Distinctive Lymphocyte Subpopulation Abnormalities in Patients With Congenital Coagulation Disorders Who Exhibit Lymph Node Enlargement

By Harry E. Prince, Joan K. Kreiss, Carol K. Kasper, Steven Kleinman, Alexander M. Saunders, Lillian Waldbeser, Gail Mandigo, and Harold S. Kaplan

The majority of patients with congenital clotting disorders who use clotting factor concentrate exhibit lymphocyte subpopulation abnormalities. A subset of these patients develop lymph node enlargement (LNE), part of the spectrum of clinical disease associated with the acquired immune deficiency syndrome (AIDS). It is therefore important to determine if these patients with LNE exhibit specific immune alterations suggestive of early infection with the AIDS agent. We used one- and two-color immunofluorescence to distinguish the lymphocyte subpopulation alterations associated with concentrate use from those associated with LNE. Patients who use concentrate had elevated levels of Leu-2* (T suppressor phenotype) cells and Leu-7* (phenotype of some natural killer) cells. These increased levels were largely caused by a dramatic (2.6-fold) increase in the number of lymphocytes co-expressing Leu-2 and Leu-7 (2*7*). A dose–response effect between amount of concentrate infused during the preceding year and level of 2*7* cells was observed. Concentrate recipients, as a group, also showed increased levels of T cells expressing Dr antigen (T' Dr' phenotype, characteristic of activated or immature T cells) and cells expressing the T10 antigen (phenotype of some null cells and activated/immature T cells). Patients with LNE showed a further increase in T10 cells as well as a distinctive decrease in Leu-3* (T helper phenotype) lymphocytes. All LNE patients exhibited either low Leu-3* levels, high T10* levels, or both. Thus, concentrate use was associated with increased levels of Leu-2* (particularly 2*7*) cells and T' Dr' cells, whereas LNE was associated with decreased levels of Leu-3* cells and high levels of T10* cells.

© 1985 by Grune & Stratton, Inc.

PATIENTS WITH congenital clotting disorders who use clotting factor concentrate represent one of the groups at increased risk for the development of acquired immune deficiency syndrome (AIDS). Laboratory assessment of immunocompetence in these patients has revealed alterations in the percentage and absolute number of cells within lymphocyte subpopulations as detected by monoclonal antibodies. These alterations include decreased levels of T helper (Leu-3/OKT4) cells,1 increased levels of T suppressor (Leu-2/OKT8) cells,1 increased levels of a population including large granular (Leu-7) lymphocytes,1 and increased levels of lymphocytes bearing the Dr antigen.1

Although the observed changes may result from blood-borne viral infection or from chronic antigenic stimulation through blood product usage, it is possible that some of these abnormalities may be associated with clinical or subclinical infection with the AIDS virus. In an effort to identify those immune system abnormalities that are caused by blood product usage and those abnormalities that are associated with AIDS-related disease (lymph node enlargement [LNE]) or full-blown AIDS, we have phenotypically characterized lymphocyte subpopulations in 54 patients with hemophilia A, hemophilia B, or von Willebrand's disease. Because some of the markers used to phenotype altered lymphocyte populations can be co-expressed by the same cells,1 we have utilized both one-color and two-color immunofluorescence and flow cytometry to obtain a more detailed phenotypic profile of lymphocyte subsets in these patients.

