PEN.012.0405

*British Journal of Haematology*, 1992, 80, 364–369

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

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*Received 4 June 1991; accepted for publication 13 November 1991*

**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 $\leqslant 0.5 \times 10^9$/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. $\beta_2$ microglobulin values $\geqslant 2.0$ 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 6/11 (54%) 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 factor VIII or factor IX usage and changes in lymphocyte subsets, $\beta_2$ 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.

In recent years there has been a growing interest in studying the effects of blood and blood product transfusion on the human immune system (Macleod *et al*, 1987). A number of abnormalities of immune function have been recognized in haemophiliacs receiving factor concentrates. Because of the high prevalence of HIV infection, it has not always been possible to distinguish the adverse effect of HIV disease on immunity from the potential influence of factor concentrate transfusion. The Edinburgh Haemophilia Centre has a relatively low rate of HIV infection (25% of treated haemophilia A patients), the majority having been infected during a single transmission incident (Ludlam *et al*, 1985; Cuthbert *et al*, 1990), and we therefore have a large group of treated haemophiliacs who have remained HIV seronegative. In some of these, absence of HIV infection has been confirmed by sensitive polymerase chain reaction (PCR) analysis (Peutherer *et al*, 1990).

We have evaluated the immune function of the HIV negative patients with a similar range of investigations that have been particularly useful in monitoring HIV disease (Moss & Bacchetti, 1989). In this study we have examined the influence of factor concentrate transfusion on these various parameters of immune function in a group of patients who are believed to be free of HIV infection.

## PATIENTS AND METHODS

Forty-four patients with haemophilia A and 15 patients with haemophilia B were studied. All patients were consistently anti-HIV antibody negative throughout the study. They had been treated for many years exclusively with Scottish National Blood Transfusion Service (SNBTS) factor VIII or factor IX concentrates. Blood samples were collected between

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Table I. Lymphocytes and lymphocyte subsets in HIV seronegative haemophiliacs

| | Haemophilia A ($n=44$) | Haemophilia B ($n=15$) | Controls ($n=50$) |
|---|---|---|---|
| Lymphocytes $\times 10^9$/l | 1·90 (0·86–3·96) | 1·98 (1·11–2·94) | 1·96 (1·23–3·0) |
| CD4+ subset $\times 10^9$/l | 0·68* (0·24–1·40) | 0·86 (0·5–1·43) | 0·81 (0·30–1·66) |
| CD8+ subset $\times 10^9$/l | 0·54 (0·10–1·45) | 0·65 (0·28–1·41) | 0·59 (0·22–1·03) |
| CD4/CD8 ratio | 1·22 (0·15–3·40) | 1·25 (0·59–2·44) | 1·41 (0·45–3·36) |

Values significantly lower than controls: * $P<0·05$ (Mann-Whitney U-test).
Values given are median with range in parentheses.

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 separated from single samples of heparinized whole blood by centrifugation over Ficoll–Hypaque SG 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 $B_2$ microglobulin concentrations were estimated by a competitive radioimmunoassay (RIA) technique (Pharmacia). Serum neopterin concentrations were estimated by a similar RIA (Henning). Serum interleukin-2 receptor was measured by ELISA (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.

Sera were screened for anti-HIV antibodies by competitive ELISA (Wellcome), and positive results confirmed by Western blotting. Patients positive for anti-HIV antibodies were excluded from the present study.

Non-parametric statistical analyses were applied. The Mann-Whitney U-test was used to assess the significance of differences in results between groups. Spearman's rank correlation was used in assessing the relationship between factor concentrate usage or liver function tests and the immune parameters. Chi-squared with Yates' correction was used to assess the significance of the number of patients with depressed CD4 counts, elevated activated T-cells, and elevated $\beta_2$ microglobulin levels, etc., compared with results from the control group.

## RESULTS

The mean annual factor VIII usage in the haemophilia A group, recorded in the year prior to study, was 19 500 units (range 500–185 500). The mean annual factor IX usage in the haemophilia B group was 15 000 units (range 1500–113 000).

