Increased responses to lymphokines are correlated with preleukemia in mice inoculated with Moloney leukemia virus

(leiukemogenesis/blastogenic factors)

JOHN C. LEE AND JAMES N. IHLE

Biological Carcinogenesis Program, National Cancer Institute–Frederick Cancer Research Facility, Frederick, Maryland 21701

Communicated by Wallace P. Rose, August 28, 1981

ABSTRACT In various mouse strains, inoculation with Moloney leukemia virus results in the establishment of an acute viremia which in most cases is followed by the induction of leukemia. Also associated with the viremia is the development of a chronic cellular immune response detectable in vitro by the ability of viral proteins to induce splenic lymphocyte proliferation. Previous studies have demonstrated that, in the absence of this cellular immune response, leukemia does not develop irrespective of whether viremia is present [Lee, J. C. & Ihle, J. N. (1981) Nature (London) 289, 497–499]. In vitro proliferative responses to antigens involve the nonspecific response of various subpopulations of lymphocytes to lymphokines produced by antigen-specific Thy 1+, Lyt 1+, 2+ lymphocytes. The studies presented here concern the effects of a chronic immune response in viremic mice on the frequency of lymphocytes capable of responding to lymphokines in vitro. The data demonstrate that the number of responsive lymphocytes is increased 30- to 100-fold in preleukemic mice and that such increases are dependent upon the induction of an immune response in viremic mice. The role of this altered immune response in leukemia is discussed.

The mechanisms by which type C viruses promote leukemogenesis are unknown. From the observations that leukemogenesis requires viremia, involves long latencies, and results in monoclonal tumors it has been postulated that direct viral transformation is not involved. Rather, as a consequence of either viral integration (1, 2) or host factors, rare events occur which give rise to transformation. The possible involvement of the immune response in the events leading to transformation has become evident from several recent observations. In both spontaneous AKR leukemia and Moloney leukemia virus (MoLV)-induced leukemia in BALB/c mice, the viremia is associated with the development of a chronic cellular immune response detectable in vitro by the presence of T-cell blastogenesis against the major virion envelope glycoprotein gp70 (3–5).

More recently it was demonstrated that, in MoLV-inoculated viremic CBA/N mice, such an immune response does not develop and these mice fail to develop leukemia (6). In subsequent experiments (unpublished data), the lack of cellular immune responses and the resistance to leukemia were found to segregate with an X-linked recessive gene and may be due to the xid gene associated with CBA/N mice (7). These results demonstrate that a cellular immune response is required for leukemogenesis. Based on these observations, it was postulated that, as a consequence of chronic immune stimulation due to viremia, the probability of somatic events giving rise to transformation is greatly increased. One such somatic “error” commonly associated with murine leukemias could be the generation of trisomy of chromosome 15 (8, 9).

In spite of the considerable evidence establishing the requirement for chronic immune stimulation in viral leukemogenesis, it has been difficult to evaluate the general effects of an antigen-specific response on the immune system. In particular, the experiments cited above utilized assays that measured the ability of lymphocytes to proliferate in vitro in response to gp70 and therefore conceivably only were measuring a minor subpopulation of lymphocytes with immunological specificity for gp70. Recent experiments, however, have demonstrated that the proliferative response to gp70 is considerably more complex (10, 11), involving different subpopulations of non-antigen-specific lymphocytes responding to various lymphokines (collectively termed “blastogenic factors”) produced by antigen-specific lymphocytes (11). Two defined lymphokines that are associated with this response are interleukin 2 (IL-2) and interleukin 3 (IL-3) (unpublished data). These factors are thought both to promote the differentiation of particular subpopulations of lymphocytes and to expand them by inducing proliferation. Examples of these dual effects are the observations that both IL-2 and IL-3 promote T-cell differentiation and can be used to establish continuous T-cell lines in vitro (12–14). The potential significance of these events for leukemogenesis is that, as a consequence of viremia, antigen-specific T cells are continually recruited and stimulated to produce various lymphokines which in turn expand the immune system by their nonantigen-specific effects.

To evaluate such potential effects, we quantitated the level of lymphocyte subpopulations capable of responding to lymphokines in preleukemic mice. We demonstrate that such populations are dramatically expanded in preleukemic mice and that this expansion is dependent upon an antiviral immune response and is correlated with the ultimate development of leukemia.

MATERIALS AND METHODS

Animals. C57BL/6 and BALB/c mice were obtained from the Animal Production Area of the Frederick Cancer Research Facility (Frederick, MD). The animals were maintained in a specific pathogen-free environment. C57BL/6 mice, 8–10 weeks of age, were inoculated intramuscularly with Moloney leukemia virus/Moloney sarcoma virus (MoLV/MoSV) complex to induce tumors and as a source of splenic lymphocytes for in vitro induction of blastogenic factors as described (10, 11).

