells with LAK cell supernatant did not appreciably influence the frequency of colony phenotype. In other words, LAK cells or their supernatant did not have a significant effect on a particular colony phenotype. Thus, augmentation of total colony number at the high LAK:BM cell ratio was nonspecific and could be explained by a nonspecific feeder effect of LAK cells on colony formation by BM cells in the irradiated host spleen. Some of the observed microscopic colony types are illustrated in Fig. 3.

In Vivo Immunotherapy-generated Killer Cells in Tumor-bearing Mice and BM CFU-S

Primary Tumor Growth

The mean s.c. tumor diameters were measured on days 15, 18, 20, and 24 after tumor cell injection (data not shown). No significant difference in primary tumor size between control

<table>
<thead>
<tr>
<th>Types of cells injected</th>
<th>BM + LAK cell, sup c</th>
<th>LAK × 10^5</th>
<th>LAK × 10^5</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM: LAK, 12.5</td>
<td>BM: LAK, 15</td>
<td>BM: LAK, 15</td>
<td>BM: LAK, 15</td>
</tr>
<tr>
<td>BM 1 × 10^5</td>
<td>Exp*</td>
<td>Comp</td>
<td>Exp*</td>
</tr>
<tr>
<td>No.</td>
<td>8.9 ± 0.7^b</td>
<td>11.6 ± 0.84</td>
<td>10.43 ± 0.44</td>
</tr>
<tr>
<td>%</td>
<td>47.7</td>
<td>54.8</td>
<td>50.8</td>
</tr>
<tr>
<td>CFU-BL</td>
<td>5.1 ± 0.5</td>
<td>3.7 ± 0.5</td>
<td>4.86 ± 0.33</td>
</tr>
<tr>
<td>No.</td>
<td>27.3</td>
<td>17.6</td>
<td>23.7</td>
</tr>
<tr>
<td>CFU-MEG</td>
<td>3.2 ± 0.2</td>
<td>3.6 ± 0.5</td>
<td>3.79 ± 0.16</td>
</tr>
<tr>
<td>No.</td>
<td>17</td>
<td>17.6</td>
<td>18.5</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>1.5 ± 0.4</td>
<td>2.0 ± 0.6</td>
<td>1.17 ± 0.2</td>
</tr>
<tr>
<td>No.</td>
<td>8</td>
<td>9.5</td>
<td>5.7</td>
</tr>
<tr>
<td>CFU-MEGE</td>
<td>0</td>
<td>0.14 ± 0.14</td>
<td>0.29 ± 0.11</td>
</tr>
<tr>
<td>No.</td>
<td>0</td>
<td>0.7</td>
<td>1.4</td>
</tr>
<tr>
<td>CFU-MIX</td>
<td>0</td>
<td>0.14 ± 0.14</td>
<td>0</td>
</tr>
<tr>
<td>No.</td>
<td>0</td>
<td>0.7</td>
<td>0</td>
</tr>
<tr>
<td>Total No.</td>
<td>18.7 ± 0.7</td>
<td>21.9 ± 1.5</td>
<td>20.53 ± 0.54</td>
</tr>
<tr>
<td>%</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*Exp, experimental group; Comp, computed group; sup, supernatant.

a Mean ± SE.
Fig. 3. Hematoxylin and eosin staining of day 7 CFU-S colonies obtained from 6-μm spleen sections: a, late CFU-E colony with basophilic erythroblasts (B), smaller normoblasts (N), and fully mature erythrocytes (E). b, CFU-GM colony showing abundant granulocytic band forms (G) and less frequent mature macrophages (Mφ) located along the periphery. c, predominantly CFU-MEG colony located along a splenic trabecula (T) and close to the capsule, illustrating polyploid megakaryocytes (M) and granulocytic band forms (G). d, CFU-MIX colonies possessing basophilic erythroblasts (B), normoblasts (N), and granulocytic band forms (G). No megakaryocyte can be seen in this section.
and treated groups existed at any of the intervals (P > 0.05). Mean tumor diameter (measured in mm) ranges were 7.7-8.4 on day 15, 8.5-10.1 on day 18, 10.1-10.9 on day 20, and 11.2-12.4 on day 24 in these animals.

