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Relationships between blood product exposure and immunological abnormalities in English haemophiliacs

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SUMMARY. Amongst 160 English haemophiliacs treated with clotting factor concentrates, abnormalities of T lymphocyte subset distribution (characterized by low T4/T8 ratios and high total T8 counts), low *in vitro* phytohaemagglutinin stimulation and raised serum IgG levels, were more common in patients with haemophilia A than B, in patients who had received heavier blood product exposure, and in adults rather than children. A slight reduction in lymphocyte and platelet counts was found in 26% and 17% of patients. In the sample of patients tested, serum α_1-thymosin levels were often raised, but β_2-microglobulin levels were usually normal. Fractionation procedures used to prepare clotting factor concentrates, and the amounts of concentrate used, are more likely to be causally related to these immunological abnormalities than the origins of source donor plasmas.

Control of bleeding in haemophiliacs depends upon repeated infusions of plasma products containing clotting factors VIII and IX. In the United Kingdom, about 95% of the factor VIII and IX used is in the form of semi-purified concentrates, generally prepared from plasma pools to which several thousand donors have contributed. Some two-thirds of factor VIII used is derived from commercial donors in the U.S.A. (U.K. Haemophilia Centre Directors annual statistics, Rizza & Spooner, 1983). Haemophiliacs are at increased risk of contracting the acquired immunodeficiency syndrome (AIDS) (Desforges, 1983; Jones, 1983; White & Lesesne, 1983; *Lancet*, 1983) and there is evidence that AIDS is transmitted by blood or blood product infusions (Ammann *et al*, 1983; Bove, 1984; Curran *et al*, 1984; Vilmer *et al*, 1984). In asymptomatic American haemophilic patients, immunological abnormalities bearing a resemblance to those detected in AIDS are common (authors cited above). However, the prognostic significance of T lymphocyte subset and other immunological abnormalities detectable in haemophiliacs is uncertain.

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Because of the long tradition of maintenance of comprehensive treatment records, and the variety of different plasma products used to treat haemophiliacs, studies of British patient populations yield information which is not readily obtainable elsewhere. We report here the results of investigations on 160 English haemophiliacs, and present an analysis of the relationships between plasma product exposure and immunological abnormalities.

PATIENTS AND METHODS

Study population and treatment analysis. The study population of 160 male patients with haemophilia A or B included 131 patients registered at the Royal Free Haemophilia Centre and 29 registered at the Oxford Haemophilia Centre. Patients' ages are shown in Table I. Treatment records are maintained in computers at both Centres, and were used as the source of data for analysis. Of the 129 patients with haemophilia A, 10 of those registered at the Royal Free Hospital and all 29 registered at Oxford had been treated only with NHS factor VIII concentrates during the 5 year period before a blood sample was obtained for analysis, or for their whole lives. These products were either prepared at the Blood Products Laboratory, Elstree, Hertfordshire, or the Plasma Fractionation Laboratory at the Oxford Haemophilia Centre. The remaining 90 patients with haemophilia A had received commercial factor VIII concentrates of U.S. origin during the preceding 5 years, or for their whole lives, as the predominant form of therapy. Virtually all factor VIII concentrates used, whether of NHS or commercial origin, was of the 'intermediate purity' type. The 31 patients with haemophilia B had all been treated with NHS factor IX concentrate, and had never been exposed to commercial blood products. All patients included in the study had received treatment with concentrates in the year before sampling. Because lifetime treatment records were not available for all adult patients, treatment exposure comparisons in most patients were based on quantities infused in the 1 year period before testing which, where lifetime records existed, reflected the intensity of treatment in previous years. Patients were arbitrarily assigned either to 'high' or 'low' treatment categories, according to whether their exposure to concentrates was greater or less than 300 u/kg/year. Total lifetime exposure was calculated only for high exposure children aged less than 10 years. The *control population* comprised 30 healthy male hospital personnel (median age 31 years, range 22–44 years).

Total white cell and platelet counts were determined using standard automated techniques, *absolute lymphocyte counts* being calculated from manually performed differential counts of 100 white cells.

