Involvement of the Spleen in Preleukemic Development of a Murine Retrovirus-induced Promonocytic Leukemia

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ABSTRACT

An acute myeloid leukemia can result from the inoculation of Moloney murine leukemia virus into BALB/c mice undergoing a 2,6,10,14-tetramethylpentadecane-induced chronic inflammatory response in the peritoneal cavity. This leukemia is ultimately observed in the peritoneal cavity as an ascites with cells infiltrating the granulomatous tissue. It has been proposed, however, that hematopoietic organs such as the spleen and bone marrow are involved in preleukemic development of Moloney murine leukemia. Therefore, to determine if the spleen plays a role in this development, mice were splenectomized at various times relative to virus inoculation. When splenectomies were performed 3 days before and 2, 4, 6, and 8 weeks after virus inoculation there was, in all cases, a decreased death rate compared to sham-splenectomized controls. The greatest difference in death rate due to promonocytic leukemia was observed when mice were splenectomized at 4 weeks after virus inoculation. The decrease in disease incidence observed as a result of splenectomy was not caused by decreased virus spread in hematopoietic organs or an alteration in the profile of the cellular infiltrate in the granuloma. It was found, however, that the spleens of 2,6,10,14-tetramethylpentadecane-treated mice, relative to those of normal mice, have a significantly increased number of granulocyte-macrophage colony-forming cells and a slightly increased number of multipotential colony-forming cells. These observations suggest that a population of target cells for transformation, consisting of granulocyte-macrophage precursor cells, may reside in the spleen. Alternatively, partially transformed cells may reside temporarily in the spleen during the developmental stages of the disease process.

INTRODUCTION

When mice receive i.p. injections of pristane, a chronic inflammatory response occurs which is evidenced by the presence of extensive granulomatous tissue throughout the peritoneal cavity. In BALB/c mice this inflammation in combination with certain retroviruses, such as Moloney MuLV, results in the formation of promonocytic leukemias (1, 2). These leukemias were previously named MML for Moloney MuLV-induced myeloid leukemia. Analyses of the phenotype of leukemic cells have shown that they have many characteristics in common with monocytes or macrophages. For example, they are positive for cell surface markers Mac-1 and Mac-2, they are adherent and phagocytic, and they produce lysozyme (1, 2). The leukemic cells as they appear in the late stages of disease in ascites are morphologically similar to the transformed cells of human acute myeloid leukemia AML-M5 (3, 4).

Molecular studies have demonstrated that 100% of MML cells examined have an altered arrangement of sequences in the c-myb locus due to proviral integration. This insertional mutagenic event invariably occurs in the 5' end of the gene, and the viral LTR promotes transcription from a site downstream of the normal promoter. Leukemic cells which have undergone insertional mutagenesis express an aberrant myb protein that is NH2-terminally truncated (5, 6).

Acute disease is manifested in the peritoneal cavity in the late stages of the leukemia at 3–4 months after virus inoculation, but it has not been determined if all neoplastic development occurs at this site or if some preleukemic development occurs at other sites as well. The fact that i.v. inoculation but not i.p. inoculation is effective in inducing disease suggests that other hematopoietic organs such as the spleen or bone marrow may be involved in the disease process.

The present study indicates that the spleen may play a role in the early development of the neoplastic disease by promoting hematopoiesis along the myeloid lineage during inflammation. The data show that the pristane-induced chronic inflammation, which is required for ascites development, increases the myeloid precursor cells in the spleen and that splenectomy can reduce the incidence of disease.

MATERIALS AND METHODS

Animal Experiments. Female BALB/cAnPt (5–8 weeks old), bred and housed in a conventional colony at Hazleton Laboratories (Rockville, MD) (National Cancer Institute Contract N01 CB-710-85), received i.p. injections of 0.5 ml pristane (Aldrich Chemical Co., Milwaukee, WI). Three weeks later they were inoculated with 0.5 ml Moloney MuLV (>106 PFU/ml) by injection into the tail vein. Mice were monitored for disease by examining smears of ascites cells that were stained using Diff-Quick (Baxter Scientific Products). If mice were positive for promonocytic leukemic cells and moribound they were sacrificed. The remaining animals were terminated 6 months after virus infection. All experimental animals were autopsied.

