A Comprehensive Study of Immunological Abnormalities in Korean Hemophiliacs

Kir-Young Kim, Chang Hyun Yang, Shin Heh Kang and Dong Soo Kim

To determine laboratory evidence suggesting immunological abnormalities in persons with hemophilia, we evaluated the immunological status of 75 Korean hemophiliacs, seronegative for human immunodeficiency virus (HIV) antibodies, who have been treated only with Korean factor VIII concentrates. From this study, it was shown that Korean hemophiliacs had decreased CD4 levels, increased CD8 levels, and decreased CD4:CD8 ratios. Diminished lymphocyte response to the mitogens, phytohemagglutinin and concanavalin A, and decreased natural killer cell activity were observed in the hemophiliacs. In addition, production of interleukin-2 in the hemophiliacs was lower than in the healthy controls. The percentage of B lymphocytes was significantly reduced but the serum levels of immunoglobulin (Ig) G were elevated. However, the serum Ig A and Ig M levels were normal. This study demonstrated a high frequency of immunological abnormalities in HIV antibody negative Korean hemophiliacs treated only with domestic factor VIII concentrates.

Key Words: Hemophiliacs, immunological abnormalities, factor VIII concentrate therapy.

Hemophiliacs treated with commercial factor concentrates have been recognized as a relatively high risk group for developing acquired immunodeficiency syndrome (AIDS) (Johnson et al. 1985). Commercial coagulation factor concentrates are derived from the pooled plasma of several thousand donors; thus, hemophiliacs treated with coagulation concentrates are thought to be at risk of infection with human immunodeficiency virus (HIV) as well as other viral agents. The rate of seroconversion has increased rapidly since 1982 (Eyster et al. 1985; Ragni et al. 1986; Ragni et al. 1987) but a recent survey by the Center for Disease Control (CDC) has shown a declining tendency of AIDS cases in hemophiliacs since mid-1985 (CDC. 1986). Patients with hemophilia need repeated transfusion of blood products, so they are frequently exposed to alloergic proteins (Matheson et al. 1987). Continuous exposure to foreign materials may stimulate the immune system. Since 1982, abnormalities of immune functions in hemophiliacs, who have no evidence of AIDS or the AIDS-related complex, have also been reported (Goldsmith et al. 1983; Landy et al. 1983; Lederman et al. 1983; Carr et al. 1984; Perelmutter and Gentlesk 1988). Although the cause of these changes remains unknown, some speculations of blood-borne infective agents in the antihemophilic factor (AHF) preparations have been suggested, with an implicit link between the putative agent and the immune abnormalities (Kimberly et al. 1984). As a result, several immunological abnormalities, including inverted T lymphocyte subset ratios, elevated serum immunoglobulin (Ig) levels, decreased lymphoproliferative responses to T cell mitogens, and diminished natural killer cell (NK) activity have been reported in otherwise asymptomatic hemophiliacs (Saidi et al. 1983; Jason et al. 1985; Seki et al. 1985; Madhok et al. 1986; Sullivan et al. 1986).

In many countries, AIDS is a serious problem, as in Korea. However, the number of registered HIV seropositive cases in Korea is approximately 30. Thus, Korean hemophiliacs currently have an extremely low risk of exposure to contaminated blood products. There is sufficient blood supply in Korea for 500 registered hemophiliacs so that foreign products up to now have not been administered. Therefore, Korean hemophiliacs are thought to be a good model for a study of immune abnormalities. We evaluated the immunological status of a group of HIV-negative
Korean hemophiliacs who have been treated only with Korean factor VIII concentrate therapy.

MATERIALS AND METHODS

Patients

Seventy-five patients with hemophilia A, aged 7 to 45 years, registered at the Comprehensive Hemophilia Center at Severance Hospital of Yonsei University College of Medicine, Seoul, Korea, were studied from June 1987 to May 1988. All patients were in good general health at the time of study, without fever or lymphadenopathy. All patients had been treated with large volumes of lyophilized factor VIII preparation (AHF, Green Cross Co., Seoul, Korea). No patient had a recognizable circulating Factor VIII inhibitor or suffered from any other chronic disorder. All patients denied intravenous drug abuse or having homosexual relations.

