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Lymphocytes of haemophilia patients treated with clotting factor concentrates display activation-linked cell-surface antigens

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SUMMARY

Peripheral blood lymphocytes from 30 patients with haemophilia A were investigated for the expression of six activation-linked cell surface antigens as well as with regard to the relative proportions and total numbers of Leu-3a and Leu-2a positive cells. Twenty-nine of the haemophilia patients showed no clinical symptoms of immunodeficiency or infection whereas one patient presented the typical symptomatology of the acquired immunodeficiency syndrome (AIDS). The proportions and total numbers of circulating lymphocytes displaying Ia antigens, the p45 protein and/or the two recently defined surface antigens VIP-4 and VIP-5 were significantly increased in haemophilia patients when compared to healthy individuals of the same age group. No such increases could be observed for transferrin receptor and IL-2 receptor expression. After the observation of depressed helper/suppressor T-cell ratios in many haemophiliacs, the expression of activation linked surface antigens represents a further lymphocyte abnormality which resembles the findings in AIDS and its prodromal stages and can also be found in certain viral and parasitic diseases.

Keywords haemophiliacs activation antigens Factor VIII concentrates

INTRODUCTION

The acquired immunodeficiency syndrome (AIDS) has recently attracted considerable interest (reviewed in Curran, 1983). Particularly striking was the observation that this syndrome seems to be confined to certain groups of individuals. Most prominent among them are homosexuals, drug addicts and Haitian immigrants (Gottlieb *et al.*, 1981; Masur *et al.*, 1981; Vieira *et al.*, 1983).

Haemophilia patients treated with commercial clotting factor concentrates have lately been added as a further risk group (Centers for Disease Control, 1982a; Ragni *et al.*, 1983; Davis *et al.*, 1983; Elliott *et al.*, 1983; Poon *et al.*, 1983; Lechner *et al.*, 1983). The number of so far reported haemophilia patients with well documented and unambiguous AIDS symptomatology is still rather small, however. There is also no direct evidence that commercial clotting factor concentrates actually contain a transmissible agent which directly can cause AIDS. It is evident, however (Jones *et al.*, 1983; Lederman *et al.*, 1983; Menitove *et al.*, 1983; Goldsmith *et al.*, 1983; Luban, Kelleher & Reaman, 1983; Poon *et al.*, 1983; Gill *et al.*, 1983; de Shazó *et al.*, 1983; Lechner *et al.*, 1983), that many clinically asymptomatic haemophiliacs and all reported haemophiliacs with AIDS symptomatology show the same relative predominance of T8 (Leu $2a^+$) cells and depletion of T4 (Leu-$3a^+$)

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cells as is regularly found in AIDS patients (reviewed by Groopman & Gottlieb, 1983) and frequently observed in clinically asymptomatic but highly promiscuous male homosexuals (Marmor *et al.*, 1982; Detels *et al.*, 1983; Moss *et al.*, 1983).

Results presented in this paper show that, besides the inverse T subset ratio, the blood lymphocytes of haemophilia patients and of AIDS patients share another abnormality. They display, in increased numbers, surface structures which are usually not found on the majority of unstimulated normal blood lymphocytes but are strongly expressed by activated T lymphocytes.

MATERIALS AND METHODS

Patients. Thirty patients with haemophilia A (mean age 29 ± 13) were compared to 20 healthy sex and age matched controls (mean age 29 ± 10). All patients were regularly treated with commercial clotting factor concentrates. Twenty-nine haemophilia A patients had severe (< 1 per cent) and one had moderate (< 5 per cent) factor VIII deficiency. One of the haemophilia patients showed clinical symptoms compatible with AIDS according to the CDC Definition (Centers for Disease Control, 1982b). He suffered from recurrent intestinal candidiasis infections, ulcerative oesophagitis and repeated episodes of unexplained fever. In addition, severe lymphopenia and thrombocytopenia, splenomegaly, lymphadenopathy and weight loss were observed.

