Immunologic Rebound After Cessation of Long-term Chemotherapy in Acute Leukemia. II. In Vitro Response to Phytohemagglutinin and Antigens By Peripheral Blood and Bone Marrow Lymphocytes

By Alexander A. Green and Luis Borella

The in vitro function of peripheral blood and bone marrow lymphocytes from 42 children with acute lymphocytic leukemia (ALL) in remission was evaluated before and after cessation of long-term combination immunosuppressive therapy. Lymphocyte function promptly recovers even after 3 yr of antineoplastic therapy, but the immunologic rebound varies, depending on whether the in vitro responses to phytohemagglutinin or various antigens are used as the index of lymphocyte function. The kinetics of immunologic rebound in three patients who relapsed following cessation of therapy did not differ from patients remaining in remission. In children off therapy there was a different distribution of antigen- and PHA-responsive lymphocytes between the peripheral blood and bone marrow compartments. This study provides further information at a cellular level on the immunologic rebound that follows cessation of long-term combination therapy. It emphasizes the need for controls in trials where immunotherapy is given after chemotherapy. Such controls are necessary before one can establish if the observed responses are due to adjuvants and/or tumor cells or are a reflection of the immunologic rebound that follows cessation of immunosuppressive therapy. The data also provide a baseline for future studies of immune impairment before leukemia relapse and the relationship between immunologic rebound and the duration of long-term leukemia-free survival.

Although there are numerous reports of immunosuppression induced by short-term courses of antineoplastic agents given alone or in combination,1-7 knowledge of the long-term effects of combination chemotherapy administered continuously for several years is limited.8-10 Moreover, it is not known whether immunosuppressive treatment selectively suppresses different immune cell compartments.

Recent results from St. Jude Children’s Research Hospital demonstrate significant improvements in the treatment and prognosis of childhood acute lymphocytic leukemia (ALL).11 Sixty-six per cent of children with ALL treated according to recent protocols12,13 have been in continuous complete remission.
(CCR) for more than 3 yr. However, there is a significant morbidity and mortality in patients with ALL in remission that is probably related to the immunosuppressive effects of the antineoplastic agents. Thus, increasingly successful control of this disease has generated concern and new questions regarding long-term ill effects of intensive and prolonged immunosuppression during and after drug treatment.

Our previous results indicated that after cessation of 2½–3 yr of combination chemotherapy in children with ALL there was an immunologic rebound expressed by enhanced synthesis of antibody in the absence of extrinsic antigenic stimulation. Although this demonstrated a rebound of cells participating in antibody production (B-cells—bursa or bone marrow-derived lymphocytes), no information is available on the functional recovery of antigen-sensitive cells (T-cells—thymus-dependent lymphocytes). In the present study, we have investigated the functional recovery of lymphocytes in patients with ALL after chemotherapy was discontinued. Functional recovery was assayed by the in vitro blastogenic response to specific and nonspecific mitogens by peripheral blood and bone marrow lymphocytes. The data to be presented demonstrate a heterogeneity in the recovery of PHA-responsive and antigen-responsive cells in children with ALL in remission and indicate that antigen- and PHA-responsive cell populations are distributed differently in the bone marrow and peripheral blood compartments.

MATERIALS AND METHODS

Patient Population

A cohort of 42 children with ALL whose antileukemia therapy had been discontinued after 2½–3 yr of CCR comprise the study group. All patients had received a combination of five drugs—prednisone, vincristine, 6-mercaptopurine, methotrexate, and cyclophosphamide—as well as cranial or craniospinal irradiation according to schedules, dosages, and route of administration previously reported. There were 23 females and 19 males ranging in age from 2½ to 16 yr at the time of diagnosis, with a median age of 6½ yr. All but three patients had remained in remission 8–24 mo following cessation of treatment. Relapse occurred in these three patients 6, 9, and 12 mo after drug therapy was discontinued, and they were excluded from the study at the time of relapse.

