A STUDY OF LYMPHOCYTE DYSFUNCTION IN MULTI-TRANSFUSED HAEMOPHILIC PATIENTS: MECHANISMS AND CLINICAL SIGNIFICANCE

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ABSTRACT

Contradictory evidence of activation and inhibition of the immune system has been described in haemophiliacs and attributed to infusion of clotting factor concentrate used in their haemostatic maintenance.

The purpose of the study was two-fold. Firstly to assess if the concentrates were activating patients' lymphocytes and thus potentially influencing the progression of HIV disease; and secondly, to assess the mechanisms of the reported concentrate-induced inhibition of T cell function *in vitro*.
Methods were established and markers of acute and chronic T and B lymphocyte activation were measured in matched groups of HIV+ve and HIV-ve haemophiliacs and controls.
HIV-ve haemophiliacs had elevated levels of soluble IL2 receptor (sIL2-R) ($p<0.05$) which were unrelated to concentrate infusion, but related to active liver disease attributed primarily to HCV infection. All other markers were normal.
HIV+ve haemophiliacs showed lymphocyte activation consistent with that seen in other HIV+ve groups with significant increases in expression of HLA-DR ($p<0.0001$), CD45RO ($p=0.0058$), sIL2-R levels ($p<0.02$) and all B cell activation markers ($p<0.001$). These did not correlate with concentrate infusion. Raised sIL2-R levels were associated with chronic liver dysfunction suggesting reactivated liver virus infection.
In conclusion infusion of concentrate did not appear to be causing lymphocyte disturbances *per se*. Immune activation was related to viral infection: either HIV or HCV.

A detailed analysis of the inhibitory effects of intermediate purity FVIII/FIX concentrates on T cell activation *in vitro* showed depression of HLA-DR and CD25 expression. This was not seen with monoclonal purified FVIII/FIX products. Inhibitory products caused a decrease in free calcium levels in the culture medium which correlated with their inhibitory effects. All products inhibited mitogen-induced calcium flux into the cells.
Reduction in calcium in culture medium by the concentrates is important in inhibiting T cell activation *in vitro*. Inhibition of calcium mobilisation suggests that events preceding calcium flux - eg, signal transduction may also be contributing to their inhibitory effects.

To conclude concentrate inhibition of T cell function *in vitro* is probably a laboratory artefact and the lymphocyte disturbances that have been reported are probably related to infection with virus.

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Abbreviations.

AIDS	Acquired immunodeficiency syndrome
AMLR	Autologous mixed lymphocyte reaction
ARC	AIDS related complex
AST	Aspartate amino transferase
ATCC	American Type Culture Collection
BCGF	B cell growth factor
BDGF	B cell differentiation factor
BPL	Bio-products laboratory
CMV	Cytomegalovirus
Ca^{2+}	Calcium ions
$[Ca^{2+}]$	Free calcium concentration
$[Ca^{2+}]_i$	Intracellular calcium concentration
CD	Cluster of differentiation
CMI	Cell mediated immunity
Con A	Concavalin A
DAG	Diacylglycerol
DMSO	Dimethy sulphoxide
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	Ethylenediamineetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
FITC	Fluorescein isothiocyanate
FIV	Feline immunodeficiency virus
FVIII	Factor VIII
FIX	Factor IX
G/M	Goat anti-mouse reagent
gamma GT	gamma Glutamyl transferase
gp120	HIV associated envelope protein
gp 41	HIV associated envelope protein
GvH	Graft versus host
HBSS	Hank's balanced salt solution
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCV-ve	Hepatitis C seronegative
HCV+ve	Hepatitis C seropositive
HDV	Hepatitis D virus (Delta particle)
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid
HIV	Human immunodeficiency virus
HIV-ve	Anti-HIV seronegative
HIV+ve	Anti-HIV seropositive
HSV	Herpes simplex virus
HTLV-III	Human T cell lymphotrophic virus III
Ig	Immunoglobulin
IL	Interleukin
IL-1	Interleukin 1
IL-2	Interleukin 2
IL-2-R	Interleukin 2 receptor

IP_3	Trisphopshoinositide
IV	Intravenous
LAV	Lymphadenopathy associated virus
LPS	(bacterial) lipopolysaccharide
Mab	Monoclonal antibody
MFI	Mean fluorescence intensity
mIg	Membrane Immunoglobulin
MLR	Mixed lymphocyte reaction
mRNA	Messenger ribonucleic acid
NANBH	Non A Non B Hepatitis
NHS	National Health Service
NK Cell	Natural killer cell
OPD	*O*-phenylenediamine
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBS-A	Phosphate buffered saline with azide and albumin
PCR	Polymerase chain reaction
PE	Phycoerythrin
PFC	Plaque forming cell
PGL	Progressive generalised lymphadenopathy
PHA	Phytohaemagglutinin
PIP_2	Phosphatidylinositol bisphosphate
PKC	Protein kinase C
PPD	Purified protein derivative
PWM	Pokeweed mitogen
RNA	Ribonucleic acid
RFH	Royal Free Hospital
RFHSM	Royal Free Hospital and School of Medicine
mRNA	Messenger ribonucleic acid
SAC	*Staphylococcus aureus* Cowen strain
SIV	Simian immunodeficiency virus
sIL-2R	Soluble interleukin 2 receptor
SLE	Systemic lupus erythematosus
SNBT	Scottish National Blood Transfusion Service
T3/Ti	T cell receptor
TNF	Tumour necrosis factor
TPA	12-*O*-tetradecanoyl phorbol-13-acetate
T4 cell	anti-CD4+ve lymphocytes, helper cell population
T8 cell	anti-CD8+ve lymphocytes, cytotoxic/suppressor cell population

1. INTRODUCTION

1.1 AIMS OF THE PROJECT

The aim of the project was to study the immunomodifying effects of treatment of haemophiliacs with clotting factor concentrate. There have been reports that infusion of these concentrates may have stimulatory or suppressive effects upon the immune system. These are dealt with in more detail in section 1.4. Both aspects of immunomodulation are important. Activation of the immune system may lead to increased progression of human immunodeficiency virus (HIV) disease in infected patients and immunosuppression may lead to susceptibility to infection.

The study was divided into two parts:

A. To assess if the infusion of concentrates led to activation of the immune system and thus influenced the progression of HIV disease.

B. To assess the mechanisms of *in vitro* inhibition of T cell function in the presence of FVIII concentrates.

1.1.1 GENERAL OVERVIEW

1.1.1.1 The *in vivo* study

There is great variability of host-virus interactions in HIV infection, some individuals exposed to the virus do not succumb to infection, or remain asymptomatic for years, whilst others progress more rapidly. One explanation for this is genetic variability where there have been reports of

increased frequency of infection related to HLA types.

Progression to AIDS in HIV infection may also be related to the virulence of the viral strain. This is illustrated in Scottish haemophiliacs: seroprevalence of HIV in Scotland in 1983 was very low and it is likely that the Edinburgh cohort of anti-HIV sero-positive (HIV+ve) haemophiliacs were probably infected from one source (Simmonds *et al,* 1988). These patients, however, have relatively high rates of morbidity and mortality indicating that the viral strain was particularly virulent (Cuthbert *et al*, 1989).

HIV disease progression may be greatly influenced by the state of immune function either at the time of infection and/or during the disease.

The importance of immune function, particularly activation in infected patients or individuals at risk, is more clearly understood when the pathogenesis of HIV is considered.

HIV disease is characterised by a progressive, selective loss of $CD4^+$ (T4) lymphocytes, which largely contributes to the immunosuppression seen in these patients.

Infection of T4 cells and replication of the virus is greatly facilitated by activation and may promote cell death (Margolick *et al*, 1987, Gowda *et al,* 1989, McDougal *et al*, 1985, Zagu ry *et al*, 1986, Stevenson *et al*, 1990). The chronic activation of HIV infected T4 cells, perhaps by other viruses or parasitic infection may lead to HIV replication, cell lysis and liberation of virus that can infect other T4 cells (Quinn *et al*, 1987). Therefore repeated stimulation of the immune system with various micro-organisms or exposure to allogeneic cells such as semen or blood, may determine the

latency of AIDS in infected patients (Zagury *et al*, 1986). Concern has been expressed that the impure clotting factor concentrates (ie < 1% FVIII) used in the treatment of haemophilia, may themselves, be activating the immune system and thus accelerating the progression of HIV disease (Landay *et al*, 1983, Vilmer *et al*, 1984).

There have been many reports of immune dysfunction in haemophiliacs, most of which was attributable to HIV infection (section 1.4.1 and 1.4.2). There is, however, evidence of both activation (increased levels of T cell lymphokines and serum immunoglobulin G (IgG)) and suppression (skin test anergy and reduced response to mitogen) of the immune system, which is unrelated to infection with HIV (section 1.4.3). Activation of cells *in vivo* may lead to a refractory status *ex vivo*. This has been demonstrated in HIV infected patients where activation of T cells determined by *in vivo* increases in gamma interferon levels and neopterin, correlated with diminished response of peripheral blood mononuclear cells (PBMC), *ex vivo*, to antigenic stimulation (Fuchs *et al*, 1990). This phenomenon has also been demonstrated in other disorders: *in vivo* and *in vitro* gamma interferon production were found to be inversely correlated in patients with graft versus host disease (GvH) (Cleveland *et al*, 1988) and similar results have been shown in patients with systemic lupus erythematosus (SLE) (Preble *et al*, 1983). This phenomenon can also occur in B cells. Stimulation of B cells by certain components, eg IgM, can partially activate the cell so that it cannot complete the cell cycle (Walker *et al*, 1986), and is then refractory to further stimulation.

Many studies suggest that the immune abnormalities in anti-HIV sero-negative (HIV-ve) haemophiliacs are related to infusion of clotting factor concentrates, in particular - antigen overload, alloantigens and virus or viral particles. We set out to test the hypothesis that activation from chronic stimulation by concentrates *in vivo* may be pushing the cells partially through the cell cycle so that they are no longer reactive to initial stimuli, and that this may render the cells refractory to antigen stimulation *ex vivo* (suppression), and possibly affecting the patients immune competence *in vivo*.

To assess if activation of the immune system was occurring we analysed the presence of both acute and chronic cellular activation markers on the T and B lymphocytes of both HIV-ve and HIV+ve haemophiliacs, and measured the levels of serum Ig and soluble interleukin 2 receptor (IL2-R). A panel of monoclonal antibodies which detected neo-antigens was used to assess if the patients lymphocytes were stimulated. The markers used detected antigens expressed at early, mid and late stages of the cell cycle and could therefore establish if partial activation was occurring. Lymphocyte activation processes and activation markers are discussed in section 1.8. Expression of these markers in the cell cycle is diagrammatically demonstrated in figure 1.3. Study of the HIV-ve patients enabled the analysis of the immune system of the haemophiliacs without the complicated influence of HIV infection. Study of the HIV infected individuals enabled investigation of the effects of administration of clotting factor concentrates upon the pattern of immune abnormalities seen in other HIV+ve patients.

1.1.1.2. The *in vitro* study

The presence of FVIII, *in vitro*, can partially inhibit the function of cultures of cell lines and peripheral blood lymphocytes from normal donors, and it is thought that this phenomenon may be contributing to the immune dysfunction reported in haemophiliacs (outlined in section 1.4). It has been suggested that this inhibition is directed at early events in T cell activation processes by a component(s) of the FVIII preparation, and that it is probable that the inhibition of proliferation is a specific effect of the down regulation of IL2 production (Lederman *et al*, 1986). Buffer preparations of the concentrates have also been demonstrated to inhibit lymphocyte function (McDonald *et al*, 1985, Wang *et al*, 1985). In addition, concentrates have been demonstrated to inhibit *in vitro* monocyte function (Eibl *et al*, 1987, Mannhalter *et al*, 1988) and inhibition in accessory cell function may be contributing to the T cell dysfunction that has been reported. The mechanisms of the T cell inhibition reported *in vitro*, however, still remain unclear. By using an *in vitro* model, investigation of the mechanism of inhibition of T cell function has been undertaken.

A series of questions were asked:

1. Do all concentrates lead to T cell dysfunction?

2. Where in the T cell cycle is the inhibition of activation occurring?

3. Is the partial inhibition seen related to specific inhibition of a T cell subpopulation?

The information gained from points 2 and 3 could then be used to understand the potential mechanisms of the inhibition.

To address these questions a range of FVIII and FIX products, of variable purity and manufacture, were assessed for their inhibitory effects. Activation markers preceding IL2 production (HLA-DR, calcium flux) and influenced by IL2 (IL2-receptor (CD25)), in conjunction with T cell subset markers - CD4 and CD8, and T4 subset specific lectins were used to assess the specificity of the inhibitory effect.

1.2 HAEMOPHILIA - AN OVERVIEW

1.2.1 HAEMOPHILIA - AN HISTORICAL PERSPECTIVE

Haemophilia is an inherited blood coagulation disorder, resulting from a deficiency in factor VIII - haemophilia A (Classic haemophilia) or from a deficiency in factor IX, - Haemophilia B (Christmas disease). The deficiencies are derived from total or partial failure to synthesise the factor, or synthesis of a functionally abnormal variant of the protein. The consequence of these deficiencies is a reduced ability to generate factor X activating complex in the clotting cascade (figure 1.1).

Both haemophilia A and haemophilia B are X-linked inherited disorders, however, a third of the haemophilia A presentations are new mutations (Tuddenham, 1989). Haemophilia A and haemophilia B are clinically indistinguishable, presenting with a bleeding tendency, the severity of which is inversely correlated with the residual factor level. The disease is recognised by repeated and spontaneous bleeding into the muscles and joints. The familial bleeding disorder was noted in the Babylonian Talmud almost 2000 years ago. Regular treatment was not available until the 1950's and before this, haemophiliacs had a poor life expectancy and suffered from recurrent bleeds leading to painful deformity. Birch, in a clinical report, in 1937, recorded the cause of death for 113 haemophiliacs. The majority of these deaths had occurred in childhood with only 6 surviving beyond the 40th year (Biggs, 1967). Twenty-five deaths resulted from minor operations, 15 of these were from circumcision and the majority of the rest resulted from tooth extraction. Twenty-three of the remaining deaths resulted from trivial injuries including cut lip and

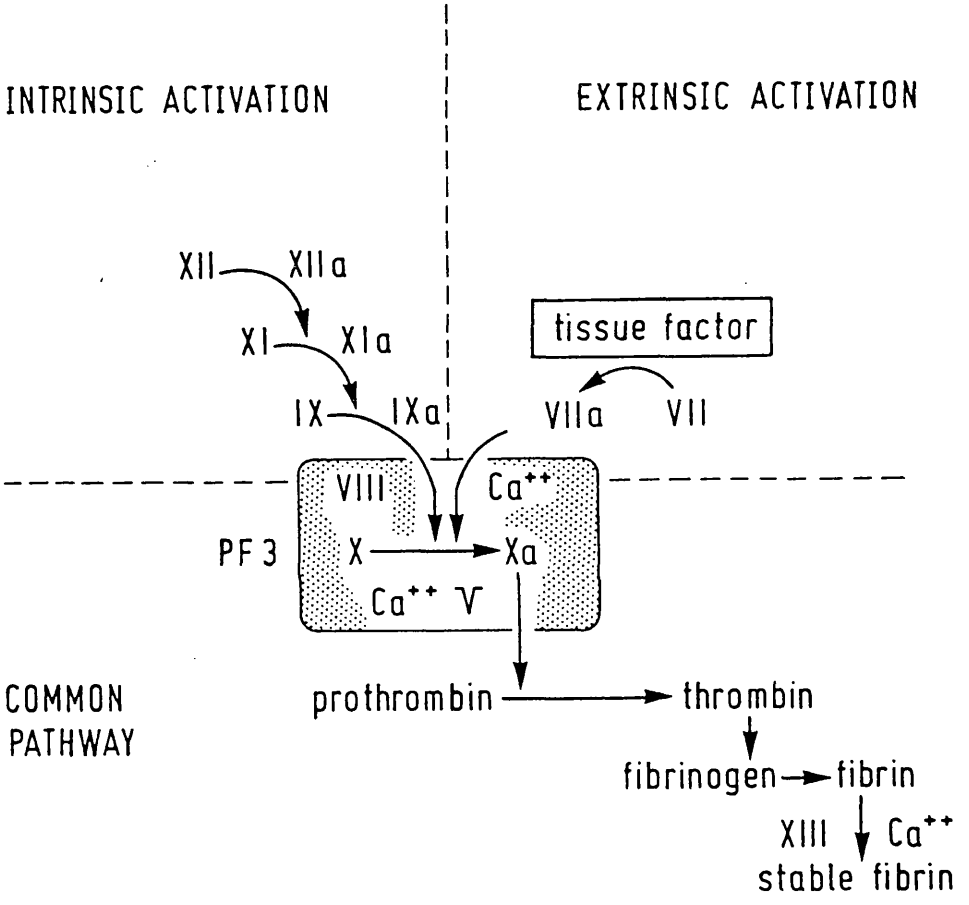


Figure 1.1: The intrinsic and extrinsic pathways of blood coagulation.

From Essential Haematology, Hoffbrand A V, Pettit J E, Blackwell Publications, 1980.

bitten tongue. The out-look for haemophiliacs was grim and it is only by making these comparisons that the impact of the availability of treatment can be fully appreciated.

1.2.2 TREATMENT OF HAEMOPHILIA

Treatment for haemophilia consisted mainly of bed rest and ice packs but unusual methods were used. For example, there is an interesting report of a bleeding episode 'for an affection of the knee joint' treated by the application of leeches from which 'bleeding .... was arrested with considerable difficulty'! (Lane, 1840). The successful transfusion of blood, recorded as early as 1840, when the same eleven year old haemophiliac reported above was treated for prolonged bleeding following a minor operation, was the break through in clinical management of this disease. Plasma transfusions were introduced later but it was the discovery of methods for concentration of factor VIII from plasma such as cryoprecipitation during the second half of this century and further purification processes enabling lyophilisation of the product, and commercial and national manufacture, that revolutionised the care of patients with haemophilia. In the 1980's, with the exclusion of patients who have become infected with HIV, life expectancy in haemophiliacs was only mildly reduced and was calculated to be 66 years (Ro sendaal *et al*, 1989). This is further substantiated by a comparison of median life expectancy in severely affected Swedish haemophilic patients where a 5-fold increase from a median of 11 years during the period 1831-1920 to 56.8 years during 1961-1980 was seen (Larsson, 1985).

1.2.2.1 Cryoprecipitate

Separation of the plasma into fractions in the late 1940's (Cohn *et al*, 1946) and further developments during the 1950's led to experimentation with concentrate therapy and the development of cryoprecipitate using single donor, or small donor pools (Poole and Shannon, 1965). These crude concentrates of plasma proteins which included FVIII and FIX, were infused parenterally for the treatment of bleeds.

1.2.2.2 Intermediate purity FVIII concentrates

Widespread treatment for haemophiliacs became available with large scale manufacture of intermediate purity FVIII by commercial and state run enterprises. This was prepared from fractionated pooled cryoprecipitate from 2,500 to 25,000 blood or plasma donors and treated with glycine-ethanol-citrate buffer and cold precipitation with aluminium hydroxide to remove protein contaminants. The final product was still relatively impure and contained large amounts of fibrinogen, however, the production of freeze-dried products greatly facilitated the treatment of patients where FVIII was now administered by intra-venous (IV) injection (Brinkhous *et al* 1968). The production of intermediate purity FVIII products from such large donor pools was expected to carry a much higher risk of infection (Giogini *et al,* 1972). As will be discussed, this has been proved to be so.

1.2.2.3 High Purity FVIII concentrates

The first generation of high purity products were developed using polyethylene glycol or ethanol cold precipitation methods of intermediate purity products (Johnson *et al*, 1971).

Later products used monoclonal antibody technology to purify cryoprecipitate. Two approachs have been used. In the first, an anti-vWF monoclonal antibody (Mab) is used to absorb the FVIII:vWF complex from cryoprecipitate and the FVIII:C is separated from the vWF by eluting the column with a high calcium buffer. A second method uses an antibody specific for the FVIII:C molecule. These high purity monoclonal antibody preparations are stabilised with plasma derived human albumin and therefore are not 'pure' products. They do, however, have a reduced mixture of proteins and are possibly less likely to be contaminated with virus (Madhok and Forbes, 1990).

1.2.2.4 Recombinant FVIII

A synthetic FVIII product would reduce the problems associated with the infusion of blood derived concentrates including viral transmission; immune disturbances (section 1.3); and availability and reliance on the 'donor system'. Following the rapid progression of the purification (Rotblat *et al*, 1985) and characterisation of the FVIII molecule (Vehar *et al*, 1984); characterisation (Gitschier *et al*, 1984) and cloning (Toole *et al*, 1984) of the FVIII:C gene; and successful transfection of the gene into mammalian cell lines (Wood *et al*, 1984 , Kaufman *et al*, 1988), the sterile recombinant FVIII product is now commercially available and undergoing clinical trials (White *et al*, 1989). The product, however, is still largely plasma derived as it is stabilised with human albumin. This protein, however, is easier to purify from plasma and is not in general associated with major protein or viral contamination (Cuthbertson *et al*, 1987).

1.2.2.5 FIX concentrates

FIX is concentrated from cryoprecipitate or citrated plasma by absorbing the vitamin K-dependent proteins onto DEAE-cellulose or sephadex. These products contain equal amounts of factors II,IX, and X. Monoclonal antibody purified products have been developed and are approaching the stage of use in clinical trial. Heparin is added to some products to prevent thrombotic complications from activated coagulation factors (Madhok and Forbes, 1990).

1.2.2.6 Heat treatment of clotting factor concentrates

One of the greatest problems associated with infusion of concentrates has been the transmission of virus and this will be discussed in section 1.3. Transfusion of hepatitis B virus (HBV) and of non A non B (NANB) hepatitis (the principle causative agent is now identified as hepatitis C virus (HCV)), and the tremendous impact of HIV infection, has led to the introduction of a 'triple- barrier' to prevent viral transmission. The first step is self exclusion of donors (by a health questionnaire related to HIV symptoms and risks); the second step comprises donor screening (measuring liver transaminase levels, detection of antibodies to HIV and to hepatitis B virus surface and core antigen and probably HCV); and thirdly, viral inactivation of products (Brettler and Levine, 1989).

Multiple methods of viral inactivation have been employed including various temperatures, duration of heat-treatment, 'dry' or 'wet' (pasteurisation) heat treatment, solvent/detergent treatment, and monoclonal antibody

purification to exclude the virus. All give a reduced yield of FVIII clotting activity (Barrowcliffe *et al*, 1986). In general, the more effective the process of decontamination, the greater the yield loss (Lee and Kernoff, 1990). HIV has been shown to be inactivated with relative ease (Dietz *et al*, 1986, Schimpf *et al*, 1989), but, it has been demonstrated that the NANB agent and HBV are more difficult to inactivate. Dry-heat (60°C for 72 hours) was demonstrated to be ineffective in the inactivation of the agent for NANB virus (Colombo *et al*, 1985). Wet heat (Alpha Profilate HT {wet method}) (60°C for 20 hours with heptane) was also shown to be ineffective but did reduce risk of hepatitis following first exposure (Kernoff *et al*, 1987). Super-dry-heating (80°C for 72 hours), used by the UK Blood Products Laboratory (8Y and 9A), in continuing trials, has shown no evidence of transmission of HIV or hepatitis (Pasi and Hill, 1989, Pasi et al, 1990, Colvin, 1990). HCV was undetectable by polymerase chain reaction methods (PCR) in 'super heat' treated products but was present in concentrates less intensively treated (dry heat 60° for 32 hours and 'wet' heat 60°C for 20 hours with heptane) (Garson *et al*, 1990). Monoclate M (60°C for 10 hours in aqueous solution) has not been shown to transmit HIV, HBV, or NANB hepatitis (Brettler and Levine, 1989).

The patients in this study were treated, virtually exclusively, with Alpha Profilate (wet heat treated), NHS 8Y and 9A or Monoclate M.

1.3 TRANSFUSION OF VIRUS BY BLOOD PRODUCTS

1.3.1 HEPATITIS - HBV, HCV (NANB)

The three etiological agents associated with blood transfusion viral hepatitis are hepatitis B virus (HBV), hepatitis C virus (HCV, NANB agent) and of less importance hepatitis D virus (HDV) (delta-particle). HBV and HCV have high morbidity and mortality levels, with symptoms ranging from acute infection and chronic sequelae of cirrhosis and primary liver cancer (Zuckerman, 1990).

Transfusion related jaundice was recognised as a problem early in the treatment with plasma products (Beeson, 1943). Adults with first exposure to concentrate (Kasper and Kipnis, 1972) or who had received blood products prepared from commercial donations had increased frequency of this problem (Giogini *et al*, 1972). In 1975, Craske et al reported that 50% of the haemophiliacs they had studied had had an attack of hepatitis, an increase from an incidence of 1.8% in the regional assessment of Haemophilia Centres from 1969-1971 (Biggs , 1974). Early reports described clinical hepatitis, and greatly underestimated the incidence. Demonstration of asymptomatic liver disease by transaminase levels revealed that 41-72% of patients tested had abnormal liver function tests (Manucci *et al*, 1975, Cederbaum *et al*, 1982) and prevalence of antibodies to HBV was shown to be 66%, illustrating the high incidence of this disease, even though only a small proportion of the patients had acute hepatitis. Liver biopsy studies indicated that 14 out of 15 patients with abnormal transaminase levels, but no clinical signs of chronic liver disease, showed histological evidence of persistent hepatitis (White *et al*, 1982). A survey at the Royal Free Hospital reported that 92%

of heavily treated, HBV-unvaccinated, haemophilia A patients had detectable antibodies to HBV (Lee *et al*, 1985).

A high incidence of hepatitis in haemophiliacs is related to 'non-A-non-B-hepatitis', with a greater risk being associated with the use of commercial products (Fletcher *et al*, 1983), and at first exposure (Kernoff *et al*, 1985). In these studies diagnoses of this disease was performed by exclusion. It is typically a mild infection but progresses to chronicity in a high proportion of cases (Lee and Kernoff, 1990).
Recent isolation of the agent for NANB hepatitis, designated hepatitis C virus (HCV) (Choo *et al*, 1989) and development of a serological assay (Kuo *et al*, 1989), have demonstrated that the majority of haemophiliacs, heavily treated with concentrate, are HCV seropositive (HCV+ve), (Ludlam *et al*, 1989). These results are in line with those in our own centre.

1.3.2 HIV INFECTION AND ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS)

In July 1982 the Morbidity and Mortality Weekly Report by the Centres of Disease Control, Atlanta, USA, reported 3 haemophilia A patients with *Pneumocystis carinii* pneumonia. Two of the patients had cellular immune dysfunction including depressed mitogen responses, depletion of T helper cells and inverted T helper/suppressor ratios. The clinical and immunological features were similar to an acquired immunodeficiency syndrome (AIDS) seen in homosexuals, IV drug abusers and Haitian immigrants. At the time the cause of the immune disturbances was unknown but it was suggested that they may be due to an agent transmitted by blood and blood

products. By the end of 1982 a further 5 haemophiliacs had been reported to have AIDS like symptoms (Centre for Disease Control, Dec 10th, 1982).

T cell abnormalities (section 1.4.1) appeared to be widespread in the haemophiliacs receiving infusion of blood products (Lederman *et al*, 1983, Menitove *et al*, 1983, Goldsmith *et al*, 1983, Kessler *et al*, 1983, Ludlam *et al*, 1983, Lee *et al*, 1983, 1985, Carr *et al*, 1984, Moffat *et al*, 1985). There was disagreement, however, whether correlation between the abnormalities and amount of concentrate administered existed.

A myriad of reports of AIDS like symptoms in haemophiliacs appeared in the remainder of 1983 (Poon *et al*, 1983, Elliott *et al*, 1983, Ragni *et al*, 1983).

In 1983, the human immunodeficiency virus (HIV) (previously designated HTLV-III or LAV) was isolated along with specific antibodies from patients with AIDS (Barre-Sinoussi *et al*, 1983, Gallo *et al*, 1984) confirming that this lymphotrophic retrovirus was the etiological agent of AIDS. In 1984 Vilner *et al*, isolated the newly reported lymphotrophic retrovirus, now designated HIV, from two siblings with haemophilia B, one of whom had AIDS. Serological estimation of the incidence of HIV infection demonstrated that 64-72% of haemophilia A patients, who had been treated with the FVIII concentrates from 1980-82, were seropositive (Kitchen *et al*, 1984, Ramsey *et al*, 1984). In these studies none of the patients who had received less than 150,000 units of FVIII concentrate over a three year period were seropositive and no antibody was detected in patients with haemophilia B.

Melbye *et al* (1984), studied seropositivity in Scottish

haemophiliacs who were largely treated with locally produced FVIII, reporting that 15.6% were seropositive for HIV. In contrast, 59.1% of the Danish haemophiliacs that they studied were seropositive. Only two of these patients had received locally produced product. Seropositivity was directly correlated with consumption of commercial concentrate, but not with locally produced material.

HIV seropositivity was correlated with transmission through the infusion of concentrates (Jason *et al*, 1985a, Gomperts *et al*, 1985, Gjerset *et al*, 1985, Goedert *et al*, 1985, Evatt *et al*, 1985, Lederman *et al*, 1985a, Daniel *et al*, 1986, Hoxie *et al*, 1986a). Very few cases of seroconversion to HIV occurred in haemophilia patients before 1979 and the rate of seroconversion increased rapidly in 1981-82 (Eyster *et al*, 1985). It was noted that the delay in symptomatic infection (AIDS) indicated that the peak of AIDS incidence was yet to come.

Analysis of serum of Scottish haemophiliacs from 1974 demonstrated no incidence of positivity prior to 1981 and it was hypothesised that infection with HIV was new in the haemophiliac environment (Madhok *et al*, 1985). There have been several recent cumulative reports of HIV infection in haemophiliacs. A survey of haemophiliacs in the UK demonstrated that of 2330 haemophilia A patients 955 (41%) had antibody directed against HIV and, in the haemophilia B patients 26 (6%) of 401 patients were HIV+ve. An increased prevalence of seropositivity was seen in severely affected haemophiliacs, with 59% of haemophilia A patients and 11% of

haemophilia B patients infected with HIV (The AIDS group of the UK Haemophilia Centres Directors, 1988). The rate of progression of disease is related to the age of the patients (Darby *et al*, 1988), with a calculated 8-year cumulative incidence of AIDS at 40% (Lee *et al*, 1989).

1.4 IMMUNE DISTURBANCES IN HAEMOPHILIACS

1.4.1 IMMUNE DISTURBANCES IN HAEMOPHILIACS - HISTORICAL PERSPECTIVE

Haemophilia patients have been reported to have immune disturbances that were thought to be related to the infusion of blood and blood products. Immune disturbances have been described in this patient group for many years including, immune complex reactions, positive Coomb's test reactions and rheumatoid arthritis (Barkagan and Yegorova, 1971).

It was following the advent of AIDS in haemophiliacs, however, that these patients were intensively investigated. These studies predated the isolation and association of HIV with the severe immune abnormalities. The patients were grouped into AIDS versus asymptomatic, many of whom were probably HIV+ve. T cell abnormalities appeared to be widespread in haemophiliacs receiving FVIII concentrates including: a relative and absolute decrease in T helper cells; a relative and absolute increase in T suppressor cells; and inverse T helper/suppressor ratios (Lederman *et al*, 1983, Menitove *et al*, 1983, Goldsmith *et al*, 1983, Kessler *et al*, 1983, Ludlam *et al*, 1983, Lee *et al*, 1983, 1985, Carr *et al*, 1984, Moffat *et al*, 1985). Lederman and Menitove noted additional abnormalities: diminished proliferative responses to the lectins phytohaemagglutinin (PHA) and Concavalin A (Con A), depressed natural killer (NK) activity and elevated IgG levels. These abnormalities did not appear to affect recipients of FIX concentrate or cryoprecipitate (Lederman *et al*, 1983, Lee *et al*, 1983, Luban *et al*, 1983, Rasi *et al*, 1983), and this further substantiated the conjecture that the

immune dysfunction was related to infusion with FVIII concentrates. These abnormalities were attributed to 'unknown properties of large donor-pool factor VIII concentrates' (Rasi *et al*, 1983). It was suggested that the differences between the patients receiving lyophilised products and cryoprecipitates were related to exposure to smaller donor-pools used in the latter product (Desforges, 1983). Immunological abnormalities, however, have been reported in haemophiliacs who were treated exclusively with cryoprecipitate, and more profound perturbations were noted in patients who had been treated for more than 15 years (Pollack *et al,* 1985).

The discrepancy in FVIII and FIX treated patients was difficult to explain as both products came from the same donor pool, however, most UK haemophilia B patients received non-commercial FIX. It was suggested that 'the higher antigenic load associated with the administration of FVIII concentrates' attributed to the fractionation methods of the two products, may have been causing the discrepancy (Lechler *et al,* 1983, Lee *et al,* 1983). At this time, the general opinion was that there were two separate phenomena leading to immune abnormalities - AIDS, probably caused by a blood borne agent; and an independent concentrate associated immune dysfunction (Lederman *et al*, 1983, Menitove *et al*, 1983).

In general those patients with AIDS had more severe immune abnormalities compared to other haemophiliacs but variability between studies existed. Asymptomatic haemophiliacs receiving treatment, although having abnormal T cell numbers, had normal

B cells, monocytes and NK cells. Haemophiliacs with AIDS, on the other hand, had abnormalities in T, B and NK cell types (Landay *et al,* 1983). More severe abnormalities were noted in those patients with lymphodenopathy (deShazo *et al,* 1983) and a one year follow up of these patients showed deterioration, indicating a progressive dysfunction of cell mediated immunity (CMI) (deShazo *et al*, 1985). No association was found between the immune abnormalities and cytomegalovirus (CMV) seropositivity, hepatitis markers, abnormal liver function tests and thrombocytopenia (Lechner *et al,* 1983).

White and Lesesne (1983), disputed the fact that concentrates caused immune dysfunction *per se* and suggested that the low incidence of AIDS reported at the time and the high incidence of abnormal T cell function in these patients suggested that the AIDS incidence at that time, was 'just the tip of the ice berg'.

Abnormalities reported in British haemophiliacs were attributed to American products. As early as 1983, however, Froebel had reported that lymphocyte abnormalities were present in Scottish patients who had not received American products which were consistent with those seen in AIDS and other acute viral infection.

Following the isolation of HIV, the etiological agent of AIDS, and the introduction of a serological assay to HIV antibodies, studies were performed in which the separate affects of concentrate infusion alone and HIV infection could be analysed. Following the isolation of HIV from two siblings

with haemophilia B (Vilmer *et al*, 1984), there were many reports associating HIV seropositivity with the immune dysfunction and AIDS in these patients (Jason *et al*, 1985a, Gomperts *et al*, 1985, Gjerset *et al*, 1985, Goedert *et al*, 1985, Evatt *et al*, 1985, Lederman *et al*, 1985a, Hoxie *et al*, 1986a). Ramsey *et al* (1984), however, reported no association between the immune abnormalities and the detection of antibody to HIV. Suggestions that large doses of FVIII concentrate might augment the manifestations of HIV infection or accentuate the immune abnormalities independently of HIV infection were put forward. Tsoukas *et al* (1984), reported that 66% of asymptomatic haemophiliacs receiving FVIII had T cell abnormalities but only half of these were seropositive for HIV. Carr *et al* (1984), reported decreased T4 numbers, abnormal T4/T8 ratios and increased serum Ig in asymptomatic haemophiliacs. These patients were later shown to be HIV-ve (Ludlam *et al*, 1985).

From the reports at this time it was evident that haemophiliacs had immune disturbances independent of HIV infection. The advent of HIV serology detection methods enabled independent studies of HIV+ve and HIV-ve haemophiliacs, and these will now be discussed in more detail.

1.4.2 IMMUNE ABNORMALITIES IN ANTI-HIV SERO-POSITIVE (HIV+ve) HAEMOPHILIACS

The pathology and mechanisms of pathology of HIV infection are discussed in more detail in section 1.7.

Reports of immune abnormalities in HIV+ve haemophiliacs suggested that both suppression and activation processes were

occurring. These will now be be listed.

1.4.2.1 T cell abnormalities

The majority of reports associated with T cell abnormalities discuss a suppression of function. HIV+ve haemophiliacs have been shown to have reduced total T cell numbers, T4 cell numbers and diminished responses to lectins (Con A, PHA and pokeweed mitogen (PWM) (Horsburgh *et al*, 1986, Sullivan *et al*, 1986), and decreased mixed lymphocyte reaction (MLR) (Smolen *et al*, 1985). PHA stimulated T4 enriched cultures were shown to be inhibited, indicating that the diminished responses were not due to an imbalance in T4 and T8 ratios (Mahir *et al*, 1988). Reduced gamma interferon production in response to PHA has also been reported (Ruffault *et al*, 1988). There were, however, no significant differences in responses to CMV or herpes simplex virus (HSV) recall antigens (Mahir *et al*, 1988). Activation of T cells, as determined by increased expression of the activation marker HLA-DR (section 1.8.4.2) has also been observed in these patients (Sullivan *et al*, 1986).

Increases in suppressor T cell activity have been reported and increases in activated T8 cells ($HLA\text{-}DR^+$) have been observed (Seki *et al*, 1985, Dianzani *et al*, 1988). In contrast, normal Con A inducible suppressor cell activity has been seen (Matheson *et al*, 1987). The $CD11^+$ and $HLA\text{-}DR^+$ T4 subpopulation has been shown to be expanded in these patients, but $CD4^+/CD28^+$ cells (thought to be suppressor/inducer cells) were significantly decreased and have been shown to be functionally defective (Sjamsoedin-Visser *et al*, 1988). This indicates that the activation of the suppressor cell

populations may be due to a defect in the control mechanisms.

1.4.2.2 B cell abnormalities

B cell abnormalities have been reported in HIV+ve haemophiliacs, and similarly to T cells, there is evidence of both activation and suppression: elevated levels of serum IgG; increased spontaneous IgG, IgM and IgA production *in vitro*; and decreased mitogen (PWM), SAC (*Staphylococcus aureus* Cowan strain), and alloantigen (MLR) induced proliferation and production of IgG, IgM and IgA (Brieva *et al*, 1985, Biagotti *et al*, 1986, Gorski *et al*, 1986, Kekow *et al*, 1986, Blomback *et al*, 1987, Sjamsoedin-Visser *et al*, 1987, Ragni *et al*, 1987).

1.4.2.3 NK cell abnormalities

NK activity has been shown to be diminished in these patients (Lederman *et al*, 1983, 1985b, Menitove *et al*, 1983, Porzsolt *et al*, 1984, Ziegler-Heitbrock *et al*, 1985, Matheson *et al*, 1986a). Immature NK cells (leu 7^+/leu 4^+) have been reported (Landay *et al*, 1983). Lederman *et al* (1985b), noted diminished NK activity in asymptomatic haemophiliacs and this was not returned to normal by monocyte depletion, but incubation with IL-2 and alpha or gamma interferon did enhance activity slightly. NK response to interferon alpha, beta, and gamma has also been shown to be diminished (Matheson *et al*, 1986a).

1.4.2.4 Monocyte abnormalities

In vivo abnormalities in monocyte function in haemophiliacs regardless of HIV serology have been reported. These include

deficiency in monocyte-T cell interaction following *in vitro* exposure to bacterial antigens (Mannhalter *et al*, 1986); selective impairment of monocyte chemotaxis to lymphocyte-derived chemotactic factor (Stasi *et al*, 1987); and phenotypic (decreased HLA-DR, LFA-1 and CR3 antigens) and functional abnormalities (decreased phagocytic function, reduced chemotactic response and reduced adherence) in heavily treated patients (Roy *et al*, 1988).

1.4.2.5 In vivo abnormalities

Impaired delayed type hypersensitivity reactions (cell mediated immunity) as assessed by cutaneous hypersensitivity skin tests to recall antigens (Brettler *et al*, 1986, McIvor *et al*, 1987, Sullivan *et al*, 1986) and dinitrochlorobenzene (DNCB) (Madhok et al, 1986) have been reported in haemophiliacs infected with HIV.

1.4.3 IMMUNE ABNORMALITIES IN ANTI-HIV SERO-NEGATIVE (HIV-ve) HAEMOPHILIACS

Immune abnormalities in haemophiliacs in the absence of HIV infection had been shown to be similar to those seen in asymptomatic HIV infected individuals. The majority of studies reported a correlation of immune defects with concentrate infusion, and those patients who had received little or no treatment were demonstrated to have normal immune function. Again the dysfunction reported included both activation and suppression of immune processes. These abnormalities are detailed below. It is possible that immunomodulation consistent with immunodeficiency or activation of the immune system may have influenced the

susceptibility of the patients to HIV infection and this immune modulation, underlying that of HIV in infected patients, may be influencing their disease state.

1.4.3.1 T cell abnormalities

Evidence of activation of T cells has been observed. Matheson *et al* (1986b), reported T cell blastogenesis in cells cultured *ex vivo* from HIV-ve haemophiliacs and exposed to FVIII, the cells recognised the concentrates as 'foreign' and thus proliferated when exposed to it. The responding cell was a T cell or T cell dependent. Matheson *et al*, therefore hypothesised that the haemophiliacs were antigenically primed to the FVIII concentrates. Other evidence of T cell activation in these patients includes: elevated spontaneous production of B cell growth factor (BCGF) and B cell differentiation factor (BCDF) *ex vivo* (Matheson *et al*, 1987); and an increase in numbers of total T cell number (Horsburgh *et al*, 1986).

The majority of T cell abnormalities reported, however, are related to immunosuppression. T4/T8 ratios have been found to be abnormal in haemophiliacs (Carr *et al*, 1984), later proved to be HIV-ve (Ludlam *et al*, 1985). This abnormality correlated to FVIII infusion and was not related to transaminase levels. Sullivan *et al* (1986), demonstrated that patients with mild deficiencies who received small amounts of concentrate had normal immune function, but heavily treated patients had significantly impaired mitogen responses (PHA, PWM, Con A), decreased T helper cells and increased T suppressor cells. As with HIV+ve haemophiliacs the responses

to PHA were decreased in unfractionated and T4 enriched peripheral blood lymphocyte (PBL) cultures (Mahir *et al*, 1988). There were no significant differences in responses to CMV/HSV recall antigens compared to normal controls. PBMC from HIV-ve haemophiliacs have been shown to have reduced production of gamma interferon in response to induction by PHA whereas the capacity to produce alpha interferon was normal. Similar suppressor cell abnormalities have been reported in the HIV-ve haemophiliacs to haemophiliacs who are infected with the virus (Ziegler-Heitbrock *et al*, 1985, Dianzani *et al*, 1988).

1.4.3.2 B cell abnormalities

B cell abnormalities in HIV-ve haemophiliacs have also been observed: elevated levels of serum IgG, reduced proliferative responses to the mitogen SAC; and reduced plaque forming cell activity (PFC) to PWM (Gorski *et al*, 1986, Kekow *et al*, 1986). Biagotti *et al* (1986), however, found no abnormality in HIV-ve patients except for significant spontaneous IgG production when compared to controls.

1.4.3.3 NK cell abnormalities

As with the HIV+ve group, NK activity has been shown to be abnormal in HIV-ve haemophiliacs (Lederman *et al*, 1983, 1985b, Menitove *et al*, 1983, Porzsolt *et al*, 1984, Ziegler-Heitbrock *et al*, 1985, Matheson *et al*, 1986a), with immaturity (Landay *et al*, 1983); diminished activity (Porzsolt *et al*, 1984, Lederman *et al*, 1985b); and diminished response to interferons (Matheson *et al*, 1986a).

Jin *et al* (1989), have reported that immune dysfunction is present in haemophiliacs who have never received treatment, however, only 5 patients were studied. The patients exhibited reduced NK activity although there was no abnormality in the percentage of cells with NK markers. They suggested, from this small study, that haemophiliacs have an intrinsic immune abnormality, although, whether they have substantiated their hypothesis is open to debate.

1.4.3.4 Monocyte abnormalities

Monocyte malfunction has been reported in haemophiliacs, in whom HIV serology did not contribute to the defects. These have been detailed in the section for the HIV+ve haemophiliacs (section 1.4.2.4). Transient, decreased phagocytic function in the circulating monocytes has also been described following FVIII infusion in HIV-ve children (Pasi and Hill, 1990).

1.4.3.5 *In vivo* abnormalities

Haemophiliacs have been reported to exhibit skin test anergy in cutaneous hypersensitivity skin tests, which did not correlate with FVIII infusion or HIV serology (Brettler *et al*, 1986, Sullivan *et al*, 1986, McIvor *et al*, 1987, Madhok et al, 1986).

Despite long term treatment with FVIII concentrates and evidence of immune dysfunction, haemophiliacs do not appear to have increased susceptibility to infection. A notable exception, however, is the report of an increased incidence (38%) of infection with *Mycobacterium tuberculosis* (Beddall *et al*, 1983, 1985), in a small number of children exposed to an index case of TB.

1.5 POSSIBLE CAUSES AND THE IMPLICATIONS OF THE IMMUNE ABNORMALITIES IN HIV-ve HAEMOPHILIACS

As previously stated, HIV infection has contributed to severe immune disturbances in haemophiliacs, but these disturbances were not isolated to HIV infected individuals alone. The general consensus has been that immune abnormalities in haemophiliacs resulted from concentrate infusion (Tsoukas *et al*, 1984), and were induced in three ways:

a) antigen overload. It was suggested that the reversal in T cell ratios could be a normal defense mechanism to antigenic load (Jones *et al*, 1983). Chronic antigenic stimulation leading to interferon gamma production by the stimulated cells was postulated to down regulate the interferon gamma and beta receptors and thus lead to impaired NK activity (Matheson *et al*, 1986a). This group also attributed secretion of spontaneous levels of BCGF and BCDF by activated T cells to antigen-specific reactivity of these cells to a component in the concentrates (Matheson *et al*, 1986b, 1987).

b) alloantigen (Jason *et al*, 1985b, Sullivan *et al*, 1986). FVIII concentrate has been shown to contain appreciable amounts of B_2 microglobulin and HLA-class I antigens (Lee *et al*, 1984). It was hypothesised that they could induce suppressor cell expansion (Dianzani *et al*, 1988), interfere with the PHA pathway (Mahir *et al*, 1988) and lead to *in vivo* T cell activation (Matheson *et al*, 1987).

c) the presence of virus or viral particles.

Concentrates may be immunosuppressive due to the infusion of virus other than HIV present in the concentrate (HBV, CMV, NANB hepatitis agent) (Jason *et al*, 1985b). Sullivan *et al* (1986), demonstrated immune abnormalities correlated with FVIII infusion and they found strong correlations with seropositivity to Epstein Barr virus (EBV) and CMV. More marked increases in T suppressor cell numbers were seen in those patients who were seropositive for HIV and CMV or EBV. Seropositivity to all three viruses strongly correlated with decreased delayed cutaneous hypersensitivity skin test responses. They concluded that 'haemophiliacs receiving commercial factor concentrate experience several step wise incremental insults to the immune system: alloantigen in FVIII concentrates, LAV/HTLV-III, and herpes virus infections. Each of these contributes to the altered cell-mediated immunoregulation observed in these patients with haemophilia'.

B cell abnormalities were thought to be related to the multiple antigens in the concentrates, including viruses contaminating the products - CMV, EBV - which are known to produce polyclonal activation of B cells and hypergammaglobulinaemia (Brieva *et al*, 1985). EBV and CMV, however, do not appear to contaminate the concentrates as the incidence of antibodies to these viruses in the patients does not exceed that in the normal population (Webster *et al*, 1989). Ragni *et al* (1987), suggested 'that chronic stimulation or activation of the immune system from repeated exposure to blood products, presumably to foreign proteins or infectious agents, for example, hepatitis B, non A non B hepatitis ....transmitted through blood' may be occurring.

General concern is expressed that infusion of clotting factor concentrate may perturb the immune system and thus influence the progression of HIV disease in infected individuals. There has been equal concern, however, that infusion of these products and the resultant immune perturbation would in fact make these patients more susceptible to the initial infection by HIV itself.

A survey in Scotland reported infection of 15 haemophiliacs with HIV from one batch of FVIII concentrate, whereas, 18 patients who had received the contaminated batch did not seroconvert (Ludlam *et al,* 1985). The HIV+ve patients had immune alterations evident in their T cell ratios which did not alter following seroconversion. The probability of seroconversion was related to pre-existing low T cell ratios, annual FVIII consumption and the amount of the contaminated batch infused.

A widely held belief at this time was that development of AIDS required impaired immune function at the time the individual was exposed to the virus (Mannhalter *et al,* 1986), supporting the idea of Levy and Ziegler that AIDS virus should be considered as an opportunistic infection in an immunocompromised host (Levy and Ziegler, 1983). They predicted that the AIDS agent caused disease only in immunocompromised individuals 'which have one common characteristic - they may be immunocompromised by antigen overload, multiple infection, drugs, or in the case of infants, immunonaive'. In the case of haemophiliacs the concentrates have been assumed to be the immunosuppressive agent. Concern had been expressed that treatment with FVIII might contribute to an immunocompromised

state facilitating infection with opportunistic infections (Mannhalter *et al*, 1988). Progression to AIDS was thought not to be determined by HIV seropositivity alone, proliferating T cells and a degree of immunosuppression were required for this progression to occur and the impairment of the immune system may prevent the haemophiliac from adequately controlling the virus (Matheson *et al*, 1987).

1.6 *IN VITRO* ABNORMALITIES CAUSED BY FVIII CONCENTRATE

It has been demonstrated that cryoprecipitate, FVIII and FIX concentrates from many sources (American (Lederman *et al*, 1986), Scottish (Froebel *et al*, 1983, McDonald *et al*, 1985), Swiss, German (Wang *et al*, 1985)) inhibit *in vitro* lymphocyte response in healthy controls and haemophiliacs, at supratherapeutic and therapeutic concentrations.

Dose related inhibition of simulation of PBMC by PHA, Con A, purified protein derivative (PPD), Tetanus toxoid and anti-CD3 stimulated normal PBMC by FVIII products was observed. These products have also been shown to partially inhibit IL2 production by stimulated PBMC and Jurkat cells (Lederman *et al*, 1986). Inhibition of proliferation was a reversible effect operating on or at an early event in blastogenesis, but prolonged incubation with the concentrates resulted in irreversible inhibition of proliferative responses. This was not due to a cytotoxic effect (Wang *et al*, 1985, Lederman *et al*, 1986).

The mechanism of this inhibition were postulated to be interference with an early event in lymphocyte proliferation and this was thought to be mediated through IL2.

The inhibitory component has been shown to be partially dialysable. In addition a similar degree of inhibition was observed with buffers used to formulate the concentrates (Wang *et al*, 1985, McDonald *et al*, 1985).

Concentrate had only minimal effects upon NK activity and only at high concentrations (Lederman et al, 1986).

Monocyte function has also been shown to be inhibited *in vitro* by the presence of clotting factor concentrates. Eibl *et al*

(1987) investigated the effects of therapeutic levels of concentrates upon function of monocytes from normal donors. A short (1 hour) pre-incubation caused significant down regulation of Fc receptors and monocyte effector functions (release of O_2 radicles, bacterial killing). The modulating effect was due to immune complexes. Modulation of monocytes was later shown by this group to be independent of product purity, IgG content or heat treatment. Gel filtration of the concentrate demonstrated that the immuno-modifying component was characterised as being of high-molecular-weight (>1270KDa) and containing IgG, IgM, FVIII and blood group substances (Eibl *et al*, 1987, Mannhalter *et al*, 1988).

1.7 HIV INFECTION - AN OVERVIEW

The human immuno-deficiency virus (HIV) has been designated as the etiological agent for the acquired immuno-deficiency syndrome (AIDS). This disease is characterised by a progressive T4 cell depletion leading to immunosuppression which is associated with many opportunistic infections (Fauci, 1988).

The HIV epidemic has now reached world wide proportions. The World Health Organisation has estimated that several million persons are now infected. In the USA and other Western countries the majority of infected persons are members of target groups - homosexuals, IV drug abusers and blood product recipients. In Africa, however, HIV is predominately a heterosexual disease (Moss and Bacchetti, 1989).

As a member of the lentivirus family of non-transforming, cytopathic retroviruses, HIV, an RNA virus, has similar biological and morphological properties to visna virus of sheep, feline immuno-deficiency virus (FIV) and simian immuno-deficiency virus (SIV). These viruses cause a slowly progressive and inevitably fatal disease in their hosts and they can remain latent within the genome of the infected cell (Fauci, 1988).

HIV has been isolated from peripheral blood T lymphocytes, bone marrow, lymph nodes, brain tissue, cell free plasma, CNS and semen (Salahuddin *et al*, 1985). CD8+ve lymphocyte cell lines have been shown to harbour the virus *in vitro* (Tsubota *et al*, 1989).

HIV is uniquely trophic for the CD4 molecule, found in

predominance on the T4 lymphocyte. The virus uses this molecule as a receptor for entry into the cell by binding via the envelope protein gp120 (Klatzmann *et al*, 1984, McDougal *et al*, 1985). Binding of HIV to the CD4 molecule results in internalisation of the virus (Maddon *et al*, 1986). Infection of the cell, by the virus, is facilitated by activation (Gowda *et al*, 1989), and viral replication is stimulated by activation of the infected T cells *in vitro* (Zagury *et al*, 1986, Margolick *et al*, 1987). More recent analysis of this process has shown that T cell activation is not required for HIV-1 infection of the cell. Viral DNA, however, does not integrate into the host genome in resting cells and is maintained extra-chromosomally. T cell activation is required for integration and leads to a productive viral life cycle (Stevenson *et al*, 1990).

After binding, HIV enters the cell by a process of receptor mediated endocytosis (Maddon *et al*, 1986), or direct viral fusion with the cell (Maddon *et al*, 1988), and the viral coat is removed. The genomic RNA is transcribed into DNA by virally encoded reverse transcriptase, and then, if activation occurs, is integrated into the DNA of the host cell. Transcription of virus can then occur, followed by protein synthesis. Viral proteins and genomic RNA are assembled at the cell surface and mature virions are formed by budding (Ho *et al*, 1987). Therefore normal T cell activation may, at different times, result in the initial establishment of HIV infection *in vivo*, enhancement of HIV replication in HIV producing cells, activation of HIV expression in latently infected cells and spread of HIV infection to newly activated $CD4^+$ T cells (Rosenberg and Fauci, 1990). In this way agents that induce T

cell activation may control the rate of HIV-1 replication and spread during AIDS progression (Stevenson *et al*, 1990). PCR analysis of PBL has demonstrated that as many as 1/100 peripheral blood $CD4^+$ lymphocytes harbour HIV-1 DNA (Schnittman *et al* , 1989) and therefore resting T4 cells may be reservoirs of HIV-1 in infected individuals.

As previously mentioned HIV disease is characterised by a progressive depletion of T4 cell numbers. As HIV can directly infect and kill T4 cells in culture (Popovic *et al*, 1984), it is probable that virus induced T4 cell destruction *in vivo* is also occurring. Several mechanisms for a direct cytopathic effect on T4 cells by HIV have been described: a) the destruction of the cell membrane during budding; b) the presence of large amounts of cytoplasmic non-integrated viral DNA, RNA and proteins interfering with cell function (Shaw *et al*, 1984); c) the complexing of HIV gp120 with intracellular CD4 molecules (Hoxie *et al*, 1986b); d) syncytium formation between infected and non-infected T4 cells induced by viral infection; e) HIV triggered T4 cell terminal differentiation processes resulting in premature death (Zagury *et al*, 1986). Destruction of T precursor cells could be occurring (Fauci 1988). Thymus infection may play a triggering role in the pathogenesis of AIDS, this is particularly relevant to paediatric cases. $CD4^+/CD8^+$ cells have been shown to be particularly sensitive to infection by HIV-1 *in vitro*. These cells are the precursors of T4 and T8 lymphocytes in the thymus. Viral destruction of these cells during HIV disease may partially explain the lack of restoration of T4 cells killed by HIV. Also virus antigens (eg gp120) entering the

thymus may induce tolerance through negative selection (De Rossi *et al*, 1990).

T4 cell depletion resulting from indirect mechanisms is also prominent in the disease. HIV could be interfering in normal cell function by the binding of gp120 to CD4, blocking MHC class II recognition during antigen presentation and inhibiting T helper cell interactions (Rosenberg and Fauci, 1990).

Homology between HIV antigen and molecules that are important for normal immunological responses may result in the generation of cross-reactive antibodies that may suppress immune function or lead to autoimmune destruction. Gp120 must share antigenic determinants with MHC class II antigen as both bind to the CD4 molecule. Homology has been demonstrated between the membrane associated envelope protein gp41 and MHC class II (Golding *et al*, 1988), and IL2 and gp120 (Reiher *et al*, 1986).

Binding of soluble gp120 to the CD4 molecule of uninfected T4 cells may lead to their destruction by targeting gp120 antibodies and killing by antibody mediated cytotoxicity. Alternatively these cells could internalise and process the gp120 and then present gp120 peptides in association with their class II molecules, and thus become sensitive to lysis by specific cytotoxic T cells (Seligmann , 1990).

Other cells in the immune system may be contributing to the decline in T4 cells in HIV infection particularly the macrophage and monocyte lineage.

Macrophage and monocyte dysfunction occurs in HIV infection (outlined in section 1.7.4). This is of particular importance

as monocytes and macrophages are regulatory cells in the immune system and produce a vast array of cytokines including IL1, IL6, and tumour necrosis factor (TNF). Clouse *et al* (1989), have demonstrated that cytokines (thought largely to be alpha TNF) from lipopolysaccharide (LPS) stimulated macrophages could actively stimulate HIV expression in a T cell clone derived from T cells infected with HIV, thus indicating that macrophages may be involved in the pathogenesis of HIV infection. Monocytes are also involved in the B cell activation seen in HIV (Amadori *et al*, 1989) and increases in soluble IL2-R levels that are reported (Kloster *et al*, 1987).

Inappropriate secretion of cytokines from monocytes may underlie some of the clinical manifestations of AIDS such as persistent fever (Fauci , 1988).

Plata *et al* (1987), hypothesised that inter-action of HIV specific cytotoxic T cells and HIV-infected macrophages may induce local inflammation of the lung and thus open the way for bacterial or fungal infections.

It is well known that T4 cells hold a pivotal position in the immune system 'helping' at all levels of immune competence, *inter alia* T cell cytotoxicity, T cell suppressor functions, macrophage killing, B cell differentiation to plasma cells, NK cell function. The progressive decline in T4 cell numbers is also associated with a decline in T cell help and the emergence of severe immune abnormalities - listed in table 1.1

TABLE 1.1: MAJOR ABNORMALITIES OF ACQUIRED IMMUNO-DEFICIENCY SYNDROME.

Characteristic abnormalities

- Depletion of $CD4^+$ T cells
- Decreased proliferative responses to soluble antigens
- Impaired delayed-type hypersensitivity reactions
- Decreased gamma-interferon production to antigens
- Polyclonal B-cell activation with increased spontaneous proliferation and immunoglobulin production (predominantly IgG1, IgG3, IgA and IgD)
- Decreased pokeweed-mitogen-induced B-cell immunoglobulin production
- Decreased humoral response to immunization (mainly primary)

Consistently detected abnormalities

- Lymphopenia
- Decreased proliferative responses to T-cell mitogens and alloantigens
- Decreased proliferative responses in Autologous MLR and to T3 antigens
- Decreased proliferative responses to specific-cell mitogens
- Decreased IL2 production
- Decreased cytotoxicity to virus infected cells
- Increased immune complex formation
- Decreased NK cell activity despite normal binding to targets
- Decreased monocyte chemotaxis
- Decreased MHC class II expression on monocytes and macrophages
- Increased acid-labile alpha interferon levels

(From: Seligmann *et al*, 1987)

1.7.1 T CELL ABNORMALITIES

The major T cell defect is the progressive decline in numbers during HIV disease. Functional abnormalities (due in part to this loss) in these cells is apparent. *Ex vivo* cultures of lymphocytes from HIV+ve patients have been shown to have defective proliferative responses to lectins (PHA, Con A, PWM) (Prince *et al*, 1984, Vaith *et al*, 1985, Gupta, 1986) allogeneic and autologous MLR (Vaith *et al*, 1985, Ebert *et al*, 1985) and to antigens (tetanus toxoid, CMV) (Antonen and Krohn, 1986, Prince and John, 1987, Krowka *et al*, 1988). These abnormalities have been associated with diminished IL-2 production and IL2-R expression (Prince *et al*, 1984, Prince and Czaplicki, 1988, Gupta, 1986). There are fewer cycling cells in G and S stages of the cell cycle in AIDS patients than in normal controls (Hornicek *et al*, 1987). Signal transduction mechanisms are also impaired (Pinching and Nye, 1990).

In contrast, to these findings, there is also evidence of T cell activation *in vivo*, *inter alia*, increased HLA-DR expression (Milvan *et al*, 1982, Salazar-Gonzalez *et al*, 1985), increased serum gamma interferon (Fuchs *et al*, 1989) and increased suppressor cell activity (Ziegler-Heitbrock *et al*, 1988).

1.7.2 B CELL ABNORMALITIES

B cell activation has been reported in patients with HIV infection. Hypergammaglobulinaemia, circulating immune complexes and autoantibodies, spontaneous IgG production and raised numbers of activated B cells have been seen (Lane *et al*, 1983, Martinez-Masa *et al*, 1987, Mizuma *et al*, 1988, Amadori and Chieco-Bianchi, 1990). These abnormalities are

related to 'T helper' dysfunction but it is evident that direct HIV induced B cell abnormalities are present. Pahwa *et al* (1985), demonstrated polyclonal B cell activation by crude, disrupted HIV preparations, but they were inhibitory for the B cell differentiation responses induced by PWM, SAC and EBV. Twenty to fifty percent of *in vitro* Ig synthesis has been shown to be directed against HIV, indicating that much of the hypergammaglobulinaemia is a specific antibody response to HIV (Amadori *et al*, 1988, 1989, Amadori and Chieco-Bianchi, 1990, Pahwa *et al*, 1989). HIV infection is also associated with an increased incidence in B cell neoplasia of which a proportion may be related to dysregulation of EBV latency (Spickett and Dalgleish, 1988).

1.7.3 NK CELL ABNORMALITIES

NK cell functional abnormalities have been described in HIV+ve patients. These can be normalised by the addition of lymphokines (interferons) to the cultures. The number of NK cells, however, is not diminished (Rook *et al*, 1983, Fauci , 1988). NK failure is probably not related to an intrinsic cell defect, but probably results from abnormalities in T cell function.

1.7.4 MONOCYTE ABNORMALITIES

The monocyte/macrophage lineage has an important role in HIV infection. They act as 'factories' and reservoirs for the virus. Usually very few peripheral blood T cells are infected with HIV (Schnittman *et al*, 1989), but infection by HIV of the macrophages is widespread. Virus has been detected in brain tissue (Koenig *et al*, 1986), and the follicular dendritic cells of lymph nodes (Cameron *et al*, 1987). In the

central nervous system, the skin and the lymph nodes, the number of productively infected macrophages is greater than that of HIV infected T cells in the blood.

Macrophages have been shown to process whole HIV via the golgi apparatus and transport the complete virus via vacuoles to the plasma membrane, in contrast to T cells, where HIV assembles and buds only from the plasma membrane (Orenstein *et al*, 1988). This is an important point as the monocyte can act as a reservoir for the virus without cell death occurring, in contrast to infected T4 cells. Virus is probably released following perturbation of the monocytes, for example, by factors released during inflammation (Meltzer *et al*, 1990). As previously mentioned, HIV interacts with CD4 in the process of infection of T cells, but, although this probably occurs in monocytes also. HIV may enter these cell by other mechanisms such as by phagocytosis, FcR-mediated endocytosis (Takeda *et al*, 1988), or interaction with receptors for mannosylated proteins (Ezekowitz *et al*, 1989). Antibody mediated-enhanced infection of monocytes with HIV has been demonstrated in the presence of certain sera from HIV infected patients (Homsy *et al* 1989).

Monocytes are probably involved in the antigenic drift of the virus seen during progression of the disease and the advancement of AIDS. HIV isolates have been shown to have distinct, strong cell trophism for T cells or monocytes and there are at least two types of virus: monocyte and T cell trophic, and T cell trophic only (Gendelman *et al*, 1988, 1990). It has also been shown that HIV isolates cultured from

T cells and macrophages/monocytes are different. The predominant viral proteins from T cell cultures are envelope proteins; in contrast, the predominant viral proteins from monocytes/macrophages are capsid. In fact progeny virus released from HIV-infected monocytes show little or no envelope projections (spikes) - the morphological representation of gp120 seen in the viral progeny from T cells.

Macrophages from AIDS patients have been shown to be abnormally activated. In this condition they are involved in the pathogenesis of AIDS.

There have been reports of increased lytic activity in cell mediated immunity (CMI) reactions and high rates of spontaneous production of monokines such as IL1 and alpha TNF (Wright *et al*, 1988, Nakajima *et al*, 1989).

Elevated levels of HLA-DR expression on monocytes from HIV+ve patients have been reported (Koethe *et al*, 1989, Allen *et al*, 1990). A strong association between the presence of a large monocyte population with increased HLA-DR expression and a decreased absolute number of $CD4^+$ lymphocytes in the peripheral blood of HIV+ve patients has been demonstrated. HLA-DR expression, however, has been shown to be reduced in monocyte populations in patients with AIDS but not with progressive generalised lymphadenopathy (PGL) (Heagy *et al*, 1984). Allen *et al* (1990), have also demonstrated $IL2\text{-}R^+$ monocytes in patients with AIDS, further evidence of monocyte activation.

Neopterin is produced by gamma interferon triggered monocytes

and has been shown to be elevated in patients with HIV infection, with gradual increases in this marker during progression of HIV disease. This is thought to be due to increased levels of gamma interferon *in vivo* produced by activated T cells (Fuchs *et al*, 1988).

Defects in monocyte function probably contribute to the immunosuppressive state in HIV infected individuals. For example defects in chemotaxis and in killing of certain micro-organisms have been reported (Fauci , 1988). Accessory cell function of monocytes to promote responses of T cells to soluble antigenic and mitogenic stimuli has also been shown to be defective in patients with HIV (Rich *et al*, 1988).

1.8 LYMPHOCYTE ACTIVATION PROCESSES - A BRIEF SUMMARY

1.8.1 T LYMPHOCYTE ACTIVATION

T cells possess a closely regulated activation process. In summary, antigen is presented, by an antigen presenting cell, to the T cell via the CD3/T cell receptor (T3/Ti) complex in conjunction with HLA. This process is governed by phosphorylation/dephosphorylation processes involving several key antigens on the cell membrane *inter alia* CD4, CD5, CD8, CD28 and CD45 (Alexander and Cantrell, 1989, Clark, 1989). Binding of the antigen to T cell receptor/CD3 complex (T3/Ti) is followed by a series of intracellular second messenger sequences which culminate in the expression of mRNA transcripts for IL2, IL2-R, gamma interferon and HLA-DR. IL2 secretion and increases in IL2-R and HLA-DR expression follow this. IL2 induced proliferation and expression of the transferrin receptor then occurs following which the cells return to a quiescent state.

1.8.1.1 Signal transduction processes through the T cell receptor

In T cells, two signals are required for cell activation to progress through to proliferation: i) perturbation of the T3/Ti receptor by ligand attachment and ii) accessory cell function including IL1 production (figure 1.2).

