Immunological studies in HIV seronegative haemophiliacs: relationships to blood product therapy

R. J. G. CUTHBERT, C. A. LUDLAM, C. M. STEEL,* DIANNE BEATSON* AND J. F. PEUTHERER†

Department of Haematology, Royal Infirmary of Edinburgh, *MRC Human Genetics Unit, Western General Hospital, Edinburgh, and †HIV and Hepatitis Reference Laboratory, Department of Medical Microbiology, University of Edinburgh

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Summary. Immunological studies were performed on a group of 44 haemophilia A and 15 haemophilia B patients who were treated exclusively with blood products manufactured by the Scottish National Blood Transfusion Service (SNBTS). All patients were HIV seronegative throughout the study.

Of the haemophilia A patients 14 (32%) had CD4+ lymphocyte subset counts ≤ 0.5 x 10^9/l, compared with one (6%) haemophilia B patient and four (8%) controls. The percentage of activated T cells was greater than 5% in 19/33 (57%) with haemophilia A, 5/9 (55%) haemophilia B and 14/50 (28%) of control subjects. β2-microglobulin values >2.0 mg/l were observed in 19 (43%) haemophilia A and four (26%) haemophilia B patients, compared with one (2%) control. No significant increases in serum interleukin-2 receptor concentrations were observed in 15 haemophilia A and one haemophilia B patients. Significantly elevated levels of IgG, IgM and IgA were observed in the haemophilia A group, but elevation of immunoglobulins was restricted to the IgG class in the haemophilia B group. Of the haemophilia A patients 16/30 (53%) and 5/11 (46%) haemophilia B patients had depression of cell-mediated immunity (CMI) as assessed by delayed-type hypersensitivity responses to intradermally injected recall antigens. There was no correlation between factors VIII or factor IX usage and changes in lymphocyte subsets, β2-microglobulin, and immunoglobulin levels. There was, however, a strong correlation between annual factor VIII usage and the degree of depression of CMI for those with haemophilia A but not for those with haemophilia B. No correlation between alterations in the immune parameters and disturbance of liver function tests was observed in either haemophilia A or haemophilia B patients.

We conclude that alloantigen or non-HIV viral exposure due to repeated administration of factor concentrates brings about alterations in the immune response, and that these changes are more marked following exposure to intermediate purity factor VIII compared with factor IX concentrate.
10 a.m. and noon from non-bleeding patients who had not recently received factor VIII concentrate infusions. Results were compared with those of a group of healthy age-matched male volunteer controls.

Total lymphocyte counts were measured by electronic counting on a Coulter S Plus counter. Peripheral blood lymphocytes were pre-separated from single samples of heparinised whole blood by centrifugation over Ficoll-Hypaque SC 1-078 and washed in phosphate-buffered saline (PBS). The CD4 and CD8 lymphocyte subsets and percentage of activated T-cells (DR +ve) were estimated by indirect immunofluorescence, using mouse monoclonal antibodies (Dako) in the first layer and FITC-conjugated F(ab) fractions of sheep anti-mouse Ig (Sigma) in the second layer. The stained cells were then resuspended in PBS with 1% formaldehyde, and scored on a Becton-Dickinson FACScan cytofluorimeter.

Serum samples were stored at −20°C and subsequently used for immunological studies. Serum β2 microglobulin concentrations were estimated by a competitive radioimmunoassay (RIA) technique (Phamacia). Serum neopterin concentrations were estimated by a similar RIA (Henning). Serum interleukin-2 receptor was measured by K1ISA (T Cell Sciences). Serum IgG, IgM and IgA were estimated by single radial diffusion using commercially prepared single radial immunodiffusion plates (Behring). Serum samples were stored at −20°C and subsequently used for immunological studies. Serum β2 microglobulin concentrations were estimated by a competitive radioimmunoassay (RIA) technique (Phamacia). Serum neopterin concentrations were estimated by a similar RIA (Henning). Serum interleukin-2 receptor was measured by K1ISA (T Cell Sciences). Serum IgG, IgM and IgA were estimated by single radial diffusion using commercially prepared single radial immunodiffusion plates (Behring).

