Immunologic Aberrations, HIV Seropositivity and Seroconversion Rates in Patients With Hemophilia B

By Doreen B. Brettler, Frank Brewster, Peter H. Levine, Ann Forsberg, Sharon Baker, and John L. Sullivan

Because there have been reports that factor IX concentrate is less immunosuppressive and therefore factor IX users have less immunologic aberrations, we have studied a group of 22 patients with hemophilia B and six patients with factor VIII deficiency and high titer inhibitors with respect to lymphocyte numbers and function, human immunodeficiency virus (HIV) serology, and factor usage. This group was compared to 111 patients with hemophilia A and a group of 28 healthy male volunteer controls. When the study began in 1983, the majority of patients with hemophilia B and with higher titer factor VIII inhibitors were seronegative, 77% and 83% respectively, as compared to only 30% of patients with hemophilia A. At that time the factor IX users also had milder immune aberrations than the hemophilia A group. However, with time and increasing clotting factor concentrate usage, seroconversion and more striking abnormalities in immune function have occurred in the hemophilia B group. In a subgroup of 16 patients with hemophilia B studied twice, the incidence of seropositivity increased from 31% in 1983 to 69% in 1985. We thus conclude that factor IX concentrate in itself is not less immunosuppressive than factor VIII concentrate. Seroconversion in factor IX concentrate users appears to be lagging behind seroconversion in factor VIII concentrate users, perhaps secondary to the lower cumulative dosage of concentrate that patients with hemophilia B utilize.

There have been numerous reports of laboratory immune abnormalities, including reduced helper/suppressor lymphocyte ratios, anergy to cutaneously applied antigens, and decreased mitogen responsiveness in patients with factor VIII deficiency (hemophilia A). The cause of these abnormalities is multifactorial, with the most important variables being the amount of factor concentrate infused and seropositivity to HIV (human immunodeficiency virus). Beginning in 1983 there have been suggestions in the literature that those patients with factor IX deficiency (hemophilia B) as well as those with high titer inhibitors to factor VIII receiving large amounts of factor IX concentrate have a lesser degree of immune dysfunction than patients with hemophilia A. However, in each of these studies the sample size was small, and correlations between seropositivity to HIV and immune data were not always available. In these studies it was implied that the factor IX concentrate was in itself less immunosuppressive. However, other reports suggested that immune aberrations were dependent on intensity of treatment. In one study, for example, patients who were intensively treated with factor IX concentrates were found to have decreased helper/suppressor cell ratios when compared to patients with hemophilia B who used minimal amounts of clotting factor concentrates. Patients with hemophilia B characteristically infuse fewer units per year than patients with hemophilia A. It remains unclear, therefore, whether factor IX concentrate is less immunosuppressive than factor VIII concentrate or if the degree of immune aberration is directly related to the amount of total units infused. We have studied a group of patients with factor IX deficiency and also a group of factor IX concentrate recipients with high titer inhibitors to factor VIII with respect to lymphocyte number and function, HIV seropositivity, and factor usage. Additionally, to test the hypothesis that factor IX-deficient patients are lagging behind in HIV seroconversion and the development of immune defects because of lower cumulative factor doses, we restudied the HIV seroconverters again after two to three years for progressive alterations in immunoregulation.

MATERIALS AND METHODS

Patient selection. Twenty-two patients with hemophilia B who were enrolled at the New England Area Comprehensive Hemophilia Center were consecutively tested at the time of their routine annual visit. All were tested at least once in 1983/1984, and 16 were tested subsequently after a two-to-three-year period from 1983 to 1985. Fourteen patients had severe hemophilia B (factor level <1%), one patient had severe hemophilia B with a high titer inhibitor, and six had moderate hemophilia B (factor IX ~2% to 5%). A final patient had severe hemophilia B by laboratory measurement but was mildly clinically and never had infused any blood products. All patients were on a home therapy program as described previously, utilizing commercial factor IX concentrate from a variety of manufacturers except for the one patient who never infused concentrate and had last been infused with plasma approximately 30 years ago. All patients began to use heat-treated factor IX in January 1985. None of the 22 patients had acquired immune deficiency syndrome (AIDS) or AIDS related complex (ARC). Six of the 22 patients were noted to have small but palpable lymph nodes in the anterior and posterior cervical node chains. One patient had a six-week episode of weight loss and fevers that have subsequently resolved. In retrospect, this particular episode was most likely related to seroconversion to HIV positivity. The mean age of the group with hemophilia B was 25.7 years, with a range of 5 to 60 years.