Antigen binding to the T3/Ti leads to hydrolysis of a membrane phospholipid, phosphatidylinositol biphosphate (PIP_2) with subsequent generation of inositol 1,4,5-triphosphate (IP_3) and diacyglycerol (DAG) (Berridge and Irvine, 1984, Nishizuka , 1984). A GTP binding G-protein has been detected in other cell second messenger systems providing a linking mechanism

Signalling pathways in lymphocytes

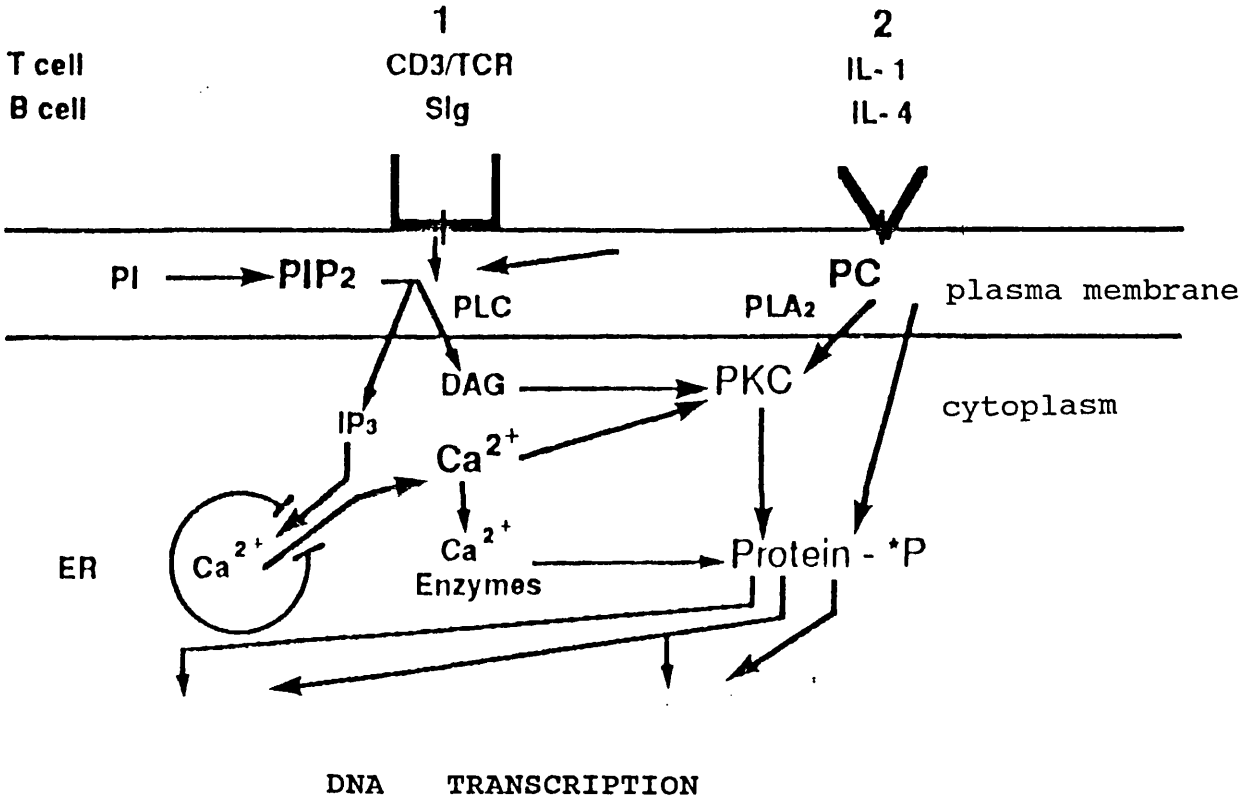


Figure 1.2: Brief summary of signalling pathways in lymphocytes. Key stimulation pathway, 1) cross linking antigen receptors in T cells, and binding of surface Ig in B cells, to ensure continuation of activation signal, a second signal is required, IL1 from monocytes to T cells, and IL4 for B cells. (P* phosphorylated protein)
(Clark , 1989).

between receptor and PIP_2 (Nishizuka , 1986). This is thought to occur in T cells also. IP_3, a water soluble compound, binds to specific receptors on the endoplasmic reticulum (ER) stimulating the efflux of calcium (Ca^{2+}) from intracellular stores (Joseph *et al*, 1984).

Perturbation of T3/Ti also activates protein kinase C (PKC) (Berridge and Irvine, 1984, Nishizuka , 1984). DAG, the product of hydrolysis of PIP_2 remains in the plasma membrane and activates PKC by markedly increasing the affinity of this enzyme for Ca^{2+} (Nishizuka , 1984). PKC is translocated from the cytosol to the plasma membrane (Farrer and Ruscetti, 1986)

1.8.1.2 Activation processes in T cells following signal transduction

Continued T cell receptor occupancy (72hour) is necessary to sustain the biochemical events associated with T3/Ti mediated signalling (Weiss *et al,* 1984).

Increased intracellular calcium increases and PKC activity are both required for mRNA transcripts of IL2, IL2-R and gamma-interferon. These appear within 2 hours of activation in T cells (Wiskocil *et al*, 1985) and peak at 9-15 hours. IL2 gene transcription is also initiated early but does not reach maximum until 24 hours following stimulation (Greene *et al*, 1986a). Binding of CD28 monoclonal antibodies have been shown to specifically enhance and stabilise mRNA for IL2 (Clark, 1989). RNA for HLA-DR (beta chain) is detectable within 30 minutes of activation and maximises at day 3 following stimulation with PHA, then falls until it is barely detectable at day 5 (Gansbacher and Zier, 1988).

Release of IL2, *in vitro*, is first observed 2-4 hours

following the initial activation signal and this reaches a maximum within 24-48 hours and progressively declines until it is undetectable at 4-5 days of culture (Weiss *et al*, 1984, Wiskocil *et al*, 1985, Gillis *et al*, 1978). It is the interaction of IL2 with the IL2-R that activates the intracellular processes leading to proliferation (Cantrell and Smith, 1984), but at least 5-6 hours of exposure of IL2 are required before any cell within a population makes the G_1-S phase transition.

IL2 increases the expression of the IL2-R (high affinity receptor or 75KDa-IL2-R (section 1.8.4.1) (Smith and Cantrell, 1985, Depper *et al*, 1985, Robb *et al*, 1987) but also leads to its internalisation (Robb and Greene, 1987). It also increases the expression of CD71 (transferrin receptor) (Neckers and Cossman, 1983).

The specificity, magnitude and duration of the T cell immune response is controlled, in part by a transient display of IL2-R (Greene *et al*, 1986a). Production of IL-2 and IL2-R expression can occur concurrently in the same cell. However, although IL2-R can be expressed by all T cells, the ability to secrete IL2 is restricted to T helper cell (Linch *et al*, 1987). The IL2-R/(75KDa) has been demonstrated to be selectively expressed on resting T8 cells compared to resting T4 cells, which predominantly express IL2-R(55KDa) (Ohashi *et al*, 1989). Further evidence of variable activation states related to subsets of cells is evident. Addition of exogenous gamma interferon (recombinant) to MLR cultures has been demonstrated to augment HLA-DR expression and proliferation in T8 cells in preference to T4 cells and augmentation of HLA-DR expression was further increased by the addition of both recombinant gamma interferon and recombinant IL2 (Siegel

1988).

IL2 binds to the high affinity receptor (detailed in section 1.8.4.1) and is internalised (Smith 1988). A rapid decrease in its expression is detectable by 24 hours associated with a relative increase in the expression of the beta chain of the IL2-R. The internalisation of the high affinity receptor is signalled by a structure on the alpha chain.

IL2 regulates increases in expression of IL2R and promotes proliferation of the cells. This is not calcium dependent and may be a dual signal event (Mills *et al*, 1985). Therefore their are two steps in the activation of T cells: the first step leads to the synthesis and secretion of IL2 and is dependent upon calcium; the second step is the expression of IL2-R and IL2 induced proliferation (Mills *et al*, 1985). The dividing cells, driven by a requirement for iron, upregulate their DNA and mRNA of the transferrin receptor and this is expressed as early as 20-24 hours, peaking at 48 hours following stimulation (Greene *et al* , 1986a) and the receptor is expressed on the cell surface during the later stages of the cell cycle, accompanying cell division.

1.8.1.3 T cell dichotomy and memory cells

Monoclonal antibodies to the leucocyte common antigen (CD45) have revealed a phenotypic and functional heterogeneity among T cells. Antibodies detecting the 200-220KDa polypeptides of the CD45 molecule have demonstrated that its presence was diminished on activation. Cells positive for this antigen were capable of suppressor/inducer activity, and were not able to 'help' B cell Ig production (Ledbetter *et al*, 1985, Tedder *et al*, 1985, Rudd *et al*, 1987). Smith *et al* (1986), described

an antibody - UCHL1 -which bound to a 180-185KDa peptide (later defined as a polypeptide belonging to the CD45 molecule family) which was shown to bind preferentially to a subset of cells that proliferated maximally to soluble antigen and provided maximum help for PWM-induced Ig synthesis. The UCHL1 negative cells did not react with soluble antigen and did not help Ig production. This functional dichotomy within the T lymphocyte population was determined to be due to the presence of 'virgin' cells (Tedder *et al*, 1985). Akbar *et al* (1988), demonstrated that the loss of the 220KDa determinant of CD45 is associated with a reciprocal, irreversible switch to the expression of the 180 KDa polypeptide of CD45, detected by UCHL1. The $UCHL1^{+}$ cells have been shown to have a high responsiveness to recall antigen (Merkenschlager *et al*, 1988) and display increased levels of cell adhesion molecules LFA1, CD2, and LFA3 (Sanders et *al*, 1988). Studies of murine T cell clones have documented the existence of two helper cell subsets based on profiles of lymphokine activity (Mos mann *et al*, 1986). These cells have characteristic 'helper' functions which for the purpose of this summary can be briefly and simply described as helpers of either T or B cell functions - T_H1-inflammatory clones, produce IL2, gamma interferon and lymphotoxin and do not help B cell Ig production; whereas the T_H2-helper clones, produce IL4 and help Ig production. The behaviour of these clones is thought to be related to the cells' memory status.

The CD45 complex has been demonstrated to be a membrane bound tyrosine phosphatase, which can regulate signal transduction and activation in both T and B cells (Ledbetter *et al*, 1988). Switching of the polypeptides on T cells may therefore

initiate changes in this regulation.

The 200-220KDa polypeptides of the CD45 molecule, are recognised by several antibodies including 2H4 and SN130 (the latter used in this study). These antibodies have been designated CD45RA (4th International Leucocyte Typing Workshop, Vienna, 1989) and UCHL1, which recognises the 180KDa polypeptide, has been designated as CD45RO.

Following the initial activation processes of signal transduction and mRNA and protein synthesis, HLA-DR, IL2-R and the transferrin receptor are among the markers which become expressed as the cells enter and progress through the cell cycle. The switch from CD45RA to CD45RO, however, is permanent. Restimulation by the presence of antigen is thought to be important in the continuation of memory (Beverley, 1990) and the presence of CD45RO is therefore an indicator of chronic activation.

1.8.2 B CELL ACTIVATION

The development of antibody specific B cell clones results from complex interactions between antigen, accessory cells, T cells and NK cells which regulate B cell growth and differentiation.

Activation of B cells is followed by proliferation (clonal expansion) and immunoglobulin gene mutation, coupled with further selection (affinity maturation, class switching). Some cells give rise to antibody secreting cells and some to memory cells (Zola , 1987).

The binding of antigen to membrane Ig (mIg) is the first signal in B cell activation and initiates inositol phospholipid metabolism in a similar way to that outlined for

the T cell (Clark , 1989) (figure 1.2).

As the cells move from G_0 through to G_1, cell size and RNA content increases and *de novo* protein synthesis follows, which is accompanied by altered antigen expression on the cell surface including increases in CD23, 4F2 and loss of IgD, (Gordon and Guy , 1987). Following the first signal, progression signals are required for the cell to move into S phase and for cell division to occur. At 24 hours after stimulation the cells become responsive to progression factors. The progression signals take the form of lymphokines (IL4, IL5, IL6, gamma interferon), the balance of which is extremely important for B cell growth and differentiation to plasma cells. The lymphokine balance also regulates isotype switching, for example, IL4 is responsible for IgG_1 and IgE responses and IL5 for IgA production (Abbas , 1988). Concentration of antigen is important in determining 'cognitive-interactions' of the T and B cells. At low concentrations B cell may present antigen to the T cell and receive the progressive signal directly, but at high antigen concentrations T cell-derived lymphokine induced B cell activation may be non-specific.

Progression of B cells through G_1 results in the acquisition the of transferrin receptor (36 hours following stimulation) as the cells prepare for cell division and differentiation (Weber and Finkelman, 1987, Gordon and Guy, 1987).

Monoclonal antibody studies have demonstrated that binding of antibody to several antigens on the cell surface can be used to trigger B cell activation (CD20, CD21) or give cell cycle progression signals (CD22, CD23) and may represent alternative or co-stimulatory pathways to antigen stimulation (Clark

and Ledbetter,1986). Triggering of different adhesion molecules, such as CD20 antigen, induce increases in CD18 expression. IL4 induces increases in CD23 and CD20 and IgM induces increases in LFA-3, suggesting that early specific signals may affect B cell migration to specific environments in the lymphoid tissue and thus their ultimate class switching (Clark , 1989).

1.8.3 LECTIN ACTIVATION OF LYMPHOCYTES

Certain lectins have been shown to facilitate preferential production of certain lymphokines in PBMC cultures. PHA has been demonstrated to induce IL2 predominately, whilst PWM stimulates IL4 (reviewed by Bottomly, 1988). The stimulation of these specific lectins reflects the functional dichotomy seen in murine T cell clones. Therefore it is possible that the lectins PHA and PWM can be used to stimulate specific T cell subsets.

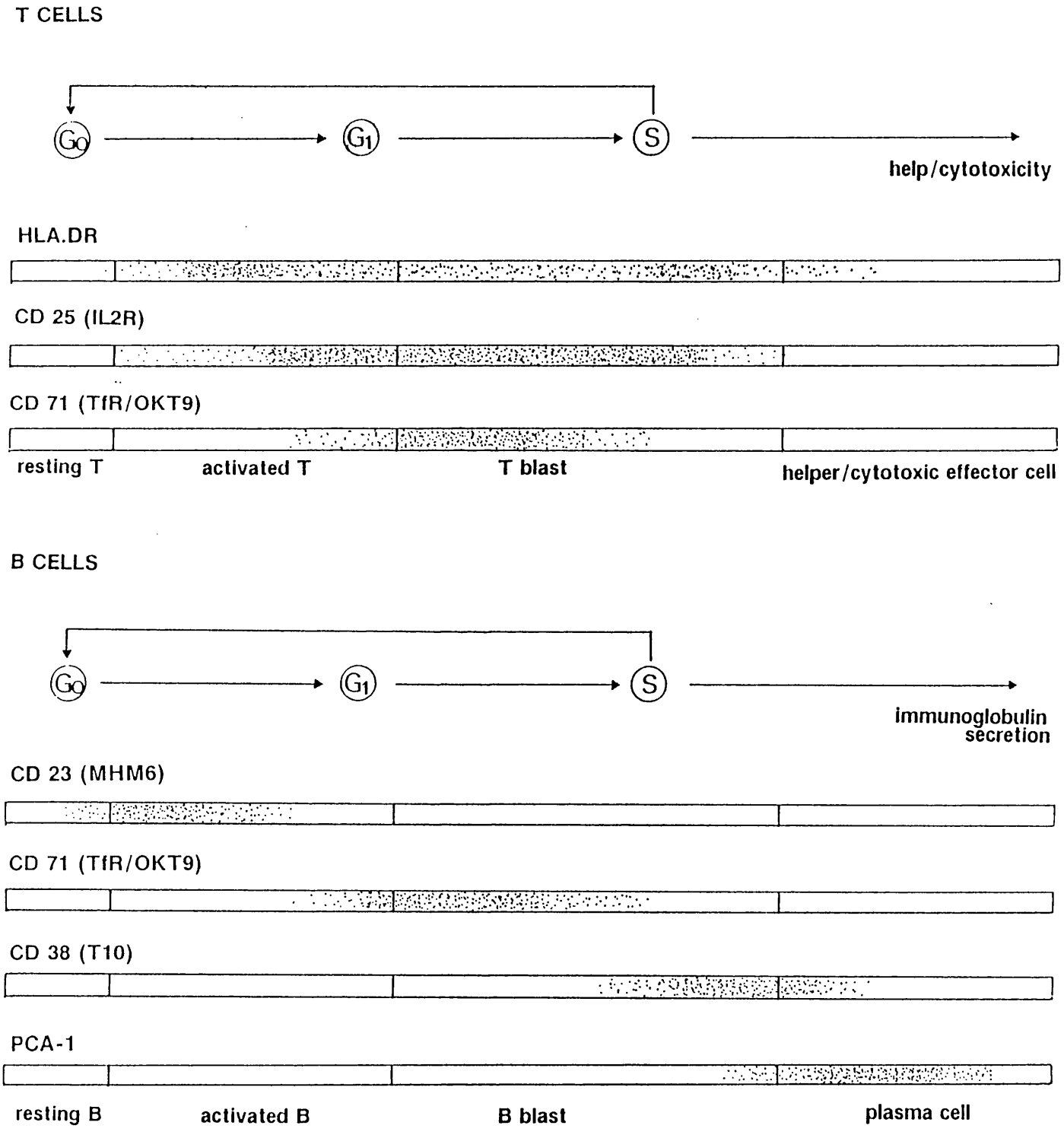
1.8.4 ACTIVATION MARKERS USED IN THIS STUDY

The appearance of these markers during the cell cycle are diagrammatically represented in figure 1.3.

1.8.4.1 The interleukin 2 receptor (IL2-R) (CD25)

The IL2-R consists of two chains (alpha and beta), both of which are capable of binding IL2 when they are expressed individually, but in each instance with a lower affinity than when they are expressed together. The beta chain (55KDa) is recognised by the anti-tac monoclonal antibody (Uchiyama *et al*, 1980), and the alpha chain (75KDa) is recognised by a recently cloned monoclonal antibody (TU27) following identification of the 75KDa chain (Takeshita *et al*, 1989). The 75KDa subunit of

Figure 1.3 **Expression of activation antigens on T and B cells**



the IL2-R is phosphorylated following binding of IL2 and is associated with tyrosine kinase activity and signal transduction (Asao *et al*, 1990). Expression of the IL2R begins within 6-8 hours of stimulation and acquisition continues slowly and asynchronously within the cell population until this reaches maximum at 72 hours when expression of the receptor declines to baseline over 10-14 days (Smith, 1988).

1.8.4.2 HLA-DR

Antigens of the HLA-DR group are MHC class II determinants. They are composed of alpha and beta chains (34KDa and 28KDa respectively), and are expressed on a large number of cell types including B cells, macrophages, and antigen-presenting cells. They are considered to be activation markers of T cells as they are not present at high density on resting T cells (Gansbacher and Zier, 1988). Hercend *et al*, (1981), demonstrated that HLA-DR was expressed on T cells 4-5 days after stimulation with various mitogens (PHA, alloantigens, soluble antigens). The extent of HLA-DR expression and the relative co-expression with CD38 and CD71 being dependent on the stimulant. More recent studies, however, have demonstrated that HLA-DR antigens can be detected on the surface of T cells within 2 hours of stimulation with mitogen. The level remains stable until 24 hours and then begins to rise, stabilising at day 4 when levels begin to decrease, accompanied by a decrease in proliferation (Gansbacher and Zier, 1988, Siegel , 1988). Therefore there appears to be two stages in HLA-DR expression.

1.8.4.3 The transferrin receptor (CD71)

The transferrin receptor (TfR) is a homodimer consisting of two 95KDa glycoprotein chains linked by disulphide bridges. It forms a transmembrane molecule of 190KDa, recognised (in this study) by the monoclonal antibody OKT9 and is found on proliferating and neoplastic cells (Judd *et al*, 1980, Goding and Burns, 1981, Sutherland *et al*, 1981, Trowbridge and Omary, 1981). Transferrin is required for iron uptake and is necessary for proliferation (Brock *et al*, 1986) and consequently is found on all dividing cells and almost all permanent cell lines (Knapp *et al*, 1989). Transferrin receptor expression is closely regulated by intracellular iron levels rather than proliferation (Pelosi *et al*, 1986). It can be upregulated by several components, including free iron, intracellular ferritin and insulin (Knapp *et al*, 1989), but its expression is mediated by IL2 (Neckers and Cossman, 1983).

Transferrin bound to iron binds to its receptor and this is internalised into a vesicle, the iron dissociates from the transferrin and the transferrin-receptor then returns to the cell surface, which may involve the golgi apparatus, and the transferrin then dissociates from the receptor. Soluble transferrin receptor is also detectable (Knapp *et al*, 1989).

1.8.4.4 CD23

CD23 is a 45-50KDa glycoprotein which is expressed at low levels on the majority of mature B cells. Its expression increases upon activation, but is reduced on B blasts and absent on B cells that have under gone isotypic changes. Several functions have been ascribed to this molecule, including the low affinity receptor for IgE, and the receptor

for 12KDa BCGF. The entire function of this molecule and the function of its soluble form (which appears to be immunoregulatory) has yet to be established (Knapp *et al*, 1989).

1.8.4.5 CD38 (RFT-10)

OKT-10 was initially defined as a T cell marker (Reinherz *et al*, 1980a) which bound to a type II integral membrane single chain glycoprotein of 45KDa (Terhorst *et al*, 1981, Knapp *et al*, 1989). OKT10 and RFT10 (used in this study) were assigned to the CD38 cluster at the Third International Antibody Workshop, Oxford, 1987. These antibodies also react with early B cells, thymocytes, activated T cells, myeloma cells and some acute leukaemic cells and, of particular relevance to this study, terminally differentiated B cells (B blasts) and plasma cells (Knapp *et al*, 1989).

1.8.4.6. 4F2

The 4F2 antibody was first described as reacting with cell lines, monocytes and activated lymphocytes (Haynes *et al*, 1981). The marker recognises the heavy subunit of a heterodimeric membrane complex (85/41KDa). Activated T cells when returning to the quiescent phase retain 4F2, whilst losing other activation markers such as transferrin receptor (Suomalainen, 1986). It has been shown to be expressed in early G_1 phase in activated B cells (Kehrl *et al, 1984)*. The marker is detectable following 6 hours of culture and is strongly expressed on all T cells in the culture following stimulation at 18 hours (Suomalainen, 1986).

1.8.4.7 Pea-nut lectin (PNL)

The expression of the PNL receptor increases as B cells differentiate from mature B cells to germinal centre blasts but is low again in differentiated plasma cells and is thus characterises proliferating differentiating B cells (Butcher *et al*, 1982, Rose *et al*, 1980).

1.8.4.8 Plasma cell associated antigen 1 (PCA-1)

PCA-1 antigen, although not B lineage specific, is expressed at the terminal stages of normal and malignant B cell differentiation. In culture, its expression is concomitant with the acquisition of plasma cell characteristics (Anderson *et al*, 1983).

1.8.4.9 Soluble IL2-R (sIL2-R)

The IL2-R has been demonstrated to be released in a soluble form both *in vivo* and *in vitro* by virtually every cell on which it is expressed (Rubin *et al*, 1985, 1986, Nelson *et al*, 1986). Soluble IL2-R is approximately 10 Kd smaller than the tac protein (55Kd polypeptide chain) (Rubin *et al*, 1985) and this corresponds to the absence of the transmembrane and intracytoplasmic domains of the molecule, indicating that proteolytic cleavage of the receptor may have occurred (Robb and Kutny, 1987). sIL2-R proteins have also been detected in the cytoplasm, indicating this molecule may be specifically produced for secretion (Symons, 1990).

The released IL2-R can bind IL2 efficiently (but at lower affinity than the high affinity IL2-R) and this suggests a potentially significant role in the regulation of IL2 dependent lymphocyte function (Rubin *et al*, 1986).

Elevated levels of sIL2-R have been reported in a variety of haematological (Pizzolo *et al*, 1987a), and immunological disorders (Symons *et al*, 1988, Pui *et al*, 1988), and during infection with virus (Greene *et al*, 1986b) and HIV (Sethi and Naher, 1986, Kloster *et al*, 1987, Pizzol o *et al*, 1987b, Lang *et al*, 1988, Prince *et al*, 1988b, Honda *et al*, 1989).

Using two colour flow cytometric methods (outlined in sections 2.3, 2.4 and 2.5) haemophilic patients' lymphocytes were analysed for elevated expression of acute activation markers: HLA-DR, CD25 (interleukin-2 receptor) and CD71 (transferrin receptor) upon T cells (using anti-CD3 for the pan-T reagent) and CD71 and CD38 upon B cells (using anti-CD20 for the pan-B reagent) (table 2.3).

HLA-DR expression on T4 and T8 cells was studied separately. Chronic activation of the lymphocytes was determined by analysis of the expression of CD45RA/RO on T4 and T8 cells and PCA-1 (plasma cell marker) upon B cells (table 2.3) and for elevated serum IgG levels (section 2.6.2). Soluble IL2-R levels in the serum were also determined (sections 2.3 and 2.6).

1.9 OBJECTIVES OF THE PROJECT

The advances in the treatment of haemophiliacs in the second half of this century have been astounding. With regular treatment by clotting factor concentrates haemophiliacs can now lead relatively normal lives and, with the exception of infection with concentrate transmitted virus, the out-look for these patients is good. The introduction and eventual transfer to use of recombinant products is a long awaited refinement of the treatment of haemophiliacs. Until this occurs, however, the use of plasma derived intermediate and high purity products will continue. Financial constraints will probably prolong their use. The viral safety and possible immunomodifying effects of these products, however, still remain major concerns.

Approximately 60% of severe haemophilia patients in the UK have been infected with HIV. The possibility that treatment of their bleeding disorders with concentrate may have an influence on the progression of HIV disease was an important factor in the impetus of this project.

In this study we set out to investigate lymphocyte abnormalities in heavily treated haemophilia patients.

The study had two objectives, which may at first appear contradictory:

1. Do clotting factor concentrates activate the immune system?

2. Is inhibition of T cell function occurring in the patients, and what are the mechanisms of the inhibition seen *in vitro*?

As we have seen, there is an equal body of evidence for clotting factor concentrate induced T cell inhibition and T cell activation.

Inhibition of the function of T cells in these patients would render the patients immunosuppressed: a problem for all patients but particularly a further stress on HIV infected individuals.

Activation of the immune system, on the other hand, could possibly lead to an acceleration of the progression of HIV disease, as it is known that activation of T4 cells infected with HIV results in an increase in viral pathogenicity and replication. Reduced lymphocyte function *ex vivo* could be reflecting partial activation of the cells occurring *in vivo*, as has been demonstrated in GvH (Cleveland *et al*, 1988) and SLE (Preble *et al*, 1983).

We have studied the expression of activation markers that appear at early, mid or late stages of the T and B cell cycles, in both HIV+ve and HIV-ve patients receiving clotting factor concentrates. In this way if activation of the immune system by the concentrates was occurring this would be seen in the HIV-ve patients. The pattern of expression of these markers would indicate if the cells were being partially pushed through the cell cycle, and thus being rendered refractory to further stimulus.

Conversely, HIV infection leads to activation of the immune system, if clotting factor concentrates were inhibiting T

cells *in vivo*, this would be reflected in the extent of activation seen in the HIV+ve patients. This part of the study is detailed in sections 4 and 5.

The second objective of the study was to elucidate the mechanisms involved in the T cell inhibition reported *in vitro*. By examining the inhibition of specific T cell activation processes, the point in the cell cycle at which inhibition was directed could be established and from this the mechanisms of the inhibition studied. This is outlined in sections 6 and 7.

2. METHODS

2.1 PRODUCTION OF MONOCLONAL ANTIBODIES

2.1.1 CULTURE OF CELL LINES

A variety of cell lines were grown in culture. The hybridoma cell lines RFT1, OKT9, RFT10, kindly provided by the Department of Immunology, RFHSM and 4F2 (from the American Type Culture Collection (ATCC), Bethesda, MD), were cultured for monoclonal antibody (MAb) production. In addition several human lymphoblastoid cell lines (table 2.1) were cultured for the standardisation of monoclonal antibody binding. Jurkat cells (obtained from NIBSC, South Mimms, Herts) were used for calcium flux studies.

All cell lines were cultured at 37°C in a 5% CO_2/95% air atmosphere in RPMI 1640 medium supplemented with 10% heat inactivated foetal calf serum (v/v) (FCS), 2mM glutamine (all from Gibco Laboratories, Uxbridge) and 50ug/ml gentamicin (Flow Laboratories, Herts, UK) (complete medium).

2.1.2 CLONING OF HYBRIDOMA LINES

OKT9 and RFT10 were cloned by limited dilution to ensure that they were completely monoclonal. One hundred microlitres of the hybridoma cells, in complete medium (50 cell/ml) were seeded into the first row of a 96 well culture plate (NUNC, Gibco Laboratories) and doubling dilutions were performed along a row of the plate. Each well was supplemented with cells isolated from the spleen of a syngeneic (Balb/c) mouse. The spleen cells were washed, by centrifugation, 3 times in RPMI (150g for 10 minutes), resuspended in complete medium at $1x10^6$cells/ml and seeded at 100ul/well. RFT10 a particularly delicate cell line, was supplemented with 20% FCS during

Table 2.1 CELL LINES USED FOR MONOCLONAL ANTIBODY TITRATIONS

CELL LINE	ORIGIN		SOURCE	DESCRIPTION
	DIAGNOSIS	SOURCE		
Nalm-6	Pre-B ALL	PBL	[a]	Pre-B
Daudi	Burkitt's	Tumour	[a]	B blast
EB-3	Burkitt's	-	[a]	B blast
Raji	Burkitt's	Tumour	[a]	B blast
LCL-16-9	EBV clone		[b]	B blast
LCL-17-6	EBV clone		[b]	B blast
HPB-ALL	T-ALL	PBL	[a]	T cell like
Molt 4	T-ALL	PBL	[a]	T cell like

[a] Kind gift from Dr J Minowada to Dr A Goodall, Haemophilia Centre, Royal Free Hospital School of Medicine, London (RFHSM); [b] raised by Dr A Goodall, RFHSM;

ALL - acute lymphoblastic leukemia; blast - lymphoblastoid; Burkitt's - Burkitt's Lymphoma; PBL - peripheral blood lymphocyte.

cloning. Cloning plates were cultured for 2-3 weeks. Wells that contained single clones were tested for antibody production (section 2.4.2) and positive clones expanded.

2.1.3 PRODUCTION OF ANTIBODY IN CULTURE

Hybridoma cell lines were seeded at $5x10^5$ cell/ml in complete medium in $80cm^2$ flasks (NUNC, Gibco Laboratories), and incubated for 2-3 days until confluent. The cells were removed from the tissue culture supernatants by centrifugation (150g for 5 minutes) and then discarded. The supernatants were stored at -20^oC until purification.

2.1.4 PRODUCTION OF ASCITES

Balb/c mice were primed with a 0.5ml peritoneal injection of pristane (2,6,10,14-tetramethyl pentadecane, Aldrich Chemical Co. Gillingham, UK). Ten to fourteen days later they received an intraperitoneal injection of $5x10^6$ hybridoma cells in 200ul 0.9% saline (w/v) (Baxter Health Care Ltd, Thetford, Norfolk). Tumour formation and ascites production followed 3-6 weeks later. Approximately 5mls of ascites were collected from each mouse on up to two occasions.

2.2. PURIFICATION OF MONOCLONAL ANTIBODIES

2.2.1 PARTIAL PURIFICATION BY AMMONIUM SULPHATE PRECIPITATION

Ascites was pooled, clarified by passage through a 0.4um filter (Millipore, Harrow) and centrifuged at 500g for 10 minutes. An equal volume of saturated ammonium sulphate (Appendix I) was added, dropwise, to the ascites, left to equilibrate for 30 minutes, at room temperature and centrifuged at 2000g for 15 minutes. The pellet was then washed by resuspending in saturated ammonium sulphate and recentrifugation. The pellet was washed twice more in 50%

saturated ammonium sulphate, centrifuging at 2600g for 20 minutes for the final wash then resuspended in distilled water at 40% of the original volume. This was dialysed at 4°C for 2 days against 4-6 changes of 2 litres of 10mM Tris-HCL pH 7.5, to remove the ammonium sulphate.

2.2.2 AFFINITY CHROMATOGRAPHY BY PROTEIN A SEPHAROSE

This was carried out essentially by the method of Ey *et al* (1978) and Goding (1986). Two buffer regimes (table 2.2) were assessed to separate mouse monoclonal antibodies from ascites and from tissue culture medium.

Table 2.2: BUFFER REGIMES FOR PROTEIN A SEPHAROSE CHROMATOGRAPHY

	METHOD 1	METHOD 2
BINDING BUFFER:	phosphate buffer pH 8.1	1.5M glycine 3M sodium chloride pH 8.9
ELUTION BUFFER:	citrate buffer pH 5.5, 4.5, 3.5	100mM citric acid pH 6.0, 5.0, 4.0
REGENERATION BUFFER:	citrate buffer pH 3.0	100mM citric acid pH 3.0

(buffer details in Appendix I)

Swelled protein A Sepharose (details of preparation in Appendix I) was packed into a 1x20cm column, equilibrated with phosphate buffer pH 8.1 (Wright Scientific Ltd, Stonehouse, Glos.) and attached to a spectrophometer and fraction collector system (Uvicord, LKB Ltd, London). The column was washed with regeneration buffer pH 3.0 (table 2.2) followed by binding buffer (table 2.2), at a flow rate of 250ul/min, prior to loading of the antibody preparation. Ammonium sulphate precipitated ascites (section 2.2.1) diluted 1:10 with binding buffer or tissue culture supernatant was loaded onto the

column at a flow rate of 50ul/min at room temperature. The flow rate was returned to 250ul/min and binding buffer applied until the chart recorder reached baseline. The antibody was eluted from the column by using a stepwise regime of buffers of decreasing pH (table 2.2). The buffers were applied individually until the trace of protein concentration on the chart recorder returned to baseline and then the next buffer, of a lower pH, was applied. The eluted material was collected using a fraction collector and neutralised by addition of Tris-HCl buffer pH 9.0 (appendix I) to prevent conformational changes to the antibody. The column was regenerated with regeneration buffer (table 2.2) or 3 bed volumes of 3M KSCN (appendix I) followed by equilibration with phosphate buffer (appendix I). The protein concentration in the eluents was determined by spectrophotometry (section 2.2.4).

2.2.3 DEAE ION EXCHANGE CHROMATORAPHY

The DE-52 gel was gently suspended in the 10mM Tris-HCL buffer pH 8.0 (appendix I), left to stand for 10 minutes at room temperature, and washed over a coarse filter, under pressure with 2.5l distilled water followed by 500ml 10mM Tris-HCL. Preparation was performed with great care to avoid the introduction of fines. Air bubbles were removed from the gel by incubating under a vacuum, at room temperature, for 20 minutes. The gel was resuspended, avoiding the generation of eddies, and poured into a 1x20cm column (Wright Scientific Ltd), attached to a fraction collector and left to settle overnight. Equilibration of the column was achieved by eluting with 100ml of 100mM Tris-HCL pH 8.0 (appendix I), at a flow rate of 250ul/min.

An ammonium sulphate precipitate of ascites was then loaded at

a slower speed of 50ul/min. The column was washed with 100mM Tris-HCL to remove excess protein and the IgG was eluted using a salt concentration gradient 0-300mM NaCl (appendix I). The column could be regenerated using 1M NaCl. Antibody concentration was determined by spectrophotometry (section 2.2.4) and the preparation concentrated to approximately 1mg protein/ml using a Minicon B15 concentrator (Amicon, Gloucestershire, UK).

2.2.4 DETERMINATION OF PROTEIN CONCENTRATION

Protein concentration was determined by spectophotometric analysis at 280nm using a deuterium lamp (ultra-violet) spectrophotometer (Pye Unicam). The concentration of the IgG was determined using the following calculation:

$$\text{IgG mg/ml} = \text{OD} \times 0.7$$

2.2.5 CONJUGATION OF ANTIBODY TO FLUOROCHROME

The purified antibody (IgG) was dialysed overnight at 4°C against carbonate buffer (pH 9.5) (appendix I) and adjusted to a concentration of 1mg protein/ml buffer. One milligram of fluorecein isothiocyanate (FITC) (Sigma, Poole) was dissolved in 1ml dimethyl sulphoxide (DMSO) (BDH AnalR, Poole) and 50ul of this was added dropwise to the 1ml of the antibody solution. The reaction was allowed to proceed at room temperature, on a rotary mixer, shielded from light, for 2 hours. The conjugated protein was separated from the free fluorochrome by passing the mixture down a Sephadex G-25 column equilibrated with PBS (appendix I). Sodium azide (w/v) (appendix I) was added to the conjugated antibody preparation to a final concentration of 0.5% (w/v).

2.2.6 CONJUGATION OF ANTIBODY TO BIOTIN

The purified antibody was dialysed overnight at 4°C with bicarbonate buffer (pH 8.4) (appendix I) and then adjusted to a concentration of 1mg/ml. One milligram of biotin succinamide ester (Calbiochem, Behring, Nottingham) was dissolved in 1ml DMSO and this was then added to 1ml of antibody solution at the following ratios: 0.12:1, 0.1:1, 0.08:1. The solutions were incubated for 4 hours at room temperature, on a rotary mixer, and then dialysed overnight, at 4°C in PBS. Sodium azide (appendix I) was added to the conjugated antibody preparation to a final concentration of 0.5% (w/v).

2.2.7 CONJUGATION OF AVIDIN WITH FLUOROCHROME

One milligram of streptavidin (Sigma, Poole) was dissolved in 1ml carbonate buffer (pH 9.5) and conjugated with the fluorochrome using the method in section 2.2.5.

2.2.8 REMOVAL OF NON-SPECIFIC STAINING FROM SECOND LAYER REAGENTS

Directly conjugated second layer reagents, eg. avidin FITC, were occasionally found to stain lymphocytes non-specifically. To prevent this, the preparations were absorbed with human peripheral blood mononuclear cells (PBMC). PBMC were isolated by density centrifugation from 10mls of blood (section 2.3.2.1). The cells were centrifuged for 2 minutes, at high speed (1000g) using a micro-centrifuge (MSE, Crawley) and then resuspended in the second layer preparation. This was incubated using a rotary mixer, shielded from light, at room temperature for 30 minutes. The mixture was recentrifuged and the supernatant aspirated. Aggregates were removed by filtering the solution through a 0.2um filter (Millipore).

2.2.9 STORAGE OF ANTIBODIES

All conjugated antibodies were stored in preservative (0.5% w/v sodium azide) at 4°C in the dark. For long term storage, small aliquots of antibody were stored at -40°C.

2.2.10 TITRATION AND EVALUATION OF MONOCLONAL ANTIBODIES

All in house antibodies, commercial antibodies and second layer reagents (table 2.3) were titrated with PBMC or cell lines, to assess optimal antibody binding. For microscopic analysis, antibody concentrations were subjectively compared for the brightest and clearest stain with low non-specific binding. For flow cytometric analysis, dilution of antibody was plotted against mean fluorescence intensity, the dilution at the start of the plateau was considered to be optimal.

2.3 LYMPHOCYTE PURIFICATION AND CULTURE

2.3.1 BLOOD COLLECTION

For the separation of lymphocytes, blood was collected into EDTA (1.5 mg/ml) (BDH Ltd, Poole, UK).

Blood for serum was collected in standard plastic monovette serum tubes (Sarstedt, UK). Due to the prolonged clotting time of blood taken from haemophiliacs, blood collected for serum was left overnight at 4°C to coagulate. Blood taken from patient groups and normal controls was treated in a similar manner. The following day the tubes were centrifuged at 1100g for 15 minutes and the serum removed and stored at -40°C until required.

2.3.2 PREPARATION OF LYMPHOCYTES FROM BLOOD

2.3.2.1 Density gradient method

Blood collected in EDTA was diluted 1:1 in PBS-A (appendix I) or when cells were to be cultured, in Hanks balanced salt

solution (HBSS), without phenol red (Gibco Laboratories). Twelve millilitres of this suspension were layered onto 8ml of Lymphoprep (Nycomed, Birmingham) and centrifuged for 25 minutes at 400g. The mononuclear cells did not enter the gradient but remained at the plasma/gradient interface as a white band, they were recovered and washed three times by centrifugation in PBS-A or HBSS (the first wash at 200g for 10 minutes and the remaining washes for 5 minutes) and with the exception of those cells prepared for *in vitro* culture (section 2.3.3) were resuspended at $2x10^7$cells/ml in PBS-A. Care was taken to maintain sterility when cells were prepared for *in vitro* culture, by carrying out all manipulations in a class II cabinet. Blood taken from HIV+ve patients was prepared in a class III cabinet.

2.3.2.2 Whole blood method

Blood was collected in EDTA and diluted 1:4 in 0.8% ammonium chloride solution (appendix I). The mixture was left at room temperature for 10 minutes for red cell lysis to occur. Red cell ghosts were removed by centrifugation at 200g for 10 minutes after which the white cell pellet was resuspended in 10mls of ammonium chloride solution and recentrifuged. The white cells were then washed, twice in PBS-A (200g for 10 minutes) and resuspended at $2x10^7$in PBS-A.

2.3.3 IN VITRO CULTURE OF PERIPHERAL BLOOD LYMPHOCYTES

from normal male and female controls (ages 20-35)

Peripheral blood mononuclear cells were fractionated from 50ml blood (section 2.3.2.1). Cells were resuspended at $1x10^6$cells/ml in complete medium (without phenol red), and cultured for 96 hours in 24x2ml well Linbro-plates (Nunc, Gibco Laboratories Ltd) in a humidified atmosphere of 5% CO_2/95% air at 37°C. The same batch of heat inactivated FCS

(06F6791S) (Gibco Laboratories) was used in all inhibition and calcium studies. The cells were stimulated with dilutions of the lectins phytohaemagglutinin (PHA) at a 1:50 dilution or pokeweed mitogen (PWM) at 1:1000 (Gibco Laboratories). To assess spontaneous activation, cells were cultured without stimulant. All reagents were prepared in sterile pyrogen free deionised water (Baxter, Thetford, Norfolk). The clotting factor concentrates were prepared according to the manufacturers' instructions. Briefly, 10ml sterile pyrogen free water was injected into the bottle and the solution was left at room temperature to dissolve. Factor VIII or factor IX preparations were added to the cultures at a 1:5 dilution [4-5u/ml].

2.3.3.1 Viability count

Viability of cells was determined by trypan-blue dye exclusion. The cells were suspended in PBS-A or HBSS at $2x10^7$cells/ml and mixed 1:1 in trypan-blue solution and counted immediately using a haemocytometer. Dead cells did not exclude the trypan-blue and were therefore clearly distinguishable from viable cells.

2.4 IMMUNOFLUORESCENT STAINING OF LYMPHOCYTES

2.4.1 LYMPHOCYTES STAINING - STANDARD METHOD

Lymphocytes (from blood or from *in vitro* cultures) or cell lines were washed 3 times in PBS-A (200g for 5 minutes) and resuspended at $2x10^7$cells/ml. Fifty microlitre aliquots ($1x10^6$ cells) were placed in either 10ml screw top centrifuge tubes (Sterilin, Hounslow, UK) for all PBMC analyses or LP-3 tubes (Luckam, Sussex) for analysis of cell lines. The specified working dilutions of monoclonal antibodies

(table 2.3) were added to the cell suspension, mixed by gentle tapping and incubated at room temperature for 15 minutes. They were then washed in PBS-A at 200g for 5 minutes to remove unbound antibody. Cells were resuspended and second layer reagents added to those antibodies that were not directly conjugated to fluorochrome: goat anti mouse antibody (G/M)-fluorochrome preparations for unconjugated antibody and avidin-fluorochrome for biotin conjugated antibody, and incubated and washed as before. Cells were resuspended and a small drop of cell suspension was placed in a well on a 12 well multislide (C A Hendley, Loughton) and fixed in formalin vapour (from 8% formaldehyde solution (appendix I) for 10 minutes. Slides were then air dried and a coverslip mounted with Citifluor (50% glycerol and PBS (v/v) with a 'secret ingredient' to preserve the fluoresence) (City University, London). The slides were examined using a standard a fluorescence microscope (Zeiss, Welwyn Garden City).

For flow cytometric analysis, the cells were fixed in solution with a 1:1 dilution of 8% formaldehyde solution prior to analysis.

2.4.1.1 Staining of cells for chronic T cell activation markers

Directly conjugated antibodies to the antigens CD45RO and CD45RA were not available and were therefore detected with a second layer - goat-anti-mouse-FITC (table 2.3). To prevent cross reactivity with the pan T cell markers a blocking reagent - mouse Ig, was incorperated into the staining procedure. Cells were incubated for 15 minutes at room temperature with optimal concentrations of CD45RO and CD45RA antibodies (table 2.3), following which the cells were washed

TABLE 2.3 MONOCLONAL ANTIBODIES AND SECOND LAYER REAGENTS USED IN THE STUDY

ANTIBODY	SOURCE		DETECTS	METHOD OF DETECTION
CD3	anti-Leu 4	a	T cells	conjugated to FITC
CD4	anti-Leu 3a	a	T4 subset	conjugated to PE
CD5	RFT1	b	T cells	conjugated to FITC
CD8	anti-Leu 2a	a	T8 subset	conjugated to PE
CD14	UCHM1	c	monocyte	G/M FITC
CD19	RFB9	b	B cell (pro-B - B blast)	G/M FITC
CD20	B1	d	B cells	G/M FITC
CD21	RFB6	b	peripheral and activated B cells	G/M FITC
CD23	B6	d	activated B cells	G/M FITC
CD24	BA-1	e	B cell subset, granulocytes	G/M FITC
CD25	clone 2A3	a	IL2 receptor	conjugated to FITC/PI
CD41	M148	f	platelets	G/M FITC
CD45RO	SN130	c	virgin/naive T cells	G/M FITC
CD45RA	UCHL1	g	memory T cells	G/M FITC

CD71	OKT9	h	transferrin receptor	conjugated to biotin
HLA-DR	clone L244	a	MHC class II	conjugated to FITC/PI
4F2	4F2	h	activation marker	conjugated to biotin
PNL	-	i	B cell subset	conjugated to biotin
PCA-1	PCA-1	d	plasma cells	conjugated to PE
W6 32	W6 32	h	MHC class I	G/M FITC

Second layer reagents:- G/M FITC: Goat anti-mouse FITC (Seralab, Crawley), detects mouse Ig; Strepatavidi
PE (Becton Dickinson, Cowley, Oxford), detects Ig bound to biotin).

[a] Becton Dickinson; [b] Dept. Immunology, Royal Free Hospital School Of Medicine, London (RFHSM); [c] Dr Poulte
Dept Rheumatology, RFHSM; [d] Coulter Immunology, Luton; [e] Hybritech Ltd, UK; [f] Imperial Cancer Research Fun
London, via Dr Goodall, Haemophilia and Haemostasis Unit, RFHSM; [g] Dr P Beverley, ICRF Tumour Immunology Grou
University College Hospital, London; [h] American Type Tissue Culture Collection, Maryland, USA; [i] Sigma Lt
Poole, Dorset.

FITC - Fluorecein isothiocyanate
PE - Phycoerythrin

in PBS-A by centrifugation (200g for 5 minutes). The cells were resuspended and incubated as before with goat-anti-mouse-FITC. Following a second wash the cells were then incubated with a 1:50 dilution of mouse Ig (Sigma, Poole, Dorset). The cells were washed again and then incubated with optimal concentrations of anti-CD4-phycoerythrin (PE) or anti-CD8-PE (table 2.3). Following the final wash the cells were fixed as outlined in section 2.4.1.

2.4.2 LYMPHOCYTE STAINING USING A MICRO-PLATE METHOD

When screening hybridoma clones for antibody production, 50ul tissue culture supernatant was removed from the individual wells of the cloning plate and placed, in the same sequence, in the 'v' shaped wells of a microtitre plate (NUNC, Gibco). Fifty microlitre aliquots of PBMC in PBS-A at $2x10^5$cells/ml, were added to into each well. The plate was covered, agitated on a plate shaker for 30 seconds, and incubated, for 15 minutes at room temperature. The plate was washed by topping up the wells with 100ul PBS-A and the whole plate was centrifuged at 200g for 30 seconds. The supernatants were removed from the plate, the cells resuspended by gentle agitation, and the plate washed a further 4 times. After the final wash the cells were resuspended in 50ul PBS-A and G/M FITC added (table 2.3). The plate was incubated and washed as before. The cells were then analysed by fluorescent microscopy as outlined in section 2.4.1.

2.5 EXAMINATION OF FLUORESCENTLY LABELLED LYMPHOCYTES BY FLOW CYTOMETRY

Fixed labelled cells were analysed using a Coulter EPICS Profile Flow Cytometer (Coulter Electronics, Luton, UK). Briefly, stained cells enter the flow chamber and are passed through a focused laserbeam in single file. As they are struck by the laser beam, scattered light and fluorescent light is emitted and separated according to wavelength by appropriate mirrors and filters, and directed to the forward angle light detector, 90°C light scatter photomultiplier, or one of the two fluorescence photomultipliers. Signals from the detectors pass to amplifying processors and then to the computer (figure 2.1). Each cell is recognisable by its forward scatter, proportional to the volume of the cell, and 90°C angle scatter, proportional to cell surface topography, nuclear:cytoplasm ratio and homogeneity of the cell cytoplsam. The lymphocyte population could be gated with an electronic bit map. T and B lyphocytes were stained with a pan T or pan B reagent. Positive cells were determined as those of which the green or red fluorescence was 98% greater than the mouse Ig controls (figure 2.2). The presence of activation marker expression was determined on the T or B cells which were identified using a 'window gate' corresponding to the cells that were positive for the pan reagent (histogram B in figure 2.2).

Diagrammatic representation of the flow cytometer

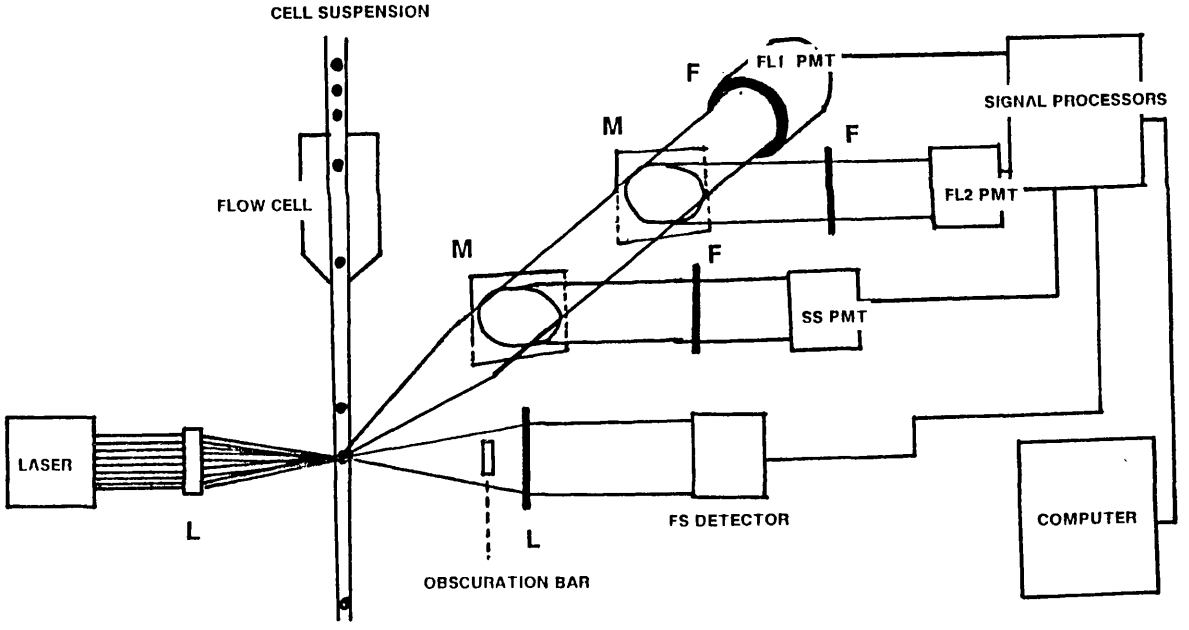


Figure 2.1: Stained cells enter the flow chamber and are passed through a focused laser beam in single file. As they are struck by the laser beam, scattered light and fluorescent light is emitted and separated according to wavelength by appropriate mirrors (M) and filters (F), and directed to the forward angle light detector (FS), 90° light scatter photomultiplier (SS), or one of the two fluorescence photomultipliers (FL1, FL2). Signals from the detectors pass to amplifying processors and to the onboard computer.

TWO COLOUR ANALYSIS OF PERIPHERAL BLOOD LYMPHOCYTES USING THE COULTER EPICS PROFILE FLOW CYTOMETER

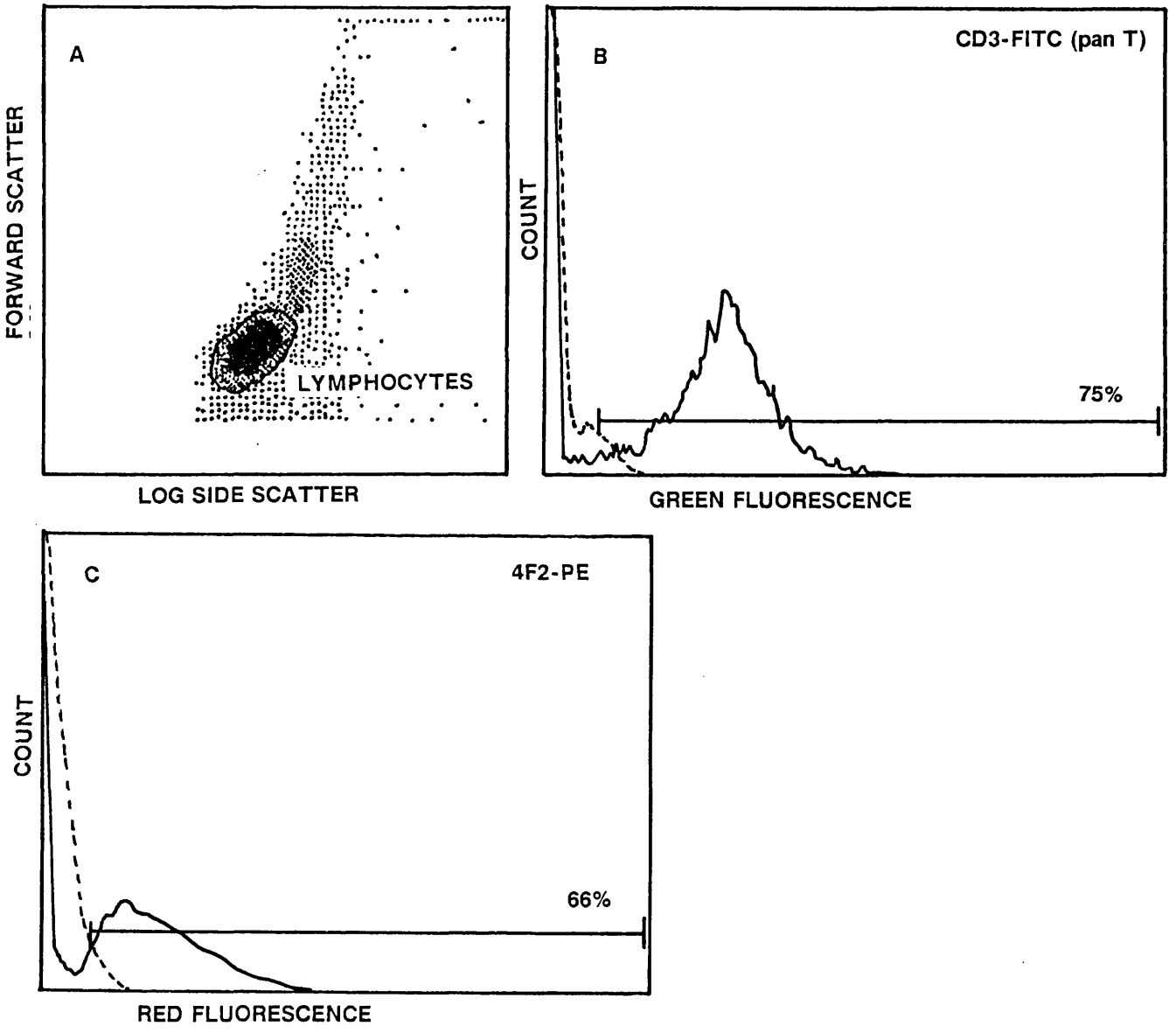


Figure 2.2: Lymphocytes were identified on the basis of their size and side scatter characteristics and isolated from other cells by the creation of a bit map (histogram A). Lymphocytes were stained with a 'pan' reagent, eg. CD3-FITC for T cells (histogram B). Positive cells were determined as those of which the green or red fluorescence was greater than 98% of the mouse Ig controls (----). The presence of activation marker expression was determined by creating a 'window gate' around the cells positive for the pan reagent. Cells that were positive for the pan reagent and the activation marker (detected directly or indirectly by phycoerythrin (PE) conjugated mabs) were then identified in histogram C, eg. 4F2.

2.6 ASSESSMENT OF LYMPHOCYTE DERIVED ACTIVATION MARKERS IN SERUM

2.6.1 DETERMINATION OF SOLUBLE IL2-R LEVELS

The soluble IL2-R levels in the serum of normal controls and patients was determined using the cell free IL2-R enzyme immunoassay (T Cell Sciences, Cambridge) using the recommended procedure. Briefly, standard controls (0-1600u/ml) and serum were incubated for 2 hours at 37°C in a 1:3 dilution in sample diluent, in wells of a 96 well microtitre plate coated with a monoclonal antibody directed against the 55KDa IL2-R (CD25) protein. After washing to remove unbound serum, a second horse radish peroxidase conjugated MAb to a second epitope on CD25, was added to the wells and the plate incubated as before. After washing, ortho-phenyldialine (OPD) substrate was added and the plates were incubated for 30 minutes at room temperature. The reaction was stopped with 100ul 2M H_2SO_4 (BDH, Anal R, Poole, Dorset) and absorbance determined at 490nm. sIL2-R concentrations in units per millilitre of serum were determined from a standard curve.

2.6.2 DETERMINATION OF LEVELS OF IMMUNOGLOBULIN G, M AND A

Patients reviewed in the centre have immunoglobulin levels determined as normal procedure by the department of Chemical Pathology, Royal Free Hospital, London (RFH) using standard nephelometry assays.

2.7 PATIENT SEROLOGY

The presence of antibody in the serum of the patients to HIV was determined by ELISA (Wellcome Diagnostics, Bromley) by the department of Virology, RFH.

Patients reviewed in the centre have the levels of serum

aspartate amino transferase (AST) and glutamyl transferase (gamma GT) determined as normal procedure and these were measured using standard colourmetric assays by the department of Chemical Pathology, RFH. T4 counts were measured with monoclonal antibodies (RFT4 and RFT8, Dept, Immunology, RFHSM) and analysed using a Becton Dickinson FACS Scan, by the department of Immunology, RFH.

Serum samples were collected at the time that blood was collected for lymphocyte analysis and was batched and stored at -40°C. At the end of the study the stored serum was analysed for the presence of antibody to HCV (c100) by the Virology department, RFH using the 'Ortho' immunoassay (Ortho Diagnostics, UK).

2.8 PREPARATION OF LYMPHOCYTES FOR CYTOPLASMIC FREE CALCIUM LEVEL MEASUREMENT

Cytoplasmic free calcium was monitored using the fluorescent dye fura-2 acetyoxymethylester (Fura-2AM), which has a high affinity for calcium and displays a change of fluorescence on binding (Grynkiewicz *et al*, 1985).

PBMC or Jurkat cells were washed twice (by centrifugation at 200g for 5 minutes) in RPMI 1640 without phenol red (Gibco Laboratories) and resuspended at $5x10^6$/ml in RPMI 1640+10% FCS (Gibco Laboratories) with Fura -2AM (Molecular Probes Inc., Oregon) (from a 1mM stock dissolved in DMSO (AnalaR, BDH, Poole)) for 30 minutes in a 37°C water bath. The fura-2AM ester is membrane permeant and therefore penetrates the cells, where it is hydrolysed by cytoplasmic esterases into the impermeable fura-2AM, thus trapping the indicator dye in the cell cytoplasm. Unfortunately, with time the fura-2AM 'leaks'

from the cells into the surrounding medium.

Following incubation the cells were centrifuged (200g for 5 minutes) and resuspended at $1x10^6$cells/ml in HEPES buffer pH 7.6 (appendix 1).

To reduce leaking of fura-2AM from the cells they were kept on ice and used within 1 hour of preparation.

2.8.1 MEASUREMENT OF CYTOPLASMIC FREE CALCIUM

A 0.5ml suspension of cells (PBMC or Jurkat cells) loaded with fura-2AM was placed in a glass cuvette in a temperature controlled (37°C) holder in a Perkin Elmer L55 fluorescence spectrometer and continuously stirred. Settings of 500nm emission and 340nm excitation were used.

Calcium flux was determined in the presence of 1mM $CaCl_2$ (BDH, Poole, Dorset), whilst calcium mobilisation was determined in the absence of free calcium in the medium which was achieved by the addition of 2mM EGTA (BDH). The effect of clotting factor concentrates (1:5 dilution equivalent to that used in the inhibition studies) upon the resting intracellular calcium levels and levels following stimulation with PHA (1:50 dilution) (Gibco Laboratories) or anti-CD3 antibody (T3b) (1:250) (kindly provided by Professor Art Sullivan, Leeds Univerity) were determined.

The fluorescence signal was calibrated in terms of levels of intracellular calcium ($[Ca^{2+}]_i$). Briefly, following each test, maximal fluorescence (f-max) was obtained by addition of 0.4% triton-x-100 (BDH) to the cells and minimal fluorescence (f-min) was obtained by quenching the signal from the lysed cells by the addition of 10mM EGTA. Cytoplasmic free calcium was determined using the equation:

$$[Ca^{2+}]_i = \frac{224 \times (F - F\text{-max})}{(F\text{-max} - F)}$$

where 224nM is the apparent dissociation constant for calcium and fura-2AM.

2.9 DIALYSIS OF CLOTTING FACTOR CONCENTRATES

Using aseptic techniques the concentrate was placed in visking tubing (Medicell International, London) and dialysed against 1 litre HBSS (without phenol red) (Gibco Laboratories Ltd). The dialysing solution was prepared in the following manner: HBSS (without phenol red and 3.5g $NaHCO_3$ (AnalaR BDH, Poole)) was dissolved in 1 litre distilled water (Baxter, Thetford, Norfolk). One hundred millilitres of this solution were filtered (0.2u) (Millipore) into a sterile 2 litre flask (with magnetic stirrer), and to this 90mls of sterile water were added. The concentrates were dialysed whilst stirring with 3 hourly changes. All manipulations were performed in a class II cabinet using sterile techniques.

2.10 MEASUREMENT OF FREE CALCIUM

Free calcium levels in RPMI 1640 (Gibco Laboratories) and RPMI 1640+10% FCS (Gibco Laboratories) were measured by the use of a calcium probe (Milivolt meter 811, Orion Research) with the kind assistance of Dr Pocock, Dept. Physiology, RFHM. The probe was calibrated with buffers with pCa levels at 4, 5.45, 6.06 and 7.1 by Dr Pocock and a calibration curve against millivolts was drawn and extrapolated.

* Patients studied were all adults with the exception of one HIV-ve 7 year old boy and one HIV+ve 12 year old boy.

Free calcium levels were determined by the following calculation:

$$pCa = \log \frac{1}{[Ca^{2+}]}$$

where Ca^{2+}(molar) = pCa x antilog x $\frac{1}{x}$

The calcium probe was immersed in 5mls of RPMI 1640+10% FCS. When the reading had stabilised (approximately 1 minute) the number of millivolts was recorded.

A 1:5 dilution of the concentrates, equivalent to that used in the inhibition studies, was added to the medium and the millivolt readings taken. The level of free calcium was then calculated using the formula above.

2.11. IN VIVO ACTIVATION STUDY

2.11.1 SUBJECTS STUDIED

2.11.1.1 Acute activation study

The presence of acute activation markers upon T and B cells was investigated in 33 anti-HIV seronegative (HIV-ve) haemophiliacs, 31 anti-HIV seropositive (HIV+ve) haemophiliacs and 22 age matched normal males. The patients attending the Haemophilia Centres of the Royal Free Hospital (RFH) and the Royal Infirmary, Edinburgh were enrolled. Subject details are given in table 2.4. Fifty eight of the patients had haemophilia A and 6 had haemophilia B.*

Of the 31 haemophiliacs with HIV infection, one was a haemophilia B patient. Three of the HIV+ve patients were from the Royal Infirmary, Edinburgh. Seven of the HIV+ve patients (determined on RFH patients only) had symptoms of HIV-related disease (ARC/AIDS) and a further three had HIV-related

TABLE 2.4: SUBJECTS STUDIED FOR ACUTE ACTIVATION MARKERS

(range ± median)

	NORMAL MALES	HAEMOPHILIACS	
		HIV-ve	HIV+ve
NUMBER	22	33	31
AGE	22-65 (32.5)	12-76 (29)	7-61 (29)
FVIII/FIX*	-	0-183,900 (35,330)	0-381,930+ (58,410)
T^4 COUNT $10^9/l$	0.6-1.7 (normal range)	0.39-1.19** (0.88)	0.03-0.94*** ++ (0.42)
ARC/AIDS	0/22	0/33	7/28+++

* Usage (in units) in previous year
** n = 21
*** n = 28
\+ No significant difference in FVIII/FIX usage HIV+ve/HIV-ve (p>0.05)
++ Significant difference from HIV-ve haemophiliacs (p=0.0001)
+++ RFH patients only

thrombocytopenia.

Although FVIII/FIX usage appeared to be higher in HIV+ve patients, there was no significant difference in FVIII/FIX usage between the two patient groups. The type and amount of concentrate used by each patient in the 12 months preceding the analysis was determined. Forty-six patients with severe haemophilia A and three with mild haemophilia A who received treatment were treated with intermediate purity, heat treated FVIII. Six haemophilia B patients, who received treatment and two severe haemophilia A patients with inhibitors (FVIII antibodies) were treated with heat-treated FIX. Forty six of the patients (20 of whom were HIV+ve) received concentrates from a single source: 17 (14 HIV+ve) were treated with Alpha Profilate ('wet' heat treated), six (2 HIV+ve) received NHS 8Y from the Blood Products Laboratory (BPL) and 14 (3 HIV+ve) received Scottish National Blood Transfusion Service (SNBT) FVIII. Seven patients received BPL 9A and one patient was treated with SNBT FIX.

2.11.1.2 Chronic activation study

HLA-DR and CD45RA/RO expression on T4/T8 subsets was analysed using a separate subject population (table 2.5).

Seventeen HIV-ve haemophiliacs, 18 HIV+ve haemophiliacs and 21 age matched normal males were studied. Two of the HIV-ve patients were diagnosed as having haemophilia B. Seven HIV-ve patients were from the Royal Infirmary, Edinburgh.

Of the 18 haemophiliacs with HIV infection, only one of these patients had haemophilia B. Four of the HIV+ve patients had symptoms of HIV-related disease (ARC/AIDS) and a further three had HIV-related thrombocytopenia.

FVIII/FIX usage appeared to be higher in HIV-ve patients,

TABLE 2.5: SUBJECTS STUDIED FOR CHRONIC ACTIVATION MARKERS
(range ± median)

	NORMAL MALES	HAEMOPHILIACS	
		HIV-ve	HIV+ve
NUMBER	21	17	18
AGE	26 - 67 (33)	22 - 62 (34)	17 - 61 (29)
FVIII/FIX*	-	3,310-142,550 (85,525)	960-268,266+ (35,590)
T^4 COUNT $10^9/l$	0.6-1.7 (normal range)	0.49 - 0.90 (0.68)	0.001 - 0.84++ (0.30)
ARC/AIDS	0/21	0/17	4/18

* Usage (in units) in previous year
\+ No significant difference in FVIII/FIX usage HIV+ve/HIV-ve (p>0.05)
++ Significant difference from HIV-ve haemophiliacs (p=0.0001)

* The haemophiliacs studied were all adults with the exception of one HIV-ve 7 year old boy.

however, there was no significant difference in factor VIII/FIX usage between the two patient groups.