We included two hemophiliacs who were found to be positive for HIV. Unfortunately, these patients had been infused with U.S.-made antihemophilic factors. Twenty normal healthy age-matched control subjects were studied in parallel with the patients.

Immunologic Evaluation

Peripheral blood mononuclear cell preparations: Peripheral blood mononuclear cells were isolated from the heparinized blood of hemophiliacs and normal controls by centrifugation over Ficoll-Hypaque (1.007 gm/ml density, Pharmacia, Piscataway, NJ, USA). White blood cell counts and absolute numbers of lymphocytes and monocytes were normal in all patients.

T lymphocyte subpopulation and B cell analysis: T cell and T lymphocyte subpopulations were analyzed using polyacrylamide beads coated with T cell monoclonal antibodies CD3 (total T cells), CD4 (T-helper cells), and CD8 (T-suppressor cells) (Bio-Rad Co., Richmond, CA, USA). The B lymphocyte percentage of positive cells was also determined using beads coated with a polyclonal antihuman immunoglobulin antibody. The Quantigen method (Bio-Rad Laboratories, Richmond, CA, USA) was used to detect CD3, CD4, CD8, and B cells. Two hundred or more bead surviving lymphocytes were counted microscopically, and rosettes containing three or more beads were considered positive.

Other immunological studies: For lymphocyte proliferation induced by the T-cell mitogens phytohemagglutinin (PHA) and Concanavalin A (Con A), standard microculture techniques were used with 1 x 10^9 mononuclear cells per well in round-bottomed microtiter plates (Costa) and RPMI 1640 medium (Gibco, Irelad, NJ, USA), containing inactivated human AB serum, 2 mM glutamine, and antibiotics. Cells were cultured for 3 days, and for the last 16 hr of culture 1 uCi of tritiated thymidine (H^3-TdR, New England Nuclear, MA, USA) was added to each well (96 well plate, Costa, Cambridge, MA, USA). After harvesting with an automated cell harvester (Skatron, VA, USA), the culture was assayed in a liquid scintillation counter.

The natural killer (NK) cell cytotoxicity was performed by the 51 Chromium release cytotoxicity assay from K 562 target cells (Gillies et al. 1978). The production of Interleukin-1(IL-1) was measured by the short term H^3-TdR uptake assay as previously described (Loizzo and Loizzo 1975). Serum immunoglobulin (Ig) G, A, and M determinations were done by radial immunodiffusion (Behringwerke AG, Marburg, W. Germany). Antibody to HIV was measured by an enzyme-linked immunosorbent assay (ELISA) technique (Behringwerke AG, Marburg, W. Germany). Selected sera with positive HIV antibody tests were confirmed by the western blot technique.

RESULTS

HIV Serology: All patients were negative for HIV antibody. We included two hemophiliacs who were found to be positive for HIV. Unfortunately, these two patients were infused U.S.-made antihemophilic factors.

T Lymphocyte subpopulations: As shown in Table 1, hemophiliacs, compared to controls, have a significantly decreased percentage of CD4 positive cells (p<0.05). Conversely, the percentage of CD8 cells in the patients was significantly higher than in normal controls (p<0.001). The mean CD4/CD8 ratio of hemophiliacs was 1.35 ±0.53 and that of controls was 2.27 ± 0.49 (p<0.001). HIV seropositive hemophiliacs tended to have a higher absolute CD8 percentage (51.5 % vs 33.2 ± 8.3 %) and lower mean CD4/CD8 ratio (0.64 vs 1.35 ± 0.35) than seronegative hemophiliacs (Table 1).

Lymphocyte function studies: The results of stimulation tests of PHA, Con A, and NK cell activity are shown in Table 2. Lymphocyte proliferative responses to optimal concentrations of PHA were significantly lower in hemophiliacs than in controls (p<0.05). The response to Con A was also significantly reduced (p<0.05) in the patients. With respect to the NK cell cytotoxicity, a significant difference between patients and controls was found (p<0.005).
Table 1. T lymphocyte subpopulations in hemophiliacs and controls