T lymphocyte subset analysis. Blood samples were collected into heparinized tubes and stored unrefrigerated before separation, which was normally carried out on the day of collection. Samples from Oxford patients were transported unrefrigerated to the Royal Free Hospital on the day of collection. Lymphocytes were separated using Ficcoll–Triosil and incubated for 10 min with fluorescein isothiocyanate (FITC)-labelled monoclonal antibodies RFT8 (produced at the Royal Free Hospital, reacting with OKT8 suppressor/cytotoxic type T cells) and Leu3a (Becton Dickinson, reacting with OKT4 helper type T cells). RFT11 (produced at the Royal Free Hospital), reacting with all E-rosetting I cells, was used as a pan T cell marker. After washing twice and formalin fixation, the percentages of T cell subsets were

determined using an EPICS-V cell sorter (Coulter Ltd, Luton, England) after lysing whole blood samples with Saponin X and counting the proportions of FITC-labelled cells within the lymphoid population. These values were compared to the total T ($RFT11^+$) counts and absolute numbers of $T8^+$ and $T4^+$ cells were then calculated from absolute lymphocyte counts.

Direct antiglobulin tests (DAT) were carried out by a conventional technique using a broad spectrum antiserum (Ortho-diagnostics).

Immunoglobulin G (IgG) levels were estimated by nephelometry or radial immunodiffusion.

Antibodies to hepatitis B surface antigen (HBsAb) were detected by radioimmunoassay (RIA) (Goodall *et al*, 1982). *Serum α_1-thymosin* was assayed by RIA (McClure *et al*, 1982). *Serum β_2-microglobulin* was measured by competitive enzyme assay (Seward Ltd, Bedford, England).

Phytohaemagglutinin (PHA) stimulation. Heparinized blood was diluted in 10 volumes of RPMI-1640 medium. Optimal concentrations of PHA were added to blood cultures in triplicate which were incubated for 72 h using U-shaped microwells. 1 μCi ^{3}H-thymidine was added, and the mixture incubated for 4 h. Results were expressed as absolute counts per minute (cpm) in stimulated and unstimulated cultures. The incorporation of ^{3}H-thymidine reflected the numbers of T cells in culture (Luquetti & Janossy, 1976). Normal responses gave counts between 30 000 and 80 000 cpm in stimulated cultures.

Statistical methods. The Mann-Whitney U test was used to test the significance of nonparametric data.

RESULTS

T lymphocyte subsets

The most frequent abnormality, seen particularly in patients with haemophilia A treated with commercial factor VIII (Table I, Fig 1), was an abnormally low value for the T4/T8 ratio. In this group the prevalence of abnormalities was related to transfusion history. Patients who had had low exposure to factor VIII (< 300 u/kg/year) had T4/T8 ratio values generally similar to controls. At higher levels of exposure, values were significantly lower (Mann-Whitney test, $P < 0{\cdot}00001$), 64% of patients having a ratio less than 1·0. A low value for the T4/T8 ratio was more often accompanied by a high absolute T8 count than a low T4 count. Both latter abnormalities were also most common in high exposure commercial factor VIII treated patients. Amongst NHS factor VIII treated patients, abnormalities of T4/T8 ratios or absolute counts were infrequent (Table I, Fig 2). However, it should be noted that only small numbers of patients were in high exposure groups. Also, nine of the 12 patients in the high exposure NHS factor VIII treated group were aged less than 10 years, and had had a lifetime exposure to factor VIII (median 58 000 u, range 2000–160 000 u) which was undoubtedly less than the majority of the high exposure commercial factor VIII treated patients who were almost always older. Patients with haemophilia B treated with NHS factor IX concentrates, who were better matched for age with both the control group and commercial factor VIII treated patients, showed a very low prevalence of T cell abnormalities (Table I, Fig 3).