**Spontaneous Lung Metastasis in Tumor-transplanted Mice Subjected to Various Protocols of Therapy**

Fig. 4 shows the mean number of spontaneous metastatic nodules on the surface of lungs isolated from variously treated tumor-bearing mice on days 24 and 25 following s.c. tumor transplantation. Lung colony counts from both days 24 and 25 were pooled within their respective groups only after similarity of values was determined by using the Kruskal-Wallis test.

Tumor-bearing control mice showed an average of 50 lung nodules. Lungs of mice treated with IL-2 possessed an average of 18 nodules, but the reduction was not significant when compared with controls. A significant reduction in nodule number (to 5) was observed in CIT + IL-2-treated mice when compared to the tumor-bearing controls.

**Effects of Incubation of Normal BM Cells with Splenocytes from Variously Treated Tumor-bearing Mice on CFU-S**

In evaluating the effects of therapeutically generated splenic effector cells on normal BM CFU-S, a statistical comparison was made between the experimental and computed CFU-S numbers in respective groups. The experimental numbers were those obtained after reconstitution with the co-incubated, mixed population of spleen and BM cells. The computed numbers were the added values of CFU-S numbers produced individually by spleen and BM cells.

**Macroscopic Colonies**. The results from the macroscopic analysis are shown in Fig. 5. The number of spleen colonies resulting from reconstitution of normal BM cells with splenocytes from normal and tumor-bearing control mice and tumor-bearing mice given immunotherapy (Fig. 5D) were not significantly different from the respective computed values (Fig. 5C), resulting from the separate injection of BM (Fig. 5A) and spleen cells (Fig. 5B). These results indicate that none of the splenic cell preparations tested in these experiments exerted any detrimental or stimulatory effect on the macroscopic spleen colony-forming ability of normal BM cells after a co-incubation in vitro.

**Microscopic Colonies**. Microscopic colony numbers are listed in Table 2 for each recognizable colony phenotype, as well as the sum total of the colony types. In all cell inoculum groups, CFU-E was the predominant colony type, with CFU-BL, CFU-MEG, and CFU-GM appearing next in the descending order of frequency. CFU-MEGE and/or CFU-MIX represented the least frequent colony types.

The total experimental colony counts resulting from reconstitution with normal BM cells in combination with splenocytes from normal mice were similar to computed values. However, combination of normal BM cells with splenocytes from tumor-bearing vehicle-treated, IL-2-treated, or CIT + IL-2-treated mice resulted in significantly lower colony numbers than their respective computed groups. The discrepancy between experimental and computed values of colony types resulting from injection of BM cells from normal mice and splenocytes from vehicle and CIT + IL-2-treated tumor-bearing mice was reflected in almost all colony types. This discrepancy was noted for the dominant colony types (CFU-E and CFU-BL) with splenocytes from IL-2-treated tumor-bearing mice.

**Femoral and Splenic CFU-S Distribution in Tumor-bearing Mice Subjected to Therapy**

BM and Spleen CFU-S Distribution in Tumor-bearing Mice Subjected to Therapy

**BM** and **Spleen** CFU-S Distribution in Tumor-bearing Mice Subjected to Therapy
Table 2. Effects of splenocytes from variously treated mice on the microscopic colony-forming ability of normal BM cells (mean CFU-S numbers ± SE)

