IMMUNOLOGIC STATUS OF HEMOPHILIA PATIENTS TREATED WITH CRYOPRECIPITATE OR LYOPHILIZED CONCENTRATE

By George F. Gjerset, Paul J. Martin, Richard B. Counts, Loren D. Fast, and John A. Hansen

We evaluated 37 patients with moderate or severe hemophilia A and six patients with severe factor IX deficiency for clinical or laboratory evidence of immune abnormalities. Patients were assigned to one of four groups according to the type of clotting factor replacement. Twenty patients had received only cryoprecipitate during the two years preceding the evaluation (group I); 11 additional patients were treated predominantly with cryoprecipitate but had also received up to nine bottles of factor VIII concentrate (group II); six patients received factor VIII concentrate (group III); six patients received factor IX concentrate (group IV). There was no clinical or laboratory evidence of immunodeficiency among the 43 patients. The mean absolute number of T₅ cells was normal in all patient groups, but the mean absolute number of T₆ cells was increased compared with controls, both in patients treated with cryoprecipitate and in patients treated with factor VIII or factor IX concentrate. There was no correlation between the T₅/T₆ ratio and patient age, alanine aminotransferase level, hepatitis serology, in vitro lymphocyte function, or amount of clotting factor administered. Our observations demonstrate that the volunteer or commercial origin of clotting factor replacement cannot fully explain the alterations in lymphocyte subset distribution previously described in patients with hemophilia A.

The recent reports of opportunistic infections in patients with hemophilia A have led to the suggestion that an agent causing acquired immunodeficiency syndrome (AIDS) can be transmitted in blood products. Additional concern about the risk of AIDS in hemophilic individuals has come from observations that many healthy hemophilic patients have alterations of T lymphocyte helper (T₅) and suppressor (T₆) cells similar to those found in the putative prodromal stage of AIDS. In previous studies, alterations in the T₅/T₆ ratio as well as absolute numbers of T₅ and T₆ were present predominantly in patients receiving lyophilized factor VIII concentrate and not in those receiving cryoprecipitate. It could not be determined in these studies whether this difference was due to the type of treatment per se or whether it reflected the relative amounts of factor VIII administered in the two groups. For over ten years we have used cryoprecipitate prepared at the Puget Sound Blood Center to treat most patients with hemophilia A, including those severely affected. The present study was undertaken to determine whether there was any clinical or laboratory evidence of immunodeficiency in our patients. The results for patients with hemophilia A treated with cryoprecipitate were compared with results for patients treated with factor VIII concentrate. An additional group of patients with factor IX deficiency was also studied.

MATERIALS AND METHODS

Subjects

Forty-three patients with factor VIII or IX deficiency, including 13 with moderate disease (1% to 5% factor VIII or IX) and 34 with severe disease (<1% factor VIII or IX), were examined between November 1982 and January 1983. Thirty-seven had factor VIII deficiency; six had factor IX deficiency. Patients were assigned to one of four groups, depending on the type of treatment received (Table 1). Twenty patients had received only cryoprecipitate during the two years preceding the study (group I). An additional group of 11 patients was treated predominantly with cryoprecipitate but had also received between one and nine bottles of factor VIII concentrate during the two years preceding the study (group II). Six patients had received factor VIII concentrate (group III), and six patients had received factor IX concentrate only (group IV). Four patients were excluded from the analysis because they had received substantial amounts of both cryoprecipitate and factor VIII concentrate and thus could not be readily assigned to any single group.

The distribution of moderately and severely affected patients was not equivalent in all groups. Twelve of the 13 moderately affected patients were either group I or group II, whereas all but one of the 12 patients in groups III and IV were severely affected. The determination of whether cryoprecipitate or factor VIII concentrate was used as the mainstay of therapy was determined primarily by the patient’s accessibility to care at the Puget Sound Blood Center. Cryoprecipitate was prepared from single volunteer donor units of whole blood as previously described, and each bag of cryoprecipitate contained approximately 100 units of factor VIII activity. Each bottle of lyophilized factor VIII concentrate contained 1,000 units and each bottle of factor IX concentrate contained 500 units. Eight patients in group I had received less than 100,000 units of factor VIII during the two years preceding the study, whereas only one patient in group II and none in group III had received less than 100,000 units of factor VIII. The medians (not shown), means, and age ranges of the four groups were comparable. The control group consisted of 38 healthy individuals (aged 13 to 66 years) who were either laboratory personnel (N = 24) or unaffected family members of patients (N = 14).

From the Puget Sound Blood Center, the Fred Hutchinson Cancer Research Center, and the University of Washington School of Medicine, Seattle.

