HAEMOPHILIA, HIV AND THE IMMUNE RESPONSE

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ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
HIV	Human Immunodeficiency Virus
HIV^+	Presence of antibody to HIV
HIV^-	Absence of antibody to HIV
T_4 or T_h	T cell expressing CD4 molecule, associated with helper function
T_8 or $T_{c/s}$	T cell expressing CD8 molecule, associated with cytotoxic/suppressor function
NK	Natural Killer
β_2M	β_2Microglobulin
IL-2	Interleukin-2
ELISA	Enzyme Linked Immunoabsorbant Assay
DNCB	2,4 Dinitrochlorobenzene
OPD	O-Phenylenediamine
IgG)	G
IgM) Immunoglobulin	M
IgA)	A
IgD)	D
PHA	Phytohaemmaglutinin
CON A	Conconavalin A
PWM	Pokeweed Mitogen
PPD	Purified Protein Derivative (from Mycobacterium tuberculosis)
STAPH. A.	Staphylococcus A. (Cowan's strain)
SI	Stimulation Index
FCS	Foetal Calf Serum
PUHS	Pooled Human Serum
PBS	Phosphate Buffered Saline
RPMI	Rosewell Park Memorial Institute 1640 Medium
"H"	"Heavy Users"
"L"	"Light Users"
"N"	Never Treated
CON	Controls
+	HIV^+

STATISTICAL ANALYSIS

Comparisons between groups were made with Wilcoxon's ranking test for unpaired data (Mann-Whitney test). Intra-group differences were detected by Wilcoxon's signed rank test for paired data. Associations between variables were assessed with the Spearman rank correlation coefficient (Seigel, 1956).

DECLARATION

The detailed planning of the work, its day to day evolution and its execution were my individual responsibility, and, except where indicated, the work was entirely personally performed.

Signed: .....

J. Alastair Gracie.

PUBLICATIONS

Some of the work presented herein has previously been presented in the following publications:-

1) Madhok, R., Gracie, A., Lowe, G.D.O. Lowe et al, 1986.

"Impaired Cell Medicated immunity in haemophilia in the absence of infection with human immunodeficiency virus".

British Medical Journal, 293, 978 - 980.

2) Madhok, R., Gracie, J.A., Lowe, G.D.O. and Forbes, C.D., 1986.

"Lack of HIV transmission by casual contact".

Lancet ii, 863.

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To Carol, and my Mum and Dad.

SUMMARY

This thesis reports the results on a variety of immunological studies on a cohort of the haemophiliac population attending the Haemophilia Centre at Glasgow Royal Infirmary.

The studies were carried out, looking for any abnormality which could be associated either with factor consumption or HIV infection.

A small percentage (around 8%) of the haemophiliac population show evidence of HIV infection as detected by the presence of antibodies to the virus. Skin testing showed an impaired cell mediated immunity *in vivo* which was related to the amount of concentrate used in therapy.

Various *in vitro* tests were carried out, the patients being grouped according to HIV antibody status or factor usage.

Lymphopenia was not present in any patient group and reduced T cell subset ratios were observed only in the HIV^+. This was due to a decrease in absolute numbers of T_4 cells. The *in vitro* response to mitogens and antigen was reduced but only in the HIV^+ group.

Assays were developed to measure Interleukin-2 and immunoglobulin production *in vitro*. HIV^+ but not HIV^- patients produced significantly less IL-2 than controls. Serum IgG, IgM and IgA were raised in all treated patient groups, especially those HIV^+. *In vitro* studies show that while HIV^- patients have normal production of immunoglobulins spontaneously, HIV^+ patients show increased levels. Neither group can be stimulated to produce levels as high as controls. β_2-Microglobulin levels were raised in all haemophiliacs, with HIV^+ being higher than HIV^-.

Finally, the effect of addition of concentrate or disrupted HIV on various *in vitro* studies was examined. In general, addition of these inhibited the response of normals.

CHAPTER ONE

HAEMOPHILIA - A REVIEW

1.1 THE HISTORY OF HAEMOPHILIA

1.1.1 The Early References

Genetically determined bleeding disorders are probably as old as time but they were not reported by the Egyptian, Roman and Greek Physicians. Written reference to what may have been human haemophilia can be traced back to the 2nd Century A.D.. Babylonian Talmud Rabbi Judah the Patriarch made a ruling which exempted a woman's third male child from being circumcised if the two previous brothers had died from excessive bleeding after circumcision (Sussman, 1967). The Rabbi's decree recognised the familial and sex-linked nature of the bleeding defect (Rosner, 1969). In the 10th century males of a certain village who bled to death from trivial wounds were described by Khalaf ibn Abbas known as Albucasis. Thereafter, down the years there are other scattered references in the scientific literature to the deaths of males who bled to death after surgery or from trivial wounds. In the older literature, various names have been used to describe the disorder e.g. haemorrhaphilia, haematophilia, haemorrhoea. The word haemophilia is derived from two Greek words "haima" - blood and "philein" - to love and its first use is attributed to Schönlein (Virchow, 1854) although the first written publication using the word "haemophilia" was in 1828 with the publication of a thesis entitled "Die Hämophilie" by Dr.Friedrich Hopff (1828), a student of Schönlein. Several years elapsed before the medical profession used this term only in the specific way that it is today. It is now realised that a variety of deficient factors may produce the same clinical picture. The commonest disease is haemophilia A or classic haemophilia and is due to deficient activity of antihaemophilic factor (AHF or Factor VIII). This accounts for 90% of bleeding defects. In their

monumental review of 1912, Bulloch and Fildes (1912) cite references back to 1519 as they carry out a scientific analysis of inheritance on all published work to that time.

The first probable case of haemophilia in America was recorded in the anonymous Obituary of Izac Zoll which appeared in the Salem (Mass) Gazette of March 22nd, 1791 (Anon,1962). The Obituary describes his death from:

> "a slight cut in one of his feet, with an axe. From the time of his receiving the wound till he expired, no method could be devised to stop the bleeding"

The Obituary goes on to describe the deceased's five brothers who had all died of exsanguination following minor trauma, e.g scratch with a comb, prick with a thorn or needle. It is of great interest that the boy's father, Henry Zoll, was twice married and only the children of his first wife were affected by the bleeding tendency.

1.1.2 Inheritance and Distribution of Haemophilia

The first significant observations on the inheritance of haemophilia are attributed to John C. Otto. In 1803 he reported (Otto, 1803) the cases of families in New England and Maryland with bleeding disorders. He coined the word "bleeders" and noted that only males in a family were bleeders but females, while exempt, could transmit the disease to their sons. Hay (1813) made what is probably the most important contribution on our knowledge of inheritance when he reported on an Ipswich, Massachusetts family who have subsequently been followed down to modern times (McKusick, 1962).

Hay made the observation:

> "Children of bleeders are never subject to this disposition but their grandsons by their daughters"

The first European notices of the disease are case reports by the Editor of Sammlung auserlesener Abhandlungen (1805) and Consbruch (1810) followed by careful clinical and post-mortem observations by Blagden (1817) and Wilson (1819). It is of interest that in 1816 Meckel (Meckel, 1816) postulated a coagulation defect in haemophiliac blood but his suggestion was ignored for eighty years. In 1820 Nasse (1820) published a collection of all cases of that time and put forward his formulation on the inheritance of haemophilia in what has become known as Nasse's Law which stated that haemophilia occurs only in males and is transmitted by unaffected females to their sons.

The first report of haemophilia in the Scottish literature is probably that of Theodore Davis, a surgeon from Nailsea in Bristol. He detailed (Davis, 1826) a family with two bleeder sons, both of whom died of haemorrhage although several daughters were unaffected. None of the maternal uncles had reached adulthood and the maternal aunts, though healthy, had transmitted the disease to their sons, but not to their daughters. One of the sons who died was noted to have swollen joints and severe rheumatic pain and this is probably one of the earliest descriptions of joint involvement in haemophilia. This report was followed by one from Alexander Murray (1826), a surgeon from Alford. He described the Coutt's family from Cobbleseat in the Parish of Keig in Aberdeenshire. Four sons were affected by recurrent oral and nasal haemorrhage, bruising and excessive bleeding from trivial cuts resulting in the deaths of three of them.

Probably the most famous of haemophiliacs are the several offspring of Queen Victoria. Without previous family history and after four normal children, Queen Victoria in 1853 gave birth to a haemophiliac son, Leopold, Duke of Albany (Rosner, 1969). He was severely affected by haemophilia, suffering recurrent life threatening

bleeds and developing arthropathy before dying at the age of 30 of cerebral haemorrhage after a fall (Anon. 1884). His only daughter was a carrier who transmitted the disease to her son, who also died of cerebral haemorrhage. Two of Victoria's daughters, Alice and Beatrice, proved to be carriers when they produced both haemophiliac sons and carrier daughters. Through the marriage of Victoria's affected grandchildren the disease was transmitted to various Royal Houses of Europe including Prussia, Russia, and Spain. The dramatic social and political consequences of the dissemination of this rare disease through the Royal European Households has been of great interest to historians. The desperate attempts to treat Victoria's great-grandson, the Tsarovich, were closely intertwined with the fall of the Tsars and the Russian Revolution. This Royal connection also heightened public interest but probably more importantly that of the medical profession. Large numbers of cases were soon reported and numerous scientific studies were commenced.

In 1855 Grandidier (1855) collected all cases published up to that time and found that there were 452 males and 32 females. However, these female cases contained ones which were probably not due to haemophilia, e.g. umbilical haemorrhage.

Legg provided his authoritative account in 1872 (Legg, 1872) in "Treatise on Haemophilia" and did as much as anyone to influence the use of the term "haemophilic" for only a heriditary and congenital bleeding disorder in males, often with swelling of the joints. However, it was not until 1890 that the involvement of joints, probably the most characteristic symptom of haemophilia, was described in detail by König (1890). Historically, the disease ranks second only to red-green colour blindness in the chronology of recognition of sex-linked abnormalities. There are five mating

combinations in sex-linked recessive inheritance. Otto (1803) described carrier female x normal male and Hay (1813) normal female x bleeder male. Brinkhous and Graham (1950) showed that mating bleeder dogs with carrier bitches produced haemophilic dogs in the expected proportions. Thus with the availability of bleeder females it was possible to test the remaining two combinations, i.e. bleeder female x normal male and bleeder female x bleeder male and results were exactly as predicted by Mendalian genetics. (Brinkhous, Graham, Penick et al, 1951). So, some 150 years after the description by Otto the pattern of sex-linked inheritance was fully confirmed. As haemophilia is sex-linked recessive an affected boy will acquire the disease from his carrier mother. The sons of a haemophiliac do not inherit his X-chromosome and are unaffected but all the haemophiliac's daughters will be carriers since they do inherit the affected X-chromosome. In the very rare instance of marriage between a haemophiliac male and a carrier female, the daughters have an equal chance of being haemophilic by inheriting the abnormal allele from each parent. At least four such authentic cases have been reported (Merskey 1951).

Classical haemophilia affects about 1 in 10,000 of the British population although only 1 in 40,000 is severely affected. A similar figure is found in Europe and the United States. The disease has now been described in every racial group (Prentice and Ratnoff, 1967) but is uncommon in some groups, e.g. the American Negro (Lewis, Didisheim, Ferguson et al, 1963) and East African Negroes (Forbes, Mackay and Khan, 1966). The severity of the bleeding tendency relates closely the plasma concentration of Factor VIII or Factor IX coagulant activity (Nilsson, Blomback and Ramgren, 1961) and the clinical classification of the severity is as follows:-

Less than 1% - severe

1-4% - moderate

5-30% - mild

1.1.3 The Coagulation System and the Defect in Haemophilia

The end stage of the coagulation process is the production of an insoluble fibrin clot. Modern understanding of blood coagulation has been based on the hypotheses independently formulated by Ratnoff (Davie and Ratnoff, 1964) and Macfarlane (1964) that the system functions as a cascade or waterfall, each enzyme releasing the active factor from its inert precursor in sequence.

The coagulation sequence functions as three interacting parts. Intrinsic activity is generated by exposure to a foreign surface leading to activation of Factor X on the platelet surface by a complex of Factor IXa, Factor VIII and calcium. Factor X is also activated through the extrinsic system by Factor VII in the presence of tissue thromboplastin. In the presence of Factor V and calcium, Factor Xa acts on prothrombin to form thrombin, which cleaves fibrinogen to form fibrin. The cascade is shown in Figure 1.1.