<table>
<thead>
<tr>
<th>Colony type</th>
<th>Normal untreated</th>
<th>Tb control (vehicle-treated)</th>
<th>Tb, IL-2-treated</th>
<th>Tb, CIT + IL-2-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU-E</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimental</td>
<td>15.17 ± 1.01</td>
<td>15.8 ± 1.5</td>
<td>16.3 ± 0.87</td>
<td>14.5 ± 0.58</td>
</tr>
<tr>
<td>Computed</td>
<td>13.38 ± 0.39</td>
<td>18.15 ± 0.37</td>
<td>24.22 ± 0.51</td>
<td>16.82 ± 0.36</td>
</tr>
<tr>
<td>CFU-BL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimental</td>
<td>6.33 ± 0.76</td>
<td>4.9 ± 0.62</td>
<td>6.0 ± 0.5</td>
<td>4.3 ± 0.72</td>
</tr>
<tr>
<td>Computed</td>
<td>6.48 ± 0.31</td>
<td>7.13 ± 0.3</td>
<td>9.4 ± 0.38</td>
<td>7.5 ± 0.88</td>
</tr>
<tr>
<td>CFU-MEG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimental</td>
<td>3.5 ± 0.96</td>
<td>4.0 ± 0.83</td>
<td>4.75 ± 0.53</td>
<td>4.6 ± 0.69</td>
</tr>
<tr>
<td>Computed</td>
<td>6.3 ± 2.17</td>
<td>7.23 ± 1.67</td>
<td>4.8 ± 0.79</td>
<td>5.05 ± 1.21</td>
</tr>
<tr>
<td>CFU-GM</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Experimental</td>
<td>2.5 ± 0.56</td>
<td>4.4 ± 0.87</td>
<td>4.13 ± 0.72</td>
<td>4.2 ± 0.77</td>
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<tr>
<td>Computed</td>
<td>4.4 ± 0.34</td>
<td>6.75 ± 0.35</td>
<td>4.45 ± 0.38</td>
<td>5.15 ± 0.35</td>
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<tr>
<td>CFU-MEGE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimental</td>
<td>1.67 ± 0.5</td>
<td>1.2 ± 0.33</td>
<td>0.25 ± 0.25</td>
<td>0.8 ± 0.33</td>
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<tr>
<td>Computed</td>
<td>0.33 ± 0.07</td>
<td>0.32 ± 0.13</td>
<td>0.75 ± 0.10</td>
<td>1.16 ± 0.01</td>
</tr>
<tr>
<td>CFU-MIX</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Experimental</td>
<td>0</td>
<td>0</td>
<td>0.25 ± 0.16</td>
<td>0.4 ± 0.16</td>
</tr>
<tr>
<td>Computed</td>
<td>0</td>
<td>0</td>
<td>0.33 ± 0.07</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimental</td>
<td>29.17 ± 2.44</td>
<td>32.3 ± 1.82</td>
<td>19.18 ± 0.64</td>
<td>29.0 ± 1.84</td>
</tr>
<tr>
<td>Computed</td>
<td>31.08 ± 1.07</td>
<td>39.68 ± 1.12</td>
<td>43.75 ± 1.79</td>
<td>35.93 ± 0.64</td>
</tr>
<tr>
<td>P value</td>
<td>0.46</td>
<td>0.0015</td>
<td>0.0029</td>
<td>0.0072</td>
</tr>
</tbody>
</table>

* Tb, tumor-bearing.

A marked increase in the spleen cell number (2.8 times) and spleen weight (2.5 times) was seen in tumor-bearing vehicle-treated mice when compared to normal controls. IL-2 treatment of tumor-bearing mice did not induce a further change in the spleen cell numbers or in the spleen weight when compared to vehicle-treated mice. However, CIT + IL-2 treatment led to an increase in spleen cell number and spleen weight, which were 3.5 and 3.1 times, respectively, the values in normal mice.

The total nucleated cell numbers per pair of femurs and the spleen were applied to the CFU-S incidence from BM or spleen cell inocula to compute the total number of macroscopic and microscopic CFU-S in the femurs and the spleens of various groups of animals (presented later in Figs. 6–9). This allowed for an approximation of the distribution of CFU-S in the marrow and the spleen of variously treated mice. In this approximation, the seeding efficiencies for spleen colony production by the CFU-S in various cell inocula were assumed to be similar and 100%. The seeding efficiency (f value) of CFU-S within the spleen has been reported to be 11% for spleen cells and 25% for BM cells (42). Thus, the presently computed total CFU-S numbers in the femurs and the spleen would be underestimated by 4–9-fold. However, the changes in CFU-S numbers in experimental animals relative to controls would remain unaffected by our computation, as long as the f values remained unchanged in experimental mice.

Macroscopic Analysis of Total CFU-S Numbers per Pair of Femurs and the Spleen. The presence of a tumor resulted in a significant decrease (to 63%) in femoral colony values as compared to normal values (values were computed as described earlier) (Fig. 6). IL-2 administered alone and in combination with CIT caused a further significant decline (to 51% and 43%, respectively) in colony numbers when compared to values in vehicle-treated control tumor-bearing mice.