Leukocyte Cultures

Peripheral blood and bone marrow cells were obtained before and at regular intervals following cessation of treatment when these specimens were required for diagnostic and prognostic purposes. Fifteen milliliters of blood and 3 ml of bone marrow containing heparin in a final concentration of 30 U/ml were obtained in plastic syringes. The syringes were held upright for 2 hr at 37°C to allow the erythrocytes to sediment; the leukocyte-rich plasma was then recovered. Leukocytes were counted using a Coulter-Selectronic Counter, and the lymphocytes were enumerated by differential counts using Wright-Giemsa-stained smears. Five X 10⁵ lymphocytes of peripheral blood (PB) or bone marrow (BM) origin were suspended in 1 ml volumes of Eagle's minimal essential medium containing 20% fresh autologous plasma (MEM-P) in loosely capped (7.5 X 1.24 cm) glass tubes. Triplicate cultures were incubated in a humid atmosphere of 95% air and 5% CO₂ at 37°C for periods of 3 or 6 days with or without mitogens. Some samples were not tested against all four mitogens, because influenza antigens were not available when the study was initiated, and low lymphocyte recovery in leukopenic patients did not permit assaying all mitogens at different doses.
Mitogens

Phytohemagglutinin (PHA) (Burroughs Wellcome, Buckingham, England) was diluted with 5 ml of sterile water; all further dilutions were made using 0.15 M phosphate-buffered saline pH 7.2 (PBS) or MEM. Dose-response curves using 0.1 ml of PHA dilutions through 1/320 were obtained with PB lymphocytes from normal adults and children and with PB and BM lymphocytes from several study patients. A broad peak response was obtained between 1/20 and 1/180 dilutions (Fig. 5). For all subsequent studies, three doses of PHA (1/20, 1/60, and 1/180) were used, and lymphocyte responses were assayed after 3 days of culture.

Keyhole limpet hemocyanin (KLH) was obtained as a powder from Calbiochem (lot No. 449), Los Angeles, Calif. Associated KLH was prepared and assayed as previously reported. A volume of 0.1 ml containing 50 µg or 100 µg was added to triplicate cultures and then was incubated for 6 days before harvesting. None of the patients in this study had been immunized with KLH, and all responses represent a primary in vitro response to associated KLH stimulation.

Candida albicans extract (Can.) was obtained as a 1:10 solution in 50% glycerine (lot No. 676040) from Hollister-Stier Laboratories, Atlanta, Ga. The optimum stimulating dose was determined using lymphocytes from normal individuals known to have a positive skin test (greater than 10 mm induration) reaction to C. albicans. As a result, 0.02 ml was added to triplicate cultures, and these were incubated for 6 days throughout this study.

Influenza antigens (Flu.) A/HK/1/68, H3N2 influenza virus, the X-31 recombinant of Kilbourne were grown in the allantoic cavity of 11-day-old chick embryos and were concentrated and partially purified by differential centrifugation, as previously reported. Hemagglutination titrations were done in plastic trays with 0.5% chicken erythrocytes as described by Fazekas and Webster. Virus was inactivated by incubation in 1/2000 formalin at 37°C for 24 hr. The vaccine was then dialyzed extensively in pH 7.2 PBS to remove all formalin and was sterilized by passage through 0.45 µ Millipore membranes. Aliquots were set up in sterile glass ampules (1 ml) and were stored at -60°C. Using PB lymphocytes from previously immunized donors, a peak stimulation on day 6 was obtained with a dose of 0.1 ml of a virus vaccine containing 10^2.0 HA/ml. This dose was used throughout this study.

Assay of In Vitro Response

Three hours before harvesting all cultures, 1.5 µCi ³Htdr (specific activity 3.0 Ci/mM) were added, and following incubation, the cultures were processed by acid precipitation for liquid scintillation counting as previously reported. All determinations were made in a Packard Tri-Carb Scintillation Spectrometer, and the results expressed as counts per minute (cpm). Net cpm (cpm cultures with PHA — cpm cultures without PHA) greater than 20,000 were considered positive response to PHA. The responses to the antigens were expressed as the ratio of cpm of cultures with antigen to cpm of cultures without antigen. Values greater than twice the unstimulated control cultures were considered positive responses to KLH, Can., and Flu.

Statistical analyses of the results were performed using both chi-square and Student's t tests. In all instances, cpm responses were converted to log10 to normalize the data before Student's t analyses. For chi-square analysis, the data were pooled as a function of time following cessation of treatment, and each time period compared independently to results obtained prior to cessation of treatment.