Haematological methods. Leucocyte counts and platelet counts were performed using an automated blood-cell analyser (TOA) from EDTA anticoagulated blood. Lymphocyte counts were determined from blood smears from EDTA anticoagulated peripheral blood.

Immunoglobulins were determined by radial immunodiffusion (Mancini) using Partigen plates (Behring Institute, Marburg, FRG).

Peripheral blood mononuclear cells. Mononuclear cells (MNC) were isolated by gradient centrifugation on Ficoll Hypaque.

Monoclonal antibodies (MoAb). The MoAb used in this study and their main specificities are listed in Table 1. Leu 4, Leu 3a and Leu 2a were kindly provided by Dr R. L. Evans (SKI, New York, USA), the anti-Tac antibody was a gift from Dr T. A. Waldmann (NCI, Bethesda, Maryland, USA), all other MoAb were raised in our laboratory. Three of them, VIP-1, VID-1 and VIP-2b, are directed against well known, cell surface structures which are also recognized by a number of other MoAb from different laboratories. VIP-1 sees the transferrin receptor and is equivalent to, for instance, T9 (Reinherz *et al.*, 1980a; Terhorst *et al.*, 1981). VID-1 reacts with a monomorphic determinant of human Ia and gives the same reaction pattern in our hands as does the Q5/13 antibody (Quaranta, Pellegrino & Ferrone, 1981). VIP-2b specifically reacts with a 45 kD protein (p45) on the surface of activated T cells, thymocytes and mature plasma cells. It is equivalent to T10 (Reinherz *et al.*, 1980a; Terhorst *et al.*, 1981). The VIB-C5 antibody detects B cells and B cell precursors (Knapp *et al.*, 1983). The two MoAb VIP-4 and VIP-5 have recently been obtained after immunizing BALB/c mice with Ia^+, $p45^+$, $T8^+$ lymphocytes from a patient with EB virus-induced infectious mononucleosis. In MNC preparations from peripheral blood both antibodies react in high percentages with lymphocytes from patients with EB virus-induced mononucleosis (VIP-4: $72 \pm 16\%$; VIP-5: $77 \pm 15\%$) and from patients with CMV-induced mononucleosis (VIP-4: $73 \pm 2\%$, VIP-5: $81 \pm 15\%$), but only in small proportions with blood lymphocytes of healthy individuals (VIP-4: $18 \pm 8\%$; VIP-5: $16 \pm 7\%$). The two antibodies differ in the molecular weight of the reactive membrane component and in their reactivity with polymorphonuclear cells (Table 1). The cell surface antigens seen by the first four antibodies have been previously shown to be expressed by *in vitro* activated lymphocytes (Fu *et al.*, 1978; Judd, Poodry & Strominger, 1980; Terhorst *et al.*, 1981; Uchiyama, Broder & Waldmann, 1981) but not in detectable quantities on the majority of unstimulated blood T lymphocytes (Fu *et al.*, 1978; Reinherz *et al.*, 1980a; Uchiyama, Broder & Waldmann, 1981). The last two of the six activation linked surface antigens studied, the VIP-4 and VIP-5, are particularly strongly expressed on *in vivo* activated Ia^+, $p45^+$, Leu-$2a^+$ T lymphocytes in acute EB virus- and CMV-induced mononucleosis. *In vitro* activation with mitogens does not lead to an increased expression of these two structures.