Cell-mediated immune responses were assessed by measuring the intradermal delayed-type hypersensitivity response to recall antigens applied by a commercial applicator (Multitest, Merieux) which includes a negative control. A positive response was indicated by a mean diameter of skin induration >2 mm at 48 h. The antigens used in this system were: tetanus toxoid, diptheria toxoid, streptococcus antigen, old tuberculin, candida albicans antigen, trichophyton mentagrophytes antigen, and proteus mirabilis antigen.

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Haemophilia A Controls
Haemophilia B

Fig 1. Absolute CD4+ lymphocyte subset count in HIV seronegative haemophiliacs and controls.

Fig 2. Percentage activated T-lymphocytes in HIV seronegative haemophiliacs and controls.

marker is shown in Fig 2. A relatively wide range of results was observed with a skew towards increased expression in a minority from each group. However, the number of haemophilia A patients with increased expression of activated T-cells was significantly greater than controls. Thus 19/33 (57%) haemophilia A patients had >5% activated T-cells compared with 14/50 (28%) controls; \( \chi^2 = 6.078, P < 0.02 \), and 9/33 (27%) haemophilia A patients had >10% activated T-cells compared with 4/50 (9%) controls; \( \chi^2 = 4.226, P < 0.05 \). Although increased expression of the T-cell activation marker was observed in some haemophilia B patients, the number of patients studied was too small to assess statistically (Fig 2).

Serum levels of \( \beta_2 \) microglobulin ranged widely in both haemophilic groups compared with controls (Table II). However, a significant number of haemophilia A patients had relatively elevated values. Thus \( \beta_2 \) microglobulin values of >2.0 mg/l were observed in 19 (43%) haemophilia A patients and four (27%) haemophilia B patients, compared with one (2%) control; \( \chi^2 = 6.216, P < 0.02 \) respectively. There was no correlation between \( \beta_2 \) microglobulin levels and amount of factor VIII or IX usage.

The ranges of values of serum neopterin concentration were wide in all three groups (Table II). There was no significant difference in serum neopterin levels between the haemophilia A or haemophilia B patients and the controls. Values above 15 mmol/l were observed in a very small number of individuals for each group. The significance of this is not clear and repeat estimations were not conducted. Results for serum interleukin-2 receptor concentration were available for 15 haemophilia A and one haemophilia B patient, and 38 controls (Fig 3). The values in the patient group did not appear to be significantly different from control values.

Serum levels of IgG, IgM and IgA were all significantly higher in the haemophilia A group compared with controls (Table II). However, only IgG was significantly increased in haemophilia B. No correlation between immunoglobulin levels and factor VIII or factor IX usage was observed and we could not confirm our previous observation of a positive correlation between IgG and annual factor VIII consumption (Carr et al. 1984).

Cell-mediated immune responses to recall antigens were assessed in 30 haemophilia A patients, 11 haemophilia B patients, and 35 controls (Fig 4). Over half the patients had diminished responses: 16 (53%) haemophilia A patients and six (54%) haemophilia B patients scored 2 or less, compared with one (2%) control. In the haemophilia A group there was a strong negative correlation between annual factor VIII usage and the cell-mediated immunity (CMI) responsiveness (Fig 5); \( r_s = 0.61, P < 0.001 \). No correlation between factor IX usage and CMI responsiveness was found in the haemophilia B group.