BALB/c mice were inoculated with Moloney virus (stock ICB.H maintained by animal passage in BALB/c mice) at birth.

Abbreviations: IL-2, interleukin 2; IL-3, interleukin 3; MoLV/MoSV, Moloney leukemia virus/Moloney sarcoma virus; 20αSDHase, 20α-hydroxysteroid dehydrogenase.
as described (4). Approximately 50% of the mice succumbed to leukemia by 8–10 weeks of age. Therefore, for the purpose of this study, preleukemic mice 4–8 weeks of age were used. Only animals showing no evidence of leukemia as judged by thymic enlargement or gross splenomegaly were used.

Production and Assay of the Blastogenic Factors. Conditioned media to be examined for blastogenic factors, IL-2 or IL-3, were prepared by incubating $10 \times 10^8$ nylon wool-fractionated T cells from immune mice in 2 ml of RPMI-1640 with 5% fetal calf serum in the presence of 20 ng of MoLV gp70 for 48 hr or the indicated times in wells of flat-bottom plates (Linbro). The MoLV gp70 was purified to homogeneity as judged by NaDodSO4/polyacrylamide gel electrophoresis as described (15). The cells were then removed by centrifugation and the supernatant was filtered through 0.45-μm-pore nitrocellulose membranes (Milllex, Millipore). The gp70 was removed from conditioned media by antibody affinity columns as described (11); by competition radioimmunoassays it was decreased to $<1$ ng/ml. Control supernatants were obtained by incubating immune or nonimmune cells without gp70 or nonimmune cells with gp70 under the same conditions.

The preparations of the blastogenic factors were evaluated for their ability to stimulate the proliferation of $5 \times 10^6$ nylon wool-fractionated T cells, the standard blastogenic assay. A 1:1 dilution of the factors in fresh RPMI 1640 with 5% fetal calf serum was used unless other concentrations were indicated. The $[^3]H$ thymidine uptake during an 8-hr period was evaluated after 2–3 days in culture.

Lymphocyte Blastogenic Assay. A standard microassay method (16) was adapted for these studies.

Partial Purification of IL-3 and IL-2. For the purification of IL-2 and IL-3, 5- to 7-liter preparations of concanavalin A-conditioned medium were used and all procedures were carried out at 4°C. Both factors were purified by using a combination of ammonium sulfate fractionation, Sephadex G-100 column chromatography, and DEAE-cellulose ion exchange as described (15).

Autoradiography. The standard blastogenic assay was used except that the system was expanded 20-fold. Therefore, $1 \times 10^7$ cells in 2 ml of medium were incubated in 24-well tissue culture plates (Linbro) with blastogenic factors. After the standard 48-hr incubation, the cultures were exposed to $20 \mu$Ci (1 Ci = $3.7 \times 10^{+6}$ becquerels) of $[^3]H$ thymidine for 8 hr. The cells were harvested and extensively washed to remove unincorporated thymidine. The cells were subsequently fixed onto microscopic slides by the use of a Cytocentrifuge (Shandon). These preparations were air dried and fixed in methanol to prepare for coating with photographic emulsion (NTB-2, Kodak). At various time intervals of exposure, the slides were developed photographically and subsequently stained by histochemical stains. The cells showing dark silver grains were counted.

RESULTS

The T-cell blastogenic response of immune mice to type C viral proteins involves at least two subpopulations of lymphocytes (11). An antigen-specific Thy 1.2+, Lyt 1−,2− lymphocyte is required for the production of various lymphokines, collectively termed "blastogenic factors," which induce the proliferation of an initially Thy 1.2+, Lyt 1−,2− lymphocyte population from either normal or immune mice (11). During experiments to define the responding populations, it was found that the response to blastogenic factor of lymphocytes from preleukemic mice was consistently higher than that of lymphocytes from normal mice. The typical differences are illustrated in Fig. 1. In this experiment a blastogenic factor preparation was obtained by stimulating lymphocytes (nylon wool-purified) from immune mice with MoLV gp70. The conditioned media were subsequently collected and the antigen was removed by antibody affinity columns and tested for ability to induce the proliferation of lymphocytes from either normal or MoLV-inoculated preleukemic BALB/c mice. There was a distinct increase in the response of lymphocytes from preleukemic mice. At the highest concentrations of blastogenic factor, lymphocytes from preleukemic mice at various ages showed a 5- to 8-fold higher response than did lymphocytes from normal mice.