For splenectomies, mice were anesthetized with 10 mg/kg sodium pentobarbital solution in 0.2 ml injected i.p. A vertical incision was made below the left costal margin, the spleen was removed after the veins and arteries were hemostatically clipped with medium 10 hemoclips (Edward Week & Co., Research Triangle Park, NC), and the incision was clipped shut with 9-mm Autoclips (Clay Adams, Parsippany, NJ). Sham splenectomies were performed on control mice by mobilizing the spleen outside of the peritoneal cavity and replacing it. Time points reported for splenectomies are relative to the time of virus inoculation, which was always 3 weeks after pristane injection. The Fisher-Irwin exact test was used for single time points (7), and the Mantel-Haenszel test was used for a combined analysis over all time points (7).

Virus and Measurement of Virus Infectivity. Moloney MuLV-infected NIH 3T3 cells were prepared as described previously (1). Twenty-four-h supernatant was collected from a confluent producer line and the titer was determined using the XC plaque assay (8). To determine the relative number of in vivo derived hematopoietic cells that were producing infectious virus, single cell suspensions of bone
were diluted in 3% acetic acid to lyse red cells. Resuspending the homogenate in KI'M1640. Prior to counting, cells (SeaPlaque; FMC, Rockland, ME). Bone marrow cells (a pool of the GM-CFCs and multipotential GEM(M)-CFCs were a modification of colonies. For the determination of GM-CFC, 50 units/ml GM-CSF colonies. For the determination of GEM(M)-CFC, medium was supplemented with 2 × 10^-4 m hemin/ml, 2 units erythropoietin/ml (step3 column-purified sheep Epo; Connaught Labs, ON, Canada), 100 units interleukin 3/ml (Genzyme), and 1 × 10^-4 m 2-mercaptoethanol/ml. Colonies were counted on day 14 and scored if there was evidence of both erythroid and myeloid components (with or without visible megakaryocytes). Positive control tissues for the GEM(M) assay were taken from mice that had received i.v. injections of 150 μg/kg 5-fluorouracil (Sigma Chemical Co., St. Louis, MO) 5 days prior to assay (data not shown).

For the determination of the proportion of cells in S phase, cell samples were set up in duplicate: one sample was incubated for 20 min at 37°C with 50 μCi of [3H]thymidine/ml (specific activity, 6.7 Ci/mmol; New England Nuclear) in RPMI 1640, and the control sample was incubated with RPMI 1640 (10). After one wash at 4°C in excess cold thymidine and multiple washings in RPMI 1640, cells were plated as for GM-CFC and GEM(M)-CFC assays described above. On appropriate days colonies were scored. The percentage of cells in S phase was determined by calculating the reduction in the number of colonies produced after samples were treated with [3H]thymidine as compared to untreated samples.

An assay for erythroid colony-forming cells used a 2-day plasma clot culture system (11). The clots were stained with benzidine and hematoxylin; benzidine-positive colonies were scored.

Preparation of Granuloma for Histology. Sections of the granulomatous tissue were excised, fixed in Fekete's modified Tellyesniczky's fluid, and stored in 70% ethanol. Samples were embedded in paraflin blocks, sliced, mounted on slides, and stained in Wright-Giemsa (Baker Histology Laboratory, Great Falls, VA).

RESULTS

Splenectomy Decreases the Incidence of MML. Since inflammation is required for the induction of leukemic ascites and the spleen is an accessory organ during inflammation (12), it was of interest to determine if the spleen played a role in the development of this leukemia. In order to assess the involvement of the spleen, splenectomies were performed on pristane-treated mice at −3 days and +2, +4, +6, and +8 weeks relative to Moloney MuLV inoculation, and the mice were monitored for the presence of leukemic ascites. At all time points the death rate due to promonocytic leukemia was decreased in splenectomized versus sham-splenectomized mice (Fig. 1). However, removal of the spleen at 4 weeks after virus infection was the most effective (P < 0.01). In establishing a control group, a previous experiment (data not shown) demonstrated that in mice receiving pristane and virus the incidence of MML was the same whether they were subjected to a sham operation or no operation.