Additionally, six patients with severe hemophilia A and high titer inhibitors, five of whom had received only factor IX products over the last five years and one who had received factor VIII concentrate in addition to factor IX concentrates, were studied. Their inhibitor titers ranged from 3.3 to 11.5 Bethesda Units. These patients ranged in age from 6 to 37 years, with a mean of 19 years. No patient in this study was infected with HIV.
various cell population per microliter of whole blood were calculated. Serum is a common determinant of human HLA-DR from Becton Dickinson. The characteristics of these group consisted of 28 healthy male volunteers on no medications who completely characterized with respect to immune function and HIV. A group had AIDS, ARC, lymphadenopathy, or thrombocytopenia. A group of 111 patients with hemophilia A that had been previously completely characterized with respect to immune function and HIV serology served as a comparison group. The nonhemophilic control group consisted of 28 healthy male volunteers on no medications who had a mean age of 33.5 years, with a range of 18 to 54 years. The characteristics of these groups are seen in Table 1.

**Lymphocyte separation.** Peripheral blood mononuclear cells (PBMC) were isolated from freshly drawn heparinized venous blood by centrifugation on Ficoll-Hypaque (FH; Pharmacia Fine Chemicals, Inc, Piscataway, NJ) density gradients. PBMC preparations contained 70% to 80% lymphocytes, 10% to 20% monocytes, and 20% polymorphonuclear leukocytes as determined by cell morphology. All studies were performed on PBMC maintained at 37°C overnight in HEPES-buffered RPMI 1640 medium (Grand Island Biological Co, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), and 25 μg/mL gentamicin (Grand Island Biological Co, Grand Island, NY).

**Lymphocyte surface marker studies.** Relative percentages of T lymphocyte populations were enumerated using indirect immunofluorescence and the following reagents: mouse monoclonal antibodies (MoAbs) OKT.11 (pan-T cell), OKT.4 (T helper/inducer) and OKT.8 (cytotoxic/suppressor; Ortho Pharmaceutical Co, Raritan, NJ). HLA-DR-bearing lymphocytes were enumerated using direct immunofluorescence with a fluorescein-conjugated mouse MoAb to a common determinant of human HLA-DR from Becton Dickinson Company (Mountain View, CA). All samples were analyzed with the use of a Becton Dickinson FACS IV. Absolute numbers of the various cell population per microliter of whole blood were calculated by multiplying their relative percentage by the absolute numbers of peripheral blood mononuclear cells as determined from complete blood counts.

**Lymphocyte proliferation studies.** Using a standard microculture method, 3 x 10⁴ PBMC in 0.25 mL of RPMI 1640 with 10% fetal calf serum (FCS) were distributed into replicate wells of Microtiter II plates (Falco Labware, Division of Becton Dickinson and Co, Oxnard, CA). Mitogens were added in the following concentrations: phytohemagglutinin (PHA; Burroughs Wellcome, Research Triangle Park, NC) 0.2 to 8.0 μg/mL; pokeweed mitogen (PWM; PL Biochemicals, St. Louis) 0.05 to 5 μg/mL; concanavalin A (Con A; Calbiochem-Boehringer Corp, American Hoechst Corp, San Diego, CA) 0.4 to 40 μg/mL. Cultures were terminated on day 3 for PHA, day 5 for Con A and PWM. All cells were cultured in a 5% CO₂ environment at 37°C. Eighteen hours before harvesting, the cultures were pulsed with 2 μCi of [³H]-thymidine, ([³H]TdR; sp act 6.7 mCi/mmol/L; New England Nuclear, Boston). Cells were harvested onto glass-fiber filters, and [³H]-thymidine content was determined by scintillation counting. All cultures were performed in triplicate.