Table 1. MoAb used in this study

Antibody designation	Ig class	Reactive membrane protein	Main specificity
Leu 4	IgG1	p19–29	Pan T lymphocytes (Evans *et al.*, 1981)
Leu 3a	IgG1	p55	Helper/inducer T lymphocytes (Evans *et al.*, 1981)
Leu 2a	IgG1	p32+33	Suppr./cytotox. T lymphocytes (Evans *et al.*, 1981)
VIB C5	IgM	n.a.	B cells and B precursors (Knapp *et al.*, 1983)
Anti-Tac	IgG2a	p50	IL-2 receptor on activated T cells (Uchiyama *et al.*, 1981; Leonard *et al.*, 1982)
VIP 1	IgG1	p90	Transferrin receptor on prolif. cells equivalent to T9 (Reinherz *et al.*, 1980a)
VID 1	IgG1	p28+32	Nonpolymorphic determinant of human Ia
VIP 2b	IgM	p45	Activated T cells, thymocytes, null cells equivalent to T10 (Reinherz *et al.*, 1980a)
VIP 4	IgG1	p150+170	T blasts in EB virus and CMV mononucleosis
VIP 5	IgG1	p100	T blasts in EB virus and CMV mononucleosis, granulocytes

Immunofluorescence. The reactivity of the various MoAb with lymphocytes was assessed by indirect fluorescence with fluoresceinated goat $F(ab')_2$ anti-mouse IgG+IgM antibodies.

Flow cytometric analysis. All cytofluorometric analyses were performed with the fluorescence activated cell sorter FACS 440 (Becton Dickinson, Sunnyvale, California; Loken & Herzenberg, 1975).

Statistical Evaluation. The Wilcoxon-Whitney-Mann test was used when controls and groups of patients were compared, for correlations the Spearman-Rang correlations coefficient was calculated.

RESULTS

In this study a total of 30 haemophilia patients and 20 healthy control persons were investigated for the expression of six activation linked lymphocyte surface antigens (Ia, p45, transferrin receptor, Tac, VIP-4 and VIP-5).

Among normal circulating lymphocytes Ia antigens are usually only expressed by B lymphocytes but not by the majority of T lymphocytes (reviewed by Winchester & Kunkel, 1979). In our control group (see Fig. 1a) the proportion of Ia positive lymphocytes ($10 \pm 3\%$) also corresponded perfectly with the simultaneously evaluated proportion of B lymphocytes ($10 \pm 3\%$, evaluated with the B-cell antibody VIB-C5). In haemophilia patients, however, we found (see Fig. 1a & e) significantly ($P < 0{\cdot}001$) higher proportions ($20 \pm 10\%$) and absolute numbers (342 ± 157 *vs* 203 ± 109 cells/ul) of Ia positive lymphocytes while the VIB-C5 reactive B lymphocyte numbers were not different from the control group. This indicates that in haemophilia patients treated with commercial clotting factor concentrates also lymphocytes of other than B cell type express Ia antigens. Activated T lymphocytes have previously been shown to express Ia antigens (Fu *et al.*, 1978; Evans *et al.*, 1978). They thus are the most likely candidates.

Another cell surface protein which has been found on activated but not on the majority of resting T lymphocytes is the p45 protein recognized by the T10 antibody (Reinherz *et al.*, 1980a). We have, therefore, included in our study the antibody VIP-2b which also recognizes this activation linked cell surface protein. As can be seen in Fig. 1b & f, the proportion and total number of VIP-2b