Supported in part by grants HL 17265, CA 29548, CA 18029, and CA 15704. Dr Gjerset is a fellow under the National Research Service Training grant.

Submitted June 23, 1983; accepted April 9, 1984.

Address reprint requests to Dr George F. Gjerset, the Fred Hutchinson Cancer Research Center, 1124 Columbia St, Seattle, WA 98104.

© 1984 by Grune & Stratton, Inc.

0006-4971/84/6403-0020$03.00/0.

Blood, Vol 64, No 3 (September), 1984: pp 715–720
Lymphocyte FITC (diluted 1:20) for 30 minutes at 4 °C. Monoclonal Cells were resuspended in tissue culture medium (RPMI 1640) with fluorescence using murine monoclonal antibodies and an affinity-purified studies and cell-mediated cytotoxicity assays. T cells were prepared by subtracting the number of non-I cells (ie, 7.2-negative) from the percentage of positively stained cells by the absolute lymphocyte count as previously described. Absolute numbers of Ia-positive cells (ie, 7.2-positive).

Hepatitis Studies

Serologic assays for the hepatitis B markers HBsAg, HBsAb, and HBeAg and measurement of serum alanine aminotransferase (ALT) levels were done by standard methods.

Preparation of Cells

Blood was collected in plastic syringes containing 20 units of heparin per milliliter. Absolute lymphocyte counts were calculated from the white blood cell count and differential. Peripheral blood mononuclear (PBM) cells were separated by Ficoll-Hypaque centrifugation and washed twice with Hanks' balanced salt solution. Cells were resuspended in tissue culture medium (RPMI 1640) with 20% human serum for lymphocyte transformation studies or in RPMI 1640 with 12% fetal calf serum for cell surface marker studies and cell-mediated cytotoxicity assays. T cells were prepared by incubating the PBM cell suspension on a nylon wool column at 37 °C for 30 minutes, as previously described.14

Lymphocyte Subsets

T cell surface markers were assessed by indirect immunofluorescence using murine monoclonal antibodies and an affinity-purified fluorescein-conjugated goat anti-mouse immunoglobulin serum (GAMlg-FITC, Tago, Inc, Burlingame, Calif). The monoclonal antibodies used are listed in Table 2. Cells (5 x 10^6) were incubated at 4 °C with 25 µL of monoclonal antibody at saturating concentration for 30 minutes, washed, and stained at 4 °C with 25 µL GAMlg-FITC (diluted 1:20) for 30 minutes at 4 °C. Monoclonal antibody 9E8, which recognizes the murine leukemia virus antigen p15(E), was used as a negative control.15 After washing and fixation with 1% paraformaldehyde, the cells were analyzed by flow microfluorometry using a FACS II (Becton Dickinson, Mountain View, Calif), as previously described.26 Absolute numbers of T6 (66.1-positive) and T8 (51.1-positive) cells were calculated by multiplying the percentage of positively stained cells by the absolute lymphocyte count. The percentage of la-positive T cells was determined by subtracting the number of non-T cells (ie, 9.6-negative) from the number of la-positive cells (ie, 7.2-positive).

Lymphocyte Transformation Assays

Lymphocyte responses in vitro were assayed in a microculture test system. Responses to soluble antigens were tested by culturing 5 x 10^6 responder cells with tetanus toxoid (Wyeth, Philadelphia, Pa) or candida antigen (Holllister-Stier, Spokane, Wash) at optimal dilution. For mitogen responses, 5 x 10^5 responders were stimulated with either phytohemagglutinin (PHA) (Burroughs-Wellcome, Research Triangle Park, NC) or concanavalin A (ConA) (Calbiochem, La Jolla, Calif). The response to stimulation with allogeneic cells in mixed lymphocyte culture (MLC) was tested by coculture of 5 x 10^6 responder cells with 5 x 10^6 irradiated pooled PBM cells from five unrelated normal donors. Mitogen-stimulated cultures were incubated at 37 °C with 5% CO2 at 100% humidity for three days; antigen-stimulated cultures and MLCs were similarly maintained for six days. Cell proliferation was assayed by terminal three-hour [3H]thymidine incorporation.