Examination of splenic CFU-S distribution in variously treated mice showed that the presence of a tumor itself resulted in a significant increase to 3.7-fold of normal controls (Fig. 7). IL-2 therapy, when compared to tumor-bearing controls, led to a small (20%) increase in colony numbers that was not significant. The combined CIT and IL-2 therapy in tumor-bearing mice resulted in an additional 110% increase, which was highly significant.

Microscopic Analysis of CFU-S. Fig. 8 presents the total number of microscopic colonies and colony phenotypes per pair...
of femurs from mice subjected to various therapeutic regimes. These values were computed as described earlier. The total femoral colony counts decreased to 75% in tumor-bearing mice when compared to normal values. In IL-2 and combined CIT + IL-2-treated tumor-bearing mice, the colony numbers declined further to 63% and 58%, respectively, compared to the values in tumor-bearing vehicle-treated mice. In all cases, the decrease in colony number was significant. These changes were generally reflected in all colony phenotypes.

Fig. 9 shows the total microscopic CFU-S colonies and colony phenotypes computed per spleen. In tumor-bearing mice, CFU-S numbers increased significantly (to 2.8 times) when compared to normal healthy mice. With IL-2 treatment, a small (20%) increase in colony numbers was seen when compared to vehicle-treated tumor-bearing mice. Combined CIT + IL-2 therapy led to a further 100% increase in splenic colony numbers when compared to vehicle-treated tumor-bearing mice. Essentially, a similar pattern of changes was seen in all colony phenotypes.

In determining the total number of CFU-S stem cells in the bodies of variously treated mice, the total number of marrow CFU-S stem cells was added to the total number of splenic CFU-S stem cells from Fig. 9 (Table 4). The total number of marrow CFU-S stem cells was calculated from the femoral microscopic CFU-S numbers shown in Fig. 8 and multiplied by 5.88, since nucleated cells in the femoral marrow represent approximately 17% of the total marrow cell number (42). Assuming that CFU-S generated from femoral marrow was representative of the marrow elsewhere in the body, the total number of CFU-S stem cells in the body rose by 32% in tumor-bearing controls. When compared to normal healthy mice, this group had a nearly 2-fold expansion in the CFU-S numbers in the body.

DISCUSSION

The combination of CIT with multiple rounds of IL-2 has been shown in our laboratory to ameliorate cancer metastasis in a number of tumor models and mouse strains (12, 20–23) and has recently been tested in a phase 2 human trial with promising results (24–26). It remains unknown whether IL-2-based therapies, designed for the purpose of activating NK cells and generating LAK cells, have any adverse effect on hemopoietic stem cells in the tumor-bearing host. Both NK and LAK cells have been reported to influence hemopoietic colony formation in vitro. However, their effect on the in vivo colony-forming ability of BM has not been investigated. For these reasons, the intent of this investigation was, first, to determine the effect(s) of NK and in vitro-generated LAK cells on normal BM CFU-S. Second, killer cells generated in vivo in tumor-bearing mice with IL-2 or CIT + IL-2 were tested for their possible effects on BM CFU-S. Finally, the effects of these therapeutic protocols on the distribution of CFU-S in the BM and the spleen of tumor-bearing hosts as well as on tumor growth and spontaneous lung metastases were examined.

Splenocytes activated for 72 h in the presence of IL-2 and subsequently injected into irradiated hosts produced few colonies despite high doses ($2.5 \times 10^5$ and $5 \times 10^5$) of cell inocula. Since few or no CFU-S survived in these cultures, even in the presence of IL-2 (which can stimulate T cells to produce colony-stimulating factors), it is likely that insufficient concentrations of colony-stimulating factors were present or also that colony-inhibiting products were released in these cultures.
Incubation of BM cells with the LAK cell supernatant had a CFU-S depressive effect at the macroscopic level but not at the microscopic level of analysis. Microscopic colony analysis includes colonies of all sizes formed on the surface as well as in the centre of the spleen (40), which are missed by macroscopic analysis. Thus, the CFU-S inhibitory activity of the culture supernatant was transient affecting the colony size rather than the colony number, either by delaying the time of onset of CFU-S proliferation or by reducing their rate of proliferation after homing in the spleen. While IL-2 alone does not have any effect on hemopoietic cells in vitro (29, 31, 34), IL-2-activated lymphocytes are known to produce potent in vitro colony-inhibiting cytokines such as tumor necrosis factor-α and interferon-γ (31, 32, 34, 43), which act synergistically and also less potent inhibitors such as transforming growth factor-β (44, 45). It is possible that any one or more of these factors were present in the LAK cell supernatant.