2.11.1.3 Soluble IL2-R study

In this study a total of 30 HIV-ve and 29 HIV+ve haemophiliacs (from RFH only); 22 HIV+ve non-haemophiliacs from the 'HIV walk in clinic' at the RFH (courtesy of Dr M Johnson, Dept. Thoracic Medicine, RFH) and 20 age-matched male controls were investigated for the presence of sIL-2R in serum (table 2.6). All of the HIV+ve hamophiliacs in this study had haemophila A. Nine of these patients had symptoms of HIV-related disease (ARC/AIDS) and three had HIV-related thrombocytopenia.*

There was no significant difference in FVIII/FIX usage between the two patient groups. As with the activation study the type and amount of concentrate used by each patient in the 12 months preceding the analysis was determined. Forty one patients with severe haemophilia A and three with mild haemophilia A were treated with intermediate purity, heat treated FVIII. The nine HIV-ve haemophilia B patients receiving treatment and two severe haemophilia A patients with inhibitors (FVIII antibodies) were treated with heat-treated FIX. One HIV+ve haemophilia A patient with inhibitors received porcine FVIII. Thirty three of the patients (14 of whom were HIV+ve) received concentrates from a single source: 15 (12 HIV+ve) were treated with Alpha Profilate ('wet' heat treated), 9 (2 HIV+ve) received NHS 8Y. Nine patients received FIX only.

The patients from the 'HIV walk in clinic' were male and age matched with the haemophilic patient group. At the time of writing this thesis further clinical details were not available.

TABLE 2.6: SUBJECTS STUDIED FOR SOLUBLE IL2-R LEVELS

(range ± median)

	NORMAL MALES	HAEMOPHILIACS HIV-ve	HAEMOPHILIACS HIV+ve	NON-HAMOPHILIACS
NUMBER	20	30	29	22
AGE	20-67 (32)	12-76 (38)	7-60 (31)	21-60 (38)
FVIII/ FIX*	-	0-183,900 (41,660)	0-381,930+ (58,410)	-
T^4 COUNT $10^9/l$	0.6-1.7 (NORMAL RANGE) (0.77)	0.39-1.40 (0.46)	0.03-0.94++	ND
ARC/AIDS	0/20	0/30	9/29	ND

* Usage (in units) in previous year

\+ No significant difference in FVIII/FIX usage HIV+ve/HIV-ve ($p>0.05$)

++ Significant difference from HIV-ve haemophiliacs ($p=0.0001$)

ND Not determined

2.12 STATISTICAL ANALYSIS

For non-gaussian data (as found in the cellular activation marker studies) the Mann-Whitney U test and Spearman Rank correlation were used for the comparison between groups.

For the remainder of the results in the study, parametric statistics were used (Student t test and Pearson correlation).

Values of $p<0.05$ were considered significant.

3. PREPARATORY EXPERIMENTS

3.1 EVALUATION OF BLOOD COLLECTION METHODS FOR THE ACTIVATION ANTIGEN STUDY

In addition to patients from the Royal Free Hospital, heavily treated haemophiliacs from the Royal Infirmary, Edinburgh, were enrolled in the study. These samples were transported overnight, and therefore, blood was occasionally analysed the day following collection.

The standard blood collection method for the separation of lymphocytes from blood utilises heparin as the anti-coagulant. For white cell analysis it is recommended that the blood is anti-coagulated with EDTA (Dacie & Lewis, 1975) as heparin may lead to white cell damage and disintegration of platelets. Blood was collected in EDTA and heparin to find the optimal blood collection method, and the following analyses were performed immediately; and after storage of blood for 24 hours at room temperature or 4°C:

i) Differential count using a Coulter Counter.

ii) Differential and morphological stain (Prodiff, Braidwood Laboratories, UK) of cytocentrifuged preparations.

iii) Viable lymphocyte count.

Damaged white cells and platelet aggregation was evident in the heparin samples as illustrated on the Coulter Counter trace (figure 3.1) resulting in increased lymphocyte and white cell numbers when compared to the EDTA sample (table 3.1), and the different cell populations were no longer distinguishable (no lymphocytes (L) or monocytes (M)).

Differential count histograms of blood collected in EDTA and heparin

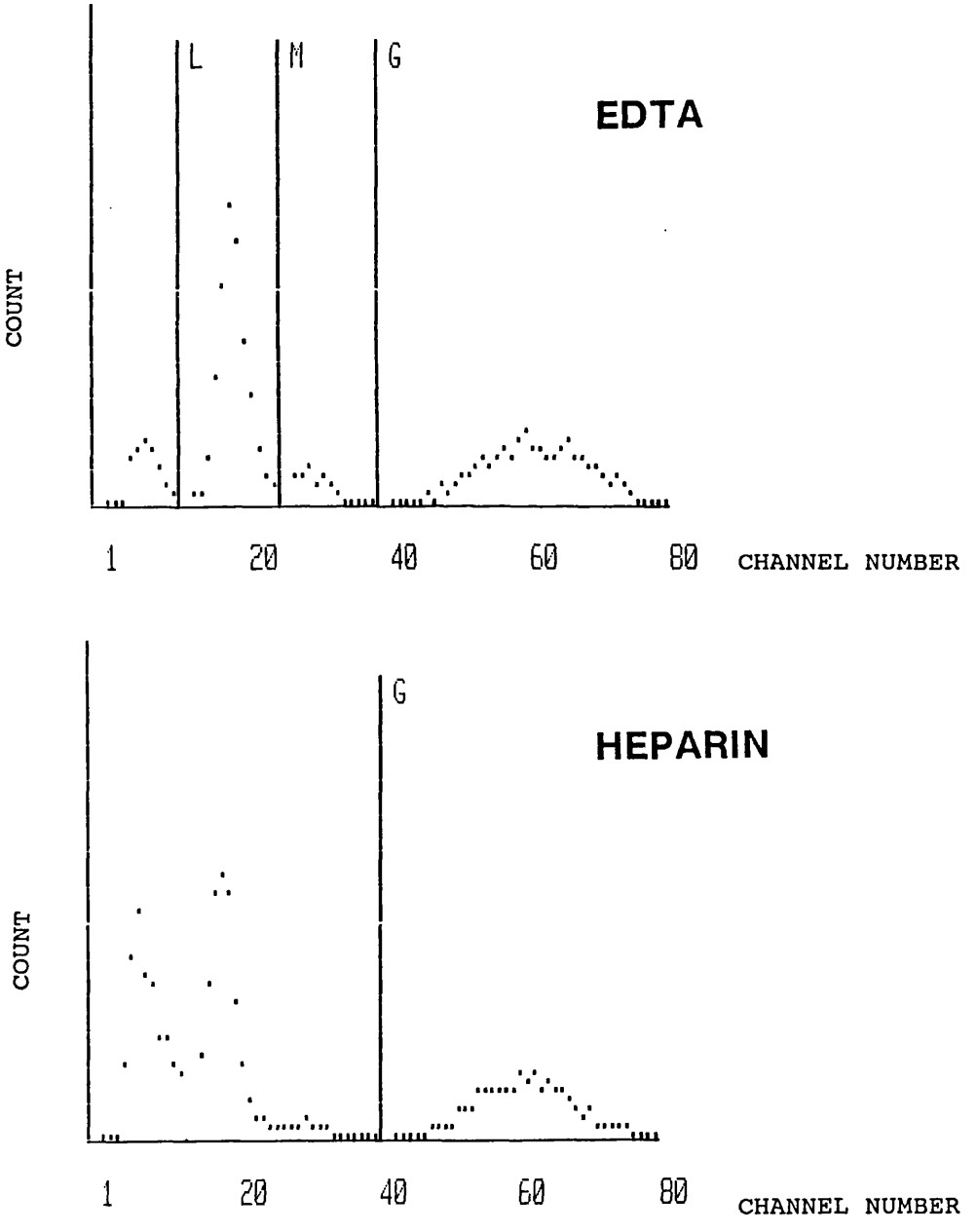


Figure 3.1: Histograms from the differential count of blood collected in EDTA and heparin, using a Coulter Counter (Coulter Electronics, Luton). (L) lymphocytes, (M) monocytes, (G) granulocytes. Figure corresponds to results in table 3.1.

Table 3.1 CELL NUMBERS FROM COULTER COUNTER, VIABILITY AND YIELD OF LYMPHOCYTES FOLLOWING VARIOUS BLOOD COLLECTION AND STORAGE METHODS

	FRESH BLOOD		BLOOD STORED FOR 24H AT: 25°C		4°C	
	EDTA	HEPARIN	EDTA	HEPARIN	EDTA	HEPARIN
CELL COUNTx10^3/ml						
TOTAL WBC	4.1	7.2	4.3	4.3	4.1	2.9
LYMPHOCYTE	1.7	3.2	*	*	1.6	*
MONOCYTE	0.4	1.2	*	*	0.4	*
GRANULOCYTE	1.9	2.8	2.0	1.2	2.1	1.2
PLATELETS	201	110	208	173	204	11
LYMPHOCYTE YIELD (x10^6cells/ml)	1.7	1.4	1.3	0.7	1.8	3.2
LYMPHOCYTE VIABILITY %	100	92	100	95	100	95

* Coulter Counter could not determine cells of this type

The alteration in size and density of the white cells probably affected the separation properties of these cell during density centrifugation.

From cell numbers and the differential stain it was evident that storage of blood at room temperature in either anti-coagulant led to cell damage.

Blood collected in heparin and stored at 4°C contained damaged neutrophils, crenulated red cells and aggregated platelets, but lymphocytes appeared undamaged. Blood collected in EDTA and stored at 4°C had comparable cell counts and lymphocyte yields to those seen in the fresh samples. Viability of the remaining separated mononuclear cells was assessed, this was unaffected in the EDTA samples but slightly reduced in the cells collected in heparin.

The was no effect upon activation marker expression on the lymphocytes from stored blood determined by fluorescence microscopy (data not shown).

Blood was collected in EDTA and heparin and the lymphocytes were stimulated with PHA (section 2.3). Analysis of the expression of HLA-DR and CD25 on $CD5^+$ lymphocytes (section 2.4, 2.5, table 2.3) following 96 hours of culture demonstrated that the expression of these markers was unaffected by the anti-coagulant (figure 3.2). The cells were also cultured in the presence of FVIII concentrate (Alpha Prolfilate) (4-5 units/ml) and this inhibited the expression of the activation markers. The inhibition was not affected by the choice of anti-coagulant.

Comparison of the Effects upon PHA Stimulated Cells, With and Without FVIII (4-5 units/ml) at 96 hours, by Two Anti-Coagulation Methods

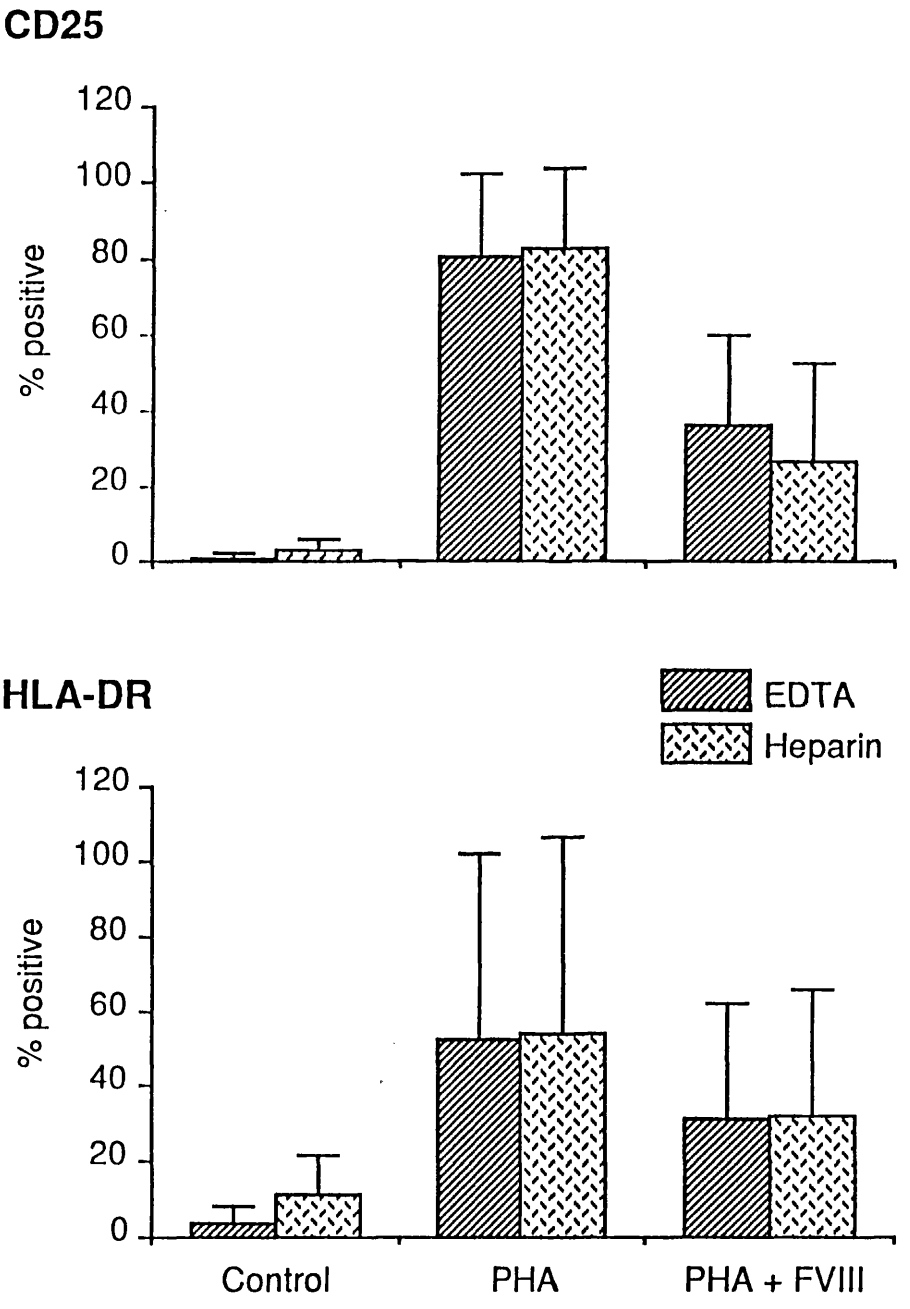


Figure 3.2: Blood was collected in EDTA or heparin and the lymphocytes stimulated with PHA and cultured for 96 hours. HLA-DR and CD25 expression on $CD5^+$ lymphocytes was determined by two colour flow cytometry with and without 4-5 units/ml FVIII (Alpha Profilate).

3.2 PURIFICATION OF IgG MONOCLONAL ANTIBODIES

The monoclonal antibodies 4F2, RFT10 and OK T9 were prepared 'in house'. Cell culture supernatant or ascites immunoglobulin preparations (section 2.1) were used for the preliminary lymphocyte activation studies. To enable dual colour analysis, the monoclonal antibodies were directly conjugated to fluorochrome or biotin, but before this could be performed the antibody preparations were purified. Several methods of purification were assessed. These included protein A Sepharose purification using 2 different buffer regimes and DEAE cellulose affinity chromatography.

Protein A, produced by *Staphylococcus aureus*, has a high binding affinity to the Fc receptor of immunoglobulins (Goding, 1986). It is commercially available covalently coupled to sepharose beads and in this form can be used for high affinity chromatography purification of immunoglobulin. Binding of mouse IgG_1 to protein A is poor, but can be substantially improved with the use of high pH buffers (pH greater than 8.0) (Goding, 1986, Separation News vol 13.5, Pharmacia). Therefore buffers with higher pH levels and incorporating glycine were used.

DEAE cellulose is a weak anion exchanger. Immunoglobulin are very basic proteins with isoelectric points in the range of 6-8. As a consequence they do not bind to DEAE cellulose very strongly, increasing the concentration of competing anions (by the addition of a salt gradient), elution of the immunoglobulin precedes other serum proteins. The binding capacity of a 10ml column is 100-200mg protein and the recovery should be virtually 100% . DEAE was eventually established as the method of choice, largely

Immunoglobulin fractionation of cell culture supernatant of 4F2 on Protein A Sepharose (buffer regime 1)

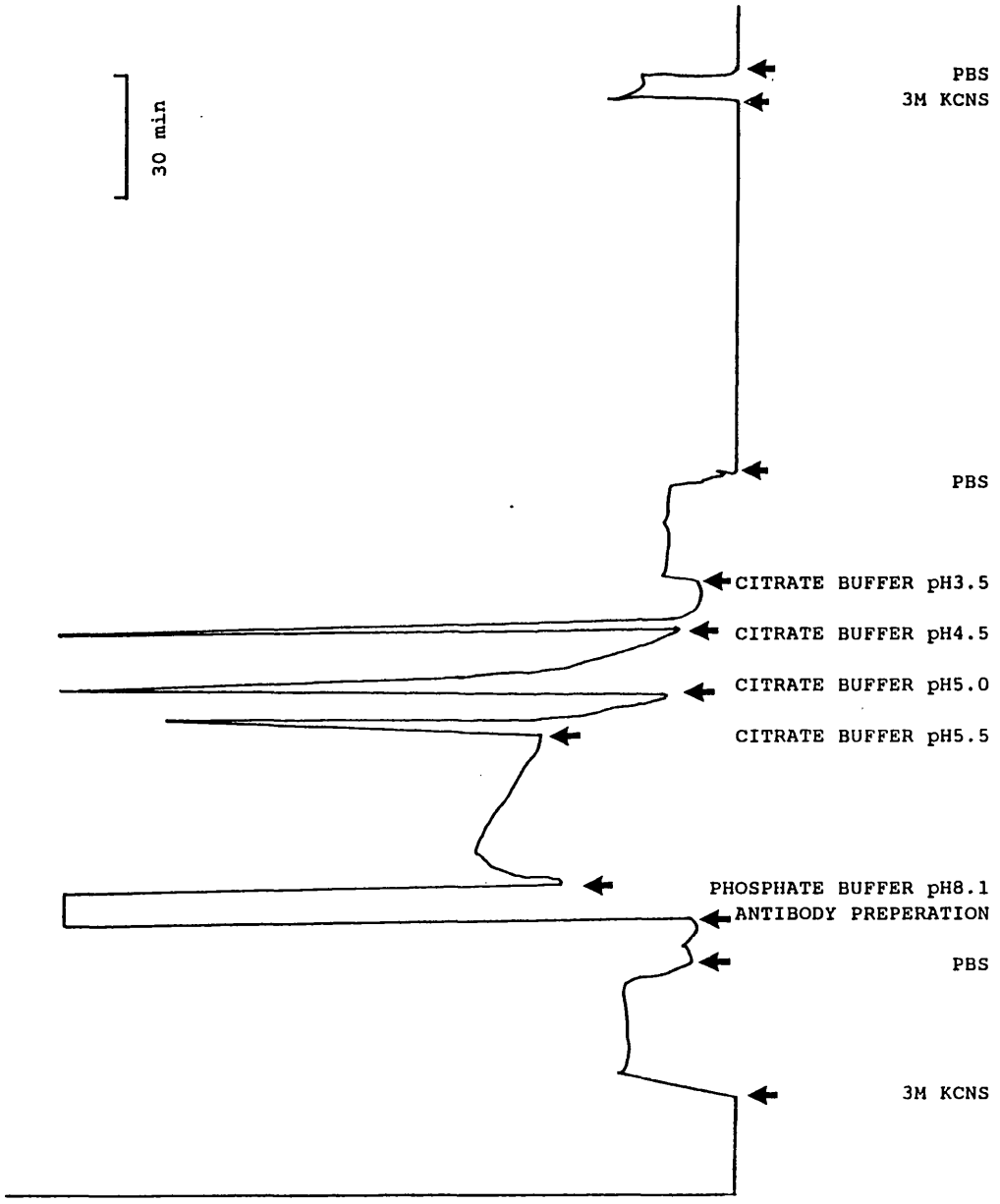


Figure 3.3: Five hundred millilitres of 4F2 cell supernatant (s/n) was purified using a protein A sepharose column and buffer regime 1 (section 2.2). Cells were removed from the s/n by centrifugation which was then diluted with phosphate buffer pH 8.1. The column was washed with 3MKCNS at a flow rate of 250ul/min. The antibody preparation was loaded onto the column at a flow rate of 50ul/min and then washed with phosphate buffer after returning the flow rate to 250ul/min. The antibody was eluted from the column by using a stepwise buffer regime of citrate buffer of decreasing pH: pH 5.5 (IgG_1), pH 4.5 (IgG_2), pH3.5 (IgG_3) and pH 3.0. The column was washed with PBS and regenerated with 3MKCNS.

Immunoglobulin fractionation of RFT1 ascites on Protein A Sepharose (buffer regime 2)

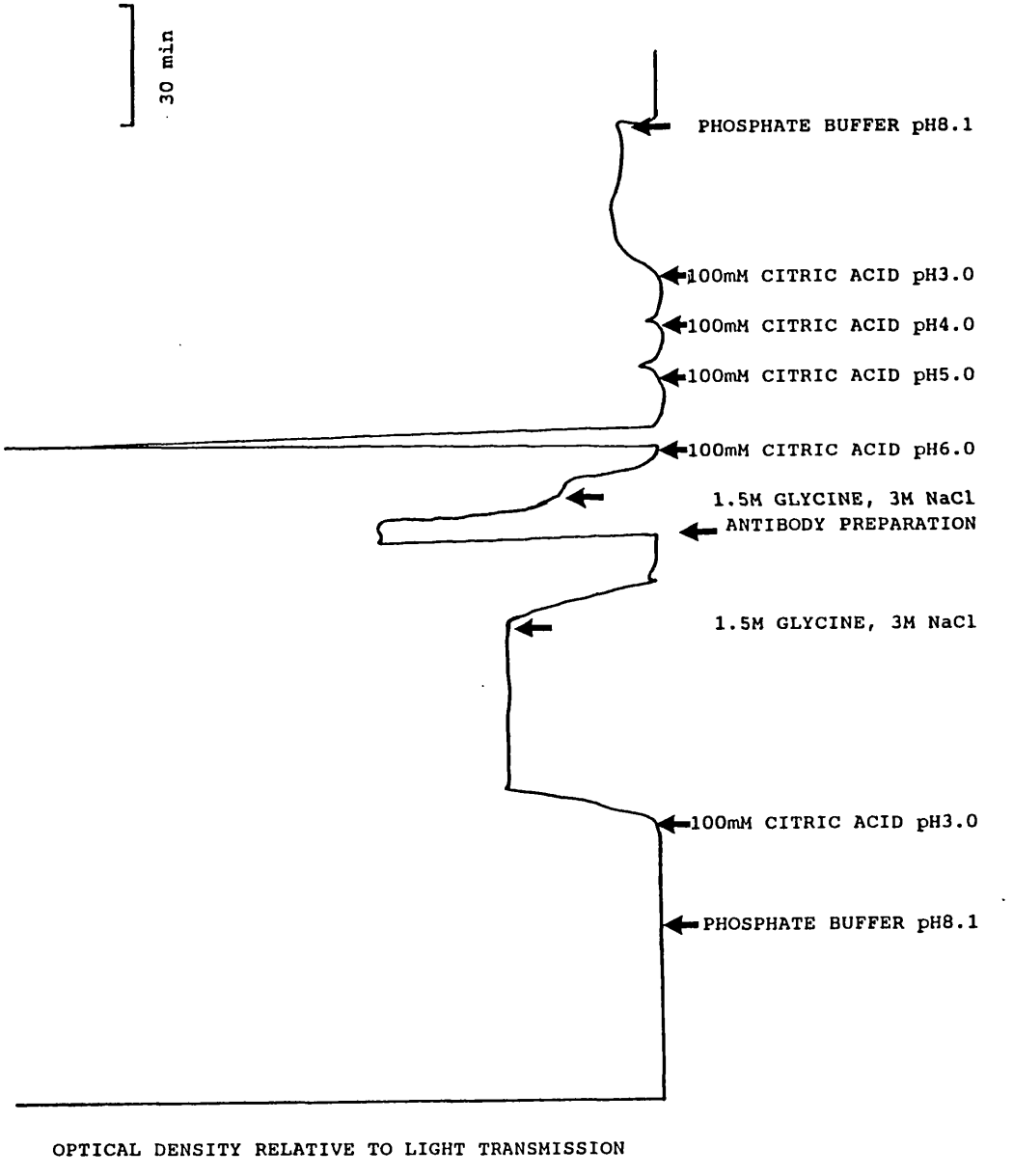


Figure 3.4: Five millilitres of RFT1 ascites was semi-purified by ammonium sulphate precipitation and then further purified by protein A affinity chromatography using buffer regime 2. This was diluted with 1.5M glycine and 3M NaCl. A protein A sepharose column was regenerated with 100mM citric acid pH 3.0, and then washed with 1.5M glycine and 3M NaCl (flow rate 250ul/min). The antibody preparation was loaded onto the column at 50ul/ml. The column was washed with 1.5M glycine and 3MNaCl at a flow rate of 250ul/min. The antibody was then eluted from the column using a stepwise regime of citric acid: pH 6.0 (IgG_1), pH 5.0 (IgG_{2a}), pH 4.0 ($IgG_{2b/3}$) and pH 3.0. The column was then washed with phosphate buffer pH 8.1.

Elution of OKT9 from a DEAE cellulose column

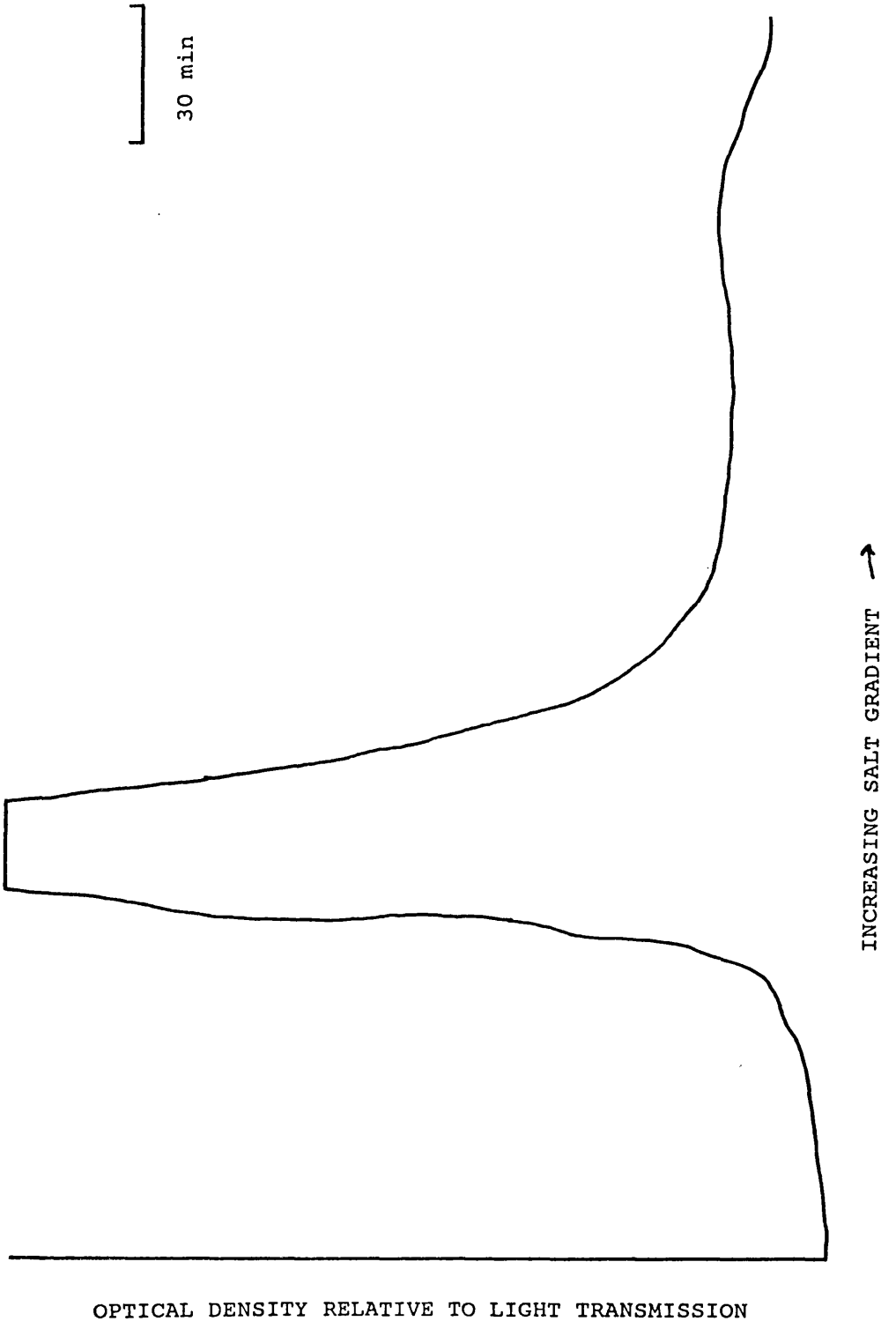


Figure 3.5: Five millilitres of OKT9 ascites was semi-purified by ammonium sulphate precipitation. The column was loaded onto an equilibrated DE52 column. This was then washed with 100mM Tris-HCl and the IgG was eluted using a salt concentration gradient 0-300mM NaCl.

because of the economy of this method, as a new column could be used for each preparation, eliminating the need to regenerate the columns and thus preventing cross contamination between MAbs.

Examples of purification of monoclonal antibodies using these three methods are shown in figures 3.3, 3.4, and 3.5.

3.3 TITRATION AND EVALUATION OF MONOCLONAL ANTIBODIES

Monoclonal antibodies were generally titrated on PBMC. Activation markers, however, are not normally expressed upon these cells and for this reason cell lines (table 2.1) were cultured and used for their titration.

The cell lines used for the titration of the monoclonal antibodies were phenotyped to verify their lineage and ensure homogeneity, using the antibodies CD19, CD20, CD21, CD24, PCA-1, CD3 and CD5 (table 2.3). The expression of these markers is shown in table 3.2).

Nalm-6 is a pre-B cell line. Of the markers tested it expressed CD19 only, a B cell antigen that is expressed on bone marrow B cell precursors which appear at the earliest stages of B-cell differentiation. EB-3, Daudi and Raji are cell lines developed from Burkitt's lymphoma patients and have lymphoblastoid features. They expressed the pan-B antigens CD19, CD20 and CD21 (table 3.2), B cell markers present on the cell throughout maturation (although CD19 is lost during differentiation to the plasma cell). CD24 expression is variable in the Daudi and Raji cell lines (Knapp *et al*, 1989). The Raji cell lines in this study did not express appreciable levels of this marker. PCA-1 was not present on the Daudi cells but 50% of the Raji cells were positive for this marker.

Table 3.2 PHENOTYPES OF THE CELL LINES

	PERCENT POSITIVE						
CELL LINES	CD19	CD20	CD21	CD24	PCA-1	CD3	CD5
NALM-6	100	0	2	0	0	0	0
RAJI	100	100	100	10	50	4	5
LCL-16-9	75	90	75	50	20	0	0
LCL-17-6	75	100	70	60	10	2	0
EB-3	100	70	75	-	-	1	5
DAUDI	100	100	100	-	3	1	4
HPB-ALL	1	2	2	-	3	90	85
MOLT 4	3	0	1	-	1	3	75

The EBV clones, again 'lymphoblastoid' in character, expressed CD19, CD20, and CD21. The B cell lines did not express either of the T cell markers, CD3 or CD5.

HPB-ALL and Molt-4 are 'T cell like' in character and were originally cloned from patients with acute lymphoblastoid leukaemia. HPB-ALL expressed high levels of both pan T markers - CD3 and CD5. Molt-4, although positive for CD5 did not express CD3. No B cell markers were expressed by the T cell lines.

These results compared favourably with the expected phenotype of these cell lines (Knapp *et al*, 1989).

For flow cytometric analysis all antibodies were titrated on the cell lines to establish optimal concentration for maximum binding. The dilution of the antibody was plotted against mean fluorescence intensity (MFI). Mean fluorescent intensity is an arbitrary measurement of the density of the antigen on the cells. It is influenced by the number of fluorochrome particles bound to the antibody and is amplified in double layer systems. Calibration of the flow cytometer and titration of the antibodies ensures consistency between experiments. A selection of the titrations performed are shown in figure 3.6 (CD3, CD4 and HLA-DR were directly conjugated to PE (Becton Dickinson, Cowley), RFT10 (CD38), 4F2 (in house) and PNL (peanut lectin) (Sigma) were directly conjugated to biotin and detected with streptavidin-PE (table 2.3) (Serotech, Oxford). Dilutions at the beginning of the plateau were taken as the optimal concentration (dotted line). At dilutions below optimum, saturation of the binding sites upon the cell was not obtained, positivity was underestimated

Titration and evaluation of Monoclonal Antibodies to detect optimum Antibody concentration

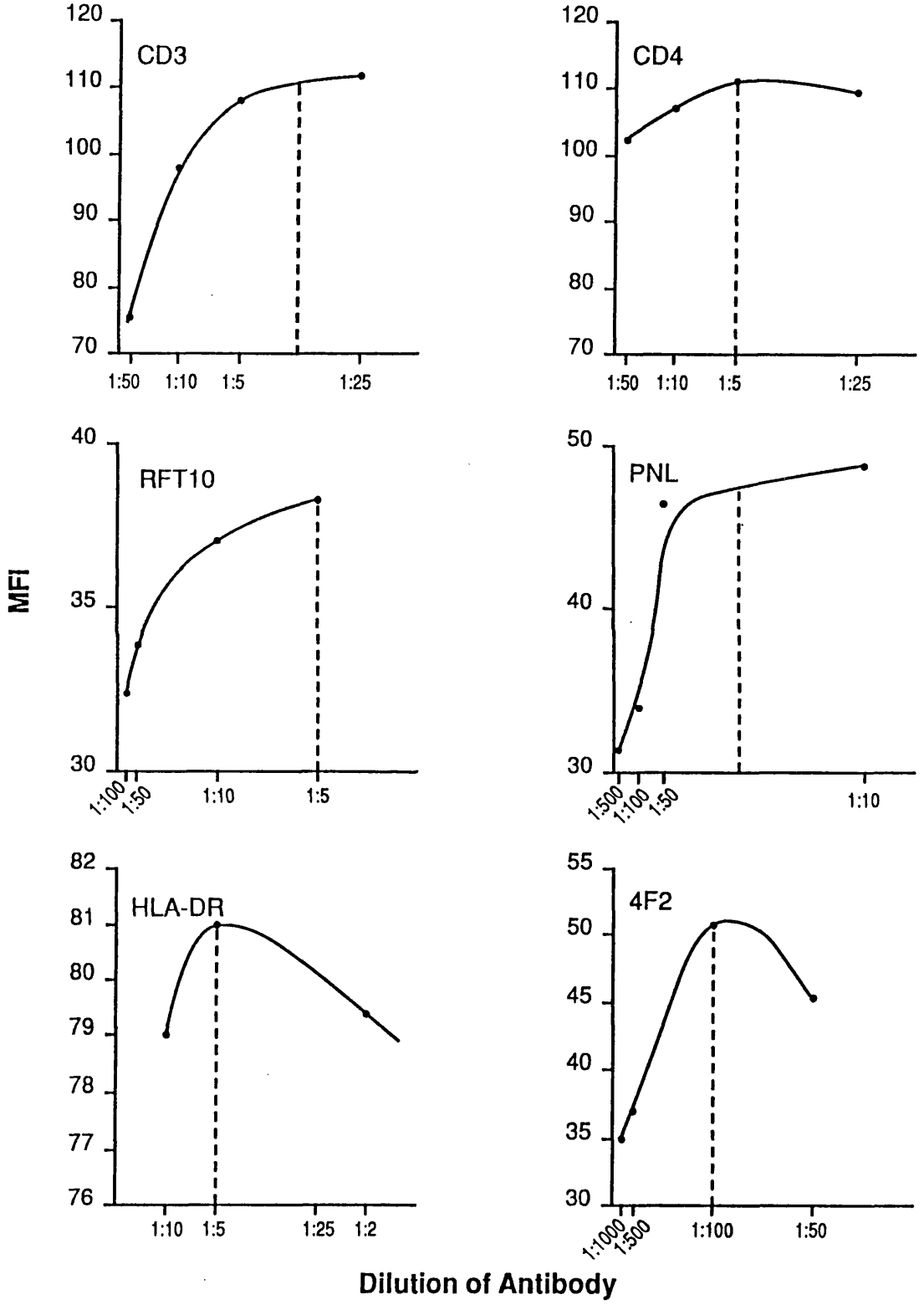


Figure 3.6: Flow cytometric analysis of titrations of monoclonal antibodies on PBMC or cell lines. CD3, HLA-DR and CD4 were directly conjugated to fluorochrome. RFT10, PNL, and 4F2 were conjugated to biotin and detected with streptavidin-PE. Optimum concentration is shown by (-----).

and the MFI was low. At dilutions above optimum, the antigen binding sites were saturated and maximum positivity and MFI were obtained. Use of excess antibody was wasteful and in some cases produced a high-dose-hook effect as seen with 4F2 and HLA-DR. The titrated antibodies were then used for the analysis of activation marker antigens on T and B lymphocytes.

3.4 ASSESSMENT OF THE MONOCLONAL ANTIBODY PANEL FOR THE ACUTE ACTIVATION STUDY

The antibody panel was designed to detect early, mid and late stages of T and B lymphocyte activation (figure 1.3) to elucidate if the infusion of concentrates activated or partially activated the lymphocytes in the haemophilic patients. Using CD3 as the pan T reagent, the T cell panel consisted of HLA-DR, 4F2, CD71 and CD25. Using CD20 as the pan B reagent, the B cell panel consisted of CD23, CD71, 4F2, PNL (peanut lectin), CD38 and PCA-1 (table 2.3).

Following the analysis of this panel on a small sample of controls and patients, the data was assessed to see if all the antibodies were equally useful in detecting activation in any of the patient groups.

Ten patients from each of the following groups: normal controls, HIV seronegative and HIV seropositive haemophiliacs, were analysed. There was no elevation of activation markers in the HIV-ve haemophiliacs, nor was there any elevation in the levels of 4F2, CD71 or CD25 on T cells or CD23 on B cells in the HIV+ve patient group compared to the control group. HLA-DR on T cells and 4F2, CD71, CD38 and PCA-1 on B cells were significantly elevated in the HIV+ve haemophiliacs compared to both the control group and the HIV-ve

Table 3.3 COMPARISON OF ACTIVATION MARKERS FROM THE ORIGINAL PANEL
(Range ± median)

	NORMAL CONTROL (n=10)	HAEMOPHILIACS HIV-ve (n=10)	HIV+ve (n=10)
T CELL			
HLA-DR	0.2-12.5 (3.4)	0.0-9.7 (3.7)	6.2-38.8* (17.8)
CD25	0.7-7.7 (2.1)	0.8-7.0 (3.0)	0.8-8.4 (3.2)
4F2	0.2-76.7 (27.3)	0.0-91.5 (27.6)	0.2-67.0 (53.4)
CD71	0.3-13.0 (0.9)	0.0-0.9 (0.3)	0.3-3.0 (1.4)
B CELL			
CD23	1.5-39.0 (9.9)	3.2-12.6 (5.9)	2.1-13.1 (6.8)
CD71	0.5-7.3 (2.4)	0.5-16.0 (3.9)	1.2-35.4* (18.1)
4F2	2.5-57.6 (31.6)	3.2-64.5 (22.4)	23.9-72.9* (60.9)
PNL	1.9-60.0 (10.5)	1.3-27.7 (14.4)	10.0-59.0 (35.5)
CD38	0.9-39.8 (8.4)	2.3-29.3 (7.7)	3.5-67.7* (31.6)
PCA-1	0.0-7.8 (4.2)	0.3-22.5 (1.5)	2.0-17.3* (10.8)

* significantly elavated compared to control and HIV-ve haemophilicas $p<0.05$

haemophiliacs ($p<0.02$).

A useful activation marker was classified as having a low expression in the control group or with a relatively small range so changes from normal would be easily identified. Several antibodies in the panel were found not to fulfil these criteria. 4F2 on T cells and PNL and CD23 on B cells, had a wide range in all patient groups and controls (table 3.3), although the median was elevated in the HIV+ve patients for 4F2 and PNL. PNL was thought to show the same cell population as CD38, however, very early two-colour, immunofluoresecence, microscope evaluations indicated that this was not the case. These antibodies did not appear to be contributing any useful information to the study so they were eliminated from the panel. 4F2 on B cells did not appear to be contributing any extra information to the panel and therefore was also eliminated.

The panel of monoclonal antibodies used for the detection of cellular activation antigen in the patient study was:

T cells - HLA-DR, CD25, CD71.

B cells - CD71, CD38, PCA-1.

3.5 PHENOTYPING CELLS SEPARATED BY THE WHOLE BLOOD METHOD

To reduce the time taken to label the cells with the T cell chronic activation antigen antibodies (using 4 layers, section 2.4.1.1) a 'whole blood' analysis was developed (section 2.3.2.2). In this method the lymphocytes were not separated from the other cells by density centrifugation, but the red cells were lysed with an ammonium chloride solution and the lymphocytes were distinguished from the other leucocytes using

a programme developed on the flow cytometer (figure 3.7). The method also enabled discreet analysis of the three white cell populations. To determine whether the cell separation method and programme could isolate true populations of leucocytes, the cells were analysed with a panel of monoclonal antibodies. The monoclonal antibodies CD5 (T cell marker), CD19 (B cell marker), CD14 (monocyte marker) show the specificity of this analysis as these antibodies do not appear on the other cell types (table 3.4). CD24 was used to detect granulocytes. This marker also appears on a percentage of peripheral B cells, but the physical differences of these cells makes contamination of their respective bitmaps unlikely.

The isolated cells were labelled with a panel of antibodies: CD5, CD3, CD20, CD19, CD14, CD24, W6 32 and M148 (table 2.3). The reactivity of these antibodies was assessed for each leucocyte (table 3.4). Both pan T reagents: CD3 and CD5 were bound to a subset of lymphocytes as expected, however, a subpopulation of granulocytes and monocytes weakly expressed these markers, but the range of expression between experiments was large. The B cell markers CD19 and CD20 were fairly specific for these cells with only negligible staining on the monocytes and granulocytes. CD14, a monocyte marker, labelled 90% of the cells present in the monocyte bitmap. There was minimal staining of lymphocyte population (5%), indicating that there may be contamination into the top end of the lymphocyte bitmap by smaller monocytes. There was also minimal granulocyte staining. CD24 is a marker for B cells and granulocytes and had 100% reactivity with both these cell populations. There was some staining of the monocytes. W6 32

EXPRESSION OF HLA CLASS I ON LYMPHOCYTES, MONOCYTES, AND GRANULOCYTES

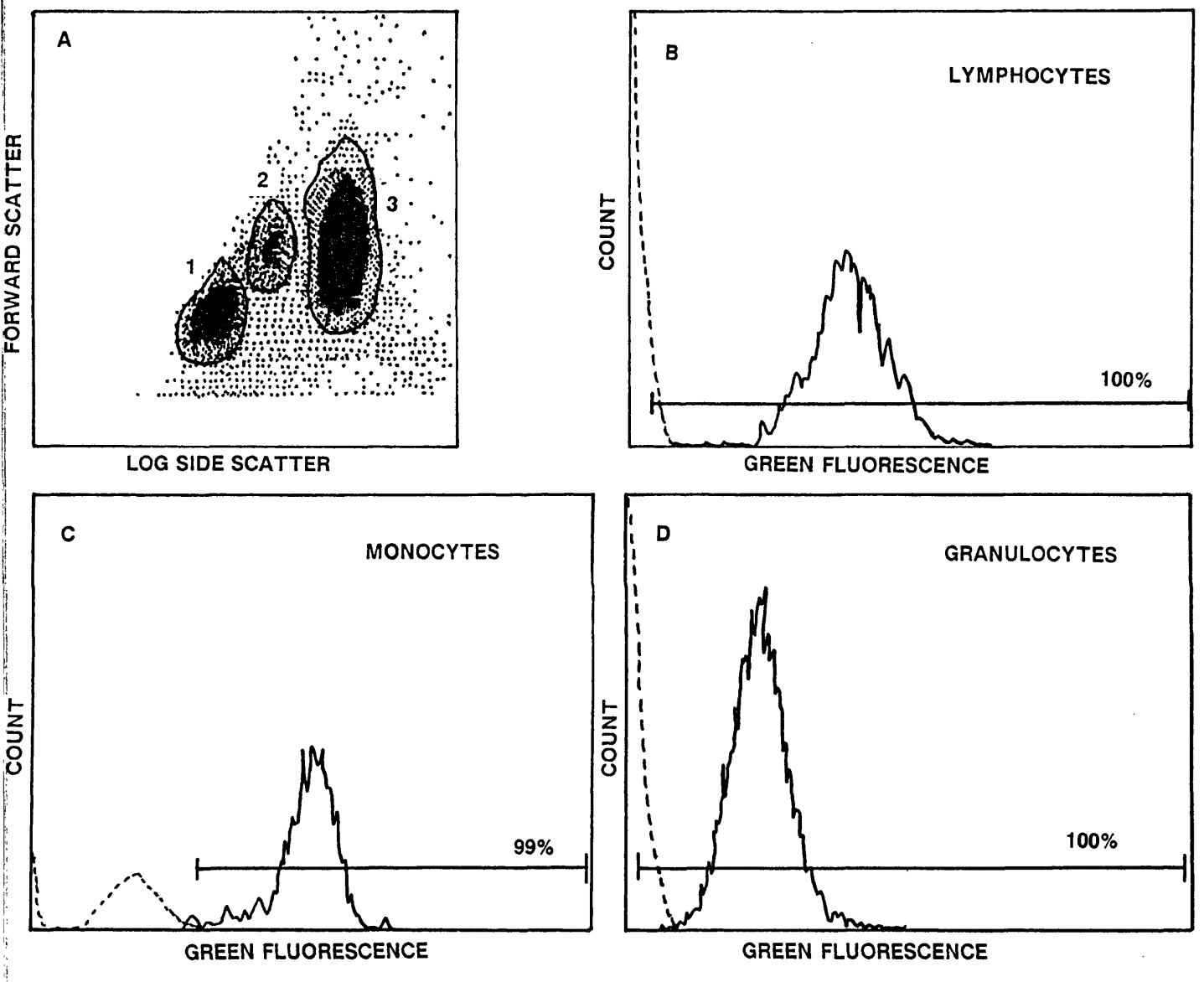


Figure 3.7: A whole blood preparation was stained with an HLA class I antibody. The leucocytes were identified on the basis of their size and side scatter characteristics, and isolated from each other by the creation of three bit maps. The fluorescent signal from each bit map was analyzed in the separate histograms: bit map 1, lymphocytes, histogram B; bit map 2, monocytes, histogram C; bit map 3, granulocytes, histogram D. Positive cells were determined as those of which the green fluorescence was greater than 98% of the mouse Ig control (----).

Table 3.4 EXPRESSION OF PHENOTYPIC MARKERS UPON LYMPHOCYTES, MONOCYTES AND GRANULOCYTES ANALYSED USING THE WHOLE BLOOD METHOD

(mean ± standard deviation of 3 experiments)

MARKER	PERCENT POSITIVE LYMPHOCYTE	MONOCYTE	GRANULOCYTE
CD5	60.0±2.0	2.4±7.6*	11.6±16.1*
CD3	61.5±3.3	16.6±7.6*	32.4±15.4*
CD20	7.7±0.7	0.4±0.5	1.1±0.2
CD19	7.5±0.7	1.5±0.2	1.0±8.4
CD14	4.1±1.1	90.0±2.5	9.8±2.1*
CD24	8.7±1.4	15.3±10.0*	99.3±1.1
W6 32	99.9±0.1	99.9±0.1	99.9±0.1
MI48	5.3±4.5	48.4±10.3*	24.2±8.7*

* low MFI

is a HLA marker and it was expressed upon all three cell types (figure 3.7). CD41, a platelet marker, was present, to some degree, upon all the cells particularly monocytes. This indicated that platelets and platelet residues were probably sticking to the cells (Pilkington *et al*, 1987).

Phenotyping of the leucocytes isolated using the whole blood method, indicated that there was little contamination of the three cell populations when they were separated by their forward angle and 90^{o} light scatter characteristics.

In general, all the antibodies bound in small amounts to the monocytes and granulocytes, but this was variable and was an indication of their 'stickiness' which becomes greater when they are activated. The method was modified by reducing the force of centrifugation to 150g. The use of fresh ammonium chloride solution also prevents endotoxin and microbial contamination and will prevent some degree of activation (P Eggleton, personal communication). These modification were not necessary for the lymphocyte studies.

The unique scattering characteristics of the three cell types, lymphocytes, monocytes and granulocytes enabled individual phenotyping of the cells in a single preparation. This proved useful in detecting the binding capacities of antibodies from the B cell workshop and antibodies to platelets that were being raised in the department.

The whole blood method was also a rapid technique for preparing the lymphocytes and a smaller amount of blood could be used than was required for density gradient preparations. This was of particular importance for anti-HIV+ve patients who had reduced lymphocyte numbers. The assay was incorporated into the study and was used for up to half of the samples

collected for the chronic activation study.

Recently both Becton Dickinson and Coulter Electronics have introduced whole blood methods, in which the red cells are lysed after staining with antibodies. Both techniques are available as packages and the Coulter method incorporates the 'Q-Prep', an enclosed mechanised lysing and fixing system.

4. *IN VIVO* ACTIVATION STUDY

There is an equal body of evidence for both activation and inhibition of the immune system of the haemophiliacs, both attributed to infusion of clotting factor concentrates. The apparent suppression of the immune response both *in vivo* and *in vitro* could be a consequence of activation *in vivo* rendering the cells refractory to further stimulus. Monoclonal antibodies were used to detect a panel of T and B cell activation markers. Their expression was studied in the HIV-ve patients to detect if activation of the cells was occurring. Conversely, if inhibition of immune function, induced by the concentrates, was occurring, then the expected elevated expression of the activation markers in the HIV+ve patients would be reduced. HLA-DR, CD25, CD71 and CD38 are all transient markers of activation, which disappear as the cell returns to its resting state. Markers of chronic activation, which occur as a result of repeated activation, would indicate if chronic and persistent stimulation was occurring. For this, the expression of CD45RO on T cells and PCA-1 on B cells and the presence in the serum of sIL2-R and elevated IgG were measured.

4.1 RESULTS FROM THE CELLULAR ACTIVATION STUDY

4.1.1 T CELLS

4.1.1.1 No T cell activation in HIV-ve haemophiliacs, but HLA-DR expression is elevated in HIV+ve haemophiliacs

There was minimal expression of the T cell activation markers in the control group (HLA-DR, 3.2%, CD25, 2.1%, CD71, 0.9%). Results are given as median values for each marker (table 4.1, figure 4.1).

TABLE 4.1: LYMPHOCYTE ACTIVATION MARKER EXPRESSION IN ALL SUBJECTS STUDIED

(Median ± range)

MARKER	NORMAL MALES	HAEMOPHILIACS	
		HIV-ve	HIV+ve
PERCENTAGE OF T CELLS POSITIVE FOR:			
HLA-DR	3.2 (0.0-27.0)	4.4 (0.0-11.4)	13.3* ** (4.6-38.8)
CD25	2.1 (0.4-7.7)	1.5 (0.3-7.0)	1.7 (0.0-8.4)
CD71	0.9 (0.3-13.0)	0.4 (0.0-2.9)	1.3*** (0.3-8.5)
PERCENTAGE OF B CELLS POSITIVE FOR:			
CD71	3.4 (0.5-16.4)	3.7 (0.0-31.0)	16.0* ** (1.2-58.2)
CD38	10.9 (0.9-39.8)	8.4 (0.2-40.5)	35.7* ** (3.5-67.7)
PCA-1	3.0 (0.0-7.8)	1.7 (0.0-22.5)	12.0** + (0.1-74.8)
Serum IgG (g/l)	8-18 (normal range)	13.5 (3.6-21.1) (n=21)	17.1++ (9.8-28.7) (n=27)

* Significantly elevated from normal p<0.0001
** Significantly different from HIV-ve haemophiliacs p<0.0001
*** Significantly different from HIV-ve haemophiliacs p<0.004
\+ Significantly elevated from normal p=0.0001
++ Significantly different from HIV-ve haemophiliacs p=0.008

Lymphocyte Activation in Normal Controls and in Anti-HIV Seropositive and Seronegative Haemophiliacs

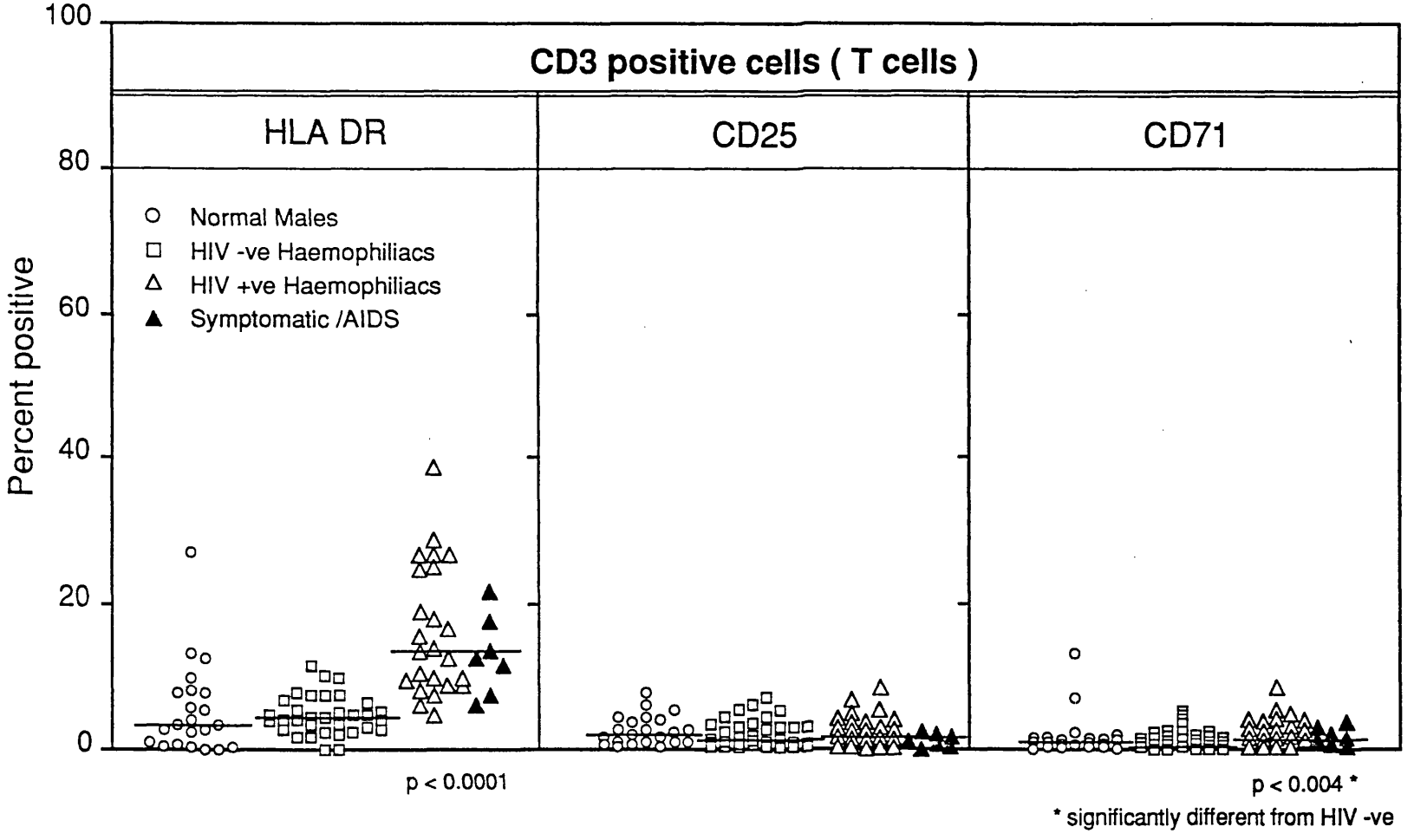


Figure 4.1

There was no elevation of transient cellular activation markers in the HIV-ve patients compared to the control group. HLA-DR, CD25 and CD71 expression on the T cells of HIV-ve haemophiliacs was minimal (HLA-DR, 4.4%, CD25, 1.5%, CD71, 0.4%). CD25 expression upon the T cells of the HIV+ve patients was also normal (1.7%), but CD71 (1.3%) although not raised above levels seen in the normal controls, was marginally significantly elevated above that of the HIV-ve patients ($p<0.004$) (figure 4.1).

HLA-DR expression, however, was significantly elevated in the HIV+ve patients (13.3%) compared to both HIV-ve haemophiliacs and the control group ($P<0.0001$) (table 4.1, figure 4.1). The level of expression was elevated on both T cell subsets, but this was seen predominately on the T8 subpopulation (T4, 8.5%, T8, 26.7%) ($p=0.0042$) (table 4.2, figure 4.2).

4.1.1.2 No Chronic T cell activation in HIV-ve haemophiliacs, but HIV+ve haemophiliacs have elevated levels of CD45RO

In the normal controls 27.7% of T4 cells expressed CD45RA and 52.0% of T4 cells expressed CD45RO. There was no evidence of chronic activation of the cells in the HIV-ve or HIV+ve haemophiliacs where expression of CD45RA/RO on these cells was not different from that seen in the normal controls.

53.4% of the T8 cells in the normal controls expressed CD45RA and 38.8% expressed CD45RO. Expression of these markers on the HIV-ve and HIV+ve haemophiliacs did not differ from the controls with the exception of CD45RO expression was significantly elevated in HIV+ve patients (44.4%) ($p=0.0058$) compared to HIV-ve haemophiliacs (31.0%). It was not

TABLE 4.2: HLA-DR EXPRESSION ON T CELL SUBSETS

(Median ± range)

	T4	T8
NORMALS (n=21)	2.2 (0.4 - 8.2)	3.8 (1.1 - 14.9)
HIV-ve (n=17)	3.0 (0.5 - 8.0)	4.3 (0.4 - 16.1)
HIV+ve (n=18)	8.5* ** (3.6 - 43.2)	26.7* + ++ (4.4 - 62.6)

* Significantly different from normal p<0.0001
** Significantly different from HIV-ve haemophiliacs p=0.0001
\+ Significantly different from HIV-ve p<0.0001
++ Significantly elevated compared to T4 in HIV+ve haemophiliacs p=0.0042

HLA-DR Expression on T Cell Subsets in Normal Controls and in Anti-HIV Seropositive and Seronegative Haemophiliacs

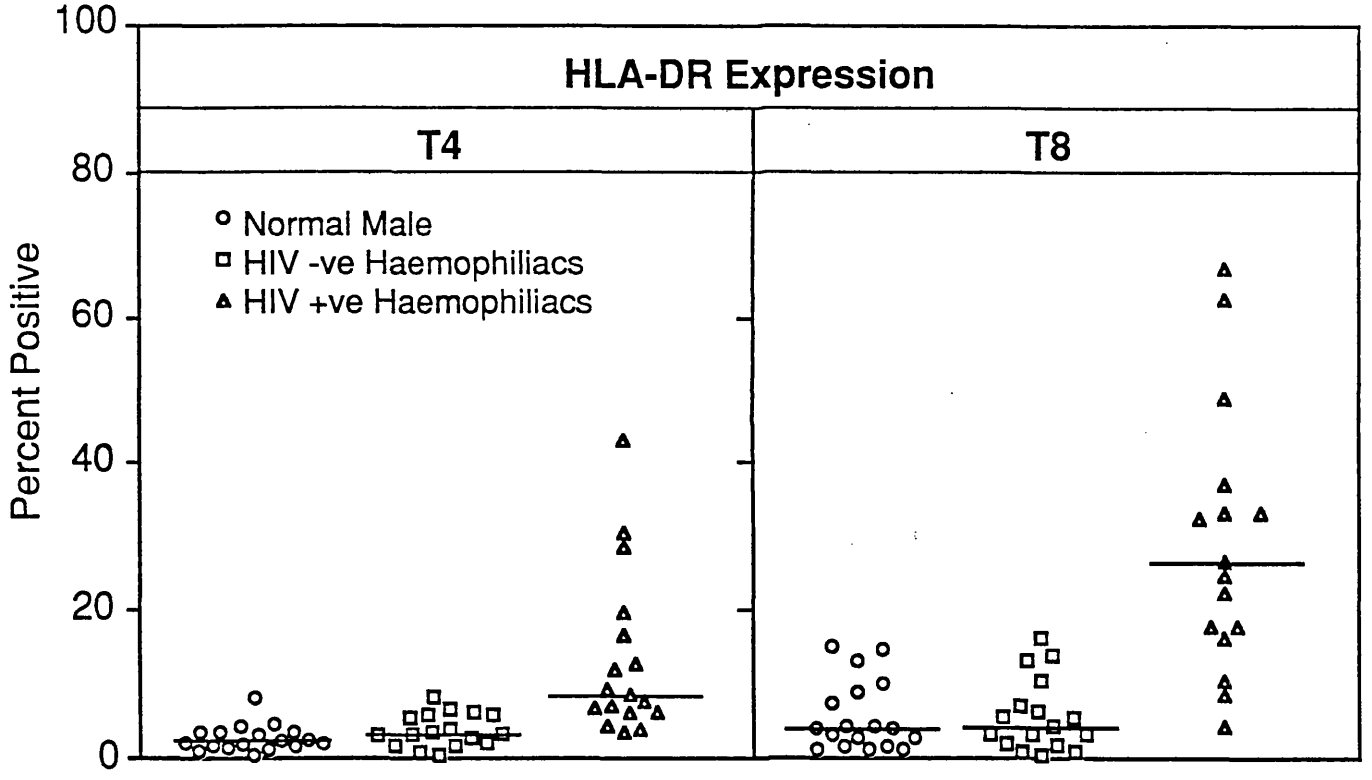


Figure 4.2

elevated, however, when compared to the normal control group (table 4.3).

4.1.2 B CELLS

4.1.2.1 No B cell activation in HIV-ve haemophiliacs

B cells of the normal controls did not express high levels of acute (CD71, 3.4%, CD38, 10.9%) or chronic (PCA-1, 3.0%) activation markers (table 4.1, figure 4.3). Neither was there was elevation of activation markers on B cells in the HIV-ve haemophiliacs (CD71, 3.7%, CD38, 8.4%, PCA-1, 1.7%). There was no elevation of serum IgG compared to the normal controls (8-18g/l) in the HIV-ve haemophiliacs (13.5g/l). IgM and IgA were also normal in these patients (data not shown).

4.1.2.2 HIV+ve haemophiliacs have activated B cells

All the B cell markers were significantly elevated in the HIV+ve patients indicating both acute (CD71, 16.0%, CD38, 35.7%) and chronic (PCA-1, 12.0%) activation in these patients ($p<0.0001$) (table 4.1, figure 4.3), when compared to normal controls and with the same degree of significance when compared to HIV-ve patients.

Serum IgG levels were significantly elevated in the HIV+ve patients (17.1g/l) compared to the HIV-ve patients (13.5g/l) ($p=0.008$). IgM and IgA levels in general, were normal (data not shown).

TABLE 4.3: CD45RA/RO EXPRESSION ON T CELL SUBSETS

(Median ± range)

	T4		T8	
	CD45RA	CD45RO	CD45RA	CD45RO
NORMAL (n=21)	27.7 (6.4-53.4)	52.0 (26.6-75.1)	53.4 (28.7-85.0)	38.8 (19.0-65.3)
HIV-ve (n=17)	26.6 (11.4-89.8)	46.6 (20.5-97.0)	63.5 (37.3-91.0)	31.0 (10.6-62.9)
HIV+ve (n=18)	34.7 (13.1-53.6)	53.5 (30.0-80.8)	54.0 (21.4-78.4)	44.4* (28.3-73.9)

* Significantly elevated compared to HIV-ve haemophiliacs p=0.0058

Lymphocyte Activation in Normal Controls and in Anti-HIV Seropositive and Seronegative Haemophiliacs

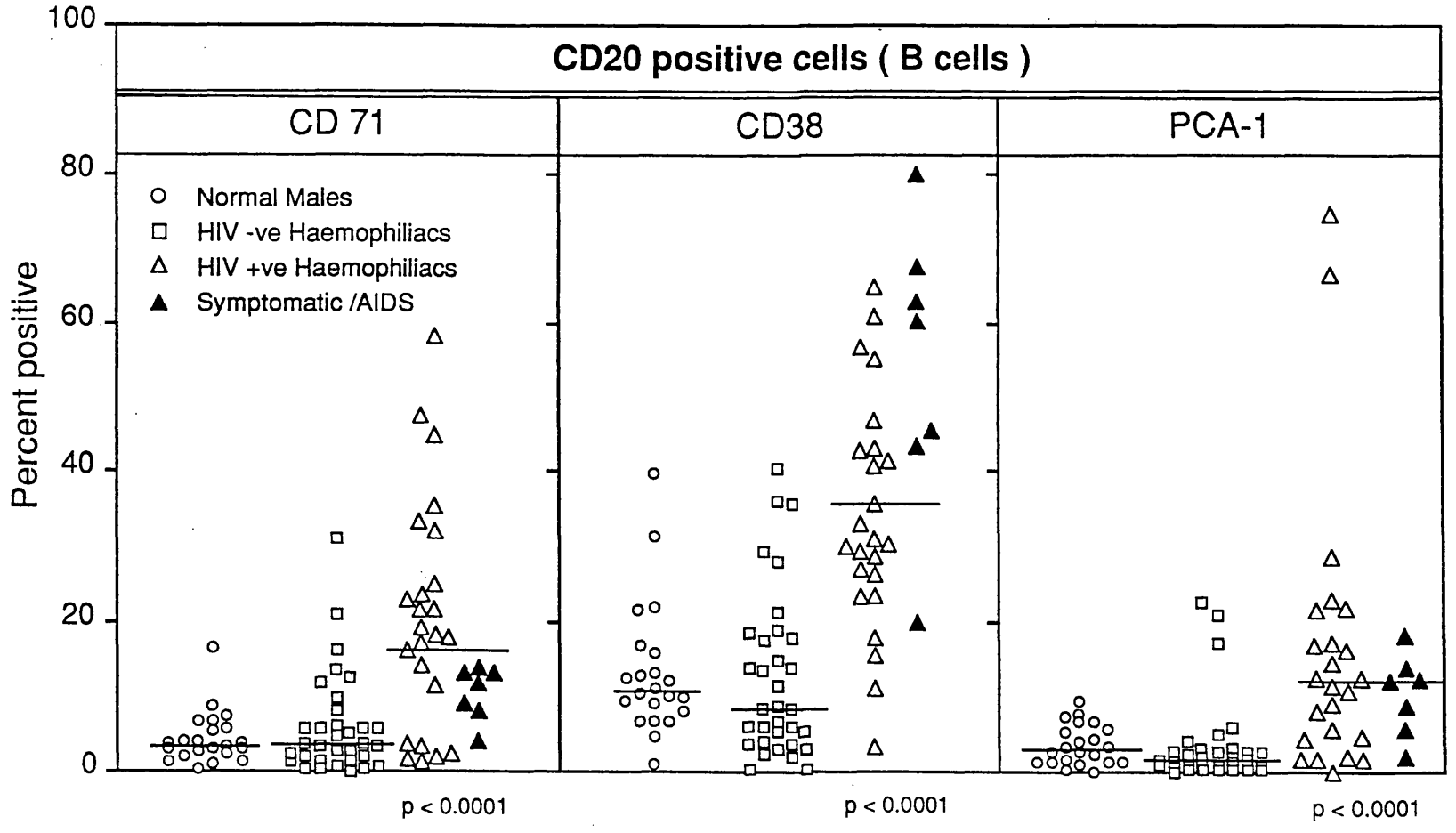


Figure 4.3

4.1.3 STATISTICAL ANALYSIS OF VARIANTS

The following clinical information was analysed to assess the effects on the activation of the patients' lymphocytes: concentrate infusion (type, amount, frequency) HCV seropositivity, liver dysfunction (determined by elevated transaminase levels), and HIV disease (CDC classification, T4 counts, months seroconverted). Due to the availability of data this analysis was performed upon the patients from the Royal Free Hospital only.

4.1.3.1 Concentrate infusion does not activate lymphocytes

There was no correlation between activation marker expression and FVIII/FIX usage (amount of concentrate the patients had received the year preceding testing) (table 4.4). The most significant correlation found was with PCA-1 where FVIII/FIX usage and expression of this marker showed a weak negative correlation ($r=-0.336$) in the HIV-ve patients. There was no correlation with concentrate usage and the elevation of activation marker expression in the HIV+ve patients (figure 4.4). This would indicate that concentrate infusion neither inhibits nor increases HIV induced lymphocyte activation.