RESULTS

Recovery of In Vitro Responsiveness to PHA and Antigens by Peripheral Blood Lymphocytes

To evaluate the recovery of in vitro lymphocyte function following cessation of drug treatment, the in vitro blastogenic responses were analyzed as the percentage of patients whose lymphocytes responded to stimulation and as
Fig. 1. In vitro response of peripheral blood lymphocytes to PHA, KLH, Can., and Flu. before and following cessation of drug treatment. Bars indicate per cent of positive responders. Fractions above each bar represent the number of positive responders over the number of patients tested for each time period. Analysis of significance was performed using chi-square. P values demonstrate the "off" treatment period where the per cent responders differs significantly from "on" treatment responders.

The magnitude of the observed response. The data presented in Fig. 1 demonstrate that the percentage of responders to all mitogens increased after chemotherapy was stopped. The magnitude of the mean response to each mitogen is illustrated in Table 1. Only patients whose in vitro responses were considered positive are included. There were no significant changes in the magnitude of response to PHA between "on" and "off" treatment periods. In contrast, the mean in vitro responses to KLH, Can., and Flu. increased by 6 wk following cessation of treatment and continued to increase through 13–36 wk.
Table 1. Magnitude of In Vitro Responses During “On” and “Off” Treatment Periods

<table>
<thead>
<tr>
<th>Mitogen</th>
<th>On Treatment</th>
<th>Weeks After Cessation of Treatment</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
<td>6-12</td>
</tr>
<tr>
<td>PHA*</td>
<td>55 (28-181)</td>
<td>46 (20-84)</td>
</tr>
<tr>
<td>KLH†</td>
<td>6.7 (2.4-18)</td>
<td>10.6 (2.3-35)</td>
</tr>
<tr>
<td>Candida</td>
<td>4.2 (2.2-9.8)</td>
<td>6.5 (2.3-25)</td>
</tr>
<tr>
<td>Flut</td>
<td>10.3 (2.7-24)</td>
<td>13 (4.1-25)</td>
</tr>
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*cpm PHA minus cpm control (× 10^3).
†cpm Ag/cpm control. Beneath each mean value, the ranges are given in parenthesis.

These increases became significantly different from the “on” treatment values by 6 wk for KLH (p <0.05) and by 7-12 wk for Candida and Flut (p <0.005).

To assess further the recovery of in vitro responses, we analyzed the recovery patterns of individual patients. Figure 2 illustrates the results of five patients who had sequential samples tested against the four mitogens. The data are expressed as net counts per minute to illustrate better the magnitude of responses to the individual mitogens when simultaneously assayed. It demonstrates the heterogeneity of response by individual patients on and off treatment to the four mitogens. The response to any one of the mitogens and its variation with time after cessation of treatment are independent of the responses to the other mitogens. It also indicates that variation of response to PHA and the antigens when tested simultaneously is real and is not an artifact of the sampling or culture technique.

In Vitro Response of Simultaneously Assayed PB and BM Lymphocytes Obtained From Patients Recovering From 2½–3 Yr of Combination Immunosuppressive Therapy

Having demonstrated that lymphocyte recovery varies depending on whether the in vitro responses to PHA, KLH, Can., or Flut are used as an index of lymphocyte function, we asked the following: “Are these responsive cell populations distributed equally between bone marrow and peripheral blood?” To answer this question, we simultaneously obtained peripheral blood and bone marrow lymphocytes from the patients after the recovery period and assayed their in vitro responses to PHA and antigens. Table 2 demonstrates the results of 78 pairs of simultaneously assayed PB and BM cultures. Twenty-four pairs were cultured with PHA and 54 pairs were cultured with the antigens KLH (17 pairs), Can. (19 pairs), and Flut (18 pairs). In one of the pairs cultured with PHA and in 27 pairs cultured with antigens, both the PB and BM lymphocytes failed to respond.