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Percentage of cells stained</th>
<th>CD4/CD8 Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD3</td>
<td>CD4</td>
</tr>
<tr>
<td>Patients (n=75)</td>
<td>67.7±9.6*</td>
<td>41.6±10.0*</td>
</tr>
<tr>
<td>Controls (n=20)</td>
<td>74.8±7.9</td>
<td>49.2±9.5</td>
</tr>
<tr>
<td>HIV(+) Patients# (n=2)</td>
<td>77.0</td>
<td>33.0</td>
</tr>
</tbody>
</table>

Results expressed as mean ± SD
* p<0.05 by Wilcoxon signed-ranks test compared to control
** p<0.001 compared to control
# HIV(+) Patients: patients with seropositive human immunodeficiency virus

Table 2. In vitro lymphocyte functions in patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Phytohemagglutinin (cpm)</th>
<th>Concanavalin A (cpm)</th>
<th>Natural killer (% cytotoxicity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients (n=75)</td>
<td>44,250±26,732*</td>
<td>40,232±32,975*</td>
<td>37.7±17.7**</td>
</tr>
<tr>
<td>Controls (n=20)</td>
<td>62,045±19,108</td>
<td>54,884±21,171</td>
<td>50.4±13.8</td>
</tr>
</tbody>
</table>

Results expressed as mean ± SD
* p<0.05 compared to control
** p<0.005 compared to control

Interleukin II production: The hemophiliacs showed a lower production of IL-II compared to healthy controls (3.43 ± 5.35 units/ml vs 8.84 ± 4.36 units/ml, p<0.001). Twenty-three of the hemophilia patients were undetected (Table 3).

Humoral immunity: Table 4 shows the results of the percentage of B cells stained and serum immunoglobulin levels in hemophiliacs and controls. The percentage of B lymphocytes was significantly reduced in hemophiliacs compared to controls (p<0.05). Mean

Table 3. Interleukin-II production of PHA-stimulated peripheral blood lymphocytes in hemophiliacs and control subjects

<table>
<thead>
<tr>
<th></th>
<th>IL-II activity (units/ml)</th>
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<tbody>
<tr>
<td>Patients (n=75)</td>
<td>3.43±5.35</td>
</tr>
<tr>
<td>Controls (n=20)</td>
<td>8.84±4.36</td>
</tr>
</tbody>
</table>

Results expressed as mean ± SD
p<0.001 compared to control
# 23 undetected patients are included.

Table 4. B lymphocytes and serum immunoglobulin levels in hemophiliacs and controls

<table>
<thead>
<tr>
<th></th>
<th>Percentage of B cells stained</th>
<th>Immunoglobulin (mg/dl)</th>
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<tr>
<td></td>
<td></td>
<td>IgG</td>
</tr>
<tr>
<td>Patients (n=75)</td>
<td>10.12±2.69*</td>
<td>1,298±136*</td>
</tr>
<tr>
<td>Controls (n=20)</td>
<td>13.00±4.63</td>
<td>1,094±142</td>
</tr>
</tbody>
</table>

Results expressed as mean±SD
* p<0.05 compared to controls

serum levels of Ig G (± 1 SD) were significantly elevated in patients (1,298 ± 136 mg/dl) as compared to normal controls (1,094 ± 142 mg/dl) (p<0.05). There was no difference in IgA and IgM levels.
DISCUSSION

The immune aberrations in hemophiliacs without any evidence of AIDS in this study may be similar to those observed in apparently healthy homosexuals, a high risk group for AIDS(Ammann et al. 1983; Schoff et al. 1983; Seki et al. 1985). Whether these T cell abnormalities in hemophiliacs predispose them to develop clinical immunosuppression and opportunistic infections remains unclear. In the present study, we found immunological abnormalities in many Korean hemophiliacs who were treated only with Korean AHF and were never exposed to foreign commercial factor concentrates. When compared to controls, hemophiliacs treated with lyophilized antihemophilic factor (AHF) had a relative decrease in helper T cells, a relative and absolute increase in suppressor T cells, and a depressed helper/suppressor T cell ratio. Functional studies demonstrated decreased NK cell activity and diminished lymphocyte proliferative responses to the mitogens PHA and Con A. The results of this study indicate significant abnormalities of cell-mediated immunity in Korean hemophiliacs, which has been reported by other investigators(Weintrube et al. 1983; Lederman et al. 1983; Moffat et al. 1985). Other studies have demonstrated alterations in T cell subsets in Scottish(Frobel et al. 1983; Ludlam et al. 1983), Australian, (Rickard et al. 1983) and English (Lee et al. 1985) patients treated only with domestic factor VIII concentrate. However, patients treated with domestic concentrate in Finland(Rasi et al. 1984) have not developed abnormal T cell subsets. From our results, we can conclude that repeated exposure to blood products may render hemophiliacs immunologically unresponsive, due to the assault on their immune system every time they receive clotting factor. It is likely that immune suppression, produced by repeated exposure to clotting factor concentrates, lowers the threshold for infection. This may lead to a gradual diminution in the ability to resist infections or neoplasms(Frobel et al. 1983; Kessler et al. 1983; Ludlam et al. 1983; Pollack et al. 1985).