MATERIALS AND METHODS

Patients. Fifty-four patients with hemophilia A or B or von Willebrand's disease from Orthopaedic Hospital, Los Angeles, were included in this study after informed consent was obtained. The mean age of study participants was 36 years, with a range of 20 to 66 years. Thirty-four patients received commercially available factor VIII concentrate as treatment for factor VIII deficiency (hemophilia A), eight patients received commercially available factor IX concentrate as treatment for factor IX deficiency (hemophilia B), and three patients with hemophilia A were treated with factor IX concentrate because of circulating anticoagulants against factor VIII. Nine patients (four with hemophilia A, two with hemophilia B, and three with von Willebrand's disease) were treated with cryoprecipitate or fresh-frozen plasma, with each bag being obtained from a single donor. The majority of concentrate recipients had severe disease (<1% normal factor VIII or IX activity), and used an average of 122,000 units of factor VIII or IX annually. Recipients of cryoprecipitate or fresh-frozen plasma tended to have mild disease (5% of normal factor activity), and used an average of 41 bags of cryoprecipitate or plasma during the preceding year. At the time of this study, 11 patients had extragluidal LNE (defined as lymph nodes >1 cm in diameter). Patients with LNE included eight patients with hemophilia A treated with factor VIII concentrate, two patients with hemophilia B treated with factor IX concentrate, and one patient with hemophilia A treated with factor IX concentrate. One patient with hemophilia A treated with factor VIII concentrate had AIDS with Pneumocystis carinii pneumonia.

Controls were 59 healthy blood donors from Los Angeles and Orange Counties in southern California. The mean age of donors was 35 years; 56% were men and 44% were women. Informed consent was obtained from donors prior to collection of blood specimens required for this study.

Blood specimens and mononuclear cell preparation. Peripheral blood collected in edetic acid tubes was used for the determination of the whole blood cell count by a Coulter counter (model M4-30, Hialeah, Fla). Blood smears were made from these same tubes, and the differential count was determined by counting 100 cells. Periph-
eral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifuga-
tion of 15 mL whole blood collected in heparinized tubes.\(^8\)

**Determination of cell surface phenotypes.** Monoclonal antibo-
dies used to determine cell surface phenotypes were conjugated with
fluorescein isothiocyanate (FITC), a green fluorescence emitter, or
phycoerythrin (PE), a red fluorescence emitter. The monoclonal
antibodies (Becton Dickenson Monoclonal Center, Mountain View,
Calif) used as markers for subsets included anti-Leu-M3 (most
mature monocytes), anti-HLA-Dr (B, activated T, most monocytes)
anti-Leu-2a (T cytotoxic/suppressor), anti-Leu-3a (T helper/
inducer, monocytes), and anti-Leu-7 (heterogeneous populations,
including some natural killer cells). Approximately 5 \(\times\) 10\(^5\) PBMCs
were placed in each of four tubes and were stained according to the
following protocol: tube 1 (monocyte control)—anti-Leu-M3-
FITC, anti-Leu-M3–PE; tube 2 (T/B/monocyte)—monocyte con-
trol tube reagents, anti-Dr-FITC, anti-Leu-2a-PE, and anti-Leu-
3a-PE; tube 3 (2/3/monocytes)—monocyte control tube reagents,
anti-Leu-3a–FITC, and anti-Leu-2a–PE; and tube 4 (2/7/mono-
cyte): monocyte control tube reagents, anti-Leu-2a–PE, and anti-
Leu-7–FITC.

After incubation for 30 minutes in an ice bath, the stained
cells were washed and resuspended in 1 mL cold phosphate-buffered
saline (PBS). Cell fluorescence was measured using a FACS Ana-
lyzer (Becton Dickinson) following the manufacturer's instruc-
tions (10,000 cells counted), with delineation of four cell populations: (1)
unstained cells; (2) cells staining with PE-conjugated reagent only;
(3) cells staining with FITC-conjugated reagent only; and (4) cells
staining with both PE- and FITC-conjugated reagents (ie, dual-
fluorescent). The percentage of dual-fluorescent cells in tubes 2, 3,
and 4 was corrected for the contribution of monocytes by subtracting
the percentage of dual-fluorescent cells in tube 1 (monocyte control)
from the observed percentage of dual-fluorescent cells. Previous
experiments have shown that Leu-3\(^-\) monocytes are found in the
dual-fluorescent area when stained with anti-Leu-M3. Thus, Leu-
3\(^-\) monocytes do not significantly contribute to the number of single
fluorescent Leu-3\(^-\) lymphocytes observed in tube 3 (A.M.S., unpub-
lished observation, July 1984).