In the 19th Century there was still no understanding as to the cause of the defect. Some writers thought bleeding was due to a vascular abnormality. Wright (1893) discovered that the clotting time of the patients blood was prolonged and Addis (1911) observed that the addition of normal blood could correct the clotting defect of haemophiliac blood. In the first few years of this century the diagnosis of haemophilia depended on clinical and family history plus the finding of a prolongation of whole blood clotting time (Liston, 1893 ; Wright, 1893).

The first major biochemical advance occurred in 1935 when

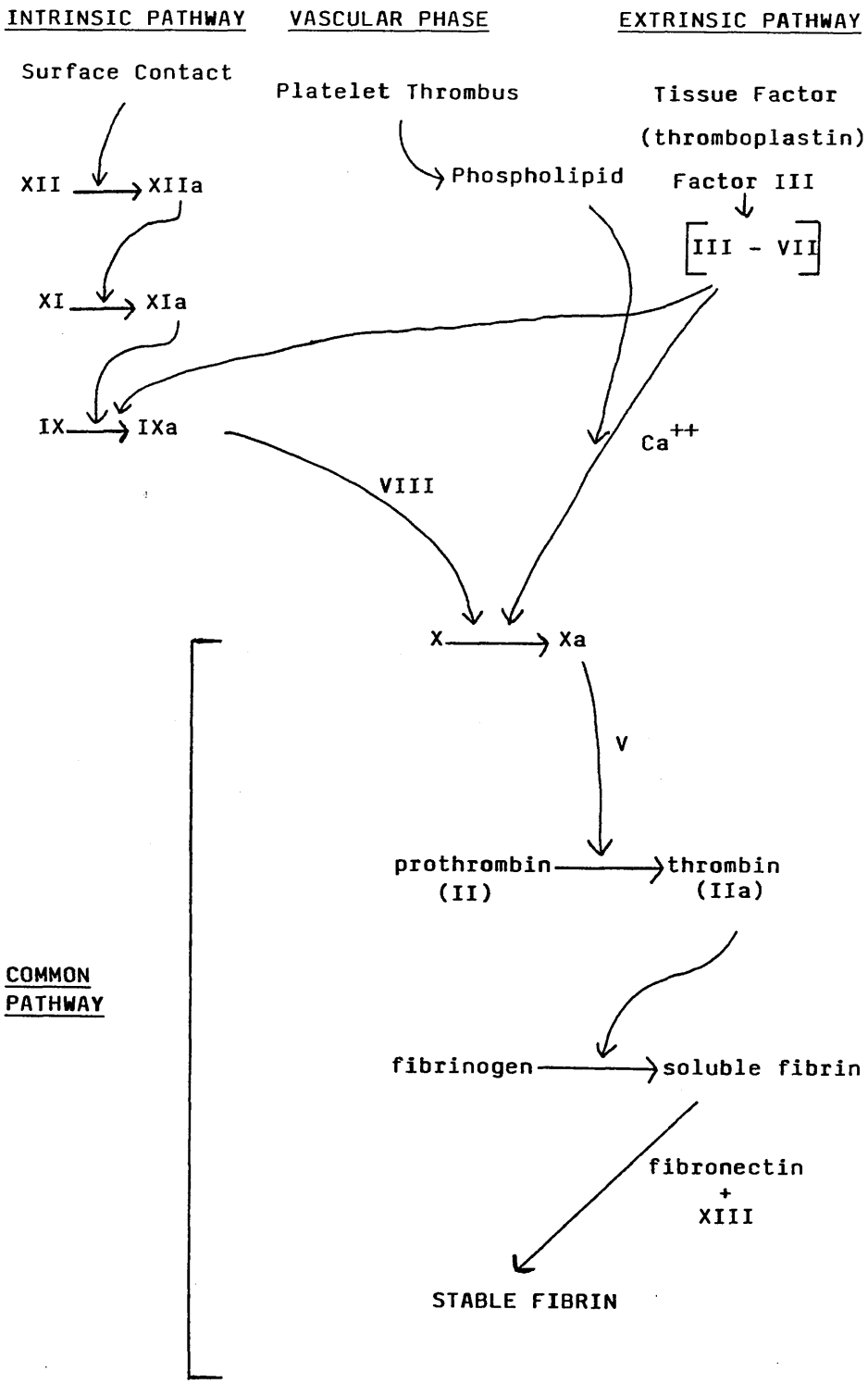


Figure 1.1: The Coagulation Cascade

Bendien and Van Creveld (1935) then Patek and Taylor (1937) found that platelet free plasma, precipitated with water at pH of 5.3 - 5.8, yielded a substance which was capable of correcting the clotting abnormality. With other colleagues, Taylor (Lewis, Tagnon, Davidson et al, 1946) later called this fraction "anti-haemophiliac globulin" which we now know as Factor VIII. Pavlovsky (1947) made the important finding that blood from one haemophiliac could correct the clotting defect in another. This started the idea that there could be more than one disorder collectively known as haemophilia. In vivo and in vitro observation by Schulman and Smith (1952) and Koller (1954) as well as the development of tests of thromboplastin generation (Biggs and Douglas, 1953) confirmed that there were two separate groups of patients. Biggs (1952) and colleagues in Oxford and Aggeler (1952) in San Francisco, and Schulman and Smith (1952) in New York all described a condition clinically and genetically similar to haemophilia but one which could be corrected by infusion of haemophiliac plasma. This new disease, first described by the Oxford group in the 1952 Christmas edition of the British Medical Journal, is associated with lack of a factor found in serum called Christmas factor (named after the first patient) or Factor IX and is now also known as haemophilia B.

As knowledge increased new tests were devised which led to bleeding disorders other than the haemophilias being described, e.g. Factor V deficiency (Owren, 1947).

Ratnoff and colleagues (Zimmerman, Ratnoff and Powell, 1971) described immunological experiments where they prepared rabbit antiserum to human Factor VIII. When they tested the serum on normal plasma they obtained a single precipitin line and demonstrated the neutralisation of Factor VIII activity by the antiserum. However,

when they tested the antiserum against haemophiliac's plasma they still found a single precipitin line indicating presence of the Factor VIII protein antigen. This work has been widely confirmed, and so haemophilia had to be redefined as a sex-linked, recessive coagulation disorder in which the biological activity of Factor VIII (VIIIC) is reduced because the Factor VIII molecule, although present in normal amounts antigenically, is functionally impaired. The formation of defective molecules of Factor VIII is probably due to the presence of an abnormal recessive allele at the gene locus on the X-chromosome controlling synthesis of this factor. Therefore, molecules possessing this one abnormal X-chromosome will produce a protein, antigenically similar and in an equal amount to normal Factor VIII but with deficient activity (Zimmerman et al, 1971). The loci for Christmas factor and Factor VIII are apparently far apart on the X-chromosome. It is thought that in Christmas disease there are two varieties, one in which there seems to be a complete absence of protein and another much rarer type in which the plasma contains Factor IX which is antigenically indistinguishable from normal Christmas factor (Hougie and Twomey, 1967) but has different activity. Cases have been recorded where both classic haemophilia and Christmas disease apparently co-exist (Soulier and Larrieu, 1953).

1.1.4 Treatment of Haemophilia

A history on the treatment of haemophilia can be divided into two easily identifiable sections: "early" and "modern" therapies.

In 1901 the United States Surgeon General's catalogue included for the first time a section on treatment of haemophilia. The list included such forms of treatment as inhalation of oxygen, administration of lime and even the use of gelatin. In 1926 the

next published list was even larger with remedies such as injection of calcium lactate or sodium citrate and even splenectomy. To this period belongs the Timperely treatment with a bromide extract of egg white (Timperely, Nash and Clark, 1936) which some haemophiliacs have believed had helped them. Most of these treatments could have been based on little more than guesswork.Their ineffectiveness is testified to by the extensive morbidity and early mortality recorded in detail by Carol Birch (1937) in her classic monograph which differed little from the natural history of the disease in the 19th Century (Grandidier, 1855) (Kerr, 1963). Probably the first treatment to be used on sound experimental result was the topical application of coagulant snake venom after work by Macfarlane (Macfarlane and Barnett 1934).

In 1840 was published one of, if not the most, historic and important papers on the treatment of haemophilia. Lane (1840) in Lancet describes the successful transfusion of blood. An 11 year old boy had bled for six days after a minor operation to correct a squint. He was given a direct transfusion of blood after which the bleeding stopped and the boy recovered. Also in this paper Lane cites the work of Wardrop who in 1835 described the cases of several people who had an inordinate disposition to bleed from slight injuries. He (Wardrop) attributed this disease to a deficiency in the coagulation power of the blood. This first case of successful transfusion came some eight years after being suggested by Schonlein (1832). The use of whole blood therapy was not rediscovered for almost sixty years (Ward 1905). Feissly (1924) and Payne and Steen (1929) then showed that the use of plasma was superior to whole blood and this treatment became standard practice for thirty years.

The key to modern treatment was suggested in 1938 by Macfarlane

when he concluded:

> "it is probable, therefore, that there is in normal blood a factor, at present unidentified that is essential for the rapid activation of prothrombin and that this factor is at fault in haemophilia."

This missing factor is now known as Factor VIII in haemophilia A and Factor IX in haemophilia B. Macfarlane had appreciated that by means of transfusion it was possible to treat a bleeding episode by temporarily replacing a missing essential component (Biggs, 1967).

Following the work of Cohn, human preparations of Factor VIII were developed in the 1950's in Britain (Kekwick and Wolf, 1957) in France (Soulier, Gobbi and Larrieu, 1957) and Sweden (Blombäck, Blombäck and Nilsson, 1958). At this time concentrates were also being produced from animal blood (Macfarlane, 1954).

Antihaemophiliac globulin (AHG) was being used to treat life threatening bleeds and to cover major surgery (Biggs, 1967) but the concept of preventative treatment for a bleed at an early stage, to stop crippling damage to joints and to maintain as normal a life for the haemophiliac, was as yet unformulated. This, however, was to change in 1965 when Professor Judith Pool reported (Pool and Shannon, 1965) that on slowly thawing frozen plasma much of the Factor VIII activity remained with the fibrinogen sludge which was slow to redissolve. This was called cryoprecipitate and could be spun down and so re-frozen for storage, and the supernatant plasma could be re-associated with the cells from the blood donation from which it was derived thus reconstituting the pint of blood for other use. With increasing availability of freeze-dried concentrates it became possible to start home-treatment whereby patients could treat themselves promptly by intravenous injections of Factor VIII

concentrates which they keep at home.

By 1980 most Haemophilia Centres in Britain had introduced home treatment. Forty-four percent of all haemophiliacs and 60% of those severely affected were being treated this way (Rizza and Spooner,1983). The increased availability of lyophilised concentrate was such that by 1980 only 14% of Factor VIII was given in form the cryoprecipitate (Rizza and Spooner, 1983).

1.2 TRANSFUSION ASSOCIATED HEPATITIS

As already mentioned one of the most severe problems affecting haemophiliacs is internal bleeding, particularly into muscles and joints, especially the knee and elbow. Bleeding episodes like these can leave the patient painfully crippled and immobile.