Cell-Mediated Cytotoxicity Assays

Assessment of natural killer (NK) cell activity and antibody-dependent cell-mediated cytotoxicity (ADCC) activity was made with and without augmentation with interferon-containing supernatants. The assay for NK cell activity was performed with 2 x 10^5 ^51Cr-labeled K562 target cells in V-bottom microculture plates. Effector (E) PBM were added to target (T) cells at E:T ratios of 12.5:1, 25:1, 50:1, and 100:1. After a four-hour incubation at 37 °C, 100 µL of supernatant fluid was counted. The ADCC activity was assayed in the same manner using as targets ^51Cr-labeled cells of the Chang epithelial cell line incubated with rabbit anti-Chang antiseraum (1:10^6) for 30 minutes at 37 °C. Interferon-augmented NK and ADCC assays were carried out by pre-incubation of effectors with human leukocyte interferon-containing supernatants (50 U/2 x 10^6 cells in 1 mL) for one hour at 37 °C. Interferon was produced by culture of PBM cells (2 x 10^6/mL) with cells of the K562 cell line (1 x 10^6/mL) overnight and assayed as previously described.27

Statistical Analysis

The T6/T8 ratio and lymphocyte proliferation data were log transformed and the cell-mediated cytotoxicity data were logit transformed in order to approximate a normal distribution.22 Comparisons were made using the raw and transformed data. For each comparison a pooled t test was used, except when the variances for the samples being compared were dissimilar, in which case a two-sample t test was used. All t tests were two sided. Data were also compared using the Wilcoxon rank sum test. When comparing dose-dependent responses, analysis of variance was used. Results for

<table>
<thead>
<tr>
<th>Table 1. Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Group Number</td>
</tr>
<tr>
<td>I</td>
</tr>
<tr>
<td>II</td>
</tr>
<tr>
<td>III</td>
</tr>
<tr>
<td>IV</td>
</tr>
</tbody>
</table>

*Less than 1% factor VIII or IX.
†One patient received 50 bags; the others received none.

<table>
<thead>
<tr>
<th>Table 2. Murine Monoclonal Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
</tr>
<tr>
<td>9.6</td>
</tr>
<tr>
<td>51.1</td>
</tr>
<tr>
<td>66.1</td>
</tr>
<tr>
<td>7.2</td>
</tr>
</tbody>
</table>
Abbreviation: HBsAg = hepatitis B surface antigen; HBcAb = hepatitis B core antibody; HBsAb = hepatitis B surface antibody; ALT = alanine aminotransferase.

**RESULTS**

**Clinical Status**

Approximately 300 patients with factor VIII or IX deficiency are treated through the Hemophilia Program of the Puget Sound Blood Center. Over the past ten years there have been no recorded episodes of opportunistic infection among these patients and, except for one death caused by acute nonlymphocytic leukemia, trauma and hemorrhage account for all mortality. Clinical history, physical examination, and relevant laboratory findings for the 43 patients in the study group are summarized in Table 3. All patients were healthy and without symptoms. Five of the patients had mild lymphadenopathy or splenomegaly, while physical examination of the remaining 38 patients was unremarkable. Forty of 41 patients tested had some serologic evidence for past hepatitis B infection and 11 of 22 tested had mild to moderate elevation in serum ALT. The frequency of positive tests for past hepatitis infection was similar in each of the patient groups.

**Lymphocyte Counts and T Cell Subsets**

The mean absolute lymphocyte count was higher in each of the patient groups than in the control group, but this difference reached statistical significance only for groups II and III (Table 4). The mean absolute number of \( T_\text{h} \) cells was significantly higher in each of the patient groups than in the control group. In contrast, the numbers of \( T_\text{s} \) cells in patients and controls were similar. Patients receiving cryoprecipitate only (group I) did not differ from those receiving factor VIII or IX concentrate in the number of lymphocytes, \( T_\text{h} \) cells, or \( T_\text{s} \) cells. The mean \( T_\text{h}/T_\text{s} \) ratio was significantly lower in groups I, III, and IV than in the controls (Fig 1). With logarithmic transformation, the \( T_\text{h}/T_\text{s} \) ratio data for the control group conformed more closely to a normal distribution. Comparisons made using log-transformed \( T_\text{h}/T_\text{s} \) ratios confirmed the statistical significance of the differences between groups I, III, and IV and the controls. Comparisons made between patient groups did not disclose statistically significant differences in the mean \( T_\text{h}/T_\text{s} \) ratios.