IL-2-activated splenocytes (inclusive of LAK cells) had no adverse effect on BM CFU-S. In fact, augmentation of colony numbers was seen at the 5:1 LAK:BM cell ratio. Since such augmentation was lacking at the 2.5:1 cell ratio, we suggest that the larger number of IL-2-activated splenocytes may have provided some feeder effect in the irradiated spleen for CFU-S growth. This feeder effect was noted for all colony phenotypes. Many laboratories have examined the effects of both IL-2-activated and -unactivated human peripheral blood lymphocytes on the in vitro colony-forming ability of BM or peripheral blood-derived hemopoietic cells and have shown either colony stimulation (27–31), colony suppression (27–29, 31–36), or no effect (27–30). A large number of methodological variables make it impossible to compare these results. These variables include: the duration of incubation of effector/hemopoietic cell mixtures before plating for colony growth, the colony type(s) examined, the source and the concentration of colony-stimulating factor(s) used for colony growth, the phenotypic and functional heterogeneity of effector cells used, and their activation states. Many of these studies did not permit a distinction between cell-mediated or factor-mediated effects on the hemopoietic colony-forming cells.

In the immunotherapy experiments, the primary tumor size remained unaltered during the observed period of 24–25 days regardless of therapy. This was in contrast to significant tumor regression noted with the same tumor model in C3H/HeJ mice subjected to CIT + IL-2 therapy in a previous study (12). This difference may have been due to a higher dose of IL-2 (2.5 × 10^3 Cetus units/injection) and indomethacin (14 μg/ml in drinking water) being used in the earlier study. We had to reduce both...
IL-2 and indomethacin dosage in C3H/HeN strain mice because of toxicity-related deaths in a pilot experiment. Furthermore, effects on the primary tumor mass would likely have been discernable at a later time point if the animals were allowed to live. Therapeutic effects were, however, noticeable in the present study in the reduction of spontaneous lung metastases. This reduction was significant with CIT + IL-2 therapy. With both immunotherapy protocols using IL-2, cells with significant LAK activity were generated in the spleens of treated mice in a parallel study (38).

When $10^5$ fresh splenocytes from normal control mice (inclusivive of NK cells, granulocytes, macrophages, and T and B lymphocytes) were incubated at a 2:1 ratio with BM cells for 4 h, the experimental CFU-S numbers scored at the macroscopic and microscopic levels matched the computed numbers. Thus, unactivated splenocytes inclusive of NK activity have no effect on day 7 CFU-S at this effector:target ratio.

The size of a CFU-S colony is dependent on the rate and the time of onset of proliferation of a hematopoietic cell following its homing in the spleen (46). As stated earlier, macroscopic analysis generally reveals the larger colonies, whereas microscopic analysis reveals both large and small colonies. Because splenocytes from control and treated tumor-bearing mice exerted a suppressive effect on colony formation (after a 4-h co-incubation) at the microscopic but not macroscopic level of analysis, we suggest that the hematopoietic stem cells, which proliferated more slowly (thereby forming the smaller colonies), were affected. The splenocyte populations used in these assays were heterogeneous. Since we showed that in vitro-generated LAK cells had no effect on stem cells as assayed by the CFU-S assay, and because splenocytes from normal mice exhibited no adverse effect on colony formation, the noted suppression of hematopoietic stem cell colony formation by spleen cells can be attributed to the presence of colony suppressor cells in the spleen of control and treated tumor-bearing mice, rather than any adverse effects of in vitro-generated LAK cells.

