Defect in B Cell Function in HTLV III/LAV Positive Hemophilia Patients

By E.J.M. Sjamsoedin-Visser, C.J. Heijnen, B.J.M. Zegers, and J.W. Stoop

The capacity of the peripheral blood lymphocytes (PBL) to generate an antibody response in vitro to T cell–dependent antigen ovalbumin was studied in 12 severe hemophilia patients who were otherwise in good health. PBL from four of 12 patients were not capable of generating such a response after stimulation in vitro, whereas all controls were normal. This negative plaque-forming cell (PFC) response coincided with the presence of antibodies directed toward human T-lymphotropic virus III/lymphadenopathy-associated virus (HTLV-III/LAV). Only one patient with antibodies against HTLV-III/LAV had a normal PFC response. The negative PFC response was not due to a deficient T helper cell activity, nor to an excessive T suppressor cell function. However, in the peripheral blood of these four patients, the presence of activated B cells that are refractory to antigen-specific T helper cell signals and secrete specific antibodies spontaneously could be demonstrated. Most of the patients showed a hyperimmunoglobulinemia. No correlation between the T4/T8 ratio and the level of the PFC response was demonstrated. From the data obtained in these investigations we raise the hypothesis that infection with HTLV-III/LAV in hemophilia patients will lead to in vivo (pre)activation of B cells that results in unresponsiveness or decreased response to antigen-specific signals.

Hemophilia patients are at an increased risk of developing the acquired immune deficiency syndrome (AIDS), caused by a lymphocytotropic retrovirus human T-lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV). Transmission occurs via biologic fluids including blood and blood products such as factor VIII and IX preparations. Aberrations of several immune functions have been demonstrated consistently in AIDS patients and notably a reversed ratio of two T cell subsets that play a pivotal role in the regulation of various types of immune responses. These subsets are characterized phenotypically by their selective affinity for the monoclonal antibodies OKT4 (T4+ cells comprising T inducer cells) and OKT8 (T8+ cells including suppressor/cytotoxic T cells).6,7 The mechanism by which an HTLV-III/LAV infection causes the immunologic aberrations observed in AIDS is still unclear. Because of these findings in AIDS patients, hemophilia patients were studied in many countries with respect to their T4/T8 ratio. In a considerable percentage of apparently healthy patients a low T4/T8 ratio was found. In addition, they nearly always showed a hyperimmunoglobulinemia, and often the mitogenic responses of their peripheral blood lymphocytes (PBL) were low.8,11

In earlier studies we showed that when normal human peripheral blood T and B cells are cultured with the antigen ovalbumin (OA), small resting B cells can differentiate into plaque-forming cells (PFC) secreting small amounts of OA-specific IgM.12 The magnitude of the PFC response is determined by the net effect of T4+ helper cell and T8+ suppressor cell activities as well as by the number of small, resting B cells present.13,15

On the basis of the observed in vivo alteration of immune regulatory T cell subsets and the hyperimmunoglobulinemia in many hemophilia patients, we studied the capacities of the cells (ie, T4+, T8+, and B cells) that are involved in the generation of an in vitro OA-induced PFC response.

MATERIAL AND METHODS

Patients. Twelve healthy patients with severe hemophilia A or B, ages 7 to 61 years and treated with various factor VIII or IX products, were investigated.

Factor VIII and IX concentrates of Dutch origin were used, except in patients 2 and 6. Patient 6 received concentrate from the United States (Hyland). Patient 2 who synthesized a factor VIII inhibitor received FEIBA, an activated factor IX preparation manufactured by Immuno-Vienna.

Serum immunoglobulin levels were determined by radial immunodiffusion (Mancini). Antibodies to HTLV-III/LAV were tested by using an enzyme-linked immunosorbent assay (ELISA), and ELISA-positive sera were subjected to immunoblot analysis.16

Controls. Included were one patient with severe hemophilia A who had not yet received any treatment at all, one patient with beta-thalassaemia major treated monthly with erythrocyte concentrate, two IgG subclass-deficient patients treated with gamma globulin subcutaneously, and two hemophilia A carriers not treated with blood products. In all experiments normal control donors were included as well.