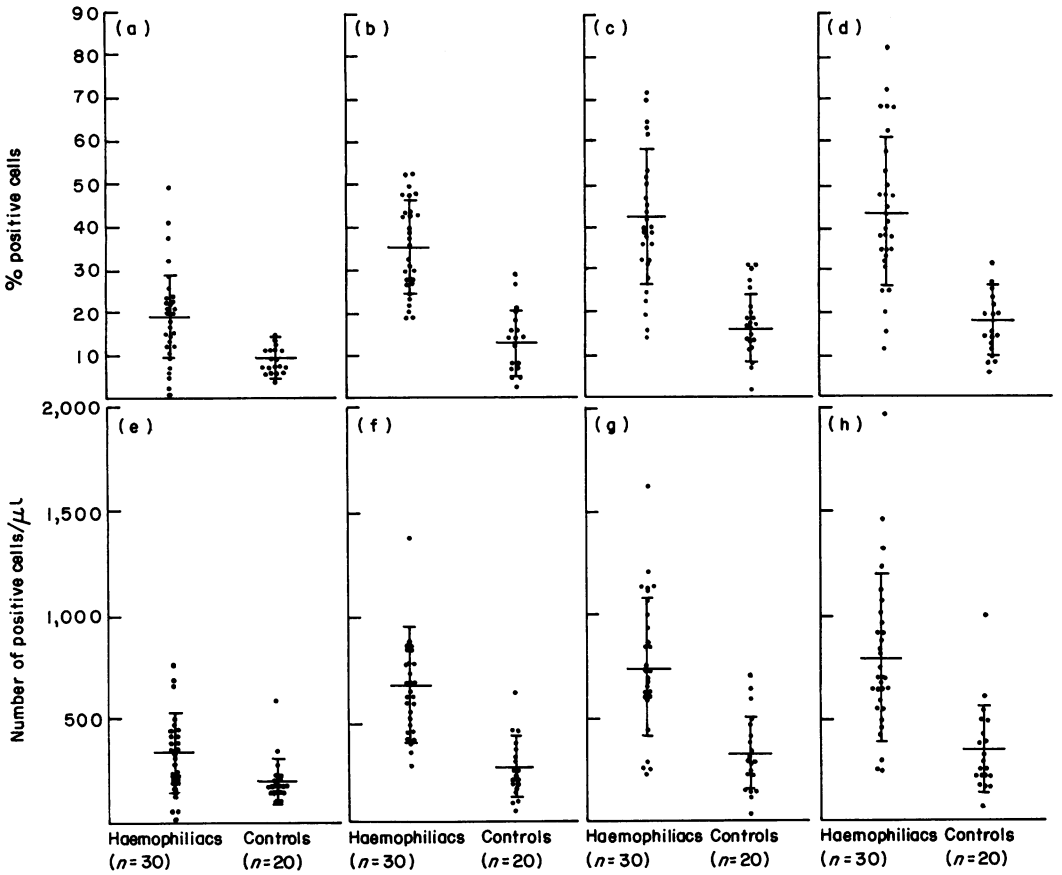

Fig. 1. Percentages (a–d) and total numbers (e–h) of peripheral blood lymphocytes from haemophilia patients and healthy control persons which react with the MoAb VID-1 (Ia) (a,e), VIP-2b (p45) (b,f), VIP-4 (p150+170) (c,g) and VIP-5 (p100) (d,h).

reactive (=p45$^+$) lymphocytes is significantly ($P < 0{\cdot}001$) higher in haemophilia patients ($36 \pm 11\%$, $624 \pm 226/\mu l$) than in healthy individuals ($13 \pm 8\%$, $262 \pm 145/\mu l$).

In contrast to *in vitro* stimulated lymphocytes, the circulating blood lymphocytes from haemophilia patients showed no detectable reactivity, however, with our monoclonal transferrin receptor antibody VIP-1. The percentages of VIP-1 reactive lymphocytes were below 5% in both groups (haemophiliacs and controls). Similarly low values were found in both groups (data not shown) with the anti-Tac antibody (Uchiyama, Broder & Waldmann, 1981) which recognizes the putative IL-2 receptor (Leonard *et al.*, 1982).

High levels of reactive cells were observed, however, with two recently established MoAb which were obtained after immunization of BALB-c mice with Ia$^+$ p45$^+$ T8$^+$ lymphocytes from a patient with EB virus-induced infectious mononucleosis.

These two antibodies, termed VIP-4 and VIP-5 react with the majority of peripheral blood lymphocytes from patients with EB virus- and CMV-induced infectious mononucleosis (see Materials and Methods) but only with a relatively small proportion of blood lymphocytes from healthy individuals (VIP-4: $18 \pm 8\%$, VIP-5: $16 \pm 7\%$).