However, the treatment of haemophilia, i.e. the use of blood derived products has also led to problems for the patients. In 1943 Beeson reported seven cases of jaundice which all occurred after the transfusion of whole blood or plasma, and he therefore suggested that they were somehow transfusion associated (Beeson, 1943). Serum hepatitis was first described by Lurman in 1855 following an outbreak of jaundice in a Bremen shipyard where workers were vaccinated with human lymph against smallpox. One hundred and ninety-one out of 1,289 people became jaundiced while 500 other workers vaccinated with a different lot of lymph remained free from disease. There have been many other reports in the literature of outbreaks of jaundice after transfusion of blood or blood products (Propert, 1938)(Spurling, Shone and Vaughan, 1946). A major advance in the study of viral hepatitis occurred in 1967 with the discovery by Blumberg of the Australia antigen (Blumberg, Gerstley, Hungerford et al, 1967) which lead to the development of tests for hepatitis B virus antigens (HbsAg) and antibodies (HbsAb). The high risk of hepatitis B associated with intravenous use of cryoprecipitate, Factor VIII or Factor IX concentrate became well documented amongst haemophiliacs (Barber, Peterson, Schulmar et al, 1973) (Hoofnagle, Aronson and Roberts, 1975) (Gerety and Barker, 1976). Since 1972 it has been a legal requirement in the U.S.A. to test all blood donors for HbsAg. However, the overall incidence of hepatitis in recipiants did not decline, although

the proportion defined as hepatitis B dropped from 2.2% to 0.5% (Seeff 1981). It has been shown that 89% of post transfusion hepatitis in the United States was caused by an unknown virus other than hepatitis type A or B and this is known as Non-A, Non-B hepatitis (Alter, Purcell and Holland, 1975). In a retrospective study, Craske and colleagues (1978) showed that non-B hepatitis developed in 52 out of 417 patients receiving commercial Factor VIII preparations. Persistent elevations in liver enzymes have been observed in haemophiliacs (Mannucci, Capitanio, Del Ninno etal, 1975). Following these findings a study of liver biopsies in selected haemophiliac populations found presence of chronic active hepatitis, chronic persistent hepatitis or cirrhosis (Mannucci, Ronchi, Rota et al, 1978) (Spero, Lewis, Van Thiel et al, 1979). These findings along with a more recent study by Hay et al, (1985) which concludes:

> "It is anticipated that liver disease in haemophiliacs will become an increasing clinical problem in the future"

all support the suggestions made in The British Medical Journal in 1981 that:

> "in some cases early death from liver disease might prove to be the price paid by haemophiliacs for the improved quality of life afforded by the easy availability of clotting-factor concentrates"
>
> (B.M.J. Editorial, 1981).

1.3 ACQUIRED IMMUNE DEFICIENCY SYNDROME

1.3.1 The New Threat

Articles, letters and comments on the Acquired Immune Deficiency Syndrome (AIDS) have rarely been absent from either medical journals (Gracie, Froebel, Madhok et al, 1985) (Gracie, Lowe and Forbes, 1985) or the popular press since the first clinical descriptions of the disease in June, 1981. These first reports recorded by the Centre for Disease Control (CDC) (Gottleib, Groopman, Schanker et al,1981) detailed cases of five young homosexuals from Los Angeles who died of Pneumocystis carinii pneumonia. This was soon followed by reports (Hymes, Cheung and Greene et al, 1981) of the deaths of a further 26 homosexuals who had developed a rapidly progressive form of Kaposi's sarcoma. It was soon apparent that these cases represented the first reports of a new epidemic, one which medicine had not seen before, and one which was to have dramatic consequences, scientifically, medically and socially. Dysfunction of the patient's cell mediated immune system was soon recognised as the characteristic feature of the disease. Early cases occurred in homosexuals (Gottleib et al, 1981) and intravenous drug abusers (Gold, Thomas and Garrett, 1982). These reports were soon followed by other cases which clearly fell into well defined groups, namely haemophiliacs (MMWR 1982) (Poon, Landay, Prasthofer et al, 1983) receiving clotting factor concentrates and recipients of blood transfusions (Curran, Lawrence, Jaffe et al, 1984).

The occurrence of a unique clinical syndrome displaying such a definite pattern of occurrence strongly suggested that the cause was a transmissible infectious agent. Human Immunodeficiency Virus (HIV)

first isolated by Montagnier in Paris (Barré-Sinoussi, Chermann, Rey et al, 1983) called lymphadenopathy associated virus (LAV) and shortly afterwards by Gallo and co-workers (Popovic, Sarngadharan, Read et al, 1984) in the United States and termed human T cell lymphotropic virus type III, is now known to be the virus responsible for a variety of clinical syndromes ranging from a glandular fever-like illness to the full blown clinical picture of AIDS with opportunistic infections, tumours and nervous system involvement. Most of the early cases of AIDS could be accounted for by the above mentioned high risk groups with only rarely a case being reported in an individual who apparently had no risk factors (Chamberland, Castro, and Haverkos et al, 1984).

The occurrence of the disease in blood transfusion recipients and haemophiliacs was soon realised to be caused by a transmissable agent in contaminated blood products. Similarly, intravenous drug abusers were at risk because they shared needles, syringes and other equipment contaminated with blood. But how was the disease spread in homosexuals? It is known that some homosexuals are more promiscuous than heterosexuals having many more sexual partners ("fast lane"). Also, anal intercourse and the use of sexual "toys" lead to tears of the anal mucosa with the possibility of semen infected with HIV entering the receptive partner's blood (Shearer 1983). This has been confirmed by anti-sperm antibodies which have been found in the serum of some homosexuals (receptive partners) (Mavligit, Talpaz, Hsia et al, 1984) and sperm has been shown to contain HIV (Ho, Schooley and Rota, 1984).

The original strategy set up in the United States, for containment and control of the AIDS epidemic was based on the

suggestion that AIDS occurred only within the defined high risk groups and it would remain as such. On the whole, to date, this has held true with only about 5% of reported cases falling outwith these risk groups. Sexual contact is believed to be the only risk factor for 64% of reported cases in the United States (MMWR,1985). Of this figure, about 2% is due to heterosexual spread with a male to female ratio of 5.5:1. Most of these were due to male to female transmission although transmission in the opposite direction has occurred. It has been suggested (Sande, 1986) that the difference between the two sexes in the rate of transmission is due to the fact that there are more male i.v. drug abusers and bisexuals capable of transmitting the disease to females than there are infected women capable of transmitting to men. HIV has now been isolated from cervical secretions of people at risk from AIDS (Vogt, Witt, Craven et al, 1986) and HIV carriers (Wofsky, Cohen, Haver et al, 1986) and this could prove to be yet another body fluid capable of transmitting the disease. If this is so then female prostitutes could pose the biggest threat in the transmission from female to male and studies from the United States cite contact with prostitutes as a high risk factor (Van de Perre, Clumeck, Carael et al, 1985). This leaves a small percentage of cases which do not fit any of the recognised high risk groups (MMWR, 1985) (Sande, 1986).

Only relatively recently has AIDS been seriously considered as a threat to the heterosexual population (Gracie, Lowe and Forbes, 1986). The incidence of heterosexual transmission seen in the USA and Europe at the moment, is somewhat different from that being experienced in certain parts of Africa where there is evidence of widespread heterosexual transmission. In early 1985 at the International Congress on AIDS in Atlanta, USA, data from central Africa (Clumeck

et al, 1985) suggested that heterosexual transmission was of great importance in the development of AIDS. The first indirect piece of evidence was the sex ratio of AIDS victims in Zaire, being 1:1.1 (male to female) (Piot, Quinn, Taelman et al, 1984). This strongly suggested that homosexual contact was not the most important factor in the spread of the disease in Africa. In addition, there is little epidemiological evidence of homosexuality. This near balanced sex ratio would suggest that transmission could be either male to female or vice versa otherwise there would soon be an imbalance of the ratio. Recent studies from cities in Zaire (Mann, Francis, Quinn et al, 1986), Zambia (Melbye, Njelesani, Bayley et al, 1986) Kenya (Kreiss, Koech, Plummer et al, 1986) and Rwanda (Clumeck, Van de Perre, Carael et al, 1985) confirm that the human immunodeficiency virus is spread mainly through heterosexual tranmsission and that the highest prevalences of antibodies to HIV are found in the most sexually active groups.

As of April 1987 there have been 45,608 cases of AIDS worldwide with an overall fatality of around 50%. This figure may rise as the time of diagnosis to full blown syndrome can vary from a few months to a few years. A higher percentage of deaths have occurred amongst the earlier recorded cases. The growth of numbers has been largely exponential. The number of diagnosed cases in the United Kingdom as of April, 1987 was 734 with 405 deaths. The marked increase in numbers in early 1987, 173 new cases alone by March 31st, highlights the growing threat of this disease to society. This concern is reflected by both the increased media attention the subject now receives and also the increased publicity and education programmes currently being set up here in the United Kingdom. The evidence from epidemiological studies to date indicates that about 10% to 30% of those infected

with HIV will develop AIDS and another 26% will develop AIDS-related symptoms. The apparently long interval during which the infection may incubate adds to the difficulty of predicting the course of the disease.

Table 1.1 gives a breakdown of recorded cases into the recognised risk groups for the United Kingdom up to the end of March, 1987.

1.3.2 Haemophilia and Transfusion Associated AIDS

It has long been known that the use of blood transfusion and blood products provide an efficient vehicle for transmission of infectious organisms. The list of infectious agents transmitted by blood has gradually expanded and now many post-transfusion viral, bacterial and parasitic diseases are well described (Soulier, 1984).

The first case of AIDS associated with cellular blood products transfusion was of a 20-month old infant who developed severe cellular immunodeficiency and multiple opportunistic infections after receiving several blood componant transfusions (Amman, Cowan, Wara et al, 1983). Subsequently, the investigation of three adults who developed Pneumocystis caninii pneumonia strengthened the hypothesis that a potentially transmittable agent was producing AIDS in transfusion recipients (Curran, Lawrence, Jaffe et al, 1984). The first haemophilia patient with AIDS was reported to CDC in January, 1982 (CDC, 1982) and workers soon suspected that non-heat treated Factor VIII preparations were the potential vehicle for HIV transmission. As of March 31st, 1987 there had been 31 cases of AIDS amongst the haemophiliac population in Britain with 23 fatalities.

The risk of HIV infection in haemophilia has been shown to be related to the amount of concentrate used (Melbye, Froebel, Madhok et

Transmission characteristic	Cumulative Cases			Number of Deaths
	Male	Female	Total	
Homosexual/bisexual	640	-	640	342
Intravenous drug abuser (IVDA)	8	2	10	5
Homosexual & IVDA	7	-	7	4
Haemophiliac	31	-	31	23
Recipient of blood : abroad	4	4	8	6
U.K.	3	2	5	5
Heterosexual:				
possibly infected abroad	13	7	20	11
U.K. (no evidence of being infected abroad)	1	4	5	4
Child of HIV antibody positive mother	3	4	7	4
Other	-	1	1	1
Total	**710**	**24**	**734**	**405**

Table 1.1: Cumulative Totals of U.K. Reports of AIDS Cases by Transmission Characteristics, to 31st March, 1987.

al, 1984; Ludlam, Tucker, Steel et al, 1985). The amount used will be directly related to the number of exposures to different donors and therefore increased risk of HIV infection.

An "average" severe British haemophiliac will probably use 40,000 I.U. of Factor VIII concentrate per year. To maintain this supply approximately 160 litres of plasma are required each year for each patient. As the production method for the concentrate involves pooling donated plasma, 160 litres could, therefore, be a result of donations by many people, perhaps into the thousands. The risk of receiving blood products from an infected donor is naturally increased if the production of the product involves the use of multiple donations from different donors.

In virtually all European studies, seroconversion has been significantly associated with the use of commercial concentrate most of which is imported from U.S.A. (Melbye et al, 1984). Cross sectional studies of HIV antibody prevalence shows presence of antibody in almost all populations studied. Retrospective studies on antibody prevalence has shown antibodies to HIV in haemophiliac serum from 1979 (Evatt, Stein, Francis et al, 1983), this is in keeping with both the first death from AIDS occurring in mid 1982 (Davis, Horsburgh, Hasiba et al, 1983) and what is now known of the time course of the disease. Since then the numbers of HIV antibody positive patients has increased significantly, particularly between 1981-83 with fewer conversions between 1984-85 (Eyster, Goedert, Sarngadharan et al, 1985). The pattern in Europe has lagged the American epidemic by 1-2 years, the first seroconversions occurring in 1980-81 (Gurtler, Wernicke, Eberle et al, 1984).

Much speculation initially surrounded the clinical significance of the presence of HIV antibody. Suggestions included innoculation

with isolated viral proteins, innoculation with whole virus and the passive transfer of antibody from factor concentrate despite the inability to detect the presence of antibody in implicated batches. HIV has now been isolated from seropositive haemophiliacs (Allain, Laurian, Paul et al, 1986) (Vilmer, Barré-Sinoussi, Rouzioux et al, 1984) implying that the presence of antibody indicates innoculation with whole virus.

As in other risk groups HIV infection in haemophilia has been associated with a glandular fever like illness, characterised by fever, macular rash, generalised lymphadenopathy and splenomegaly (Tucker, Ludlam, Craig et al, 1985). The most frequent infectious agents in haemophiliac cases of AIDS have been Pneumocystis carinii pneumonia, Toxoplasmosis , candidiasis, Myobacterium avium and Cryptococcus meningitis. There is no evidence to suggest that the spectrum of opportunistic infections reported in haemophiliac cases differs from other risk groups. Kaposi's sarcoma, the most frequently occurring neoplasm in AIDS patients has been infrequently reported amongst haemophiliac cases although it has been documented in one patient with Factor V deficiency (Vélez-Garcia, Robles-Cardona, and Fradera (1985).