One notable alteration in cell surface markers was the presence of more than 10% Ia-positive T cells in 12 of 35 patients tested. In contrast, only 1 of 16 control

### Table 3. Clinical Status of Hemophiliacs

<table>
<thead>
<tr>
<th>Clinical Finding</th>
<th>Group</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>History of infection*</td>
<td></td>
<td>2/20 (10†)</td>
<td>2/11 (18)</td>
<td>0/6 (0)</td>
<td>3/6 (50)</td>
</tr>
<tr>
<td>Symptoms</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Palpable spleen tip</td>
<td></td>
<td>1/20 (5)</td>
<td>1/11 (9)</td>
<td>0/6 (0)</td>
<td>0/6 (0)</td>
</tr>
<tr>
<td>Lymphadenopathy§</td>
<td></td>
<td>0/20 (0)</td>
<td>1/11 (9)</td>
<td>1/6 (17)</td>
<td>1/6 (17)</td>
</tr>
<tr>
<td>Laboratory</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBsAg</td>
<td></td>
<td>2/20 (10)</td>
<td>0/10 (0)</td>
<td>0/5 (0)</td>
<td>0/6 (0)</td>
</tr>
<tr>
<td>HBsAb</td>
<td></td>
<td>13/19 (68)</td>
<td>9/11 (82)</td>
<td>4/5 (80)</td>
<td>5/6 (83)</td>
</tr>
<tr>
<td>HBcAb</td>
<td></td>
<td>13/18 (68)</td>
<td>9/11 (82)</td>
<td>4/5 (80)</td>
<td>6/6 (100)</td>
</tr>
<tr>
<td>ALT &gt; 40 IU</td>
<td></td>
<td>3/10 (30)</td>
<td>5/8 (62)</td>
<td>2/2 (100)</td>
<td>1/2 (50)</td>
</tr>
</tbody>
</table>

*Clinical hepatitis one to 17 years before the study.
†Denominator, number of patients tested; percentages in parentheses.
§A 1-cm posterior cervical node was noted in two patients and a 2 x 2-cm posterior auricular node was noted in one patient.

### Table 4. Lymphocytes and T Cell Subsets in Hemophiliacs

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Patients</th>
<th>( T_\text{h}/T_\text{s} ) Ratio</th>
<th>Number*</th>
<th>Lymphocytes per ( \text{mm}^3 )</th>
<th>( T_\text{h} ) Cells per ( \text{mm}^3 )</th>
<th>( T_\text{s} ) Cells per ( \text{mm}^3 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>20</td>
<td>1.0 ± 0.5†</td>
<td>18</td>
<td>2,143 ± 1,214‡</td>
<td>840 ± 517‡</td>
<td>873 ± 303†</td>
</tr>
<tr>
<td>II</td>
<td>11</td>
<td>1.3 ± 0.8‡</td>
<td>9</td>
<td>2,687 ± 880§</td>
<td>1,114 ± 437‡</td>
<td>1,205 ± 474‡</td>
</tr>
<tr>
<td>III</td>
<td>5</td>
<td>0.6 ± 0.6§</td>
<td>4</td>
<td>2,504 ± 103†</td>
<td>818 ± 612‡</td>
<td>1,267 ± 407§</td>
</tr>
<tr>
<td>IV</td>
<td>6</td>
<td>0.7 ± 0.4†</td>
<td>4</td>
<td>2,201 ± 358‡</td>
<td>745 ± 503‡</td>
<td>1,019 ± 226§</td>
</tr>
<tr>
<td>Controls</td>
<td>38</td>
<td>1.6 ± 0.6</td>
<td>20</td>
<td>1,749 ± 565</td>
<td>888 ± 288</td>
<td>611 ± 211</td>
</tr>
</tbody>
</table>

Results are given as the mean ± 1 SD.

*Number of patients on whom absolute counts were obtained. Ratios were obtained on all patients and controls.
†\( P < .01 \), comparison with control group, by all tests.
‡\( P < .05 \), comparison with control group.
\( \text{§} P < .05 \), comparison with control group, by all tests (see Materials and Methods).
subjects had more than 10% la-positive T cells (Fig 2). All patients with more than 10% la-positive T cells had Th/T, ratios less than 0.9. Patients with more than 10% la-positive T cells were found in each of the four treatment groups, but the mean percentage of la-positive T cells differed significantly from controls only in group III.

The Th/T, ratio was compared with other clinical and laboratory findings to determine whether patients with a low ratio had any other distinctive characteristics. There was no correlation between the ratio and age in patients or controls. There was likewise no correlation between the ratio and the number of bags of cryoprecipitate received, or the serum ALT level, examined independently of treatment group assignment. Similarly, there were no differences in the mean Th/T, ratio of patients separated according to the results of each serologic test for hepatitis B (HBsAg, HBsAb, or HBCAb).