**Delayed cutaneous hypersensitivity.** Delayed cutaneous hypersensitivity (DCH) was measured in both hemophiliacs and controls using the Multitest CMI (Merieux Institute Inc, Miami) skin test set. Seven test recall antigens (tetanus toxoid, diphtheria toxoid, streptococcus, proteus, tuberculin, candida, and trichophyton) and a glycerine-vehicle control were simultaneously applied on the volar surface of the arm with the multipuncture set. The skin tests were read at 48 hours, and the presence of induration of greater than 2 mm for each antigen was recorded. A patient was considered anergic if no skin tests were positive after 48 hours and a responder if one or more skin tests were positive.

**HIV serology.** Serum samples were assayed for IgG antibody to HIV using a modification of an indirect immunofluorescent assay described previously. Patient sera were also assayed for antibody to HIV viral proteins by the Western blot method. Briefly, sodium dodecyl sulfate (SDS)-disrupted HIV (kindly provided by R.C. Gallo) was fractionated on a discontinuous polyacrylamide-SDS gel system and then electrophoretically transferred to a sheet of nitrocellulose (Schleicher and Schuell, Keene, NH) by methods described previously. After blocking with 5% nonfat dry milk saline solution, the Western blot was then cut into strips and reacted with a 1:100 dilution of patient serum overnight at room temperature. The strips were first reacted with biotinylated goat antihuman IgG and then avidin D-conjugated horseradish peroxidase (Vector Labs, Burlingame, CA). A 4-chloro-l-naphthol substrate solution was added for ten minutes, then the color reaction was stopped and the banding pattern compared to patterns obtained with HIV positive sera, with

<table>
<thead>
<tr>
<th>Table 1. Characteristics of Each Group</th>
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<tbody>
<tr>
<td>Mean Age (yr) (range)</td>
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<td>-----------------------</td>
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<tr>
<td>Normal controls</td>
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<tr>
<td>Hemophilia A</td>
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<tr>
<td>Hemophilia B</td>
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<tr>
<td>Factor VIII inhibitor patients</td>
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**Table 2. Lymphocyte Markers in Each Group at Outset of Study**

<table>
<thead>
<tr>
<th>Normal Controls (n = 28)</th>
<th>Hemophilia A Patients (n = 111)</th>
<th>Hemophilia B Patients (n = 22)</th>
<th>Inhibitor Patients (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helper cells</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Absolute cells/μL</td>
<td>965 ± 321</td>
<td>789 ± 560*</td>
<td>851 ± 384</td>
</tr>
<tr>
<td>Percent</td>
<td>48.1 ± 8.3</td>
<td>32.4 ± 11.6†</td>
<td>36.7 ± 7.9†</td>
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<tr>
<td>Suppressor cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute cells/μL</td>
<td>513 ± 195</td>
<td>722 ± 868†</td>
<td>760 ± 285††</td>
</tr>
<tr>
<td>Percent</td>
<td>25.9 ± 7.3</td>
<td>37.1 ± 10.6†</td>
<td>34.1 ± 11.8*††</td>
</tr>
<tr>
<td>Helper/suppressor ratio</td>
<td>2.0 ± 0.7</td>
<td>1.0 ± 0.6†</td>
<td>1.3 ± 0.7†</td>
</tr>
<tr>
<td>HLA-DR positive cells (%)</td>
<td>23.6 ± 3.0</td>
<td>30.4 ± 1.1*</td>
<td>23.7 ± 2.2††</td>
</tr>
</tbody>
</table>

Results expressed as mean ± SD.