1.3.3 Reducing the Risk

What has been done to curtail the AIDS problem in the use of blood and blood products?

Blood donor screening has now been introduced. All blood donations are tested for antibodies to HIV with any positive samples being discarded from the donor pool. However, the possibility of a donor being infected but not seropositive will mean that there is still a risk of infected blood being used. Heat-treated clotting factor

concentrates were developed several years ago with the unfulfilled goal of decreasing hepatitis infectivity of the factor concentrates. These products have been commercially available, but were not widely used until studies showed that HIV is heat sensitive (Spire, Dormont, Barré-Sinoussi et al, 1985). All factor concentrate now being used is heat treated. However, many patients were treated with non heat treated material and because of the long incubation period of AIDS, cases will continue to occur. The gene for Factor VIII has been cloned (Wood, Capon, Simonsen et al, 1984). However, there are no definite predictions as to when bio-engineered factor products will be widely available. Most investigators indicate that several years delay is probable.

1.3.4 The Causative Agent

An infectious agent, in particular a virus was strongly suspected in the aetiology of the acquired immune deficiency syndrome from the onset of the epidemic (Curran, Morgan, Hardy et al, 1985). Retroviruses in particular were considered to be prime candidates because an animal retrovirus, feline leukaemia virus, was known to cause a disease similar to AIDS in cats (Hardy, Hess, McEwan et al, 1976). In addition, the mode of transmission, the spread of the disease and the selective T cell depletion was reminiscent of the patterns previously seen with the then known T cell tropic human retrovirus (Wong-Staal, Shaw, Hahn et al, 1985). It has now been well established that the Human Immunodeficiency Virus (HIV) is the causal agent of AIDS.

The earliest report of a retrovirus as the possible cause of AIDS was from Barré-Sinoussi and colleagues (1983). They described C-type retrovirus particles isolated from T lymphocytes from a patient

with generalised lymphadenopathy. The subsequent availability of permanent T cell lines allowed recovery of high titres of virus (Popovic, Sarngadharan, Read et al, 1984). Virus isolation could be achieved from over 80% of pre-AIDS and 30-50% of AIDS patients (Gallo, Salahuddin, Popovic et al, 1984;) (Salahuddin, Markham, Popovic et al, 1985). This in turn allowed the production of test reagents for epidemiological surveys. The high prevalence of HIV antibody in patients with overt disease and in the aforementioned high risk groups, with subsequent virus isolation established the final link between HIV infection and AIDS. Retrospective studies confirm this and show that 15 to 50% of HIV infected persons in high risk groups have developed AIDS or AIDS-related symptoms after a period of 3 to 4 years (Goedert, Biffer, Weiss et al, 1986). HIV as mentioned is a retrovirus. Therefore, it carries its genetic information in the form of RNA. On infecting a target cell the virus makes use of the unique enzyme Reverse Transcriptase (RT) to convert its own RNA into DNA (pro-viral) which is then integrated into the DNA of the host cell.

The genomes of replication competent retroviruses are similar similar in organisation, most posessing three genes:-

1. gag : coding for the group specific antigen proteins which are found in the core.
2. pol : coding for reverse transcriptase.
3. env : coding for viral envelope proteins.

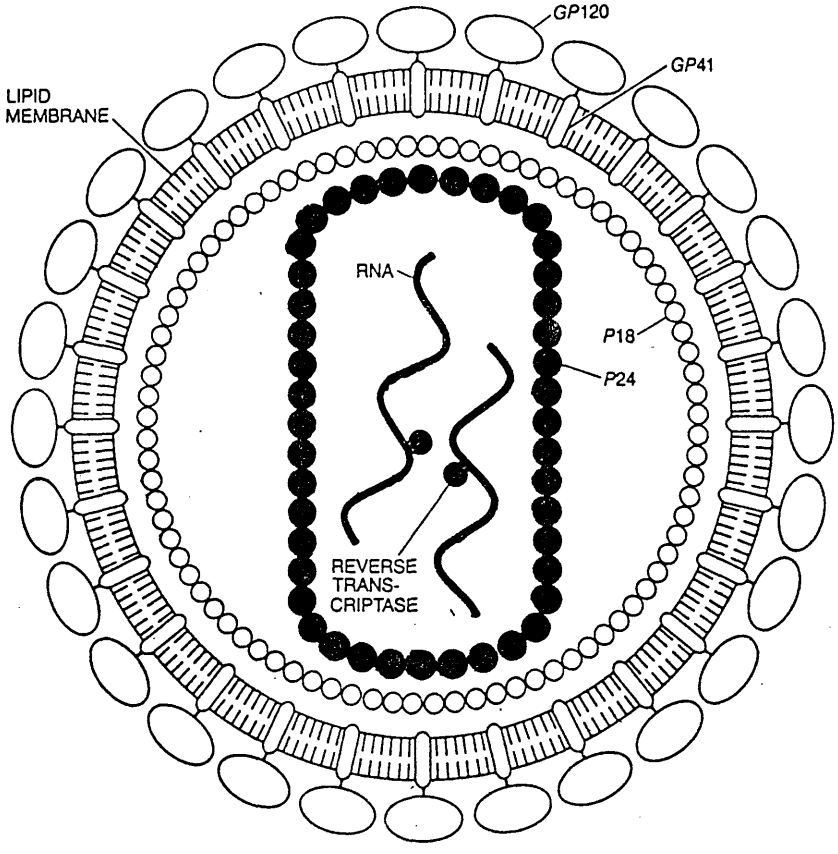
They are arranged gag-pol-env in the direction from 5' to 3' end of the genomic RNA. In the virion RNA of HIV these genes are flanked by parts of long terminal repeat sequences (LTR) (Starcich, Ratner, Josephs et al, 1985) and are interspersed by sequences with open reading frames (ORF) called sor, 3'orf and tat. Both sor and 3'orf are expressed during replication and antibody to their products have

been found (Kan, Frachini, Wong-Staal et al, 1986) but the functional property of these proteins is still unknown. Between sor and 3'orf is tat which codes for a protein with trans-activated transcription activity directed by the HIV LTR (Sodroski, Patarka, Rosen et al, 1985) (Seigel, Ratner, Josephs et al, 1986). Transactivation genes and mechanisms have also been observed in HTLV I and II (Sodroski, Rosen and Haseltine, 1984) and in visna virus (Hess, Clements and Narayam, 1985) and may play a crucial role in determining the biological actvity of these viruses (Sodroski et al, 1984, Wong-Staal and Gallo, 1985). A representative diagram of the structure of HIV is shown in Figure 1.2.

1.3.5 Retrovirus Replication and the Origin of HIV

The replication of retroviruses is unique and will be briefly described with specific relevance to HIV. The first stage is the adsorption of the virus to a specific receptor on a susceptible cell. There is strong evidence that for HIV part of the T helper cell antigen termed CD4 is involved in the process (Dalgleish, Beverly, Clapham et al, 1984). Binding of the virus is mediated by its own envelope glycoprotein, gp110 (McDougal, Kennedy, Sligh et al, 1986). After adsorption comes penetration (endocytosis) and uncoating of the viral genome (possibly in lysosomes).

Reverse transcriptase then converts the viral RNA into a double-stranded DNA molecule which can be incorporated into the DNA of the host cells. The pro-viral DNA thus becomes a part of the genetic material of the cells which, upon activation, programmes the further manufacture of viral components and their assembly into the whole virus. The new viruses leave the cell by budding out through the cell membrane and are capable of infecting other cells with the



HTLV-III VIRION, or virus particle, is a sphere that is roughly 1,000 angstrom units (one ten-thousandth of a millimeter) across. The particle is covered by a membrane, made up of two layers of lipid (fatty) material, that is derived from the outer membrane of the host cell. Studding the membrane are glycoproteins (proteins with sugar chains attached). Each glycoprotein has two components: *gp*41 spans the membrane and *gp*120 extends beyond it. The membrane-and-protein envelope covers a core made up of proteins designated *p*24 and *p*18. The viral RNA is carried in the core, along with several copies of the enzyme reverse transcriptase, which catalyzes the assembly of the viral DNA.

Figure 1.2: Representative Structure of HIV.

(from Gallo, 1987).

same host. Therefore, infection once acquired, probably persists for life and infected subjects remain infectious to others (Curran, et al, 1985).

The origins of HIV are not yet clear. It has been suggested that the virus might be man-made by laboratories in the U.S.A. or Soviet Union, in an attempt to engineer biological warfare (Medvedev, 1986 ; Seale, 1986). Alternatively, there is more support for the theory that HIV first occurred in remote polulations in Africa and subsequently spread via population movement (Pinching,1986). Research has been largely directed at the possibility that HIV in humans may have originated from the animal kingdom.

Sequences of HIV have been compared to other HTLV viruses and with other retroviruses (Wong-Staal & Gallo,1985). HIV has been shown to have close homology with a simian virus called STLV-III MAC which causes immunodeficiency in the Asian macaque monkey. Attention has additionally focused on the African green monkey. A virus related to HTLV-III has been isolated from healthy animals of this species. Antibodies to this virus known as STLV-III AGM have been found to cross react with HTLV-III.

Recent observations have indicated the presence of at least one other retrovirus, genetically closer to the simian virus in human populations in West Africa. Barin and colleagues (1985) have reported that antibodies to the AIDS virus found in a number of healthy subjects were more closely related to STLV-III ASM than to reference strains of HTLV-III. Whether or not this virus which has been called HTLV-IV (Kanki, Barin, M'Boup et al, 1986) or LAV-2 (Montagnier,1986) causes disease in humans is not yet clear.

The HIV genome has also been shown to have close homology to that of equine infectious anaemia virus (Stephens, Casey and Rice,

1986) and to members of the Lentivirus subfamily such as visna (Gonda, Wong-Staal, Gallo et al, 1985). A considerable degree of genomic diversity, especially in the envelope gene, has been found among different HIV isolates (Wong-Staal et al, 1985) (Starcich, Hahn, Shaw et al, 1986).

The origins of HIV are thus shrouded in uncertainty but evidence still favours the theory that it somehow originated in Africa.

1.4 IMMUNOLOGY OF AIDS

Before discussing the immune defects found in AIDS it is necessary to first of all give a brief overview of the workings of the intact immune system.

1.4.1 Organisation of the Immune System

The sole function of the immune system is to distinguish self from non-self and, if necessary, to mount a response against that which is foreign to eliminate it from the body. The initial discrimination is made by a distinct set of thymus derived T cells known as T helper/inducer (Th) cells. The cell does this when antigen that has been processed by an antigen presenting cell such as a macrophage is presented to it in the context of self components known as Class II structures coded for by the Major Histocompatibility Complex (MHC). This is probably done by a single receptor on the T helper cell surface which recognises the antigen in conjunction with the Class II structures. T helper cells also express a 55 - kilodalton cell surface molecule known as CD4. The use of anti-CD4 monoclonal antibodies is a convenient way of enumerating this cell type (Reinherz, Kung, Goldstein et al, 1979). The precise function of CD4 is not yet known but it is thought to be part of the T cell receptor involved in secondary or associative recognition of class II structures.

As a consequence of T helper cell recognition, they then switch on various arms of the immune response as appropriate. They may induce antigen-specific B cells to proliferate and differentiate into plasma cells secreting antigen specific antibody. They can also induce T cytotoxic (Tc) which recognise antigens on cells surfaces in

conjunction with Class I MHC products and lyse cells. The cytotoxic/suppressor cell populations express the CD8 (T8) molecule (Reinharz and Schlossman,1981). The immune system can function either through direct cell to cell contact or by the release of lymphokines such as Interleukin 2 (IL-2), gamma interferon (γ-IFN), B cell growth factor (BCGF) etc., which are all produced by the CD4 T helper cells. These lymphokines have potent effects on other cells in the immune system such as natural killer (NK) cells and macrophages.