As shown in Table 2, of the 23 pairs responding to PHA, only one pair had a greater response by BM lymphocytes than by PB lymphocytes. Seven pairs had equal responses of PB and BM lymphocytes, and in the remaining 15 pairs,
Fig. 2. In vitro response of peripheral blood lymphocytes simultaneously assayed against PHA (open circles), KLH (black circles) Candida (black triangle), and Flu (black square) before and after cessation of drug treatment. Each graph illustrates results from a single individual. Results are expressed as net cpm (cpm cultures with mitogen minus cpm cultures without mitogen) and are the mean of triplicate cultures.

the PB lymphocytes had significantly greater responses than did the BM lymphocytes. Figure 3 graphically depicts the responses of pairs stimulated with PHA. Along the ordinate are the responses of PB lymphocytes and on the abscissa are the responses of BM lymphocytes. The slanted line distinguishes the point where PB and BM responses are equal. Six pairs had essentially no response by BM lymphocytes, and the bulk of the pairs cluster on or above the line of equivalence. Only a single point falls below the line.

As seen in Table 2, most of the pairs had significantly different responses between PB and BM lymphocytes to antigenic stimulation. However, the results are in contrast to the results of PHA stimulation, in that ten pairs or
Table 2. In Vitro Response of Simultaneously Cultured Peripheral Blood Lymphocytes (PBL) and Bone Marrow Lymphocytes (BML)

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<tr>
<th>PHA</th>
<th>Antigens</th>
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<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>No response</td>
<td>1</td>
<td>42</td>
<td>27</td>
</tr>
<tr>
<td>PBL = BML</td>
<td>7</td>
<td>30.5</td>
<td>2</td>
</tr>
<tr>
<td>PBL ≠ BML</td>
<td>16</td>
<td>69.5</td>
<td>25</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td></td>
<td>54</td>
</tr>
<tr>
<td>PBL &gt; BML</td>
<td>15</td>
<td>94</td>
<td>15</td>
</tr>
<tr>
<td>PBL &lt; BML</td>
<td>1</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td></td>
<td>25</td>
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Blood and bone marrow lymphocytes obtained simultaneously from the same individual were assayed. The definitions PBL = BML, PBL ≠ BML, PBL > BML, and PBL < BML are given in the text.

40% had significantly greater response by BM lymphocytes than by PB lymphocytes to antigenic stimulation. The results of antigen-stimulated pairs are graphically displayed in Fig. 4. The results for each antigen are scattered above and below the line of equivalence.

We conclude from these results that PB and BM lymphocytes do not respond similarly to either PHA or antigenic stimulation.

Relationship Between Dose of Mitogen and/or Numbers of Nonlymphoid Cells and the Difference in Response Between PB and BM Cultures

To evaluate why these two populations respond differently, we determined if the dose of PHA or antigen necessary to stimulate optimally BM lymphocytes was different from the dose that produced optimum stimulation of PB lympho-

Fig. 3. In vitro response of simultaneously assayed PB and BM cultures to PHA stimulation. Results are expressed as net cpm (CPM cultures with PHA minus CPM cultures without PHA) and are the mean of triplicate cultures. The slanted line is present only to distinguish the point where PB and BM responses are equal.
cytes. Figure 5 illustrates the responses of PB and BM lymphocytes from four patients simultaneously assayed with different doses of PHA. The peak response of both PB and BM lymphocytes occurred at the same dose of PHA. Similar experiments using different doses of each of the antigens provided identical results, in that the peak response of both PB and BM lymphocytes occurred with the same dose. We, therefore, conclude that the different magnitude of response by PB and BM lymphocytes to PHA and antigens is not due to different dose requirements.

Fig. 4. In vitro response of simultaneously assayed PB and BM cultures to antigenic stimulation. Black circles, KLH; black triangles, Candida; black squares, Flu. Explanation of net cpm and the diagonal line are as in Fig. 3.

Fig. 5. In vitro response of simultaneously assayed PB and BM cells to different doses of PHA. Each section represents results from an individual patient. Open bars represent PB responses and closed bars the BM responses. Net cpm are as in Fig. 3.
Since bone marrow lymphocytes and peripheral blood lymphocytes had similar dose requirements, it is possible that the lymphocytes assayed in the bone marrow were peripheral blood lymphocytes that contaminated the bone marrow aspirates. However, this explanation could be ruled out for the following reasons: (1) an equal number of lymphocytes were obtained from 2 to 3 ml of bone marrow as from 15 ml of peripheral blood; (2) in 40% of assayed pairs the BM cells responded to antigens better than the PB lymphocytes, and in 90% of assayed pairs the PB lymphocytes responded better to PHA than the BM lymphocytes; and (3) Park et al. reported that in normal individuals when a 3 ml volume or less of bone marrow is aspirated little, if any, response to PHA could be detected, indicating that contamination by PB lymphocytes did not occur.19