There was no significant difference in total lymphocyte counts between haemophiliac groups and controls (Table I). A relatively wide range of results for the CD4+ lymphocyte subset was observed in both haemophilia groups and controls (Fig 1). The distribution of CD4+ counts in the haemophilia A group appeared to be bimodal with the majority having results comparable to haemophilia B patients and controls, but a significant minority having relatively lower values (Fig 1). Thus CD4 counts $\leqslant 0·5 \times 10^9$/l were observed in 14 (32%) haemophilia A patients compared with one (6%) haemophilia B patients, and four (8%) controls; $\chi^2=7·106$, $P<0·01$. There was no correlation between CD4 counts and factor VIII usage. CD4 lymphocyte counts in the haemophilia B group were not significantly different from the control group. The distribution of the CD8+ lymphocyte subset results was similar in haemophilia A patients, haemophilia B patients and controls (Table I). Although the CD4/CD8 ratios in the patient groups were somewhat lower than the control group, these changes did not reach statistical significance at the 5% level.

Expression of the activation marker, HLA DR (MCH class II), on T-lymphocytes was assessed in 33 haemophilia A patients, nine haemophilia B patients and the 50 controls. The distribution of percentage T-lymphocytes positive for this

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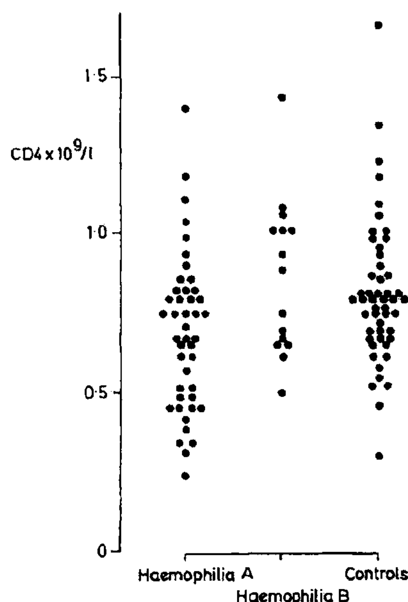


Fig 1. Absolute CD4+ lymphocyte subset count in HIV seronegative haemophiliacs and controls.

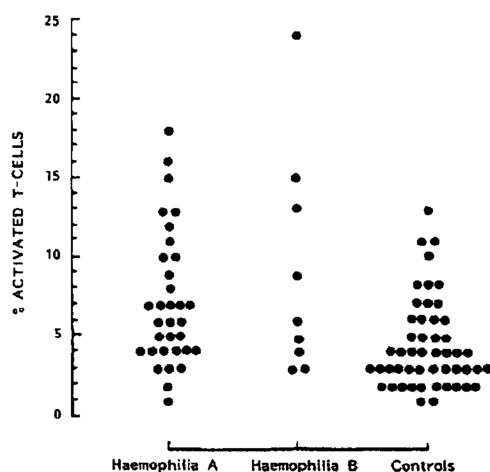


Fig 2. Percentage activated T-lymphocytes in HIV seronegative haemophiliacs and controls.

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; $\chi^2 = 6{\cdot}078$, $P < 0{\cdot}02$, and 9/33 (27%) haemophilia A patients had $\geqslant$10% activated T-cells compared with 4/50 (9%) controls; $\chi^2 = 4{\cdot}226$, $P < 0{\cdot}05$. 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 haemophiliac 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; $\chi^2 = 21{\cdot}302$, $P < 0{\cdot}001$ and $\chi^2 = 6{\cdot}216$, $P < 0{\cdot}02$ 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); $r_s = 0{\cdot}61$, $P < 0{\cdot}001$. 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 II.** $\beta_2$ microglobulin, neopterin and immunoglobulins in HIV seronegative haemophiliacs