1.4.2 Infections and Immune Defects

Before the advent of AIDS, it was known that certain types of infection could be associated with specific immune defects. Viral, fungal and protozoal infections tend to occur in patients with a T cell defect whereas bacterial infections are more of a problem for people with B cell dysfunction (Rosen, Cooper and Wedgewood, 1984). Some infections like Pneumocystis carinii pneumonia are opportunistic occurring only in patients with immune dysfunction, whereas other such as Herpes can occur in both normal and immunodeficient patients.

1.4.3 HIV Infection in AIDS

From the immunologic standpoint, the AIDS phenomenon is best viewed as an HIV infection affecting T cells. AIDS is characterised by progressive lymphopenia predominantly of T helper/ inducer cells which renders the patient immunologically compromised and thus susceptible to a variety of opportunistic infections and malignancies. A prime determinant of susceptibility to HIV infection is the presence of the CD4 molecule on the cell surface which acts as a receptor for the virus and which binds the gp110 envelope glycoprotein of the virus. Certain CD4 monoclonal antibodies can block viral binding and

infection (Dalgleish et al, 1984). Similarly, virus infected cells bind and fuse with uninfected $CD4^+$T cells (but not $CD4^-$T cells) resulting in syncytia formation that can be inhibited by anti-CD4 monoclonal antibodies (Lifson, Reyes, McGrath et al, 1986). Direct demonstration of a bimolecular complex of the CD4 molecule and viral gp110 has been obtained in radioimmunoprecipitation experiments (McDougal et al, 1986). Also when human cell lines which do not express CD4 and cannot be infected by HIV are rendered CD4-positive by transfection with the human CD4 gene then virus can bind to and replicate within these cells (Maddon, Dalgleish, McDougall et al, 1986).

While infection and depletion of $CD4^+$ T cells is likely the major contributor to quantitative viral replication and pathogenesis of immunodeficiency in the host, infection of other cell types has been demonstrated. Monocytes have been shown to express low levels of CD4 (Wood, Warner & Warnke,1983) and HIV particles have been found in certain monocyte derived cell types (Gyorkey, Melnich, Sinkovics et al, 1985). Montagnier (1984) described HIV replication in B cell lines which have subsequently been shown to be $CD4^+$ (Dalgleish and Clapham, 1985). Neuropsychiatric symptoms may be a prominent clinical feature of HIV infection, and HIV has been isolated from brain tissue (Shaw, Harper, Hahn et al, 1985).

1.4.4 Immunological Abnormalities in AIDS

The first abnormality to be associated with AIDS was the reversal of the normal T helper to T suppressor (T_4/T_8) ratio found in the blood (Gottleib et al,1981). It is now known that the inverted ratios are due to a marked decrease in the absolute number of helper/inducer T cells combined with a variable number (increased, decreased or normal) of suppressor/cytotoxic T cells (Schroff,

Gottleib, Prince et al, 1983). The decrease in T_4 cells is a more specific marker for severe immunodeficiency as defined by the CDC surveillance definition of AIDS (Fauci, Macher, Longo et al, 1984). T_4 cell depletion is consistent with results of *in vitro* tests for T cell function. When tested as isolated T cells with normal B cells in a pokeweed mitogen driven assay for immunoglobulin production T helper cell function has been reported to be normal or depressed depending on severity of T cell depletion in the patients selected (Lane, Masur, Edgar et al, 1983). It is rarely totally abrogated except in AIDS patients with severe T cell depletion. On the other hand, proliferative responses to soluble antigen and autologous mixed lymphocyte response are more consistently depressed or even abrogated, and, these may be the earliest qualitative defects in the immune system of the HIV infected patient. Responses to T cell mitogens tend to be depressed when assayed on unseparated lymphocyte cultures, but are generally normal in cell separation studies where the different subpopulations are reconstituted in normal numbers (Lane et al, 1983). Response to soluble antigen however is abnormal in reconstitution experiments and Lane (1985) has suggested an intrinsic defect in the capacity of surviving T cells to recognise soluble antigen. Antigen-specific proliferative responses are an *in vitro* correlate of Delayed Type Hypersensitivity (DTH), and skin testing shows impairment in HIV-infected patients. Total anergy is most likely to occur in those with severest T cell depletion (Lane and Fauci, 1985).

The T_4 helper cell is akin to the conductor of an orchestra. Without him the rest do not know what to do and depend on him for instructions. It is well known that the T_4 cell is central to the immune response and controls many of the other functions. This is shown in Figure 1.3.

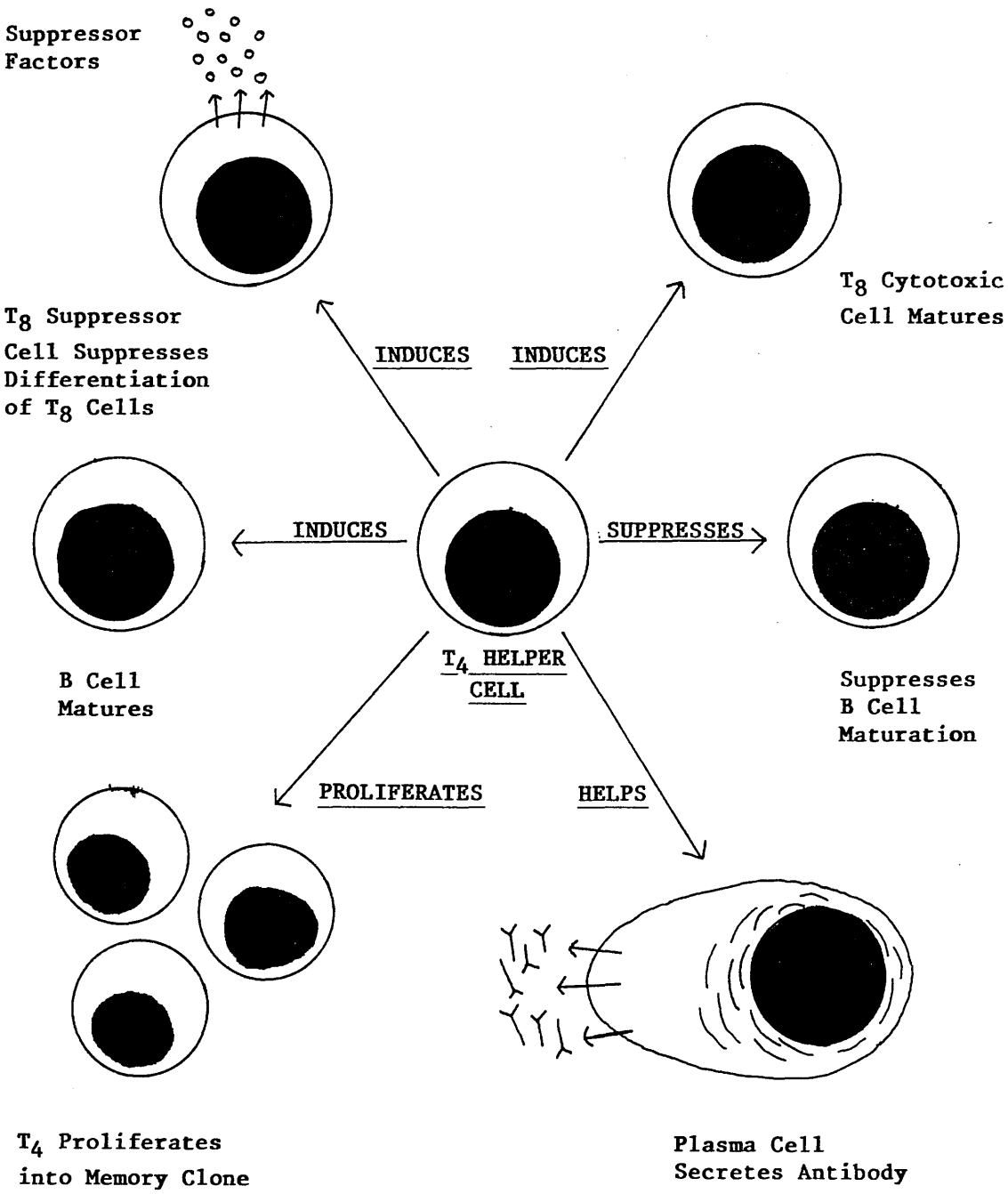


Figure 1.3: Central Role of the T_4 Helper Cell.

The numbers of T_8 cells can be normal, elevated or depressed. These cells comprise the T suppressor and T cytotoxic subsets. There is some evidence that asymptomatic HIV-infected subjects have higher levels of T_8 cells than normal controls. This elevation is due to a subset of T_8 cells associated with cytotoxic precursor and effector function (Clement, Dagg and Landay, 1984). HIV-infected subjects mount poor cytotoxic effector responses in vitro but presumably have relatively normal precursors. The specificity and function of the increased number of T_8 cells identified as cytotoxic cells and their relevance to clinical course or prognosis remains to be determined. A poor immune system could result from either a loss of helper function or an excess of suppressor function. Much work has, therefore, focused on the second possibility in patients with AIDS. In vitro work (Lane et al, 1983) (Lane and Fauci, 1985) has shown that in fact there is no evidence of excess suppressor activity. Although there does not seem to be excessive suppressor activity by cells, several workers have described suppressor factors. Laurence et al (1983) have described the spontaneous production in vitro of such a suppressor factor dependent on T cell - monocyte interactions. Other workers (Cunningham-Rundles, Michelis and Masur, 1983), (Siegel, Djeu, Stocks et al, 1984) have described factors in the sera of AIDS patients with suppressive or immunomodulating activity. Pahwa (1985) suggested that retrovirus products such as the gp110 envelope glycoproteins themselves could be immunosuppressive.

Another population of cells studied have been Natural Killer (NK) cells. These cells reside largely, but not exclusively, within a population of lymphocytes known as large granular lymphocytes. Much like the results of MHC-restricted and allogeneic cytotoxic cells, NK

cell function is normal in asymptomatic HIV-infected individuals but declines in severe immunodeficiency and clinical complications (Lopez, Fitgerald and Siegel, 1983). Poli (1985) suggested that the functional defect is in the killing process itself. Another two characteristic findings in AIDS patients are hypergammaglobulinaemia and elevated levels of immune complexes (Fauci, et al, 1984) (McDougal, Hubbard, Nicholson et al, 1985). Despite this polyclonal immunoglobulin elevation, AIDS patients mount a poor antigen-specific antibody response after in-vivo immunisation. Primary responses are affected to a greater degree than secondary responses (Lane et al, 1983). Patients can show a high titre of antibody against HIV which drops only with end stage disease (Sarngadharan, Popovic, Bruch et al, 1984). Much of the work on B cell function has been done by Lane, Fauci and co-workers . B cells from AIDS patients produce a poor immunoglobulin response in vitro to pokeweed mitogen stimulation (Lane et al, 1983). This is not due entirely to a defect in immunoregulatory T cells because purified B cells from patients are poorly responsive when mixed with normal T cells, nor can they be stimulated by T cell independent stimulus such as Staphylococci (Lane et al, 1983). There seems to be a high degree of spontaneous polyclonal B cell stimulation rendering the HIV infected patients poorly responsive to normal early activation signals. The reason for this B cell activation is still a matter of speculation. One suggestion is that the abnormality of T cell control allows infections or reactivation of other viral infections, e.g. Epstein Barr Virus, which are B cell activators. Another possibility is that HIV-infected T cells elaborate B cell stimulating factors.

Blood monocytes and tissue macrophages form one of the first lines of defence against infection by migrating towards site of entry,

phagocytosing foreign material, and in most cases degrading and inactivating the foreign micro-organisms. Many of the monocyte functions have been examined in AIDS patients. Numbers of monocytes are usually normal but those from AIDS patients may display defective chemotaxis (Pinching, McManus, Jeffries et al, 1983). H_2O_2 and superoxide production are generally normal (Murray, Rubin, Masur et al, 1984) but results on phagocytosis and intracellular killing have been reported both as normal (Murray et al, 1984) and abnormal (Pinching et al, 1983). Other abnormal responses have been reported for monocyte-mediated, antibody-dependent, cellular cytotoxicity and Fc-mediated clearance of IgG-coated autologous erythrocytes (Bender, Auger, Quinn et al, 1986).