An alternate explanation for the different responses of PB and BM lymphocytes to PHA and antigens is interference by contaminating nonlymphoid marrow cells. These cells might release in the culture medium inhibitors of the blastogenic response of bone marrow lymphocytes. To investigate this possibility, we first assumed that, if contaminating cells interfere with the response of BM lymphocytes, they would also interfere with the response of PB lymphocytes. The following mixing experiment was performed. Blood and bone marrow cells were obtained from two patients and cultured under four experimental conditions. The first group consisted of cultures of PB cells containing $5 \times 10^5$ lymphocytes per culture. The second group consisted of cultures of BM cells containing $5 \times 10^5$ lymphocytes per culture. The third group contained equal mixture of the first (PB) and second (BM) groups in 1 ml volume—therefore, twice as many cells per culture. The fourth group contained an equal number of PB cells as group one and twice as many BM cells as group two in 1 ml cultures. For each experimental situation, cultures were run in triplicate and with three dosages of PHA.

Table 3 demonstrates that the addition of BM cells to cultures of PB lympho-

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Control (cpm)</th>
<th>PHA Stimulation</th>
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<tr>
<td></td>
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<td>PHA 1:20</td>
</tr>
<tr>
<td>PBL*</td>
<td>204</td>
<td>59,825</td>
</tr>
<tr>
<td>BML*</td>
<td>4679</td>
<td>35,515</td>
</tr>
<tr>
<td>PBL* + BML*</td>
<td>3407</td>
<td>93,954</td>
</tr>
<tr>
<td>PBL* + BML†</td>
<td>6693</td>
<td>110,381</td>
</tr>
<tr>
<td>PBL*</td>
<td>532</td>
<td>75,689</td>
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<table>
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<th>Experiment 2</th>
<th>Control (cpm)</th>
<th>PHA Stimulation</th>
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<tr>
<td></td>
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<td>PHA 1:20</td>
</tr>
<tr>
<td>BM*</td>
<td>7586</td>
<td>8,508</td>
</tr>
<tr>
<td>PBL*</td>
<td>1811</td>
<td>81,804</td>
</tr>
<tr>
<td>BML†</td>
<td>947</td>
<td>79,330</td>
</tr>
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Results are expressed as mean cpm from triplicate cultures. In PHA-stimulated cultures cpm in controls were subtracted from cpm in cultures with PHA. All cultures were maintained in 1 ml volumes.

*Represents $5 \times 10^5$ lymphocytes from peripheral blood (PBL) or bone marrow (BML).
†Represents $1 \times 10^6$ lymphocytes from bone marrow.
cytes did not interfere with the response to PHA and, in fact, the sum of the
response of BM lymphocytes and PB lymphocytes was obtained. The results
also illustrate the similar dose requirements of PB and BM lymphocytes for
optimum response to PHA. In experiment 1, both the PB and BM lymphocytes
of DMCE responded to PHA and, except at the lowest dose of PHA, there was
no inhibition of peripheral blood response by the bone marrow cells. In ex-
periment 2 (MP), although the response of BM lymphocytes to PHA was
lower than in patient No. 1, no interference of the response of PB lymphocytes
to PHA was again observed by the addition of BM cells.

Since the differences between the in vitro responses to PHA and antigens
by peripheral blood and bone marrow cells cannot be explained either by
differences in dose requirements or by an inhibitory effect of nonlymphoid
cells, we conclude that in vitro responses to PHA and to antigens are expressed
by different populations of cells and that these cells are distributed differently
in the peripheral blood and bone marrow compartments.

DISCUSSION

This study demonstrates a rapid recovery of in vitro lymphocyte function
in patients with ALL in remission after 2½–3 yr of immunosuppressive
therapy. The recovery varies depending on whether the in vitro responses to
PHA or to antigens are used as an index of lymphocyte function. The hetero-
genesis of recovery best illustrated by the sequential assays of individual
patients suggest that the blastogenic response to PHA and to antigens are
expressed by different populations of cells. Comparison of the magnitude of
stimulation in peripheral blood and bone marrow cell cultures supports this
contention and demonstrates a different distribution of PHA-responsive and
antigen-responsive cells between the peripheral blood and bone marrow
compartments.