In haemophilia patients $43 \pm 16\%$ of the blood lymphocytes (in absolute numbers 749 ± 345 cells/μl) reacted with the VIP-4 antibody (Fig. 1c & g) and $44 \pm 18\%$ (779 ± 402) with the VIP-5

antibody (Fig. 1d & h). These proportions and total numbers are not as high as in EB virus-induced mononucleosis, but significantly ($P<0{\cdot}001$) higher than in healthy controls (Fig. 1).

Several groups, including ourselves, have recently reported a relative increase in the proportion of circulating T lymphocytes bearing the Leu 2a/T8 antigen and a relative reduction of Leu 3a/T4 positive cells in asymptomatic haemophiliacs (Lederman *et al.*, 1983; Menitove *et al.*, 1983; Goldsmith *et al.*, 1983; Luban, Kelleher & Reaman, 1983; Poon *et al.*, 1983; Ragni *et al.*, 1983; Davis *et al.*, 1983; Gill *et al.*, 1983; de Shazo *et al.*, 1983; Lechner *et al.*, 1983). These abnormalities result in an inversion of the normal ratio of these two lymphocyte subpopulations. Also in the present study half of the patients showed an inverse ($=<1{\cdot}00$) Leu 3a/Leu 2a ratio and in the other patients the ratio was lower (mean: $1{\cdot}48\pm0{\cdot}63$) than in the control group but not inverted. The mean total number of circulating T lymphocytes bearing the Leu 2a/T8 antigen is significantly ($P<0{\cdot}05$) increased (765 ± 296 cells/μl) and the mean total number of Leu 3a/T4 positive cells is significantly ($P<0{\cdot}01$) decreased (504 ± 296 cells/μl *vs* 964 ± 298 cells/μl). With $1{\cdot}08\pm0{\cdot}61$ the mean Leu 3a/Leu 2a ratio of all haemophilia patients is also significantly ($P<0{\cdot}001$) lower than the respective value of the control group ($2{\cdot}15\pm0{\cdot}58$).

In order to see whether there is a possible association between the expression of the various activation linked cell surface antigens presented above and the observed changes in total numbers of Leu $2a^+$ cells and Leu $3a^+$ cells and in the Leu 3a/Leu 2a ratios, four groups of haemophilia patients were separately evaluated and compared. Group A consisted of 14 haemophilia patients with more than 1,192 cells/μl Leu-2a positive cells (mean $\pm$ 1 s.d. value of the control group). It was compared with group B, which included the other 16 patients who had total numbers of Leu $2a^+$ cells within the mean $\pm$ 1 s.d. range of the control group.

In group A the numbers of lymphocytes positive for VIP-2b (712 ± 244 cells/μl), VIP-4 (919 ± 352 cells/μl) and VIP-5 (953 ± 472 cells/μl) were significantly ($P<0{\cdot}05$) higher than in group B. There, the according values were 548 ± 183 for VIP-2b, 595 ± 256 for VIP-4 and 624 ± 263 for VIP-5. Group C ($n=15$) consisted of patients with inverse Leu 3a/Leu 2a ratios (below 1), Group D ($n=4$) of patients with Leu 3a/Leu 2a ratios above 1·6 (=above the mean value -1 s.d. of the control group). The proportions of VID-1, VIP-4 and VIP-5 reactive cells are somewhat higher in group C that in group D. These differences are, with the exception of VIP-4, statistically not significant, however. The mean proportion of $VIP\text{-}4^+$ cells in group C is with $50\pm14\%$ significantly ($P<0{\cdot}02$) higher than in group D (mean value $30\pm6\%$).

A number of other parameters which were also compared with the expression of the activation linked cell surface antigens under study showed no significant correlations.