Table I. T cell subsets in patients with haemophilia

Patient population		Age	T4/T8 ratio	Absolute T4 count	Absolute T8 count
Definition of abnormality			$<1{\cdot}0$	$<0{\cdot}3\times10^9$/l	$<0{\cdot}8\times10^9$/l
Haemophilia A < 300 u/kg/year	*	27 (4–81)	1·7 (0·9–4·9)	0·7 (0·4–2·1)	0·5 (0·1–1·1)
(Commercial treatment, $n=32$)	†		4/32 (13%)	0/31 (0%)	4/31 (13%)
Haemophilia A > 300 u/kg/year	*	25 (4–64)	0·9 (0·2–2·8)	0·7 (0·2–3)	0·8 (0·2–2·8)
(Commercial treatment, $n=58$)	†		37/58 (64%)	6/54 (11%)	28/54 (52%)
Haemophilia A < 300 u/kg/year	*	29 (1–75)	2·0 (0·7–3·8)	0·9 (0·2–3·6)	0·4 (0·2–1·6)
(NHS treatment, $n=27$)	†		2/27 (7%)	2/27 (7%)	3/27 (11%)
Haemophilia A > 300 u/kg/year	*	8 (4–29)	2·5 (0·8–3·5)	1·0 (0·6–1·2)	0·4 (0·2–1·3)
(NHS treatment, $n=12$)	†		1/12 (8%)	0/12 (0%)	2/12 (17%)
Haemophilia B < 300 u/kg/year	*	30 (5–60)	2·0 (0·7–3·3)	1·0 (0·6–2·6)	0·5 (0·2–1·3)
(NHS treatment, $n=17$)	†		2/17 (12%)	0/16 (0%)	4/16 (25%)
Haemophilia B > 300 u/kg/year	*	33 (3–55)	2·0 (0·7–2·7)	0·9 (0·51–1·9)	0·51 (0·2–1·4)
(NHS treatment, $n=14$)	†		2/14 (14%)	0/11 (0%)	2/11 (18%)
Normal subjects	*	31 (22–44)	1·7 (1·0–3·3)	0·9 (0·3–1·6)	0·5 (0·1–1·0)
	†		0/30 (0%)	0/30 (0%)	2/30 (7%)

n = Total number of patients within treatment group.
* Median values with ranges in parentheses.
† Proportion of patients showing abnormality with percentages in parentheses.

Antibodies to HBsAG

No patients had previously been vaccinated against HBV. A large majority of patients, particularly in high exposure groups, had detectable HBsAb (Table II). There was a higher seroconversion rate amongst patients treated with commercial concentrate.

Lymphocyte counts, platelet counts and antiglobulin tests

Low lymphocyte counts ($<1{\cdot}5\times10^9$/l) were detected in 39/151 patients (26%), but only in four cases were the counts less than $1{\cdot}0\times10^9$/l. Low platelet counts ($<150\times10^9$/l) were observed in 19/112 patients (17%), but only one patient had a count below 100×10^9/l. There were no obvious relationships between depressed lymphocyte or platelet counts and either exposure to blood products or T lymphocyte subset distributions (Table I). Direct antiglobulin tests were performed in 55 patients with haemophilia A and 21 with haemophilia B. Only one patient, treated with commercial factor VIII concentrate, showed a positive result. Elution studies demonstrated the presence of anti-B, the patient's blood group being B.

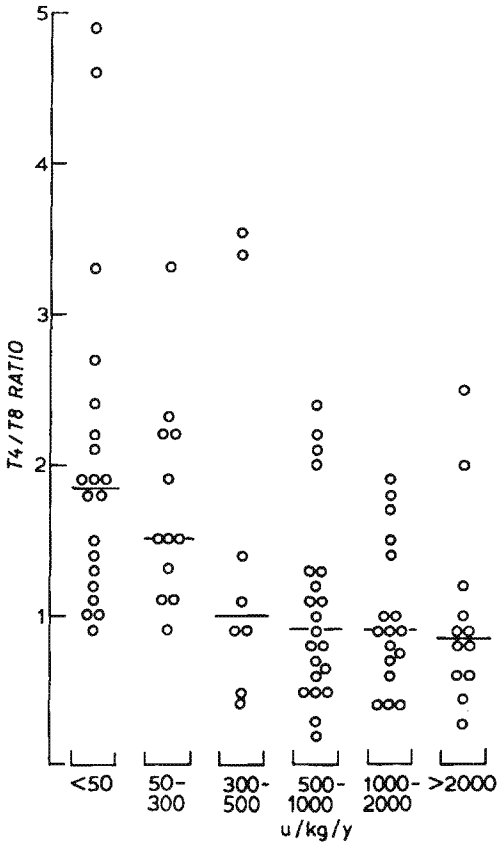

Fig 1. T4/T8 ratios in patients with haemophilia A treated with commercial VIII.

α_1-thymosin, β_2-microglobulin and IgG levels

The median serum α_1-thymosin level was higher amongst high exposure patients receiving commercial factor VIII concentrates (median 818 pg/ml, range 372–2691, $n=20$) than in low exposure patients (median 551 pg/ml, range 468–1106, $n=6$) or control subjects (median 597 pg/ml, range 395–1107, $n=16$). However, using a Mann-Whitney test these differences were not significant. Amongst factor IX treated patients levels were significantly higher amongst high exposure patients (median 910 pg/ml, range 459–1613, $n=9$) than in the low exposure group (median 770 pg/ml, range 504–1125, $n=6$) (Mann-Whitney test $P<0{\cdot}03$). α_1-thymosin levels were not measured in patients treated with NHS factor VIII concentrate.