Splenectomy Does Not Overtly Affect Pristane-induced Granuloma Formation. To determine if splenectomy affected the development of the pristane-induced granuloma in the peritoneal cavity and, thus, indirectly affected a developing leukemia in this region, granuloma tissue from the peritoneal cavity of comparably treated mice was examined. Mice were pristane-treated for 3 weeks, received injections of virus, and splenectomized at −3 days or +4 weeks relative to the virus inoculation. Granuloma samples were collected at 5, 10, and 20 weeks after virus inoculation and fixed for histological preparation. Morphological observation of tissue sections indicated that splenectomy had no obvious effects on the content or proportion of monocyte-macrophage cells, neutrophils, or lymphocytes.

Splenectomy Does Not Affect Virus Spread to Other Hematopoietic Organs. An explanation for the decreased incidence of leukemia in splenectomized mice could be that the removal of the spleen affects the levels of replicating virus in the remaining hematopoietic tissues. It was of interest, therefore, to determine the level of virus replication by comparing the number of infected cells in the hematopoietic organs of mice that received virus and pristane but did or did not undergo splenectomy.

A determination was initially made of the time course of infection in several hematopoietic organs in the absence of splenectomy (Fig. 2). Using the XC plaque assay, cells producing virus were quantitated in the bone marrow, spleen, and liver of pristane-treated mice at 1, 2, 3, 4, 5, 6, 8, 10, and 20 weeks after Moloney MuLV inoculation. The results are presented in Fig. 2, with the results shown on three different scales to emphasize that while the number of infected cells (infectious centers) are highest in the bone marrow and lowest in the liver, the kinetics are the same in all three tissues. At each time point the organs from four mice (two mice at 20 weeks) were assayed, and the number of infectious centers for each organ from each mouse
and liver from mice that were splenectomized at either -3 days or +4 weeks (the time point at which splenectomy exhibited the greatest inhibitory effect on disease) relative to virus inoculation. Fig. 3 shows that, regardless of whether the spleen was removed before or after virus inoculation, both 6 and 10.5 weeks later, there was no significant difference in the number of infectious centers in the bone marrow or the liver of splenectomized mice as compared to sham-splenectomized mice.

Peritoneal inflammation does not increase virus replication but causes an increase in splenic myelopoiesis. Since pristane has been shown to be necessary for the induction of the acute phase of MML, we wanted to see if the presence of a granuloma or the activity of forming or sustaining the granuloma increase number of infected cells in hematopoietic organs. The number of infected cells (infectious centers) in the bone marrow, spleen, and liver were compared in virus-inoculated mice that either received pristane or did not receive pristane. The data in Fig. 4 indicate that at 6 weeks there is little difference in the number of infectious centers obtained for pristane-treated or untreated mice. But by 10.5 weeks there was a substantial difference between the two groups, which was evident from the infectious center data obtained for both the bone marrow and the spleen. However, instead of increasing the number of infected cells in these organs, pristane decreased the number. It can be concluded from this experiment that the inflammatory response is not required for leukemia induction simply because it increases virus spread.