Isolation of peripheral blood mononuclear cells. The materials and methods that concern the isolation of PBL, monocytes or adherent cells (AC), T cells, and B cells have been described extensively elsewhere.16

Production of antigen-specific T cell–derived helper factor (ThF120-OA) and T cell–derived suppressor factor (TsF120-OA). The preparation as well as the functional and biologic properties of ThF120-OA and TsF120-OA have been described earlier.17,18 For the preparation of ThF120-OA, isolated T cells were cultured in a Marbrook-Diener system with OA (3 μg/mL) in a concentration of 5 x 10^6 cells in 2 mL of culture medium that consisted of RPMI 1640 (GIBCO, Grand Island, NY) with 100 mmol/L glutamine, 5 x 10^-5 mol/L 2-mercaptoethanol, antibiotics, and 2% AB serum in the presence of 10% AC for 96 hours. After the culture period the cells were washed and recultured in a concentration of 5 x 10^6 cells/mL in the presence of 3 μg OA/mL. After 24

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5% of the patients had serological evidence of past infections with hepatitis B virus, cytomegalovirus (CMV), and Epstein-Barr virus (EBV), respectively.

The generation of PFC in vitro. The results depicted in Fig 1 demonstrate that the PBL of four out of 12 patients could not mount a PFC response with the optimal dose (3 μg/mL) of the antigen OA: mean, 151.5 ± 96.2 PFC/10^6 lymphocytes. The eight patients with a positive PFC response showed the same level of PFCs (mean, 1,250 ± 281.2) as the normal control donors: mean, 1,052 ± 216 PFC/10^6 lymphocytes. The control patients also responded normally (mean, 1,199 ± 264). Since a negative PFC response may be caused by a dysfunction of antigen-presenting monocytes, T helper cells, T suppressor cells, or B cells, we designed experimental protocols in order to dissect functionally the various cellular activities. The activity of T4+ and T8+ cells was determined by their capacity to generate T helper factor and T suppressor factor respectively. T helper factor is produced by T cells after culturing the cells for 120 hours with autologous monocytes and 3 μg of the antigen OA. The supernatants of these cultures contain a factor that can only induce an OA-specific PFC response. T suppressor factor is produced by T cells when they are stimulated by a high dose of the antigen (100 μg OA/mL), preferably in the absence of monocytes. Under these conditions human T suppressor cells secrete an antigen-specific T suppressor factor that is capable of decreasing the PFC response by the fact that it inhibits antigen-specific T helper cell function. The B cell function of the patients was tested by the capacity to differentiate into PFC in the presence of OA-specific T helper factor derived from normal donor T cells.

T cell function: T helper cell activity. When B cells and 10% monocytes from a normal donor were cultured for six days in the presence of the ThF120-OA preparations of the four patients, it turned out that the ThF120-OA of each of the patients was perfectly capable of inducing the differentiation of normal B cells into PFCs (Fig 2). Because antigen presentation by monocytes is essential to generate T helper cell activity, these results prove also that the antigen-presenting cell function is normal.

Table 1. Clinical and Laboratory Features of 12 Patients With Severe Hemophilia

<table>
<thead>
<tr>
<th>No.</th>
<th>Age (yr)</th>
<th>Hemophilia</th>
<th>A/B</th>
<th>Therapy</th>
<th>IgG* (g/L)</th>
<th>IgM* (g/L)</th>
<th>IgA (g/L)</th>
<th>Ly total number/μL</th>
<th>T4* (μL)</th>
<th>T8* (μL)</th>
<th>T4/T8</th>
<th>PFC Response</th>
<th>HTLV-III/LAV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>A</td>
<td>FVII conc†</td>
<td>13.2</td>
<td>1.6</td>
<td>1.4</td>
<td>2,537</td>
<td>1,909</td>
<td>608</td>
<td>1.79</td>
<td>318</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>A</td>
<td>FVIII conc†</td>
<td>24.7</td>
<td>1.2</td>
<td>1.7</td>
<td>2,704</td>
<td>946</td>
<td>1.082</td>
<td>0.87</td>
<td>102</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>54</td>
<td>B</td>
<td>PCC‡</td>
<td>26.4</td>
<td>3.1</td>
<td>3.1</td>
<td>1,906</td>
<td>431</td>
<td>1,519</td>
<td>0.36</td>
<td>91</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>61</td>
<td>B</td>
<td>PCC‡</td>
<td>18.4</td>
<td>2.6</td>
<td>2.9</td>
<td>1,110</td>
<td>477</td>
<td>610</td>
<td>0.78</td>
<td>95</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>A</td>
<td>FVIII conc‡</td>
<td>23.2</td>
<td>1.8</td>
<td>2.0</td>
<td>2,310</td>
<td>993</td>
<td>785</td>
<td>1.26</td>
<td>1,485</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>A</td>
<td>FVIII conc‡</td>
<td>16.4</td>
<td>1.4</td>
<td>1.3</td>
<td>1,960</td>
<td>1,038</td>
<td>764</td>
<td>1.36</td>
<td>1,275</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>23</td>
<td>A</td>
<td>FVIII conc + cryo‡</td>
<td>20.0</td>
<td>4.0</td>
<td>2.9</td>
<td>2,145</td>
<td>1,094</td>
<td>1,030</td>
<td>1.06</td>
<td>656</td>
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<td></td>
</tr>
<tr>
<td>8</td>
<td>27</td>
<td>A</td>
<td>cryo‡</td>
<td>12.9</td>
<td>2.7</td>
<td>2.3</td>
<td>1,750</td>
<td>892</td>
<td>700</td>
<td>1.27</td>
<td>1,250</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>28</td>
<td>A</td>
<td>cryo‡</td>
<td>15.0</td>
<td>1.5</td>
<td>3.1</td>
<td>2,162</td>
<td>757</td>
<td>800</td>
<td>0.95</td>
<td>2,045</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>33</td>
<td>B</td>
<td>PCC‡</td>
<td>16.0</td>
<td>1.3</td>
<td>2.8</td>
<td>2,584</td>
<td>1,111</td>
<td>468</td>
<td>2.29</td>
<td>1,186</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>48</td>
<td>A</td>
<td>cryo‡</td>
<td>12.4</td>
<td>1.7</td>
<td>3.6</td>
<td>2,400</td>
<td>912</td>
<td>888</td>
<td>1.03</td>
<td>1,200</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: Ly, lymphocytes; conc, concentrate; cryo, cryopreservation; FVII, factor VIII.