Treatment with FVIII/FIX type (table 4.5) appeared to have no significant effect upon activation of the lymphocytes in both the HIV-ve and HIV+ve patients, with the exception of CD71 and CD38 on B cells, which were elevated in HIV+ve patients receiving commercial FVIII and Scottish FVIII, and PCA-1 expression in HIV+ve patients receiving NHS FVIII which was low compared to the other groups. The numbers of patients who received one product was small in some of the groups so significance values could not be attained.

TABLE 4.4: CORRELATION (r_s) OF FACTOR VIII/FIX USAGE WITH LYMPHOCYTE ACTIVATION MARKERS IN ANTI-HIV NEGATIVE AND ANTI-HIV POSITIVE HAEMOPHILIACS

	Percentage of T cells positive for:	Percentage of B cells positive for:			Serum IgG (g/l)
	HLA-DR	CD71	CD38	PCA-1	
HIV NEGATIVE PATIENTS	-0.120	-0.071	0.098	-0.336	0.082*
HIV POSITIVE PATIENTS	-0.151	-0.213	-0.081	-0.214	-0.182**

* n=20
* n=27

Correlation Between Elevated Activation Markers and FVIII Usage in Anti HIV Seropositive Haemophiliacs

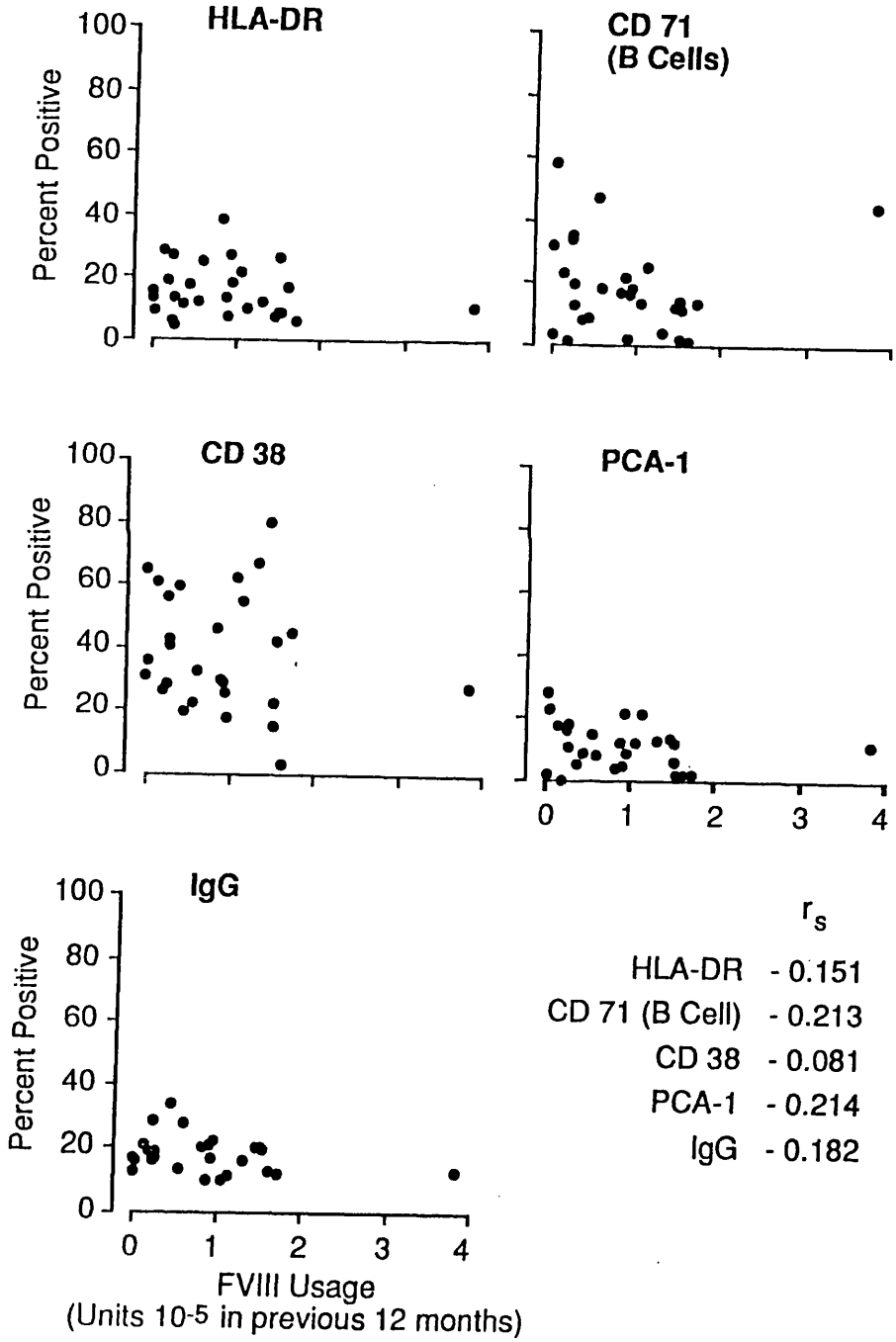


Figure 4.4

TABLE 4.5: COMPARISON OF ACTIVATION MARKERS WITH TREATMENT (FVIII/FIX) IN ANTI-HIV NEGATIVE AND ANTI-HIV POSITIVE HAEMOPHILIACS (median + range)

Treatment	Percentage of T cells positive for:		Percentage of B cells positive for:			Serum IgG (g/l)
	HLA-DR	CD71	CD71	CD38	PCA-1	
a) Anti-HIV negative patients						
Commercial FVIII (n=3)	4.4 (4.2-4.8)	1.1 (0.1-2.1)	5.9 (0.5-11.8)	14.8 (3.8-27.9)	1.9 (1.2-4.1)	11.6 (10.9-14.2
NHS FVIII (n=4)	4.2 (3.3-10.1)	1.4 (0.3-2.3)	4.9 (2.7-13.5)	12.1 (6.2-35.9)	3.1 (0.5-17.1)	12.9 (3.6-17.8)
NHS FIX (n=7)	3.9 (0.0-9.7)	0.4 (0.0-2.7)	5.7 (1.2-20.8)	11.4 (0.2-40.5)	1.4 (0.3-20.8)	13.4 (10.2-21.1
Scottish FVIII (n=11)	5.2 (1.8-11.4)	0.9 (0.1-2.9)	3.4 (0.0-8.2)	6.0 (0.3-35.7)	2.4 (0.3-3.5)	-
Scottish FIX (n=1)	4.7	1.8	2.1	8.4	1.2	-
b) Anti-HIV positive patients						
Commercial FVIII (n=14)	13.5 (7.4-38.8)	1.3 (0.3-3.6)	15.6 (1.2-58.2)	57.8 (3.5-80.3)	13.2 (2.0-28.5)	16.5* (9.8-21.1)
NHS FVIII (n=2)	18.5 (18.0-19.0)	5.8 (3.0-8.5)	9.8 (1.6-18.0)	22.0 (18.0-26.0)	4.6 (0.1-9.0)	20.6 (18.9-22.3
NHS FIX (n=1)	8.9	1.4	2.0	15.4	12.3	20.0
Scottish FVIII (n=3)	9.4 (8.1-24.5)	1.3 (0.4-1.8)	21.7 (3.5-23.7)	40.6 (11.1-43.1)	66.6 (1.6-74.8)	- (* n=1

The patients were grouped into those who were treated prophylactically (greater than once per week), infrequently (less than once per week but greater than once per month) or irregularly (less than once a month). This analysis showed no significant effect upon activation of the lymphocytes in either the HIV-ve or HIV+ve patients (table 4.6).

There was no correlation between the expression of the activation markers with the time that had elapsed since the patients were last treated prior to testing (table 4.7), but there were very weak associations with HLA-DR (r=0.460), CD71 (B cells) (r=0.433) and PCA-1 (r=0.497) expression in HIV-ve haemophiliacs. The majority of patients were treated more than once a month but the range of activation marker expression in these patients was large questioning these associations (figure 4.5).

There were no correlations with FVIII/FIX usage with CD45RO, CD45RA expression upon T cell subsets in either HIV-ve or HIV+ve haemophiliacs (table 4.8).

4.1.3.2 No relationship with HCV seropositivity

Haemophiliacs have been assaulted with several transfusion transmitted viruses. 79.6% of the haemophiliacs in this study were found to be seropositive for hepatitis C virus (HCV) and similar numbers were seen in both HIV-ve and HIV+ve patients (80.6% and 78.3% respectively).

There was no apparent difference in lymphocyte activation marker expression in either HIV-ve or HIV+ve haemophiliacs who

TABLE 4.6: COMPARISON OF ACTIVATION MARKERS WITH FREQUENCY OF FVIII/FIX INFUSION IN ANTI-HIV NEGATIVE AND ANTI-HIV POSITIVE HAEMOPHILIACS

(median ± range)

Frequency of infusion	Percentage of T cells positive for:		Percentage of B cells positive for:			Serum IgG (g/l)
	HLA-DR	CD71	CD71	CD38	PCA-1	
a) Anti-HIV negative patients						
> once/week (n=7)	3.4 (0.1-4.8)	0.3 (0.1-2.1)	5.9 (0.4-16.0)	13.8 (2.3-29.3)	1.8 (0.0-22.5)	13.8 (11.6-15.9)
<once/wk>once/month (n=8)	5.0 (0.0-10.1)	0.5 (0.0-2.9)	3.2 (1.2-20.8)	14.5 (3.1-40.5)	1.0 (0.3-20.8)	13.6 (3.6-21.1)
<once/month (n=4)	3.1 (2.8-4.2)	0.2 (0.1-0.3)	8.8 (5.0-16.0)	12.2 (0.2-29.3)	9.2 (1.0-22.5)	11.5 (10.9-12.3)
b) Anti-HIV positive patients						
> once/week (n=11)	13.7 (7.4-38.8)	1.4 (0.8-2.8)	18.0 (2.0-44.7)	32.9 (15.4-67.7)	11.4 (4.3-21.6)	20.0 (9.8-27.9)
<once/wk>once/month (n=9)	16.6 (6.0-26.7)	1.3 (0.4-8.5)	13.2 (1.2-47.6)	29.2 (3.5-62.9)	12.0 (0.1-21.9)	17.1 (9.9-28.7)
<once/month (n=6)	10.6 (4.6-28.7)	1.2 (0.3-2.7)	27.4 (8.1-58.2)	58.6 (35.7-65.1)	17.2 (5.7-28.5)	16.5 (13.1-21.2)

TABLE 4.7: CORRELATION (r_s) OF DAYS NO TREATMENT PRIOR TO TESTING WITH LYMPHOCYTE ACTIVATION MARKERS IN ANTI-HIV NEGATIVE AND ANTI-HIV POSITIVE HAEMOPHILIACS

	Percentage of T cells positive for:	Percentage of B cells positive for:			Serum IgG (g/l)
	HLA-DR	CD71	CD38	PCA-1	
HIV NEGATIVE PATIENTS	0.460	0.433	-0.040	0.497	-0.113*
HIV POSITIVE PATIENTS	0.014	-0.004	0.277	-0.072	-0.042**

* n=20
** n=27

Correlation Between HLA-DR, CD71 (B Cells) and PCA-1 Expression and Days Without Treatment Prior to Testing in Anti-HIV Seronegative Haemophiliacs

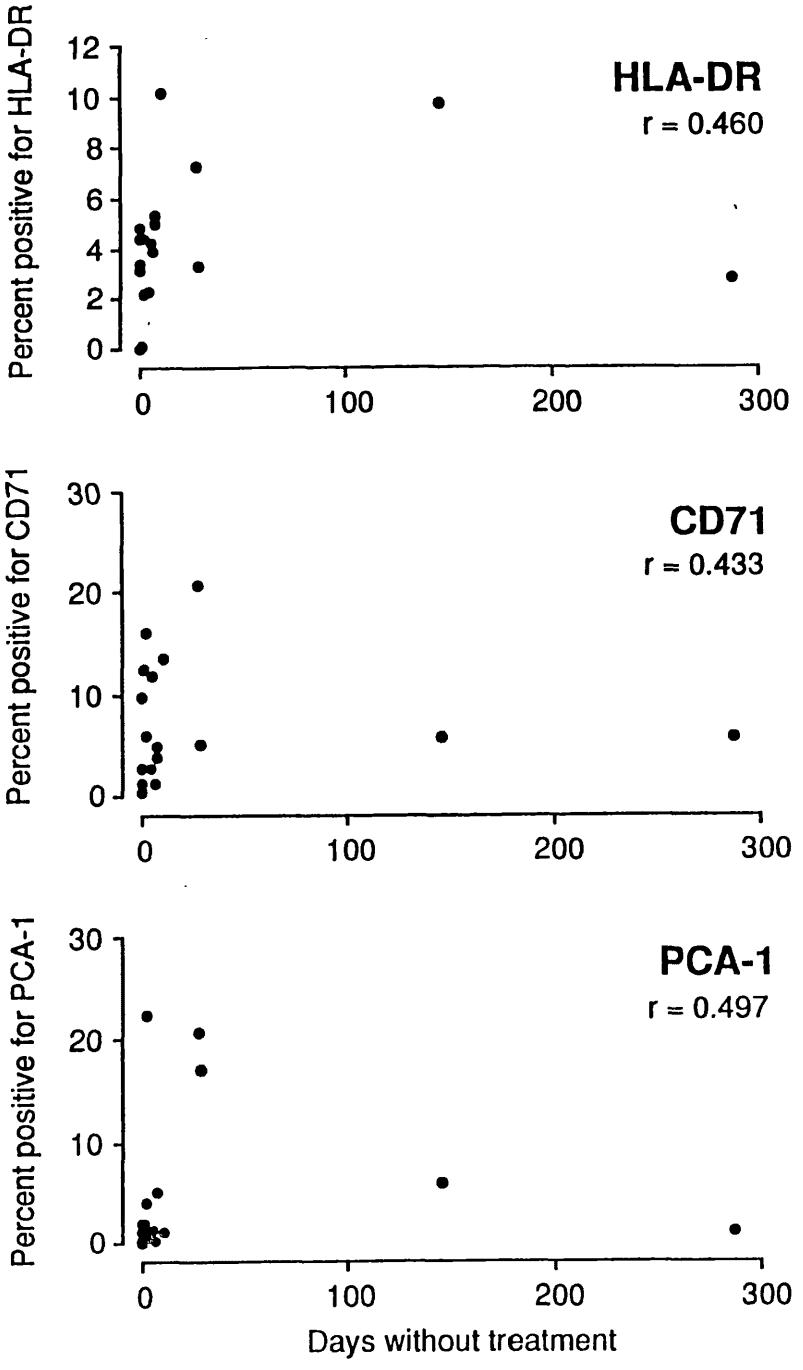


Figure 4.5

TABLE 4.8 CORRELATION (r_s) OF VARIOUS PARAMETERS WITH CD45RA, CD45RO AND ON T4 AND T8 SUBSETS IN HIV+ve HAEMOPHILIACS

	T4		T8	
	CD45RA	CD45RO	CD45RA	CD45RO
T4/ CD45R0	-0.379			
T8/ CD45RA	0.606	-0.342		
T8/ CD45RO	-0.101	0.333	-0.575	
T4/ HLA-DR	-0.261	0.371	-0.422	0.653
T8/ HLA-DR	0.024	0.424	-0.266	0.487
FVIII USE	-0.009	-0.202	-0.045	-0.085
T4 COUNT	-0.101	0.136	0.575	-0.598
MoS	-0.157	-0.139	0.008	0.064

MoS months of serconversion

were seropositive or seronegative for HCV (table 4.9). Statistical analysis of these data was difficult due to low numbers of HCV-ve patients. Expression of antigens did not appear to be different between the patient groups, with the exception of CD38 in the HIV-ve patients (HCV-ve, 16.2%, HCV+ve, 7.7%) and CD71 on B cells in HIV+ve patients (HCV-ve, 34.5%, HCV+ve 16.6%), where expression of the markers was greater in the HCV-ve individuals. CD38 (HCV-ve, 26.8%, HCV+ve, 38.7%) and serum IgG levels (HCV-ve, 11.6g/l, HCV+ve, 18.0g/l), in the HIV+ve haemophiliacs were higher in those individuals who were HCV+ve also. Although the medians of these values were different, HCV+ve and HCV-ve groups had corresponding ranges.

4.1.3.3 No relationship with liver disease

Aminotransferase levels are well documented for their usefulness in the analysis of active liver damage related to infection with hepatic virus in haemophiliacs. Aspartate amino transferase (AST) and glutamyl transferase (gamma GT) levels were measured as standard clinical procedure in the Haemophilia Centre. AST levels were utilised in preference to gamma GT levels in the analysis due to their wider application within the patients studied.

There were no significant correlations with AST levels and the activation markers (table 4.10) with the exception of HLA-DR and AST (r=-0.424) in the HIV-ve patients and CD38 and gamma GT levels (r=0.420) in the HIV+ve patients where small associations were observed. There was also a slight correlation between gamma GT and T4 cell numbers (r=-0.474) in

TABLE 4.9: COMPARISON OF ACTIVATION MARKER EXPRESSION AND HCV POSITIVITY IN ANTI-HIV NEGATIVE AND ANTI-HIV POSITIVE HAEMOPHILIACS

(median ± range)

HCV Positvity	Percentage of T cells positive for:		Percentage of B cells positive for:			Serum IgG (g/l)
	HLA-DR	CD71	CD71	CD38	PCA-1	
a) Anti-HIV negative patients						
HCV NEGATIVE (n=4)	3.8 (2.3-10.1)	0.5 (0.3-2.9)	7.3 (0.4-13.5)	16.2 (6.0-35.9)	0.8 (0.0-1.2)	11.6 (3.6-17.8)
HCV POSITIVE (n=14)	4.4 (0.0-9.7)	0.4 (0.0-2.7)	5.4 (0.5-31.0)	7.7 (0.2-40.5)	1.9 (0.2-20.8)	13.4 (10.2-17.6
b) Anti-HIV positive patients						
HCV NEGATIVE (n=2)	13.1 (12.5-13.7)	1.3 (1.2-2.3)	34.5 (21.4-47.6)	26.8 (23.3-30.3)	13.6 (12.5-14.6)	11.6 (9.8-13.4)
HCV POSITIVE (n=10)	15.0 (4.6-38.8)	1.2 (0.3-8.5)	16.6 (1.2-58.2)	38.7 (3.5-56.8)	9.5 (0.1-22.8)	18.0 (13.1-27.9

TABLE 4.10: CORRELATION (r_s) OF AST AND GAMMA GT LEVELS WITH ACTIVATION MARKER EXPRESSION IN ANTI-HIV NEGAT AND ANTI-HIV POSITIVE HAEMOPHILIACS

	Percentage of T cells positive for:	Percentage of B cells positive for:			Serum IgG (g/l)
	HLA-DR	CD71	CD38	PCA-1	
a) Anti-HIV negative patients					
AST U/l	-0.424	-0.074	0.045	-0.032	0.13
gamma GT U/l	-0.119	0.023	0.017	0.025	-0.08
b) Anti-HIV positive patients					
AST U/l	-0.027	0.038	0.229	-0.082	0.27
gamma GT U/l	0.088	-0.056	0.420	-0.179	0.08

HIV+ve patients, indicating that HIV infection may be involved in liver dysfunction. gamma GT and AST levels only correlated in those patients who were HIV+ve (r=0.639), no relationship was seen in the HIV-ve patients (r=0.399).

Analysis of AST levels was performed in two ways: either by analysis of the enzyme on the day of activation marker testing, to assess acute liver dysfunction, and grouping them as normal (<40U/l), elevated (40-80U/l) or high (>80U/l) (table 4.11), or, by long term AST level analysis over the previous 2 years, to assess chronic liver dysfunction, where the patients were grouped as normal (AST<40), elevated or fluctuating or consistently high (AST>80) (table 4.12).

No relationship was found with activation marker expression and acute or chronic liver dysfunction in either the HIV-ve patients or the HIV+ve patients, although CD38 expression was higher in those patients with high AST levels (<80U/l) compared to patients with lower AST levels (<80U/l) in either HIV-ve or HIV+ve patients (table 4.11) (HIV-ve: AST<40U/l, 12.1%, 40-80U/l, 10.1%, <80U/l, 17.7%; HIV+ve AST<40U/l, 31.0%, 40-80U/l, 30.3%, <80U/l, 43.5%). CD71 and CD38 expression on B cells in HIV-ve haemophiliacs with consistently high AST levels was low (table 4.12) (2.7%, 6.2% respectively) in comparison with those patients with normal (7.7%, 11.8% respectively) or elevated/fluctuating AST levels (4.8%, 12.6% respectively). In contrast, HIV+ve patients who had elevated/fluctuating AST levels had high levels of CD71 and CD38 on their B cells (15.1%, 45.1% respectively) as did those patients with consistently high AST levels (18.0%, 41.5%

TABLE 4.11: COMPARISON OF ACTIVATION MARKERS AND AST LEVELS IN ANTI-HIV NEGATIVE AND ANTI-HIV POSITIVE HAEMOPHILIACS

(median ± range)

AST level	Percentage of T cells positive for:		Percentage of B cells positive for:			Serum IgG (g/l)
	HLA-DR	CD71	CD71	CD38	PCA-1	
a) Anti-HIV negative patients						
<40 (n=8)	4.8 (3.3-10.1)	0.5 (0.1-2.7)	7.4 (0.1-31.0)	12.1 (3.8-40.5)	1.5 (0.0-20.8)	13.0 (11-1-17.8)
40-80 (n=8)	4.2 (0.0-9.7)	0.4 (0.0-2.1)	4.7 (0.8-12.5)	10.1 (2.3-27.9)	1.5 (0.3-5.9)	13.7 (10.9-19.5)
>80 (n=5)	2.8 (2.2-5.0)	0.2 (0.1-2.9)	4.8 (1.2-16.0)	17.7 (0.2-35.9)	1.0 (0.3-22.5)	13.7 (3.6-21.1)
b) Anti-HIV positive patients						
<40 (n=7)	13.4 (9.7-21.6)	1.8 (0.3-8.5)	13.0 (1.2-58.2)	31.0 (3.5-62.9)	11.4 (0.1-22.8)	16.5 (9.9-19.2)
40-80 (n=15)	12.5 (6.2-38.8)	1.3 (0.3-3.0)	16.0 (2.0-47.6)	30.3 (15.4-80.3)	12.3 (1.8-21.9)	20.1 (9.8-33.6)
>80 (n=6)	14.0 (4.6-26.7)	1.0 (0.3-1.6)	18.7 (4.2-33.5)	43.5 (29.2-67.7)	11.7 (2.1-28.5)	16.6 (12.2-27.9)

TABLE 4.12: COMPARISON OF ACTIVATION MARKERS AND AST FUNCTIONAL GROUPS IN ANTI-HIV NEGATIVE AND ANTI-HIV POSITIVE HAEMOPHILIACS
(median ± range)

AST Group	Percentage of T cells positive for:		Percentage of B cells positive for:			Serum IgG (g/l)
	HLA-DR	CD71	CD71	CD38	PCA-1	
a) Anti-HIV negative patients						
NORMAL (n=10)	4.8 (3.3-10.1)	0.4 (0.1-2.7)	7.7 (0.4-31.0)	11.8 (5.4-40.5)	1.6 (0.2-20.8)	12.5 (10.9-17.8)
ELEVATED/ FLUCTUATING (n=8)	3.4 (0.0-5.3)	0.3 (0.0-2.1)	4.8 (0.8-16.0)	12.6 (0.2-29.3)	1.0 (0.3-22.9)	15.7 (10.2-21.1)
HIGH (>80) (n=3)	3.1 (2.3-5.0)	2.3 (0.2-2.9)	2.7 (1.2-4.8)	6.2 (2.3-35.9)	0.5 (0.3-5.1)	13.7 (3.6-13.8)
b) Anti-HIV positive patients						
NORMAL (n=7)	12.5 (9.7-19.0)	1.1 (0.3-8.5)	3.7 (1.2-58.2)	28.7 (3.5-35.7)	4.6 (0.1-22.8)	16.5 (12.6-21.0)
ELEVATED/ FLUCTATING (n=14)	14.7 (6.2-38.8)	1.3 (0.4-3.6)	15.1 (2.0-35.4)	45.1 (15.4-80.3)	13.1 (1.8-28.5)	19.3 (9.9-33.6)
HIGH (>80) (n=7)	12.3 (4.6-26.7)	1.3 (0.3-3.0)	18.0 (4.2-33.5)	41.5 (18.0-60.4)	9.0 (2.1-12.6)	20.0 (12.2-28.7)

respectively) compared to HIV+ve patients with consistently normal AST levels (3.7%, 28.7% respectively). Although the medians of these values were different, the groups all had corresponding ranges.

4.1.3.4 CD38 expression correlates with HIV disease progression

The only evidence of activation in the haemophiliacs was in patients who were seropositive for HIV, therefore the effect of progression of HIV disease was assessed. This was determined in several ways: by classification into the CDC grouping system (CDC II, CDC IV_A and CDC IV_C) to ascertain the effects of symptomatic infection, or correlations with the number of months seroconverted and T4 counts.

Progression of HIV infection did not influence acute activation marker expression, with the exception of CD38 on B cells, which was significantly elevated in the patients in group CDC IV_C (65.3%) when compared to the group CDC II (30.7%) patients (p=0.006) (table 4.13, figure 4.6). Numbers were probably too low in this analysis, to determine true significance, but when the analysis was repeated, grouping the patients into asymptomatic, CDC II/II_B and symptomatic, CDC IV_A/IV_C, CD38 was still marginally significantly elevated in the symptomatic patients (30.3%, 60.4% respectively) (p=0.03) (table 4.13).

Expression of CD71 on B cells in the symptomatic patients was below the median for the HIV+ve group, this is illustrated in figure 4.3, however, although there a trend of low CD71

TABLE 4.13: COMPARISON OF ACTIVATION MARKERS WITH SYMPTOMS OF ARC/AIDS IN ANTI-HIV POSITIVE PATIENTS
(Median ± range)

CDC Group	Percentage of T cells positive for:		Percentage of B cells positive for:			Serum IgG (g/l)
	HLA-DR	CD71	CD71	CD38	PCA-1	
II (n=19)	14.7 (6.2-38.8)	1.3 (0.3-8.5)	20.0 (1.2-58.2)	30.7 (3.5-65.1)	12.4 (0.1-28.5)	17.0 (9.8-28.7)
II_B (n=3)	7.5 (4.6-26.7)	0.3 (0.3-1.6)	19.3 (2.2-33.5)	29.8 (29.2-41.5)	10.7 (4.6-16.1)	20.2 (17.1-21.0)
IV_A (n=3)	13.3 (6.0-17.5)	1.8 (0.3-3.0)	13.0 (9.0-13.7)	43.3 (20.0-45.4)	8.8 (2.1-18.2)	19.2 (12.2-33.6)
IV_C (n=4)	11.9 (7.4-21.6)	1.3 (0.8-3.6)	10.0 (4.2-13.2)	65.3* (60.4-80.3)	12.3 (5.7-13.7)	16.0 (9.9-20.0)
II/II_B (n=22)	13.7 (4.6-38.8)	1.2 (0.3-8.5)	19.3 (1.2-58.2)	30.3 (3.5-65.1)	12.3 (0.1-28.5)	17.1 (9.8-28.7)
$IV_{A/C}$ (n=7)	12.3 (6.0-21.6)	1.3 (0.3-3.6)	11.9 (4.2-13.7)	60.4** (20.0-80.3)	12.0 (2.1-18.2)	17.6 (9.9-33.6)

* Significant elevated compared to CDC II p=0.006
** Significantly elevated compared to II/II_B p=0.03

Comparison of Lymphocyte Activation in asymptomatic and symptomatic Anti- HIV seropositive Haemophiliacs

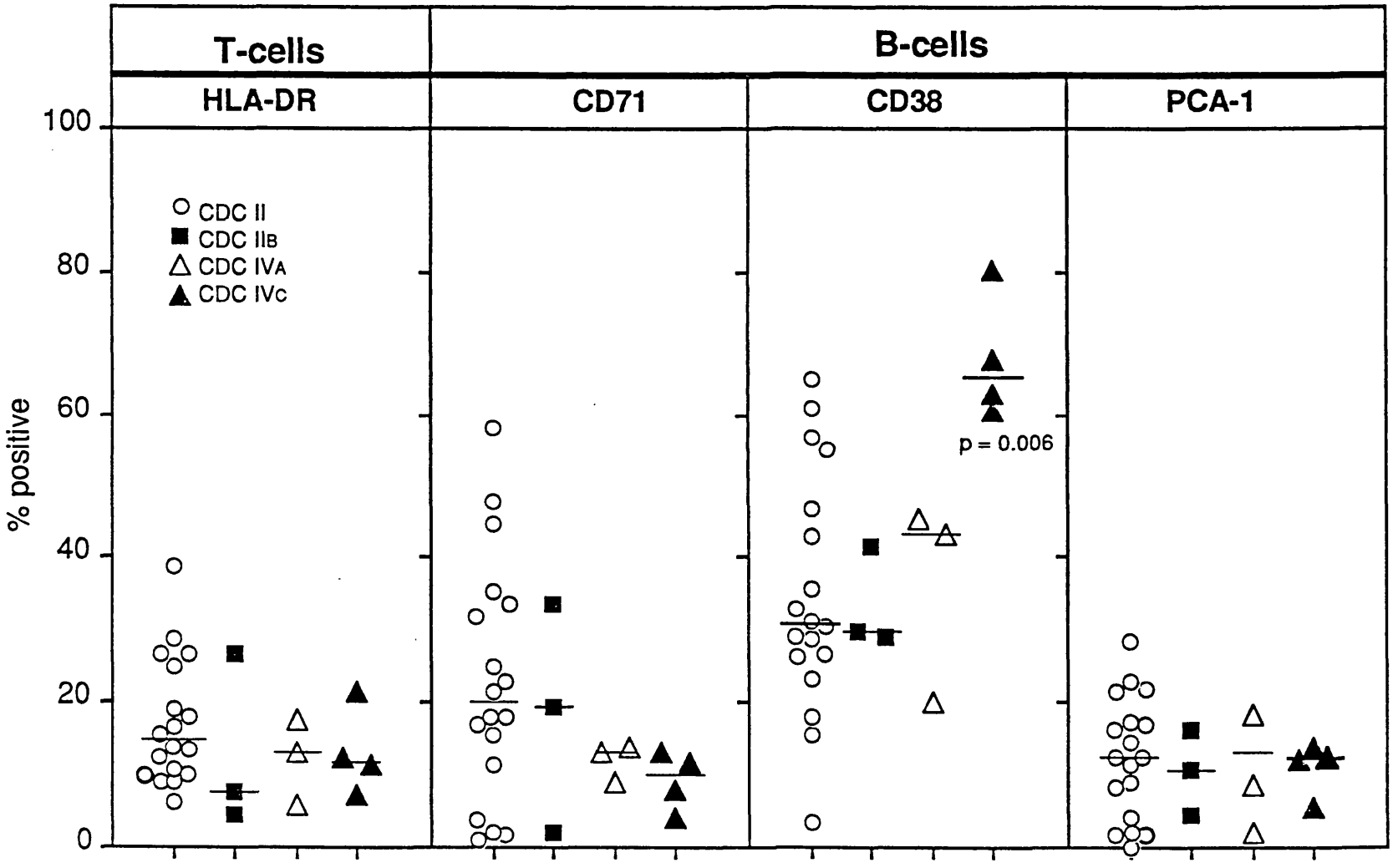


Figure 4.6

expression was evident this was not significantly different from that seen in the asymptomatic patients in the CDC classification analysis (CDC II/II_B, 19.3%, CDC $IV_{A/C}$, 11.9%).

Progression of disease, as determined by correlation of the activation markers with T4 count or months seroconverted (table 4.14), did not influence the levels of expression of the activation markers with the exception of CD25 (r=0.438), and CD71 (B cell) (r=0.415) with T4 count where there was a slight relationship.

There was a significant difference in the T4 counts between the HIV-ve and the HIV+ve haemophiliacs ($0.88x10^9/l$ and $0.42x10^9/l$ respectively) (p=0.0001) (table 2.4) of the sample group tested in this study.

Analysis of HLA-DR expression on T4 and T8 subsets demonstrated that the marker increased as T4 count decreased, this was seen as the negative correlation between T4 count and T4/HLA-DR expression (r=-0.530) (table 4.15) and by the increases in the expression of the HLA-DR upon T4 cells in those patients with levels of T4 less than $0.4x10^9/l$ (T4, 12.6%, T8, 32.9%), compared to patients with higher T4 counts (T4, 7.1%, T8, 19.2%) although this was not statistically significant (table 4.16), which probably related to the low numbers of patients in this analysis. This elevation was also seen in T8 cells but there was no correlation with T4 count and T8/HLA-DR expression (r=-0.390), however, HLA-DR expression on T4 and T8 cells was significantly correlated (r=0.835) (table 4.15). This increase in HLA-DR expression on

TABLE 4.14: CORRELATION (r_s) OF LYMPHOCYTE ACTIVATION MARKERS WITH HIV DISEASE IN ANTI-HIV POSITIVE HAEMOPHILIACS

	Percentage of T cells positive for:		Percentage of B cells positive for:			Serum IgG (g/l)
	HLA-DR	CD25	CD71	CD38	PCA-1	
T4 COUNT (10^9/l)	0.173	0.438	0.415	-0.371	0.268	-0.094
Months Seroconverted	0.074	0.178	0.132	0.384	0.202	0.269*

* n=26

TABLE 4.15: CORRELATION (r_s) OF VARIOUS PARAMETERS WITH HLA-DR EXPRESSION ON T4 AND T8 SUBSETS IN HIV+ve HAEMOPHILIACS

	HLA-DR	
	T4	T8
T8/HLA-DR	0.835	
T4 COUNT	-0.530	-0.390
MoS	0.282	0.353

MoS months of serconversion

TABLE 4.16: INFLUENCE OF T4 COUNT UPON CHRONIC ACTIVATION MARKER AND HLA-DR EXPRESSION ON T4 AND T8 SUBSETS OF ANTI-HIV SEROPOSITIVE HAEMOPHILIACS

(median ± range)

	T4 COUNT	
	$>0.4x10^9/l$ n=6	$<0.4x10^9/l$ n=12
T4/CD45RA	39.8 (15.0-53.6)	34.7 (13.1-51.7)
T4/CD45RO	53.7 (43.9-60.6)	52.1 (30.0-69.8)
T8/CD45RA	59.9 (51.8-78.4)	48.1 (21.4-76.7)
T8/CD45RO	38.0 (28.3-45.8)	45.7* (31.9-75.9)
T4/HLA-DR	7.1 (3.6-9.2)	12.6 (6.0-43.2)
T8/HLA-DR	19.2 (4.4-24.6)	32.9 (8.3-67.0)

* P=0.028

T4 and T8 cells was also seen in those patients with symptomatic disease (CDC IV) (T4, 30.4%, T8, 40.2%) compared to healthy patients (T4, 8.0%, T8, 25.7%) (table 4.17). Numbers of patients are very low in this analysis, no relationship is seen in the larger study of HLA-DR expression on $CD3^+$ T cells (table 4.13).

Analysis of chronic activation of T4 cells in the HIV+ve haemophiliacs demonstrated that the elevated levels of CD45RO on T8 cells were found predominately in those patients who had low T4 counts (table 4.16). This marker was significantly elevated ($p=0.028$) in patients whose T4 counts were less than $0.4x10^9/l$ (45.7%), compared to patients whose T4 cell levels were higher than this (38.0%).

There was a correlation between $T4/CD45RA^+$ and $T8/CD45RA^+$ expression in the HIV+ve patients ($r=0.606$) (table 4.8). As levels of $T8/CD45RA^+$ reduced, this correlated with an increased expression of $T8/CD45RO^+$ ($r=-0.575$). CD45RO expression on T8 cells correlated with HLA-DR expression of the T4 cells ($r=0.653$) and also slightly with HLA-DR expression on the T8 cells ($r=0.487$).

There was no correlation with T4 count and T4 chronic activation marker expression, however, there were relationships with $T8/CD45RA^+$ expression ($r=0.575$), and $T8/CD45RO^+$ expression ($r=-0.598$). Therefore as T4 count decreased the percentage of $T8/CD45RO^+$ cells increased.

TABLE 4.17: COMPARISON OF CHRONIC ACTIVATION MARKER EXPRESSION AND HLA-DR EXPRESSION ON T4 AND T8 SUBSETS WITH SYMPTOMS OF AIDS/ARC IN ANTI-HIV POSITIVE HAEMOPHILIACS

(median ± range)

	CDC CLASSIFICATION	
	CLASS II n=12	CLASS IV n=4
T4/CD45R	37.6 (13.2-53.6)	17.0 (13.1-46.1)
T4/CD45RO	53.7 (40.8-69.8)	44.9 (30.0-80.8)
T8/CD45RA	59.9 (21.9-78.4)	47.4 (21.4-51.5)
T8/CD45RO	43.7 (28.3-75.1)	47.2 (44.0-69.2)
T4/HLA-DR	8.0 (3.6-28.4)	30.4* (6.0-43.2)
T8/HLA-DR	25.7 (4.4-67.0)	40.2** (17.8-62.6)

* n=3
** n=2

There was no relationship with chronic activation markers and progression of HIV disease determined by months seroconverted (table 4.8) or CDC classification (table 4.17).

Many of the HIV+ve patients were either using AZT or were members of the Concorde trial, where a double blind testing regime was implemented, because of this it was difficult to perform analyses on the data regarding AZT treatment.

4.1.3.5 Inter-relationships between the activation markers

There were inter-relationships between expression of the different acute activation markers (table 4.18). In HIV-ve patients these were seen as slight correlations between HLA-DR and CD25 (r=0.419), HLA-DR and CD71 (T cell) (r=0.452), CD38 and CD71 (B cell) (r=0.616); and in the HIV+ve patients: HLA-DR and CD25 (r=0.593), HLA-DR and CD71 (T cell) (r=0.475) and CD71 (B cell) and PCA-1 (r=0.677) and CD25 and CD71 (B cell) (r=0.577).

The correlation of CD25 with HLA-DR expression in the HIV+ve patients is interesting as HLA-DR expression is elevated above normal levels in these patients and CD25 is not.

TABLE 4.18: CORRELATION (r_s) OF LYMPHOCYTE ACTIVATION MARKERS IN ANTI-HIV NEGATIVE AND ANTI-HIV POSITIVE HAEMOPHILIACS

		Percentage of T cells positive for:			Percentage of B cells positive for:		
		HLA-DR	CD25	CD71	CD71	CD38	PCA-1
a) Anti-HIV negative patients							
T cell	CD25	0.419					
	CD71	0.452	0.341				
B cell	CD71	0.157	0.281	0.294			
	CD38	-0.072	0.129	0.248	0.616		
	PCA-1	0.148	0.258	0.201	0.370	0.097	
	Serum IgG (g/l)	0.205	-0.239	-0.293	-0.022	-0.034	-0.007
b) Anti-HIV positive patients							
T cell	CD25	0.593					
	CD71	0.475	0.187				
B cell	CD71	0.097	0.557	0.022			
	CD38	-0.145	-0.040	-0.189	0.229		
	PCA-1	0.082	0.375	-0.051	0.677	0.353	
	Serum IgG (g/l)	0.323	0.031	0.321	-0.192	-0.265	-0.173

4.2 THE SOLUBLE IL-2 RECEPTOR STUDY

4.2.1 ALL PATIENT GROUPS HAVE ELEVATED sIL2-R LEVELS

To assess activation of non-circulating T cells soluble IL2-R levels were assessed.

Soluble IL2-R levels were raised in all patient groups. Elevation of sIL2-R was seen in the HIV+ve patients: HIV+ve haemophiliacs (mean and standard deviation: 762.4 $\pm$ 375.8 u/ml) and HIV+ve non-haemophiliacs (883.9 $\pm$ 518.3 u/ml), when compared to the normal controls (433 $\pm$ 241.4 u/ml) ($p<0.01$), however, in contrast to the cellular activation markers, significant elevation of sIL2R was also seen in HIV-ve haemophiliacs (658.9 $\pm$ 352.2 u/ml) ($0.05>p>0.02$). There were no significant differences between the patient groups (table 4.19, figure 4.7). The expected normal range for male and female donors was 273u/ml with an upper limit of 477u/ml as determined by the kit manufacturer (T cell Sciences, Cambridge), however, the normal range in this study was found to be 200-935 u/ml, somewhat higher than expected.

4.2.2 Statistical analysis of variants

As with the cellular activation markers these data were analysed to determine the influence of various parameters upon the elevation of sIL2-R levels.

4.2.2.1 Concentrate infusion did not cause elevation in sIL2-R levels

FVIII/FIX infusion did not affect sIL2-R levels in the haemophiliacs.

Treatment with the different types of FVIII/FIX was assessed but no statistical analysis was performed due to low patient

TABLE 4.19: SOLUBLE IL2-R LEVELS (u/ml) IN ALL SUBJECTS STUDIED

(Mean ± standard deviation)

	NORMAL MALE	HAEMOPHILIACS		NON-HAEMOPHILIACS
		HIV-ve	HIV+VE	HIV+ve
NUMBER	20	30	29	22
MEAN	433 ± 241.4	658.9 ± 352.2	762.4 ± 375.8	883.9 ± 518.3
RANGE	200 - 935	160 - 1860	335 - 1490	285 - 2010
p value*		p<0.05	p<0.01	p<0.01

* significance compared to normal

Soluble IL-2 ® levels in Normal Controls, Anti-HIV seronegative and seropositive Haemophiliacs, and in Anti-HIV seropositive Non-Haemophiliacs

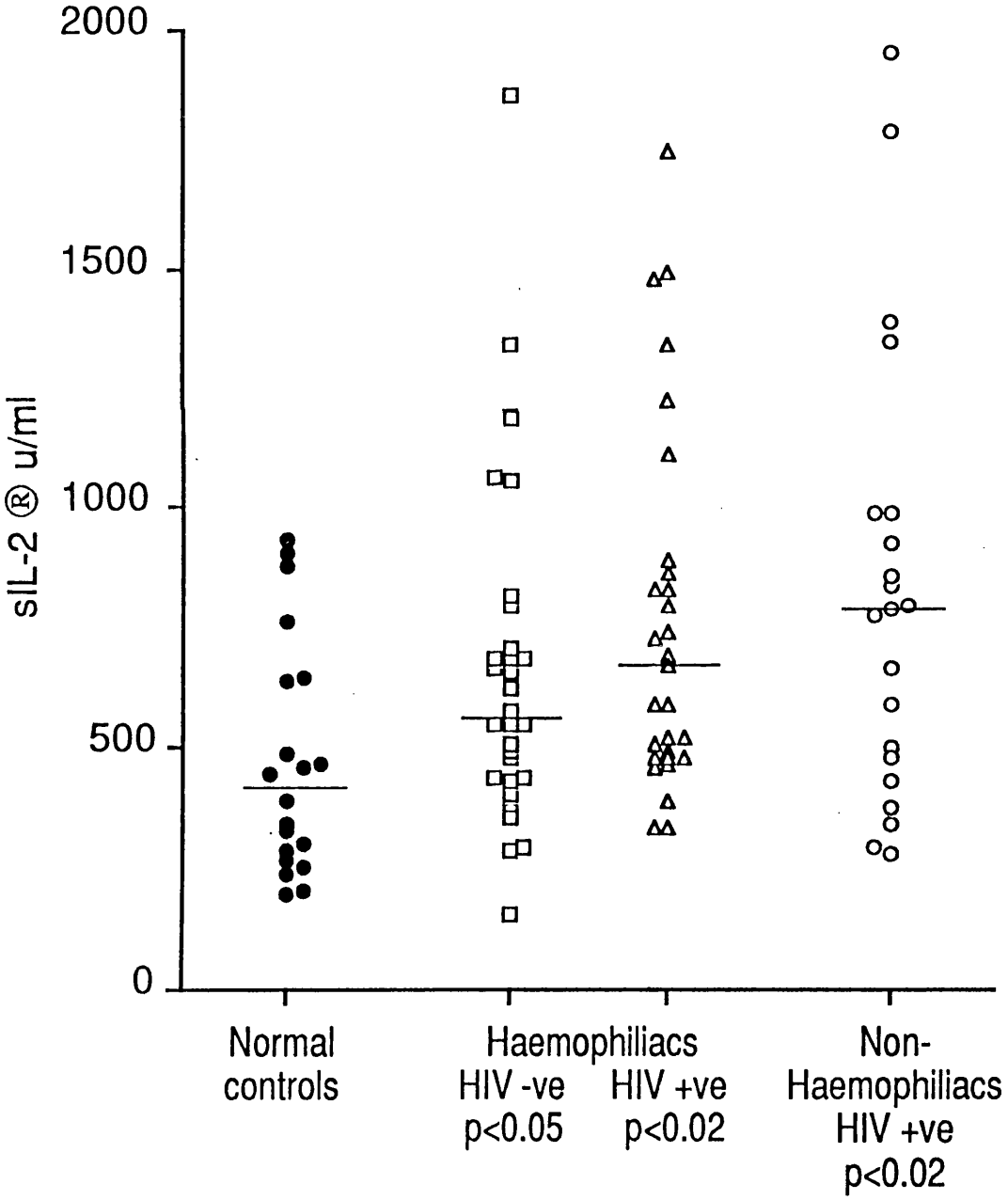


Figure 4.7

numbers in some groups (table 4.20). As with the patients analysed in the activation marker study there appeared to be no difference in IgG levels between the patients receiving the different FVIII/FIX types.

In HIV+ve haemophiliacs mean sIL2-R levels appeared to be higher in those patients treated with commercial FVIII (911.3 ± 414.9u/ml) compared to those receiving NHS FVIII (457.5 ± 95.5u/ml). This was also seen in the HIV-ve patients (810 ± 336.5u/ml and 669.3 ± 221.5u/ml respectively). The ranges of sIL2-R levels in both groups were, however, comparable.

The frequency of treatment, where statistical analysis could be performed (table 4.21), did not affect sIL2-R levels.

There was no significant correlation between FVIII/FIX usage (amount consumed in year before testing) with sIL2-R or between sIL2-R levels and days without treatment in both the HIV-ve patients ($r=0.355$ and $r=-0.303$ respectively) and HIV+ve patients ($r=-0.029$ and $r=-0.043$ respectively) (table 4.22).

4.2.2.2 HCV seropositivity influences sIL2-R levels in HIV+ve haemophiliacs only

The haemophiliac patients were analysed for HCV positivity to determine if there was a relationship between elevated sIL2-R levels and HCV infection. Although the majority of the haemophiliacs were positive for HCV making statistical analysis difficult, sIL2-R levels were significantly elevated in the HIV+ve haemophiliacs seropositive for HCV (913.0 ± 429.9u/ml) compared to those who were not (598.8 ± 161.7u/ml) ($0.05>p>0.02$) (table 4.23), no such relationship was found

TABLE 4.20: COMPARISON OF SOLUBLE IL2-R WITH TREATMENT (FVIII/FIX) IN ANTI-HIV NEGATIVE AND ANTI-HIV POSITIVE HAEMOPHILIACS

(Mean ± standard deviation and range)

TREATMENT	sIL2-R (u/ml)	sIgG (g/l)
a) Anti-HIV negative patients		
Commercial FVIII (n=3)	810 ± 336.5 (550 - 1190)	12.2 ± 1.7 (10.9 - 14.2)
NHS FVIII (n=7)	669.3 ± 221.5 (405 - 1060)	13.1 ± 4.6 (3.6 - 17.8)
NHS FIX (n=9)	648.7 ± 350.1 (160 - 1343)	13.6 ± 3.5 (9.9 - 21.1)
b) Anti-HIV positive patients		
Commercial FVIII (n=12)	911.3 ± 414.9 (335 - 1490)	17.0 ± 5.3 (9.8 - 28.7)
NHS FVIII (n=2)	457.5 ± 95.5 (390 - 525)	20.6 ± 2.4 (18.9 - 22.3)
Porcine FVIII (n=1)	590	19.2

TABLE 4.21: COMPARISON OF SOLUBLE IL2-R (u/ml) WITH FREQUENCY OF TREATMENT(FVIII/FIX) IN ANTI-HIV NEGATIVE AND ANTI-HIV POSITIVE HAEMOPHILIACS

(Mean ± standard deviation and range)

FREQUENCY	>3 TIMES/WEEK	<ONCE/WEEK >ONCE/MONTH	<ONCE/MONTH
a) Anti-HIV negative patients			
	746.0 ± 457.7 (335 - 1890) n=10	613.1 ± 302.8 (290 - 1343) n=9	627.5 ± 335.9 (160 - 1065) n=8
b) Anti-HIV positive patients			
	763.2 ± 379.6 (335 - 1490) n=12	862.6 ± 466.6 (480 - 1743) n=7	786.1 ± 342.0 (460 - 1480) n=7

TABLE 4.22: CORRELATION (r_s) OF VARIOUS PARAMETERS WITH SOLUBLE IL2-R LEVELS IN ANTI-HIV NEGATIVE AND ANTI-HIV POSITIVE HAEMOPHILIACS

	HIV-ve HAEMOPHILIACS	HIV+ve HAEMOPHILIACS
FVIII/FIX USAGE (units)	0.355	-0.029
DAYS WITHOUT TREATMENT WITH FVIII/FIX	-0.303	-0.043
IgG (g/l)	0.025	0.165
T4 (10^9/l)	-0.007	0.019
AST (U/l)	0.408	0.173
gamma GT (U/l)	0.148	0.033

TABLE 4.23: COMPARISON OF SOLUBLE IL2-R (u/ml) WITH HCV SEROPOSITIVITY IN ANTI-HIV NEGATIVE AND ANTI-HIV POSITIVE HAEMOPHILIACS

(Mean ± standard deviation and range)

	HCV-ve HAEMOPHILIACS	HCV+ve HAEMOPHILIACS
HIV-ve HAEMOPHILIACS	565.8 ± 155.6 (355 - 710) n=6	662.5 ± 383.5 (160 - 1860) n=20
HIV+ve HAEMOPHILIACS	598.8 ± 161.7* (480 - 830) n=4	913.0 ± 429.9* (390 - 1743) n=15

* Significant difference between HCV-ve and HCV+ve ($0.05>p>0.02$)

with the HIV-ve haemophiliacs. Although significance values were attained with the data it should be noted that the number of HCV-ve patients was very low.

Although liver transferase levels indicate liver dysfunction, probably related to infection, there was no relationship between the AST or gamma GT levels, on the day of analysis, and HCV positivity but again numbers of negative patients were too low for statistical evaluation (table 4.24).

4.2.2.3 AST levels influence sIL2-R levels in both HIV-ve and HIV+ve haemophiliacs

Correlation of sIL2-R levels between AST and gamma GT in HIV-ve and HIV+ve patients (r=0.408, r=0.148, r=0.173 and r=0.033 respectively) (table 4.22) showed only a slight association between AST levels and sIL2R in HIV-ve patients.

Analysing this data in a slightly different way, by grouping the patients by their AST levels on the day of sIL2-R testing: <40U/l, 40-80U/l and >80U/l to determine acute liver dysfunction (table 4.25), demonstrated that sIL2-R levels were related to AST levels only in those patients not infected with HIV. There was a marginally significant elevation of sIL2R in HIV-ve patients with levels between 40-80U/l (824.1 $\pm$ 421.9u/ml) (0.05>p>0.02) and patients with AST levels >80/l (796.6 $\pm$ 325.3u/ml) >80U/l (p=0.01) when compared to patients with AST levels <40U/l (527.1 $\pm$ 202.6u/ml) (figure 4.8). There was no significant difference, however, between the HIV+ve patient groups in this analysis.

The HIV+ve patients with normal AST levels, however, had higher sIL2-R levels compared to the HIV-ve patients with

TABLE 4.24: COMPARISON OF AST AND GAMMA GT LEVELS WITH HCV POSITIVITY IN ANTI-HIV NEGATIVE AND ANTI-HIV POSITIVE HAEMOPHILIACS

(Median ± range)

	HCV POSITIVE		HCV NEGATIVE	
	AST LEVEL (U/l)	gGT LEVEL (U/l)	AST LEVEL (U/l)	gGT LEVEL (U/l)
HIV-ve	40.0 (21.0-141.0) n=20	37.5 (6.0-132.0) n=20	41.0 (25.0-83.0) n=6	39.5 (7.0-345.0) n=6
HIV-ve	44.0 (4.0-206.0) n=15	60.0 (4.0-132.0) n=13	52.5 (41.0-123.0) n=4	66.0 (19.0-114.0) n=4
HIV+ve/HIV-ve	42.0 (4.0-206.0) n=35	48.0 (4.0-132.0) n=33	43.5 (25.0-123.0) n=10	39.5 (7.0-345.0) n=10

(patients in soluble IL2-R study)

TABLE 4.25: COMPARISON OF SOLUBLE IL2-R (u/ml) WITH AST LEVELS IN ANTI-HIV NEGATIVE AND ANTI-HIV POSITIVE HAEMOPHILIACS

(Mean ± standard deviation and range)

AST LEVEL (U/l)	HIV-ve HAEMOPHILIACS	HIV+ve HAEMOPHILIACS
<40	527.1 ± 202.6 (290 - 1060) n=14	834.7 ± 481.5 (360 - 1743) n=7
40-80	824.1 ± 421.9* (355 - 1860) n=11	668.9 ± 288.9 (335 - 1480) n=15
<80	796.6 ± 325.3** (520 - 1343) n=5	893.3 ± 426.4 (465 - 1490) n=7

* Significantly elevated from HIV-ve normal liver function group (AST <40) (0.05>p>0.02)

** Significantly elevated from HIV-ve normal liver function group (AST <40) (p=0.01)

Comparison of soluble IL-2® levels with AST levels in anti-HIV seronegative and anti-HIV seropositive Haemophiliacs

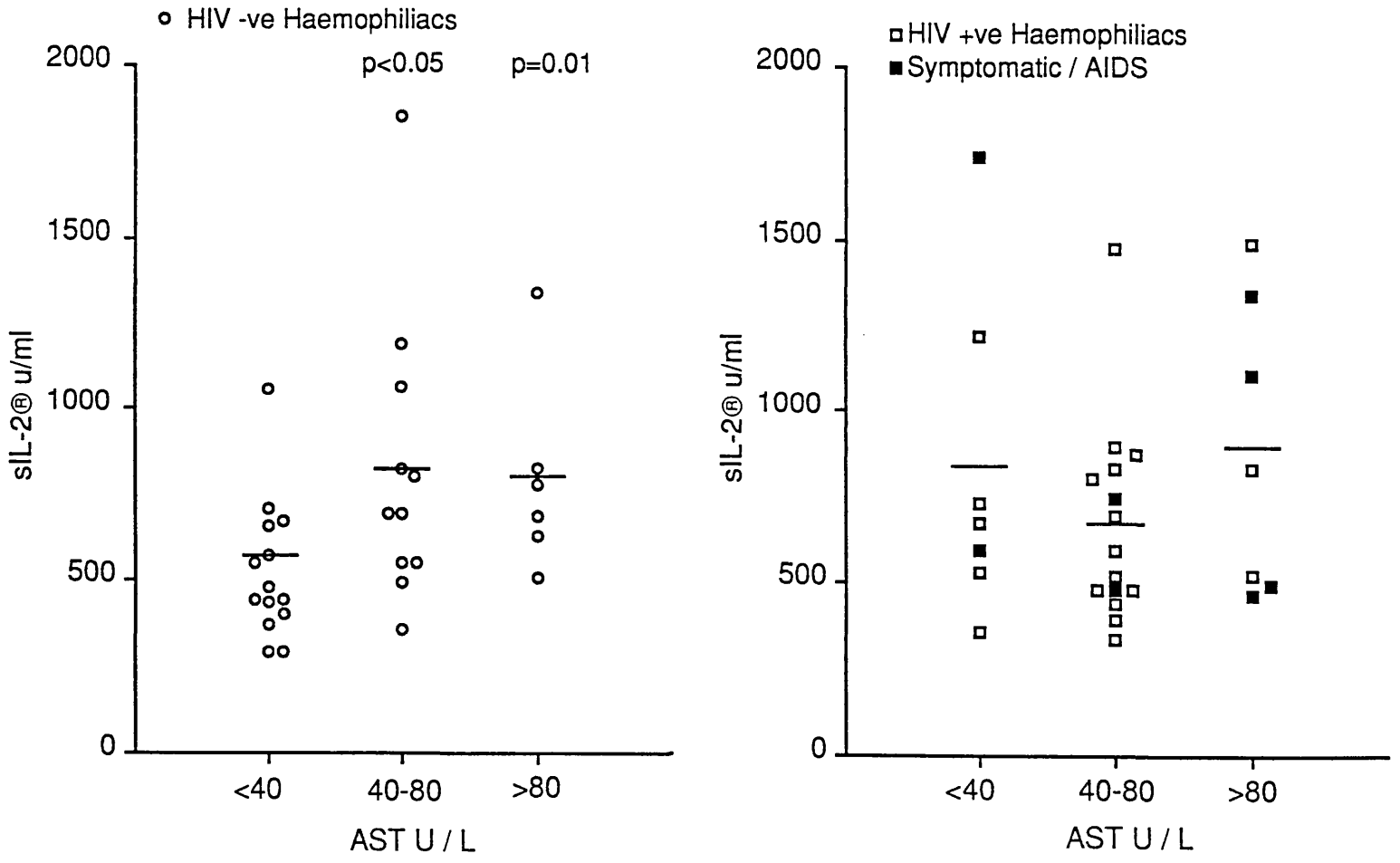


Figure 4.8

normal liver enzyme levels (834.7 ± 481.5 and 527.1 ± 202.6 respectively) but this was not statistically significant.

Evaluation of the AST levels over a two year period, to determine chronic liver damage, classified the patients into groups determined as: normal, (<40U/l); elevated/fluctuating or high (>80U/l) (table 4.26, figure 4.9). In contrast to the previous evaluation, no significant difference between the HIV-ve haemophiliac groups was found, but a significant relationship in patients seropositive for HIV was observed. HIV+ve patients with AST levels that were consistently high had elevated sIL2-R levels (915.4 ± 375.5u/ml) when compared to HIV+ve patients with normal liver function (598.6 ± 159.8u/ml) ($0.05>p>0.02$). In contrast to the acute AST level analysis (table 4.25), where sIL2-R levels were high in HIV+ve patients with normal liver function, in the chronic AST analysis patients with normal liver function had similar levels of sIL2-R regardless of their HIV seropositivity. This was due to regrouping two patients, with high sIL2R levels, who had consistently elevated or fluctuating AST levels but on the occasion of the analysis were below 40U/l, and the placing of two patients into the normal group with evidence of abnormal AST levels which did not exceed 45U/l.

Patients who were HIV+ve tended to have abnormal levels of AST that were higher than those seen in the HIV-ve patients (table 4.27). When the patients were classified by CDC groups there appeared to be a predominance of high AST levels in those patients classified as CDC IV (113.3 ± 65.3U/l) compared to asymptomatic HIV+ve haemophiliacs (65.5 ± 28.9U/l) and HIV-ve

TABLE 4.26: COMPARISON OF SOLUBLE IL2-R (u/ml) WITH AST FUNCTIONAL GROUPS IN ANTI-HIV NEGATIVE AND ANTI-HIV POSITIVE HAEMOPHILIACS

(Mean ± standard deviation and range)

AST GROUP	HIV-ve HAEMOPHILIACS	HIV+ve HAEMOPHILIACS
NORMAL	566.6 ± 197.9 (295 - 1060) n=16	598.6 ± 159.8 (360 - 830) n=7
ELEVATED/ FLUCTUATING	817.1 ± 470.0 (290 - 1860) n=11	758.3 ± 431.7 (335 - 1743) n=14
HIGH	791.7 ± 238.6 (625 - 1065) n=3	915.4 ± 375.5* (465 - 1490) n=8

* Significantly elevated from normal liver function group (0.05>p>0.02)

Comparison of soluble IL-2 ® levels with AST Functional Groups in anti-HIV seronegative and anti-HIV seropositive Haemophiliacs

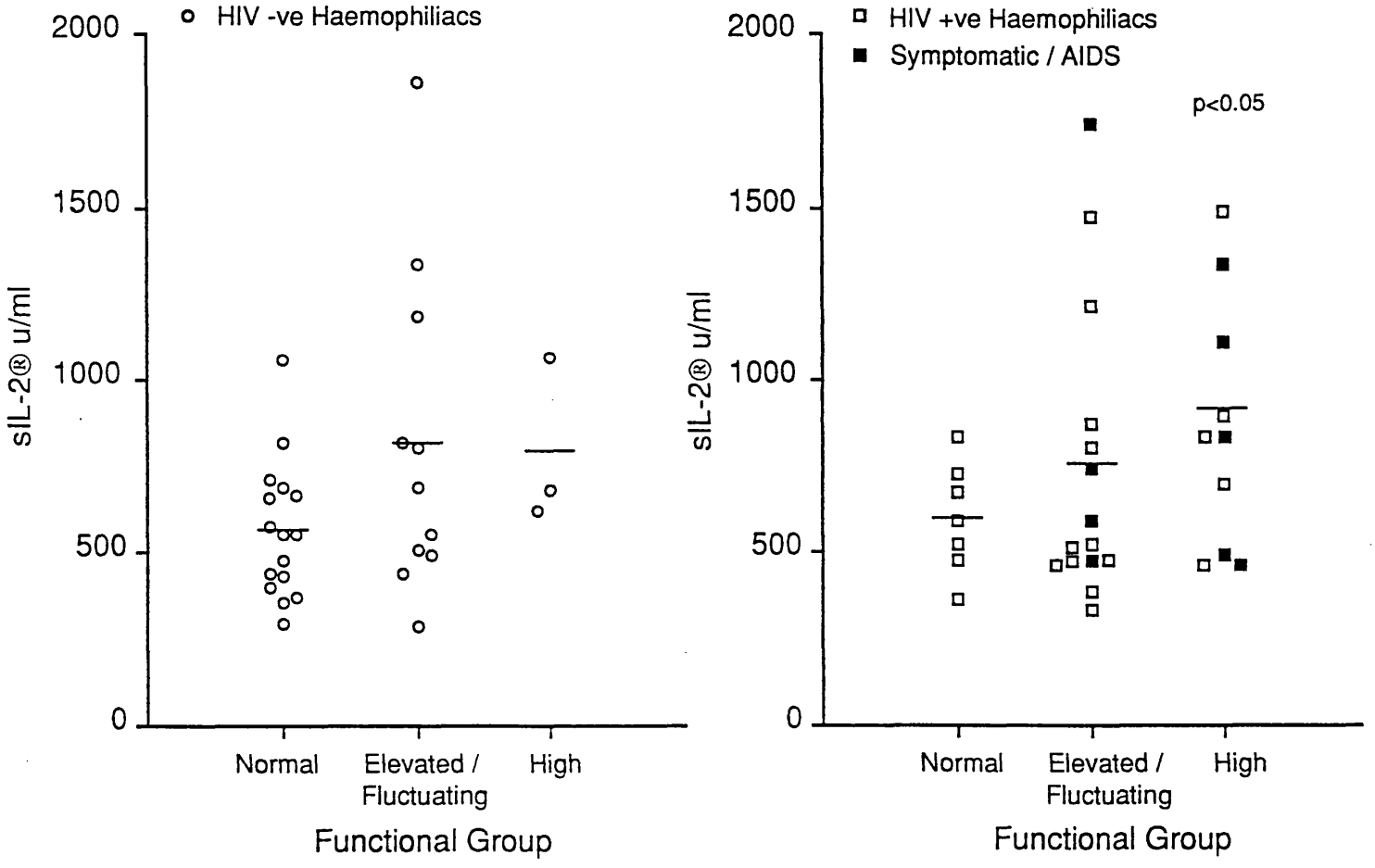


Figure 4.9

4.27: RELATIONSHIP BETWEEN HIV DISEASE STATUS AND ELEVATION OF AST LEVELS (U/l) IN HAEMOPHILIACS WITH ABNORMAL LIVER FUNCTION (AST<40U/l)

(Mean ± standard deviation and range)

HIV-ve	HIV+ve	HIV+ve	
		CDC_{II}	CDC_{IV}
71.3 ± 29.6 (40 - 141) n=16	82.1 ± 49.3 (41 - 206) n=23	65.5 ± 28.9 (41 - 118) n=15	113.3 ± 65.3 (44 - 206) n=8

haemophiliacs (71.3 ± 29.6U/l), however, this was not statistically significant.

4.2.2.4 Progression of HIV disease does not influence sIL2-R levels

T4 levels were significantly reduced in the HIV+ve haemophiliacs ($0.46x10^9$) when compared to the HIV-ve haemophiliacs ($0.77x10^9$/l) ($p<0.0001$) (table 2.6), in the patients analysed in this study, as was found in the other study samples.

Classification of the haemophiliacs into CDC groups showed no significant relationship between the progression of HIV disease and sIL2-R levels (table 4.28). Although levels were higher in those patients classified with CDC IV_C (922.0 ± 377.8u/ml) and CDC II (744.0 ± 361.5u/ml). Patient numbers in the groups in this analysis, however, were low. Similarly there was no correlation between sIL2-R levels and time since seroconversion ($r=0.089$), nor with T4 count ($r=0.019$) (table 4.29, 4.22).

There was no correlation between months since seroconvertion and IgG levels ($r=-0.240$) of T4 counts ($r=-0.389$) in this sample group of patients (table 4.29).

TABLE 4.28: COMPARISON OF SOLUBLE IL2-R LEVELS (u/ml) WITH SYMPTOMS OF AIDS/ARC IN ANTI-HIV NEGATIVE AND ANTI-HIV POSITIVE HAEMOPHILIACS

(Mean ± standard deviation and range)

CDC CLASSIFICATION			
II	II_B	IV_A	IV_C
744 ± 361.5 (335 - 1490) n=17	561.7 ± 116.4 (480 - 695) n=3	821.6 ± 535.4 (465 - 1743) n=5	922 ± 377.8 (495 - 1343) n=4

CDC CLASSIFICATION	
II/II_B	IV_A/IV_C
716.7 ± 340.5 (335 - 1490) n=20	866.2 ± 446.8 (465 - 1343) n=9

CDC CLASSIFICATION	
II	$II_B/IV_A/IV_C$
744.0 ± 361.5 (335 - 1490) n=17	790.1 ± 399.2 (465 - 1343) n=12

TABLE 4.29: CORRELATION (r_s) OF VARIOUS PARAMETERS WITH MONTHS SEROCONVERSION IN ANTI-HIV POSITIVE HAEMOPHILIACS

	sIL2-R (u/ml)	T4 count (10^9/l)	IgG (g/l)
MONTHS SEROCONVERSION	0.089	-0.389	-0.240

4.2.2.5 Relationships between sIL2-R and other immune activation markers

There was no correlation between sIL2R and IgG serum levels in either the HIV-ve or HIV+ve haemophiliacs ($r=0.025$ and $r=0.165$ respectively) (table 4.22).

No relationship was found between sIL2R levels and the elevated activation antigens seen in the HIV+ve haemophiliacs (table 4.30).

TABLE 4.30: CORRELATION (r_s) OF ACTIVATION MARKERS WITH SOLUBLE IL2-R LEVELS IN ANTI-HIV POSITIVE HAEMOPHILIACS

	ACTIVATION MARKERS			
	T CELL HLA-DR	CD71	B CELL CD38	PCA-1
sIL2-R	0.123	0.083	-0.031	0.214

5. DISCUSSION FOR THE *IN VIVO* STUDY

5.1 THE IMPORTANCE OF LYMPHOCYTE ACTIVATION IN HAEMOPHILIACS

There have been reports of immunomodulation in haemophiliacs which have been associated with infusion of clotting factor concentrate. Many of the abnormalities in these patients are attributable to infection with HIV. Immune disturbances, however, have been reported in HIV-ve haemophiliacs also. Lymphocyte function is typically suppressed in respect to mitogen responses and delayed cutaneous skin test responses, but indications of lymphocyte activation have also been reported (section 1.4.3).