**DISCUSSION**

Several abnormalities of immune function were observed in this study. Depression of the CD4+ subset of T-cells (T-helper cells) was observed in about one third of the recipients of factor VIII, but not in recipients of factor IX. This confirms the observations from our previous study (Carr et al. 1984). Although, at the time of that study, the then putative viral
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Table 1. β₂ microglobulin, neopterin and immunoglobulins in HIV seronegative haemophiliacs

<table>
<thead>
<tr>
<th></th>
<th>Haemophilia A (n=44)</th>
<th>Haemophilia B (n=13)</th>
<th>Controls (n=50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β₂ microglobulin (mg/l)</td>
<td>1-85* (1-10-3-10)</td>
<td>1-70$ (0-9-3-1)</td>
<td>1-30 (0-90-2-30)</td>
</tr>
<tr>
<td>Neopterin (nmol/l)</td>
<td>9-0 (3-2-25-0)</td>
<td>7-0 (3-8-15-5)</td>
<td>7-0 (3-8-15-0)</td>
</tr>
<tr>
<td>IgG (g/l)</td>
<td>13-81* (9-00-25-70)</td>
<td>12-7 (9-5-17-4)</td>
<td>11-30 (6-13-14-40)</td>
</tr>
<tr>
<td>IgM (g/l)</td>
<td>2-03† (0-81-4-75)</td>
<td>2-03 (1-0-4-25)</td>
<td>2-30 (0-74-1-74)</td>
</tr>
<tr>
<td>IgA (g/l)</td>
<td>2-60°F (0-71-5-15)</td>
<td>2-10 (1-0-4-25)</td>
<td>2-19 (0-81-4-70)</td>
</tr>
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Values significantly higher than controls; * P<0.001, † P<0.01, $ P<0.05 (Mann-Whitney U-test). Values given are median with range in parentheses.

Fig 1. Serum concentrations of interleukin-2 receptor in haemophilia A patients (●), haemophilia B patient (□), and controls.

Fig 2. Intradermal delayed-type hypersensitivity responses to recall antigens in HIV seronegative haemophiliacs and controls. A positive response is indicated by a mean diameter of skin induration > 2 mm at 48 h.

Fig 3. Haemophiliacs (●) and controls (□).

Fig 4. Intradermal delayed-type hypersensitivity responses to recall antigens in HIV seronegative haemophiliacs and controls. A positive response is indicated by a mean diameter of skin induration > 2 mm at 48 h.

Aetiological agent for AIDS had not been identified, subsequent testing of stored serum samples confirmed that all the patients were HIV seronegative. Depression of T-helper cell numbers has also been reported by other groups treating patients with factor VIII concentrate, lyophilised factor VIII cryoprecipitate, and single donor cryoprecipitate (Gan et al. 1983; Ceuppens et al. 1984; Pollack et al. 1985). In these earlier studies it was not always possible to exclude the presence of asymptomatic HIV disease as a cause of T-helper cell depression. However, Teitel et al. (1989) recently showed mild depression of CD4+ lymphocyte counts in a group of haemophilia A patients who were consistently HIV seronegative. Interestingly, following a change in factor VIII preparation to a product with less risk of non A non B (NANB)
hepatitis they observed a slight, but not statistically significant, rise in CD4+ lymphocyte counts.

Elevated levels of \( \beta_2 \) microglobulin occurred in 43% of haemophilia A patients and 20% of haemophilia B patients. High concentrations of \( \beta_2 \) microglobulin in association with other HLA related proteins have been found in factor VIII concentrates (Lee et al., 1984). This may partly explain the increased levels observed in haemophilia A patients. However, factor IX concentrates do not contain such high concentrations of \( \beta_2 \) microglobulin. It seems likely, therefore, that continuing in vivo production of \( \beta_2 \)M is contributing significantly to the levels observed in haemophils. \( \beta_2 \) microglobulin, being a polypeptide sub-unit of HLA class I molecules, is secreted by most cells. The serum level rises in conditions associated with increased B-lymphocyte turnover (Mescon, 1986). Elevated levels are related to disease activity in several conditions including viral infection (Forman, 1982). The increased levels in our patients may reflect the presence of chronic hepatitis; however, we were unable to detect any correlation with changes in liver transaminases.