The annual consumption of factor VIII concentrate varied among our patients between 111 u/kg and 3507 u/kg. A direct comparison between the annual concentrate consumption and the expression of four activation linked cell surface antigens as shown in Fig. 2 gave no significant

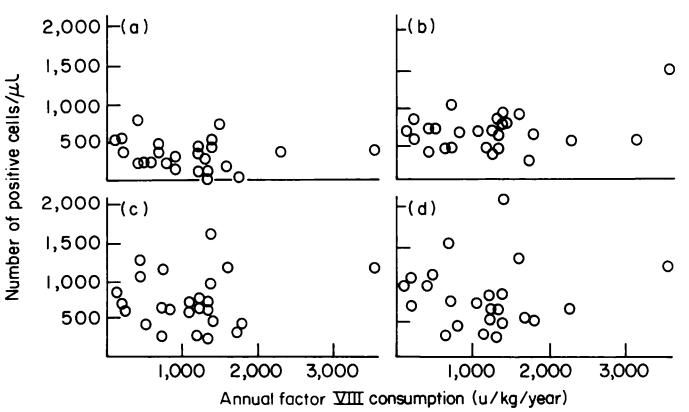

Fig. 2. Relation between the expression of activation linked surface antigens and annual factor VIII consumption. (a) VID-1(Ia), (b) VIP-2b (p45), (c) VIP-4 and (d) VIP-5.

correlation. Also, the presence or absence of lymphadenopathy and the serum IgG levels were not correlated with the expression of these surface structures.

Twenty-nine of the 30 haemophilia patients under study including 14 of the 15 patients with inverse Leu 3a/Leu 2a ratios showed no clinical symptoms of immunodeficiency. One of our haemophilia patients showed clinical symptoms compatible with AIDS. This patient differed from the clinically asymptomatic patients by the low number of lymphocytes and had—in contrast to the asymptomatic haemophiliacs with the same ratio—a decrease of the total number of Leu $2a^+$ cells (despite a relative increase) and a greatly reduced absolute number of Leu $3a^+$ cells.

In this patient the relative values for the various activation linked antigens were particularly high. In a longitudinal study of the expression of these antigens during a time period of 13 weeks the values for Ia (VID-1) amounted up to 46%, for p45 (VIP-2b) up to 70%, for VIP-4 up to 69% and for VIP-5 up to 74%. A FACS profile of this patient's lymphocytes with seven MoAb from one of these investigations is shown in Fig. 3 and compared with a typical example of an asymptomatic

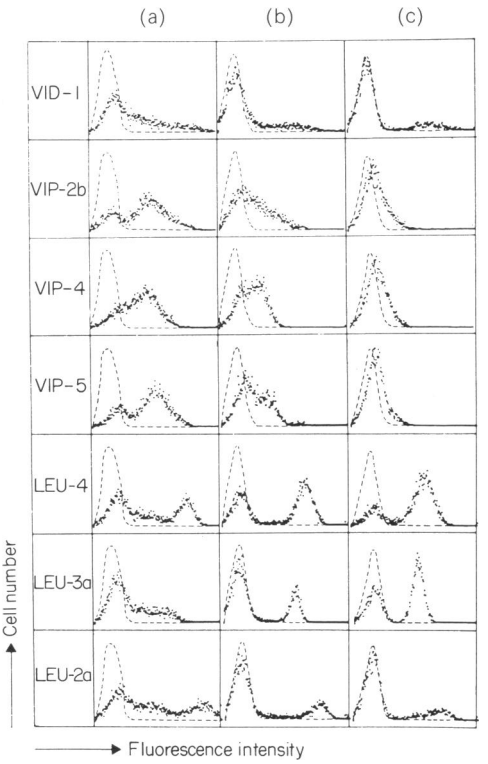

Fig. 3. Typical FACS profiles of lymphocytes from the haemophilia patient with (a) AIDS symptomatology, (b) an asymptomatic haemophilia patient and (c) a healthy control person when tested with the monoclonal antibodies VID-1 (Ia), VIP-2b (p45), VIP-4, VIP-5, Leu-4, Leu-3a, and Leu-2a.

haemophilia patient and a control person. It has to be mentioned, however, that the listed rises are only of a relative nature and that to the low lymphocyte counts the absolute counts were at no time above average.