In vivo activation has been demonstrated to lead to a refractory state *in vitro* in other immune disorders, GvH disease (Cleveland *et al*, 1988) and SLE (Preble *et al*, 1983). So it is possible that the immune defects that have been observed in HIV-ve haemophiliacs may be related to activation of their immune system by concentrate infusion. This is of particular importance in HIV infected haemophiliacs as activation of HIV infected T cells may influence the progression of HIV disease.

Most early reports of immune abnormalities in haemophiliacs were performed without the benefit of serological markers for HIV, and comparisons were made between asymptomatic patients and those with AIDS. Later studies, although determining HIV seropositivity, were studying patients who were receiving insufficiently heat treated products in which the hepatic viruses were not neutralised.

We sought to discover whether concentrates used in the treatment of haemophilia were activating the immune system, resulting in a refractory state and the immune disturbances reported.

5.2. IMMUNE STATUS OF HIV-ve HAEMOPHILIACS

5.2.1. ACTIVATION OF THE IMMUNE SYSTEM

There have been reports of T cell activation in HIV-ve haemophiliacs. Matheson *et al* (1986b), reported T cell blastogenesis in the cells cultured *ex vivo* from haemophiliacs in the presence of therapeutic concentrations of FVIII, demonstrating that their PBMC were antigenically primed to a component(s) in the FVIII concentrate. Elevated spontaneous production of B cell growth factor (BCGF) and B cell differentiation factor (BCDF) was also observed in cultures of PBMC from haemophiliacs (Matheson *et al*, 1987).

Other reports of activation of T cells in HIV-ve haemophiliacs include elevated HLA-DR expression (Nashida *et al*, 1989) and an increased expression of CD11 indicating the presence of a differentiated or activated subset of T cells (Antonaci *et al*, 1988).

In our study, there were no increases in the expression of any of the cellular markers of activation in HIV-ve haemophiliacs, but there was a slight negative correlation ($r=-0.424$) with high AST levels and HLA-DR expression and may indicate viral induced suppression. The r value was very low and expression of the marker minimal in the HIV-ve patients, therefore it is difficult to assess the relevance of this association. There

was no evidence of an increase in a switch from CD45RA to CD45RO in the HIV-ve patients analysed in this study, thus indicating that no chronic activation of the T cells was occurring. These patients have been found to have normal T4 levels (Dr Christine Lee, Haemophilia and Haemostasis Unit, RFH).

Activation of lymphocytes in the *in vivo* situation mostly occurs in lymph nodes. 'Blood can only be regarded as a convenient, haphazard and incomplete sample of tissue bound samples' (Janossy *et al*, 1989). Soluble IL2 receptor, used as a marker of activation of T cells residing in the tissue, indicated that the HIV-ve haemophiliacs in our study had immune activation. There was no relationship between the elevated levels of sIL2-R detected and FVIII/FIX infusion (amount, type, frequency), nor was there an association with general liver function or HCV positivity. The levels did correlate, however, with active liver damage associated with significant elevation of AST levels on the day of testing. This finding is substantiated in the study of Kloster *et al* (1987) where one of the two HIV-ve patients with elevated levels of sIL2-R had a history of chronic active hepatitis.

T cells were thought to be activated by antigen overload or alloantigen from infusion of concentrates (Jason *et al*, 1985b, Matheson *et al*, 1986b, 1987, Sullivan *et al*, 1986), but our own results indicate that activation is unrelated to concentrate infusion. T cell activation, determined by sIL2-R levels, was present in those patients with acutely elevated AST levels, presumably related to hepatic virus infection.

B cell activation in HIV-ve patients observed in previous studies, included increased serum IgG and spontaneous IgG production (Biagotti *et al* (1986), Gorski *et al* (1986)), but these studies pre-date the introduction of sufficiently heat treated products that are virucidal for HCV.

In our study there was no evidence of B cell activation in the HIV-ve haemophiliacs, and in contrast to many previous studies, serum IgG levels were normal.

The causes of B cell activation had been attributed to FVIII/FIX infusion by the study groups and this was summarised by Antonaci *et al*, 1988 where they found a variable B cell response in HIV-ve and HIV+ve haemophiliacs. They attributed *ex vivo* and *in vitro* B cell abnormalities in haemophiliacs to several factors: concentrate-induced immunisation of these patients; the imbalance in the T cell repotoire; CMV and EBV induced chronic antigen stimulation; or maximal activation resulting from viral infection. It is unlikely, however, that CMV and EBV were important in contributing to the abnormalities as haemophiliacs do not have a raised incidence of these viruses, therefore it is improbable that these viruses survive the concentrate preparation procedure (Webster *et al*, 1989).

T cells are known to have a pivotal position in the control of B cell function. Matheson *et al* (1987), showed that B cell defects may have been due to alterations in the production of the T cell mediators BCGF and BCDF, indicating a T cell abnormality. However, SAC, a T cell independent B cell mitogen was shown to be inhibited also which indicated that

non T cell related B cell abnormalities were also present in the HIV-ve patients. This was substantiated by a report that pre-vaccination Ig levels were elevated for pneumococcal antigens but not for influenza antigens (Ragni *et al*, 1987). Pneumococcal antigens induce T cell independent B cell responses, whilst influenza is a T cell dependent B cell antigen and this indicated that administration of blood products had been leading to polyclonal activation of B cells independently of T cell help.

NK cells have also been shown to regulate the terminal stages of humoral responses: NK cells are preferentially localised in the B cell areas of lymphoid tissue (Banerjee & Thi bert, 1983) and it has been proposed that they regulate late stages of B cell differentiation (James *et al*, 1984, Brieva *et al*, 1984). Diminished NK activity has been reported in HIV-ve haemophiliacs (Lederman *et al*, 1983, 1985b Menitove *et al*, 1983, Porzsolt *et al*, 1984, Ziegler-Heitbrock *et al*, 1985, Matheson *et al*, 1986) and loss in regulation by these cells may be contributing to polyclonal B cell activation.

B cell dysfunction related to T suppressor cell function will be discussed later.

5.2.2 INHIBITION OF THE IMMUNE SYSTEM

Inhibition of immune function is well documented in HIV-ve haemophiliacs. The responses of T cells to PHA and PWM have been shown to be inhibited (Mahir *et al*, 1988) and production of gamma interferon in response to induction by PHA was also reduced (Ruffault *et al*, 1988).

Suppression of B cell function has also been observed including reduced proliferative responses to SAC and reduced plaque forming cell activity to PWM (Gorski *et al*, 1986), and this had been attributed to either an inability of stimulated T helper cells to produce B cell help or an inability of the B cells to respond to the T cell lymphokines. T cell independent B cell antigen proliferation and Ig production, however, have also been reported to be diminished indicating that the B cells were malfunctioning (Kekow *et al*, 1986). gotti *et al* (1986), however, did not find suppression of B cell function in the HIV-ve patients.

If inhibition of the immune system of the HIV-ve haemophiliacs in our study is occurring, it would not be detected as the baseline levels of the cellular activation markers upon resting lymphocytes are very low. There were some indications of inhibition in the study, however, CD71 expression upon T cells was significantly reduced in HIV-ve patients when compared to HIV+ve patients, but expression of this marker in both groups of haemophiliacs was not significantly different from the control group. PCA-1 expression was also low in the HIV-ve patients but not significantly so. Furthermore there were no negative correlations with sIL2-R levels and the infusion of clotting factor concentrates, which one would expect to see if the concentrates were inhibiting T cell activation in these patients.

The HIV+ve patients had increased expression of HLA-DR on T cells and elevated levels of CD71, CD38, PCA-1 and serum IgG, indicating activated B cells. This immune activation is found

in other HIV+ve groups (section 5.4) and therefore indicated that HIV induced activation of the immune system was not inhibited in these patients by the infusion of clotting factor concentrates. It should be noted that HIV induced abnormalities are generally very severe, and it is possible that if the concentrates cause inhibition, this may be over-ridden by the effects of HIV.

5.2.2.1 *In vivo* suppression of immune function in HIV-ve haemophiliacs

The evidence for suppressed immune function in HIV-ve haemophiliacs is not accompanied by increased susceptibility to infection with the exception of one report (Beddall *et al*, 1983, 1985). An increased number (38%) of haemophilic children exposed to an index case of TB contracted the infection, similar to that seen in immunocompromised patients (48%). Susceptibility was correlated to FVIII usage but the overriding association to infection was proximity in the ward to the index case. In addition, the incident involved a small number of boys, 6 out of 38, reducing the significance of this observation.

Inhibition of the immune system *in vivo*, as determined by delayed cutaneous hypersensitivity skin tests, has also been observed in HIV-ve haemophiliacs who have been reported to have a decreased response. The significance of the skin tests as a method of assessing the immune system was disputed by the study of Sharp *et al* (1987) which found that poor skin test responses to tuberculin and tetanus in HIV-ve haemophiliacs were the result of not having received prior vaccination,

rather than an inability to mount an immune response. In the studies of Brettler *et al* (1986) and McIvor *et al* (1987), however, these two antigens did not give the poorest response compared to normal. DNCB response was also inhibited and this test does not involve prior vaccination (Madhok *et al*, 1986). The contribution of concentrate infusion to skin test anergy has been disputed by the groups who studied this phenomenon. Only some of the groups found a direct correlation, but the greatest contribution to the inhibition was the presence of multiple virus infection (EBV, CMV, HIV) (Brettler *et al*, 1986, Sullivan *et al*, 1986). Lowe *et al* (1989), failed to demonstrate an abnormality in the microanatomical distribution of lymphocytes and macrophages of the lesions following a cutaneous tuberculin response. They did, however, find an absence of increasing T4/T8 ratio in the diffusely infiltrating lymphocytes with increasing dermal depth suggesting a subtle qualitative defect, which they proposed may be an indicator of impending immunosuppression.

Although there have been reports of suppression of cell mediated immunity in HIV-ve haemophiliacs they do not appear to have a reduced response with vaccination to pneumococcal polysaccharide or influenza virus (Ragni *et al*, 1987). From our own studies (Oon *et al*, 1990), there was no statistically significant difference in antibody response to hepatitis B vaccine in HIV-ve patients compared with that seen in normal controls. Therefore only one arm of the immune response, that of cell mediated immunity, may be affected.

5.2.2.2 Suppression related to monocyte dysfunction

Competent cell mediated immunity and defence against intracellular parasites like *Mycobacterium tuberculosis* require both healthy T cells and monocyte/macrophages. It is possible monocyte dysfunction is contributing to the cell mediated defects. Monocytes from haemophiliacs have been shown to have phenotypic and functional abnormalities (Mannhalter *et al*, 1986, Stasi *et al*, 1987, Roy *et al*, 1988, Pasi and Hill, 1990), attributed to concentrate infusion, which *in vitro* were related to the modulating affect of immune complexes (Mannhalter *et al*, 1986, Eibl *et al*, 1987).

5.2.2.3 Inhibition related to suppressor cell activity

It is possible that the reported inhibition of immune function in haemophiliacs may be related to increased suppressor cell activity or to abnormalities in their function. An imbalance in T4/T8 ratios, with increases in T8 cell numbers, both relative and absolute, has been observed in HIV-ve haemophiliacs (Carr *et al*, 1984, Tsoukas *et al*, 1984, Ludlam *et al*, 1985, Sullivan *et al*, 1986). Zeigler-Heitbrock *et al* (1985) reported an increase in the suppressor cell population $CD8^+/CD57^+$ ($T8^+/leu\ 7^+$) in the haemophiliacs and found no correlation with HIV seropositivity. They later showed that a proportion of these cells express elevated levels of HLA-DR (Zeigler-Heitbrock *et al*, 1988). The function of these cells is unclear although there is evidence of cytopathic activity and of suppressor activity for B cell differentiation and NK activity. This again indicates that the B cell dysfunction may have been due to abnormalities in their control mechanisms.

Suppressor cell expansion related to FVIII concentrate consumption has also been reported by Dianzani *et al* (1988). CD73 (ecto-5 'nucleotidase) activity (which is a marker of maturation in lymphocytes and is involved in cytotoxic T lymphocyte activation) was significantly decreased compared to healthy controls in HIV-ve haemophiliacs (T4/T8 ratios <1) and this deficiency was attributable to the expansion of $CD8^+/CD11^+$ suppressor cell populations. In keeping with this alloreactive cytotoxicity was also decreased. The suppressor cell expansion was attributed to FVIII infusions. Other studies, however, have not demonstrated defective T suppressor/inducer cell ($CD4^+$) function (Sjamsoedin-Visser *et al*, 1988),in HIV-ve haemophiliacs or an expansion $CD8^+/CD57^+$ suppressor cell population (Kaplan *et al*, 1988). There was no elevation in T8 cell numbers or activated T8 cells in our study.

5.2.2.4 Inhibition related to activation

The reports of suppression of immune response in HIV-ve haemophiliacs was usually linked with evidence of activation, particularly in B cells. For example, Gorski *et al*, have demonstrated activation: increased serum IgG and spontaneous IgG production, accompanied by reduced proliferative responses to lectin. It would appear then that the original hypothesis that the B cells are partially activated and are therefore refractory to stimulus may have true in this instance.

5.2.3 IMPORTANCE OF VIRUSES IN THE IMMUNE DYSFUNCTION IN HIV-ve HAEMOPHILIACS

Many viruses have been demonstrated to have immunomodulating effects (Rinaldo *et al*, 1980) and to be immunosuppressive (Young & Mortimer, 1984). EBV has been demonstrated to activate a T cell sub-population and suppress overall immune responses (Reinherz *et al*, 1980b). Abnormal lymphocyte subpopulations have been demonstrated in EBV and CMV infections. As previously discussed, there is evidence of abnormal T cell populations and increased suppressor cell function in the HIV-ve haemophiliacs. This may indicate that immune abnormalities in HIV-ve haemophiliacs may be virally induced (White & Lesesne, 1983). Increases in and activation of suppressor cells have been described in infection by HBV (Barnaba *et al*, 1983), CMV (Carney *et al*, 1981,83) and EBV (Reinherz *et al*, 1980b). The presence of multiple viral (EBV, CMV and HIV) infection led to the greatest reduced skin test response (Brettler *et al*, 1986, Sullivan *et al*, 1986). The $CD8^+/CD57^+$ suppressor cell population, demonstrated to be expanded in the HIV-ve haemophiliacs, has been shown to be increased in CMV infection, and CMV infected bone marrow transplant recipients (Zügler-Heitbrock *et al*, 1988). These points further demonstrate that the abnormalities in the haemophiliacs may be related to viral infection. Although these patients do not have an increased incidence of CMV or EBV infection (Webster *et al*, 1989) they did, at the time relevant to the previous studies, have a high incidence of HBV and NANB hepatitis and received live virus during infusion of concentrates. Not all the early studies of T cell abnormalities found associations with the abnormalities and CMV, HBV, EBV or aminotransferase

levels, however (Ceupens *et al*, 1984, Weintrub *et al*, 1983). HTLV-1 has also been implicated in causing immune disturbances in haemophilic patients, but as with CMV and EBV it does not survive the purification processes of concentrate manufacture (Chorba *et al*, 1985).

There was no evidence of peripheral blood lymphocyte activation in the HIV-ve haemophiliacs in our study, there was, however, elevation of sIL2-R levels which correlated to liver function abnormalities and probably HCV infection. The release of sIL2-R appears to be a characteristic marker of T lymphocyte activation (Rubin *et al*, 1985). The elevated levels in the HIV-ve patients are probably related to a normal immune response to viral infection of the liver, and the immunopathology that may result is shown by the raised AST levels (Dudley *et al*, 1972, White *et al*, 1982, Aledort *et al*, 1985, Alexander , 1990).

The patients in our study received BPL (NHS) 8Y, BPL 9A (NHS FIX) (products which have been shown not to transmit HIV or HCV in ongoing trials (Pasi & Hill, 1989, Pasi *et al*,1990)), and Alpha Profilate (reduced transmission of HCV) (Kernoff *et al*, 1987), and were probably not receiving large amounts of live virus. It is possible that the patients in the earlier studies were treated with non-heat-treated, or inadequately heat-treated clotting factor concentrates and therefore did receive live virus. Thus correlations with immune abnormalities and concentrate infusion would be expected in these studies.

The incidence of HCV in this study was approximately 80% in

both HIV-ve and HIV+ve patients, this is in keeping with other studies (Ludlam *et al*, 1989, Kuo *et al*, 1989). Hepatitis C virus (HCV) (the agent responsible for virtually all cases of NANB hepatitis) has been seen to interfere with the immune responses to other viral infections. This was demonstrated in chimpanzees when HCV had a profound effect on superinfection of Hepatitis A (HAV). Elevated AST levels did not develop as expected and the virus delayed anti-HAV responses. NANB infection has also been shown to depress the replication of HBV and delays the appearance of serological markers (R Wright, 1990). Chronicity of HBV infection is related to defective cell mediated immunity (Dudley *et al*, 1972). Therefore the presence of HCV probably had profound effects upon the immune response to HBV infection in the haemophiliacs and one would expect that a large proportion of the haemophiliacs are undergoing, or have been subjected to concentrate-transmitted-viral-induced immune alterations. The presence of multiple viral infection will further complicate this picture.

5.2.4 VARIABILITY OF IMMUNE DYSFUNCTION STUDIES IN HIV-ve HAEMOPHILIACS

There have been studies from many countries giving conflicting reports of immunomodulation in haemophiliacs. Not until HIV serology testing was it possible to separate the effects of HIV infection from those of FVIII/FIX infusions. There are, however, reports of immune dysfunction where HIV serology has been determined and immune abnormalities have been seen in both HIV+ve and HIV-ve patients. During the last decade donor pools and product manufacture have changed considerably with

the introduction of donor screening and solvent and heat treatment of products. These changes have possibly caused variations of the study groups and led to the conflicting reports of the presence or absence of lymphocyte modulation in HIV-ve patients. New products may not perturb the immune system to the same degree as that seen in earlier products. Teitel *et al* (1989), in a 2 year evaluation of immune function (1986-1988), observed a transient rise in T4 count in the HIV-ve haemophiliacs and a period of 'stabilisation' in T4 numbers in the seropositive group which coincided with a change to products where virucidal methods had been improved to eliminate the agent responsible for NANB hepatitis. This suggested that the reduction in repeated exposure to these virus was responsible for the transient effects on the T4.

The patients in our study were analysed between December 1987 and December 1989. All patients in the study have received heat treated products - Alpha Profilate and BPL (NHS) 8Y and BPL 9A (NHS FIX) since their introduction in 1985. These products have been shown to transmit little or no HCV. Therefore the patients had a reduced challenge with live virus for at least 3 years prior to evaluation. This would therefore explain why our patients do not display the high degree of activation demonstrated in other studies and why the activation observed (raised sIL2-R levels) did not correlate with concentrate infusion.

5.2.5 DO CONCENTRATES LEAD TO LYMPHOCYTE ABNORMALITIES IN HIV-ve HAEMOPHILIACS?

As we have seen, there is conflicting evidence that the infusion of clotting factor concentrates lead to lymphocyte abnormalities. The majority of the observations of immune abnormalities in HIV-ve haemophiliacs were published prior to the introduction of HCV neutralised products. Variability in the findings is probably related to the type of concentrate received. There was no cellular activation in the HIV-ve patients in our study, but sIL2-R was elevated correlating with transaminaemia, related to viral infection. Therefore we can conclude that it is unlikely that the concentrates are causing non-specific lymphocyte dysfunction due to antigen overload and that problems are probably related to viral infection transmitted by the products prior to heat treatment. This contrasts with patients who receive multiple whole blood transfusions and this will be discussed later. Pasi and Hill (1989), have shown no immunomodulation in their trial of previously untreated patients (pup) where all products received are 'super-heat-treated,' and whilst trials are ongoing, there is no evidence that such products transmit viral infection. Patients who have been previously infected, however, may respond to antigenic epitopes of the inactivated virus which are still present in the products following sterilisation.

5.3 EVIDENCE OF IMMUNE ABNORMALITIES IN BLOOD TRANSFUSION RECIPIENTS

Immune abnormalities, both suppression and activation have been seen in patients who receive multiple blood transfusions. These include increases in suppressor cell numbers accompanied by suppression of immune function resulting in reduced allograft rejection; increased incidence of cancer (reviewed by MacLeod *et al*, 1987); increased rate of growth of chemically induced sarcoma in rats and decreased lymphocyte reactivity to PPD and PHA (Francis *et al*, 1981); decreased NK cell activity and an increase in HLA-DR expression on T cells (Gascon *et al*, 1984).

Thalassaemics, who also receive multiple blood transfusions, also show evidence of immune modulation: increased levels of late stage differentiated B cells (Akbar *et al*, 1985); elevated serum Ig levels, increased T8 cells (Grady *et al*, 1985); decreased NK activity (Akbar *et al*, 1986) and increased CD45RO expression (Akbar *et al*, 1990). Those patients who receive multiple transfusions are repeatedly exposed to chronic stimuli from large doses of foreign cellular or soluble antigen, in particular histocompatibility antigens. Non-specific immunosuppression is thought to be mediated by a suppressor cell population which has been shown to be expanded (Fischer *et al*, 1980) transfusion of anti-idiotypic antibodies (MacLeod *et al*, 1987) and non-cytotoxic Fc receptor blocking anti-lymphocytic antibodies (MacLeod *et al*, 1982, Forwell *et al*, 1986). Iron plays an important role in the regulation of lymphoid cell migration and modulation of expression of cell markers and iron overload in thalassaemic patients is thought

to contribute to some of the immune disturbances reported (Kapadia *et al*, 1980). Immune dysfunction related to NK activity (both regulatory and cytotoxic) has also been attributed to iron overload (Akbar *et al*, 1986). Patients who receive filtered blood (removal of white cells) or platelet infusions are not immunologically assaulted to the same degree and do not exhibit such profound immune disruption. Transfusions of filtered blood pre-treatment are ineffective in preventing renal allograft rejection (Persijin *et al*, 1981). Therefore, immune abnormalities in these patients are mainly related to iron overload and exposure to foreign MHC antigens on the infused leucocytes. It should be noted that the reactivity of cells in culture in a MLR, which is a response to MHC, far exceeds that for antigen, therefore the reactivity of the immune response to foreign cells infused during the transfusion will be very high. This contrasts with patients who receive blood products, as there is no infusion of iron or whole cells, just plasma proteins. If these include MHC antigens, the response of the recipient will be similar to that induced by other antigens.

Patients who receive blood and blood product transfusion have been exposed to the same blood transmitted viruses and a large proportion of patients have been infected with HIV (Curran *et al*, 1984) and it has been suggested that viruses are also involved in the suppression seen in allograft recipients (Woodruff and Van Rood, 1983). Therefore transfusion recipients are probably subject to multiple immune modulators - iron, alloantigen, and viruses.

5.4 THE IMMUNE STATUS OF HIV+ve HAEMOPHILIACS

HIV infection is characterised by a progressive decline in T4 lymphocyte numbers associated with an increase in T8 lymphocyte numbers, immunosuppression results in an increased susceptibility to frequent opportunistic infections leading to AIDS.

Haemophiliacs do not seem to differ from non-haemophiliac HIV infected individuals. Both, have profound T and B lymphocyte, monocyte and NK cell abnormalities.

In our study the HIV+ve haemophiliacs had an increased expression of most of the cellular activation markers on their peripheral blood lymphocytes: increased HLA-DR expression (predominately on T8 cells) and increased CD71, CD38 and PCA-1 on B cells. Serum IgG was elevated and high levels of sIL2-R were also observed (discussed in section 5.5).

The overriding cause of activation was related to HIV positivity but only CD38 expression correlated with disease progression. There was no correlation between activation and FVIII/FIX infusion (amount, type, frequency), HCV positivity, or liver damage related to transaminase levels and cellular activation, however there was a slight association between gamma GT and CD38 expression and slight negative correlation between T4 counts and gamma GT levels. This may be due to reactivation of hepatic liver virus infections due to HIV induced-immunosuppression or may be related to direct HIV liver damage.

5.4.1 T CELL ABNORMALITIES

5.4.1.1 HLA-DR expression and increased suppressor cell activity - evidence of activation

The HIV+ve haemophiliacs in our study had increased HLA-DR expression and this has also been described by Koller *et al*, 1985, Sullivan *et al*, 1986 and Nashida *et al*, 1989, substantiating our results. Koller's study did not define HIV serology, although, one patient had AIDS. As with the HIV-ve haemophiliacs, suppressor cell increases have been reported in HIV+ve haemophiliacs. Ziegler-Heitbrock *et al* (1985) and Kaplan *et al* (1988), reported an increase in suppressor cell population $CD8^+/CD57^+$ ($T8^+/Leu\ 7^+$) in the HIV+ve haemophiliacs and as previously mentioned, Dianzani *et al* (1988), reported a significant decrease in CD73 activity and the $CD11^+$ and $HLA\text{-}DR^+$ T4 subpopulation was expanded.

HLA-DR expression has been shown to be increased in other HIV infected patients (Milvan *et al*, 1982, Salazar-Gonzalez *et al*, 1985): Lin *et al*, 1988 found a significant concordant relationship between the percentages of $Leu2^+7^+$ (CD8/CD57) and $Leu4^+DR^+$ ($CD3/HLA\text{-}DR^+$) cells in asymptomatic HIV+ve IV drug abusers. Expansion of a CD8/CD57 expressing cell population has also been reported in HIV+ve haemophiliacs (Kaplan *et al*, 1988, Ziegler-Heitbrock *et al*, 1985). Three colour flow cytometric analysis of the $CD8/CD57^+$ cells in both homosexual and haemophilic HIV+ve patients, demonstrated that they were also expressing HLA-DR. It is, therefore, possible that these cells are specifically activated by HIV and were involved in the immune dysfunction of AIDS. We can assume that Lin observed an expansion of suppressor T cells, which was later

confirmed by Ziegler-Heitbrock, a phenomenon observed in this study also. Bogner *et al* (1990), has demonstrated that the expansion of $CD3^+/HLA\text{-}DR^+$ paralled by the increases in $CD8^+/CD57^+$ cells occurs very early during HIV disease preceding depletion of T4 cells.

Other evidence of T cell activation in HIV infected individuals, apart from the presence of the increase in suppressor cell numbers and HLA-DR expression include elevated serum levels of gamma interferon (Fuchs *et al*, 1989); an acid labile alpha-interferon in haemophiliacs with AIDS (Eyster *et al*, 1983); and elevated lymphotoxin levels thought to be secreted by activated T4 cells (Fuchs *et al*, 1987). Antonaci *et al* (1988), observed an increase in CD25 expression in his HIV+ve haemophiliacs, in contrast to our own study and that of others (see below). Activated monocytes have also been described (determined by elevated secretion of IL-1 (Weiss *et al*, 1989), neopterin (Fuchs *et al*, 1988), TNF (Wright *et al*, 1988) and elevated expression of HLA-DR and IL2-R (Allen *et al*, 1990) and are thought to be related to the presence of activated T cells. The increase in activated T cells, as indicated by HLA-DR expression, may relate directly to HIV infection, or to processes occurring secondary to the immunodeficiency induced by HIV infection, for example, antigenic stimulation by other pathogens: CMV, HBV, HSV or allogeneic stimulation by exposure to semen, blood or allografts (Ho *et al*, 1987).

5.4.1.2 CD25 and CD71 expression are not elevated - evidence of immunosuppression

Although HLA-DR expression is elevated in HIV+ve patients most immune modulation reported is of a suppressive nature associated with the decline in T4 cell numbers. We found that the markers of T cell activation CD25 and CD71 were not significantly elevated in our HIV+ve patients. This has also been reported in other studies of HIV+ve haemophiliacs (Koller *et al*, 1985) and in other HIV+ve, non-haemophiliac patients (Salazar-Gonzalez *et al*, 1985, Gupta, 1986). Ta1 (a T cell activation marker) has also been shown to be reduced on T cells in patients with low T4 counts compared to controls (De Martini *et al*, 1988).

As referred to in the introduction, reduced proliferation to mitogens and alloantigens has been demonstrated in HIV+ve individuals. The dysfunction is attributed to the reduced IL2 production responses to lectins (PHA, Con A, PWM) (Prince *et al*, 1984, Vaith *et al*, 1985, Gupta, 1986) allogeneic, autologous MLR (Vaith *et al*, 1985, Ebert *et al, 1985)* and antigens (tetanus toxoid) (Prince & John, 1987, Antonen & Krohn, 1986). $CD4^+/CD28^+$ cells (thought to be suppressor/inducer cells) have been shown to be significantly decreased in number in HIV+ve haemophiliacs (Dianzani *et al*, 1988). The T suppressor inducer cell CD4 has also been demonstrated to have defective function in HIV+ve haemophiliacs (Sjamsoedin-Visser *et al*, 1988). They hypothesised that activated B cells in these patients activated the T suppressor inducer cell subset *in vivo* in the absence of antigen. This activation *in vivo* would then impede reactivation *in vitro*.

5.4.1.3 Possible causes of inhibition of IL2 and IL2 receptor in HIV infection.

One would expect elevated expression of IL2-R (CD25) to accompany activation of the immune system, however, in HIV infection this is not the case, and may imply that activation is not occurring in HIV after all. It has been postulated that HIV directly inhibits the production of IL2 and the expression of its receptor.

It has been demonstrated (Wainberg *et al* 1987), that addition of HIV to cultures of normal lymphocytes impeded the response to PHA, this was dose dependent and due in some part to interference with IL2 production. IL2-R expression was unaffected. The inhibition was not a result of active infection of cells.

Serum and plasma from patients with AIDS have been shown to inhibit IL2 production and IL2-R expression in lectin induced normal lymphocyte cultures (Siegel *et al*, 1985, Farmer *et al*, 1986, Donnelly *et al*, 1987). Donnelly reported that inhibition was primarily mediated at the level of the IL2 response but it did not involve blocking of the IL2-R. Fractionation of the inhibitory component indicated that it eluted in the Ig fraction and was probably an auto-antibody. In contrast, Farmer demonstrated that the inhibitory component had a MW of >70KDa and was not an immunoglobulin. The increases in sIL2-R levels during HIV infection could have a role in the inhibition of IL-2 dependent cellular functions observed in these patients and could be the inhibitory component in the serum. sIL2-R is capable of binding its own

ligand efficiently (Rubin *et al*, 1986, Robb and Kutny, 1987). It is thought to compete with the cellular IL2-R for IL2 binding and therefore exert an immunoregulatory role (Reske-Kunz *et al*, 1987). The excess of the receptor released *in vivo* by activated and/or infected cells may remove the circulating endogenous IL2, thus contributing towards the defective IL2 dependent functions (Kloster *et al*, 1987, Pizzolo *et al*, 1987a).

Increased serum neopterin in patients with HIV-1 infection is correlated with reduced *in vitro* IL2 production (Fuchs *et al*, 1990). Therefore reduced IL2 production and IL2-R expression may be reflecting T cell activation where raised gamma interferon levels have stimulated increased neopterin levels (Fuchs *et al*, 1989) and the cells have become refractory to activation *in vitro*.

Monocytes, in patients infected with HIV have been shown to express IL2-R (Allen *et al*, 1990). The monocytes were found to adsorb and therefore deplete IL2, and may contribute to the reduced proliferative response of T cells detected *ex vivo*.

HIV infection of cell lines has been shown not to affect transcription of IL2 (Arya & Gallo, 1985), but reduced IL2 production and IL2-R expression *in vitro* may be related to cell death. Zagury *et al* (1986), showed that HIV harboured in T4 cells was activated during mitogen stimulation and that this followed the peak production of IL2 and preceded a decrease in cell viability. Cell death in his cultures occurred 7-10 days after exposure to mitogen. Therefore it

appeared unlikely that cell death was causing the inhibition. Professor Janossy Dept. Immunology, RFH (personal communication), and Hornicek *et al* (1987), however, have demonstrated a rapid fall in cell viability at 72 hours. This cell death may explain reduced proliferation *ex vivo* and may contribute to the reduced IL2 production and receptor expression reported.

Direct HIV-induced suppression may play an important role in the pathogenesis of AIDS. Pahwa *et al* (1985), demonstrated that high concentrations of crude disrupted HIV preparations could inhibit T cell proliferation and IL2-R expression. Donnelly *et al* (1987)suggested that the inhibitory nature attributed to some patients serum could be due to the presence of viral particles.

Regions of homology (six amino-acid peptide) have been identified between the HIV and IL2 and the IL2-R (Reiher *et al*, 1986), and have been shown to interfere with IL2 binding to its receptor (Weigent *et al*, 1986). HIV could also be involved in suppression by competitive binding or cytopathy by crossreactive antibodies produced in these patients. Homology has been demonstrated between the transcription control mechanisms for the IL2-R alpha-gene and those for HIV-1 (Bohnlein *et al*, 1988) and between a 50KDa silencer protein involved in the negative regulation of IL2-R alpha gene and the HIV-1 gene (Smith and Greene, 1989). This homology between IL2, IL2-R genes and HIV may explain why IL2 and IL2-R expression are specifically affected by HIV.

Several HIV peptides and recombinant HIV peptides have been demonstrated to inhibit T cell function. HIV lysates have been shown to be inhibitory for T cell function, and fractionation has demonstrated that the inhibitory component had a molecular weight of 67KDa (Hofmann *et al*, 1989). Chanh *et al* (1988), have reported suppressive properties of large amounts of peptides of gp41. Purified gp120 has been demonstrated to bind to CD4 receptor, and suppress the normal PHA response (Mann *et al*, 1987). Bank and Chess (1985), have shown that perturbation of the T4 molecule can transmit negative signals to $CD4^+$ cells. gp120 binds to CD4 (Klatzmann *et al*, 1984, Krowka *et al*, 1988) and may inhibit responses by switching on the CD4 molecules as a 'negative' receptor, or by non-specifically blocking receptor-ligand interactions. HIV interaction with the CD4 molecule is discussed in the introduction and is evidence that inhibition of T cell function by HIV may not be specific for IL2. This is further substantiated by reports that signal transduction mechanisms in HIV+ve patients have also been shown to be abnormal (Pinching and Nye, 1990).

5.4.1.4 Why is HLA-DR expression elevated?

A. Elevation related to T cell activation

HLA-DR expression in our study was increased on T cells but this was predominately seen on the T8 subset. HLA-DR expression on T8 cells is increased in the presence of gamma interferon (Siegel 1988). This lymphokine has been shown to elevated in HIV+ve individuals (Fuchs *et al*, 1989) so activated T cells could be upregulating this marker directly. Changes in the HLA-DR expression have also been reported on

monocytes and on dendritic cells (Heagy et al, 1984, Koethe *et al*, 1989). *In vitro* gamma interferon production in HIV+ve patients, however, is inhibited (Ruffault *et al*, 1988, Murray *et al*, 1985), but this inhibition may be a further indication of *in vivo* activation rendering the cells refractory to further stimulus as has been shown in studies of GvH disease (Cleveland *et al*, 1988) and SLE (Preble *et al*, 1983). Arya *et al* (1985), however, has shown that in HIV infected cell line cultures the cells could not synthesise mRNA for gamma interferon, nor could they be induced to do so.

In our study, (data not shown) raised gamma interferon levels were not detected although this probably reflects a poor sensitivity of the assay used.

B. Does HIV directly induce HLA-DR expression?

We may not be seeing T cell activation *per se*. Kannagi *et al* (1987), have demonstrated specific induction of MHC class II antigens *in vitro* by infection of cell lines with simian immunodeficiency virus (SIV). SIV and HIV are morphologically indistinguishable and antigenically related, they both share the T4 tropism and both induce analogous immunodeficiency syndromes. HIV has been shown to directly induce HLA-DR mRNA and increase its expression in T cell clones (Altmann et al, 1987). They suggested that the antigen may have an important role in the pathogenesis of HIV as certain class II antibodies were able to partially inhibit syncitium formation in infected lines. Kannagi *et al* (1987), demonstrated that MHC class II expression occurred long after virus production and was therefore probably unrelated to gamma interferon production and more likely to be directly induced by the virus.

MHC class II is acquired by the SIV during budding and this may be a passive or active phenomenon. Either way, due to class II/CD4relationships, the presence of class II on the virus may increase the tropism of these virus to CD4.

C. HLA-DR expression could be related to specific T cell subsets

Sala zar-Gonzalez *et al* (1985), demonstrated a reduction in CD73 activity and enhanced CD38 and HLA-DR expression upon T8 cells in patients with AIDS. They suggested that these data indicated a qualitive change in the T8 cells in AIDS, the presence of CD38 and HLA-DR indicating immaturity. There are several clinical situations which follow this pattern. Epstein Barr virus induced infectious mononucleosis features reduced levels of CD73 and a transient increase in the number of CD8 cells as well as increases in CD38 and HLA-DR on T cells (De Waele *et al*, 1981). However, circulating T cells bear CD71, but lack CD25. These elevations in T cell activation markers during infection with EBV are transient but this is not the case for AIDS.

Pantaleo *et al* (1990), has shown an increase in a subpopulation of $CD3^+CD8^+HLA\text{-}DR^+CD25^-$ cells in patients with HIV infection, the proportion of these cells increased during disease progression. Sorting of the cells into $CD8^+/HLA\text{-}DR^+$ and $CD8^+/HLA\text{-}DR^-$ demonstrated that the $CD8^+/HLA\text{-}DR^+$ cells had reduced responses to anti-CD3, anti-CD2, anti-CD28, PHA and Phorbol myristate acetate (PMA), this was not augmented by the addition of IL-4 or IL-2. This inhibition was not seen in the $CD8^+/HLA\text{-}DR^-$ cells. Only the $CD8^+/HLA\text{-}DR^-$ cells expressed

significant levels of CD25 following culture. The $CD8^+$/HLA-DR^+ cells also had decreased clonogenic potential compared to the $CD8^+$/HLA-DR^- cells. VLA-2 antigen, a marker of chronic activation, was shown to be present on a large proportion of those cells with $CD3^+CD8^+$HLA-DR^+CD25^-. They suggested that these results demonstrate an activated CD8 population which is refractory to further *in vitro* signals for proliferation and that this may contribute to the decrease in the HIV-specific cytotoxic T8 cells in AIDS patients. It is possible, however, that the presence of these cells may be showing partial activation induced anergy. $CD8^+$HLA-DR^+CD25^- cells are a common finding in other viral infections such as EBV (Tomkinson *et al*, 1987) and CMV (Carney *et al*, 1983) suggesting that this cell may be a normal immune response to viral infection and this has been substantiated by Panteleo *et al* (1990), where they have shown that the cells cytotoxic to HIV are infact $CD8^+$ HLA-DR^+ $CD25^-$ in healthy HIV+ve individuals and suggested that the proliferative defects may be indicative of a maturational stage rather than an intrinsic cellular defect. It is unclear if the presence of VLA-2 indicates a state of non-specific chronic activation or reflects an ongoing effector response that at least in part is HIV-specific. Tomkinson also observed an elevated level of sIL2-R which correlated with the increased percentages of T8/HLA-DR^+ cells. We were unable to determine if this was occurring in our studies as the patient groups for the two parameters were not the same.

5.4.1.5 Chronic activation of T cells in HIV infection

Chronic activation of lymphocytes was also observed. The HIV infected haemophiliacs had elevated levels of CD45RO, compared to the HIV-ve haemophiliacs, on T8 cells but not on T4 cells. These were found predominately on those patients with T4 counts below $0.4x10^9$ cells/l. We are seeing a switch to primed cytotoxic/suppressor cells in these patients. This data substantiates the findings of De Martini *et al* (1988), where a switch from $CD4^+/CD45R^+$ (using 2H4 = CD45RA) to $CD45R^-$ in HIV+ve homosexuals with T4 counts below $0.4x10^9$ cells/l was observed.

From our study, correlations between CD45RO on T8 cells and T4 counts demonstrated that during disease progression as the T4 count decreases the level of CD45RO on T8 cells increases along with the expression of HLA-DR on T4 cells. This indicates that the $CD45RO^+$ T8 cells may be associated with the removal of activated T4 cells (ie those expressing HLA-DR).

Excessive infiltration of the germinal centres of lymph nodes by $CD8^+/CD45RO^+$ cells in HIV+ve patients, before the onset of AIDS, has been reported (Janossy *et al*, 1989, 1990). This is an unusual phenomenon as lymph nodes are normally populated with T4 cells and T8 cells sited in the lymph node are rare and are generally unprimed. $CD8^+/CD45RO^+$ cells have been shown to have exceptionally high cytotoxicity and Jannosy has hypothesised that these cells, by autoaggressive killing of T4 cells, may be contributing to the pathology of AIDS. An interesting observation by Levacher *et al* (1990) was that during treatment of ARC/AIDS patients with AZT there was a

transient rise in T4 lymphocytes associated with a decline in $CD3^+/HLA\text{-}DR^+$ cells which reached statistical significance at 24 weeks of the trial. T8 cells remained unchanged. This suggests that AZT, by reducing the viral load, reduces the level of HLA-DR positivity upon T cells, either because of the reduction in the requirement for activated specific subsets of cells or a reduction in cytotoxic cells ($T8^+/HLA\text{-}DR^+$).

Activated/proliferating T cells are more easily infected with HIV, one would therefore expect to see preferential infection of memory cells (Stevenson *et al*, 1990). Evidence of this is seen in SIV infected macaques where the virus could not be isolated from naive T cells, this preference also exists *in vitro* (Willerford *et al*, 1990).

5.4.2 B CELL ABNORMALITIES

B cell abnormalities have been reported in HIV+ve haemophiliacs: elevated serum levels of IgG, IgM and IgA; spontaneous IgG production; and decreased proliferation and antibody production with PWM, SAC and MLR (Biagotti *et al*, 1986, Brieva *et al*, 1985, Gorski *et al*, 1986, Kekow *et al*, 1986, Sjamsoedin-Visser *et al*, 1987, Blomback *et al*, 1987). The B cells of the HIV+ve haemophiliacs in this study were activated, this was evident from the elevation in the expression of acute (CD71, CD38) and chronic (PCA-1, IgG) activation markers.

HIV+ve non-haemophiliac patients also display phenotypic changes associated with activation (Lane *et al*, 1983, Martinez-Masa *et al*, 1987) with elevation seen in CD71, PCA-1

and serum Ig. The elevation was associated with HIV disease progression.

5.4.2.1 Possible causes of B cell activation

In vitro inhibition of B cell function may be related to a refractory state induced by the *in vivo* activation that has been reported. HIV+ve haemophiliacs have been reported to have a negative response to the soluble antigen ovalbumin (a T cell dependent B cell stimulant) (Sjamsoedin-Visser *et al*, 1987), which was due to a selective inhibition of secretion of immunoglobulin subclasses. Only resting B cells bearing IgM and IgD are stimulated *in vitro* by ovalbumin in the presence of T help. When B cells are activated to a stage where they have lost their surface IgD (*in vivo/in vitro*) they are no longer sensitive to antigen specific T helper signals. This suggests that inhibition of B cell function was due to the fact that B cells were no longer resting.

HIV+ve patients have been shown to have a disproportionate increase in serum levels of the different Ig isotypes (Mizuma *et al*, 1988), reflecting both polyclonal B cell activation and alterations in various homeostatic mechanisms that control serum Ig levels. Lane *et al*, (1983) have shown that Ig secretion is increased only in a small proportion of B cells.

Paradoxically hypergammaglobulinaemia and spontaneous IgG secretion is generally seen to accompany severe reduction of T cell numbers and is perhaps an indication of loss of control of B cell homeostatic mechanisms.

Direct or indirect mechanisms could be responsible for the B cell activation observed in HIV infection.

A. Direct mechanisms

Pahwa *et al* (1985), demonstrated polyclonal B cell activation by crude disrupted HIV preparations but these were inhibitory for the B cell differentiation responses induced by PWM, SAC and EBV and therefore may be reflecting the *in vivo* situation. Recent reports indicate that spontaneous HIV-1 specific Ig production and total Ig synthesis were highly correlated and that 20-50% of *in vitro* Ig production from HIV+ve patients was directed against the virus itself (Amadori *et al*, 1988, 1989, Amadori and Chieco-Bianchi, 1990, Pahwa *et al*, 1989) suggesting that HIV-1 antigens rather than indiscriminate polyclonal activation underlies the B cell hyper-reactivity in HIV+ve patients. The presence of activated B cells to HIV-1 determinants may have an important role in the pathogenesis of this infection by: enhancement of viral infection by cytokine release; cytotoxic damage to uninfected cells by uptake of virus-antibody complexes; and B cell activation involvement in lymphoma development (Amadori and Chieco-Bianchi, 1990).

B. Indirect mechanisms

It has been suggested that the activation of B cells is caused by an indirect method (. . Breen et al, 1990). B cell activation and immunoglobulin secretion is not consistently induced on exposure of purified B cells to HIV-containing supernatant. IgG and IgM stimulation correlated with alterations in numbers of T cell subsets where IgG and IgM secretion increased with the reduction of $CD4^+$ and rise in $CD8^+$

cells (Mizuma *et al*, 1988) perhaps indicating a control of B cell responses by T cell induced lymphokines. Increases in spontaneous BCGF and BCDF reduction in haemophiliacs has been demonstrated but this phenomenon was not related to HIV seropositivity (Matheson *et al*, 1987). Significant increases in spontaneous IL6 production have been demonstrated in HIV+ve patients, compared to normal individuals (Breen *et al*, 1990), and this overproduction of IL6 *in vivo* may be contributing to the B cell activation seen in HIV+ve patients. It has been shown that IL6 is essential for spontaneous Ig production, although this is dependent on IL6 from monocytes not T cells (Amadori *et al*, 1989). IL-6 production has been demonstrated in cultures of mononuclear cells from normal donors exposed to live and inactivated HIV and was observed in the monocytes in the absence of T cells (Nakajima *et al*, 1989). This monocyte-released lymphokine may be important in lymphoma production in HIV infections and may be another example of monocyte-derived immunopathy in this disease. Monocytes have been demonstrated to produce increased TNF following stimulation with LPS and gamma interferon *ex vivo* from patients with AIDS. Therefore they may be involved in the B cell suppression seen *ex vivo* (Wright *et al*, 1988).

NK cells have also been shown to regulate the terminal stages of humoral responses. Diminished NK activity has been reported in HIV+ve patients (Rook *et al*, 1983, Sirianni *et al*, 1990) and HIV+ve haemophiliacs (Lederman *et al*, 1983, 1985b, Menitove *et al*, 1983, Porzsolt *et al*, 1984, Ziegler-Heitbrock *et al*, 1985, Matheson *et al*, 1986a) and loss in regulation may be contributing to polyclonal B cell activation. The

presence of elevated levels of $CD8^+/CD57^+$ cells which have suppressive activities for B cell differentiation and NK activity (Ziegler-Heitbrock *et al*, 1988) may also be contributing to B cell dysregulation.

5.4.3 CONCLUSION

HIV+ve haemophiliacs have evidence of immune activation similar to that reported in other HIV+ve individuals, where there is a progressive loss in T4 cells associated with a decline in immune function. HLA-DR expression is increased, however, but other T cell markers of T cell activation with the exception of sIL2-R (discussed in next section) are not increased. We may not be witnessing 'activation' of the T cells *per se* but this elevation of HLA-DR may represent activation of specific subpopulations of T cells. Another possibility is that the T cells are chronically activated *in vivo* and express gamma interferon induced HLA-DR, predominately on T8 cells, but no longer express CD25 (due to sloughing off of the receptor) or CD71 and are refractory to stimulation *ex vivo*.

B cell abnormalities are also present in individuals with HIV infection. As with the T cells, activation and suppression are evident. B cell activation has been shown to be directly induced by HIV and probably contributes, in part, to suppressed function *ex vivo*. Breakdown in the control mechanisms related to T cells, monocytes and NK cells also contribute to B cell dysfunction to a large degree.

5.5 IMMUNE ACTIVATION STATUS DETERMINED BY SOLUBLE IL2 RECEPTOR LEVELS

In this study we detected elevated sIL2R levels in HIV-ve and HIV+ve haemophiliacs and in HIV+ve non-haemophiliacs from the 'AIDS walk in clinic' at the Royal Free Hospital. Levels were higher in the HIV+ve patients, although this was not significant. These results compared with reports that sIL2-R levels were elevated in patients with HIV infection (Sethi & Naher, 1986, Kloster *et al*, 1987, Pizzolo *et al*, 1987b, Prince *et al*, 1988ab, Lang *et al*, 1988, Honda *et al*, 1989 and Schulte & Meurer, 1989). The levels reported in the patients differ from study to study, and is probably related to the method of detection. All studies, however, included internal controls to which the patient groups were compared.

The discrepancy between our study and that of Kloster and Honda may be related to the method of blood collection utilised in our study. To allow adequate time for clotting, blood was stored overnight at 4°C before separating the serum. To maintain uniformity, however, the blood collected from the controls was treated in a similar manner. The method of separating the serum may explain why our normal control group had high levels of sIL2-R when compared to the normal levels that were quoted by the kit manufacturer. This may have resulted from the sloughing off of receptors from the cells during storage, but Kloster *et al* (1987) reported that this phenomenon does not occur.

The elevated levels of sIL-2R detected in the HIV+ve haemophiliacs did not correlate with FVIII/FIX infusion

(amount, type, frequency), and therefore it is unlikely that they were induced by the infusion of concentrates *per se*. HIV+ve haemophiliacs had higher mean levels of sIL2-R when compared to HIV-ve haemophiliacs but this was not statistically significant. The elevated sIL2-R levels in the HIV+ve haemophiliacs were higher in those patients who were HCV+ve, however, numbers were probably too low for true significance. In contrast to the HIV-ve patients, classification of the patients into groups related to their AST levels on the day of testing did not show significance. Grouping the patients according to the general liver function over a period of time, however, demonstrated significant elevation in those patients with consistently high AST levels (chronic infection) when compared to those patients who had normal AST levels, conversely no relationship of this kind could be found in the HIV-ve haemophiliacs. This discrepancy between HIV-ve and HIV+ve haemophiliacs is probably related to HIV infection. In the HIV+ve patients it is probable that the patients have a gradual loss of control of their viral infection as they become more immunosuppressed, and the latent infection is being reactivated, as has been reported in other HIV+ve haemophiliacs with HBV infection (Waite *et al*, 1988). Patients with symptomatic HIV infection have higher abnormal levels of AST compared to those with less severe disease, an indication of their loss of control of the HCV infection. Presence of HCV antibody does not appear to correlate with active viral infection as there was no relationship between AST/gamma GT levels and HCV positivity, but assessment of anti-HCV titres or PCR studies for HCV may indicate if the HIV+ve patients with high AST are in fact experiencing reactivation.

Further evidence that this may be occurring has been demonstrated by Madhok & Forbes, 1990, in HIV+ve haemophiliacs who had reduced *ex vivo* production of IL2 which correlated with the severity of liver disease, but only in the HIV+ve individuals.

5.5.1 POSSIBLE CAUSES OF sIL2R LEVEL ELEVATION

The release of sIL-2 receptor appears to be a characteristic marker of T cell lymphocyte activation (Rubin *et al*, 1985).

In the HIV-ve haemophiliacs, elevated levels were related to acute elevated AST levels. The sIL2-R levels are probably related to normal immune responses to viral infection of the liver, and the immunopathology that may result is shown by raised AST levels (Dudley *et al*, 1972, White *et al*, 1982, Aledort *et al*, 1985, Alexander, 1990).

Elevated sIL2-R levels have been observed in a variety of inflammatory, infectious, immune and neoplastic disorders. The levels correlate with rheumatoid disease activity, where there are elevated levels in both the serum and synovial fluid (Symons *et al*, 1988), and elevation of sIL2-R has been demonstrated in childhood acute lymphoblastoid leukaemia (Pui *et al*, 1988). Patients with acute infectious mononucleosis had increased sIL2-R as compared to normal controls and this correlated with increased percentages of $CD8^+/HLA\text{-}DR^+$ cells (Sethi & Naher, 1986). It has been observed that sIL2-R levels were increased in hepatitis B infection, lung cancer and patients with acute lymphoblastic leukaemia.

As previously mentioned, sIL2-R levels have been reported to be elevated in individuals infected with HIV. The non-haemophiliac HIV+ve individuals, in our study, had higher levels of sIL2-R than was seen in the HIV+ve hamophiliacs, however, this was not statistically significant. It is possible that this elevation is related to the later presentation of non haemophiliacs, as these patients probably present much later than the HIV+ve haemophiliacs, who are a closely monitored patient group; and the high incidence of EBV or CMV or Kaposi's sarcoma, aspects of HIV infection not seen in the haemophiliacs (Christine Lee, Haemophilia centre and Haemostasis unit, RFH, personal communication). Honda *et al* (1989), demonstrated an association between sIL2R and the clinical classification of HIV disease in haemophiliacs. A relationship between T4 count, T4/T8 ratio and clinical disease has also been reported in other HIV+ve patients (Prince *et al*, 1988b, Lang *et al*, 1989, Schulte and Meurer, 1989). Honda *et al* (1989), found significant correlations of sIL2-R with T4/T8 ratios and decreased T4 counts, and related this finding to chronic activation, perhaps by other viral or parasitic infections leading to T4 cell loss and increases in levels of sIL2-R.

Other studies (Pizzolo *et al*, 1987b, Kloster *et al* (1987), Lang *et al* (1988), and our own, however, found there was no association between sIL2-R levels and HIV disease progression or decreased T4 cell numbers. Moreover in the present study sIL2-R levels did not correlate with activated T cells (HLA-DR), this data verifies the study by Lang et al (1989). Switching on of the T4 cells could be facilitated by the

presence of opportunistic viral infections such as HTLV-I and HTLV-II, as suggested by Honda *et al* (1989), and this could be indicated by the sIL2-R levels. Large increases in sIL-2R levels were reported by Kloster *et al* (1987), in HTLV-I+ve adults with T cell leukaemia where the mean was 10 fold higher than that seen in AIDS. HTLV-1, however, is not transmitted in the concentrates and the haemophiliacs do not have an increased incidence of this virus (Chorba *et al*, 1985). Elevated levels of sIL2-R observed in groups at risk of HIV (drug abusers/homosexuals) raises the possibility that at least in some cases the T cell activation, as determined by sIL2-R release, could be associated with other infections.

5.5.2 HIV RELATED INCREASES IN sIL2-R - CAUSES

Although sIL2-R is a characteristic marker of T cell activation, it is probable that the reasons for its elevation in HIV infection are multifarious and that this is related to the complexities of HIV infection itself.

sIL2-R may be emanating from T4 cells that are lysed as a result of infection with HIV. The proportion of infected mononuclear cells is <0.01% (Harper *et al*, 1986) and it is unlikely that T cell lysis would lead to the levels of sIL2-R seen. Schnittman *et al* (1989), demonstrated that approximately 1:1000 $CD4^+$ T cells in AIDS patients expressed HIV. This is in line with other studies (1/100,000 to 1/10,000 PBMC expressing viral mRNA), but Schnittman has shown, using PCR techniques, that at least 1/100 $CD4^+$ T cells in AIDS patients contain HIV-1 DNA. In contrast, asymptomatic patients have a reduced viral load, with viral detectible DNA of about 1/10,000 $CD4^+$ T cells. This indicates that AIDS patients have a much higher viral load than at first

anticipated and in the latter stages of the disease the viral DNA could be switched on and could be responsible for the demise of the T4 cells seen at this stage of the disease. Therefore it is possible that the gradual rise in sIL2-R, seen in some studies, may be reflecting this.

It is important to characterise the contribution of the lymphocytes within the lymphoid tissues, a major site of T cell activation in HIV, to sIL2-R levels in the serum. Janossy *et al* (1989), have reported enrichment of the lymph nodes with exceptionally cytotoxic T cells ($CD45RO^+CD8^+$) and he hypothesised that they may be contributing to the decrease in T4 cell numbers.

Prince *et al* (1988ω) failed to find support for the hypothesis that sIL2R levels reflected HIV associated decreases in T cell numbers caused by activation. Production of sIL2-R was shown to parallel cellular IL2-R expression and DNA synthesis. HIV related defects affecting cellular IL2-R also affected sIL2-R production. As IL2 production is diminished in HIV infection this evidence strongly suggests that sIL2-R in HIV+ve patients may not be emanating from activated T cells.

It has been demonstrated that sIL2-R is actively released from living cells and is not the sequelae of cell death (Kloster *et al*, 1987). Studies have shown that sIL2-R is released by $T4^+$, $T4^-$ and B cells following activation by mitogen and specific antigens (Nelson *et al*, 1986, Wagner *et al*, 1986).

sIL2-R has been shown to correlate with B_2 microglobulin and neopterin levels (Schulte & Meurer, 1989, Lang *et al*, 1988,

1989). Neopterin is a sensitive indicator of monocyte/macrophage activation (Fuchs *et al*, 1988). Monocytes have been shown to express cell surface IL2-R and have also been found to release sIL2-R under conditions different from those required for T and B cells (Holter *et al*, 1987). Monocytes play a very important role in HIV infection (section 1.7.4). There is evidence of activated monocytes in HIV infected individuals. Increased IL2-R on monocytes from patients with AIDS has been recently reported and shedding of IL2-R may contribute to the increased levels of sIL2-R in AIDS patients (Allen *et al*, 1990). HIV-1 binding to the CD4 receptor of monocytes induces IL2-R and HLA-DR genes. Thus in the monocytes, sIL2-R production may be a direct result of infection with HIV-1. Therefore, monocytes and possibly antigen presenting cells may be the sources of sIL2-R in HIV infection (Kloster *et al*, 1987).

Serum B_2 microglobulin reflects B cell proliferation, and this too, as previously mentioned correlates with sIL2-R levels. As activated B cells express and release sIL2-R (Green *et al*, 1986b) B lymphocytes may represent another source of sIL2-R in HIV infection. It is therefore possible that serum sIL2-R reflects monocyte and possibly B cell alterations rather than T cell activation and destruction alone (Lang *et al*, 1988).

Clearance of sIL2-R is decreased in disturbances of liver or renal function by HIV infection (Honda *et al*, 1989), and this is probably very relevant in HCV and HBV infection. In both HIV+ve and HIV-ve haemophiliacs, elevation of sIL2-R could be a result of reduced clearance as well as increased production.

5.6 CONCLUSION

The initial question of the study was 'do FVIII concentrates activate the immune system and thus accelerate the progression of HIV disease?'. While there was no evidence of elevation of cellular activation markers of the peripheral blood lymphocytes in the HIV-ve haemophiliacs, there was elevation of sIL2-R levels which correlated to liver function abnormalities and thus probably to HCV infection. We can conclude from this that there is activation of the immune system in these patients but it is unlikely to be caused by antigen overload, as no correlations were seen with concentrate infusion, but is related to infection with blood transmitted virus. Since the introduction of effective heat treatment, live viruses are (hopefully) no longer present in the concentrates. The patients, however, may still react to viral antigens to which they have been primed by earlier infection. New heat treated products have been demonstrated, in ongoing trials not to have immunomodulating effects on the lymphocytes of previously untreated patients (Pasi *et al*, 1989, 1990a). From this we can assume that antigen overload does not lead to lymphocyte dysfunction, and that the defects are from viral transmission alone.

One would expect activation of the immune system to lead to an alteration in the progression of HIV disease due to the acceleration of viral replication. HIV+ve haemophiliacs do have evidence of lymphocyte activation, but they do not appear to differ significantly from other HIV+ve patient groups. Both HIV-ve haemophiliacs and other HIV 'at risk' groups have been demonstrated to have high levels of sIL2-R, suggesting

both populations had activated immune systems prior to infection with HIV. The high incidence of viral infection (HCV, HBV) reported in haemophiliacs is also seen in homosexual and IV drug abusing groups, along with high incidence of other infections eg, EBV, CMV, syphilis and allogeneic assault eg, semen (Ho *et al*, 1987).

Therefore we may be seeing comparable patient groups in terms of HIV disease with chronic lymphocyte activation from infections accelerating the progression of the disease in both patient groups. Comparison of the HIV infected haemophiliac cohorts of England and America with the American non-haemophiliac HIV infected cohort has demonstrated that the two groups do not have different rates of disease progression. This further substantiates the evidence that the immune system of haemophiliacs is not being activated to a greater degree, than other HIV+ve patients, by the infusion of clotting factor concentrates (Giesecke *et al*, 1988). It will be interesting to follow progression of disease in patient groups who do not have a history of multiple infection eg, occupational HIV infections in hospital staff.

The rate of progression of HIV disease in haemophiliacs is lower than that in transfusion recipients (Giesecke *et al*, 1988). This is probably related to increased triggering of HIV replication by allogeneic reactions resulting from infusion of high levels of MHC antigens on the blood cells, which has been shown to occur *in vitro* in monocytes and T cell cultures (Schrier *et al*, 1990). It should be noted that patients receiving blood transfusions are by definition 'sick'

and their increased mortality may relate to their underlying disease state.

HBV can lead to activation of the lymphocytes and HCV, CMV and EBV all have immunomodulating capabilities so it is plausible that multiple infection by these virus in HIV+ve patients is affecting the progression of HIV disease. Indeed, Webster *et al* (1989), has shown that progression of disease to AIDS is facilitated by the presence of CMV. We can conclude that infusion of virally contaminated concentrate in the past may have influenced the progression of HIV disease in these patients. Teitel *et al* (1989), reported a stabilisation in the T4 count of the HIV+ve patients and a normalisation of the T4 count in the HIV-ve patients which coincided with the introduction of new products in which the attenuation of HCV was achieved and with the introduction of newer, safer products, viral induced activation in previously infected patients should no longer be occurring.

Concentrates are now heat treated and are unlikely to transmit live virus, but, activation of the immune system may be occurring in the previously infected patients because of the presence of viral antigen to which they are already primed. We have seen no evidence of this, however, when analysing vaccine induced antibody response to hepatitis B surface antigen. There was no correlation with titre of antibody and FVIII/FIX infusion. Nor was there a difference between those patients who received or did not receive infusion of concentrate (Oon *et al*, 1990) indicating that the infusions of attenuated HBV in the concentrates was not increasing antibody response.

HIV+ve patients, however, switching from intermediate purity (Kryobulin TIM3, Immuno, Austria) to high purity FVIII products (Hemophil M, Baxter Hyland Division, USA) have been shown to have a stabilisation of T4 counts following 48 weeks of treatment. These patients had significantly higher T4 counts than the control group of patients who remained on intermediate purity products, where the T4 numbers continued to decline (Rocino *et al*, 1990). This report indicates that a component in the intermediate purity concentrates is influencing the progression of HIV disease. This report, however, only showed preliminary data from 20 patients (10 per group). It is possible that with greater numbers of patients the statistical significance of this will decline as has been found in other long term studies of high purity versus intermediate purity concentrate studies (Brettler and Levine, 1989 Brettler, 1991). The intermediate purity product was heat treated at 60°C in the presence of solvents, which is similar to the method of heat treatment used in the production of Alpha Prolfilate (wet heat treated). This product has been demonstrated to transmit HCV, although at a reduced rate, (Kernoff *et al*, 1987) and therefore it is possible that the Kryglobulin TIM3 may not be 'live virus free'. This study, therefore may be comparing 'live virus' versus 'no live virus'.

The possibility that recognition of attenuated virus may lead to an immunomodifying effect, will only be eliminated with the introduction of purer products or recombinant product.
Although we may not be seeing evidence of non-specific activation or specific activation of viral particles one

problem that should be addressed is suppression induced by the presence of viral particles eg CMV, EBV, HCV, HBV, or HIV. As previously discussed peptides of HIV have been shown to lead to immune-modulation, it is possible that the other viruses are doing this also. The presence of these viral particles may disrupt the immune responses to infection. In studies of mice models of infection with *Listeria monocytogenes*, Virgin and Unanue, (1984), have shown that secondary challenge with a protein antigen suppressed responses to listeria and introduced a transient state of high susceptibility to infection. The mechanisms of this suppressive state were attributed to the formation of immune complexes. Viral particles should not be present in the concentrate, however, due to fractionation processes following their heat treatment (Dr J Smith, BPL, Oxford, personal communication).

Therefore immunomodulation in haemophiliacs is probably occurring in those patients who have previously been infused with contaminated products and the problem will probably remain until the widespread introduction of synthetic products.

Although concentrate induced modulation may not be important now, in terms of the haemophiliac immune system, it may have been important at the time when these patients were being infected with HIV, when live virus (HBV, HCV etc) was present. Thus the supposition that these patients were more susceptible to infection by HIV as referred to in the introduction, may have had some grounds. We still await the effects of the possible transmission of slow virus or as yet undefined

infectious agents in these patients. We conclude that the concentrates *per se* are not causing modulation of lymphocyte function *in vivo* and that abnormalities that exist are due to viral infection. The presence of clotting factor concentrate in *vitro* lymphocyte cultures, however, has been demonstrated to be immunomodifying. The relevance of this will be discussed in the next section.

Although the concentrates do not appear to cause lymphocyte abnormalities *per se*, there is evidence that they can affect monocyte function. Pasi & Hill(1990), have demonstrated that the infusion of concentrate caused transient inhibition of monocyte function *ex vivo*. Eibl (1991), has demonstrated that HIV-ve haemophiliacs have a defective response to vaccine. Patients were vaccinated against tick borne encephalitis virus. Primary responses (determined from antibody response and T cell proliferation assays) were normal, but, secondary responses were virtually absent. The study demonstrated that the lymphocytes were probably functioning normally as the primary response was intact.

The secondary response is dependent upon antigen presentation to memory cells sited in the lymph nodes. Primary responses may involve antigen presentation to T cells by B cells, thereby bypassing monocytes/antigen presenting cells. Therefore it is probable that the defect in the response to booster vaccine is related to defective antigen presentation rather than defective lymphocyte function. Further studies are in progress to verify this hypothesis.

In vitro studies have demonstrated that the monocyte dysfunction is related to the presence of immune complexes (Eibl *et al*, 1987, Mannhalter *et al*, 1988). Purification of the concentrates will reduce Ig and immune complex levels. Pasi (1991), has demonstrated that products of a high specific activity are less inhibitory for monocyte function.

Monoclonal antibody-purified and recombinant products, however, will not necessarily eliminate immune complex formation *in vivo*, because of the presence of mouse monoclonals used in their production. Therefore unless there are advances in the use of 'humanised' monoclonal antibodies, switching to these more pure products may not affect the capacity of the concentrate to inhibit monocyte function (Eibl, 1991).

To conclude, clotting factor concentrates *per se* probably inhibit monocyte function. The clinical significance of this, however, appears to be minimal. In most studies haemophiliacs do not appear to have an increased risk of infection and the lymphocytes (in the absence of virus) do not appear to be affected.

6. *IN VITRO* STUDY

6. IN VITRO STUDY

6.1 INHIBITION OF T CELL FUNCTION BY CLOTTING FACTOR CONCENTRATES - AN OVERVIEW

As discussed in section 1.6, FVIII concentrates can partially inhibit mitogen-induced T cell proliferation and IL2 production *in vitro*. It has been postulated that this phenomenon may be causing the immune dysfunction reported in HIV-ve haemophiliacs (Froebel *et al*, 1983, Wang *et al*, 1985, McDonald *et al*, 1985, Lederman *et al*, 1986).

We sought to examine potential mechanisms involved in the inhibition of T cell function *in vitro* by clotting factor concentrates.

There have been several suggestions put forward to explain the cause of inhibition of T cell function by the concentrates.

The partial inhibitory effect on IL2 production by PBMC and Jurkat cells by the FVIII concentrates was shown not to be due to cytotoxicity. It has been suggested that the inhibition of early events in T cell activation processes was a specific consequence of the down regulation of IL2 production by a component(s) of the FVIII preparation (Lederman *et al*, 1986).

Buffer used in the formulation of the concentrates have been demonstrated to inhibit lymphocyte function, and the inhibitory component has been shown to be at least partially dialysable (McDonald *et al*, 1985, Wang *et al*, 1985). McDonald *et al*, have suggested that this phenomenon may be related to the presence of citrate in the buffers. They have shown that

citrate, at levels similar to those present in the concentrates could inhibit T cell proliferation in a variable manner, and that plasma products formulated without citrate in the buffer were not inhibitory.

The addition of FVIII concentrates to cultures of monocytes from normal donors *in vitro,* has been shown to down-regulate Fc receptor expression and inhibit monocyte function (Eibl *et al*, 1987, Mannhalter *et al*, 1988).
Monocytes play an important role in preliminary events in T cell activation and the inhibition in accessory cell function may be contributing to the T cell dysfunction that has been reported.