The percentage of lymphocytes bearing the T10 antigen (null
cells, activated/immature T cells) was determined by immunofluor-
estence using whole blood samples and the monoclonal antibody
OKT10 (Ortho Diagnostic Systems). Lymphocyte populations were
distinguished from monocytes and granulocytes by correlated analysis of forward
scatter and right-angle scatter.\(^9\)

The absolute number of cells expressing a given phenotype was
determined by calculating the product of the WBC count, the
percentage of lymphocytes from the differential, and the percentage
of lymphocytes positive by immunofluorescence for that phenotype.

**Statistical analysis.** Means testing was performed using the
Mann-Whitney U test (two-tailed). The Spearman rank test was
used to determine correlation coefficients (and their significance
levels) between the amount of concentrate used and levels of a given
lymphocyte subpopulation. Comparisons between groups of the
proportion showing an abnormal value were performed using Fisher's
exact test.

**RESULTS**

**Lymphocyte subpopulation levels.** Patients with con-
genital clotting disorders were divided into three groups for
the purpose of data analysis. One group was composed of
fresh-frozen plasma or cryoprecipitate (FFP/cryo) recipients,
a second group of factor VIII or factor IX concentrate recipients
without LNE, and a third group of concentrate recipients with LNE.
Comparison of the levels of lymphocyte phenotypes between factor VIII and factor IX concentrate recipients, and between patients with factor VIII and factor IX deficiencies revealed no significant differences (data not shown).

As shown in Table 1, FFP/cryo recipients exhibited an
elevation in the number of Leu-7\(^+\) lymphocytes when com-
pared with controls; this increase was caused by increased
levels of Leu-7\(^+\) cells not expressing the Leu-2 antigen (ie,
2\(^7\)\(^+\) cells). However, the elevated mean 2\(^7\) level was
attributed to one individual with a value of 612 cells per
microliter. When this level was omitted, a mean value was
obtained that was not significantly different from controls.

As shown in Table 1, FFP/cryo recipients exhibited increased
levels of T10\(^-\) lymphocytes. The levels of all other lymphocyte sub-
populations were not significantly different from controls.

Concentrate recipients without LNE exhibited increased
levels of Leu-2\(^-\) cells. Two-color analysis showed that this

<table>
<thead>
<tr>
<th>Table 1. Lymphocyte Subpopulations in Control and Patient Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
</tr>
<tr>
<td>----------------------------</td>
</tr>
<tr>
<td>Lymphocytes</td>
</tr>
<tr>
<td>Leu-3(^-) lymphocytes</td>
</tr>
<tr>
<td>Leu-2(^+) lymphocytes</td>
</tr>
<tr>
<td>2(^7)</td>
</tr>
<tr>
<td>2(^7)</td>
</tr>
<tr>
<td>Leu-7(^-) lymphocytes</td>
</tr>
<tr>
<td>Dr(^-) lymphocytes</td>
</tr>
<tr>
<td>T(^+) Dr(^+)</td>
</tr>
<tr>
<td>T(^-) Dr(^+)</td>
</tr>
<tr>
<td>T10(^+) lymphocytes</td>
</tr>
</tbody>
</table>

*Values are mean cell number per microliter ± 1 SD.
†Significantly different from concentrate recipients without LNE group value, \(P < .05\).
‡Significantly different from control group value (\(P < .05\).
§Significantly greater than concentrate recipients without LNE group value, \(0.05 < P < .1\).
group had elevated levels of Leu-2" cells coexpressing Leu-7 antigen (2"7"), as well as Leu-2" cells not expressing Leu-7 (2'7'). However, the 2"7" elevation was more marked, being 2.6 times greater than the control value, while the 2'7" increase was only 1.4 times greater than the control value. The 2"7" elevation also contributed to the elevation in Leu-7" lymphocytes observed in concentrate recipients without LNE. Levels of 2"7" cells, however, were not significantly different from control levels. This group also exhibited increased levels of T cells co-expressing the Dr antigen (T'Dr') and lymphocytes expressing the T10 antigen (Table 1).