Lymphocyte Responses In Vitro and Cell-Mediated Cytotoxicity

There was no statistically significant difference between any patient group and controls in the response of lymphocytes to stimulation with the mitogen PHA, soluble antigens candida and tetanus, or allogeneic cells (Figs 3 and 4). The lymphocyte response to stimulation with concanavalin A was lower in group III than in the controls (Fig 5), but this difference was of borderline statistical significance.* Natural killer lytic activity in all patient groups was normal both with and without interferon augmentation (Table 5). Cells from patients in all four groups demonstrated normal lytic activity in the ADCC assays. For each assay there was a group of outliers, but the t test and rank sum test showed no differences between the patients and the controls.

DISCUSSION

We have studied the immune function of patients with factor VIII or factor IX deficiency treated with large amounts of cryoprecipitate prepared from healthy volunteer blood donors or with commercially prepared factor VIII or factor IX concentrate. Among the approximately 300 patients treated by the hemophilia program of the Puget Sound Blood Center, frequent or unusual infections have not been noted. The clinical histories of the 43 patients studied in detail were unremarkable. Three of these patients had localized lymphadenopathy and two had mild splenomegaly, but the physical examinations were otherwise normal. Alterations in the proportions of T cell subsets

---

* A P value less than .05 was detected using the pooled t test, which disregards unequal variances, but not using the two-sample t test, which assumes unequal variances. The wide scatter of the data in group III, along with the small number of patients, make a conclusive comparison with controls difficult.
did occur and appeared more marked in the group treated with large amounts of commercial factor VIII concentrate. However, neither lymphopenia nor depression of the number of Th cells was found in any patient group. The in vitro proliferative and cytolytic functions of cells from our patients were normal, with the possible exception of the ConA response of patients treated with factor VIII concentrate. We thus found no clinical or laboratory evidence of immunodeficiency in our patients.

Our results confirm previous reports that the Th/T, ratio is often decreased in otherwise healthy individuals with hemophilia A and that this finding is due to an increased number of Tc cells rather than a decreased number of Th cells. In contrast to the previous studies, we found decreased Th/T, ratios both in patients treated with cryoprecipitate and in those treated with factor VIII or IX concentrate. This finding was detectable despite the fact that more of our patients treated with cryoprecipitate were moderately affected and had received less total factor VIII replacement than the patients treated with factor VIII concentrate. Our observations in patients with moderate or severe factor VIII or factor IX deficiency demonstrate that the type of clotting factor replacement per se cannot fully explain the alterations in lymphocyte subset distribution.

An increased number of Ia-positive T cells was found in some patients. This observation was most marked in the group treated with factor VIII concentrate but was also present in the other groups. Circulating Ia-positive T cells can be found in a variety of disease states, but they can also be found in healthy individuals after immunization with a specific antigen such as tetanus toxoid. Further work is needed to determine the clinical significance of this finding in our patients. In the absence of demonstrable immunodeficiency, it may represent a normal physiologic response to antigenic stimulation.

The significance of decreased Th/T, ratios in otherwise healthy hemophiliacs is not known. There is the possibility that if there are carriers for a putative AIDS-causing agent, exposure to blood products from even one such donor could represent the principal cause of the lymphocyte subset alterations seen in our patients treated both with cryoprecipitate and with concentrate. For the patients in group I who received only cryoprecipitate, there was an approximately 50% prevalence of abnormalities and a mean of 2,000 donor exposures during the two years preceding the study. As of May 1983, however, only 13 cases of AIDS had been reported (H. Handsfield, personal communication) in the entire western Washington region, which has a population of more than 2 million adults. This apparent discrepancy suggests that alterations in lymphocyte subset distribution do not necessarily reflect exposure to an AIDS-causing agent. This conclusion is supported by observations that alterations in Th/T, ratios are frequent among patients with classical hemophilia exposed only to local volunteer blood products in Scotland and Australia, where the risk of exposure to an AIDS-causing agent appears extremely remote. We hypothesize that the altered ratio in most hemophiliacs could be related to some undefined antigenic stimulation caused by frequent transfusions, ie, alloantigens (serum proteins or soluble major histocompatibility antigens), viruses, immune complexes, DNA, or RNA. Whether the decreased ratio we observed indicates that our patients are at increased risk for developing AIDS can be determined only by further observation. From the data available at present, however, there is little, if any, basis on which to recommend a change in current therapeutic regimens using clotting factor concentrates.

ACKNOWLEDGMENT

We thank Gary Longton and Mariel Clements, RN, for their assistance and Pauline Marsden for help in manuscript preparation. We thank Dr Eloise Giblett for helpful discussions and critical review of the manuscript.
REFERENCES


Immunologic status of hemophilia patients treated with cryoprecipitate or lyophilized concentrate

GF Gjerset, PJ Martin, RB Counts, LD Fast and JA Hansen