* P < 0.025 by Student’s t test when compared to control.
† P < 0.005 when compared to control.
‡ P < 0.025 when compared to patients with hemophilia A.
special note made of antibody reactivity to p24 (viral core antigen) and gp120 (envelope glycoprotein).

Statistical analysis. Statistical differences between groups were evaluated by the use of Student's t test.

RESULTS

Surface markers. As can be seen in Table 2, patients with hemophilia B had significantly decreased percentages of T helper cells (36.7 ± 7.9% vs 48.1% ± 8.3%, P < 0.005) when compared to the nonhemophilic control group. The absolute number and the percentages of suppressor cells were increased and the helper/suppressor ratio significantly decreased when compared to controls. When compared to the group with hemophilia A, however, absolute number and percentage of helper cells were increased, and relative amounts of suppressor cells were significantly decreased, making the helper/suppressor ratio higher and more similar to the normal controls. Percentages of HLA-DR cells were also not significantly different from the control population. The six patients with factor VIII deficiency and high titer inhibitors, although utilizing large amounts of factor IX, had in vitro immunologic tests that were not significantly different from the normal controls.

Mitogens. The responses to the mitogens PHA, Con A, and PWM were all significantly decreased in the patients with hemophilia B when compared to controls, as can be seen in Table 3. The responses were similar to those patients with hemophilia A.

Delayed cutaneous hypersensitivity. As we have reported previously,17 no patient with hemophilia B was anergic, as compared to 51% of patients with hemophilia A. Of the 17 patients on whom results were available, all had at least one skin test that was positive. The mean number of tests that were positive for hemophilia B patients was 2.5 ± 1.2 as compared to 1.2 ± 0.1 for hemophilia A patients and 3.5 ± 1.5 for controls (data not shown).

Factor concentrate usage. The group of patients with hemophilia B used significantly less clotting factor concentrate than those with factor VIII deficiency (757 ± 910 units factor IX/kg/yr vs 1384 ± 252 units factor VIII/kg/yr, P < 0.005). Interestingly, those with inhibitors to factor VIII, although normal from an immunologic standpoint, used significantly increased amounts of factor concentrate when compared to both those with factor IX deficiency (5315 ± 3436 units factor IX/kg/yr vs 758 ± 909 units factor IX/ kg/yr, P < 0.005) and those with factor VIII deficiency (5315 ± 3435 units factor IX/kg/yr vs 1384 ± 1252 units factor IX/kg/yr, P < 0.005).

HIV serology. When comparing serology among the different groups, it is notable that in 1983, the first year that the patients were studied, 17/22 or 77% of patients with hemophilia B were seronegative as well as 5/6 patients with inhibitors to factor VIII. This is compared to only 30% of patients with factor VIII deficiency who were initially seronegative. The five patients with hemophilia B who were initially seropositive did use significantly higher amounts of factor IX concentrate than those with hemophilia B who were seronegative (Table 4). Thus it appears that in the hemophilia B population, increased amounts of clotting factor concentrate usage increases the incidence of seropositivity. In contrast to what has been reported in the group with hemophilia A,4 seropositivity to HIV in the group with hemophilia B studied in 1983 did not correlate with either decreased responsiveness to PWM or an increased amount of circulating HLA-DR-positive lymphocytes (Table 4).

In the patients with inhibitors to factor VIII who use large amounts of factor IX, the initial seropositivity rate was only 1/6 or 16.6%. It remains unclear why the patients had an initial low rate of seropositivity; this may only be a reflection of the small sample size for this group.

Longitudinal studies. Sixteen patients with hemophilia B were studied in year I (1983) and then in a subsequent year (1984). It was noted that during this period no patient became seropositive to HIV. The seropositivity rate was 17/22 (77%) in 1983 and 16/22 (73%) in 1984.