Concentrate recipients with LNE were the only patient group to exhibit decreased levels of Leu-3" (T helper) cells. This decrease was significant compared with both controls and concentrate recipients without LNE. Like concentrate recipients without LNE, those with LNE showed increased levels of Leu-2" and Leu-7" lymphocytes, primarily as a result of an increase in 2"7" cells; they also showed increased T'Dr" levels. Cell levels of T10" were also increased in this group as compared with controls, as was observed for FFP/cryo recipients and concentrate recipients without LNE. However, there was a distinct trend toward higher T10 levels in concentrate recipients with LNE as compared with concentrate recipients without LNE; this increase was significant at the P < .1 level (Table 1).

**Correlation of lymphocyte alterations with concentrate use.** Table 2 presents correlation coefficients between the lymphocyte population abnormalities in concentrate recipients and the number of concentrate units used in the previous year. Significant correlation was observed only between the number of 2"7" lymphocytes and the number of yearly concentrate units.

**Prevalence of abnormal values in concentrate recipients.** Table 3 presents the number of patients within the concentrate recipient groups who exhibited abnormal values. The frequencies of abnormal values for 2"7", 2'7", and T'Dr" levels were similar in concentrate recipients with and without LNE. Notable, although statistically nonsignificant, are the increases in the prevalence of abnormal Leu-3" values or abnormal T10 values observed in LNE + no LNE concentrate recipients. Prevalence rates for FFP/cryo recipients are not presented in Table 3 because, with one exception, mean values for these parameters were not significantly different from control values. The exception was T10 values, for which nine (44%) FFP/cryo recipients exhibited abnormal (elevated) values.

Table 4 presents data for concomitant Leu-3" and T10" levels in the concentrate recipients with or without LNE. In the group consisting of concentrate recipients without LNE, eight patients showed low Leu-3 levels only, 15 patients showed high T10 levels only, and ten patients showed normal values for both markers. None of the concentrate recipients without LNE exhibited abnormal values for both Leu-3 and T10. Five concentrate recipients with LNE exhibited high T10 levels only, three showed low Leu-3 levels only, and three showed both high T10 levels and low Leu-3 levels. The one hemophilia A patient with AIDS also showed a concomitant high T10 value and a low Leu-3 value. None of the concentrate recipients with LNE exhibited normal values for both Leu-3 and T10 levels.

The marked differences between LNE and no LNE concentrate recipient groups in prevalence rates for concomitant normal and abnormal values for both Leu-3 and T10 were significant, using Fisher's exact test (Table 4).

**Discussion**

The findings presented here demonstrate that concentrate use by patients with congenital clotting disorders is associated with abnormalities in the number of cells within various lymphocyte subpopulations. Although others have

**Table 2. Correlations Between Amount of Concentrate Used and Altered Lymphocyte Subpopulations**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Correlation Coefficient (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu-2&quot; lymphocytes</td>
<td>0.24</td>
</tr>
<tr>
<td>2&quot;7&quot; lymphocytes</td>
<td>0.16</td>
</tr>
<tr>
<td>2'7&quot; lymphocytes</td>
<td>0.31*</td>
</tr>
<tr>
<td>Leu-7&quot; lymphocytes</td>
<td>0.11</td>
</tr>
<tr>
<td>Dr&quot; lymphocytes</td>
<td>0.20</td>
</tr>
<tr>
<td>T'Dr&quot; lymphocytes</td>
<td>0.06</td>
</tr>
<tr>
<td>T10&quot; lymphocytes</td>
<td>0.24</td>
</tr>
</tbody>
</table>

*Significant at the P < .05 level (Spearman rank correlation).

**Table 3. Prevalence of Abnormal Values in Concentrate Recipients With or Without LNE**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Concentrate Recipients Without LNE (n = 33)</th>
<th>Concentrate Recipients With LNE (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu-3&quot; lymphocytes</td>
<td>8 (24%)</td>
<td>6 (55%)</td>
</tr>
<tr>
<td>Leu-2&quot;7&quot; lymphocytes</td>
<td>5 (15%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Leu-2'7&quot; lymphocytes</td>
<td>9 (27%)</td>
<td>3 (27%)</td>
</tr>
<tr>
<td>T'Dr&quot; lymphocytes</td>
<td>6 (18%)</td>
<td>4 (36%)</td>
</tr>
<tr>
<td>T10&quot; lymphocytes</td>
<td>15 (45%)</td>
<td>8 (73%)</td>
</tr>
</tbody>
</table>

Abnormal values: less than control fifth percentile value for Leu-3" lymphocytes, or greater than control 95th percentile value for all other parameters.