DISCUSSION

This study demonstrates that increased proportions of peripheral blood lymphocytes from haemophilia patients treated with clotting factor preparations express certain activation linked cell surface antigens.

Particularly elevated were the percentages of Ia, p45, VIP-4 and VIP-5 positive lymphocytes. No such increases could be observed for the transferrin receptor and the putative IL-2 receptor (Tac antigen).

After the observation of depressed helper/suppressor T cell ratios and diminished proliferative T cell responses in many haemophiliacs (Lederman *et al.*, 1983; Davis *et al.*, 1983; Elliott *et al.*, 1983; Poon *et al.*, 1983; De Shazo *et al.*, 1983), the expression of activation linked cell surface antigens represents a further lymphocyte abnormality which resembles the findings in AIDS and its prodromal stages (Gottlieb *et al.*, 1981; Schroff *et al.*, 1983; de Shazo *et al.*, 1983). Apart from AIDS the combination: decreased helper suppressor cell ratio and expression of activation linked cell surface antigens, including Ia, by circulating blood lymphocytes has so far been reported in the following clinical situations: EB virus-induced infectious mononucleosis, CMV-induced infectious mononucleosis, acute-phase toxoplasmosis with lymphadenopathy (Reinherz *et al.*, 1980b; de Waele, Thielemans & Van Camp, 1981a, 1981b; Carney, Iacoviello & Hirsch, 1983), and the regeneration phase of following allogeneic bone marrow transplantation (de Bruin *et al.*, 1981). This lymphocyte phenotypic combination thus seems to be associated with certain viral and parasitic infections and/or with clinical conditions which predispose to infections.

Haemophiliacs have a high risk for viral infection as indicated by a high incidence of jaundice and frequent findings of antibodies to CMV, EB virus, Hepatitis B, as well as to HTLV associated membrane proteins (Craske *et al.*, 1978; Enck *et al.*, 1979; Evatt *et al.*, 1983). It may well be, therefore, that the observed lymphocyte alterations in haemophilia patients are a consequence of such infections.

The most likely cause of infections in haemophiliacs are of course contaminations of clotting factor concentrates. One would therefore expect that the incidence of (re)infections and, if related to it, also the incidence of lymphocyte alterations, is correlated to the amount of factor VIII concentrate administered. Such a correlation between lymphocyte alterations and factor VIII consumption does not exist, however. Neither the inversion of the helper–suppressor ratio (Lederman *et al.*, 1983; Lechner *et al.*, 1983) nor the expression of activation-linked cell-surface structures (this paper) are correlated with the amount of annually administered factor VIII concentrate.

We must assume, therefore, that either, (a) factor VIII concentrates and their infectious or non-infectious contaminations have nothing at all to do with the observed alterations, (b) that such contaminations play a role, but are not the only cause of the observed changes and other factors, probably patient related, are also involved, or (c) that such contaminations play the decisive role but are only included in certain lots of factor VIII concentrates. In the latter case it should be the exposure to these specific lots and not the annual consumption that is associated with the observed abnormalities.

We consider assumption (a) as being rather unlikely. It would imply that the observed abnormalities reflect the presence of an intrinsic immunoregulatory disorder in haemophilia patients, an assumption for which, so far, no evidence could be found (Menitove *et al.*, 1983; de Shazo *et al.*, 1983).

We cannot distinguish between possibilities (b) and (c) at the moment on the basis of lymphocyte marker studies.

One of the key questions is, of course, whether the demonstration of such lymphocyte alterations identifies persons at risk for the development of an immunodeficiency syndrome or simply shows secondary and clinically meaningless immunologic changes associated with sexually or blood product transmitted viral infections. The precise answer to this question can only be given by prospective studies. It may be worthwhile to remember, however, that the activation of circulating lymphocytes seen in the acute phase of certain viral diseases (Reinherz *et al.*, 1980b) is associated with an impaired overall human immune response. One therefore might speculate that the lymphocyte activation demonstrated in this study, be it specific or not, is also a predisposing factor for secondary infections.

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