The mechanisms of the T cell inhibition reported *in vitro*, however, still remains unclear. By systematically studying the effects of concentrates upon *in vitro* T cell activation, this study has attempted to gain an understanding of the potential mechanisms of concentrate-induced inhibition.

6.2 OUT-LINE OF STUDY

To elucidate the mechanisms of inhibition of T cell function by clotting factor concentrates, the following questions were asked:

1. Do all concentrates lead to T cell dysfunction?

2. Where in the T cell cycle is the inhibition occurring?

3. Is the partial inhibition seen related to a specific subset of T cells?

The information gained from points 2 and 3 could then be used to understand the potential mechanisms of the inhibition.

A variety of products were studied to establish if all the concentrates were capable of inhibiting T cell function *in vitro*.

The expression of activation markers which precede IL2 production, namely HLA-DR, and those that are influenced by IL2 production (CD25 and CD71), were measured on normal T cells stimulated with mitogens in the presence of FVIII concentrates. This would establish where in the T cell cycle the inhibition of activation was occurring and if the inhibition was specifically directed at IL2 production alone.

Two colour flow cytometric analysis of the expression of the activation markers on T4 or T8 cells would elucidate the specificity of the inhibition on T cell subsets. To determine

specificity of the inhibition against T helper cell subsets the lectins PHA and PWM were also used (section 1.8.3).

6.3 PRELIMINARY EXPERIMENTS

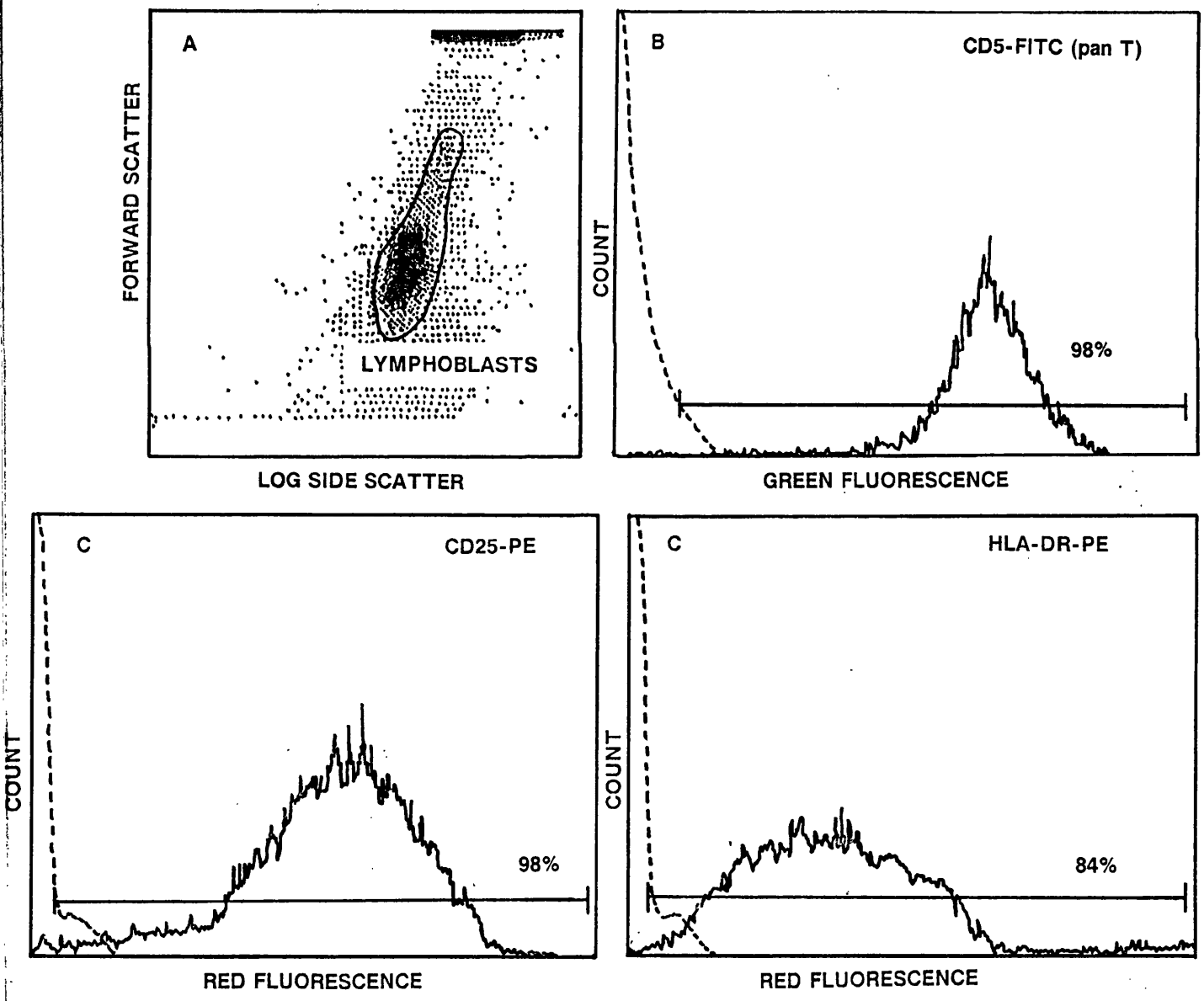
Peripheral blood mononuclear cells were separated and cultured for 96 hours in the presence of either PHA or PWM as described in section 2.3. Base line levels of the activation markers during a 96 hour lectin stimulated culture of PBMC was established. The expression of a preliminary panel of T cell activation markers HLA-DR, CD25, CD71 and 4F2 was measured. T4 and T8 cells were determined by two colour flow cytometry using methods described earlier (section 2.4, table 2.3).

Stimulation of the PBMC with lectin was accompanied by cell clumping. Blastogenesis of the T cells occurred and they acquired lymphoblastoid characteristics. Analysis by flow cytometry involved the identification of these cells by an electronic bitmap (figure 6.1). The activated cells were indistinguishable from the monocytes by their size and side scatter characteristics. Using two colour analysis (section 2.5), the T lymphocytes were further identified using a 'window gate' corresponding to the cells that were positive for the pan T reagent (histogram B in figure 6.1). The standard pan T reagent used in the activation marker study (section 4) was anti-CD3.

Analysis of the expression of CD3 throughout the 96 hour culture established that its expression declined during the early stages (figure 6.2) and it was therefore unsuitable as a pan T reagent in this study.

Figure 6.1: PBMC were stimulated with lectin and cultured for 96 hours. Blastogenesis occurred accompanied by cell clumping. The blasts were identified on the basis of their size and side scatter characteristics and isolated by the creation of an electronic bit map (histogram A). The blasts were further identified with a pan-reagent, CD5, for the detection of T cells (But CD4 or CD8 were also used for the detection of specific T cell subsets (histogram B). Positive cells were determined as those where the fluorescence was greater than 98% of the mouse Ig controls (-----). The presence of activation marker expression was determined by creating a 'window gate' around the cells positive for the pan-reagent. Cells that were positive for the pan-reagent, in this case CD5-FITC, and the activation marker (using an alternative fluorochrome to the pan-reagent) were then identified in histogram C. Two examples of histogram C are shown in the example: HLA-DR and CD25, directly conjugated to phycoerythrin (PE).

TWO COLOUR ANALYSIS OF 96 HOUR, PHA STIMULATED LYMPHOBLASTS USING THE COULTER EPICS PROFILE FLOW CYTOMETER



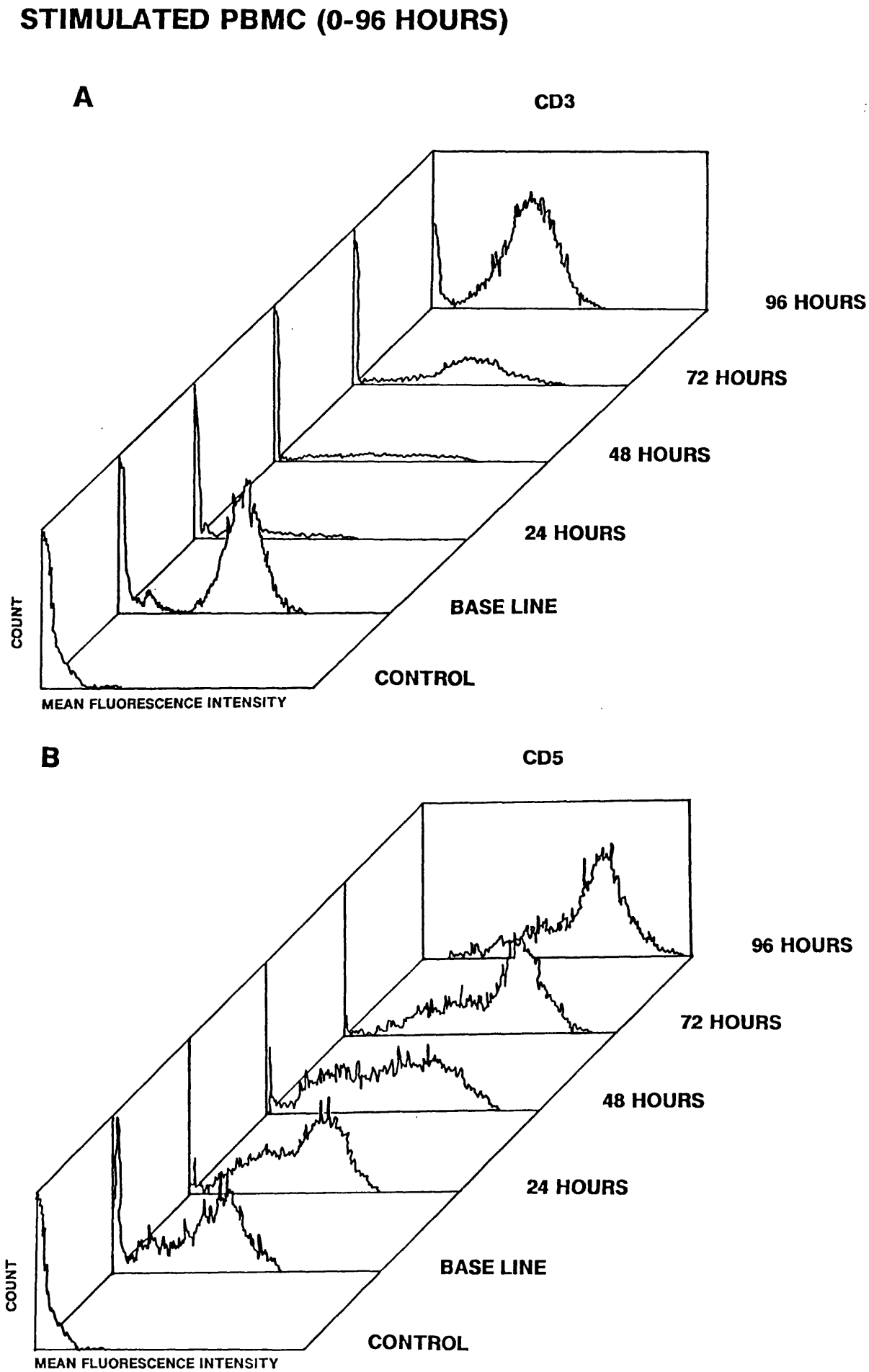


Figure 6.2: PBMC were stimulated with PHA and cultured for 96 hours. The cells were labelled with the pan-T reagents anti-CD3 or anti-CD5 at 24 hourly intervals and analyzed by flow cytometry.

The reduction in expression of CD3 may be related to binding of PHA to this antigen (Leca *et al*, 1986). The density of CD5, an alternative pan T marker, however, increased three fold over the 96 hour period ($p<0.05$) (figure 6.2) and was subsequently used as the pan T marker throughout the *in vitro* study.

The expression of the activation markers on T cell subsets was established with the reagents anti-CD4 and anti-CD8 (table 2.3). The expression of activation markers upon T helper subsets was determined by specific stimulation with PHA and PWM. The cells were analysed at 24 hourly intervals over a 4 day period. Expression was determined by the percentage of the cells in the population positive for the marker and the density of antigen upon these cells (mean fluorescence intensity).

6.3.1.1 Expression of activation markers on $CD5^+$ lymphocytes

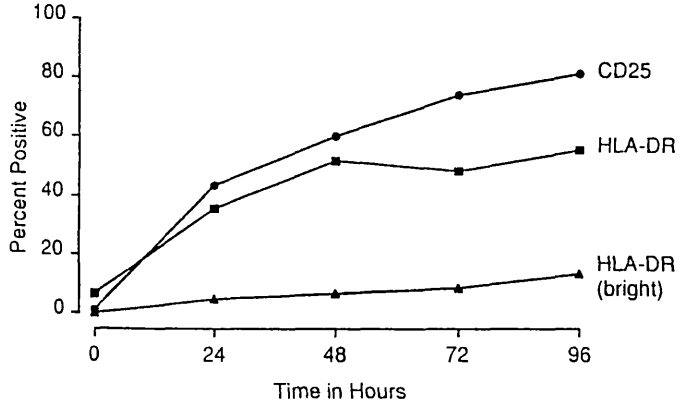
Activation markers are expressed following the activation of the T cells and were only found at low levels pre-stimulation.

A. HLA-DR antigen and CD25 antigen expression

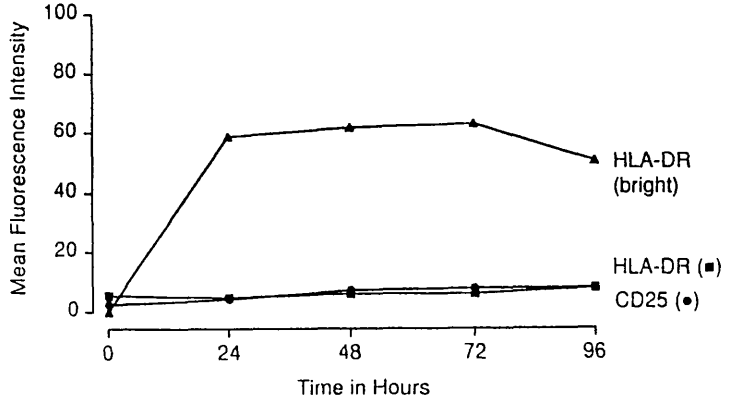
At twenty-four hours of culture, following stimulation with PHA, approximately 40% of $CD5^+$ lymphocytes were expressing HLA-DR and CD25 (figure 6.3a).

The percentage of cells that were HLA-DR^+ reached a maximum and stabilised at 48 hours, whereas the number of cells expressing CD25 continued to increase until 96 hours when the experiment was stopped. Although the number of cells

a) Expression of HLA-DR and CD25 upon CD5 +ve Lymphocytes Stimulated with PHA (% positive)



b) Expression of HLA-DR and CD25 upon CD5 +ve Lymphocytes Stimulated with PHA (mean fluorescence intensity)



c) Expression of CD71 and 4F2 upon CD5 +ve Lymphocytes stimulated with PHA (% positive)

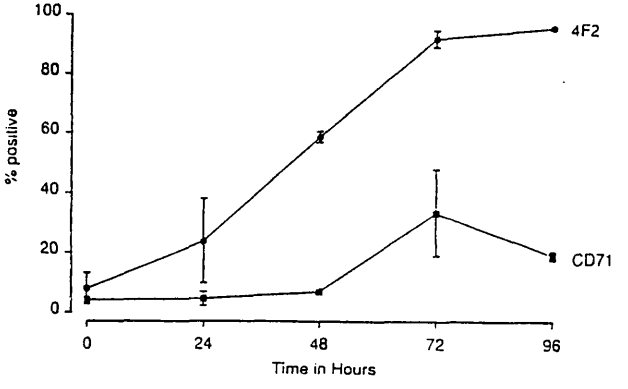


Figure 6.3: PBMC were stimulated with PHA and cultured for 96 hours. Two colour analysis of the expression of HLA-DR, CD25, 4F2 and CD71 was determined at 24 hourly intervals on PHA stimulated $CD5^+$ lymphocytes.

expressing these markers increased, the antigen density (determined by the mean fluorescence intensity) per individual cell remained low (figure 6.3b). A small subpopulation of the lymphocytes, however, expressed an increased density of HLA-DR up to 20 times higher than that seen on the other HLA-DR$^+$ cells. The proportion of these cells, referred to as 'HLA-DR bright' increased gradually throughout the culture, but did not exceed more than 10% (figure 6.3a). Maximum density of HLA-DR on this subset of cells was achieved by 24 hours after which it stabilised, beginning to diminish by 72 hours of culture (figure 6.3b).

B. 4F2 antigen expression

4F2 expression on increased CD5$^+$ lymphocytes at a steady rate throughout the 96 hours of culture (figure 6.3c), appearing after CD25 and HLA-DR.

C. CD71 antigen expression

CD71 was found on 20-40% of the cells at 72 hours, before and after this, expression of this marker was minimal (figure 6.3c).

CD71 and 4F2 did not appear to be giving any additional information to HLA-DR and CD25, and therefore were not used in the detailed analyses.

6.3.1.2 Expression of activation markers on CD4$^+$ lymphocytes

There was a slight increase in the percentage of T4 cells and expression of T4 antigen following 96 hours of culture in lectin stimulated cells. This increase was not statistically

significant.

PHA: The proportion of T4 lymphocytes expressing CD25 at 24 hours in the PHA cultures was greater than those expressing HLA-DR (figure 6.4a). All T4 cells (99%) expressed CD25 by 48 hours of culture, but the density of antigen, determined by the MFI, did not maximise until 72 hours (figure 6.4b). From this point the level began to decline.

The percentage of cells expressing HLA-DR was maximal at 48 hours when 93% of the cells were positive, following this numbers diminished slightly but remained fairly stable (figure 6.4a). Mean fluorescence intensity of HLA-DR (representing antigen density) was constantly low throughout the culture (figure 6.4b).

Again as seen with the $CD5^+$ cells there were two distinct subpopulations of $HLA\text{-}DR^+$ cells. The percentage of cells expressing high levels of this antigen did not exceed 10% and maximised at 48 hours (figure 6.4a). Mean fluorescence intensity of the HLA-DR 'bright' cells increased rapidly during the first 24 hours of the culture, and then stabilised (figure 6.4b).

PWM: The expression of HLA-DR and CD25 upon T4 lymphocytes stimulated with PWM was distinctly different from that seen when the cells were stimulated with PHA (figure 6.4c/d).

There was greater donor variability in reaction to this lectin than was seen with PHA and the response was slower. Approximately 60% of cells expressed these markers at 96 hours. Both markers were expressed synchronously with a

Expression of HLA-DR and CD25 on CD4+ve and CD8+ve lymphocytes stimulated with PHA and PWM

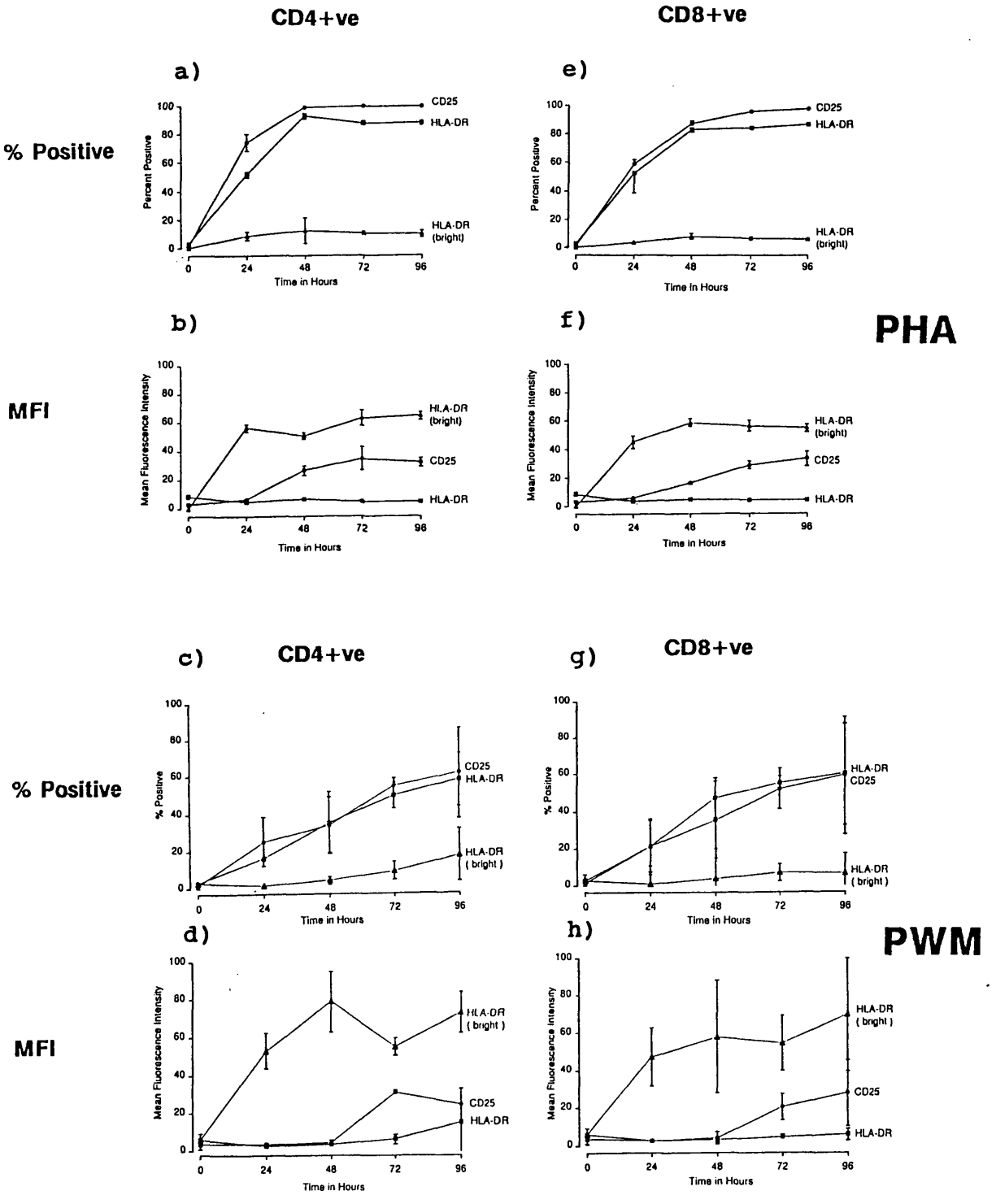


Figure 6.4: PBMC were stimulated with PHA or PWM and cultured for 96 hours. Two colour analysis of the expression of CD25 (●—●), HLA-DR (■—■), and 'HLA-DR bright' (▲—▲) was determined at 24 hourly intervals on $CD4^+$ and $CD8^+$ lymphocytes. (Mean ± standard deviation of three experiments).

linear increase throughout the culture. The HLA-DR 'bright' population also increased gradually throughout the culture with almost 20% of the cells expressing a high density of this marker at 96 hours (figure 6.4c).

The density (mean fluorescence intensity) of CD25 did not increase from base line levels until 48 hours (figure 6.4d), in contrast to the PHA blasts where expression of this marker was almost maximal at this point. It maximised, however, at 72 hours and then started to decline in a manner similar to that seen with PHA (figure 6.4d). In contrast to PHA stimulated T4 cells there was a marked increase in the density of HLA-DR at 96 hours in the PWM cultures (figure 6.4d). Maximum density of the HLA-DR antigen in the 'bright' population was slightly higher in the culture stimulated with PWM than in the PHA stimulated cells. Expression of this marker maximised at 48 hours, dropped at 72 hours but had increased again at 96 hours.

6.3.1.3 Expression of activation markers on $CD8^+$ lymphocytes.

PHA: T8 lymphocytes stimulated with PHA followed a similar pattern of expression of activation antigens to that seen in the in T4 lymphoblasts. The percentage of cells positive for CD25, however, was lower at 24 and 48 hours (figure 6.4e). The density of the markers on the T8 cells was also similar to that seen in PHA stimulated T4 cells (figure 6.4f), but density of the CD25 antigen did not reach maximum until 96 hours of culture, compared to T4 cells stimulated with PHA which had maximum expression of this marker at 72 hours.

PWM: Expression of HLA-DR and CD25 on T8 cells stimulated

with PWM was similar to that on T4 cells stimulated with this lectin and was delayed in comparison to PHA stimulated T8 cells (figure 6.4g). Again as was seen with PHA stimulated cells, CD25 antigen density did not reach maximum until 96 hours compared to 72 hours in PHA stimulated T4 cells. The percentage of cells expressing high intensity HLA-DR was higher in the T4 cultures than in the T8 cultures and the peak mean fluorescence intensity at 48 hours was also higher (figure 6.4h).

6.4 THE EFFECTS OF FVIII CONCENTRATE ON THE EXPRESSION OF THE ACTIVATION MARKERS

Prior to the start of this study there had been several reports that clotting factor concentrates of American, German and Scottish origin, inhibited T cell proliferation and IL2 production (Jurkat cells) in a dose dependent manner.

A simple experiment was performed to determine whether the activation markers HLA-DR, CD25, CD71 and 4F2 were inhibited in the presence of FVIII concentrate. Normal PBMC stimulated with PHA were cultured for 96 hours in the presence of high dose (2u/ml) and low dose (0.001u/ml) Alpha Profilate. The expression of HLA-DR, CD25, 4F2 and CD71 was determined. The results are shown in figure 6.5. HLA-DR, CD25, 4F2 and CD71 expression on $CD5^+$ cells was inhibited in the presence of 2u/ml of FVIII over the 96 hour period. The percentage of cells positive for HLA-DR expression was low throughout the culture and may represent donor variation. Culture with low doses of FVIII (0.001u/ml) inhibited CD25 and 4F2 expression, but the inhibition was less than that seen at the higher

Expression of Activation Markers in the Presence of High and Low Concentrations of FVIII Concentrate

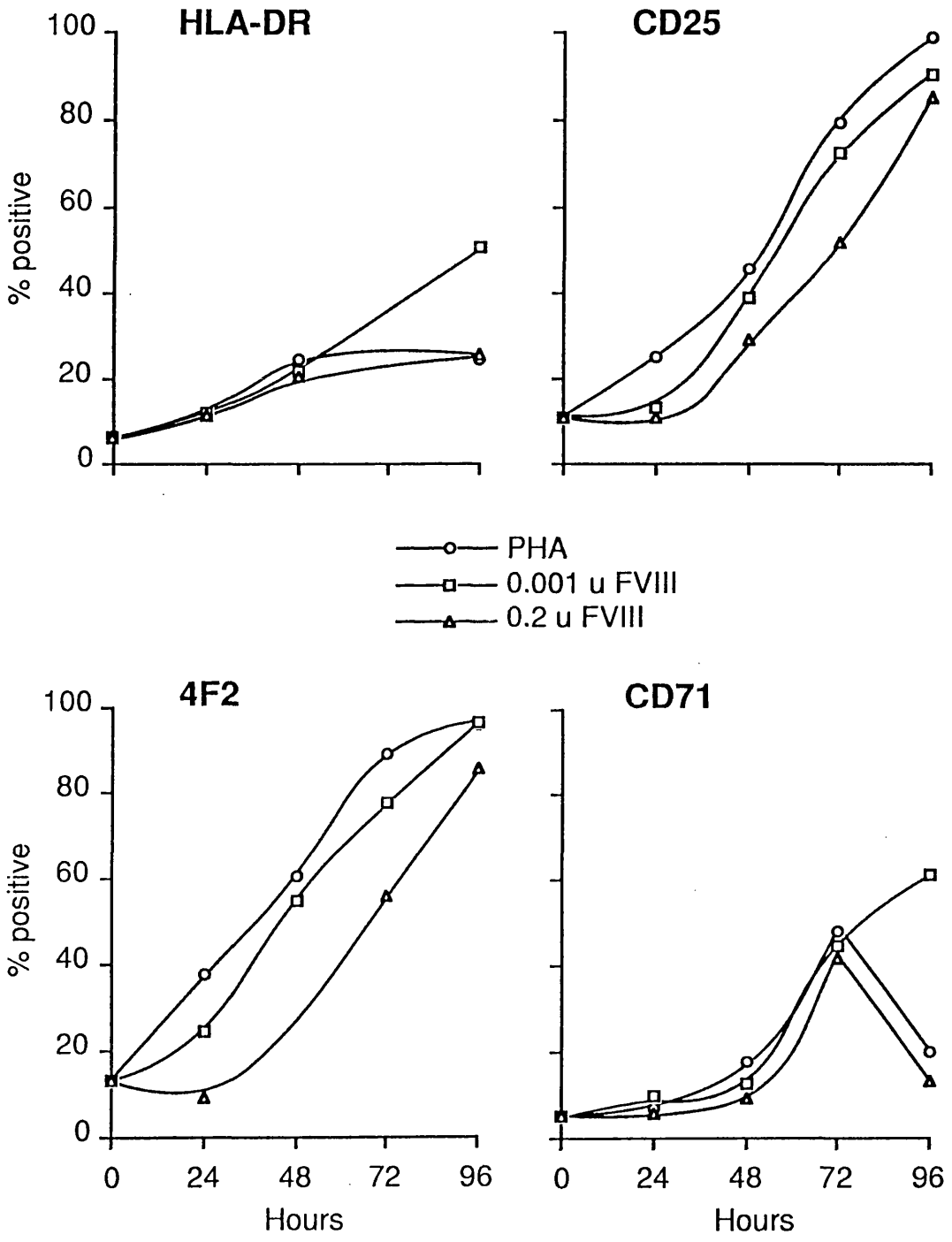


Figure 6.5: Alpha Profilate was added to PHA stimulated cultures (O—O) at 0.001u/ml (□—□) and 0.2u/ml (Δ—Δ). The cells were cultured for 96 hours and the proportion of $CD5^+$ lymphocytes expressing the activation markers HLA-DR, CD25, 4F2 and CD71 was determined at 24 hourly intervals.

concentration. The proportion of cells expressing CD71 and HLA-DR, although slightly inhibited for up to 48 hours in the presence of 0.001u/ml FVIII concentrate, then increased up and above that seen in the control (PHA stimulated cells without FVIII) until at 96 hours the percentage of cells positive for these markers was twice that seen in the control and may be evidence of stimulation.

6.4.1 TITRATION OF LECTINS PHA AND PWM IN THE PRESENCE OF FVIII

To study the mechanism of the inhibition of T cell activation by clotting factor concentrates all experiments were conducted in situations where maximum inhibition was attained. Preliminary studies had established that the FVIII concentrates inhibited T cell function in a dose dependent manner (data not shown) and confirmed the results of Lederman *et al* (1986). Therefore to achieve maximum inhibition we decided to use very high doses of concentrate. A 1:5 dilution of FVIII concentrate was used throughout the inhibition studies, and this corresponded to approximately 4-5 units of FVIII/FIX/ml of culture medium, ie, 4-5u/1x10^6 cells.

Oppenheim *et al* (1970), demonstrated that maximal inhibition of the response to lectins was achieved at suboptimal concentrations of lectin. PHA and PWM were titrated, in duplicate, in the presence of 1:5 FVIII to determine the concentration of lectin where maximal inhibition could be achieved. These results are shown in figure 6.6.

CD25 expression was determined on $CD5^+$ lymphocytes following 96 hours of culture. Ninety-five percent of cells expressed CD25 in the presence of 1:50 PHA (figure 6.6a): however, a 1.5

Effect of the titration of lectins (PHA and PWM) upon CD25 expression with and without FVIII concentrate

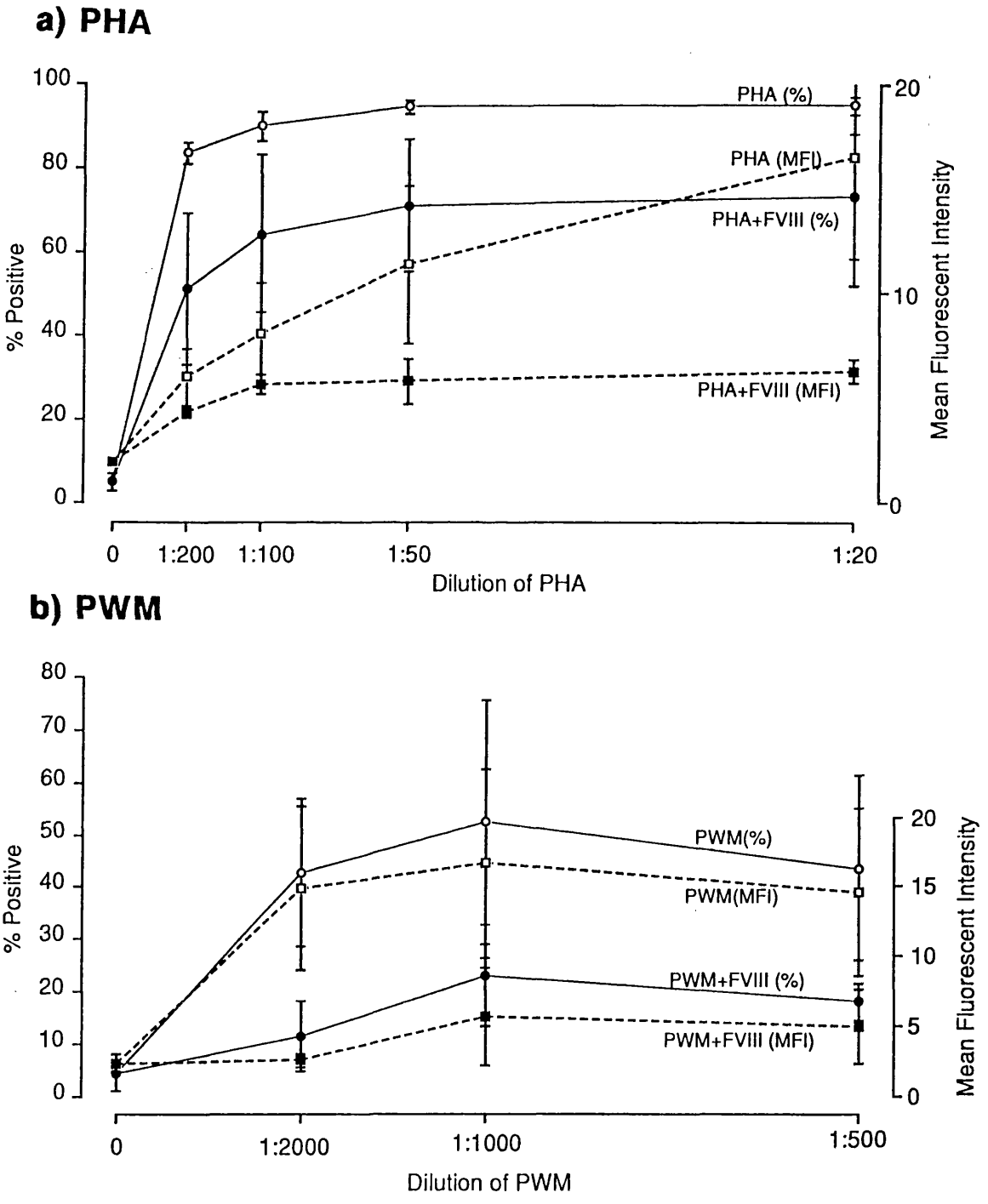


Figure 6.6: PHA and PWM were titrated in PBMC cultures with and without 4-5 units/ml FVIII (Alpha Profilate). Following 96 hours of culture, two colour analysis of CD25 expression on $CD5^+$ lymphocytes was determined. The percentage of cells positive for CD25 (○—○) and CD25 antigen density (□--□) were measured. The effect of FVIII concentrate upon the expression of CD25 (percent positive cells (●—●) and antigen density (■---■) was determined. (Mean ± standard deviation of three experiments).

fold increase in the intensity of antigen expression was achieved by increasing the amount of PHA (a 1:20 dilution). Inhibition of the number of cells expressing CD25 by FVIII concentrate (Alpha Profilate) was greatest at 1:200 dilution ($p<0.05$). In contrast the inhibition of CD25 antigen density (determined by MFI) was greatest at a 1:20 dilution of PHA ($p<0.05$). Therefore a 1:50 dilution was chosen as this gave maximal activation and inhibition of CD25 was high.

In experiments where PWM was titrated (figure 6.6b), maximal expression of CD25 (percentage of cell and antigen density) was attained at a dilution of 1:1000. Maximal inhibition of expression of CD25, by FVIII was attained at the lowest concentration of PWM (1:2000). Inhibition of CD25 expression by FVIII concentrate was not statistically significant, but this was probably because of the high standard deviations in this experiment. These were presumably caused by variation in individual donor response to PWM and the difference in the inhibitory capacity of the products tested.
Due to variable donor response to this lectin the titration that gave the greatest activation (1:1000) was chosen.

6.4.2 INHIBITION OF ACTIVATION MARKERS BY VARIOUS FVIII AND FIX PRODUCTS

It has been postulated that the capacity of clotting factor concentrates to inhibit T cell function *in vitro* may be causing the immune dysfunction reported in HIV-ve haemophiliacs (Froebels *et al*, 1983, McDonald *et al*, 1985, Wang *et al*, 1985, Lederman *et al*, 1986). As discussed in section 5, however, we do not think this is the case. We set

out to establish if the clotting factor concentrates used at the RFH could inhibit T cell function or if this was a property exclusive to those products (American, German and Scottish) previously tested in other studies.

Six FVIII products: Alpha Profilate, BPL (NHS) 8Y, Scottish National Blood Transfusion (SNBT) FVIII, Cutter Koate, Octa VI and Armour Monoclate (monoclonal purified), and three FIX products: BPL 9A (NHS FIX), NHS FIX with heparin and Armour Mononine (FIX) (monoclonal purified) were assessed for their capacity (at 1:5 dilutions) to inhibit the expression of CD25 and HLA-DR on $CD5^+$ lymphocytes in 96 hour PHA cultures.

There was great variability in the inhibitory capacity between products and between the batches of the products (figure 6.7). The inhibitory capacity of those products tested more than once is listed in table 6.1.

Alpha Profilate and NHS 8Y were significantly more inhibitory than Armour Monoclate ($p<0.01$). These two products significantly inhibited CD25 to a greater degree than SNBT ($p<0.02$), but the inhibitory capacity of SNBT was not significantly different from either BPL (NHS) FVIII of Armour Monoclate.

The greatest inhibition of the activation markers was seen in those cultures with BPL 9A (NHS FIX) (with and without heparin). BPL 9A (NHS FIX) was significantly more inhibitory than the above products for both markers ($p<0.01$) with the

Inhibition of HLA.DR and CD25 Expression on CD5 +ve T Lymphocytes, by F VIII / F IX Products in 96 hour PHA Cultures

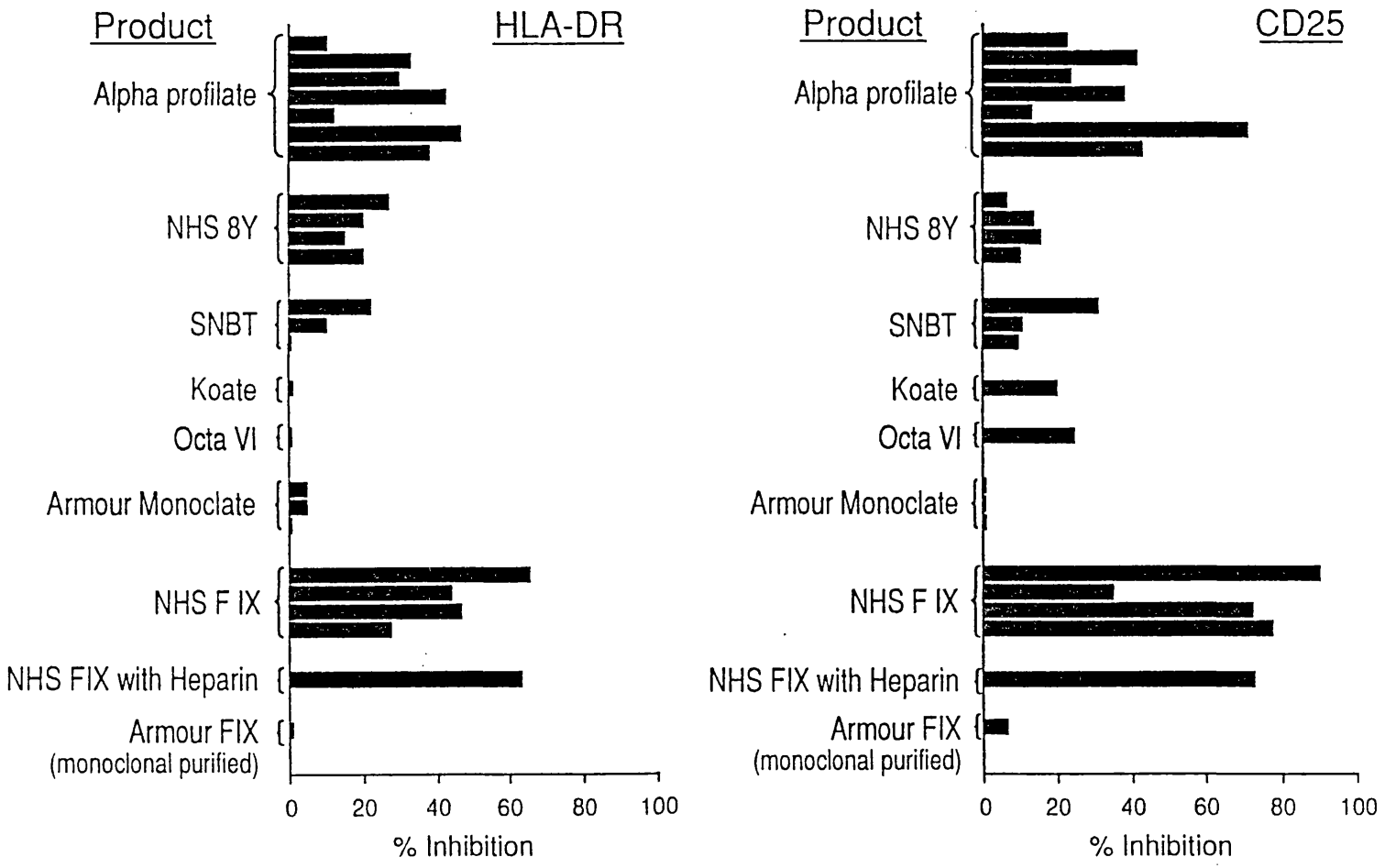


Figure 6.7: Percentage inhibition of HLA-DR and CD25 expression on $CD5^+$ lymphocytes, in 96 hour PHA stimulated cultures of PBMC, by clotting factor concentrates (4-5 units/ml).

TABLE 6.1: INHIBITION OF HLA-DR AND CD25 EXPRESSION ON $CD5^+$ LYMPHOCYTES BY CLOTTING FACTOR CONCENTRATES

(Mean ± standard deviation)

Product	% INHIBITION	
	HLA-DR	CD25
Alpha Profilate n=7	30.2 ± 14.1	36.1 ± 18.8
NHS 8Y n=4	20.5 ± 4.8	11.1 ± 3.7
SNBT n=3	10.8 ± 11.2	17.0 ± 12.1
Armour Monoclate n=3	3.3 ± 2.9	0.0
NHS FIX n=4	45.8 ± 15.4	70.3 ± 24.2

exception of Alpha Profilate and HLA-DR expression.

There was a strong significant correlation between inhibition of HLA-DR and CD25 expression ($r=0.819$) ($p<0.001$) indicating that the inhibition was not directed at specific markers but was related to overall inhibition of T cell function. In general the products inhibited the expression of HLA-DR to a lesser degree than CD25 expression, 23.3 $\pm$ 19.8 and 30.1% $\pm$ 27.6 respectively, but this difference was not statistically significant. In contrast NHS 8Y significantly ($p<0.01$) inhibited HLA-DR expression compared to CD25 expression.

The Koate and Octa products were unusual in that they inhibited CD25 expression but not HLA-DR expression, this pattern of inhibition was also seen in one of the Scottish products and may indicate that some products may specifically inhibit CD25.
The monoclonally purified FVIII (Monoclate) and FIX (Mononine) products were not significantly inhibitory for activation marker expression.

Therefore clotting factor concentrates have varying capacities to inhibit T cell function. BPL (NHS) 8Y, Alpha Profilate, FVIII products used at the RFH are inhibitory at supra-therapeutic concentrations *in vitro*.

6.5 AT WHAT POINT IN THE T CELL CYCLE IS THE INHIBITION OCCURRING AND IS THE INHIBITION DIRECTED AT A T CELL SUBSET?

By establishing where in the T cell cycle the inhibition of activation was occurring, information about the potential

mechanisms of the inhibition could be obtained. The expression of activation markers that precede IL2 production, namely HLA-DR, and those that are influenced by IL2 production (CD25), were measured in the presence of the concentrates. This would enable us to establish if the inhibition was specific to IL2 production.

Previous reports, and results from our own studies, have demonstrated that the inhibition of T cell function is only a partial effect. This could correspond to specific inhibition of a T cell subset. To investigate this, T4 and T8 cells were analysed by using anti-CD4 and anti-CD8 pan-reagents. As previously described in section 1.8.3, certain lectins have been demonstrated to stimulate certain lymphokines preferentially and it is thought that the specific production of these lymphokines represents the activation of specific T helper cell subsets. Therefore the effects of concentrate upon virgin T helper cells, by stimulating with PHA, and memory T helper cells, by using PWM, could be measured.

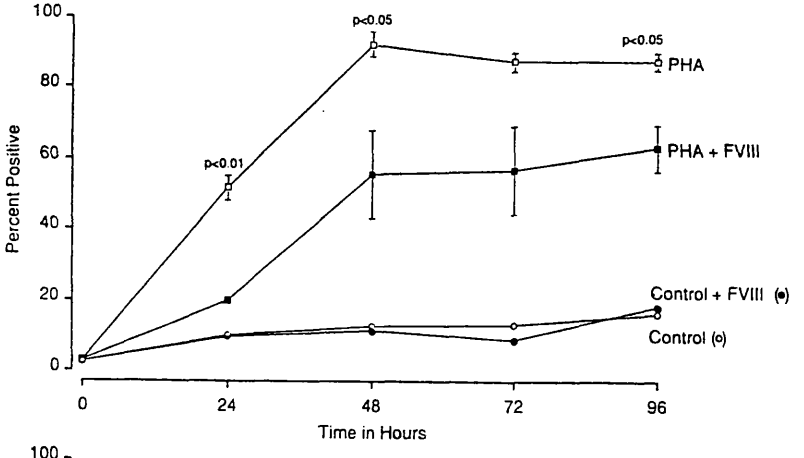
PBMC were stimulated with PHA or PWM in the presence or absence of FVIII (Alpha Profilate) and using two colour flow cytometry, the presence of HLA-DR and CD25 was determined on $CD4^+$and $CD8^+$ cells at 24 hourly intervals. These experiments were repeated with three batches of Alpha Profilate.

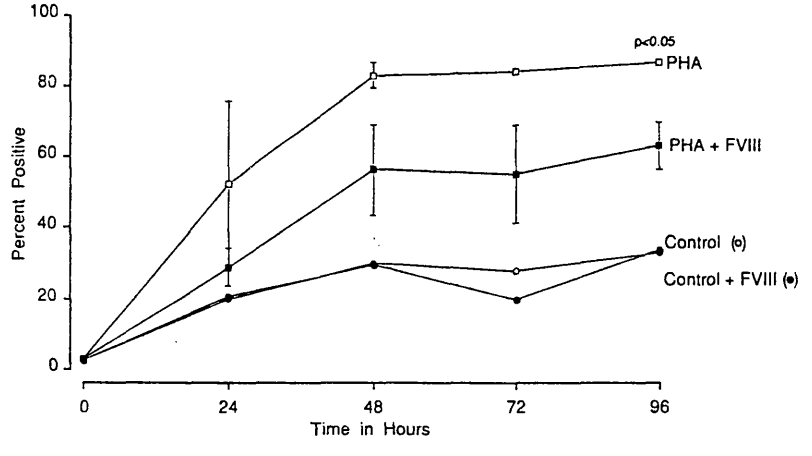
There was a similar inhibition of the expression of HLA-DR and CD25 in both T4 and T8 cell subsets stimulated with either mitogen (figures 6.8). In all cases the inhibition was

The effect of FVIII concentrate on HLA-DR and CD25 expression on T4 and T8 lymphocytes stimulated with PHA and PWM

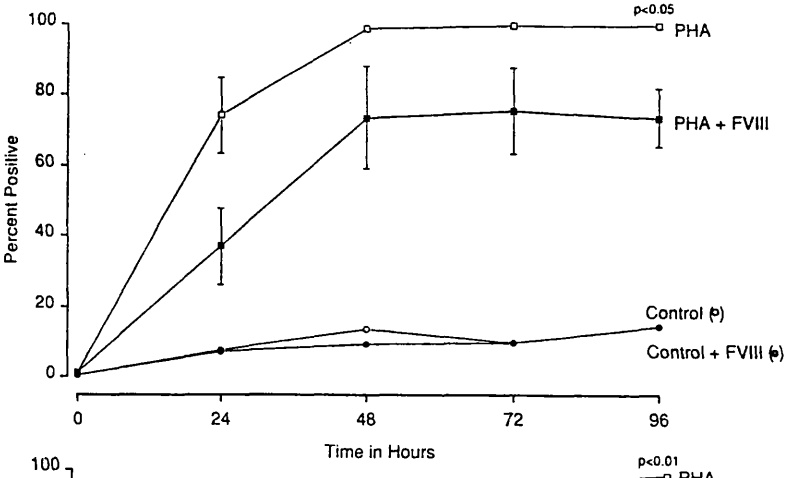
PHA

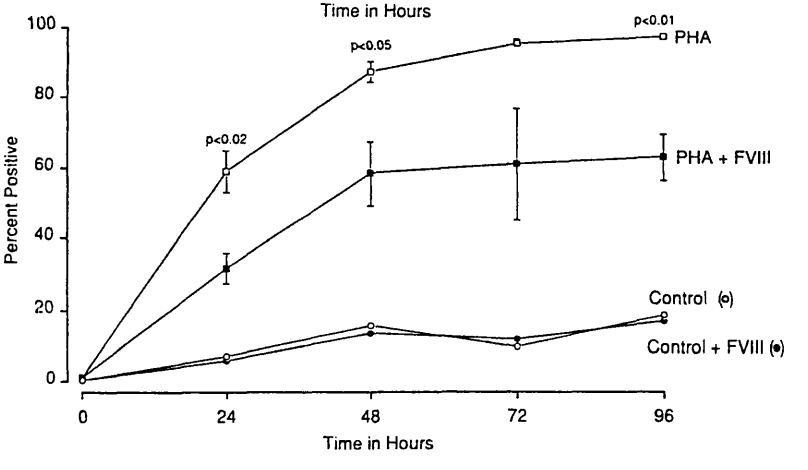
HLA-DR





CD25





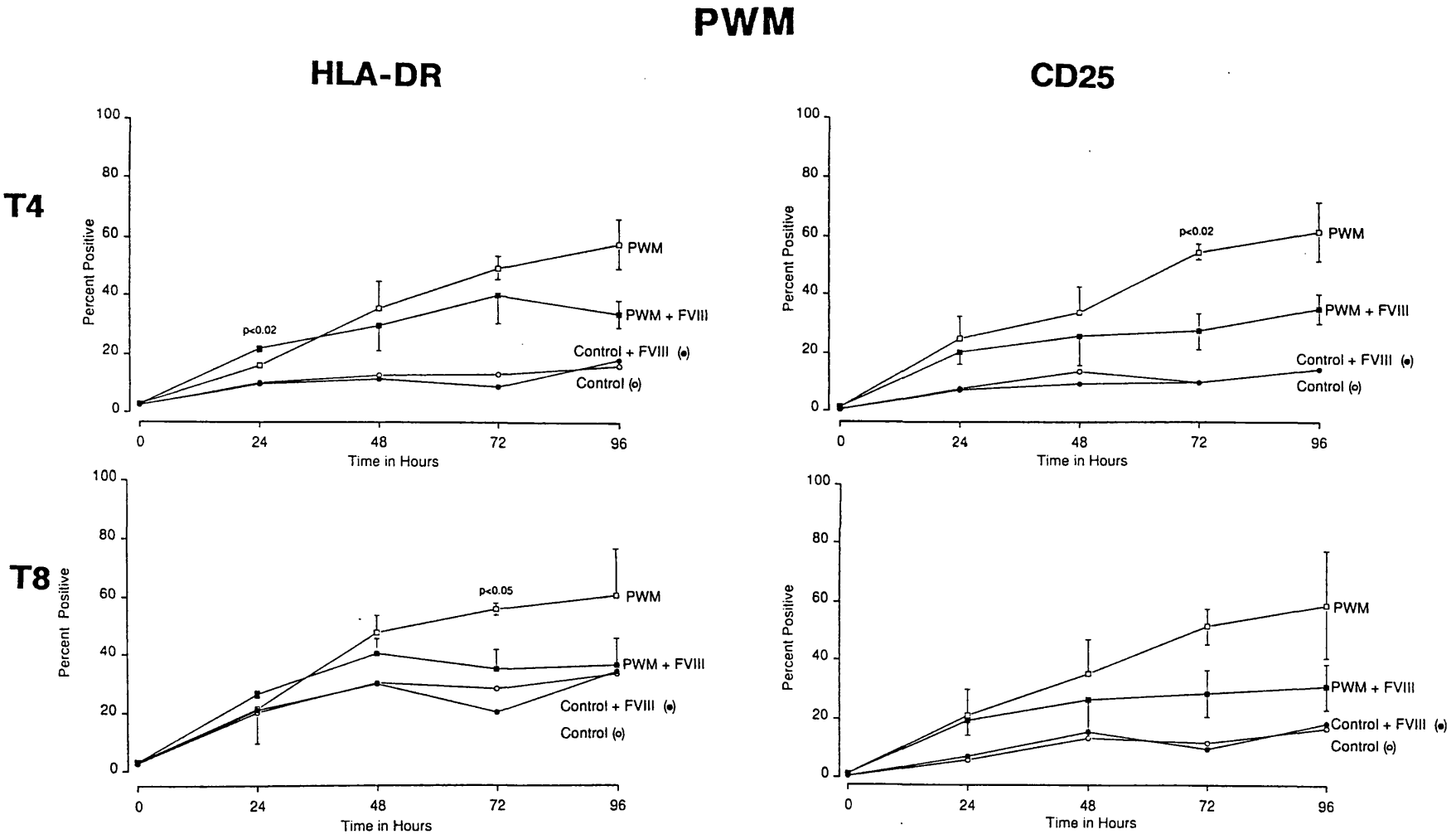


Figure 6.8: PBMC cultures were stimulated with PHA or PWM and cultured for 96 hours with (■—■) and without (□—□) FVIII concentrate (Alpha Profilate) (4-5 units/ml). PBMC were also cultured in the absence of lectin with (●—●) and without (○—○) FVIII. HLA-DR and CD25 expression on $CD4^+$ and $CD8^+$ lymphocytes was determined at 24 hourly intervals using two colour analysis.

partial, therefore the partial nature of inhibition does not reflect specific inhibition of a T cell subset nor was the inhibition specific for IL2 production.

Inhibition occurred from the onset of expression of these markers and continued throughout the culture, thus indicating that the inhibition occurred at a very early stage in the T cell cycle. In the PHA cultures, inhibition of the expression of the activation markers was significant at 96 hours, but the inhibition was not statistically significant all the time during the culture and was dependent on the T cell subset (details on figure). This variability in the statistical significance of inhibition was greater in the PWM cultures. Inhibition was not seen within the first 24 hours of PWM cultures, but expression of the activation markers was relatively low at this point. Although there was a reduced expression of CD25 on T8 cells stimulated with PWM, this was not statistically significant.

HLA-DR expression in PWM stimulated T4 cells was increased in the presence of FVIII at 24 hours ($p<0.02$). This FVIII concentrate-induced stimulation, however, was transitory and the marker was inhibited during the remainder of the culture. The variability in inhibition in these experiments reflected the large standard deviations in the three experiments which was due to differences in the inhibitory capacities of the concentrate batches and donor responses.

The expression of the activation markers in the absence of lectin was established. This was found to be minimal throughout the cultures. The T8 cells, however, did show marginal increases in expression of HLA-DR which plateaued at

48 hours. The addition of FVIII to the control cells was neither inhibitory or stimulatory.

Figure 6.9 illustrates the inhibition of the markers by FVIII concentrate upon PHA and PWM stimulated T4 and T8 cells over a 96 hour period. From this we can see that HLA-DR and CD25 expression were inhibited to a greater degree early on in the PHA stimulated cultures and that this inhibition then declines. In contrast there was no inhibition of activation marker expression early in the PWM stimulated T cell cultures. The contrasting inhibition between the PWM stimulated cells and PHA stimulated cells reflected the delayed expression of the markers on these cells which was described earlier.

6.6 IS THE INHIBITION OF T CELL FUNCTION RELATED TO CALCIUM CHELATORS IN THE CONCENTRATE PREPARATIONS?

We have established that the mechanism of inhibition was directed at an early stage in the T cell cycle. Calcium flux, a very early event, is an obligatory signal for T cell activation. Disturbances in the calcium flux would affect T cell function. Previous studies have already eluded to the possibility that calcium regulation is an important mechanism in concentrate induced T cell inhibition. Dialysis can reduce the inhibitory capacity of the concentrates. Similarly it has been found that citrate in the buffers used to formulate the concentrates can, by themselves, be inhibitory. Therefore we analysed the effects of clotting factor concentrate upon calcium flux in the lymphocytes.

Percentage Inhibition by FVIII Concentrate (4-5 units/ml) of HLA-DR and CD25 Expression on PHA and PWM Stimulated T-cells

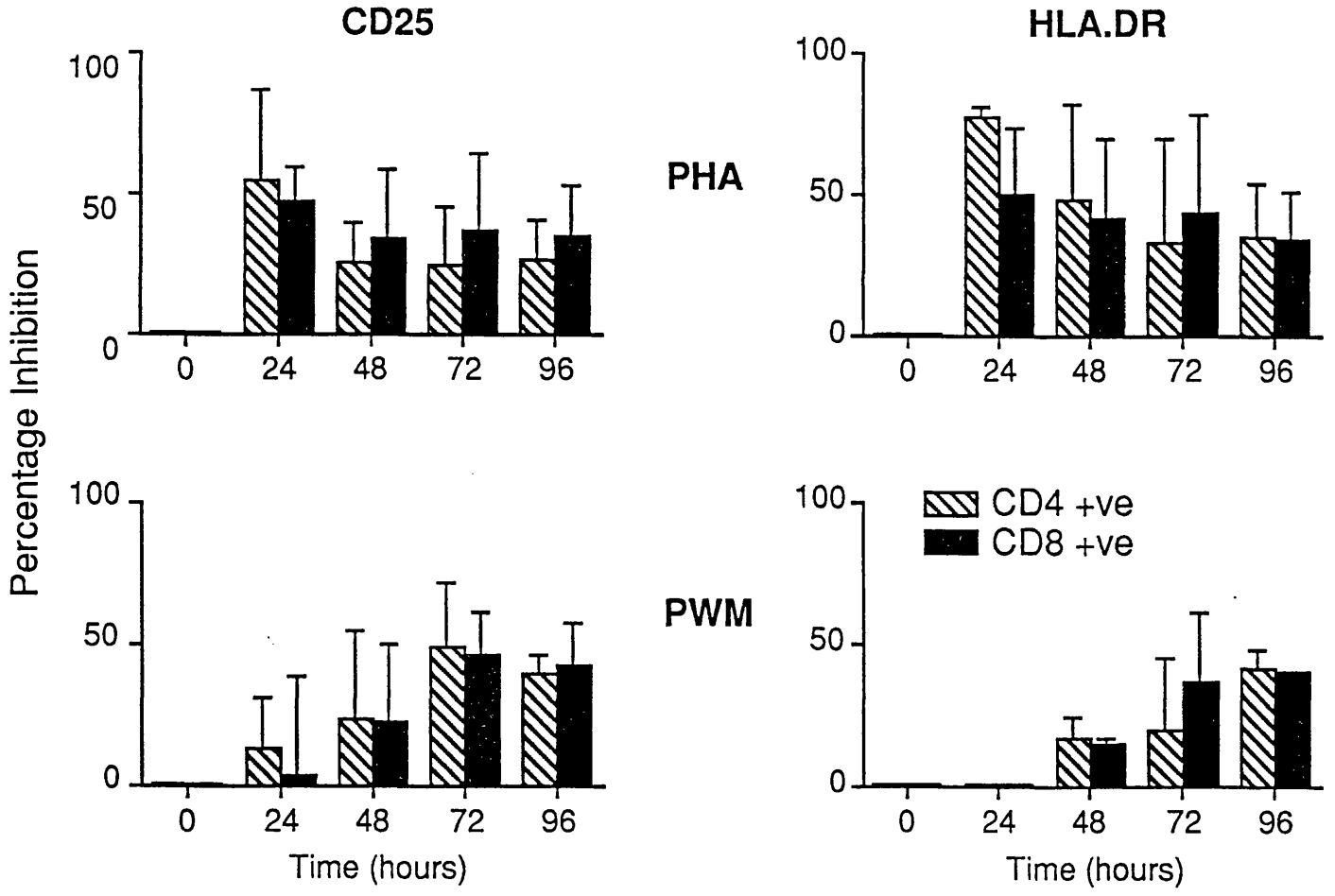


Figure 6.9: Percentage inhibition by Alpha Profilate (4-5 units/ml) of HLA-DR and CD25 expression on $CD4^+$ (hatched bars) and $CD8^+$ (closed bars) lymphocytes stimulated with PHA and PWM. This data is a alternative representation of figure 6.8, with the mean and standard deviation from three experiments.

6.6.1 TITRATION OF IONOMICIN

Maximum calcium flux and mobilisation in Jurkat cells were determined by titrating ionomicim in the presence of 1mM calcium chloride and 2mM EGTA respectively.

Ionomicin, like all ionophores, enhances the uptake of alkali metal ions, across the plasma membrane, and induces calcium movement into the cell in a dose-dependent manner. Maximum calcium flux was obtained following the addition of 0.8um ionomicin, addition of concentrations greater than this did not increase intracellular calcium levels ($[Ca^{2+}]_i$) (figure 6.10). This may reflect the saturation level of the dye within the cells. It is unlikely that this level reflected the maximum capacity for calcium uptake by the cells.

Ionophores alter membrane permeability and can transport ions across the membrane against concentration gradients to high levels which are harmful to the cell. Ionomicin did induce some calcium mobilisation but this was not dose dependent.

6.6.2 THE EFFECT OF CLOTTING FACTOR CONCENTRATE UPON CALCIUM FLUX

Calcium flux levels were determined by measuring the internal calcium levels ($[Ca^{2+}]_i$) in PBMC and Jurkat cells loaded with Fura-2AM (section 2.9).

Stimulation of CD3/TCR (by PHA (figures 6.11 and 6.12) or anti-CD3 (figure 6.13)) induces second messenger pathway signals and increases in intracellular calcium levels ($[Ca^{2+}]_i$). Influx of calcium into the cell is determined in buffers containing calcium (1mM) and generally represents a large increase in $[Ca^{2+}]_i$. Calcium mobilisation from intracellular stores is also induced by CD3 agonists. To

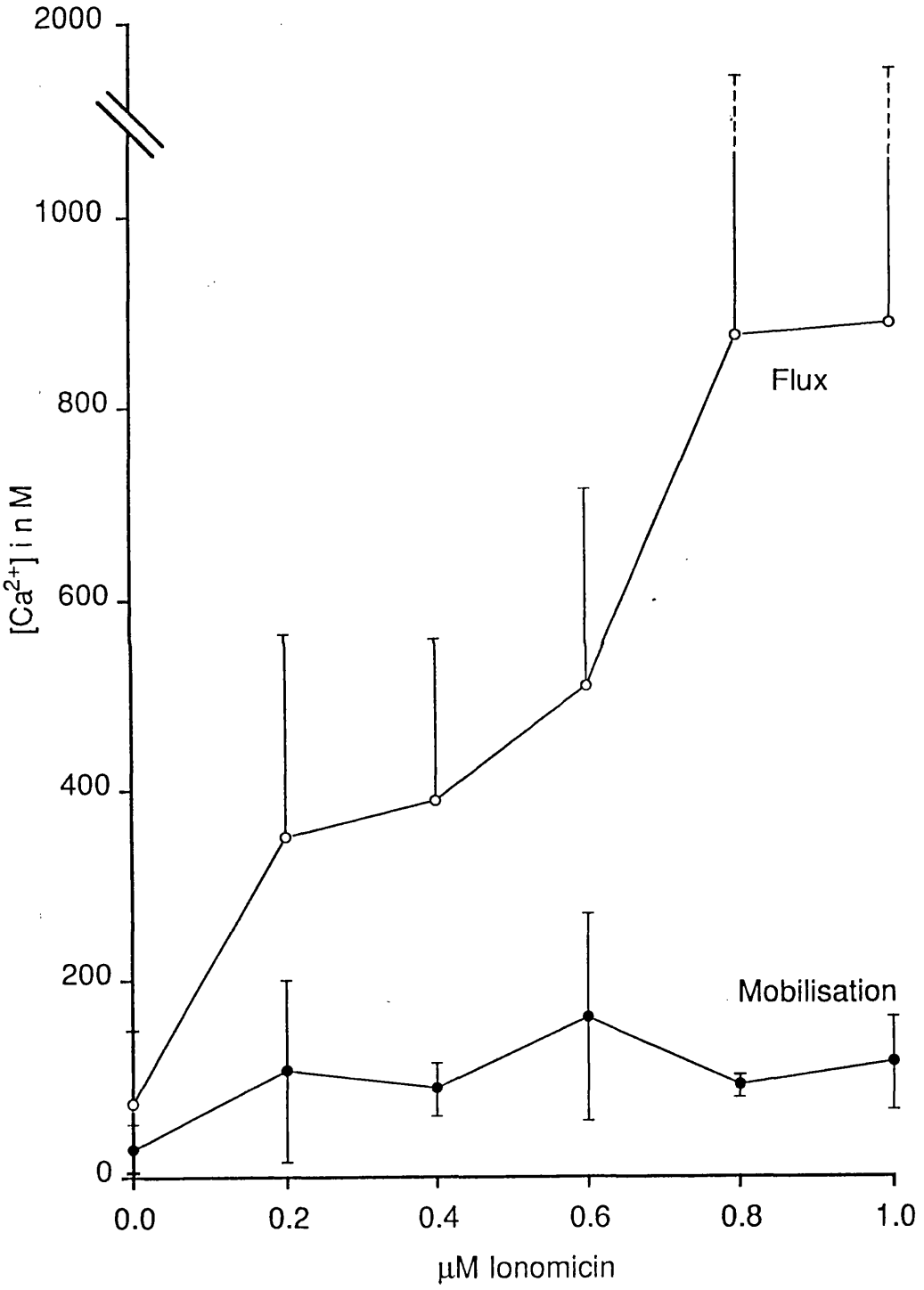


Figure 6.10: Intracellular calcium levels ($[Ca^{2+}]_i$) were determined in fura-2AM loaded Jurkat cells when titrated with ionomicin (0-1uM). Calcium flux was induced in the presence of 1mM Ca^{2+}. calcium mobilisation was performed in the presence of 2mM EGTA. Mean and standard deviation of 3 experiments.

determine increases in calcium mobilisation, calcium influx is prevented by performing experiments in calcium free buffers by adding 2mM EGTA.

6.6.2.1 Clotting factor concentrates reduce calcium flux and mobilisation in PHA stimulated PBMC

PHA, at levels used in the *in vitro* experiments (1:50), induced a three-fold increase in $[Ca^{2+}]_i$ (figure 6.11) and caused the release of calcium from intracellular stores (figure 6.12).

The peak levels of both flux and mobilisation occurred within one minute of stimulation.

Increased cytoplasmic calcium levels were maintained upon the addition of PHA for over 30 minutes. The cells began to aggregate and this was indicated by the increased disturbance in the trace (figure 6.12, upper trace). The addition of a 1:5 dilution of Alpha Profilate reduced the level of free calcium ($[Ca^{2+}]$) in the medium (figure 6.11) shown in the reduction of the lower trace before the addition of PHA. It prevented the calcium flux peak that occurred in the control, slowing down the accumulation of calcium into the cell so that $[Ca^{2+}]_i$ did not reach the levels seen in the control until over 30 minutes of incubation. Aggregation was also prevented. Addition of FVIII to cells where experiments were performed in EGTA, so that there were no free extracelluar calcium ions, indicated that the concentrates could slightly reduce basal $[Ca^{2+}]_i$ (figure 6.12). The presence of FVIII concentrates also inhibited calcium mobilisation from intracellular stores as shown in figure 6.12, in the lower trace.