*None of the differences between concentrate recipients with or without LNE was significant.

Table 4. Analysis of Concomitant Leu-3 and T10 Values in Concentrate Recipients

<table>
<thead>
<tr>
<th>Leu-3</th>
<th>T10</th>
<th>Concentrate Recipients Without LNE (n = 33)</th>
<th>Concentrate Recipients With LNE (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Normal</td>
<td>10 (31%)</td>
<td>0 (0%)*</td>
</tr>
<tr>
<td>Normal</td>
<td>High</td>
<td>15 (45%)</td>
<td>5 (45%)</td>
</tr>
<tr>
<td>Low</td>
<td>Normal</td>
<td>8 (24%)</td>
<td>3 (27%)</td>
</tr>
<tr>
<td>Low</td>
<td>High</td>
<td>0 (0%)</td>
<td>3† (27%)*</td>
</tr>
</tbody>
</table>

See Table 3 for definition of abnormal values.

*Significantly different from concentrate recipients without LNE value (P < .03).
†In addition, the one hemophilia patient with AIDS fell into this category.
shown that Leu-2 (T suppressor) and Leu-7 (some natural killer cells) levels are elevated in concentrate recipients, our data revealed that an elevated level of a lymphocyte subpopulation simultaneously expressing Leu-2 and Leu-7 markers (2⁺7⁺ cells) was primarily responsible for increased Leu-2 and Leu-7 levels. The number of 2⁺7⁺ cells was also increased, but not as dramatically as the number of 2⁺7⁻ cells.

Furthermore, the 2⁺7⁺ population was the only cell population whose number was significantly correlated (albeit weakly) with concentrate use. This population may therefore be an indicator of the extent of chronic exposure to antigens through administration of blood products. This marker may also prove to be an indicator of chronic antigenic exposure from other sources, such as viral infections. D. Stites (University of California at San Francisco, personal communication, July 1984) has found that 2⁺7⁺ levels are elevated in healthy homosexuals, who tend to have a history of frequent exposure to viruses such as cytomegalovirus and hepatitis B. The exact functional role played by 2⁺7⁺ cells in immune response remains unknown.

Concentrate use was also associated with an increase in the number of T cells expressing the Dr antigen. Increased levels of Dr⁺ lymphocytes in hemophilia patients using factor VIII concentrate were demonstrated by deShazo et al. Our data, obtained by two-color immunofluorescence, extend these findings by demonstrating that it is the Dr-bearing T cell population (T⁺Dr⁺), rather than the B cell population (T⁺Dr⁻), that is abnormal.

T cells express Dr antigen in vitro after activation with antigen or mitogen. The Dr⁺T cells in our patient groups may therefore represent T cells activated in vivo by chronic antigenic stimulation with concentrate. However, the lack of correlation between T⁺Dr⁺ levels and concentrate use suggest that additional factors are involved in Dr antigen expression by T cells in patients treated with concentrate.

Elevated levels of Dr⁺ lymphocytes have been observed in AIDS patients, and Mildvan et al. have further shown that the T cells expressing Dr are Leu-2⁺ cells in these patients. In our study, good correlation was observed between T⁺Dr⁺ levels and levels of both Leu-2⁺ subpopulations (2⁺7⁻, 2⁺7⁺) (data not shown). This observation provides indirect evidence that selective expression of Dr antigen on Leu-2⁺ cells may occur in patients being treated with clotting factor concentrate as well as in AIDS patients. Longitudinal studies are necessary to determine whether Dr expression by T cells is reflective of concentrate use or is an early indicator of AIDS infection.