*Normal value for adults (mean ± 1 SD): IgG, 9.0 ± 2.17; IgM, 1.32 ± 0.46; IgA, 1.44 ± 0.55.
†Greater than 50,000 U/Lyr.
‡Zero to 50,000 U/Lyr.

RESULTS

Serological and immunologic characteristics in patients and controls. Table 1 summarizes the various data on the patients that are relevant to the present study. The data show normal lymphocyte counts in all patients except two who showed decreased numbers, ie, lower than 1,500/μL (patients 4 and 12). The T4+/T8+ ratio was below the control value of 0.93 (range, 0.93 to 3.43; mean, 1.79; n = 50) in three patients, and most patients showed hyperimmunoglobulinemia. In five patients, specific antibodies against HTLV-III/LAV were demonstrated. In addition, all patients had serological evidence of past infections with hepatitis B virus, cytomegalovirus (CMV), and Epstein-Barr virus (EBV), respectively.
I. In previous papers we have shown that in normal donors the decline of the response is due to the activation of T suppressor-effector cells (T8+ cells). In normal patients with a negative PFC response (n=5) this phenomenon is demonstrable when B cells are cultured for six days in the absence of antigen.

To demonstrate the antigen specificity of ThF120-OA, normal unprimed B cells were cultured with the various ThF120-OA preparations in the presence of the antigen SRBC. The results of the PFC response were as follows: B cells + ThF120-OA of the patients + SRBC, 142 ± 7 PFC/10^6 B cells; B cells + ThF120-OA of normal donors + SRBC, 134 ± 12 PFC/10^6 B cells. These results are only slightly above the background PFC response of B cells of normal donors cultured without ThF120-OA (94 ± 11, see Table 2). We can conclude therefore that ThF120-OA produced by the patients' T cells or T cells of normal donors was specific for the antigen OA.

T suppressor cell activity. When PBL are cultured for six days in the presence of a high dose of antigen, the PFC response decreases to baseline levels (see Fig 1). In previous papers we have shown that in normal donors the decline of the response is due to the activation of T suppressor-effector cells (T8+ cells).

Because of the fact that the PFC response of four of the hemophilia patients was found to be negative, the possibility existed that the low response was due to an excessive T suppressor-effector cell function. Therefore we determined the capacity of patients' T8+ cells to produce TsFl20-OA. The T suppressor cell activity was determined by adding the TsFl20-OA-containing supernatant in graded quantities to normal PBL cultured with an optimal dose of the antigen OA.

The results depicted in Fig 3 show that TsFl20-OA from these four hemophilia patients caused a subnormal suppression of the PFC response in low concentrations but a normal suppression in concentrations of 5% and 10% vol/vol.