β_2-Microglobulin levels showed no significant differences beteen control subjects (median 2·6 mg/l, range 1·8–4·4, $n=20$), and high exposure patients treated with commercial factor VIII (median 2·8 mg/l, range 2·7–3·2, $n=9$), NHS factor VIII (median 2·9 mg/l, range 2·2–3·8, $n=8$) or factor IX (median 2·5 mg/l, range 1·7–2·9, $n=9$).

Abnormally high levels of serum IgG were found more commonly in patients who had

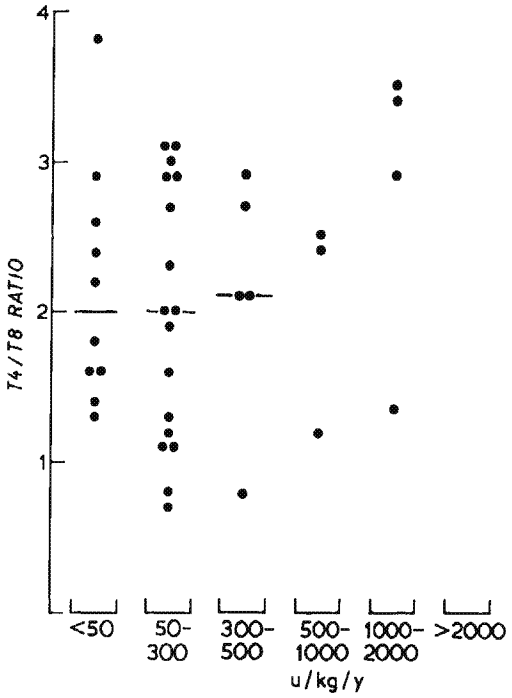

Fig 2. T4/T8 ratios in patients with haemophilia A treated with NHS VIII.

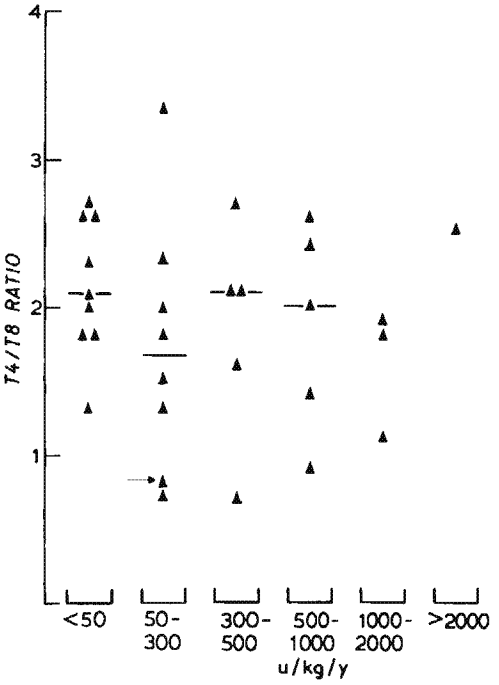

Fig 3. T4/T8 ratios in patients with haemophilia B treated with NHS IX. The patient marked with the arrow was a known heroin addict.

Table II. Lymphocyte and platelet counts, immunoglobulin concentrations and antibody to hepatitis B in patients with haemophilia

Patient population		Lymphocyte count	Platelet count	IgG	HBsAb
Definition of abnormality		$<1{\cdot}5\times10^9$/l	$<150\times10^9$/l	>18 g/l	†
Haemophilia A<300 u/kg/year	*	1·7 (0·8–4·8)	160 (109–341)	13·9 (6·4–22·7)	
(Commercial treatment, $n=32$)	†	12/31 (39%)	5/31 (16%)	2/28 (7%)	20/28 (71%)
Haemophilia A>300 u/kg/year	*	2·2 (0·9–6·2)	203 (101–341)	16·4 (6·4–28·7)	
(Commercial treatment, $n=58$)	†	12/54 (22%)	11/54 (20%)	15/43 (35%)	44/48 (92%)
Haemophilia A<300 u/kg/year	*	1·9 (0·8–10·3)	218 (149–384)	13·7 (8·6–50·8)	
(NHS treatment, $n=27$)	†	6/27 (22%)	1/25 (4%)	2/22 (9%)	10/25 (40%)
Haemophilia A>300 u/kg/year	*	2·4 (1·3–3·5)	224 (120–356)	13·7 (7·5–14·6)	
(NHS treatment, $n=12$)	†	2/12 (17%)	1/12 (8%)	0/12 (0%)	9/12 (75%)
Haemophilia B<300 u/kg/year	*	2·3 (1·2–5·1)	230 (74–307)	11·1 (7·1–18·4)	
(NHS treatment, $n=17$)	†	3/16 (19%)	2/16 (13%)	2/12 (17%)	9/16 (56%)
Haemophilia B>300 u/kg/year	*	2·1 (1·2–5·5)	200 (110–298)	11·4 (7–31)	
(NHS treatment, $n=14$)	†	4/11 (36%)	1/11 (9%)	2/12 (17%)	12/14 (86%)
Normal range		$1{\cdot}5$–$4{\cdot}0\times10^9$/l	150–400×10^9/l	8–18 g/l	