Elevated serum neopterin levels were observed in only a small proportion of patients. Neopterin is an intermediate produced during the synthesis of the co-enzyme tetrahydrobiopterin by macrophages. Increased levels are observed following stimulation of macrophages by T-cell derived cytokines including interleukin-2 (Fuchs et al., 1988). Thus neopterin concentration indirectly reflects the degree of T-cell activation. By this indirect method of assessment, there was little evidence of excessive T-cell activation in the patients in our study. However, a significant proportion of haemophilia A patients (and possibly haemophilia B patients—our numbers were too small to confirm statistically) had an increased proportion of activated T-cells in comparison with age-matched healthy male controls. These T-cell changes parallel the observations of elevated \( \beta_2 \) microglobulin levels reflecting increased B-cell activity. This further suggests that factor VIII concentrates may give rise to abnormalities of immune regulation. It will be necessary to repeat this type of investigation in a group of factor concentrate recipients who are free of NANB hepatitis, since this group of infections may be implicated as a cause of increased T-cell (and B-cell) activation. Interleukin-2 receptor is expressed on the surface membrane of activated T-lymphocytes and some activated B-lymphocytes (Ichihara et al., 1981; Faudoa et al., 1984). Subsequently a proportion is shed from the cell surfaces into the plasma where it can be detected using immunohchemical methods (Rubin et al., 1985; Greene et al., 1986). Increased levels of circulating plasma IL-2 R have been detected in certain viral infections including HIV and some B-cell malignancies, in which it may act as a marker for disease activity (Durno et al., 1986; Medina Ibarrondo et al., 1987). Our patients had no significant increases in plasma IL-2 R levels. Thus, although there is some evidence of T-cell activation in these patients, this does not appear to be associated with IL-2 receptor expression. Furthermore, in vitro studies have shown that factor VIII impairs the secretion of IL-2 by T-cells in response to mitogens (Thorpe et al., 1989).

Elevation of serum immunoglobulin levels was an almost universal finding in haemophilia A patients. In our previous study, increases in immunoglobulins were shown to correlate with the activity of NANB hepatitis reflected by increases in liver transaminases (Carr et al., 1984). In haemophilia B patients changes in immunoglobulins were confined to elevation of the IgG class. The reason for the different patterns in haemophilia A and haemophilia B patients is not clear, since no significant differences in NANB hepatitis activity were observed.

A significant degree of depression of CMI manifested by diminished delayed-type hypersensitivity responses to recall antigens, was observed in recipients of factor VIII concentrate, and to lesser extent recipients of factor IX concentrate. Similar observations have been made by other workers (Teitel et al., 1989; Madhok et al., 1986). There was a striking positive correlation between impaired CMI and annual factor VIII usage, further suggesting that factor VIII concentrate itself may influence immune regulation. However, no such correlation was observed in recipients of factor IX concentrate. Thus some component of the factor VIII concentrate not present in factor IX concentrate may diminish the cell-mediated response. Madhok et al. (1986) showed that the CMI response to a pentastigm dimethyloxobenzene was impaired in HIV seronegative haemophils. The response was most severely inhibited in recipients of higher doses of factor VIII concentrate. Subsequently they demonstrated a diminished proportion of T-helper lymphocytes infiltrating the inflammatory lesion (Lowe et al., 1989; Thorpe et al., 1989) have shown that factor VIII concentrates impair the capacity of T-cells to secrete IL-2 in response to antigen challenge but this effect was not related to the purity of the product. The immunosuppressive component of factor VIII concentrate has not been isolated. However, Lederman et al. (1986) have shown that suppression is mediated by a low molecular weight component, and also a component which is of similar molecular weight to factor VIII:C.

The results reported in these and other studies, on the effect of factor concentrates on immune function in vitro, provide good evidence for the importance of evaluating the immune...
modulating potential of new clotting factor concentrates prior to their general use in patients.

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