The effect of FVIII concentrate upon PHA stimulated calcium influx in Jurkat cells

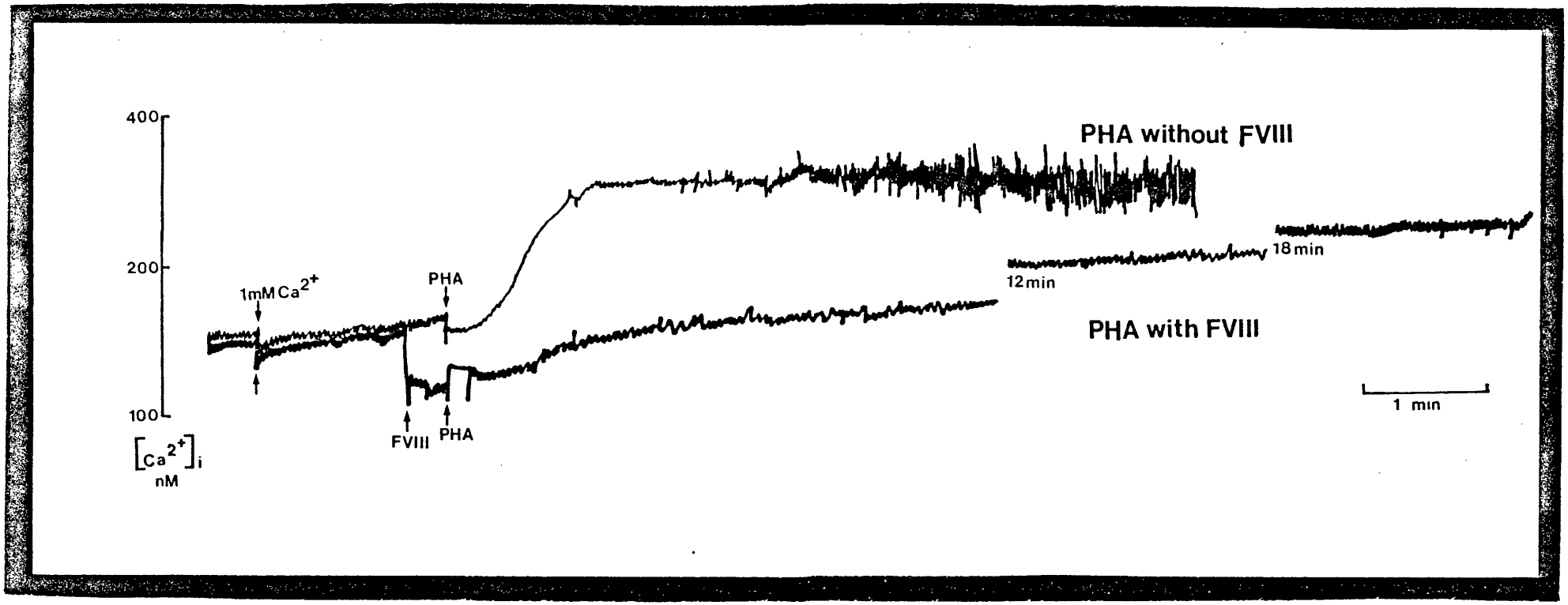


Figure 6.11: PHA (1:50 dilution) induced changes in $[Ca^{2+}]_i$ were determined in fura-2AM loaded Jurkat cells in the presence of 1mM Ca^{2+} (upper trace). 4-5 units/ml FVIII concentrate (Alpha Profilate) were added to the cells in the lower trace before stimulation with PHA.

The effect of FVIII concentrate upon PHA stimulated calcium mobilisation in Jurkat cells

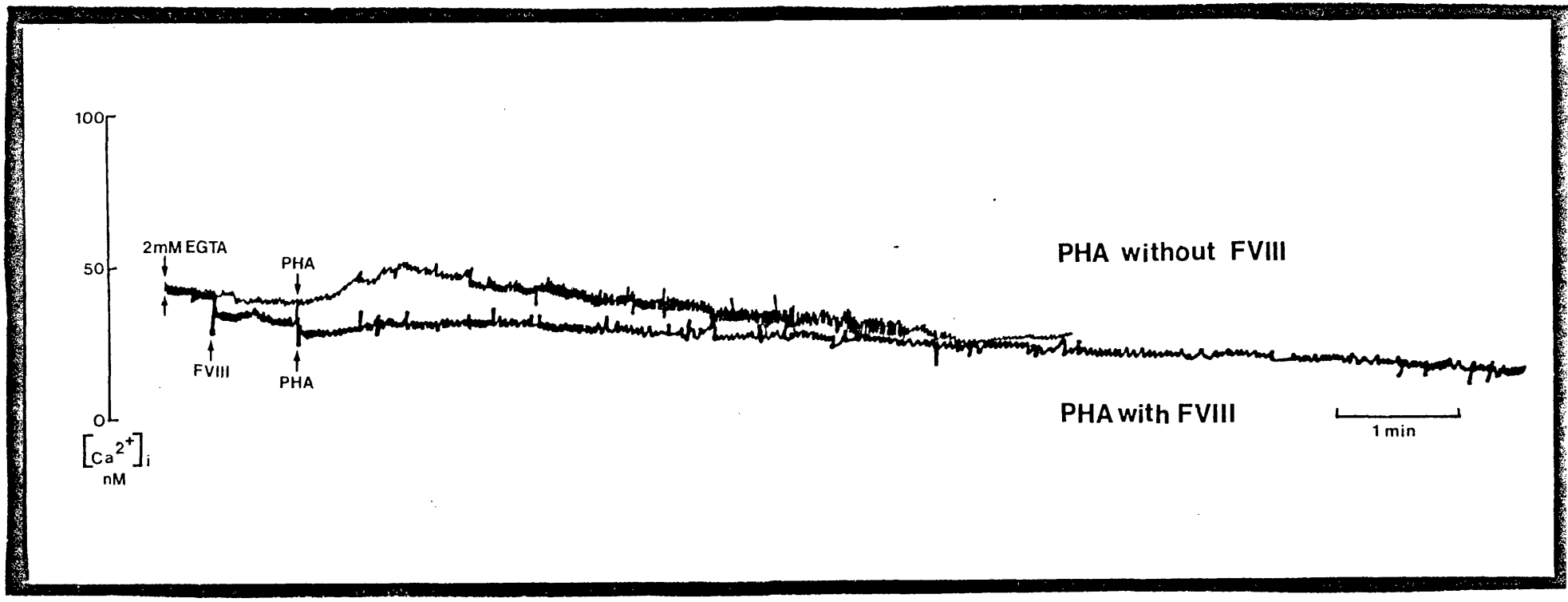


Figure 6.12: PHA (1:50 dilution) induced calcium mobilisation was determined in fura-2AM loaded Jurkat cells in the presence of 2mM EGTA (upper trace). 4-5 units/ml FVIII concentrate (Alpha Profilate) were added to the cells in the lower trace before stimulation with PHA.

6.6.2.2 FVIII concentrates inhibit calcium flux and calcium mobilisation in Jurkat cells stimulated with anti-CD3 antibody

Stimulation of Jurkat cells with anti-CD3 led to rapid calcium influx and an approximate 5-fold increase in $[Ca^{2+}]_i$ and mobilisation from intracellular stores (figure 6.13).
The changes in $[Ca^{2+}]_i$ were biphasic, consisting of a large transient peak, and a smaller sustained plateau, measurement of which was made at 90 seconds following stimulation (plateau).

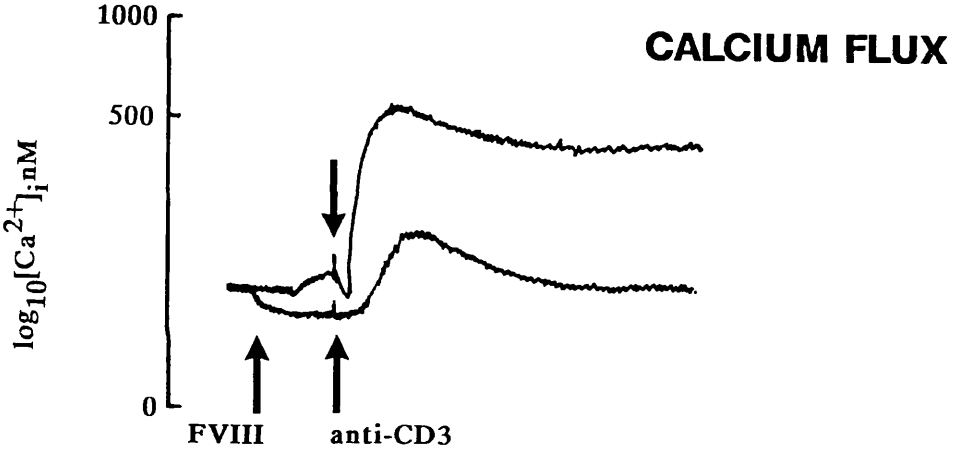
These measurements were also made for calcium mobilisation (determined in the presence of 2mM EGTA), however, at 90 seconds post stimulation (plateau) the $[Ca^{2+}]_i$ calcium levels had returned to base line (figure 6.13).

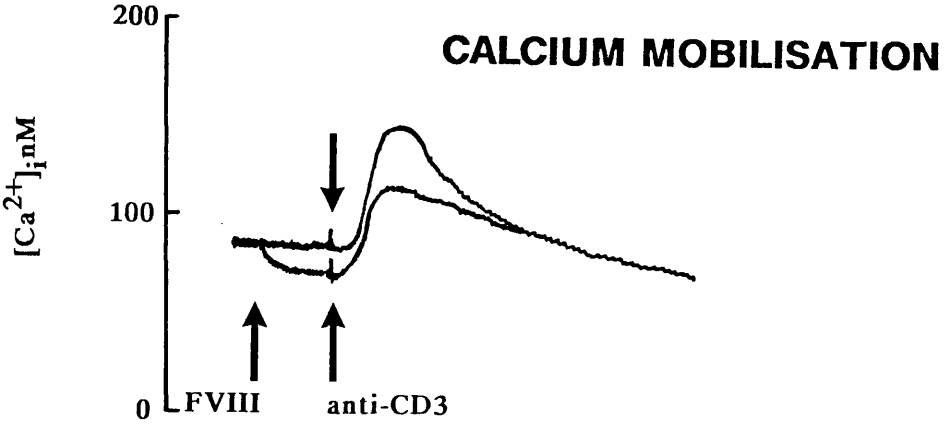
The levels of calcium in the cell preparation were found to be 554.2 $\pm$ 1093.7 (138 tests). There was great variability of this level between the tests and this probably represented leaking of the fluorescent dye from the cells which varied with each preparation of cells. The base line level of cytoplasmic calcium in the cells was determined in the presence of 2mM EGTA and was found to be 112.4 $\pm$ 86.7nM (77 tests).
The anti-CD3 antibody was titrated and maximum stimulation of calcium flux was achieved with the addition of a 1:250 dilution of the antibody.

Following stimulation with anti-CD3 (in the presence of calcium), intracellular calcium levels increased to a peak of 639.5 $\pm$ 265.7nM (14 tests) and then dropped to a plateau

Figure 6.13: $[Ca^{2+}]_i$ levels were measured in fura-2AM loaded Jurkat cells.
Figure A (upper trace) is an example of anti-CD3 antibody stimulated cells in the presence of 1mM Ca^{2+}. Upon stimulation there was a biphasic change in $[Ca^{2+}]_i$, comprised of a transient peak, where there was a five-fold increase in $[Ca^{2+}]_i$, followed by a smaller sustained plateau.
Figure B (upper trace) is an example of calcium mobilisation where $[Ca^{2+}]_i$ increases induced by anti-CD3 antibody were determined in the presence of 2mM EGTA. There was a rapid small increase in $[Ca^{2+}]_i$, which quickly reduced to normal levels, indicating that the large increase in $[Ca^{2+}]_i$ in the above figure was predominately due to calcium influx.
FVIII concentrate (4-5 units/ml) was added to the cells suspension and the anti-CD3 induced calcium levels are shown in the lower traces.

EFFECT OF FVIII CONCENTRATE (4-5 units/ml) ON ANTI-CD3 INDUCED CALCIUM FLUX IN JURKAT CELLS





level of 416.3 ± 218.3nM within 90 seconds (calcium flux) (figure 6.13). Peak calcium mobilisation of intracellular stores (determined in the presence of 2mM EGTA) was found to be 180.7 ± 86.1nM which dropped to below normal base line levels (92.0 ± 31.4nM) within 90 seconds of stimulation (14 tests). Therefore the peak calcium flux measurement is predominately due to release of calcium from intracellular stores and the plateau calcium levels represent influx into the cell.

All FVIII products reduced the levels of calcium in the cell preparations in the presence of 1mM Ca^{2+} (figure 6.14). The addition of 2mM EGTA removed the calcium from the medium and gave true basal levels of calcium in the cytoplasm of the cells. This too was reduced by approximately 50% in the presence of FVIII (figure 6.14). There was no statistical difference between the different products in the ability to reduce the calcium base line levels.

Inhibition by FVIII concentrates of anti-CD3 induced calcium flux (peak and plateau) in Jurkat cells are shown in figure 6.15. All FVIII concentrates tested inhibited calcium flux in the cells (both peak and plateau). There was batch to batch and product to product variation in the inhibitory capacity of these products, but this was not statistically significant.

FVIII concentrates inhibited calcium mobilisation (figure 6.16). There was batch to batch variation between concentrates but once again this was not statistically significant.

Suppression of Base Line Levels of Calcium by Clotting Factor Concentrates

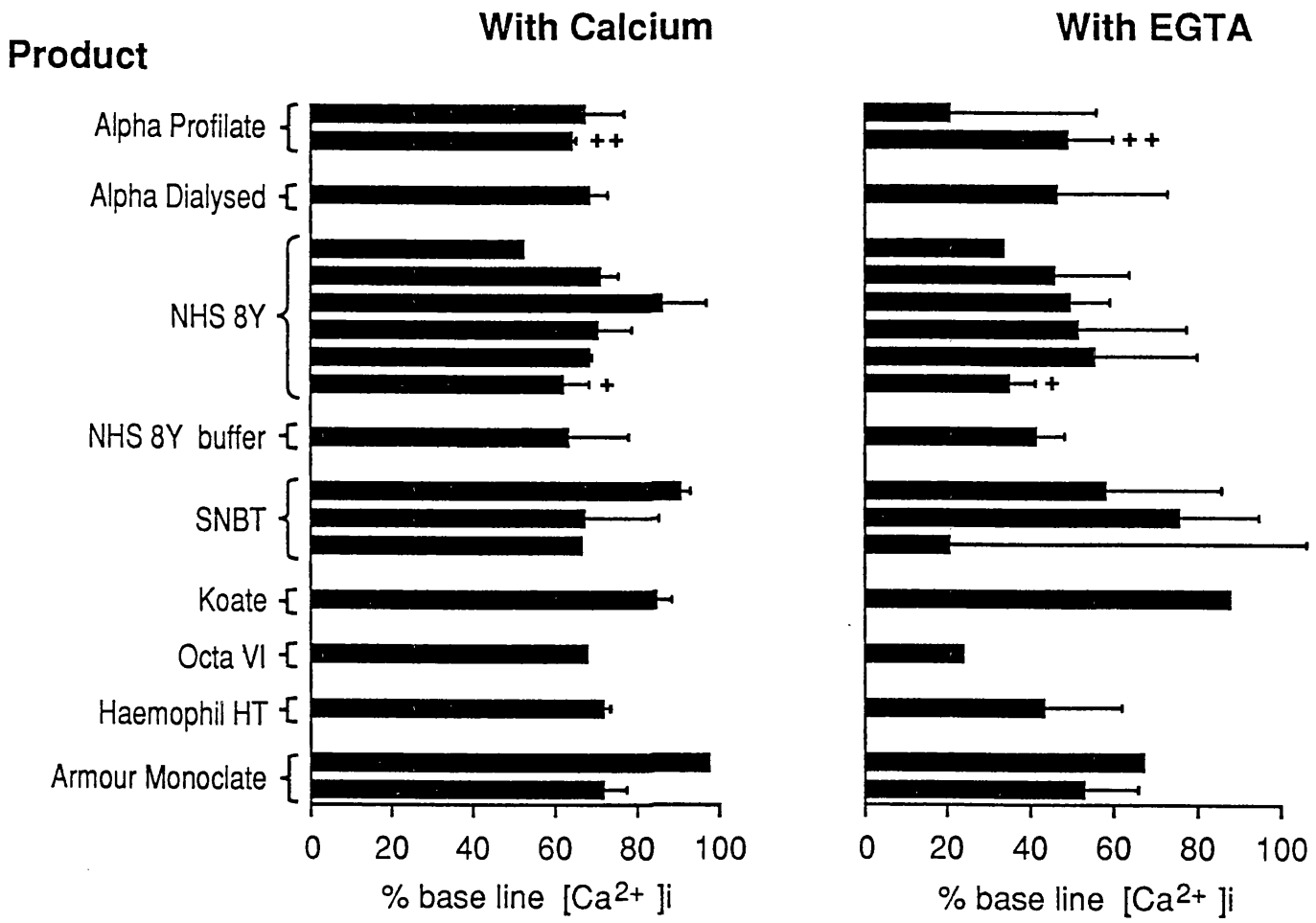


Figure 6.14: The effect of the addition of FVIII concentrate (4-5 u/ml) to base line [Ca^{2+}] in fura-2AM Jurkat cell preparations in the presence of 1mM Ca^{2+} and 2mM EGTA. Mean and standard deviation of at least two experiments. + concentrate corresponding to buffer preparation, ++ Alpha Profilate pre dialysis

Inhibition of Anti-CD3 Induced Calcium Flux in the Presence of Clotting Factor Concentrates

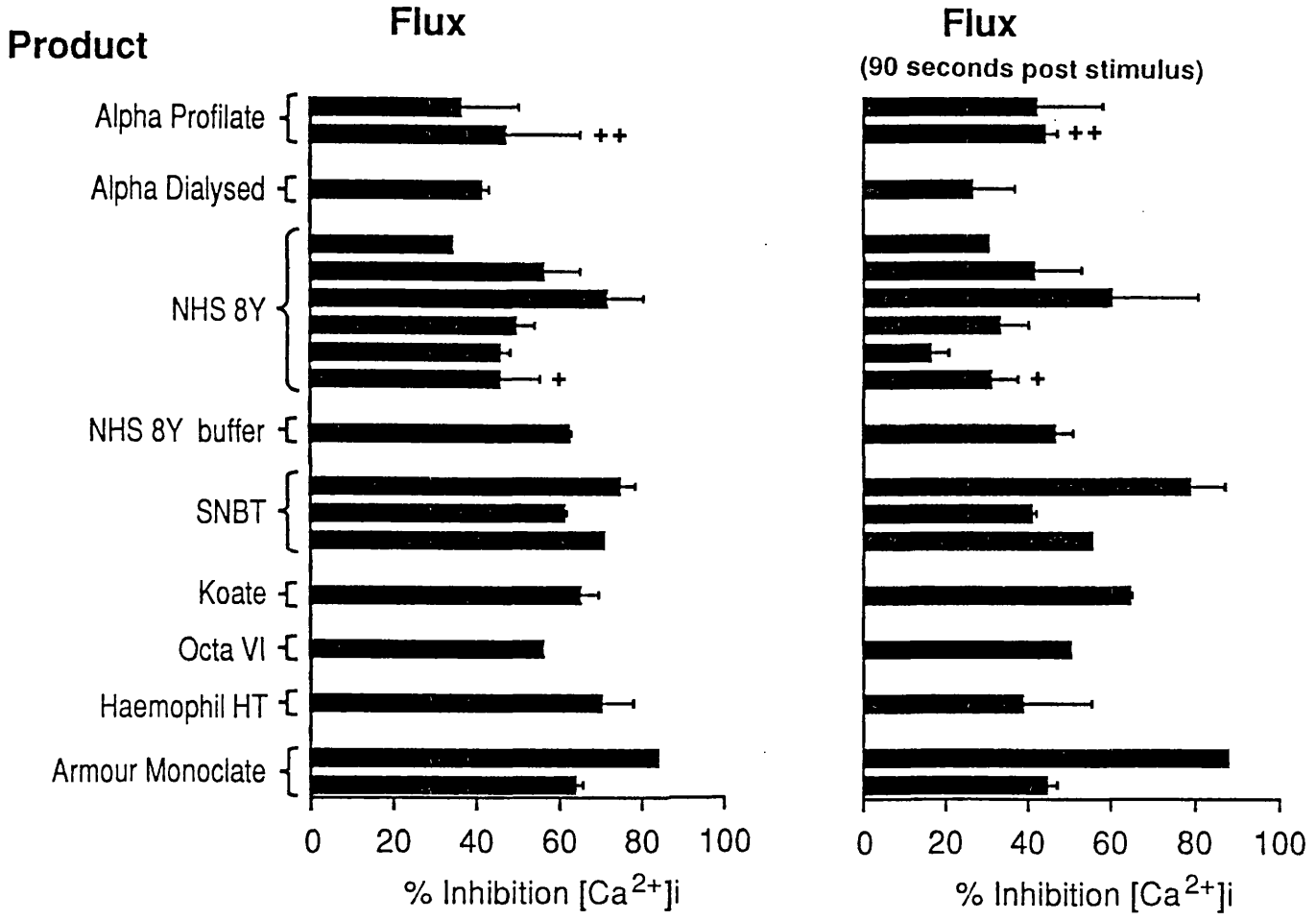


Figure 6.15: Percent inhibition of peak calcium flux (determined in the presence of 1mM Ca^{2+}), and plateau calcium flux (determined 90 seconds post stimulation by anti-CD3 antibody), by the addition of FVIII concentrates (4-5 units/ml), in fura-2AM loaded Jurkat cells. Mean and standard deviation of at least two experiments. + concentrate corresponding to buffer preparation, + + Alpha Profilate pre dialysis

Inhibition of Anti-CD3 Induced Calcium Mobilisation in the Presence of Clotting Factor Concentrates

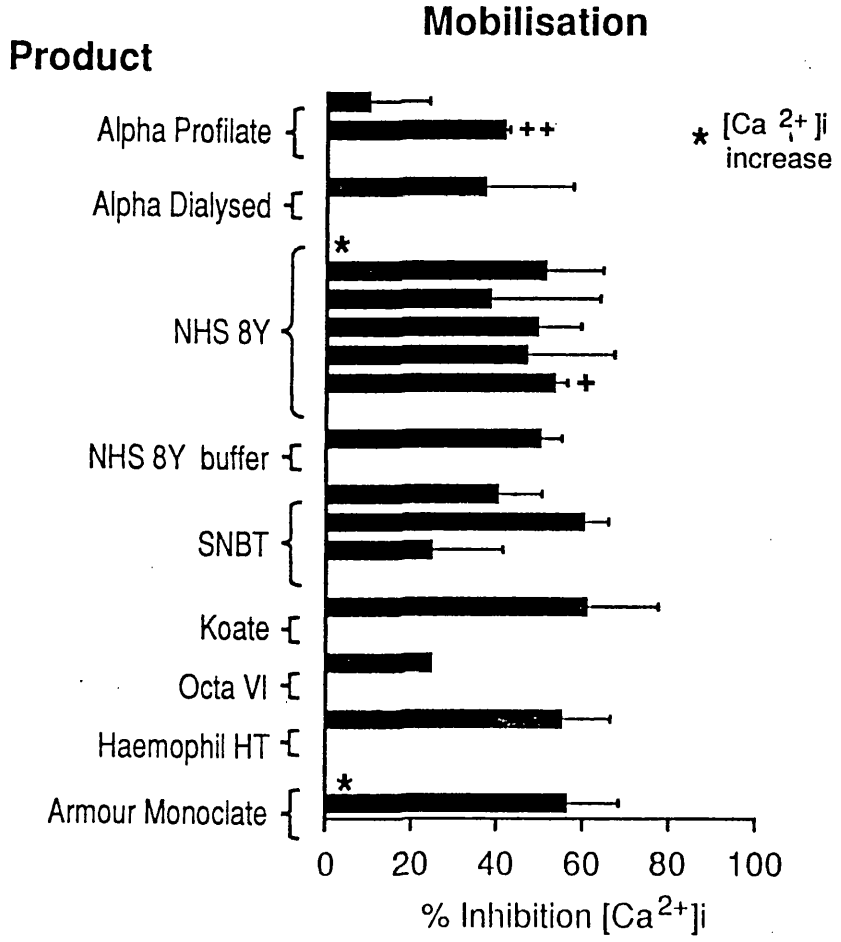


Figure 6.16: Percent inhibition of calcium mobilisation (determined in the presence of 2mM EGTA) by the addition of FVIII concentrates (4-5 units/ml) in anti-CD3 antibody stimulated, fura-2AM loaded Jurkat cells. + concentrate corresponding to buffer preparation, ++ Alpha Profilate pre dialysis

One BPL (NHS) 8Y concentrate and one Armour Monoclate product increased calcium peak mobilisation.

There was a good correlation between the percentage inhibition by the FVIII concentrates of peak and plateau antibody flux levels (r=0.850). There was, however, no correlation of these values with the inhibition of calcium mobilisation within the cells.

This indicates that the peak calcium flux value is not wholly due to calcium mobilisation from intra-cellular stores.

6.6.2.3 There is no correlation between inhibition of calcium flux and inhibition of activation marker expression

The correlations between the ability of the FVIII concentrates to inhibit activation marker expression and inhibit calcium flux and mobilisation are demonstrated in figures 6.17 and 6.18.

In the sample of products analysed in the calcium flux study there was no correlation between inhibition of HLA-DR and CD25 (r=0.390), in contrast to the whole study where the correlation was highly significant (r=0.819) ($p<0.001$) (section 6.4.2). There was no correlation with the inhibition of CD25 expression and inhibition of calcium flux/mobilisation. There was, however, a significant negative correlation between the inhibition of HLA-DR expression and the inhibition of calcium flux (peak (r=-0.648) ($p<0.01$) and plateau (r=-0.515) ($p<0.05$)), but there was no relationship with calcium mobilisation. This indicates that although FVIII products inhibit calcium flux and mobilisation, this

Correlation of the Percentage Inhibition of Anti-CD3 Induced Calcium Flux with the Percentage Inhibition of HLA-DR Expression by Clotting Factor Concentrates

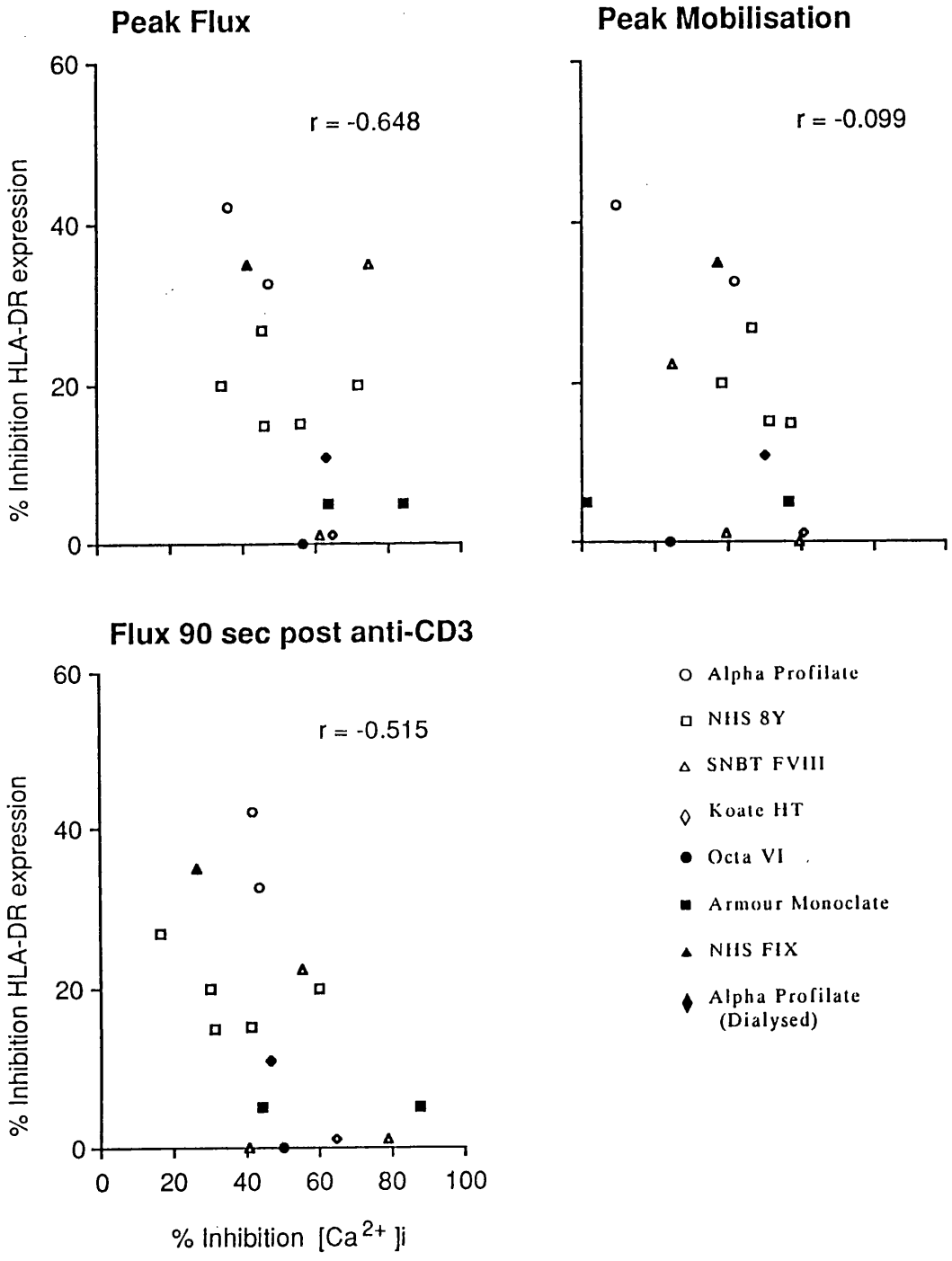


Figure 6.17: The percent inhibition of $[Ca^{2+}]_i$ by FVIII concentrates (4-5 units/ml) was determined in fura-2AM loaded Jurkat cells, in the presence of 1mM Ca^{2+} (flux) and 2mM EGTA (mobilisation). These parameters were correlated with percent inhibition of HLA-DR expression. There were significant correlations with the inhibition of HLA-DR expression and peak calcium flux ($r=-0.648$) ($p<0.01$), plateau calcium flux ($r=-0.515$) ($p<0.05$).

Correlation of the Percentage Inhibition of Anti-CD3 Induced Calcium Flux with the Percentage Inhibition of CD25 Expression by Clotting Factor Concentrates

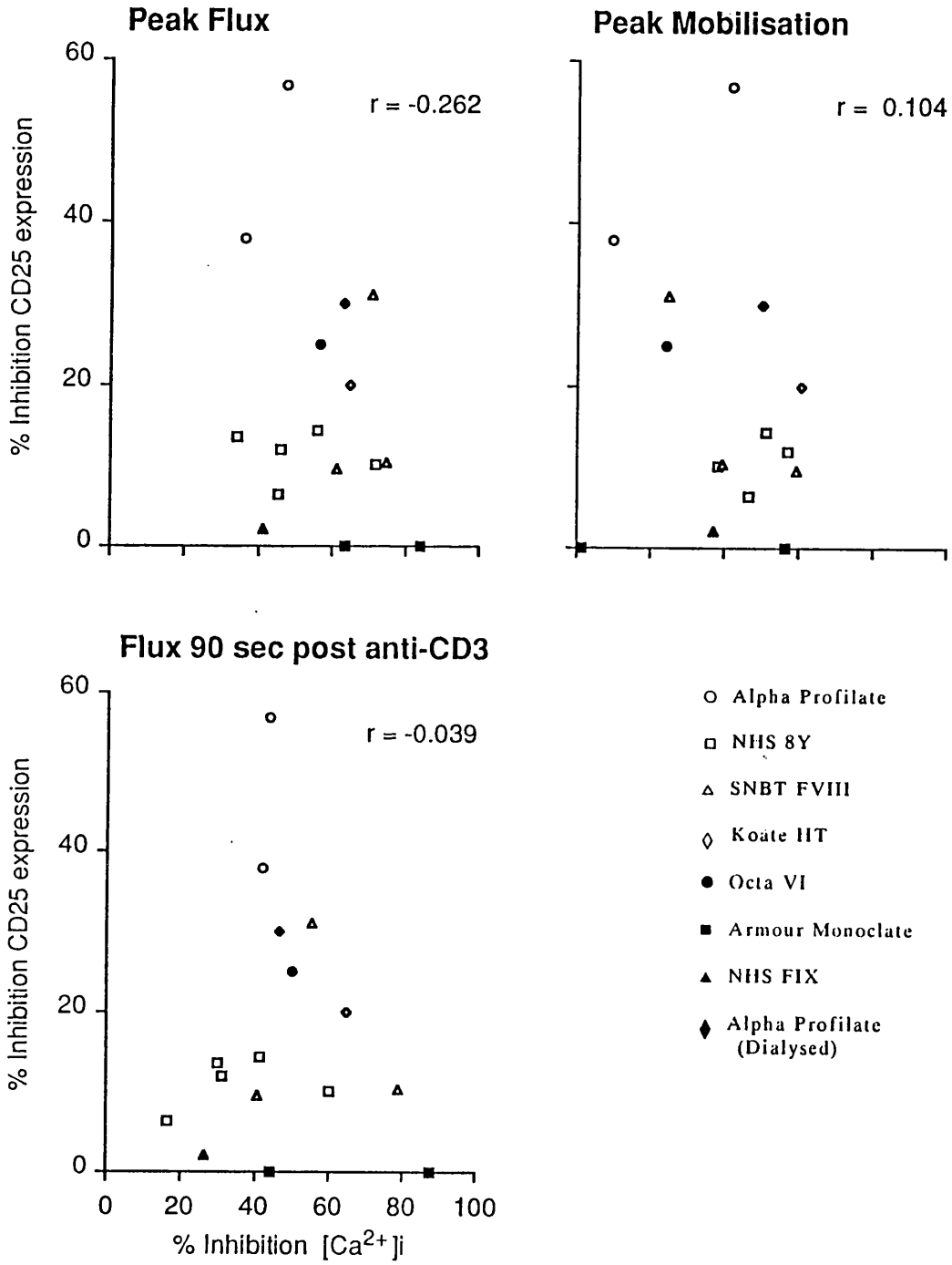


Figure 6.18: The percent inhibition of $[Ca^{2+}]_i$ by FVIII concentrates (4-5 units/ml) was determined in fura-2AM loaded Jurkat cells in the presence of 1mM Ca^{2+} (flux) and 2mM EGTA (mobilisation). These values were correlated with percent inhibition of CD25 expression. There were no significant correlations.

inhibition does not appear related to the inhibition of CD25 expression. Inhibition of HLA-DR, however, was inversely proportional to the inhibition of calcium flux.

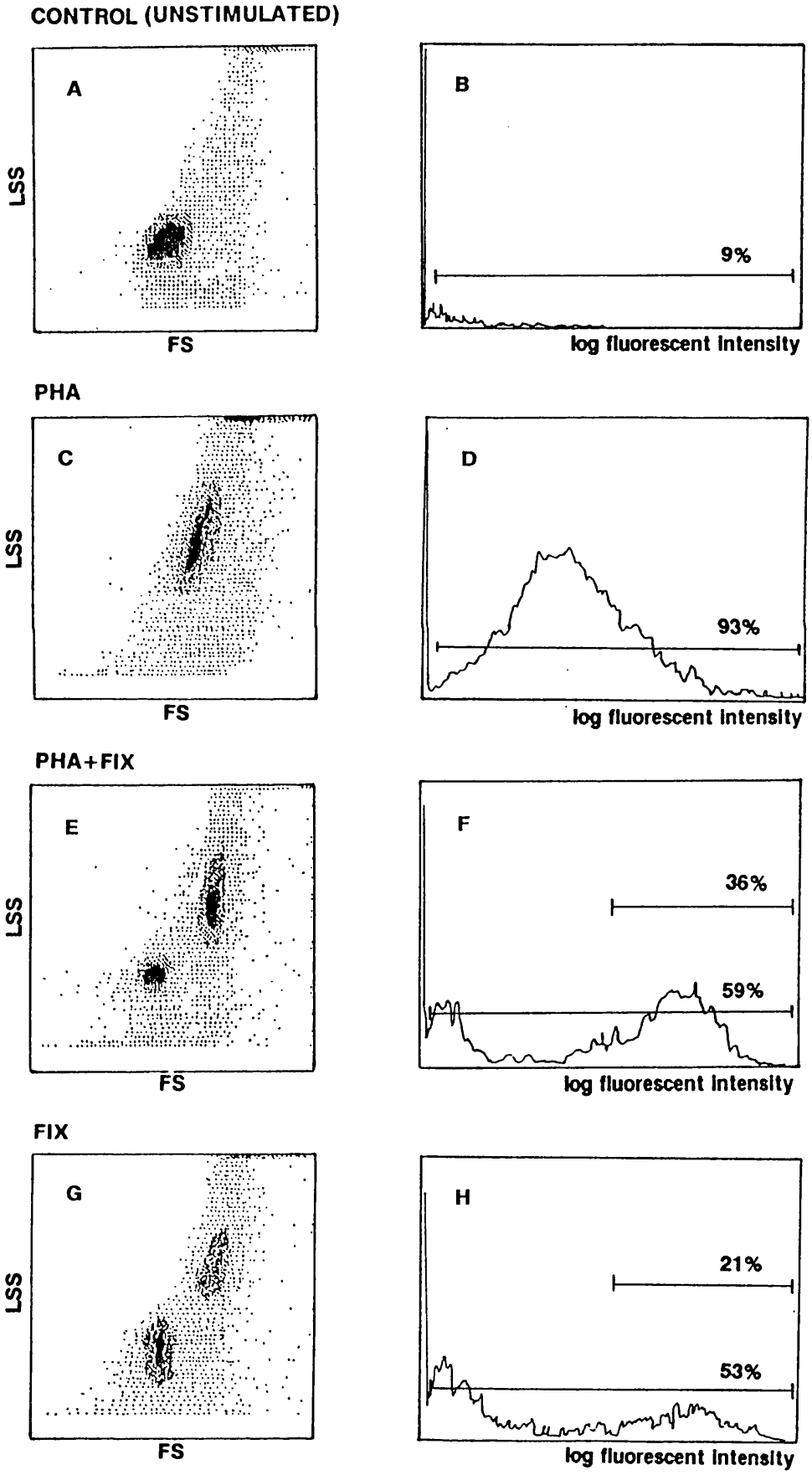
6.7 EFFECTS UPON T CELL ACTIVATION BY BPL 9A (NHS FIX)

The most inhibitory product in our analyses was BPL 9A (NHS FIX) (figure 6.7).

It was significantly more inhibitory for HLA-DR and CD25 expression than the other products tested, with the exception of the inhibition of HLA-DR expression by Alpha Profilate. Surprisingly, not only was this product inhibitory in the presence of lectin, but was found to be mitogenic in normal PBMC control cultures (ie unstimulated cells) (figure 6.19). Blastogenesis occurred and the expression of HLA-DR and CD25 was increased. The effect was variable between the batches but on average there was an approximate 30% increase in expression of the markers, however, this was found not to be statistically significant compared to the control levels (6 tests). An example of a particularly mitogenic product is shown in figure 6.19. Here four examples of flow cytometric histograms are illustrated showing the size and side scatter characteristics of the cells (first column) and HLA-DR expression on the blasts (right hand column). In normal cultures there were low levels of HLA-DR but no evidence of blastogenesis. Stimulation by PHA resulted in activation of the cells with blast and clump formation and high levels of HLA-DR expression at 96 hours of culture. Addition of FIX inhibited the activation of the cells by PHA. This inhibition was partial and reduced HLA-DR expression. Two populations of cells were evident - blasts and normal lymphocytes. The

Figure 6.19: PBMC have a distinctive LSS/FS profile (histogram A) and express low levels of HLA-DR (histogram B). Stimulation with PHA leads to blast formation and following 96 hours of culture the cells are clumped and enlarged (histogram C) and express HLA-DR (histogram D). Addition of NHS FIX to the PHA cultures greatly inhibited the bast formation following 96 hours of culture, where two populations of cells are clearly visible in the particularly inhibitory products (histogram E), there was also a reduction in the expression of HLA-DR (histogram F). Addition of NHS FIX to cells in the absence of lectin led to blast formation following 96 hours of culture, indicating that the NHS FIX itself was mitogenic (histogram G). There was also an increase in activation marker expression (HLA-DR in figure) (histogram H).

HLA-DR EXPRESSION ON CONTROL (UNSTIMULATED) AND PHA STIMULATED PBMC AT 96 HOURS WITH AND WITHOUT FIX



induction of two populations of cells only occurred in cultures with very inhibitory FIX products. The addition of this particularly mitogenic FIX concentrate to PBMC from a normal control produced notable blastogenesis and HLA-DR expression by 96 hours of culture. Calcium flux studies (as out lined in the previous section) were performed with the NHS FIX products but it was found that the FIX product itself was fluorescent even at dilutions of 1:100. This phenomenon could not be overcome even by dialysis and therefore these studies of calcium measurements were not performed with the FIX concentrates.

6.8 SOLUBLE IL2-R IS NOT AN INHIBITORY COMPONENT IN THE CONCENTRATES

Synovial fluid from patients with rheumatoid arthritis has been demonstrated to inhibit T cell function *in vitro* and this has been attributed in part to the presence of high concentrations of sIL2-R (Symons *et al*, 1988). It is possible that sIL2-R in plasma may be concentrated with the FVIII and FIX during the production of these concentrates, and that its presence may be leading to the inhibitory effect. Examination of two inhibitory products (Alpha Profilate and NHS 8Y), at dilutions of 1:10, 1:100 and 1:1000, for the presence of sIL2-R (section 2.6.1) indicated that sIL2-R was not present.

6.9 ADDITION OF SODIUM HEPARIN TO CULTURES TO PREVENT CLOTTING

The study of the mechanisms of inhibition by the clotting factor concentrates *in vitro* required the manipulation of the levels of free calcium ($[Ca^{2+}]$). Relative increases in $[Ca^{2+}]$ resulted in clot formation in the culture plates containing the concentrate. To prevent this, the anti-coagulant sodium heparin (C P Pharmaceutical, Wrexham, Wales) was added to the cultures. Heparin acts as an anti-coagulant by interfering with factor X formation without disturbing the calcium concentration.

Sodium heparin was titrated (0-10 units/ml) in PHA stimulated PBMC cultures and the expression of HLA-DR and CD25 on $CD5^+$ lymphocytes was assessed following 96 hours of culture. The experiment was performed in the presence of a 1:5 dilution of FVIII (Alpha Profilate).

Addition of heparin at concentrations of 0.5 units/ml and above prevented the concentrates clotting in the presence of 1mM $CaCl_2$ (AnalaR, Poole, Dorset), however, addition of heparin itself increased the expression of HLA-DR (figure 6.20). In the presence of FVIII, however, this increase did not occur.

As with HLA-DR, CD25 expression was slightly increased with increasing concentration of heparin, however, further stimulation was undetectable with this marker when expression reached 100%. CD25 expression was diminished in the presence of FVIII, addition of 2 units/ml and above of heparin partially abrogated this inhibition.

Effects of Heparin upon the Expression of HLA-DR and CD25 Expression with and without FVIII

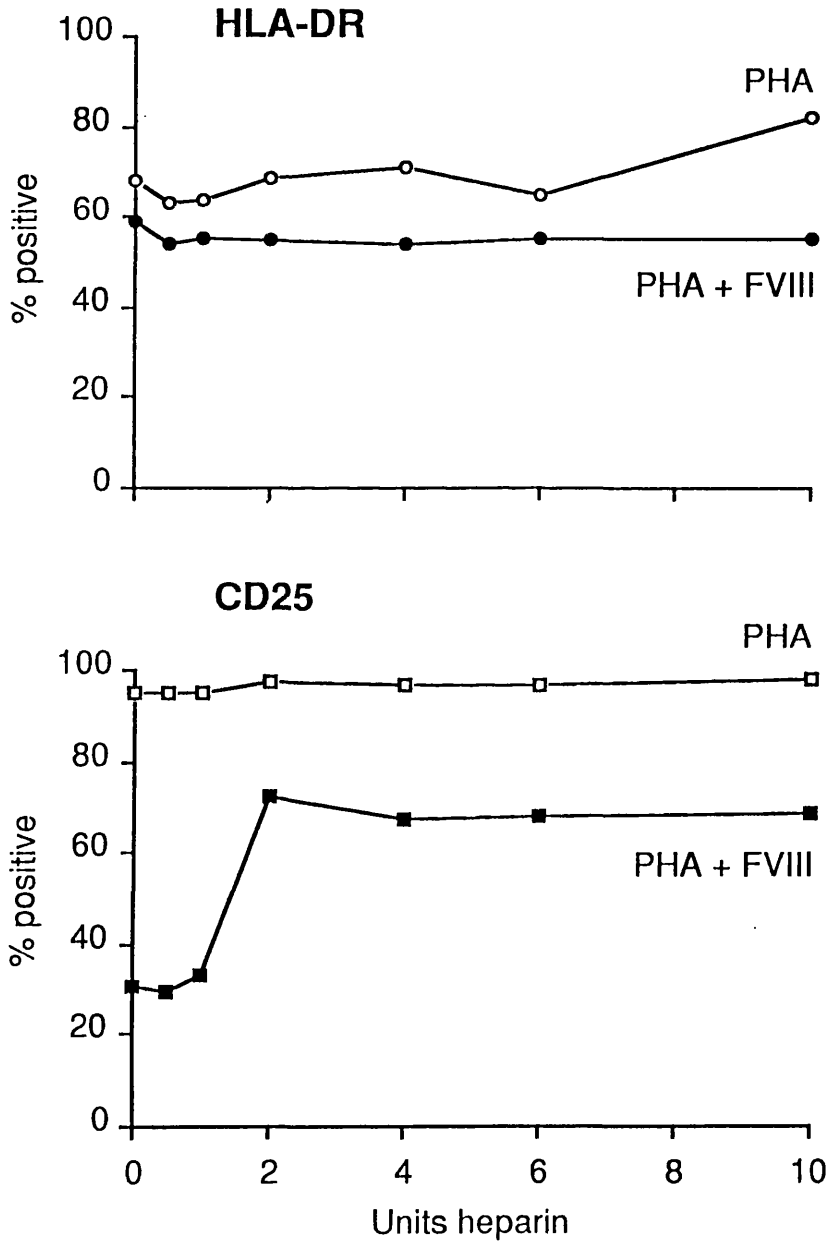


Figure 6.20: The effects of increasing concentration of heparin (0-10u/ml) upon HLA-DR (○—○) and CD25 (□—□) expression on $CD5^+$ lymphocytes at 96 hours of culture was measured by 2 colour flow cytometry. FVIII concentrate (4-5 units/ml) was added to the cultures and the inhibition of the expression of HLA-DR (●—●) and CD25 (■—■) in the presence of heparin was determined.

6.10 IS THE INHIBITORY COMPONENT RELATED TO THE BUFFER USED TO FORMULATE THE PRODUCT

As described earlier, several studies have shown that the buffers used to formulate the concentrates can inhibit T cell function. Dialysis has also been shown to reduce the inhibitory capacity of the products. Two confirmatory experiments were performed to assess if the buffers from BPL (NHS) 8Y products were inhibitory, and to see if dialysis removed the capacity of the concentrates to inhibit T cell function as determined by methods in this study.

6.10.1 BUFFERS INHIBIT THE EXPRESSION OF HLA-DR AND CD25

A BPL (NHS) 8Y product (FHC 02320) and its corresponding buffer, (kindly provided by Dr J Smith BPL, Oxford) were tested at a 1:5 dilution, in PHA stimulated cultures of PBMC, to establish their capacity to inhibit CD25 and HLA-DR expression upon $CD5^+$ lymphocytes following 96 hours of culture.

The buffer and concentrate inhibited HLA-DR expression to a similar degree (table 6.2). Inhibition of CD25 expression by the buffer, however, was more than two times that produced by the concentrate.

The buffer and its corresponding concentrate were assessed for their ability to reduce calcium flux (section 6.6.3). The buffer and corresponding concentrates suppressed base line levels to an equal degree (+ on figure 6.14) and there was no difference in the inhibition of calcium mobilisation (+ on figure 6.16).

TABLE 6.2: INHIBITION OF HLA-DR AND CD25 EXPRESSION BY FVIII CONCENTRATE AND ITS BUFFER.

	% INHIBITION	
PRODUCT	HLA-DR	CD25
CONCENTRATE	15	12
BUFFER	11	30

The buffer, however, significantly inhibited calcium flux, both peak and plateau ($p<0.01$), to a greater degree than that seen by the concentrate (+ on figure 6.15). This may explain why the buffer had a greater capacity to inhibit CD25 than the concentrate.

6.10.2 DIALYSIS OF CLOTTING FACTOR CONCENTRATES

Three clotting factor concentrates were dialysed against Hank's buffer (section 2.10): Alpha Profilate A61140, NHS 8Y 3455 and NHS FIX 3444, and stored at -70°C until they were used in culture and calcium experiments. Dialysis of the NHS 8Y product resulted in the concentrate clotting. The inhibitory capacity of the dialysed Alpha Profilate and BPL 9A (NHS FIX) on HLA-DR and CD25 expression on $CD5^+$ lymphocytes, was measured in 96 hour PHA cultures of PBMC in the presence of 0.5u/ml heparin (figure 6.21), and was found to be diminished compared to the undialysed products. This was greatest for the FIX product where inhibition of the activation markers was reduced by half. Dialysis of the Alpha product virtually removed the capacity to inhibit CD25 expression. This was not the case for HLA-DR expression.

Alpha Profilate and dialysed Alpha Profilate were assessed for their ability to reduce calcium flux (section 6.6.3). There was no difference in the inhibition of peak calcium flux (++ on figure 6.15) or calcium mobilisation (++ on figure 6.16) or in the suppression of base line levels (++ on figure 6.14) between the dialysed product and the pre-dialysed concentrate.

The ability to inhibit plateau calcium flux, however, was

The effect of dialysis upon the inhibitory capacity of clotting factor concentrates

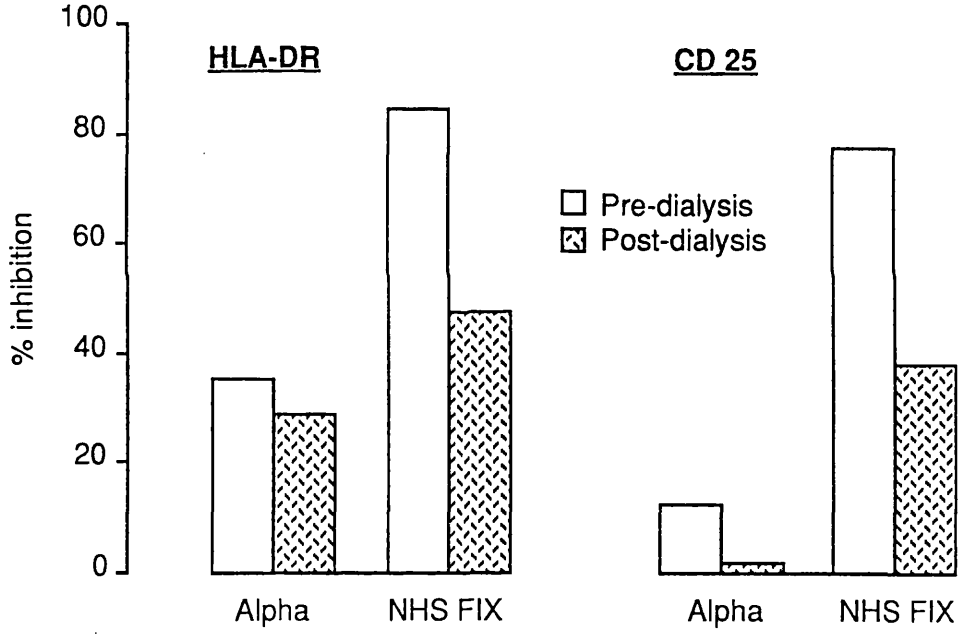


Figure 6.21: Alpha Profilate and NHS FIX (4-5 units/ml) and the products following dialysis in Hank's Buffer (hatched bars) (1:5 dilution), were cultured with PBMC for 96 hours. The inhibition of the expression of HLA-DR and CD25 on $CD5^+$ lymphocytes was determined by two colour flow cytometry.

reduced upon dialysis (41.8 ± 16.5 and 26.6 ± 10.3 respectively) (++ on figure 6.15), but this was not statistically significant ($p>0.05$).

6.11 BUFFERING CAPACITY OF AND CONCENTRATES

The preceding data has demonstrated that the concentrates were inhibiting very early events in T cell activation. Calcium flux and mobilisation were inhibited and the levels of calcium in the cell preparations and basal cytoplasmic levels were also suppressed. Formulation buffers were equally inhibitory for T cell function. There was a dialysable inhibitory component in the concentrate. Collectively this evidence indicated that chelators in the buffers may be causing the inhibition of T cell function by reducing free calcium levels, and thereby affecting calcium flux and all T cell activation events that follow. Therefore the calcium buffering capacity of the concentrates was assessed.

The free calcium level of RPMI 1640+10% FCS was determined using a calcium probe (section 2.11) and found to be $2.2x10^{-4}M$. Calcium chloride was added to the medium to establish a final concentration range of $0.5-8.0x10^{-3}M$, and the level of free calcium ions ($[Ca^{2+}]$) was measured. When this was plotted (figure 6.22), $[Ca^{2+}]$ was lower than expected and did not correspond to the amount of calcium added (dotted line) indicating that the medium was buffering the level of calcium.

The addition of 1:5 dilution of various concentrates (Alpha Profilate, BPL (NHS) 8Y, BPL 9A (NHS FIX)) reduced $[Ca^{2+}]$ in

Relationship Between the Addition of Calcium Chloride and Free Calcium Levels in RPMI 1640 + 10% FCS in the Presence of Clotting Factor Concentrates

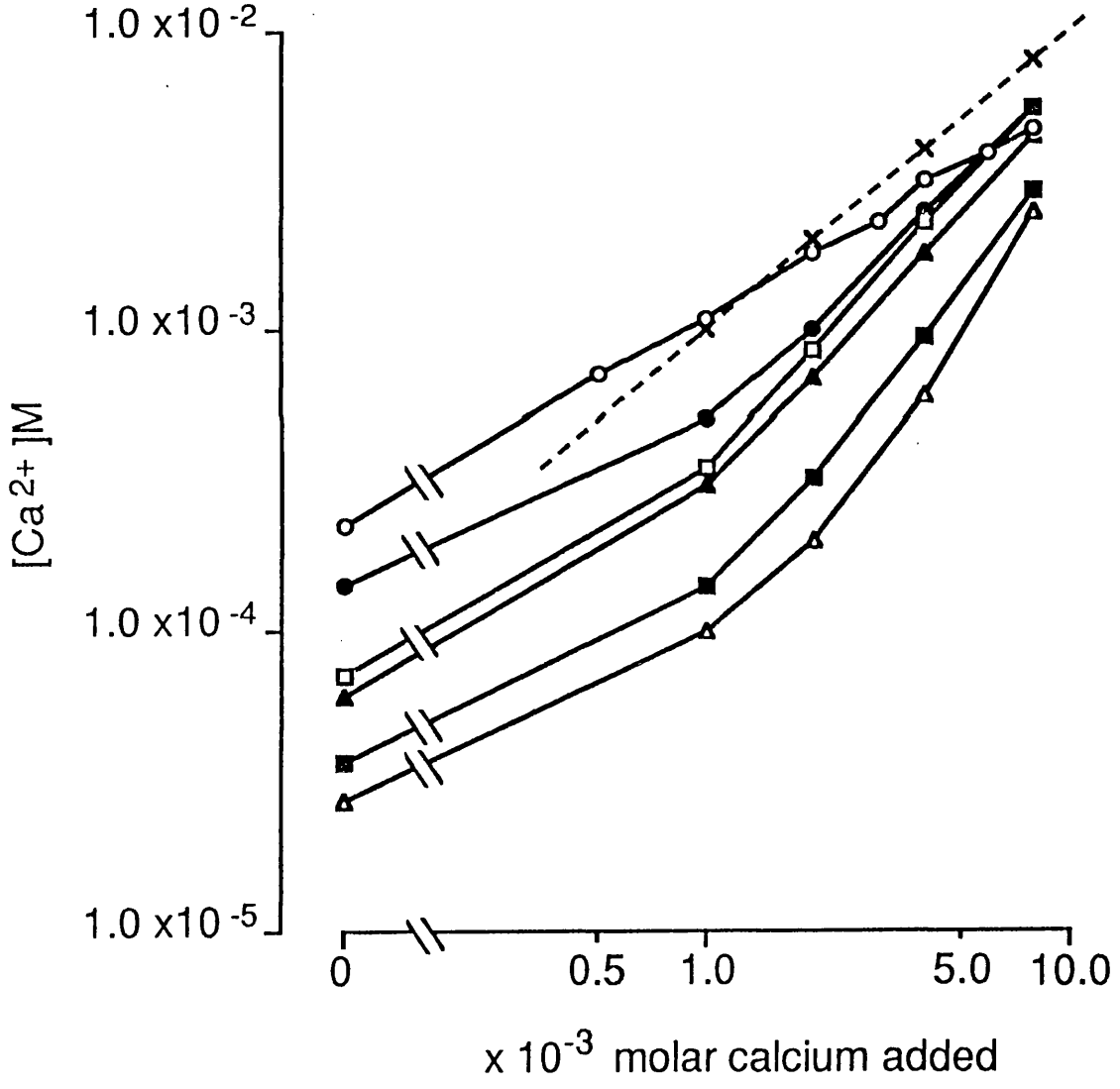


Figure 6.22: Free calcium levels in RPMI 1640+10% FCS were measured with a calcium probe. $CaCl_2$ was added to the medium (○—○) and in the presence of several clotting factor concentrates: Alpha Profilate (□—□) (△—△) (▽—▽), NHS 8Y (●—●) and NHS FIX (■—■), to a final concentration of $8.0x10^{-3}$M. The expected concentration of calcium is depicted by (-----).

the medium. The calcium buffering capacity of the concentrates when $CaCl_2$ was added, corresponded with the initial reduction of the level of calcium that the products induced. In other words the greater the capacity for the concentrate to reduce $[Ca^{2+}]$ the greater its buffering capacity.

6.12 FREE CALCIUM LEVELS CORRELATE WITH INHIBITION OF ACTIVATION MARKER EXPRESSION

Free calcium levels were determined in the presence of RPMI 1640+10%FCS. A 1:5 dilution of the concentrates was added to the medium and with the exception of Armour Monoclate all FVIII and FIX concentrates tested reduced the levels of free calcium (table 6.3).

The capacity of these products to inhibit the expression of the activation markers (figure 6.7) significantly correlated with the free calcium levels ($[Ca^{2+}]$) induced by the addition of the concentrates (HLA-DR $r=-0.535$ ($p<0.05$) and CD25 $r=-0.621$ ($p<0.01$)) (figures 6.23 and 6.24). BPL 9A (NHS FIX) inhibited the expression of CD25 expression to a greater level than that of the other inhibitory products with equivalent capacities to reduce $[Ca^{2+}]$. The correlations of $[Ca^{2+}]$ with the inhibition of activation marker expression were repeated excluding the FIX products from the analysis.

Now the correlations were slightly different in that there was a slightly better correlation of CD25 expression and reduction in $[Ca^{2+}]$ ($r=-0.690$) ($p<0.01$) but no significant relationship between the levels of $[Ca^{2+}]$ and the inhibition of HLA-DR

TABLE 6.3: LEVELS OF FREE CALCIUM IN RPMI+10% FCS IN THE PRESENCE OF CLOTTING FACTOR CONCENTRATES

PRODUCT	$[Ca^{2+}]M$ (mean ± standard deviation)
RPMI 1640+10% FCS	2.2×10^{-4}
Alpha Profilate n=8	$4.7 \times 10^{-5} \pm 1.3 \times 10^{-5}$
NHS 8Y n=8	$7.3 \times 10^{-5} \pm 3.0 \times 10^{-5}$
SNBT n=3	$7.1 \times 10^{-5} \pm 2.2 \times 10^{-5}$
Cutter Koate n=1	8.9×10^{-5}
Octa VI n=1	7.4×10^{-5}
Armour Monoclate n=3	$1.7 \times 10^{-4} \pm 5.5 \times 10^{-5}$
NHS FIX n=5	$3.1 \times 10^{-5} \pm 5.0 \times 10^{-6}$

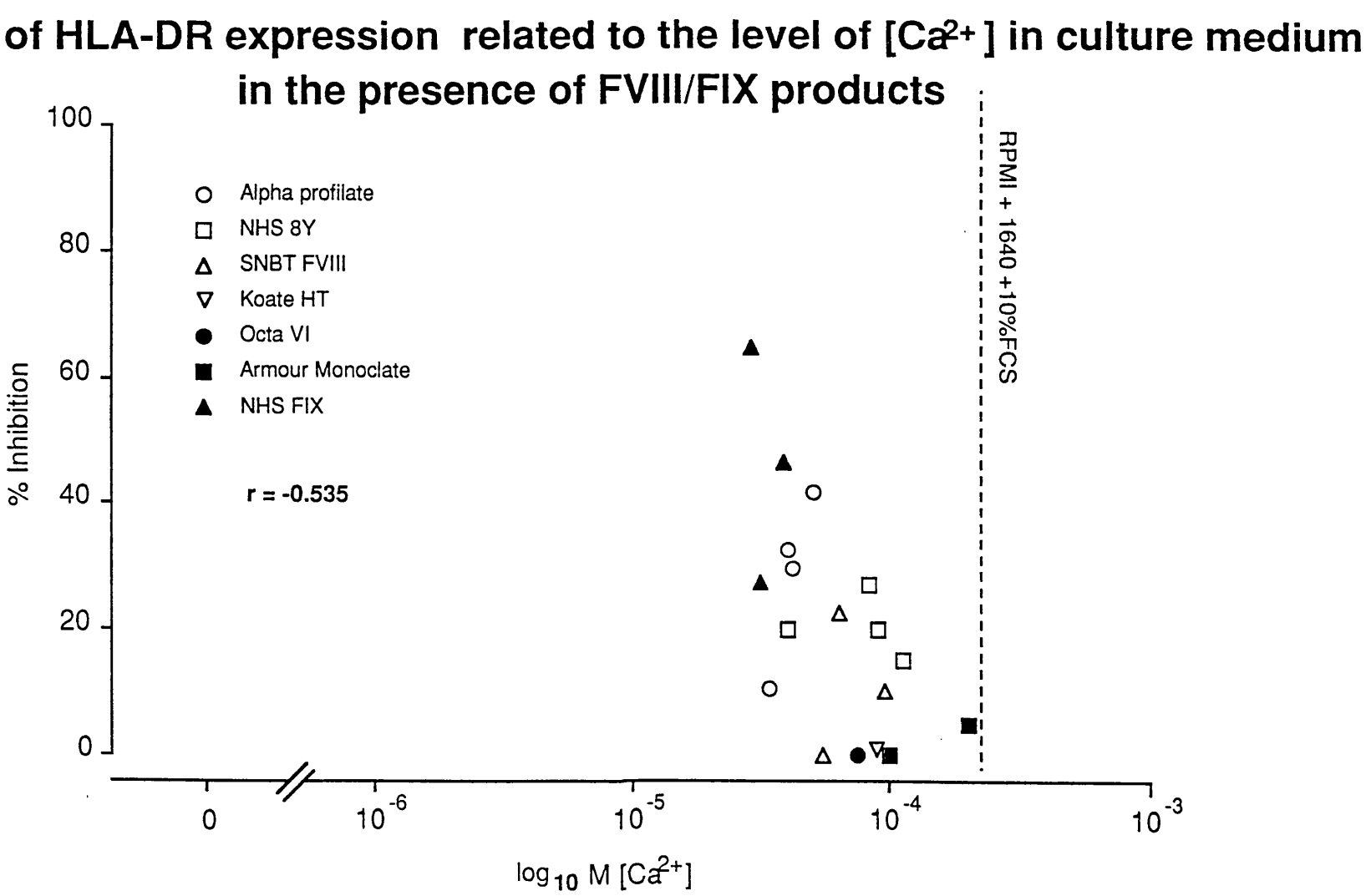


Figure 6.23: The capacity of FVIII and FIX concentrates (4-5 units/ml) added to RPMI+10% FCS to reduce $[Ca^{2+}]$ was measured with a calcium probe. The $[Ca^{2+}]$ correlated with the level of inhibition of HLA-DR expression on $CD5^{+}$ lymphocytes ($r=-0.535$) ($p<0.05$) at 96 hours in PHA stimulated PBMC cultures induced by an equal dilution of concentrate. Correlation of $[Ca^{2+}]$ and inhibition of HLA-DR expression by the FVIII concentrates only, indicated that there was no significant relationship between these two parameters ($r=-0.450$).

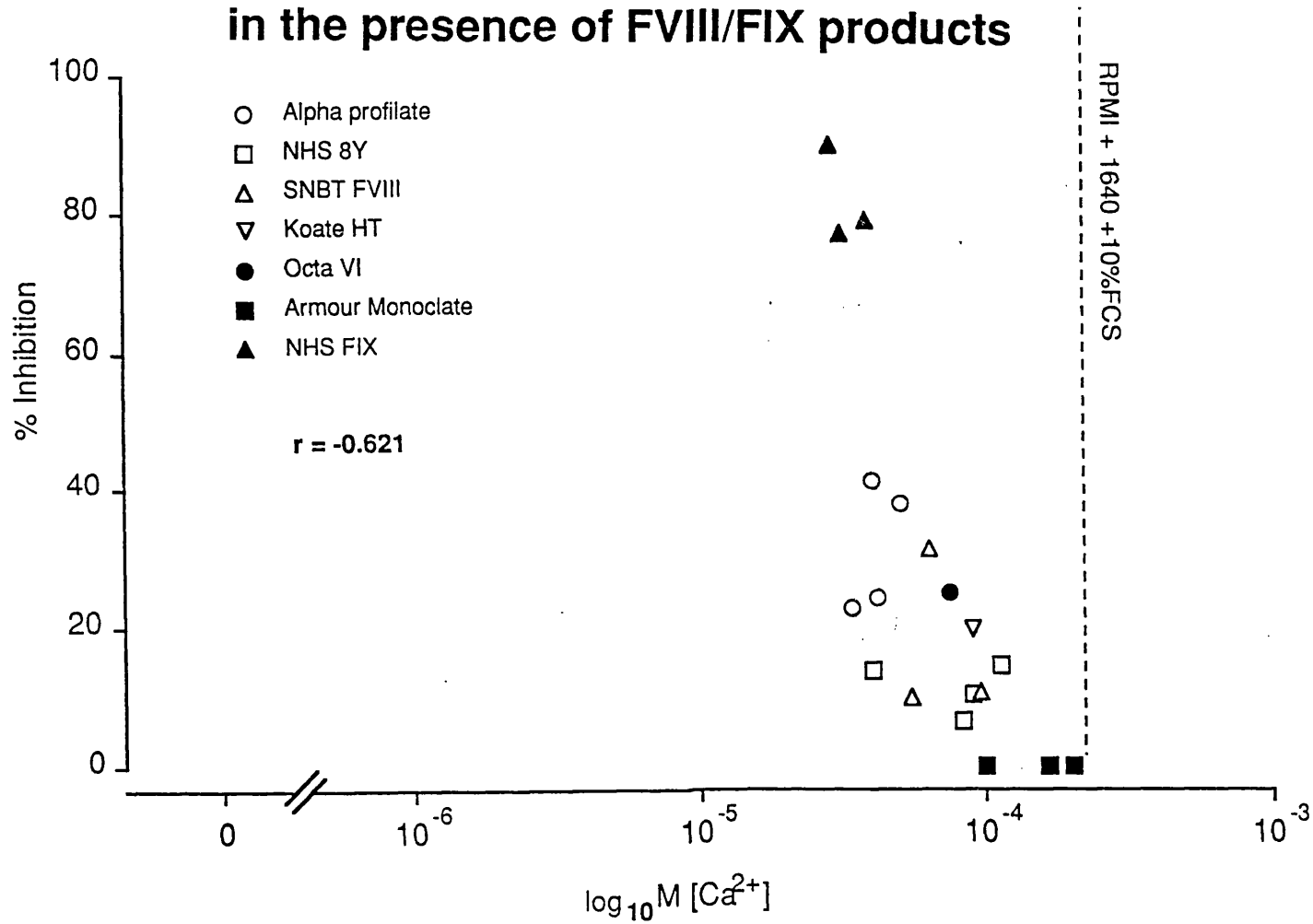


Figure 6.24: The capacity of FVIII and FIX concentrates (4-5 units/ml) added to RPMI+10% FCS to reduce $[Ca^{2+}]$ was measured with a calcium probe. The $[Ca^{2+}]$ correlated with the level of inhibition of CD25 expression on $CD5^{+}$ lymphocytes (r=-0.621) ($p<0.01$) at 96 hours in PHA stimulated PBMC cultures induced by an equal dilution of concentrate. Correlations with the FVIII products alone, increased the value slightly (r=-0.690) ($p<0.01$).

expression ($r=-0.450$) ($p>0.05$).