n = Total number of patients within treatment group.
* Median values with ranges in parentheses.
† Proportion of patients showing abnormality with percentages in parentheses.

received high exposure to commercial factor VIII concentrate, than in low exposure patients or those treated with factor IX (Table II).

PHA stimulation tests

An abnormally low response to PHA stimulation ($<30\times10^3$ cpm) was observed in 8/28 (29%) and 1/19 (5%) of patients who had received high and low exposures, respectively, to commercial factor VIII. Of patients with haemophilia B, 3/9 (33%) in the high exposure group, but none of six patients in the low exposure group showed similar abnormalities. Abnormalities were therefore more common amongst more intensively treated patients. NHS factor VIII treated patients were not tested for PHA stimulation.

Clinical observations

None of the patients studied had clinical evidence of AIDS, as defined by the Centers for Disease Control, Atlanta, and none were known to have other risk factors for AIDS except one

Table III. Patients with haemophilia A showing clinical abnormality

Patient	Age	T4/T8 ratio	Absolute T4 count $\times 10^9$/l	Absolute T8 count $\times 10^9$/l	Lymphocyte count $\times 10^9$/l	Platelet count $\times 10^9$/l	PHA stimulation	IgG (g/l)	Treatment (u/kg/year) Comm VIII
1	24	0·4	0·4	0·9	2·2	274	Normal	21	4700
2	55	0·2	0·2	0·9	1·6	125	Low	31	2700
3	53	0·6	0·4	0·7	1·8	101	—	28	740
4	34	0·3	0·6	2·0	2·9	165	—	22	700
5	40	1·0	1·0	1·0	2·4	188	—	11	1070

patient with haemophilia B who was a known heroin addict. A few patients have, however, shown clinical abnormalities.

Patient 1 had had persistent painless bilateral cervical lymphadenopathy of unknown cause for 2 years, associated with intermittent fever, malaise and fatigue. A lymph node biopsy showed a predominant T8 cell population in the paracortical areas but no destruction of architecture or involution of germinal centres. At the time of writing both his lymphadenopathy and abnormalities of T cell subsets (Table III) have resolved spontaneously.

Patient 2 has had cervical lymphadenopathy of at least 1 year's duration and has recently developed severe *ophthalmic zoster*. A lymph node biopsy has not been carried out.

Patient 3 has lost weight for no apparent reason and has also suffered episodic malaise and fever.

Patient 4 suffered a severe attack of *Streptococcal pneumoniae* pneumonia with septicaemia which responded to treatment with cefuroxime.

Patient 5 developed *herpes zoster* in the upper thoracic region unilaterally, but was receiving steroids for treatment of vasculitis.

DISCUSSION

Although the number of haemophiliacs known to have contracted AIDS is small, the approximate prevalence of 1–2 per 1000 treated patients is similar in Britain and the U.S. (PHLS unpublished, 1984; MMWR, 1984). 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 prodromal or forme fruste of AIDS (Desforges, 1983; Jones, 1983; White & Lesesne, 1983; *Lancet*, 1983). Whether or not this is the case, it seems beyond doubt that the abnormalities we and others have found in haemophiliacs without AIDS are caused by exposure to blood products.