In order to determine whether this increased response to blastogenic factor was correlated with the ultimate development of leukemia, we compared the responses of lymphocytes from individual MoLV-inoculated BALB/c, CBA/J, and CBA/N mice. MoLV inoculation in newborn mice readily induces viremia in these strains of mice but only CBA/J and BALB/c develop leukemia (6). The lack of leukemia in CBA/N mice is correlated with the lack of development of a cellular immune response against the virus. There were distinct differences in the response of lymphocytes from these mice to blastogenic factor (Fig. 2). Lymphocytes from individual MoLV-inoculated BALB/c or CBA/J mice showed a high-level response compared to lymphocytes from normal un inoculated mice of comparable ages. The responses of CBA/J mice were more variable than those of BALB/c and may be associated with the longer latency for leukemia observed in this strain. With lymphocytes from the leukemia-resistant MoLV-inoculated CBA/N mice,
however, the responses were similar to those seen with lymphocytes from uninoculated mice. These results strongly suggest that the increased responses to blastogenic factor preparations are correlated with the development of leukemia.

The blastogenic factors produced in viral antigen-stimulated cultures are known to consist of various lymphokines including IL-2 and IL-3. IL-3 has been shown to induce the expression of 20a-steroid dehydrogenase (20acsDHas) in nu/nu splenic lymphocyte preparations (17), and IL-2 promotes the proliferation of relatively more mature cytotoxic T cells (13, 14). To determine whether the increased responses of preleukemic mice were due to specific T-cell subpopulations or specific lymphokines, we compared the responses of splenic lymphocytes from normal and MoLV-inoculated BALB/c mice to partially purified IL-2 and IL-3. Nylon wool-purified lymphocytes from MoLV-inoculated BALB/c mice responded consistently higher to either factor than did lymphocytes from un inoculated mice (Fig. 3). Therefore, the increased responses to blastogenic factors appear to involve a general increase in responsiveness to functionally distinct lymphokines.

The increased responses of lymphocytes from preleukemic mice could be due to either increased sensitivity to the various factors or increased frequency of lymphocytes capable of responding. To test this we used autoradiographic techniques. For these experiments, nylon wool-purified splenic lymphocytes were initially incubated for 48 hr with an antigen-depleted preparation of blastogenic factors. The cells were subsequently pulsed with [3H]thymidine and prepared for autoradiography and stained with Giemsa stain. Typical preparations are illustrated in Fig. 4. There was a dramatic difference between normal lymphocytes and lymphocytes from preleukemic, MoLV-inoculated mice. In the absence of blastogenic factors, an average of approximately 0.1% of the lymphocytes from normal mice were labeled whereas approximately 0.3% of lymphocytes from preleukemic mice were labeled (Table 1). In the presence of blastogenic factor the frequency of labeled cells from normal mice increased to 0.8% whereas the frequency of labeled cells from preleukemics increased to 15.9%. Therefore, in response to blastogenic factors, normal mice gave a 10-fold increase and preleukemic gave approximately a 50-fold increase. More importantly, however, the frequency of lymphocytes capable of responding to blastogenic factors was increased approximately 20-fold in preleukemic mice relative to normal mice.

**DISCUSSION**

Our results demonstrate that lymphocyte preparations from preleukemic mice have increased responses to blastogenic factor preparations or to partially purified IL-2 and IL-3. In these and additional experiments, we have not observed any apparent alteration with regard to the concentrations of factor or specific
lymphokines required for 50% of maximal response. This suggests that the lymphocytes are not altered with regard to the presumed receptors involved in lymphokine binding and induction of proliferation. Therefore, the increased responsiveness is most likely associated with an increase in the frequency of lymphocytes capable of responding to the various factors involved.

Estimates of the relative increase of responsive lymphocytes were obtained from the cell dilution and autoradiography experiments. In autoradiography the increased frequencies in these experiments and in others have ranged from 10- to 20-fold. The actual absolute increase in the number of these lymphocytes in vivo, however, is considerably higher because the total number of splenic lymphocytes in spleens from preleukemic mice is generally increased 3- to 5-fold. Therefore, the total relative increase of responsive lymphocytes is approximately 30- to 100-fold.

The increased frequency of lymphocytes responding to blastogenic factors is clearly dependent upon a cellular immune response against the virus rather than being a more direct effect of viremia. This is demonstrated by the absence of an expanded population of responding cells in MoLV-inoculated viremic CBA/N mice. As previously demonstrated, this mouse strain differs from most strains in that MoLV inoculation in newborns fails to induce a cellular immune response. The lack of a response segregates in genetic crosses with an X-linked recessive gene which also confers resistance to the development of leukemia. For these reasons it appears that the in vivo expansion of lymphocyte populations capable of responding to lymphokines requires an ongoing chronic immune response.