Table 3. Mitogen Responses in Each Group at the Outset of the Study (1983)

<table>
<thead>
<tr>
<th>Mitogen</th>
<th>Normal Controls (n = 28)</th>
<th>Hemophilia A (n = 111)</th>
<th>Hemophilia B (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytonegglutinin (PHA)</td>
<td>80,740 ± 6,489</td>
<td>46,028 ± 2,999*</td>
<td>46,386 ± 6,333*</td>
</tr>
<tr>
<td>Concanavalin A (Con A)</td>
<td>130,194 ± 10,481</td>
<td>50,808 ± 4,247*</td>
<td>56,598 ± 7,411*</td>
</tr>
<tr>
<td>Pokeweed mitogen (PWM)</td>
<td>46,079 ± 5,212</td>
<td>20,404 ± 1,697*</td>
<td>22,697 ± 3,158*</td>
</tr>
</tbody>
</table>

*P < 0.005 when compared to controls. All results are expressed as mean CPM ± SEM.

Table 4. Variables Correlating With HIV Seropositivity in 1983

<table>
<thead>
<tr>
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<th>Hemophilia A: HIV Serology</th>
<th>Hemophilia B: HIV Serology</th>
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<tbody>
<tr>
<td></td>
<td>Negative (n = 28)</td>
<td>Positive (n = 83)</td>
</tr>
<tr>
<td>Factor usage U/kg/yr</td>
<td>1.033 ± 339</td>
<td>1.732 ± 150*</td>
</tr>
<tr>
<td>Response to PWM (Cpm)</td>
<td>29.752 ± 4,847</td>
<td>17.413 ± 1,482*</td>
</tr>
<tr>
<td>% HLA-DR positive</td>
<td>24.0 ± 1.9</td>
<td>32.7 ± 1.3*</td>
</tr>
</tbody>
</table>

All results expressed as mean ± SD except for response to PWM expressed as mean ± SEM.

*P < 0.001 when compared to seronegative counterpart.
†P < 0.005 when compared to seronegative counterpart.
year, year 2 (1984 and/or 1985) with respect to immune function and HIV serology. In this subgroup of patients the number of those who were seropositive increased from 5/16 (31%) in 1983 to 11/16 (69%) in 1985. There were six seroconverters during that time period. When these six patients were compared with respect to immunologic parameters between year 1 and year 2, there was a decrease in T helper/suppressor ratio from 1.65 ± 0.95 to 1.15 ± 0.50, which did not reach significance (Fig 1), and a trend toward a decrease in absolute T helper cells and an increase in absolute T suppressor cells (Fig 2). One patient who seroconverted did have an acute but prolonged febrile illness in retrospect associated with seroconversion. Otherwise the group of seroconverters did not differ clinically from the group as a whole.

The five patients who remained seronegative actually had an increase in the helper/suppressor cell ratio from 1.28 ± 0.69 to 1.46 ± 0.76 secondary to a slight increase in helper cells (data not shown). They also used clotting factor concentrates from a variety of manufacturers and did not differ clinically from the patients who seroconverted. Interestingly, it is of note that the utilization of factor concentrate over the study period 1983 to 1985 was not significantly different between the patients who seroconverted and the patients who remained seronegative (330 ± 323 units/kg/yr v 706 ± 497 μ/kg, P is not significant). However, these subsets represent very small patient groups, and thus statistical comparison is difficult.

The five patients who were initially seropositive remained the same immunologically with decreased helper/suppressor ratios secondary to both decreased helper cells and increased suppressor cells. It should be noted that the intensity of treatment had not changed in these patients during the interval between the two sets of studies.

The six patients with inhibitors were also studied longitudinally. Three patients seroconverted. The mean helper/suppressor cell ratio of the six patients at the outset of the study was 1.70 ± 0.96, whereas in 1985, after the three seroconversions occurred, the mean ratio was 0.77 ± 0.26, a significant downward trend (P < 0.025).