The results of our studies suggest that the prevalence and severity of T lymphocyte subset distribution abnormalities in haemophiliacs are related to the magnitude of previous plasma product exposure, and the type of product infused. In children aged less than 10 years the

prevalence of abnormalities was much lower, a finding noted by others (Ragni *et al*, 1983) and this could be attributed to a lower total and shorter duration of exposure. We are unable to draw firm conclusions about the relative frequences of abnormalities in NHS and commercial factor VIII treated patients because insufficient NHS factor VIII is available to treat high usage adults. However, studies in Scotland, Australia and America have clearly shown that patients treated with factor VIII concentrate prepared from volunteer donor plasma can show T cell subset abnormalities (Carr *et al*, 1984; Rickard *et al*, 1983; Cable *et al*, 1983).

We have previously pointed out that the relative normality of results amongst our factor IX treated patients is unlikely to be attributable to characteristics of the source plasma (Lee *et al*, 1983). Similarly normal results have been found in patients treated with commercial factor IX concentrates of both American and European origins (Kessler *et al*, 1983; Saidi *et al*, 1983; Lechner *et al*, 1983). It therefore seems probable that characteristics of the final product, and the fractionation methods used to make it, render it less likely to cause T cell abnormalities in patients. AIDS is known to have occurred in patients with haemophilia B (Vilmer *et al*, 1984), but whether the incidence is similar or less than that in haemophilia A is unclear.

Various mechanisms have been proposed for the T lymphocyte disturbances in haemophiliacs. Reduced T4/T8 ratios associated with increased T8 counts have been noted after infection with several viruses (Crawford *et al*, 1981; Reinherz *et al*, 1980; Schooley *et al*, 1983; Thomas *et al*, 1982). However, the similarly high prevalence of HBsAb amongst our patients treated with different therapeutic products and the very high incidence of acute non-A, non-B hepatitis after a first exposure to both factor VIII and IX concentrates (Kernoff *et al*, 1984) does not suggest any lesser degree of contamination of factor IX. We have shown that the concentration of β_2-microglobulin, a probable measure of HLA A, B and C antigenic content, is much lower in factor IX concentrates (Lee *et al*, 1984). T8 lymphocytes mainly respond to antigens in association with the HLA, A, B and C antigens (*Lancet*, 1984a), and it therefore seems possible that the T cell subset abnormalities which occur in patients with haemophilia A, but less commonly in haemophilia B, may be causally related to this response.

The occurrence of immune thrombocytopenia and DAT positive haemolytic anaemia in haemophiliacs with evidence of impaired cellular immunity has suggested possible causal relationships (Ratnoff *et al*, 1983; Harris *et al*, 1983). We did not look for platelet-associated IgG in our patients, but none of the 76 tested showed evidence of autoantibodies to red cell antigens. The mild thrombocytopenia and lymphopenia which were found in 17% and 26% of our patients seem less likely to be due to specific immune mechanism than, for example, the splenomegaly which is common amongst haemophiliacs (Levine *et al*, 1977; Meyer *et al*, 1983). The raised levels of serum IgG, found particularly in high exposure patients treated with factor VIII in contrast to those treated with factor IX, may represent development of antibody to foreign protein, as suggested by Wardle (1967). Factor VIII concentrates contain substantial amounts of protein, including IgG, and considerably more than factor IX. Elevated levels of both β_2-microglobulin and α_1-thymosin have been reported in homosexuals with or without AIDS (Reuben *et al*, 1983; Biggar *et al*, 1983; Bhalla *et al*, 1983), and possible pathogenetic relationships have been suggested (Kreiss *et al*, 1984; Reuben *et al*, 1983).

Unlike Kreiss *et al* (1984), we found β_2-microglobulin levels to be normal, but α_1-thymosin levels to be raised in high exposure haemophiliacs.

There is increasing evidence that the abnormalities of T cell subset distribution represent a functional immunological defect. In patients without clinical evidence of immunodeficiency, we and others have found defective lymphocyte proliferative responses to PHA, and defective responses to concanavalin A and reduced natural-killer activity have been reported (Lederman *et al*, 1983; Froebel *et al*, 1983). In a preliminary report, Beddall *et al* (1983) noted an outbreak of tuberculosis amongst children hospitalized with haemophilia. The clinical events observed in our patients are probably related to mild or moderate immunodeficiency.

It is increasingly likely that AIDS is caused by an infectious agent which may be transmitted by blood product infusion (*Lancet*, 1984b) and that immune suppression produced by repeated exposure to clotting factor concentrates lowers the threshold for infection. With improvements in plasma fractionation technology and the production of 'genetically engineered' factor VIII, the prospects for resolution of these problems in haemophiliacs seem good.

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