An important question is whether the increased frequency of lymphocytes responding to blastogenic factors seen in preleukemic mice is observed in other immune responses not associated with leukemia. In this regard, we have examined the frequencies of responsive lymphocytes during the course of MoLV/MoSV tumor growth and regression. Tumor regression in this system has been shown to require a vigorous cellular immune response (10, 15). In comparing these systems, the effects seen in preleukemic mice show two distinct differences. First, at the peak of tumor regression, when the highest increase in the frequency of responsive lymphocytes occurs, the increases observed are approximately 1/2 to 1/3 those seen in preleukemic mice (11). Second, during MoLV/MoSV tumor regression, the increased frequencies are transient; after tumor regression, they return to normal levels.

The basis for the increased frequency of responsive lymphocytes is not known. One possibility is that these cells are trans-

**Table 1.** Frequency of labeled lymphocytes responding to blastogenic factors in normal and preleukemic BALB/c mice

<table>
<thead>
<tr>
<th>Sample</th>
<th>Normal BALB/c</th>
<th>MoLV-inoculated BALB/c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without blastogenic factor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unlabeled</td>
<td>Labeled</td>
</tr>
<tr>
<td>1</td>
<td>5786</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>6170</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>6253</td>
<td>5</td>
</tr>
<tr>
<td>Mean</td>
<td>6235</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>With blastogenic factor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unlabeled</td>
<td>Labeled</td>
</tr>
<tr>
<td>1</td>
<td>6235</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>5150</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>6752</td>
<td>48</td>
</tr>
<tr>
<td>4</td>
<td>4831</td>
<td>57</td>
</tr>
<tr>
<td>Mean</td>
<td>0.8</td>
<td></td>
</tr>
</tbody>
</table>

Individual normal or MoLV-inoculated BALB/c mice 2–3 months of age were used. Splenic lymphocytes were prepared and the T-cell subpopulation was partially purified on nylon wool columns. The lymphocytes were subsequently incubated in the presence or absence of a blastogenic factor preparation. The cells were then pulsed with [3H]thymidine and prepared for autoradiography. Random fields were counted for total number of lymphocytes and number of labeled lymphocytes. Samples 1–3 and 1–4 represent the results obtained with individual mice.
formed. Although we cannot directly rule out this possibility, several observations argue against it. First, the increased responses are evident in the majority of mice as early as 1 month after inoculation, the earliest examined. Because a number of BALB/c mice survived for 3–4 months, and CBA/J mice for 5–6 months, a large population of transformed cells would have to exist for long periods without overt pathological manifestations. Second, the proliferation observed is dependent on lymphokines, whereas we know of no lymphoma cell lines that require lymphokines for growth. Indeed, transformation may involve loss of the regulation of proliferation by lymphokines. Last, as discussed below, the responses observed are general and involve different subpopulations of lymphocytes. Nevertheless, because leukemogenesis may involve a multistep process, the lymphokine-dependent proliferation may represent a premalignant step.

The more likely possibility for the increased responses involves the mechanisms by which the immune system is normally regulated. First, the increased involve lymphocytes responding to two different lymphokines, IL-2 and IL-3, indicating a general effect on lymphocyte subpopulations. Second, as noted above, similar increases occur transiently during cellular immune responses to the virus under conditions that do not give rise to leukemia. Last, both IL-2 and IL-3 appear to influence the immune system by promoting differentiation and proliferation (12–14). Thus, in vivo with antigen excess and antigen-specific helper T cells present, both IL-2 and IL-3 would be produced. This in turn would have an effect on the recruitment and expansion of lymphocytes responding to these factors and could result in the increased frequencies observed here. This is supported by the correlation of an absence of such increases in CBA/N mice which also fail to develop gp70 specific helper T cells.

Although previous studies have shown that chronic immune stimulation is required for leukemogenesis, this response could be required to provide appropriate target cell populations for virus infection and transformation. Although not directly addressed here, our previous work strongly suggests that the majority of the expanded subpopulations are not virus-infected (4). This comes from the observation that gp70-induced proliferation of splenic lymphocytes from preleukemic mice is not inhibited by treating the cells with monocolonal antibodies against gp70 and complement. Therefore, neither the antigen-specific lymphocytes responding to gp70 and producing blasticogenic factors nor the populations responding to these factors appear to express the virus.

An alternative possibility is that, as a consequence of viremia, the induction of a cellular immune response, and the concomitant non-antigen-specific expansion of subpopulations responding to lymphocytes, the probability of somatic errors associated with transformation is greatly increased. This possibility appears to be especially likely because the increases are in the order of 30- to 100-fold and persist over periods of 3–4 months prior to the actual onset of leukemia. Although suggestive, our data do not permit us to distinguish which elements of the immune response are most important. Based on the hypothesis developed by McGrath and Weissman (18), the most important components would be the generation of antigen-specific lymphocytes which would be the primary targets for transformation. Conversely, if somatic events are involved, the more general effects on lymphocytes mediated by the lymphokine responses described here would be the most significant.

This research was sponsored by the National Cancer Institute under Contract NO1-CC-75380 with Litton Bionetics, Inc.

References