Since pristane is required for the induction of leukemic ascites and our data have shown that hematopoietic organ(s) are involved in disease development, we thought it was important to determine the effects of pristane on myelopoiesis in the bone marrow, spleen, and liver. To examine the impact of pristane, or the inflammatory response, on the myeloid compartment, colony assays were performed on cells derived from pristane-treated or untreated mice. Myeloid precursor cells from the...
bone marrows, spleens, and livers of these mice were assayed 10, 21, 45, and 87 days after pristane injection. The cells were quantified by scoring the number of colonies formed in soft agar in response to growth factors. Tables 1 and 2 summarize the differences between numbers of colonies formed from cells of pristane-treated mice as compared to cells from untreated controls which were age matched and assayed in parallel in several different experiments. Spleens of pristane-treated mice were demonstrated to have increased numbers of GM-CSF-responsive colonies (Table 1). The fold increase ranged from 7.3 to 84; the number varied depending on the time of assay. The number of multipotential cells, responsive to interleukin 3 and represented by GEM(M)-CFC, also increased in the spleens of pristane-treated mice (4.7-fold) (Table 2). This increase was not as dramatic as the increase observed for GM-CSF-responsive cells. A record was made of spleen weights at days 10, 21, 45, and 87 from mice that were pristane treated. The average weights were from 4–5 mice/time point and ranged from 0.16 g at day 10 to 0.20 g at day 87. The average spleen weight for untreated adults was 0.10 g. This indicates that the actual total increase per spleen in the numbers of CSF-responsive cells in pristane-treated mice was 1.6- to 2-fold more than that indicated by the data in Tables 1 and 2.

In the bone marrow, the number of GM-CFCs (Table 1) and GEM(M)-CFCs (Table 2) did not change significantly as a result of pristane treatment. It was found, however, that although the numbers of myeloid precursor cells in the bone marrow did not increase in pristane-treated mice, there was a slight increase in the number of GM-CFCs in S phase (Table 3). The total number of GEM(M) colonies in Table 3, in the suicide experiment, were consistently lower than those in Table 2. It is possible that the manipulations of these cells for thymidine treatment affected colony-forming potential.

For the livers of pristane-treated and untreated mice, GM-CFC and GEM(M)-CFC were determined and compared also. The numbers of colonies are presented in Tables 1 and 2. It demonstrates that in the liver there is also an apparent increase of colony-forming cells in pristane-treated mice.

In order to determine whether the cellular response to pristane represented a generalized increase in hematopoietic cells we also quantitated precursor cells committed to the erythroid lineage in the bone marrow and the spleen. Erythroid colony-forming cells were enumerated 2 days after plating cells in plasma clot cultures. As shown in Table 4 there was no increase in the number of erythroid colony-forming cells in pristane-treated mice, indicating that pristane-induced chronic inflammation more specifically expands the granulocyte-macrophage compartment.

Because we have been interested in knowing if myeloid precursor cells could be targets for insertional mutagenesis we wanted to determine if individual multipotential precursor cells could be directly infected by virus. Therefore, we took individual GEM(M) colonies generated from precursor bone marrow cells of two virus-inoculated mice and assayed them for virus production. For this analysis, cells from each colony (5 from one mouse and 4 from the other) were overlayed on a plate containing NIH 3T3 cells. The NIH 3T3 cells were passaged three times, and the medium was analyzed for the presence of virus by the XC plaque assay. Nine of 10 colonies were virus positive, even though 2 negative control plugs picked from the agar in the same plates and overlayed in a similar manner were negative.

**DISCUSSION**

The data presented here show that splenectomy effectively decreases the incidence of MML induced by Moloney MuLV in pristane-treated mice. The data also show that splenectomy does not affect virus spread or overtly influence the cellular make-up of the peritoneal granuloma. Therefore, to understand the role of the spleen we examined the effects of the inflammatory response, which is required for disease, on the spleen. A major effect of the pristane-induced inflammatory response on the spleen was shown to be a large increase in the number of precursor cells committed to the granulocyte-macrophage lineage and a moderate increase in multipotential stem cells. We therefore propose that the spleen may provide a source of cells that are targets for the primary transforming event (e.g., insertional mutagenesis of c-myb by Moloney MuLV; see Fig. 5). The initial target for insertional mutagenesis into the c-myb gene is most likely an early multipotential cell or a myelomonocytic progenitor cell, because c-myb transcription is associated primarily with immature hematopoietic cells (13, 14, 15). Since retroviruses integrate preferentially into genetic regions that are actively being transcribed (16), it is possible that the increased number of GM-CFCs and GEM(M)-CFCs seen in the spleens of pristane-treated mice provide an additional number of target cells in which this transforming integration can occur. Interestingly, we have only been successful in inducing this promyelocytic leukemia when the i.v. route of virus inoculation is utilized; we have not been successful in inducing MML by i.p.