Dialysis of the products (section 6.10.2) reduced the capacity of the concentrates to reduce $[Ca^{2+}]$. Free calcium levels were reduced from normal by approximately one order of magnitude when the clotting factor concentrate (pre-dialysis) was added (table 6.4). The dialysed Alpha Profilate, however, did not affect calcium levels, but still had a marked, although reduced inhibitory effect on T cell activation. The dialysed FIX product reduced $[Ca^{2+}]$ but not to the same degree as the non-dialysed concentrate. Therefore dialysis of the concentrates reduced or removed the agent(s) that reduced $[Ca^{2+}]$. In the BPL 9A (NHS FIX) product this corresponded to a reduction in the inhibitory capacity of the concentrates. However, dialysed Alpha Profilate concentrate was still inhibitory for HLA-DR expression even though $[Ca^{2+}]$ was normalised by dialysis. It should be noted that $[Ca^{2+}]$ did not appear to be normalised in fura-2AM preparations before stimulation, however, these base line levels of the cell preparations include basal cytosol calcium. Therefore dialysis removed the component in the Alpha product that reduced free calcium in the medium but not the component that reduced basal calcium levels in the cell.

6.13 FREE CALCIUM LEVEL REDUCTION BY CONCENTRATES DOES NOT CORRELATE WITH INHIBITION OF CALCIUM FLUX

When the levels of free calcium in the medium induced by the addition of concentrate were compared with the percentage inhibition of calcium flux, no relationship was found (figures 6.25). Therefore the reduction in free calcium did not cause the inhibition of calcium flux alone.

TABLE 6.4: INHIBITION OF FREE CALCIUM LEVELS BY CLOTTING FACTOR CONCENTRATES AND THEIR DIALYSATES

PRODUCT	PRE-DIALYSIS			POST-DIALYSIS		
		PERCENT INHIBITION			PERCENT INHIBTION	
	$[Ca^{2+}]M$	HLA-DR	CD25	$[Ca^{2+}]M$	HLA-DR	CD25
ALPHA PROFILATE A61140	$3.5X10^{-5}$	35.1	12.4	$2.1X10^{-4}$	28.8	2.0
NHS 8Y 3455	$4.0X10^{-5}$	13.3	21.2	clotted		
NHS FIX 3444	$2.6x10^{-5}$	77.5	84.2	$7.4x10^{-5}$	47.6	38.1

FREE CALCIUM LEVELS IN RPMI 1640+10%FCS = $2.2x10^{-4}$

Correlation of Levels of Free Calcium with % Inhibition of Anti-CD3 Induced Calcium Flux in the Presence of Clotting Factor Concentrates

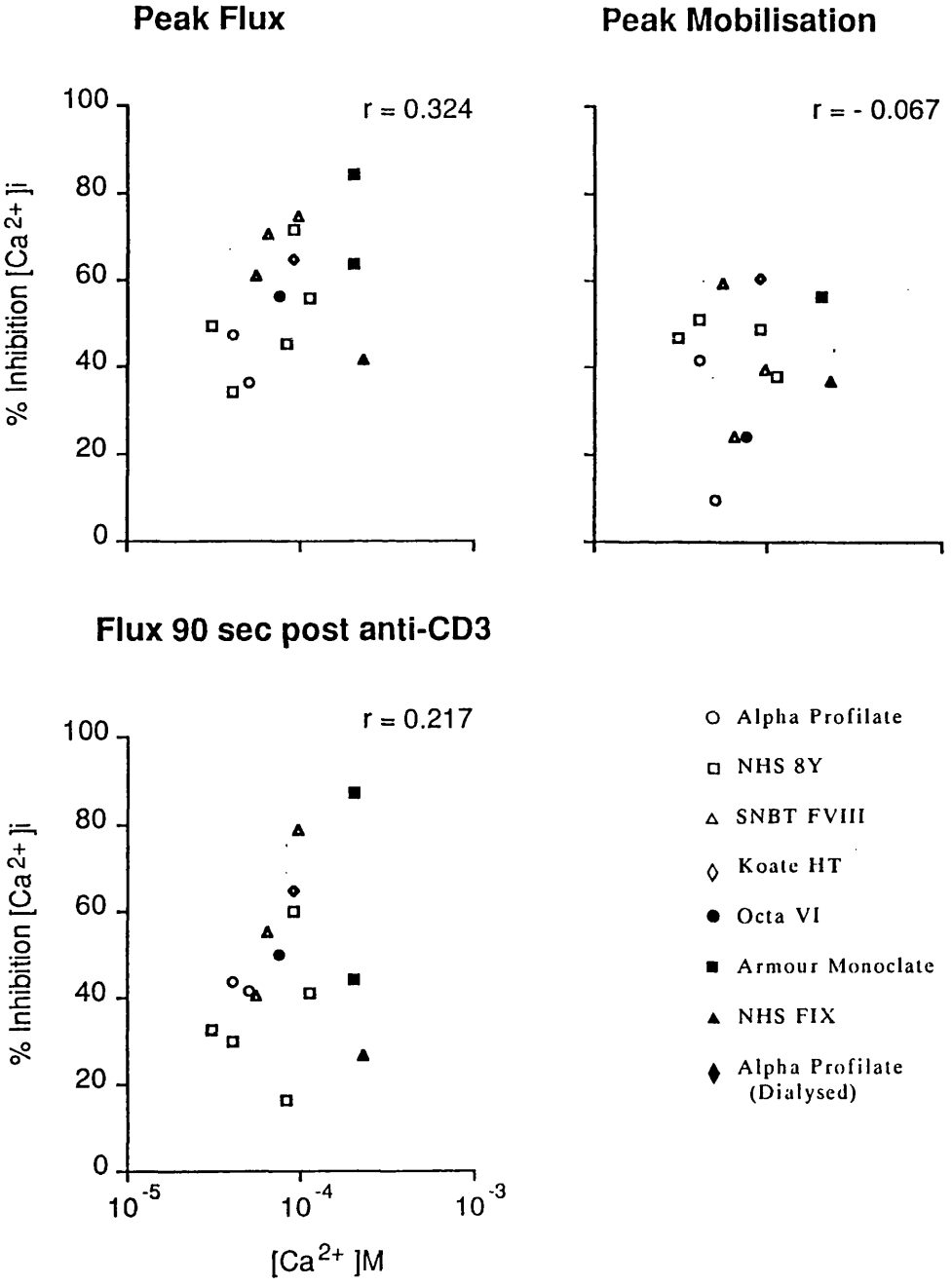


Figure 6.25: Inhibition of free calcium levels ($[Ca^{2+}]$) in RPMI 1640+10% FCS, in the presence of FVIII concentrates was determined with a calcium probe. The percentage inhibition of calcium flux (determined in the presence of 1mM Ca^{2+}) and calcium mobilisation (determined in the presence of 2mM EGTA) was measured in fura-2AM stimulated Jurkat cells. There was no correlation between the two parameters.

6.14 EFFECT OF THE ADDITION OF CALCIUM CHELATORS ON [Ca^{2+}] AND EXPRESSION OF HLA-DR AND CD25

We have suggested that the calcium chelators in the formulation buffers are important in the inhibition of T cell function by the concentrates. To establish the effects of reducing calcium levels in the medium on T cell function, the chelator EGTA was studied.

EGTA was added to RPMI 1640+10% FCS to give a final concentration range of 0-500nM/ml and free calcium levels were measured using a calcium probe. The addition of EGTA reduced the [Ca^{2+}] (figure 6.26) but this was not a linear relationship. The addition of 500nM EGTA reduced [Ca^{2+}] by an order of magnitude. Extrapolation of the curve demonstrated that the [Ca^{2+}] was rapidly diminished above this concentration, indicating that threshold levels were important.

Sterile EGTA was added to PHA cultures of PBMC at final concentrations of 10nM - 1.7mM and the expression of HLA-DR and CD25 on $CD5^{+}$ lymphocytes was determined at 96 hours (figure 6.27).

Concentrations of EGTA as low as 10nM reduced the expression of HLA-DR by 50% and CD25 by approximately 25% of normal levels. The results have been plotted so that the concentration of EGTA corresponds to the level of free calcium in the medium (determined from figure 6.26). This indicates that the inhibition of CD25 increased with decreases in free calcium levels, however, this was not a true dose dependent relationship. The results were similar for HLA-DR. For

Levels of Free Calcium in RPMI 1640 + 10% FCS in the Presence of Increasing Concentrations of EGTA

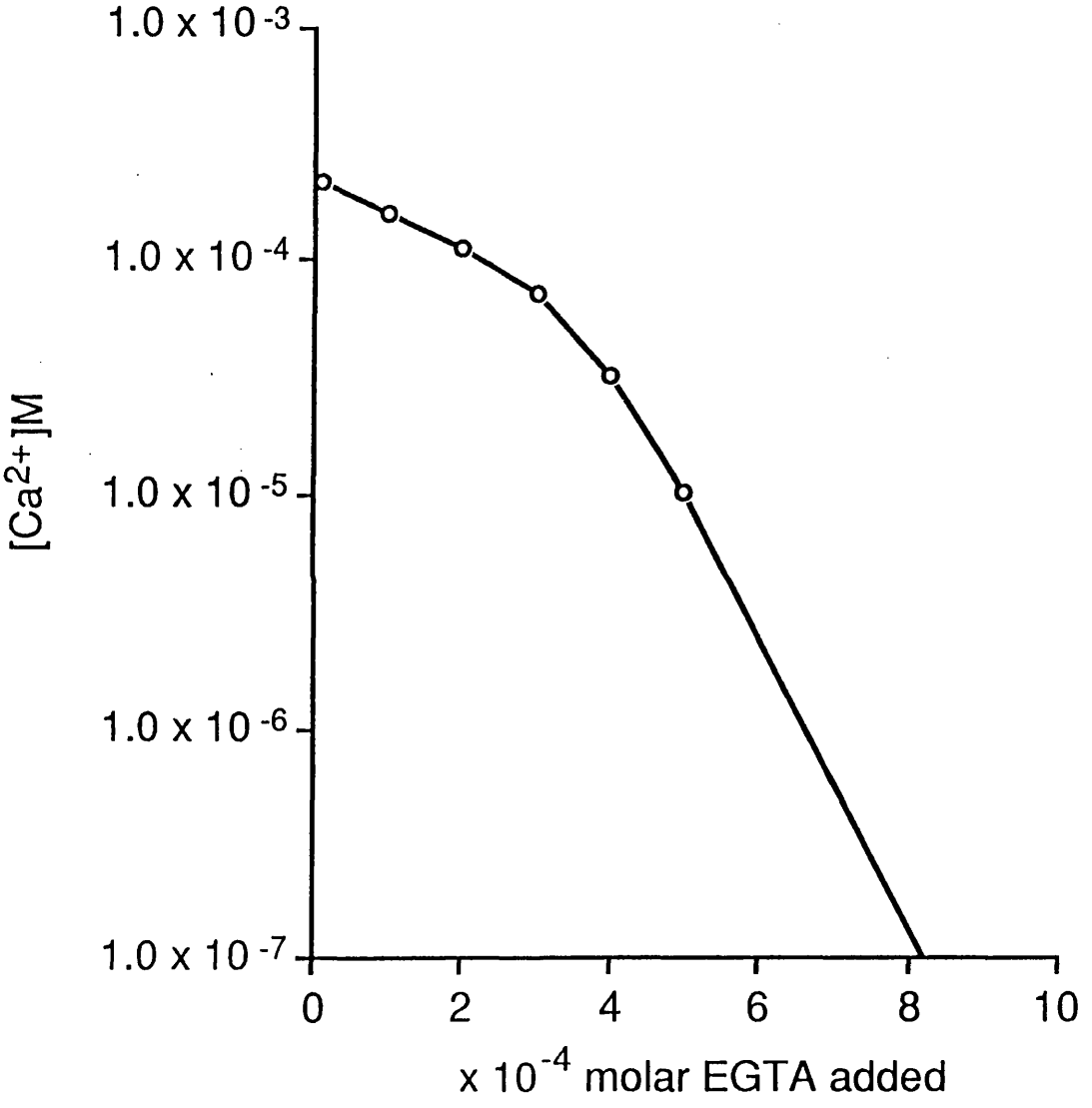


Figure 6.26: Free calcium levels were measured with a calcium probe in RPMI+10% FCS in the presence of increasing concentrations of EGTA (0-500nM/ml) and were found to be reduced.

Inhibition of HLA-DR and CD25 expression in the presence of increasing concentrations of EGTA, related to the calcium levels in culture medium

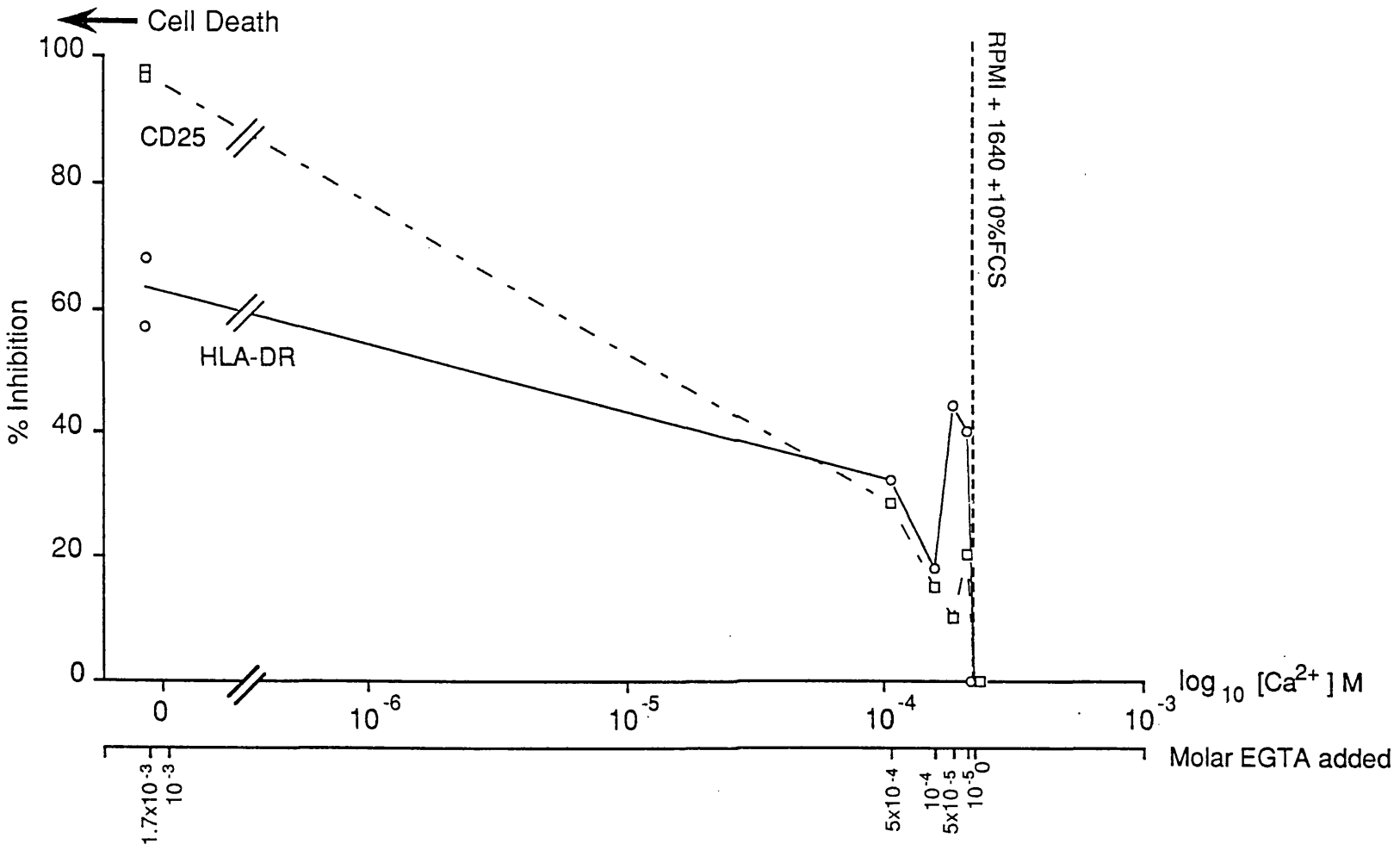


Figure 6.27: The effects of addition of increasing concentration of EGTA (0-1.7mM/ml) on the expression of HLA-DR (O—O) and CD25 (□—□) of 96 hour PHA stimulated $CD5^+$ lymphocytes was determined by two colour flow cytometry. The concentration of EGTA (x axes) was related to [Ca^{2+}] in the medium (determined from figure 6.26).

example, 50nM EGTA was less inhibitory than 10nM EGTA. High doses of EGTA inhibited the expression of the activation markers in a dose dependent manner, but this may be related to cell death.

Comparison of reduction in $[Ca^{2+}]$ induced by the concentrates and EGTA with their effects upon the expression of HLA-DR and CD25 (figures 6.23, 6.24 and 6.27), indicated that most of the concentrates inhibited the expression of the activation markers to an equal or lesser degree than EGTA when corresponding levels of free calcium were induced. The FIX products, however, inhibited activation marker expression to a greater degree than could be accounted for by the reduction in $[Ca^{2+}]$ alone.

6.15 REPLACEMENT OF CALCIUM

If the inhibition of activation of lymphocyte function is related to the calcium buffering capacity of the concentrates, which in turn diminishes calcium flux and all activation processes that follow, then one would expect that replacement of calcium in the medium would prevent this inhibition. Addition of concentrates to RPMI 1640+10% FCS reduced the level of free calcium, but this did not correlate with reduction in calcium flux, nor did it correlate with inhibition of expression of the activation markers.

To test this hypothesis three products - Alpha Profilate, BPL (NHS) 8Y and BPL 9A (NHS FIX) were added to PHA cultures in the usual manner, in the presence of sterile $CaCl_2$ at final concentrations of 0.1, 0.5, 1.0 and 2.0mM and the expression

of HLA-DR and CD25 upon $CD5^+$ lymphocytes at 96 hour of culture determined.

Addition of 2mM calcium increased the level of HLA-DR expression from 75% to 82% in PHA cultures, this increase was not seen at lower concentrations of calcium, nor were increases seen in the expression of CD25, but this marker was maximally expressed in the absence of additional calcium.

As previously mentioned, the clotting factor concentrates buffered the levels of calcium when $CaCl_2$ was added. The Alpha and NHS 8Y products performed in a similar manner (figure 6.28), but NHS FIX had a greater capacity to reduce the level of free calcium and consequently had a greater buffering capacity.

In the experiments where calcium was added back into the system to assess if calcium replacement abolished the inhibition, it was important to establish the true level of calcium rather than the concentration that had been added. Figures 6.29 and 6.30 represent the inhibition of expression of the activation markers in the presence of the three clotting factor concentrates, this is plotted in relationship to the true concentration in the cultures following the addition of calcium. Using figure 6.28, the true concentration of calcium in the culture medium was determined and plotted against the level of inhibition of the activation markers that the concentrates induced. The additional *x* axes refer to the concentration of $CaCl_2$ added to the cultures. Addition of calcium to levels that were equivalent to the

Relationship Between the Addition of Calcium Chloride and Free Calcium Levels in RPMI 1640 + 10% FCS in the Presence of Clotting Factor Concentrates

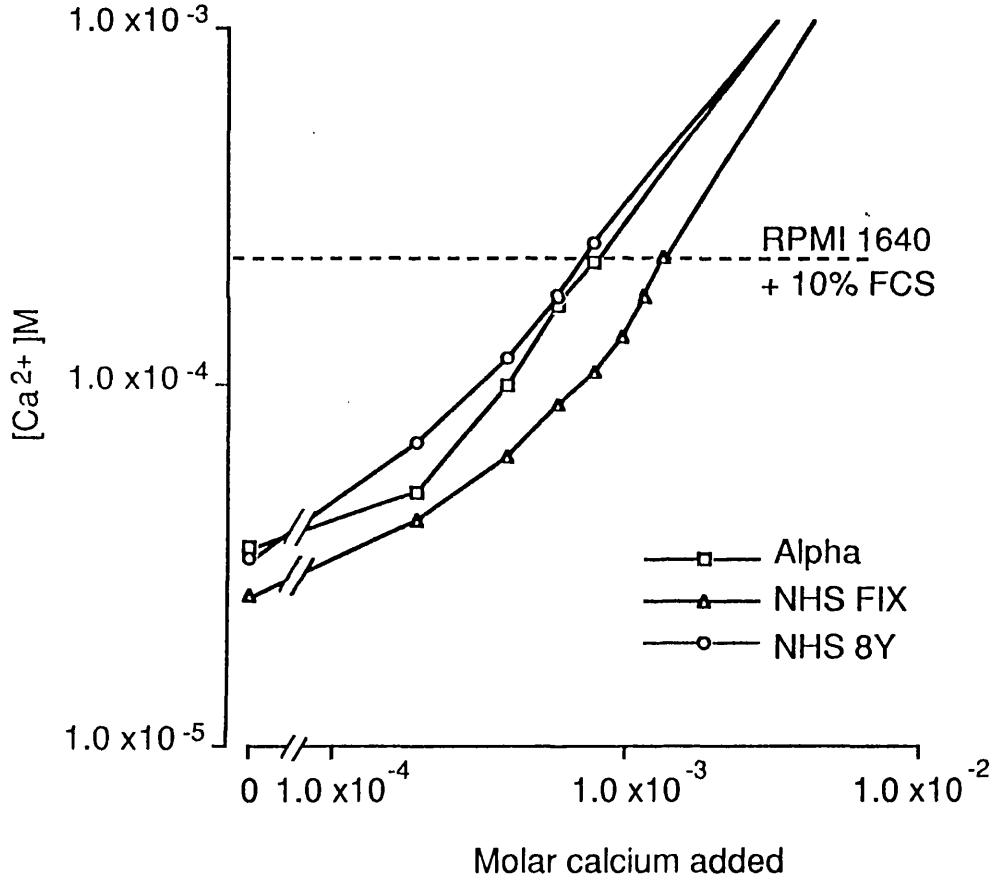


Figure 6.28: Free calcium levels in RPMI 1640+10% FCS were measured with a calcium probe. $CaCl_2$ was added to the medium in the presence of the three concentrates which were studied in detail: Alpha Profilate (□—□), NHS 8Y (○—○) and NHS FIX (△—△), to a final concentration of 2.0×10^{-3}M. The medium and concentrates buffered the level of $[Ca^{2+}]$ so that it was lower than the concentration that was added and this was related to the initial $[Ca^{2+}]$.

The effect of addition of calcium upon inhibition of HLA-DR expression in the presence of clotting factor concentrates

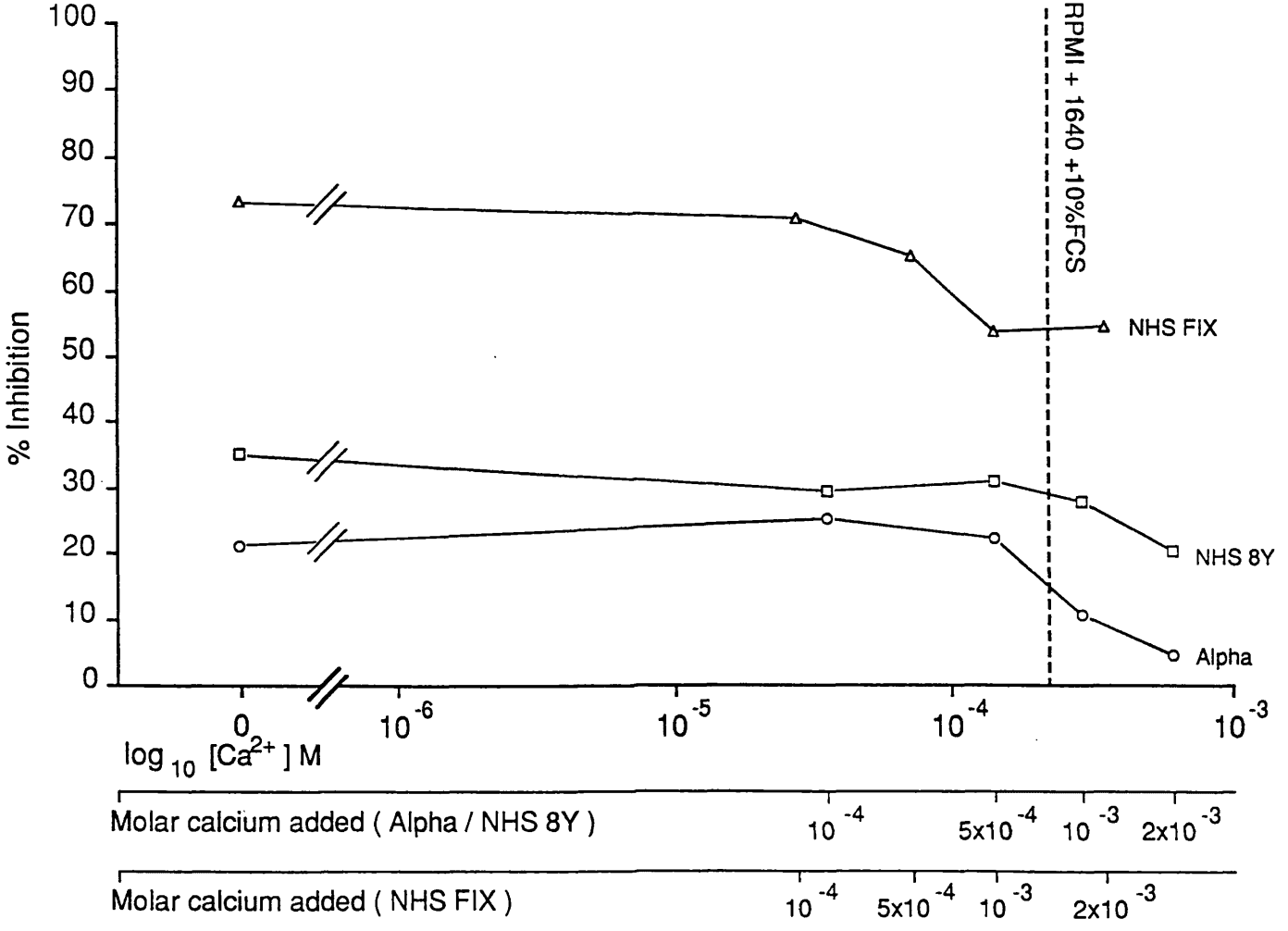


Figure 6.29: The calcium levels in cultures of PHA stimulated PBMC with clotting factor concentrates (Alpha Profilate (○—○), NHS 8Y (□—□) and NHS FIX (△—△)) were replaced with the addition of $CaCl_2$. The buffering capacity of the concentrates resulted in lower $[Ca^{2+}]$, therefore the data was plotted against the true $[Ca^{2+}]$ determined from figure 6.28. The additional x axes refer to the amount of calcium added to the cultures. $[Ca^{2+}]$ in RPMI 1640+10% FCS were $2.2x10^{-4}$ (-----). The effects of replacing the calcium levels in the medium on HLA-DR expression on 96 hour cultures of PHA stimulated $CD5^+$ lymphocytes was determined by two colour flow cytometry.

The effect of addition of calcium upon inhibition of CD25 expression in the presence of clotting factor concentrates

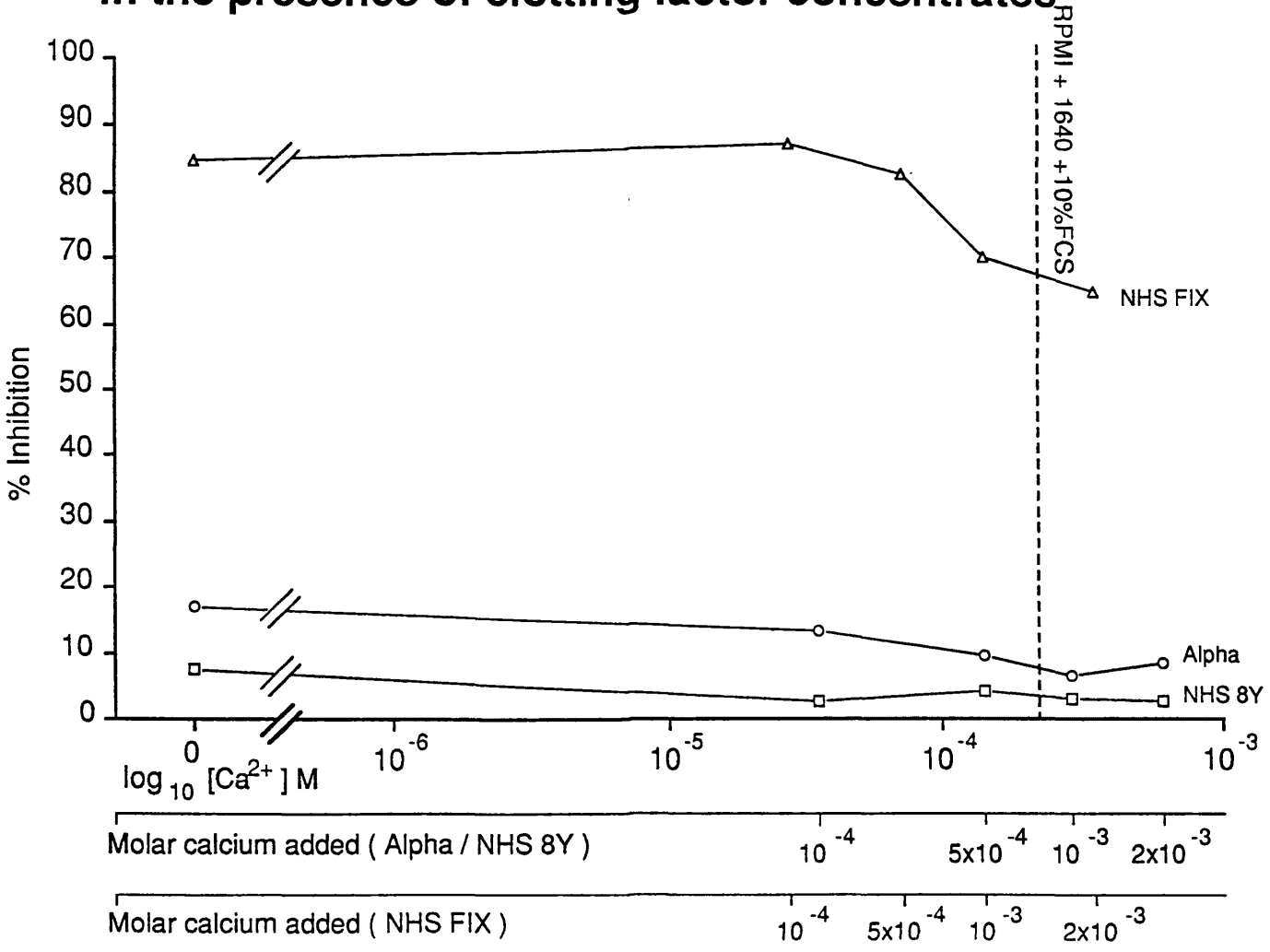


Figure 6.30: The calcium levels in cultures of PHA stimulated PBMC with clotting factor concentrates (Alpha Profilate (O—O), NHS 8Y (□—□) and NHS FIX (△—△)) were replaced with the addition of $CaCl_2$. The buffering capacity of the concentrates resulted in lower [Ca^{2+}], therefore the data was plotted against the true [Ca^{2+}] determined from figure 6.28. The additional x axes refer to the amount of calcium added to the cultures. [Ca^{2+}] in RPMI 1640+10% FCS were $2.2x10^{-4}$ (-----). The effects of replacing the calcium levels in the medium on CD25 expression on 96 hour cultures of PHA stimulated $CD5^+$ lymphocytes was determined by two colour flow cytometry.

concentration of free calcium in RPMI 1640+10% FCS ($2.2x10^{-4}M$) only slightly reduced the level of inhibition of HLA-DR expression (figure 6.29) by the BPL (NHS) 8Y. Replacement of calcium to these levels in the culture containing the Alpha product, reduced inhibition by a quarter. Increasing the level of free calcium to above that seen in the medium to near physiological (1mM) continued to decrease the level of inhibition seen by these products. At a concentration of $6.0x10^{-4}M$, inhibition of HLA-DR expression by the Alpha product was reduced by over 75%, but reduction in inhibition by BPL (NHS) 8Y at this concentration was not as high being of the order of 30%.

Referring to the BPL 9A (NHS FIX) product, inhibition of HLA-DR expression was comparatively much higher. At levels of calcium equivalent to that seen in RPMI 1640+10%FCS inhibition was reduced by only a quarter, the experiment did not replace calcium to concentrations higher than $4.0x10^{-4}$.

Inhibition of CD25 by Alpha and NHS 8Y was low (<20%), and addition of $CaCl_2$ reduced it slightly (figure 6.30).

Again BPL 9A (NHS FIX) was very inhibitory for the expression of CD25 and addition of calcium to levels equivalent to those seen in RPMI 1640 +10% FCS reduced this inhibition by approximately 20%.

In conclusion replacement of calcium did reduce the level of inhibition but did not completely remove it.

7. DISCUSSION FOR *IN VITRO* STUDY

7. DISCUSSION FOR THE IN VITRO STUDY

Clotting factor concentrates have been demonstrated to inhibit T cell function *in vitro*. At the onset of this study it was suggested that this inhibition was specifically directed at IL2 and IL2-R proliferative responses. To establish if this is so, the expression of activation markers that precede IL2 production, namely HLA-DR and calcium flux, and are influenced by IL2 production (CD25, CD71) were examined.

7.1 EXPRESSION OF ACTIVATION MARKERS IN LECTIN STIMULATED PBMC CULTURES

The kinetics of the expression of the activation markers HLA-DR, CD25, 4F2, and CD71, upon lectin stimulated T cells was evaluated. These results were comparable to earlier studies of the expression of IL2 (reveiwed by Smith, 1988), 4F2 (Suomalainen , 1986), and CD71 (Pelosi *et al*, 1986). Maximal expression of the markers on the cells was generally at 72-96 hours following stimulation with PHA. We demonstrated, however, that CD25 was detectable before this on the T4 cells.

Our own experiments demonstrate that expression of CD25 is greater at 24-48 hours of culture on T4 cells compared to T8 cells. The delayed expression of CD25 on these cells probably demonstrates the requirement for T cell help in the form of IL2 from the T4 cells for complete activation.

Recent studies, have shown that HLA-DR mRNA and antigen are detectable very early (2 hours following stimulant) in culture

(Gansbacher and Zier, 1988, Siegel, 1988). Our own results demonstrated that the majority of cells expressed HLA-DR by 48 hours but the density of antigen remained low. A small proportion of the cells, however, expressed high levels of HLA-DR antigen. This proportion did not exceed 20% of the cells in the culture and was higher in PWM cultures, particularly on T4 cells. Taniguchi *et al* (1983), have shown that HLA-DR follows CD71 expression during S phase of the cell cycle and suggested that its expression was dependent on CD25 expression. Hercend *et al* (1981) demonstrated that HLA-DR was expressed following 4-5 days of culture after stimulation with various mitogens (PHA, alloantigen, soluble antigen) and that it co-expressed (depending on the stimulant) with CD38 and CD71. The expression of HLA-DR at the latter stages of a PHA culture (5-6 days) is of a high density (Dr M Moore, ICRF Human Tumour Immunology Group, UCH, London, personal communication). Therefore there appear to be two stages in HLA-DR expression. The first, when the expression of this marker expands to the majority of cells in the culture; and the second, when the expression of the antigen increases on each cell. In this study the first stage of HLA-DR expression was studied.

Some of this study was based on the hypothesis that PWM preferentially stimulated memory T cells, based on information available at the commencement of the study, and reviewed by K Bottomly (1988). The hypothesis stated that lectins could preferentially facilitate the production of specific lymphokines related to naive or to memory T cell subsets in both humans (Ledbetter *et al*, 1985, Tedder *et al*, 1985, Smith

et al, 1986, Rudd *et al*, 1987) and murine clone experiments (Mos mann *et al*, 1986) (section 1.8.3).

A more recent review of the available data on memory in T cells (Akbar *et al*, 1991) indicates that these definitions oversimplify very complex events. Naive and memory cells react equally to lectins. This cytokine production is not an all or none phenomenon as both T4 subsets can produce the cytokines but more IL2 is produced by naive cells and more IL4 is produced by memory cells. Therefore IL2 is produced initially in cultures followed by production of IL4, as the stimulated cells undergo a functional transition to memory cells. It should be noted, however, that there is variability between studies.

PWM stimulation of PBMC, in our experiments, only stimulated a subpopulation of cells and there was greater donor variability and slower kinetics of the activation marker expression. PWM is a T cell dependent stimulator of B cells (Janossy *et al*, 1977), and although PHA has been described as a B cell stimulant, PWM can stimulate the B cells to synthesise large quantities of Ig. Memory T cells have been demonstrated to 'help' Ig production (Tedder *et al*, 1985, Smith *et al*, 1986) and therefore it is possible that the subpopulation of cells stimulated in PWM cultures may be memory T cells. On the other hand, it is possible that the lectin non-specifically stimulates both B and T cells and the B cell help that occurs is coincidental. Never-the-less, in this study, all T cells regardless of stimulant or subclass, were inhibited by clotting factor concentrates.

7.2 CLOTTING FACTOR CONCENTRATES INHIBIT ACTIVATION MARKER EXPRESSION

At the commencement of this study, American, German and Swiss and Scottish FVIII and FIX products had been demonstrated to inhibit PBMC proliferation in response to lectin and antigen. FVIII had also been shown to inhibit IL2 production in normal cells and in Jurkat cells (Lederman *et al*, 1986). These inhibitory effects were dose dependent. We have further demonstrated that BPL 8Y (NHS FVIII) and BPL 9A (NHS FIX) are also inhibitory for T cell function, and that the BPL 8Y product was less inhibitory than Alpha Profilate (an American product).

There was a variability in the capacity to inhibit activation marker expression between different products and between batches of product of FVIII and FIX. A similar finding was reported by Thorpe *et al* (1989), who demonstrated that these same products variably inhibited IL2 production by Jurkat cells. The most inhibitory FVIII product was Alpha Profilate and the least inhibitory FVIII product was Armour Monoclate. The BPL 9A (NHS FIX) product inhibited activation marker expression to a greater extent than all the FVIII products that were tested. But as with the FVIII product, the monoclonally produced FIX product was not inhibitory. These results were verified by Dr Robin Thorpe, NIBSC, South Mimms, Herts (personal communication).

Alpha Profilate was found to inhibit the expression of HLA-DR, CD25, 4F2 and CD71 in PHA stimulated cultures but low doses of FVIII were found to increase the expression of HLA-DR and CD71

at 96 hours of culture above that seen in the control. This experiment was only performed once, but supports data reported by Matheson *et al* (1986), who showed that low doses of FVIII stimulated PBMC from haemophiliacs *ex vivo* and Batchelor *et al* (1990), who demonstrated a co-stimulatory effect by one batch of Scottish FVIII at therapeutic concentrations. BPL 9A (NHS FIX) stimulated PBMC in culture in the absence of lectin.

It has been suggested by Batchelor (1991), that the activation of PBMC by concentrates *in vitro* is related to the presence of MHC antigens in the concentrates. This implies that the concentrates may be stimulating an allogeneic response. The activation of lymphocytes in MLR is very complex. The MHC is recognised in the context of whole cell and the response is stronger and more widespread in the T cell population than is seen for antigen. In contrast, MHC peptides in the concentrates are not presented in the context of whole cell and therefore it is likely that they would be presented as ordinary antigen following processing by antigen presenting cells in the culture.

In our experiments we have shown that FVIII inhibited the expression of HLA-DR and CD25 on T cells from an early stage in the cell culture and this inhibition was greatest at the beginning of the PHA cultures and delayed in the PWM cultures. This presumably reflects the delay in the expression of these activation markers observed in PWM stimulated cells. A recent publication (Hay *et al*, 1990), has demonstrated inhibition of HLA-DR, CD25 and CD71 expression on T4 and T8 PHA stimulated

cells at 72 hours of culture, and these verify our results.

Lederman *et al* (1986), had demonstrated that maximal inhibition occurred when concentrate was added at time 0 and was reduced by 75% when concentrates were added at 24 hours. This pattern of inhibition was also observed by Wang *et al* (1985), where inhibition was greater than 50% when concentrate was added within 8 hours of stimulant, but had diminished at 20 hours and was almost completely abolished by 32 hours.
Lederman *et al* (1986), demonstrated that pre-culture with FVIII resulted in a time dependent inhibition of proliferation of PBMC stimulated by PHA, which was reversible on washing the cells following incubation with FVIII for 1 hour, partially reversible after incubation for 4 hours and irreversible following 48 hours of incubation.
The inhibitory effect seen in these experiments was not related to the cytotoxicity of the concentrates (Lederman *et al*, 1986).

Observations published before and during this study verify results we have obtained. They indicate that the mechanism of inhibition is occurring very early in T cell activation. CD25 (Smith and Cantrell, 1985) and CD71 (Neckers and Cossman, 1983) are dependent upon IL2 for their expression. HLA-DR, however, can be upregulated by gamma-interferon (Siegel , 1988) and is not modulated by addition to rIL2 on T4 cells. HLA-DR expression on T4 cells is inhibited by the concentrates. Therefore the inhibition was not directed at IL2 production and IL2 dependent proliferation alone. This is further substantiated by the observation that inhibition of

proliferation is not corrected by the addition of exogenous IL2 to the cultures (Hay *et al*, 1990). Therefore the inhibition of T cell function is a general phenomenon and not directed specifically at IL2.

Binding of lectin by FVIII concentrates could account for, or partially account for the inhibition seen. Direct lectin-glycoprotein interaction (Con A-FVIII concentrate, PHA-FIX concentrate) has been shown (McDonald *et al*, 1985), but inhibition can be induced by prolonged pre-incubation and removal of FVIII concentrate before the addition of lectin. This indicates that binding of lectin by components in the products is not important in FVIII concentrate-induced inhibition of T cell function.

7.3 IMPORTANCE OF CALCIUM IN THE INHIBITION OF T CELL FUNCTION BY THE CLOTTING FACTOR CONCENTRATES

In this study buffers have been shown to be equally as inhibitory as the concentrates themselves, confirming the results of others (McDonald *et al*, 1985, Batchelor *et al*, 1990). We and other groups (McDonald *et al*, 1985, Wang *et al*, 1985) have also demonstrated that dialysis of the concentrates reduced their capacity to inhibit activation marker expression and proliferation of PBMC. Thorpe *et al* (unpublished data) also found that dialysis reduced inhibition of IL2 production by Jurkat cells.

Gel filtration of the products revealed that there were two peaks of inhibitory activity in the concentrates with a molecular weight of >200KDa (co-migrating with FVIII coagulant

activity and FVIII:Ag) and a molecular weight of approximately 60KDa (Lederman *et al*, 1986). McDonald *et al* (1985), however, demonstrated that the inhibitory component was not present in the protein fractions and was less than 10KDa in size.

The observations that the buffers have an equal inhibitory effect as the concentrates and that the inhibitory component is partially dialysable indicates that the inhibition may be related to the buffers rather than the protein content of the products. The buffers contain anti-coagulants. Lederman *et al* (1986), studied heparin and plasmin at concentrations similar to those seen in the products, but these were not inhibitory. Wang *et al* (1985), studied the influence of glycine and citrate at concentrations equivalent to those in the products but these did not consistently inhibit proliferation. Although inhibition with citrate was seen in experiments using RPMI medium. McDonald *et al* (1985), demonstrated that sodium citrate inhibited proliferation in a variable manner over a concentration range of 5mM-40mM/ml. They suggested that this anti-coagulant could be a major inhibitory component in the products and demonstrated that other plasma products containing citrate (stable plasma protein solution and salt poor albumin) were also inhibitory and that this too could be reduced by dialysis, while human normal immunoglobulin, which is a citrate free product, was not inhibitory.

The binding of PHA to CD3 and CD2 is a calcium dependent phenomenon. Observations of PBMC stimulated with PHA, in the presence of FVIII concentrates revealed that there is less

clumping of cells. This may be related to the diminished activation, but it is possible that the reduction in calcium induced by the products could also be reducing binding of PHA to the cells, and thus contributing to the inhibition seen. It is unlikely, however, that this is an important phenomenon for the reasons outlined earlier, namely that prolonged preincubation of the cells with FVIII concentrates followed by washing is inhibitory.

We have demonstrated that FVIII products inhibit calcium flux, both influx and mobilisation. They also reduce the levels of free calcium when added to the cell culture medium culture and reduce basal calcium levels.

There was no relationship between the reduction in free calcium levels in the medium and the inhibition of calcium flux. This indicates that the relationship of the inhibition of calcium flux with reduction in free calcium levels inhibition is complex and is probably governed by threshold levels rather than a dose dependent relationship.

Dialysis of Alpha Profilate eliminated the capacity of the product to reduce free calcium levels. Therefore it is unlikely that the presence of calcium binding proteins in the FVIII concentrate is contributing to the reduction of free calcium. This indicates that the component that reduces free calcium levels may be a calcium chelator in the buffers, presumably citrate.

Dialysis of Alpha Profilate also reduced the capacity of the

products to inhibit plateau calcium flux, ie true calcium influx, which is affected by extracelluar calcium levels, but calcium mobilisation and suppression of base line levels of calcium were unaffected. Therefore the dialysable inhibitory component only affected free calcium levels and contributed to the reduction in calcium influx from the extracelluar environment. There was a strong correlation with free calcium levels induced by the clotting factor concentrates and inhibition of the activation markers, particularly CD25, but it was evident that this was not the whole story as replacement of calcium in the cultures only partially removed the inhibition.

Dialysis of the FIX product, however, did not eliminate the capacity to reduce free calcium levels. This may be because the duration of dialysis was not long enough, but it is possible that these products contain calcium binding proteins. This is further evidence that the FIX products were operating in a different manner to that of the FVIII products.

Comparison of reduction in $[Ca^{2+}]$ induced by the concentrates and EGTA with their effects upon the expression of HLA-DR and CD25 indicated that the FIX products inhibited these markers to a greater degree than could be accounted for by the reduction in $[Ca^{2+}]$ alone.

Collectively these data demonstrate that the inhibition of calcium levels in the buffers is not the only mechanism involved in the inhibition of T cell function by the concentrates.

Reduction of free calcium levels in the medium would not be expected to reduce the levels of calcium mobilisation from intracellular stores in the cells, but this phenomenon was observed. This indicates that the FVIII products may have an ability to affect other very early events in T cell activation as well as calcium flux.

Calcium mobilisation and calcium flux are probably inhibited by a second component in the concentrates that is interfering with events that precede calcium flux. One possibility is interference with the second messenger systems by the concentrates. Preliminary experiments by Dr R Thorpe and Dr T Mire Sluis of the National Institute of Biological Standards, South Mimms, have shown that concentrates inhibit inositol phosphate turnover, thus confirming this hypothesis.

Activation of lymphocytes is via the CD3/TCR. This results in the generation of second messengers culminating in calcium mobilisation and influx. Production of DAG leads to translocation of PKC. Both these events lead to production of mRNA for IL2, IL2R and gamma interferon. Phorbol ester activates PKC directly, bypassing the second messenger and calcium mobilisation systems.

Hay and McEvoy (1989), demonstrated that concentrates can inhibit phorbol ester (TPA) stimulated PBMC, indicating that the PKC pathway was specifically inhibited.

Mills *et al* (1985), state that there are two steps in the activation of T cells. The first step leads to the synthesis

of IL2 and is associated with, and dependent upon calcium. The second step is the expression of IL2-R, this does not require calcium and is produced by an alternative pathway, perhaps the PKC pathway. Conversely, Gelfand *et al* (1986), when stimulating PBL with calcium ionophore achieved IL2-R (CD25) expression without detectable production of IL2. Therefore mRNA for IL2-R is upregulated by either the IP_3/calcium or the PKC pathways. Conversely IL2 may require both signals simultaneously.

It is proposed that the concentrates could be inhibiting IL2 production and IL2-R expression from both sides of the CD3/TCR activation pathway - inhibition of calcium flux by reducing extracelluar calcium and by inhibiting second messenger systems; and inhibition of PKC translocation. This would in part, explain why reduction in calcium flux does not correlate with inhibition of CD25 expression, as stimulation with PHA may bypass calcium primary signals and directly stimulate PKC.

There was a very complex relationship between HLA-DR expression and the calcium parameters determined in the study. Replacement of calcium levels in the medium reduced the inhibition of HLA-DR expression by the concentrates, and addition of 2mM Ca^{2+} to normal cultures increased its expression. In contrast, there was an inverse relationship with inhibition of HLA-DR expression and inhibition of calcium flux. These paradoxical observations may indicate calcium is directly involved in regulation of HLA-DR expression.

Although activation of T cells is inhibited by FVIII

concentrates, we have demonstrated that the switching of the cells from virgin to memory cells is not (data not shown). This probably means that a 'slowing down' or 'delay' of the activation response is occurring. In addition, intra-cellular calcium levels in the presence of FVIII concentrates, following simulation by PHA, eventually normalised, but this was delayed by over 30 minutes. Perhaps this delay in primary activation signals (second messenger signals and calcium) cascades through the activation processes delaying gamma interferon and interleukin 2 production; both of which have an obligatory requirement for both calcium and PKC translocation. In turn, IL2-R expression would be delayed by reduction of the autocrine effect of IL2 on its receptor. This would lead to reduced activation and adhesion molecule expression, resulting in reduced cell contact and thus reduced proliferation. Therefore in such a complex pathway the effect of slowing down the primary signal, would serve to conceal the relationships of cause and effect.

7.4 IMPORTANCE OF MONOCYTES IN THE INHIBITION OF T CELL FUNCTION BY CONCENTRATES

Functional defects have been described in monocytes exposed to FVIII both *in vivo* (Mannhalter *et al*, 1986, Stasi *et al*, 1987, Roy *et al*, 1988, Pasi& Hill, 1990) and *in vitro* (Eibl *et al*, 1987, Mannhalter *et al*, 1988).

In vitro, therapeutic concentrations of FVIII were shown to down regulate Fc receptor and reduce monocyte effector functions, this was unrelated to product purity or heat treatment and was shown to be due to the large molecular weight component (>1270KDa) which corresponded to immune

complexes (Eibl *et al*, 1987, Mannhalter *et al*, 1988). Stimulation of PBL by PHA cultures is dependent upon perturbation of the CD3/TCR but activation of T cells via this pathway also requires accessory cell function in the form of IL1. If *in vitro* inhibition of monocyte function was an important factor in concentrate inhibition of T cell function *in vitro,* then the addition of IL1 would abrogate inhibition by the concentrates. Hay & McEvoy (1989), however, has shown that addition of IL1 to the cultures does not reduce inhibition. Activation of T cell with phorbol ester bypasses the CD3/TCR signals and therefore bypasses the requirement for IL1, however, this mechanism of activation is also inhibited in the presence of concentrate (Hay & McEvoy 1989). Finally Jurkat cells do not require IL1 for their activation processes and they too are inhibited in the presence of concentrates (Lederman *et al,* 1986, Thorpe *et al*, 1989). This data demonstrates that the inhibition of T cell activation by concentrates is not a result of the observed monocyte dysfunction.

It should be noted that second messenger systems/calcium flux/PKC translocation pathways are relevant activation pathways in all cells. FVIII concentrates have been demonstrated to inhibit these three pathways in T cells. Therefore it is probable that this inhibition is occurring in monocytes *in vitro* also.

7.5 WHAT IS CAUSING INHIBITION OF T CELL FUNCTION?

Inhibition of T cell function by FVIII concentrates is due to several interrelated mechanisms including:

- inhibition of second messenger (inositol phosphate pathways.

- reduction in extracelluar calcium levels.

- inhibition of PKC pathway.

The protein components of the concentrates may be causing inhibition but the buffers themselves are equally as inhibitory. It has been demonstrated that equivalent concentrations of BSA did not cause inhibition of T cell function (Lederman *et al*, 1986), and that fibrin (the major contaminating protein), fibrinogen and IgG are not inhibitory (McDonald *et al*, 1985, Thorpe *et al*, 1989). Other plasma proteins have been demonstrated to inhibit T cell function *in vitro*, these include certain lipoproteins (Curtiss and Edgington, 1976) and C-reactive protein (Mortensen *et al*, 1975). It is possible that similar inhibitory or immune modulating proteins may be present in the products, we have ruled out sIL2-R but there are many other possibilities. The presence of live virus (in those concentrates not sufficiently heat treated) may also influence T cell function *in vitro*.

It is possible that heat treatment of the concentrates may be degrading both protein and virus to release inhibitory components. Peptides of HIV are immunomodulating (Reiher *et al*, 1986, Weig ent *et al*, 1986, Reuben *et al*, 1986, Chanh *et*

al, 1987, Hofmann *et al*, 1989), this may also be true for peptides of HCV and HBV. It has been demonstrated that peptides of fibrin and fibrinogen can inhibit T cell blastogenesis and proliferation (Plow *et al*, 1982), and that the mechanisms of inhibition are directed at amino acid transport and protein synthesis, suggesting that inhibition of both early and late events of lymphocyte responses may occur. Following heat treatment, however, the products are fractionated to remove small peptide compounds so it seems unlikely that this is occurring.

7.6 RELEVANCE OF THE IN VITRO STUDIES

Inhibition of T cell function by the FVIII concentrates was only seen at supra-therapeutic levels (>2u/ml). At levels below this inhibition was minimal or absent. The more inhibitory FVIII concentrates - Alpha Profilate - are no longer available and those products regularly used in this centre - BPL 8Y and Armour Monoclate, were not inhibitory at therapeutic levels (1u/ml) *in vitro*. Although BPL 9A (NHS FIX) was the most inhibitory product in the *in vitro* studies, its mechanisms of inhibition appeared to be different to the FVIII products. Our *in vivo* studies suggest that there is no difference in the immunomodifying effects between these products however.

It is important to assess if these abnormalities are clinically significant and not an *in vitro* artifact. *In vitro* experiments do not relate to the complexities of cell to cell interactions or microenviroments. If the inhibitory components are purely buffer related, normal homeostasis will be expected to correct the presence of the buffers immediately

upon infusion. If the inhibition is related to proteins in the concentrates, high concentration of the components seen in the *in vitro* experiments will not be attained in the blood. There is the possibility that the proteins may be concentrated in the lymph nodes, for example, in which case their immunoregulatory capacity will be enhanced.

There is little clinical evidence to substantiate the hypothesis that the concentrates are inhibitory for T cell immune function *in vivo*. There are very few reputable *in vivo* studies, post heat treatment, that compare the effects of concentrate infusion upon the function of T cells. To test whether the inhibition seen *in vitro* is of any clinical significance *in vivo*, and to assess if previous reports of immune modulation are related to the infusion of non-HIV virus, we are currently studying T cell function in HIV-ve patients pre and post infusion with heat-treated FVIII concentrate, by measuring expression of HLA-DR, CD25 and IL2 production. Age and viral infection, both important contributory parameters to T cell *ex vivo* function, can be then be assessed properly.

In conclusion, T cell inhibition by clotting factor concentrates *in vitro* is probably a laboratory artefact and is unlikely to cause immune disturbances *in vivo*. We have not observed lymphocyte activation in our haemophilic patients nor have we seen inhibition related to concentrate infusion. Many of the studies of immune abnormalities in these patients were performed before the introduction of heat treated products in which hepatic viruses were not neutralised. The minor immune abnormalities that do exist are probably related to concentrate-induced monocyte dysfunction.

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9. APPENDIX I

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REAGENTS AND MATERIALS

AMMONIUM SULPHATE PRECIPITATION

saturated ammonium sulphate (prepared at room temperature)

50% ammonium sulphate (w/v)

10mM Tris-HCL pH 7.5

PROTEIN A SEPHAROSE PURIFICATION

Phosphate buffered saline (PBS) pH 7.4 (Mercia Diagnostics, UK)

Protein A gel: 1.5mg Protein A sepharose CL-48 (Pharmacia, Milton Keynes) was swelled at 4°C overnight in PBS with 100mM sodium azide producing 5.5ml gel.

Phosphate buffer pH 8.1

-100mM disodium hydrogen orthophosphate

-100mM sodium dihydrogen orthophosphate

1M Tris-HCl buffer pH 9.0

3M Potassium thiocyanate

Buffer Regime Method 1

Binding Buffer: Phosphate buffer pH 8.1

Elution Buffer: Citrate buffer pH 5.5, 4.5, 3.5.

-100mM citric acid

-100mM disodium hydrogen orthophosphate

Regeneration buffer: Citrate buffer pH 3.0

Buffer Regime Method 2

Binding buffer: 1.5M glycine, 3M sodium chloride, adjusted to pH 8.9 using 5M sodium hydroxide.

Elution buffer: 100mM citric acid adjusted to pH 6.0, 5.0, 4.0 using 5M sodium hydroxide.

Regeneration buffer: 100mM citric acid adjusted to pH 3.0 using 5M sodium hydroxide.

{buffers made with sterile, filtered distilled water to prevent microbial contamination, optional addition of 0.02% sodium azide (w/v) to buffers}

DEAE ION EXCHANGE CHROMATOGRAPHY

DE52 Cellulose (Waterman, Biosystems Ltd, Kent)

10mM and 100mM Tris-HCL pH 8.0

300mM and 1M NaCl

(with 0.02% sodium azide (w/v))

CONJUGATION OF ANTIBODIES

Carbonate buffer pH 9.5

-80mM anhydrous sodium carbonate

-200mM sodium hydrogen carbonate

10% sodium azide

100mM sodium bicarbonate pH 8.4

Sephadex G-25 gel: 50ml of preswollen G-25 sephadex (Pharmacia) was washed, by filtering, under pressure, with 1l distilled water followed by PBS.

100mM sodium bicarbonate pH 8.4

PBS pH 7.4 with 0.02% sodium azide (w/v)

FLUORESCENT LABELLING OF CELLS

PBS-A -PBS with 0.2% sodium azide (w/v) and 0.2% bovine serum albumin (w/v) (Sigma, Poole)

8% formaldehyde in PBS (v/v)

WHOLE BLOOD METHOD

Ammonium chloride solution

-0.8% ammonium chloride (w/v)

-10mM sodium hydrogen carbonate

-116nM NaEDTA

Trypan Blue solution

-0.2% trypan blue (w/v)

-4.25% sodium chloride (w/v)

-prepare by mixing trypan blue 4:1 with sodium chloride solution.

DETERMINATION OF CYTOPLASMIC FREE CALCIUM

HEPES buffer pH 7.6

-145mM NaCl, 5mM KCl, 0.5mM $MgSO_4$, 1mM NaH_2PO_4, 10mM hepes (Sigma, Poole, Dorset), 10mM glucose.

-1mM calcium was added to each individual test as required.

All reagents, unless specified, supplied by BDH Ltd, Poole, Dorset.

10. Appendix II

Summary

Plasma derived, intermediate purity concentrates have, until recently, been the treatment of choice for the management of Haemophilia. These concentrates contain less than 1% factor VIII (FVIII) in a milieu of plasma proteins. There are contradictory reports that repeated exposure to the contaminants in the concentrates induces immune abnormalities. With the advent of human immunodeficiency virus (HIV) testing in the mid 1980's it became clear that the immune defects in the patients were attributable to infection with HIV. Since then, however, reports of immune abnormalities in HIV sero-negative (HIV-ve) haemophiliacs have persisted. These abnormalities were thought to be mainly of a suppressive nature: decreases in CD4+ve lymphocyte numbers (Carr *et al*, 1984, Ludlam *et al*, 1985); reduction in T and B cell responses to mitogen (Smolen *et al*, 1985, Horsburgh *et al*, 1986, Sullivan *et al*, 1986, Mahir *et al*, 1988, Ruffault *et al*, 1988); reduced skin test reactions (Brettler *et al*, 1986, Madhok *et al*, 1986, Sullivan *et al*, 1986, McIvor *et al*, 1987); and reduced monocyte function *in vivo* (Pasi and Hill, 1990). However, there have also been reports of immune activation: primed T cells in HIV-ve haemophiliacs (Matheson *et al*, 1986a, 1987) and increased serum IgG levels and spontaneous IgG production (Biagotti *et al*, 1986, Gorski *et al*, 1986, Kekow *et al*, 1986, Ragni *et al*, 1987). This study tested the hypothesis that some of the apparent inhibition of the immune system reported in these patients could be the result of activation of their lymphocytes. Refractory immune states in graft versus host disease (Cleveland *et al*, 1988) and systemic lupus erythematosus (Preble *et al*, 1983) resulting from activation *in vivo* have been reported. To determine whether infusion of FVIII concentrates was causing a direct activation of lymphocytes, *in vivo* markers of T and B cell activation were analysed in a group of haemophilia patients receiving intermediate purity FVIII/FIX

concentrates. Thirty three HIV-ve patients were compared with 31 HIV sero-positive (HIV+ve) patients matched for age, sex and amount of treatment recieved. A group of 22 age matched, normal healthy males was used as a control.

In the HIV-ve patient group the activation status of the T and B lymphocytes was no different from the controls, as determined by the expression of activation-specific antigens on the cell surface by two-colour flow cytometry (HLA-DR, CD25 and CD71 on T cells and CD38 and CD71 on B cells). Contrary to several earlier reports, serum Ig; a marker of chronic B cell activation, was also normal in the HIV negative patients, as were two cell surface markers of chronic B and T cell stimulation; the PCA-1 antigen and CD45RO expression respectively. In contrast, serum levels of soluble interleukin 2 receptor (sIL2R), used to assess the activation status of lymphocytes sequestered in the tissues, were significantly elevated in the HIV-ve patients compared to controls ($p<0.05$). Non-parametric statistical analysis found that the increases in sIL2R in these patients was unrelated to FVIII usage; either the amount, type, frequency of infusion, or time since last treated. Measurement of serum aspartate transaminase (AST), however, showed a marked association between high levels of AST on the day of testing and raised levels of sIL2R ($p=0.01$). The HIV+ve haemophiliacs did have activated, circulating lymphocytes consistent with observations in other HIV+ve groups (Salazar-Gonzalez *et al*, 1985, Gupta, 1986, Sullivan *et al*, 1986, Nashida *et al*, 1989). Their T cells had increased HLA-DR expression ($p<0.0001$), predominantly on CD8+ve lymphocytes ($p=0.004$), whilst other markers of T cell activation were unaffected. CD45RO a marker of memory T cells, was, however, elevated on CD8+ve cells ($p=0.006$). The activation status of the HIV+ve patients was not influenced by the type or amount of FVIII concentrate administered, nor, with

the exception of CD38 expression on B cells, was there a correlation with severity of disease. sIL2R was also raised in this group compared to the controls ($p<0.02$) but, was not statistically significantly different from that in the HIV-ve group. As with the HIV-ve patients, sIL2R levels in the HIV+ve patients were unrelated to FVIII infusion, nor were they related to paramateres of HIV disease (CD4 count, duration of seropositivity and disease status), but they were associated with persistant elevation of serum AST, an indication of ongoing, active liver disease in these patients ($p<0.05$).

In conclusion, lymphocyte activation observed in haemophiliacs was not affected by FVIII infusion but was related to ongoing infection with virus; HIV and probably hepatitis C virus (HCV). In the HIV-ve patients sIL2R was the only activation marker to be raised and this may reflect a normal immune response to viral infection. Since >80% of the patients have been infected with HCV, and since sIL2R levels correlated with serum AST levels, HCV is principle candidate for invoking this response. The discrepancy in the serum Ig levels between this study and earlier reports may substantiate this hypothesis since many such studies were carried out on patients receiving incompletely sterilised concentrate, who were then subjected to repeated challenge with live hepatitis virus.

The HIV+ve patients showed an 'abortive' pattern of T cell activation, characterisitic of other HIV+ve groups. This phenomenon of elevated HLA-DR expression but unchanged expression of CD25 on T cells of HIV+ve patients has been attributed to either a subset of HLA-DR+ve/CD25-ve T cells (Pantaleo *et al*, 1990); direct regulation of HLA-DR by HIV (Altmann *et al*, 1987); and increases in gamma-interferon levels from activated T cells (Fuchs *et al*, 1989). Raised levels of sIL2R have been attributed to activated B cells (Lang *et al*, 1988) and macrophages (Kloster *et al*, 1987), and activation of B cells has been linked

directly to HIV (Pahwa et al, 1989, Amadori and Chieco-Bianchi, 1990) and to lymphokines, in particular IL6, from activated macrophages (Amadori *et al*, 1989, Breen *et al*, 1990).

The concept that FVIII concentrates could suppress the immune response, in a large part, rests on observations that high concentrations of these products can cause partial inhibition of T cell function *in vitro* (Froebel *et al*, 1983, McDonald *et al*, 1985, Wang *et al*, 1985, Lederman *et al*, 1986). The second part of the study was an investigation of the mechanism of the inhibition of T cell function *in vitro* by analysing early events in T cell activation. A number of FVIII and FIX concentrates were tested and all inhibited the expression of CD25 and HLA-DR antigens. The degree of inhibition varied between different types of concentrate and between batches of the same concentrate. High purity products (monoclonal purified FVIII and FIX), however, had no effect. The inhibition affected the CD4+ve and CD8+ve T cell subsets to a similar extent and was similar for PHA- and PWM-stimulated cultures. CD25 and HLA-DR expression was inhibited by the concentrates soon after initial appearance on the cell surface and expression was significantly reduced over the 96 hour culture period. This indicated that the inhibition was not restricted to a subset of T cells, and that it occurred at the pre-transcriptional or transcriptional level.

Calcium flux studies in Jurkat cells demonstrated that the inhibitory concentrates reduced calcium mobilisation and influx in PHA or anti-CD3 stimulated lymphocytes. In the presence of FVIII products the calcium concentration in the culture medium was reduced to below physiological levels, attributed to the presence of chelators in the products. Indeed, the buffers of the concentrates were inhibitory themselves and dialysis of inhibitory concentrates partially abrogated their inhibitory effects. Reconstitution of the

calcium levels in the culture system partially reversed the inhibition. The free calcium levels in medium containing the concentrates correlated with the inhibition of the expression of HLA-DR and CD25 ($r=-0.5$ and $r=-0.6$ respectively) although not with inhibition of calcium flux ($r<0.3$). Inhibition of calcium mobilisation suggests that events preceding calcium flux eg. signal transduction, may also be contributing to their inhibitory effects.

The inhibition of T cell function occurred only in the presence of supra-therapeutic concentrations of concentrates. This fact, together with the expected restoration of physiological calcium levels *in vivo* by normal homeostatic mechanisms suggests the observed reduction in calcium levels in the culture media is only effective *in vitro*.

To conclude, it is proposed that lymphocyte disturbances reported in haemophiliacs are related to infection by virus (either HIV or HCV), whilst the inhibition of T cell function *in vitro* is largely a laboratory artefact.