| | Haemophilia A ($n$=44) | Haemophilia B ($n$=15) | Controls ($n$=50) |
|---|---|---|---|
| $\beta_2$ microglobulin (mg/l) | 1·85* (1·10–3·30) | 1·70‡ (0·9–3·1) | 1·30 (0·90–2·30) |
| Neopterin (nmol/l) | 9·0 (3·2–25·0) | 7·0 (3·8–15·5) | 7·0 (3·8–15·0) |
| IgG (g/l) | 13·81* (9·00–25·70) | 12·7 (9·5–17·4) | 11·30 (6·33–14·40) |
| IgM (g/l) | 2·03† (0·83–4·75) | 2·03 (1·0–4·25) | 1·64 (0·74–3·74) |
| IgA (g/l) | 2·60† (0·71–5·15) | 2·10 (1·0–4·25) | 2·19 (0·81–4·70) |

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.

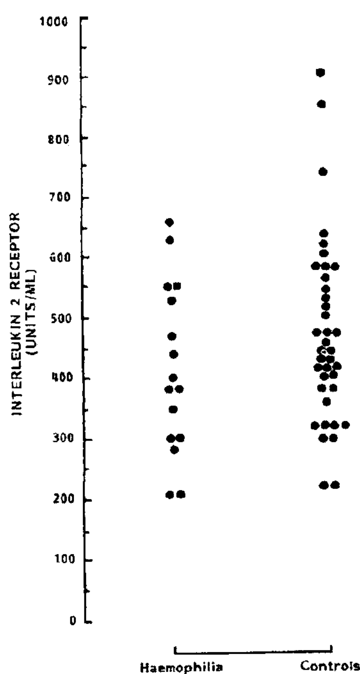


Fig 3. Serum concentrations of interleukin-2 receptor in haemophilia A patients (●), haemophilia B patient (■), and controls.

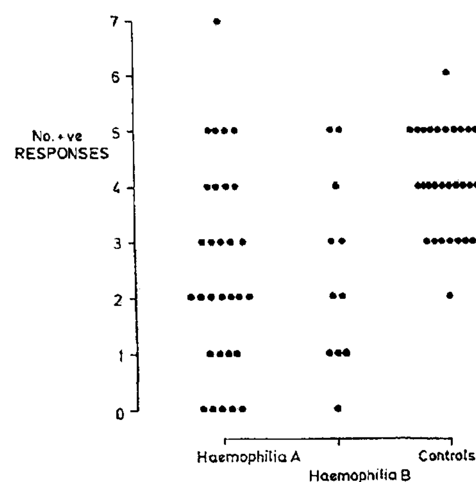


**Fig 4.** Intradermal delayed-type hypersensitivity responses to recall antigens in HIV seronegative haemophiliacs and controls. A positive response is indicated by a mean diameter of skin induration >2 mm at 48 h.

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, lyophilized 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)

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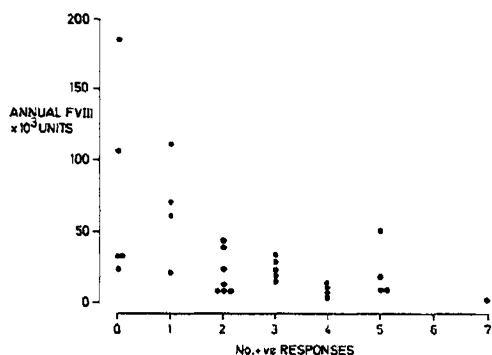


**Fig 5.** Relationship between intradermal delayed-type hypersensitivity responses to recall antigens and annual factor VIII usage in haemophilia A patients.

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 haemophiliacs. $\beta_2$ 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 (Messner, 1984). 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 tetra hydrobiopterin 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 (Uchiyama *et al*, 1981; Tsudo *et al*, 1984). Subsequently a proportion is shed from the cell surfaces into the plasma where it can be detected using immunochemical 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 neoantigen dinitrochlorobenzene was impaired in HIV seronegative haemophiliacs. 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

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modulating potential of new clotting factor concentrates prior to their general use in patients.

ACKNOWLEDGMENTS

We wish to thank Miss Audrey Trotter for the invaluable secretarial assistance. This work was supported by the Medical Research Council and the Scottish Home and Health Department.

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