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**Table 1** GM-CSF-responsive colony-forming cells in bone marrow, spleen, and liver of pristane-treated and untreated mice

<table>
<thead>
<tr>
<th>Bone marrow</th>
<th>Spleen</th>
<th>Liver</th>
</tr>
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<tbody>
<tr>
<td><strong>Day</strong></td>
<td><strong>Pristane</strong></td>
<td><strong>n</strong></td>
</tr>
<tr>
<td>10</td>
<td>–</td>
<td>4</td>
</tr>
<tr>
<td>21</td>
<td>+</td>
<td>24</td>
</tr>
<tr>
<td>45</td>
<td>+</td>
<td>28</td>
</tr>
<tr>
<td>87</td>
<td>+</td>
<td>250.0 ± 53.8</td>
</tr>
</tbody>
</table>

* Day of assay relative to pristane injection.
* a Colony-forming cells, the mean no. of combined G-, M-, and GM-CFC/5 × 10⁵ cells ± 1 SEM.
* b Fold differences between GM-CFC from pristane-treated mice (P+) and GM-CFC from untreated mice (P-).
* c P < 0.001 Student's t test.
* d ND: Not done.
* e Second set of plates were at numbers too high to count.
ROLE FOR SPLEEN IN PRELEUKEMIA

Table 2 Interleukin 3 and EPO-responsive GEM(M) colony-forming cells in the bone marrow, spleen, and liver of pristane-treated and untreated mice

<table>
<thead>
<tr>
<th>Bone marrow</th>
<th>Spleen</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pristane</td>
<td>CFC ± SEM</td>
<td>P*/P*</td>
</tr>
<tr>
<td>−</td>
<td>24</td>
<td>81.5±19.1</td>
</tr>
<tr>
<td>+</td>
<td>29</td>
<td>116.9±16.9</td>
</tr>
</tbody>
</table>

*This assay was performed 21 days post-pristane injection.

Inoculation, although a retrovirus carrying c-myb can cause macrophage leukemias in pristane-treated mice when introduced by this method (1). This observation together with the observations made in the present report implies that virus directly introduced into the circulation can more efficiently infect areas where early precursor cells reside.

An alternative explanation of why splenectomy decreases disease incidence is that the spleen may provide an attractive environment for cells that are initially transformed in the bone marrow and will be passing into the peritoneal cavity for further development after virus inoculation. It is well accepted that monocytic-like cells are attracted to factors released by cells involved in an inflammatory response in the peritoneal cavity (12). The pristane-induced inflammatory granuloma is most likely providing a favorable environment for the recruitment and proliferation of preleukemic or leukemic cells; further transforming events may occur there as well.

In support of our data presented here, which suggest that at some time during leukemia development, transformed or partially transformed cells reside in the spleen, we have recently demonstrated that cells with insertional activation of c-myb can reside in the spleen as early as 3 weeks after virus inoculation (this is the subject of another manuscript in preparation).

![HYPOTHETICAL PATHWAYS LOCALIZED EVENTS](image)

Fig. 5. Hypothetical pathways for the development of MML. I, hypothetical route preleukemic cells might take through hematopoietic organs during tumor development. In this model cells that are partially transformed in the bone marrow by Moloney MuLV insertional mutagenesis of the c-myb gene might travel through inflammatory organs. During tumor development, these cells are attracted to the peritoneal cavity through inflammatory stimulation. In association with the pristane-induced granuloma, they expand and manifest into the late stages of the disease. In II, an alternate model is depicted whereby an initial event of insertional mutation is released during the inflammatory response in the peritoneal cavity.
ACKNOWLEDGMENTS

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