DISCUSSION

In numerous reports, patients with hemophilia B have been shown to have less immunologic aberrations than patients with factor VIII deficiency. It has been found that the factor IX concentrates have decreased amounts of beta-2 microglobulin protein when compared to factor VIII concentrates. This has been postulated as a reason why these concentrates might cause less immunosuppression than do factor VIII concentrates. Recently it has been reported that a prolonged exposure to ethanol during plasma fractionation can inactivate HIV virus. Since certain factor IX products, especially activated prothrombin complex used intermittently by patients with inhibitors, are made utilizing such a step, less immune aberrations have been postulated to occur in patients using these products. In one study, patients treated only with activated prothrombin complex were shown to have no seroconversion over a three-year period. Another reason put forth that patients with hemophilia B may have more normal immune function is that a smaller percentage of patients with hemophilia B are HIV antibody positive. In Great Britain only 6.3% of 316 hemophilia B patients studied were seropositive.

In our group of 22 patients with hemophilia B and six patients with inhibitor to factor VIII studied initially in 1983, immunologic abnormalities were less striking than in a comparison group of patients with hemophilia A and more similar to a normal control group. When compared to patients with hemophilia A, we found, as previously reported by others, that helper/suppressor ratios were higher as a result of increased amounts of helper cells and decreased numbers of suppressor cells. Skin test anergy was also absent in all patients with factor IX deficiency and also the inhibitor patients.

In 1983 the seropositivity rate in the patients with hemophilia A was 72%, in contrast to a rate of 31% in patients with hemophilia B and 16.6% in patients with high titer inhibitors. Seropositivity to HIV was correlated with higher clotting factor concentrate usage in the population with hemophilia B found to be seropositive at first testing. This finding is comparable to that of Mannucci et al as well as to data from other European and American hemophilia centers. Overall, the amount of factor infused was less than in the
population with factor VIII deficiency. Based on the above information, one could hypothesize that the factor IX itself is not less immunosuppressive but that less of it was used per patient. Seropositivity and subsequent immunologic aberrations should emerge later, in time, as either the cumulative dose increases or the probability of exposure to an HIV-contaminated lot increases.

Supporting this hypothesis is the data on the 16 patients with hemophilia B who were studied over at least two consecutive years. There was a 38% seroconversion rate, with 6/16 becoming seropositive. Correlated with seroconversion was a trend toward worsening of immune function. The incidence of seropositivity in the hemophilia B population may never reach that in patients with hemophilia A, since future seroconversion may well be prevented by the newer heat-treated concentrates that will likely eliminate further HIV exposure.

Thus it appears that factor IX concentrate itself is not less immunosuppressive but that less per year has been utilized in the hemophilia B patients when compared to factor VIII usage in hemophilia A patients. The correlation between amount of factor infused, conversion to HIV antibody positivity, and subsequent immunologic aberrations when examined in 1983 is similar to that seen in patients with factor VIII deficiency, only delayed. It is of note that the patients who seroconverted during the study years did not use more factor concentrate over the three-year span than those who continued to remain HIV antibody negative. Thus not only cumulative dose but chance exposure to a HIV-contaminated lot could also be important. These results also suggest that a more purified factor IX preparation is needed to decrease the immunosuppression observed in patients with hemophilia B receiving concentrate infusions.

The data concerning the factor VIII inhibitor patients are difficult to interpret, since the group represents a small subset. Because the patients appeared at the outset of the study to have normal immune studies, they may have been, in some manner, protected from the immune suppressant effects of large doses of factor IX concentrate. However, over the course of two years, 3/6 patients with inhibitors seroconverted, and their immune function deteriorated. This is consistent with the immunologic deterioration seen in patients with hemophilia A or B following HIV infection.

ADDITIONAL

Since this paper was originally submitted, 21 of the 22 original patients with hemophilia B have seroconverted as well as five of the six inhibitor patients.

REFERENCES


Immunologic aberrations, HIV seropositivity and seroconversion rates in patients with hemophilia B

DB Brettler, F Brewster, PH Levine, A Forsberg, S Baker and JL Sullivan