Another of the important functions of monocytes is to process and present antigen in the context of Class II MHC structures to T cells. Monocytes recovered from AIDS patients express Class II MHC structures of a lower density than that from normals. It has not been determined whether monocyte defects result from direct infection with HIV or as a result of immunoregularity T cell abnormalities. As mentioned earlier recent papers (Gyorkey et al, 1985) have shown direct infection of monocytes which express the CD4 antigen which could result in viral persistence as monocytes are long lived compared to T cells and thus could pose problems in therapeutic strategies for elimination of HIV. As well as affecting cells of the immune system, HIV infection has resulted in abnormalities in lymphokines and other soluble serum substances. The production of IL-2, the T cell derived T cell growth factor, is generally regarded to be deficient in response to mitogen (Hauser, Bino, Rosenberg et al, 1984) though some workers have suggested normal production (Lane, Depper, Greene et al, 1985). This deficiency is proportionate to the amount of T cell

depletion as remaining T cells respond normally to the addition of exogenous IL-2 (Gluckman, Kaltzmann, Cavaille-Coll et al, 1985). There is decreased production of gamma interferon, a T cell derived protein with potent antiviral and immunopotentiating effects. Again, this is due to quantitative depletion of T cells rather than a qualitative defect in production or responsiveness (Murray et al, 1985). Levels of a unique form of alpha interferon that is acid labile are higher in AIDS patients especially those with more severe immunodeficiency (Eyster, Goedert, Poon et al, 1983). Its role is unknown but could be produced by the polyclonally activated B cells (Boumpas, Harris, Hooks et al, 1984). Elevated levels of immune complexes are also found in AIDS patients (Gottleib, Groopman, Weinsten et al, 1983).

Beta-2 microglobulin (β_2M) is a low molecular weight glycoprotein associated with Class I MHC structures found on the surface of all nucleated cells. Neoptrin is a cellular purine metabolite. Serum levels of both of these substances correlate with cell destruction and turnover and an increased level of both are found in AIDS patients (Grieco, Reddy, Kothari et al, 1984) (Wachter, Fuchs, Hausen et al, 1984) and increased levels of β_2M have been reported in haemophiliacs positive for antibody to HIV (Madhok, Gracie, Lowe et al, 1986).

1.4.5 The Haemophiliac Problem

The intensive use of potent clotting factor concentrates obtained from large plasma pools in the treatment of haemophiliacs has markedly improved the life expectancy of these patients (Ikkala, Helske, Myllyla et al, 1982). However, the possible existence of undesirable side-effects such as excessive antigenic load and

transmission of viral diseases has become a matter of great concern. As already mentioned, hepatitis is well recognised as being closely associated with the use of factor concentrates. Also, more recently, haemophiliacs have been well recognised as a risk group for contracting AIDS through the use of factor concentrate contaminated with HIV. This has prompted many laboratories to study carefully the immune system of the haemophiliac. Previous work from many laboratories, including our own, have reported immunologic abnormalities in haemophiliacs who are apparently asymptomatic showing no signs of HIV infection. These abnormalities have included inverted T lymphocyte subset ratio, elevated serum immunoglobulin levels, decreased lymphoproliferative responses to T cell mitogens and diminished NK activity (Stein, Evatt, McDougal et al, 1985). It should be noted that many of these studies were carried out before the serological kits were available for measuring anti HIV antibodies. Therefore, many of the patients, although asymptomatic clinically, could in theory have been infected with HIV.

Since the advent of the antibody screening tests for HIV it has been possible to group the patients according to HIV status and therefore follow their immunological status with a clearer understanding of underlying infection. Broadly speaking the immunological defects seen in HIV antibody positive haemophiliacs are similar to those seen in the HIV positive patients belonging to the other at risk groups (Stein et al, 1985).

1.4.6 TREATMENT OF AIDS

There have been endless numbers of publications on several treatment regimes for the various opportunistic infections and tumours, as well as other problems encountered by a patient suffering from AIDS.

However, the ultimate goal is a cure for AIDS, (i.e. against HIV) and also a way of preventing infection, e.g. by vaccination. Work towards producing a vaccine is currently intense and it is hoped to soon have a prototype vaccine which should be available for human trials within the next 12 months. There seems to be many problems involved in trying to produce a vaccine. HIV isolates from different sources although often quite similar, can show distinct differences in the genome. If these differences occur in areas of the genome encoding, for example the envelope proteins, then different HIV isolates will express different envelope proteins. This could mean that antibodies produced in response to vaccination will recognise the strain of virus in the vaccine but not other genetic variants which could infect the individual.

For a vaccine to be effective it must safely evoke two different types of immunologic response. The B cells must be stimulated to produce neutralising antibodies which bind to the envelope proteins of the virus and prevent it from entering cells. Also the cellular immune system involving the T cells must be activated and must be capable of attacking and destroying cells already infected with virus. Although people infected with HIV do make antibodies to it, the amount of effective neutralising antibody is disturbingly low, and cellular immunity is severely impaired by the death of the T_4 cells. A successful vaccine must boost both responses greatly.

A vaccine is not a cure for people already infected. Various lines of research have been followed in looking for treatment or a cure. Much work is looking at an anti-viral approach. These may include inhibition of binding of the virus with its receptor on target cell membrane. This could be achieved by the use of

monoclonal antibodies directed against either viral proteins (especially envelope glycoproteins) or the antigen (viral) receptor on the T_4 lymphocyte. Another important anti-viral approach is to use drug therapy. Many of the drugs being tested, e.g. AZT, have anti-reverse transcriptase activity. Therefore, the virus cannot replicate. However, these drug therapies, although sometimes effective in halting progression of the disease, cannot remove the virus from the body and if therapy is stopped then deterioration associated with viral infection usually recurs. Other drug therapy angles have tried to augment the bodies immune response. Isoprinosine is a drug with both anti-viral and immunopotentiating properties. Early clinical studies proved hopeful as did some in vitro studies. Some studies showed enhanced natural killer activity, γ-interferon and IL-2 production, and an increase in T_4 positive cells. However, on the whole, results do not suggest that this agent will be of significant use in the treatment of AIDS.

Possibly the most ambitious approach has been that of immunorestorative therapy. These have included bone marrow transplants (Lane and Fauci, 1985), white cell transfusion (Lane, Masur, Longo et al, 1984) and thymic transplantations, but they have met with little or no success. Attempts have been made to augment any remaining immune response by injections of the missing lymphokines, e.g. IL-2 and γ-interferon but although the in vitro studies were promising, the clinical trials have not been encouraging. Recent observations have suggested that HIV infected cells activated by IL-2 show enhanced expression of virus which is undesirable (Zagury, Bernard, Leonard et al, 1986).

It is evident from early clinical trials that any of the currently available anti-viral or immunorestorative approaches alone

will not be curative for patients with HIV infection and immune deficiency. However, as an effective vaccine, the ultimate goal, is as yet some time off, a combination of anti-viral and immunorestorative therapy is possibly the best approach currently available. Anti-viral agents should preferably be virucidal, or, if they are virustatic, they should be easily administered and should have no or minimal side effects so that they could be given for prolonged periods of time. A realistic approach would be to develop an effective pharmacologic immunopotentiator and administer it along with a safe and effective antiviral drug.

1.5 SCOPE OF STUDY

The initial purpose of this study was as a more comprehensive and larger follow-up study to the pilot study already carried out in our department on a small cohort of the haemophiliac population.

I wished to examine more closely the immunological status of our haemophiliacs. This meant setting up new assays to measure various immunological parameters. I also wanted to find out if the patients immune system was altered solely as a result of HIV infection or if there was some underlying immunological defect associated with the use of factor concentrate in the absence of HIV infection. Each of the various immunological parameters examined will be dealt with separately in this thesis and will be discussed in relation to any other findings where relevant.

1.6 AIMS OF THE STUDY

The aims of the study are as follows:-

1. To determine the degree of HIV infection in our group of patients.

2. To establish the effect of HIV infection on the immunological status of the patients.

3. To examine the immune status in the absence of HIV infection.

4. To investigate the effect of Factor VIII concentrate and HIV antigen on various *in vitro* immunological assays.

CHAPTER TWO

HIV STATUS OF THE HAEMOPHILIAC POPULATION

2.1 INTRODUCTION

Early in the AIDS epidemic it was realised that from epidemiological data that the cause was an infectious agent which is horizontally transmitted by intimate contact or exposure to contaminated blood or certain blood products (Chamberland et al, 1984) (Curran et al, 1984). The causative agent which we now know to be a retrovirus belonging to the family of human T lymphotrophic viruses (HTLV) was given the name HTLV-III by Gallo and colleagues (1984). As described earlier other workers described the isolation of retroviruses which they gave different names to and the standard term HIV is now being used to describe the virus, causative of AIDS.

It was necessary to develop some means of detecting whether or not a patient was infected by HIV. The ideal way would be to try and look for live virus from an individual's blood sample. It has proved difficult to develop such assays, which would be both readily available, and also relatively easy and safe to carry out. The other possibility was to develop a test which would detect evidence of infection by HIV. In developing such a test it would be necessary to have an assay system which was reproducible, easy to perform, safe, specific and, perhaps more important for the company who developed and produced the assay, an assay system which would have financial rewards. Nearly all assay systems developed made use of the fact that an infected person will have in his bloodstream circulating antibodies to HIV. Therefore, an assay has to be able to detect antibodies to HIV from serum or plasma samples. There are many such kits now available on the market which are variations on this fact. Some are radioimmunoassays and others are enzyme immunoassays (ELISA). The kit used to detect antibodies to HIV in our patients

was the ELISA developed by Abbott Laboratories (Wokingham, Berkshire, U.K.). If a sample is found to be positive it must be re-tested by the same or another similar assay and if again found to be positive it is confirmed by a more sensitive technique such as Western Blotting.

2.2 MATERIALS AND METHODS

2.2.1 Serum Samples

Serum was separated from clotted blood by centrifugation at 650g and stored at -20°C until assayed.

2.2.2 Biological Principles of the Assay

The Abbott ELISA is manufactured from HIV virus propagated in the T lymphocyte cell line H9/HTLV III . Briefly isolated virus is disrupted and inactivated with detergent and sonication prior to coating of plastic beads. Coated beads are then incubated with the sample being tested. Also included are appropriate positive and negative controls, i.e. samples known to be positive or negative for antibodies to HIV. Any antibody to HIV in the serum sample will bind to the HIV which is coating the beads. The second step, after washing to aspirate unbound sample, is to incubate the bead with a goat antibody to human IgG conjugated with horseradish peroxidase. This antibody will in turn bind to any human IgG which is bound to the HIV on the surface of the bead. After a further incubation the beads are again wahsed to remove any unbound material. Next, O-Phenylenediamine (OPD) solution containing hydrogen peroxide is added to the bead. This substrate solution reacts with the horseradish peroxidase bound to the bead complex and a yellow colour develops which is proportional to the amount of anti-HIV antibody originally present in the serum and now bound to the bead. The colour change can be read on a spectrophotometer and inclusion of appropriate controls allows determination of a positive or negative result.

2.2.3 Problems Associated with such an Assay

There are many problems associated with assays of this type. Although very straightforward to carry out there is still an element of risk for the person who has to handle the specimen. Care is required. The biggest problem with such assays is the possibility of false positive and false negative results. A result which comes back positive is, of course, re-checked and if necessary re-checked again. The implications to the patient of a positive result are obvious, therefore it is not desirable to have "false positive" results which could produce unnecessary problems, psychological or otherwise, to the patient. However, perhaps more important is the desire to avoid false negative results. This would come about if an assay was not sensitive enough to pick up anti-HIV antibodies in the serum, or, if there are technical problems with an assay run. It is important to have an assay which is reliable, easy to carry out and one which contains the appropriate controls, e.g. "positive controls" which will tell you that the assay itself is working. The consequences of a false negative result are also obvious, for example, in the screening of blood for the use of in transfusions or the manufacture of blood products such as Factor VIII.

It should be remembered that a negative result does not guarantee that a sample is free from HIV. In some cases it may be possible to have been exposed to HIV and not produced antibodies to it. This could occur if, for example, blood was taken for testing soon after exposure to HIV and before the body has had time to produce antibodies to it. Therefore, although the kits for screening antibody are all that is readily available at the moment, the ideal assay would be to look for virus in the blood sample.

As already mentioned a positive sample is re-checked, and if

still found to be positive, is confirmed by a more sensitive procedure such as Western Blotting.