B cell function: Capacity of resting B cells to differentiate into PFC. Since the monocyte, T helper and T suppressor cell functions of the patients appeared to be intact, we wondered whether the negative PFC response was due to a defect in the B cell compartment. In order to test the capacity of B cells of our patients to differentiate into PFC, we prepared ThF120-OA from T cells and monocytes of normal donors and determined the capacity of the patients' B cells to

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**Table 2. Generation of a PFC Response by B Cells of Hemophilia Patients in the Presence of ThF120-OA From Normal Donors (Mean ± SEM)**

<table>
<thead>
<tr>
<th>B Cell Source</th>
<th>PFC/10^6 B Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>ThF120-OA +</td>
<td></td>
</tr>
<tr>
<td>Normal donors (n=5)</td>
<td>2.005 ± 39 (44 ± 3)*</td>
</tr>
<tr>
<td>Patients with a negative PFC response (n=4)</td>
<td>144 ± 7 (2 ± 0.4)*</td>
</tr>
<tr>
<td>Patients with a positive PFC response (n=3)</td>
<td>1.899 ± 21 (22 ± 4)*</td>
</tr>
<tr>
<td>ThF120-OA -</td>
<td></td>
</tr>
<tr>
<td>Normal donors (n=5)</td>
<td>94 ± 11</td>
</tr>
<tr>
<td>Patients with a negative PFC response (n=4)</td>
<td>45 ± 6</td>
</tr>
<tr>
<td>Patients with a positive PFC response (n=3)</td>
<td>64 ± 5</td>
</tr>
</tbody>
</table>

*The anti-SRBC PFC responses are given in parentheses. No background response is demonstrable when B cells are cultured without ThF120-OA (94 ± 11). The results of the PFC response of four of the hemophilia patients were found to be negative, the possibility existed that the low response was due to an excessive T suppressor-effector cell function. Therefore we determined the capacity of patients' T8+ cells to produce TsFl20-OA. The T suppressor cell activity was determined by adding the TsFl20-OA-containing supernatant in graded quantities to normal PBL cultured with an optimal dose of the antigen OA.

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differentiate into PFC by culturing them with 10% normal
ThF120-OA. The results of the PFC assay (Table 2) demon-
strate that the patients whose PBL could not be induced to
the formation of PFC in the presence of 3 μg OA/mL (Fig 1)
did not appear to have B cells in their peripheral blood that
could differentiate into PFCs in the presence of ThF120-
OA.

Spontaneous IgM secretion. When normal B cells are
applied directly to an OA-SRBC monolayer without any
culture procedure, no specific antibody response is observed.
In Table 3 it is clearly shown that, unlike the patients with a
positive PFC response, the B cells present in the peripheral
blood of the four hemophilia patients with a negative PFC
response spontaneously secrete anti-OA antibodies. This
spontaneous antibody response is not limited to an anti-OA
response. We could also demonstrate the presence of these B cells
secreting antibodies spontaneously with specificity for sheep
erthrocytes and TT (Table 3).

DISCUSSION

To obtain a better understanding of the biologic signifi-
cance of the immunologic alterations found in healthy hem-
ophilia patients, we studied the capacity of the lymphocytes of
12 patients to generate an OA-induced PFC response. The results
show that in four out of 12 patients the specific antibody response
in vitro against antigens like OA was negative (Table 1 and Fig 1), which could be confirmed on
several occasions (data not shown). It came out that in each
of these four patients an infection with HTLV-III/LAV
could be shown. Only one patient with specific antibodies
against this virus had a normal PFC response (Table 1).

The negative PFC response was not due to a dysfunction of
the T helper cells, since every patient was capable of generat-
ing antigen-specific T helper cell factor (Fig 2). The latter
finding implies that the capacity of monocytes to present
antigen to T helper cells also is not affected. Furthermore
the proliferative response of the lymphocytes to either of the
antigens TT or Candida albicans appeared to be positive in
the patients with a negative PFC response (data not shown).
We may conclude that in contrast to the data obtained in
AIDS patients23 no selective defect in the recognition of
soluble antigen by T4+ cells could be demonstrated. The
negative PFC response in four patients was not due to an
excessive T suppressor-effector cell function either because
we could show that the T cells of these patients did not
produce an excess of TsF120-OA activity as compared with
normal control donors, but on the contrary, they exhibited a
lower TsF120-OA activity.

We investigated also whether the low TsF120-OA activity
was a result of a prior activation in vivo in such a way that the
cells could no longer be restimulated in vitro for the produc-
tion of TsF120-OA. If so, spontaneous T suppressor cells,
can be found as in the peripheral blood of agammaglo-
bulinemic patients, for example,24 should be present. How-
ever, no such activity could be demonstrated (data not
shown) that supports our aforementioned conclusion that the
negative PFC response is not caused by a direct activity of T
suppressor cells.