Tumor growth in a host frequently results in reduced immune competence and an increased formation of immune suppressor cells (47, 48). Previous studies in this laboratory have shown that, during the development of transplanted or spontaneous tumors in mice, splenic NK cells were progressively suppressed by a population of PGE2-producing macrophages (2, 4), which were I-A$^b$ in cell surface phenotype (5, 6). PGE2 has been reported to inhibit in vitro colony formation of hematopoietic cells (37). Whether PGE2-producing macrophages in the tumor-bearing host can suppress colony formation by CFU-S remains unknown. However, the fact that the suppressive effects were not abrogated with splenocytes from tumor-bearing animals subjected to chronic indomethacin therapy indicates that the present results cannot be explained by PGE2-mediated suppression of CFU-S.

Young et al. (14, 48) have reported a second class of immune suppressor cells in the murine spleen and BM during the later stages of tumor progression. These “natural suppressor” cells are considered to be immature monocytoid cells associated with active hemopoiesis (14, 48). They have been shown to suppress T cell blastogenesis (48, 49) and NK cell cytotoxicity against YAC-1 lymphoma cells (48, 49) in a PGE2-independent manner. It is likely that the activity of natural suppressor cells in the tumor-bearing host is not abrogated by indomethacin and IL-2 therapy and is responsible for the macroscopic colony inhibition seen with splenocytes recovered from both control and treated tumor-bearing mice. Furthermore, it is quite possible that enhanced splenic hemopoiesis in IL-2 or CIT + IL-2-treated mice also expanded natural suppressor cells to a similar degree.

The distribution of stem cells in the femur and spleens of variously treated mice was shown to be altered. A decrease in the number of femoral stem cells and an increase in the number of splenic stem cells were seen at both the macroscopic and microscopic levels of analysis in control and treated tumor-bearing mice when compared to normal mice. These findings can be attributed to CFU-S migration from the BM to the spleen, followed by CFU-S expansion in the spleen. Present data are consistent with our earlier reports of the effects of tumor bearing on hemopoietic distribution, showing a decrease in the number of femoral CFU-S and CFU-C followed by a sequential increase of these colony-forming cells in the peripheral blood and later in the spleen (50, 51). These effects were interpreted as a redistribution of stem cells from the femur to the spleen in response to an increased hemopoietic demand (51). Hemopoietic demand in the tumor-bearing host may have been due to leukocyte migration to the tumor site and hemorrhaging within the tumor (51). Since rodent marrow is incapable of expanding to accommodate increased hemopoietic demand, the spleen expands and becomes highly hemopoietic in its place (46). Furthermore, tumor cells have been shown to secrete colony-stimulating factors in vivo (50), thereby aiding hemopoietic expansion.

IL-2 administration to tumor-bearing mice resulted in a further redistribution of CFU-S from the marrow to the spleen without a change in the total CFU-S numbers in the body. IL-2 therapy in combination with CIT led to an additional rise in the CFU-S content of the body, as evidenced by their expansion in the spleen. The abrogation of PGE2 production by certain suppressor cells may have allowed further expansion of LAK cells and consequently hemopoietic cells in the spleen due to production of colony-stimulating cytokines by LAK cells in vivo (52).

Thus, even though splenocytes from control and treated tumor-bearing mice exerted a suppressive effect in vitro on normal femoral CFU-S, this effect was not identified in vivo on the overall CFU-S numbers. A major expansion of CFU-S in the spleen indicated that such suppressive effects are greatly overshadowed by other stimulatory factors allowing for CFU-S amplification in vivo.

In conclusion, the present study revealed that tumor immunotherapy with IL-2 alone or in combination with chronic indomethacin therapy had no adverse effect on hemopoietic stem cells. Indeed, the latter therapies led to a substantial expansion of the total stem cell reservoir in vivo. Thus, IL-2-based immunotherapy and in particular, IL-2 in combination with indomethacin, can be considered safe for the hemopoietic tissue of the cancer-bearing host.

REFERENCES

IL-2-BASED TUMOR THERAPY AND HEMOPOIETIC CELLS


Effects of Cancer Immunotherapy with Indomethacin and Interleukin-2 on Murine Hemopoietic Stem Cells

Mary Nel Saarloos, Nelson K. S. Khoo and Peeyush K. Lala


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