2.2.4 Western Blotting

Very briefly, Western Blotting technique involves running disrupted HIV on polyacrylamide gel electrophoresis. The protein bands corresponding to the various HIV proteins can then be blotted onto Nitro-cellulose strips. These are then overlayed with test sera and any antibody to the viral proteins will bind to the Nitro-cellulose strips. Bound antibody can then be detected by further incubation steps. This technique is more specific than others and it is possible to detect antibodies against the various HIV proteins. Disease state has been associated with a particular serological profile, and a reduced incidence of detectable antibodies to the major HIV core protein, p24, in patients with AIDS compared with that in other people infected with HIV has been reported (Kalyanaraman, Cabradillo, Getchell et al, 1984) (Schupbach, Hallar, Vogt et al, 1985). Loss of intensity of bands and total loss of antibodies to p24 may therefore herald the onset of AIDS (Lange, Coutinho, Krone et al, 1986) and in recent studies AIDS seemed to be strongly associated with the expression of HIV antigen in the serum as detected by a new test (Goudsmit, de Wolf, Paul et al, 1986).

2.3 RESULTS

At regular clinic appointments blood was taken for HIV status determination. Most of our haemophiliac population (around 250) have now been tested for antibodies to HIV. Twenty-one of the patients have been found to be seropositive for antibodies to HIV by ELISA. These results have all been confirmed by Western Blotting. Therefore, approximately 8.4% of our haemophiliac population show evidence of exposure to HIV or HIV antigens.

2.4 DISCUSSION

As stated 8.4% of our haemophiliacs have been shown to be HIV^+ (i.e. have antibodies to HIV). Many workers believe that if a patient is shown to have antibodies to HIV then it will be possible to isolate infectious virus from that individual. It was originally suggested that perhaps haemophiliacs were HIV^+ because of carry over of immunoglobulins to HIV in the Factor VIII concentrate. However, this does not seem to be the case as anti-HIV antibodies have not been detected in these preparations. Another possibility is that haemophiliacs have been exposed to disrupted viral antigens, in the concentrates, rather than to live virus. If this were the case then the concentrate would be acting in the same was as a vaccine does with the patient producing antibodies to a harmless form of the virus which would in turn confer immunity against infection with the live virus. This seems very unlikely as there have now been many cases of AIDS amongst haemophiliacs whose only exposure to live HIV must have been through the use of Factor VIII preparations. HIV has now been isolated from HIV antibody positive haemophiliacs (Allain et al, 1986). As infection with the virus results in its DNA being inserted into the cells' genes, then it has to be assumed that the virus can lie dormant and it could be possible to reactivate the virus. Therefore, anyone who is HIV^+ should be treated as potentially infectious. Very recently we have managed to assay our HIV^+ haemophiliacs for HIV antigen and four of them have proved to be positive. It will be necessary to monitor these patients' clinical condition very carefully as it has now been conclusively shown that they are infected with live virus. In fact, one of these patients has now lost antibodies to the p24 protein on Western Blotting.

The percentage of patients we have who are HIV^+ is much lower than has been reported by many other groups (Jones, Hamilton, Bird et al, 1985). This is probably due to the fact that here in Scotland we are self sufficient in the production of Factor VIII concentrate from local blood donations. To date, this donor pool has been largely free from infection with HIV, thus allowing the production of relatively safe concentrates. However, there has been a report in the literature of at least one batch of "home produced" concentrate which was contaminated resulting in several seroconversions (Ludlam et al, 1985). In contrast, are the commercially available concentrates, mostly from the USA, which are produced from paid blood donors. As a result, many of the high risk groups, e.g. intravenous drug abusers will donate blood to receive money which will in turn be used to obtain more drugs. All of our haemophiliacs who have seroconverted have at some time received commercially available concentrates and the one patient who died of AIDS received heavy prophylactic treatment with these commercial products while living in England.

In summary, therefore, a small percentage of our patients (8.4%) have been exposed to HIV. The majority of them seroconverted around 1981-82 which is in keeping with the lag phase seen in European countries compared to the USA experience. With this information available it will be possible to monitor various aspects of the immune system of haemophiliacs (as described in the following chapters) to look for any defects which may be attributable through infection with HIV, the causative agent of AIDS.

CHAPTER THREE

SKIN TESTING

3.1 INTRODUCTION

Abnormalities *in vivo* in AIDS have been observed. Skin test anergy to common recall antigens that usually reveal delayed-type hypersensitivity (DTH) is generally found (Gottleib et al, 1981) (Siegel, 1984). Anergy suggests that some part of the response from antigen recognition through to recruitment of inflammatory cells causing the reaction is defective. Primary immunisation to elicit DTH commonly employs skin-sensitising haptens such as 2,4-Dinitrochloro-benzene (DNCB). The DNCB skin test is considered to be the best single test to evaluate both the afferent and efferent arms of the cell mediated immune response *in vivo* as it measures a person's ability to respond to a new antigen, as DNCB is rarely encountered (Aisenberg, 1962). DNCB binds to skin proteins or perhaps directly to antigen presenting, Ia-bearing cells (Langerhans cells) in the skin leading to clonal selection and expansion of antigen reactive T cells. The development of contact dermatitis on re-challenge suggests intact cellular immunity.

In this study we have measured the patients response to DNCB and have tried to compare their test score with HIV antibody status and Factor VIII concentrate consumption.

3.2 MATERIALS AND METHODS

3.2.1 Patients

We studied 29 patients with clinically severe haemophilia (Factor VIII or IX concentration <50 U/L). Twelve of these patients were known to be HIV antibody positive. All patients were examined for features of disease related to HIV. The mean annual consumption of factor concentrate was calculated from case records. The presence of transaminasaemia and previous exposure to hepatitis B virus, assessed by antigen and antibody testing (Abbott, Wokingham, U.K.) was also noted.

3.2.2 Serological Tests

Blood was withdrawn to test for antibodies to HIV and routine screening for viruses including CMV, Herpes, and EBV.

3.2.3 Lymphocyte Subsets

At the time of re-challenge with DNCB, blood was withdrawn for enumeration of T cell subsets as by method described in Chapter 4.

3.2.4 Dinitrochlorobenzene (DNCB) Test

The principle of this test is to sensitise the patient with an antigen he has not encountered before and then re-challenge and measure the response. If the patient has an intact cell mediated response then he should recognise the DNCB as foreign and a varying degree of reaction will be seen at exposure sight. However, on re-challenge patients with intact cell mediated response will recognise the DNCB as something met previously (i.e. immunological memory) and their response will correlate to the degree of intactness

of the immune system and will give a measure in vivo of cell mediated immunity.

A modification of the DNCB test described by Watson and colleagues (1979) was used. DNCB was dissolved in acetone to a final concentration of 20 mg/ml. 0.1 ml was applied to an area on the dominant forearm measuring 2.5 cm in diameter, where the acetone evaporated leaving DNCB on the skin. After 14 days the patient was re-challenged on the opposite forearm. Varying doses of DNCB (30, 15, 7.5, 3.7 and 1.8 µg) dried onto 1 cm diameter felt pads (A1 test patches, Astra Chemicals, Watford, U.K.) were applied to the skin. The patient's response was then measured 48 hours later.

The criteria for scoring was as follows:-

Score

0 : No reaction or erythema only
1 : Erythema and induration confined to site of test patch
2 : Erythema and induration spreading beyond test patch
3 : As for 2 plus blistering.

The minimum score obtainable was 0 and could range to a maximum of 15 (3 score x 5 test patches).

In a previous study from our area (Watson et al, 1979) using the same method of testing, the median score in a group of 15 normal subjects was 9 [range 4-14]. Obtaining a normal population is difficult as it is undesirable to sensitise people to DNCB if they may meet it again, e.g. laboratory personnel. Therefore, as the previous study used the same methodology and the normals were sex and age matched to our population, it was decided to use the previous result as a control population for our study.

3.3 RESULTS

3.3.1 Clinical

The DNCB response is shown in Figure 3.1. Two patients had appreciable cervical lymphadenopathy but no other features of HIV related disease. One patient had persistent mild thrombocytopenia, the lowest recorded count being 96 x 10^9/L. No other patient had clinical features related to HIV.

3.3.2 DNCB Testing

The DNCB response is shown in Figure 3.1. The bar value in this and all subsequent scatter plots throughout the Thesis represents the median value. The median score in normal subjects was 9 (range 4-14). All the patients had a score below 9. The median score in all patients was 3 (range 0-8) which was significantly less than normals ($p<0.001$). In the HIV^+ patients the median score was 1 (range 0-8) which was also significantly less than normals ($p<0.001$). The seronegative patients had a median score of 4 (range 0-8). This too was significantly different from the normals ($p<0.001$) but no different compared to the HIV^+ patients. Among the 3 patients with clinical features of disease related to HIV, the score was 0 in the patient with thrombocytopenia and 0 and 4 in the patients with lymphadenopathy.

3.3.3 Serological

Twelve of the patients had antibody to HIV as tested by ELISA (Abbott). No patient had circulating IgM to any other virus tested. Previous exposure to hepatitis B virus and the presence of transaminasaemia was similar in both patients positive and negative for HIV.

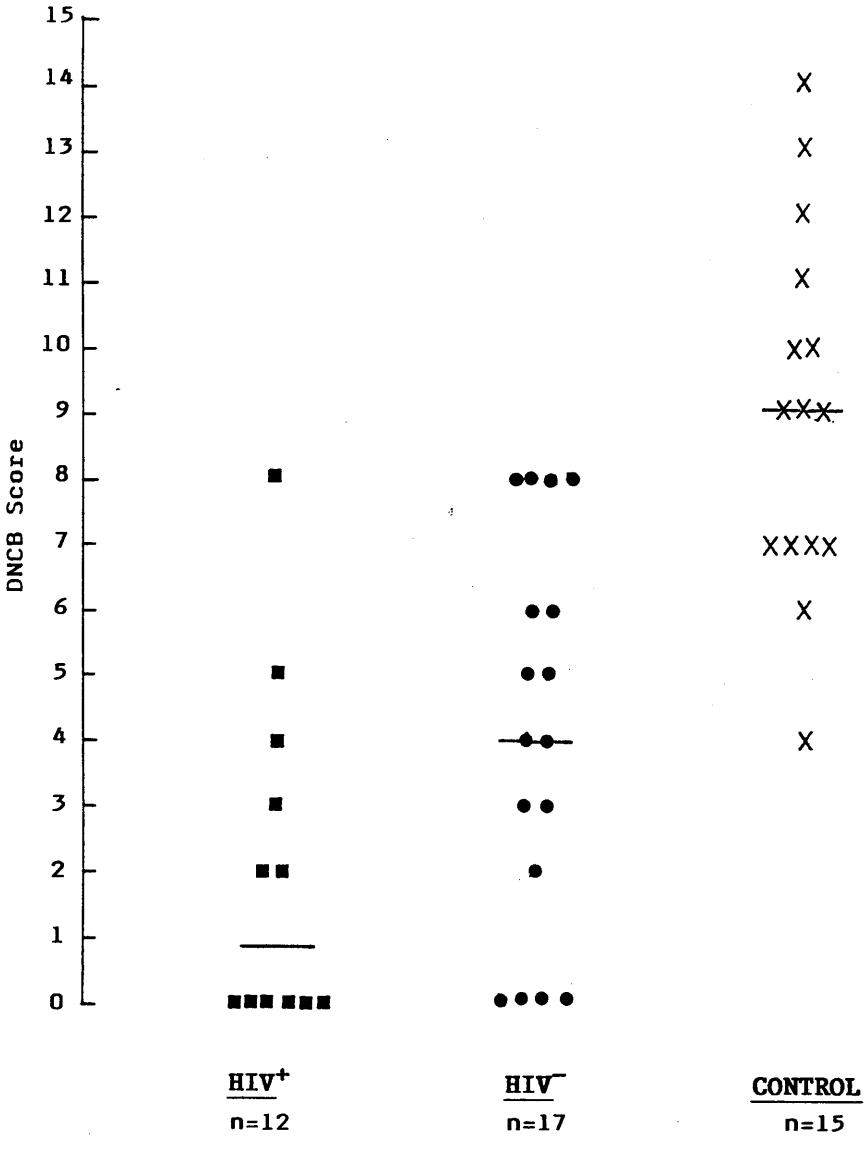


Figure 3.1: Response (Score) to DNCB.