Various mechanisms have been proposed for the lymphocyte disturbances in hemophiliacs. Firstly, reduced CD4/CD8 ratios associated with increased CD8 counts have been noted after infection with several viruses, including CMV, EBV, and herpes(Reiner et al. 1980; DeWaele et al. 1981; Carney et al. 1983). It has been suggested that many hemophiliacs might have been exposed to inactivated virus in clotting factor preparations. Because factor VIII concentrates are derived from a large pool of financially remunerated donors, there should be an increased risk of viral contamination in this form of treatment. (Stein et al. 1985; Melbye 1986; Andrews et al. 1987; Eagleson et al. 1988). Secondly, it is possible that long term immunoregulatory defects caused by alloantigens contained in factor VIII concentrates will, in the future, be associated with clinically significant immunodeficiency in the absence of HIV infection(Sullivan et al. 1986). Other studies also support the notion that factor VIII has an immunosuppressive effect which is independent from HIV(Madkhot et al. 1986).

There is increasing evidence that the abnormalities in T cell subset distribution represent a functional immunological defect. In patients without clinical evidence of immunodeficiency, we and others have found significant impairment of the lymphocyte proliferative responses to PHA and Con A, and reduced NK cell activity(Frobel et al. 1983; Lederman et al. 1983; Lee et al. 1985). In vitro incubation of factor VIII concentrate with lymphocytes inhibits the proliferative response to PHA and Con A in both hemophiliacs and normal subjects(Frobel et al. 1983). Preliminary studies also suggest that factor VIII concentrates may directly suppress the proliferation of normal lymphocytes(DeShazo et al. 1985). There is possibly some component of factor VIII concentrate which interferes with the lymphocyte activation pathway by PHA. It has been shown that a component of factor VIII concentrate has a high affinity for the CD2 receptor, thus interfering with lymphocyte activation by PHA but not by viral and other antigens(Mahir et al. 1988). It is therefore not likely that inhibition of interleukin II (IL-II) production is mediated through interference with the production or response to IL-II, because IL-II production by Jurkat cells proceeds independently of IL-II(Lederman et al. 1986). LAH inhibited production of IL-II in human lymphocytes and infusions of LAH concentrates in patients with hemophilia caused severe impairment of NK cell activity and affected the function of in vivo mononuclear phagocyte systems. The most relevant effect of factor VIII infusions may be exhibited directly on the monocyte-macrophage. Similarly, monocytes are a source of a factor which can decrease natural killer cell activity(Kimberley et al. 1984).

Apart from T lymphocyte abnormalities, this study also demonstrates that certain B cell functions are abnormal in patients with hemophilia. In addition, hemophiliac serum Ig G has been found to be elevated in this as well as other reports(DeShazo et
The mechanism by which clotting factor concentrate administration induces the B cell alterations is not known. Therefore, it might be concluded that repeated exposure of hemophilicics to multiple antigens in the concentrate of clotting factors induces these phenomena. The recognition that many asymptomatic patients have laboratory abnormalities resembling those detectable in AIDS has raised the possibility that these patients may have a condition representing a prodrome or formerefractory of AIDS. There is no doubt that these abnormalities found in hemophilics without AIDS are caused by exposure to blood products (Stein et al. 1985).

In conclusion, this study demonstrated a high frequency of immunological abnormalities in HIV seronegative patients treated only with domestic factor VIII concentrate in Korea. Further studies are required to clarify the role of LAHF in immunological abnormalities and hemophilia.

REFERENCES

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Volume 30
Study of Immunological Abnormalities in Hemophiliacs