We had shown previously that an immunologic rebound occurs after cessa-
tion of long-term immunosuppressive treatment.9 This phenomenon was
characterized by an early rise of bone marrow lymphocytes followed by a
delayed increase in peripheral blood lymphocytes. In addition, without
extrinsic stimulation, there was a significant rise of serum immunoglobulins
and of antibody titers to an antigen to which these children had been exposed
earlier during the immunosuppressive phase of treatment.8 The present study
demonstrates similar recovery of function at the cellular level. Without evi-
dence of extrinsic stimulation, there was an increase in the per cent of
responders and in the magnitude of the responses by peripheral blood lympho-
cytes to Candida antigens, hemocyanin, and influenza virus.

These results indicate the need for cautious interpretation of data on the
stimulatory effect of various forms of immunotherapy given after drug therapy
is stopped. Adequate controls should be included before one can establish
whether specific or nonspecific stimulation of immunocompetent cells is due
to adjuvants and tumor cells or is mainly a reflection of the immunologic
rebound that follows cessation of chemotherapy.

Although there are several reports of in vitro blastogenic stimulation of
blood lymphocytes during and after short-term courses of chemotherapy,\textsuperscript{1-7} no information is available on the recovery of cell-mediated responses after several years of combination chemotherapy. We have shown that even after prolonged immunosuppression there is a prompt recovery of immune function, as assayed by antibody synthesis\textsuperscript{9} and by the blastogenic response of antigen-sensitive cells. In vitro assessment of functional recovery of immunocompetent cells correlates well with clinical observations. Children with ALL in remission and "off therapy" grow normally, and the incidence and severity of infections is not different from that observed in normal children.

Hersh et al.,\textsuperscript{6} Cheema and Hersh,\textsuperscript{4} and Harris and Stewart\textsuperscript{7} had reported that in patients with neoplasias, recovery of in vitro lymphocyte function after short-term chemotherapy correlates with the response to therapy and with prognosis. At the present time, we are unable to reach similar conclusions, because all the patients studied but three remain in their initial remissions from $3\frac{1}{2}-4\frac{1}{2}$ yr. Furthermore, no significant differences were noted between the patterns of immunologic recovery in these three patients when compared to those still in remission.

Published results of stimulation of human bone marrow lymphocytes with phytomitogens are in conflict with each other, and only three studies\textsuperscript{10,19,20} evaluated simultaneously the response of PB and BM lymphocytes to the same stimulant.\textsuperscript{19-23} Pegrum et al.\textsuperscript{21} failed to observe any response of BM lymphocytes to PHA as did Abdou and Abdou\textsuperscript{20} and Park et al.\textsuperscript{19} In contrast, Dicke et al.\textsuperscript{22} and Amato et al.\textsuperscript{23} demonstrated that BM lymphocytes do respond in vitro to PHA stimulation. Abdou and Abdou\textsuperscript{20} observed that pokeweed mitogen could stimulate both PB and bone marrow cells, but there is no information on bone marrow response to antigens. Our experiments indicate that the difference in response between PB and BM is not artifactual and that assessment of PB lymphocyte recovery may not correlate with functional recovery of other immune cell compartments such as BM.

Although one should be cautious in extrapolating these data to immunosuppressed patients without lymphoproliferative disorders, we believe these studies are pertinent to a basic understanding of the effects of chemotherapy on various immune cell compartments in man.

Data from experimental leukemia in animals indicate that immunosuppression may precede leukemogenesis.\textsuperscript{24-26} When these clinical studies were initiated, one of the main goals was to elucidate whether in patients with leukemia and off chemotherapy, immune impairment may occur before leukemia relapse. The answer to this question remains obscure. However, integral immunologic assessment and follow-up of individual patients with ALL in remission and "off therapy" should establish whether there is a relationship between immunosuppression and leukemogenesis. In addition, these studies will determine whether immunologic rebound without intrinsic stimulation plays a role in the long-term leukemia-free survival of children with ALL.

\textbf{ACKNOWLEDGMENT}

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REFERENCES


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