However, we could demonstrate that the peripheral blood
of patients with a negative PFC response contained B cells
that spontaneously secreted antibodies of various specificities
(Table 3). In each case the presence of these antibody-
secreting cells in the peripheral blood coincided with an
absence of a response of patients' B cells to ThF120-OA.
Comparable phenomena have been described by Lane et al,25
who reported in vivo polyclonal B cell activation and a lack of
or a deficient antibody production in AIDS patients and in
HTLV-III/LAV—positive patients, respectively; Pahwa et
al26 could also demonstrate a severe impairment of B cell
function in these patients. More recently, increased sponta-
neous immunoglobulin secretion by B cells of hemophiliacs
has been found in addition to decreased pokeweed mitogen-
duced immunoglobulin production.27

't Hart et al23 have shown recently that in our culture
system only resting B cells bearing IgM and IgD on their
surface are activated in vitro by OA in the presence of T
helper cells or T helper cell factor. When B cells are activated
(in vivo or in vitro) to the stage that they have lost their
surface IgD, they are no longer sensitive to antigen-specific T
helper cell signals.28 When we consider the results presented
in this paper in the light of these data, we are inclined to
hypothesize that the negative PFC response is due to the fact
that the B cells of these patients are no longer in a resting
phase but are already activated in vivo and consequently no
longer sensitive to the signals derived from OA-specific T
helper cells.

Characterization of B cells secreting antibodies sponta-
neously as well as of the PFC that are generated in our assay
system have revealed that they are not high-rate-secreting
plasma cells with a blastlike appearance but are B cells that
have only differentiated into the excited state (the G1 phase)
of the cell cycle.13 This transition is accompanied by a
decrease in cell density and some rounds of replication during
which the cell loses its surface IgD and the expression of
HLA-DR increases.13 Unanswered is the question whether
these activated B cells present in HTLV-III/LAV—positive
hemophilia patients play a regulatory role in vivo. Recently
De Kruijff et al29 have shown in the murine system that
activated B cells play a regulatory role by the fact that they
can stimulate antigen-specific T cells in the absence of
antigen. The authors showed that only B cells in the G1
phase of the cell cycle possess this property. Preliminary
experiments in our laboratory have already shown that B
cells in an activated state can trigger T4+ cells in the
absence of antigen to become T suppressor-inducer cells.30

| Table 3. Spontaneous IgM Antibody Secretion by Peripheral |
| Blood B Cells of Hemophilia Patients Without Prior In Vitro |
| Activation (Mean ± SEM) | |
| B Cells | PFC/10⁶ B Cells | αOA | αSRBC | αTT |
| Normal donors | 0 | 2 | 0 |
| Patients with a negative PFC response (n = 4) | 205 ± 69 | 304 ± 71 | 423 ± 46 |
| Patients with a positive PFC response (n = 8) | 0 | 5 | 2 |
| Abbreviation: α, anti.- |
(data not shown). Second, we have evidence that in hemophilia patients the presence of activated B cells in the peripheral blood coincides with a functional absence of T4+ suppressor-inducer cells (manuscript in preparation).

In view of the data presented here we raise the hypothesis that treatment of hemophilia patients induces activation of the immune system by transfused blood products such as factor VIII or IX concentrates and by contaminating viruses such as EBV and CMV. This stimulation of the immune system may perturb the normal homeostasis, and sometimes hyperimmunoglobulinemia and a reversed T4/T8 ratio will be the result. This state of the immune system might be susceptible to further dysregulation but need not be characterized as a pathological status per se. When, however, the treated hemophilia patient is confronted with a HTLV-III/LAV infection, this may lead to a further perturbation of the newly achieved equilibrium, which will result in more severe functional abnormalities as described here for the four patients with a negative PFC response and a positive antibody response to HTLV-III/LAV. The one patient with antibodies against HTLV-III/LAV and a normal PFC response may represent the status of a recent infection with this virus.

ACKNOWLEDGMENT

We are indebted to Annemarie Meekes for technical assistance in the immunologic studies, to Drs J.W.M. van der Meer and J.M. Vossen for submitting two control patients, to Dr J. Goudsmit for testing HTLV-III/LAV antibodies, to Helene van Moorsel and Linda Gramberg for secretarial assistance, and to Elis Haan for supplying several patients’ samples.

REFERENCES

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