Notably, all 11 LNE patients and the AIDS patient in our study received concentrate, indicating a clear association between exposure to concentrate and changes in clinical status that may be associated with AIDS. Many of the alterations observed in concentrate recipients without LNE were also seen in those with LNE. The most remarkable difference noted between these two groups was a significant decrease in the number of Leu-3⁺ (T helper) cells, an abnormality that is the major laboratory manifestation of AIDS. Another marker associated with LNE is T10, also a marker of activated or immature T cells, as well as some natural killer cells. Although T10 levels were elevated in concentrate recipients as a whole, they tended to be higher in those with LNE than in those without LNE (P < .1). Thus, although antigenic exposure appears to result in elevated levels of T10⁺ cells, a change in clinical status of the patient using clotting factor concentrate is associated with a further increase in T10. Although the in vivo function of these cells is unknown, recent reports of decreased natural killer activity in concentrate recipients indirectly suggest that increased numbers of T10⁺ lymphocytes in these patients do not represent functional natural killer cells.

All LNE patients in our study exhibited an abnormal value for at least one of these two markers (Leu-3 or T10), with 33% exhibiting abnormal values for both markers. In contrast, none of the concentrate recipients without LNE exhibited alterations in both markers. Thus, the lymphocyte alteration most clearly associated with LNE was a concomitant decrease in Leu-3 levels and an increase in T10 levels.

Longitudinal studies of concentrate recipients without LNE are required to determine the kinetics of lymphocyte alteration development and the value of these alterations as indicators of clinical changes associated with AIDS. One such preliminary study has shown that lymphocyte alterations are not transient and were sometimes further altered upon remeasurement. Follow-up of LNE patients is similarly required to determine which combination of lymphocyte alterations is associated with the actual development of AIDS. The AIDS patient included in this study exhibited abnormal values for both T10 and Leu-3, possibly indicating a poor prognosis for concentrate recipients with LNE who exhibit alterations in both T10 and Leu-3 levels.

In addition to numerical and statistical analyses of results, visual examination of two-dimensional dot plot patterns yielded important characteristics of the cell populations identified. In the clotting disorder patients with high 2⁺7⁺ levels, the majority of these cells stained brightly with anti-Leu-2 and dimly with anti-Leu-7. This pattern is very different from that found in homosexual populations, whose cells stain brightly with both anti-Leu-2 and anti-Leu-7 (H. Perkins, Irwin Memorial Blood Bank, San Francisco, personal communication, July 1984). The significance of this difference is not yet known and is under study.

Ragni et al. recently described a T lymphocyte colony assay that can discriminate between hemophilia A or B patients with LNE and those without LNE. Thus, T cell function, as well as the levels of T cells expressing the Leu-3 or T10 cell surface markers, is abnormal in patients with clotting disorders who exhibit LNE. Taken together, these findings indicate that patients with LNE constitute a group with distinctive immune system alterations. Clinical and laboratory follow-up is indicated to determine whether these alterations represent the beginning of AIDS or the endpoint of a "normal" physiologic response of these patients to chronic antigenic stimulation.

In summary, our findings show that lymphocyte subset alterations that may reflect chronic antigenic stimulation by clotting factor concentrate in patients with clotting factor disorders can be distinguished from those reflective of specific clinical changes associated with AIDS-related condi-
tions. Changes in Leu-2 levels (particularly 2'7' cells) and possibly in T'Dr cell levels are associated with concentrate use per se. In addition, changes in Leu-3 levels and TlO levels may prove valuable as prognostic markers for LNE and/or AIDS among patients with congenital coagulation disorders.

ACKNOWLEDGMENT

The authors wish to thank Drs Shelby Dietrich and June Marshall for helpful discussions, and Ortho Diagnostic Systems for providing monoclonal antibodies and the Spectrum III flow cytometer. The FACS analyzer and Leu reagents were supplied by Becton Dickinson, and Ruth Isenberg provided method development.

REFERENCES

Distinctive lymphocyte subpopulation abnormalities in patients with congenital coagulation disorders who exhibit lymph node enlargement

HE Prince, JK Kreiss, CK Kasper, S Kleinman, AM Saunders, L Waldbeser, G Mandigo and HS Kaplan