3.3.4 Lymphocyte Subsets

Table 3.1 gives the results of T lymphocyte subsets. The median number of T helper cells in the HIV antibody positive patients was 0.76 [range 0.45-1.05] x 10^9/L. This was not significantly different from the number in the patients negative for antibody to HIV, (median 0.8 [range 0.3-1.67] x 10^9/L. The score on DNCB testing inpatients positive for HIV antibody showed an inverse correlation with the T suppressor cell count ($r=0.45$, $p<0.05$) but no correlation with the T helper cell count ($r=0.04$, $p>0.05$). In HIV negative patients the DNCB score showed a direct correlation ($r=0.49$, $p<0.05$) with the T helper cell count but no correlation ($r=0.15$, $p>0.05$) with the T suppressor cell count. The difference in the median number of T helper cells in patients negative for the antibody between those with DNCB scores in the normal range and those with scores below the normal range was significant ($p<0.05$).

3.3.5 Consumption of Clotting Factor

The median annual consumption of clotting factor over four years in all patients was 49,068 units [range 5,104-190,925]. The median consumption in seropositive patients was 97,250 [range 5,104 - 190,925] and in seronegative patients 32,375 [range 9925-92,500]. This difference was significant ($p<0.05$). An inverse correlation was noted between increasing usage of clotting factor and decreasing score on DNCB testing as shown in Figure 3.2. ($r=-0.3$, $p<0.05$). In seronegative patients with a score less than normal (<4) the median consumption of clotting factor was 59,685 units [range 21,175-92,500]. this was significantly more than in seronegative patients with a DNCB response in the normal range (median 22,899 units [range 9,925-91,925] $p<0.02$. An inverse correlation was noted in all seronegative patients

	HIV$^-$		HIV$^+$	
DNCB SCORE	<4	≧4	<4	≧4
NUMBER OF PATIENTS	7	10	9	3
T HELPER CELLS ($x10^9/L$)	0.72† (0.3 - 1.67)	1.18 # (0.74 - 1.65)	0.69 (0.45 - 1.05)	0.79 (0.77 - 0.9)
T SUPPRESSOR CELLS ($x 10^9/L$)	0.56 (0.18 - 1.39)	0.8 (0.48 - 1.10)	0.5 (0.33 - 0.65)	0.48 (0.37 - 0.48)

† Median (range)

# $p<0.05$ Vs. HIV$^-$ with a Score <4.

Table 3.1: T Cell Subset Numbers in HIV and HIV Patients.

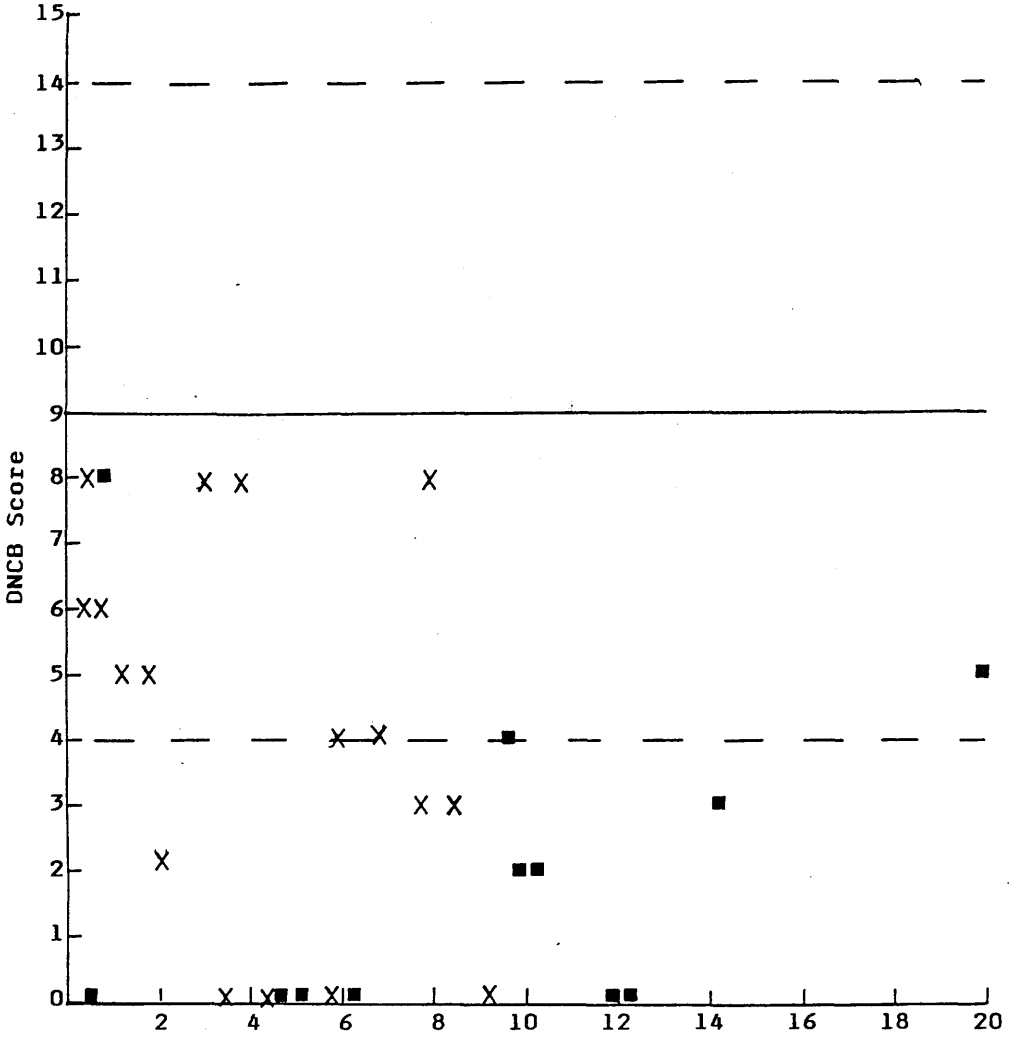


Figure 3.2: Consumption of Clotting Factor and Score on DNCB Testing in Patients Positive (■) and Negative (X) for Antibodies to HIV.
Broken Horizontal Lines Indicate Normal Range.
Solid Horizontal Line Indicates Median Normal Value.

between clotting factor usage and DNCB score ($r=-0.41$, $p<0.05$), but the correlaton in seropositive patients was not significant ($r=0.08$). There was no significant difference, however, between the correlations in the seropositive and seronegative groups.

3.3.6 Hepatic Dysfunction

There was no significant association between DNCB and the presence of transaminasaemia or exposure to hepatitis B virus.

3.4 DISCUSSION

As the response to DNCB evaluates both the afferent and efferent arms of the immune response, it gives a measure of the patient's current cell mediated immune state. The results show that all patients with severe haemophilia treated with Factor VIII concentrate had a response below the median normal value, and in 19 of the 29 patients studied, the response was on or below the lower limit of the normal range (that is ≤ 4). In addition, the response was impaired in patients seronegative for antibody to HIV. The publication of this study (Madhok, Gracie, Lowe et al, 1986) was the first such report to show a depressed cell mediated response in vivo in haemophiliacs in the absence of apparent infection with HIV as determined by the absence of antibodies to HIV.

In patients negative for antibody to HIV the response to DNCB was inversely related to consumption of clotting factor. In particular, patients who had a subnormal response had used significantly more factor concentrate than those patients with a response which fell within the normal range. This assoiciation of consumption of clotting factor with reduced cell mediated immunity may be due to one of, or a combination of, a variety of reasons; (i) transmission of viral infection, (ii) a direct chemical effect of concentrate, or (iii) an indirect effect mediated via the immune system.

Concomitant serological studies ruled out the possibility of an incidental viral infection. Infection with HIV in the absence of antibody was considered unlikely, as none of our seronegative patients had HIV related disease features. In our patient population HIV seroconversion occurred during 1980-84, especially 1981-82 (Madhok et al, 1985) and in a previous report our group have shown that

seroconversion was associated with use of imported American concentrates (Melbye et al, 1984). No seroconversions have been recorded in these patients since 1984, when all the patients began to receive only locally prepared factor concentrate from an area with a low prevalence of disease related to HIV. In addition, isolates of virus from seronegative patients are exceptional (Salahuddin, Groopman, Markham et al, 1984). This study, therefore, suggests that the depression in cell mediated immunity is probably due to either a direct effect or an immunological consequence of infusion of factor concentrate.

In a previous study of our patients Forwell et al (1984) have reported high circulating levels of Fcγ receptor antibody. This antilymphocytic antibody develops after repeated blood transfusions or use of blood products. The Fcγ receptor is an important immuno-regulatory protein (Unkeless, Fleit and Mellman, 1981) and blocking of this receptor with antibody or immune complexes can stimulate a non-specific T suppressor cell response (Moretta, Mingari, Moretta et al, 1979) which could then additionally inhibit macrophage activation or T helper functions. Excessive activity of T suppressor cells may therefore account for the abnormalities seen in our seronegative patients on DNCB testing.

In the twelve seropositive patients, the DNCB score was significantly below normal ($p<0.001$), and nine of the patients had a score below the normal range. The score did not differ significantly, however, from that found for the seronegative patients. There was no correlation in the seropositive patients between the score and the consumption of clotting factor. Both of these negative findings may have been due to the small numbers in this group.

In conclusion, therefore, the results suggest that a decrease in cell mediated immunity in the severe haemophiliacs is related to the amount of Facor VIII used in therapy. Whether this abnormality is related to risk of infection with HIV, or to its sequelae, or both, is as yet unknown. Though no significant difference in DNCB score was observed between the HIV seropositive and seronegative groups, infection with HIV was fairly recent. Future studies of DNCB response in the HIV seropositive patients, who have been infected for a longer period, may show lower responses, as HIV induced immunosuppression may take many years to become firmly established.

CHAPTER FOUR

T CELL SUBSETS

4.1 INTRODUCTION

Since their discovery, monoclonal antibodies have been used extensively in many areas of medical and scientific research (Köhler and Milstein, 1975). One of the most common uses has been to raise monoclonal antibodies to the various cell subpopulations of the immune system. As these monoclonal antibodies are specific for only one antigenic moiety, it has been possible to produce antibodies specific to such populations as B cells, T helper cells, T cytotoxic/suppressor cells, moncytes etc. In clinical research in recent years it has been very popular to enumerate the various cell populations, especially the T helper cells or T cytotoxic/suppressor cells in various clinical conditions or disease states. The most popular way to express the results is as a ratio of the number of T helper cells to the number of T cytotoxic/suppressor cells. This ratio has been expressed differently but the most common forms are $T_h/T_{c/s}$ or the T_4/T_8 ratio. The latter got its name from the commercially available monoclonal antibodies which recognise the different cell types, OK-T_4 for T helper cells and OK-T_8 for T cytotoxic/suppressor cells.

In the normal population, free from illness, the T helper cells and the T cytotoxic/suppressor cells constitute about 64 and 36 per cent respectively, of the total T cells giving a $T_h/T_{c/s}$ ratio of approximately 1.7. However, this figure can change quite dramatically even in a seemingly normal population. In disease states, however, the ratio is often abnormal. Even in something quite trivial like a common viral infection such as influenza, the ratio can be reduced. This is due to an imbalance caused by an increase in the T_8 population relative to the T_4 population. There are many references

in the literature of such imbalances caused by ratio can be viral infections, e.g. cytomegalovirus (Carney, Iacoviello and Hirsch, 1983).

It is thought that by measuring this ratio it is possible to find out if for instance the immune system is intact or even activated in fighting an infection.

In AIDS, the T cell subset ratio was one of the first immunological tests examined. A profound deficiency in immune function underlies the ongoing epidemic of AIDS. A reversed $T_h/T_{c/s}$ cell ratio (from about 1.7 in normal to about 0.5 or less in AIDS patients) is one of the characteristic findings when studying the patients immune status (Gottleib et al, 1981).

In full blown AIDS, this decrease or reversal of the normal ratio is due to the lyphopenia, or more specific, the marked reduction in absolute numbers of T helper cells. As previously mentioned the decrease in T helper cell count is due to the tropism of HIV for T_4^+ cells.

Altered ratios have also been reported to be a common occurrence in haemophiliac subjects treated with lyophilised Factor VIII concentrates (Ludlam, Carr Veitch et al, 1983) and this has been found even in the apparent absence of HIV infection by Ludlam and colleagues (Carr, Veitch, Edmond et al, 1984). Although in AIDS patients the reduction in ratio is due primarily to a reduction in T_4 numbers in haemophiliacs, the cause has sometimes been found to be a combination of reduced T_4 and increased T_8 numbers, a point which will be discussed later.

In the AIDS free haemophiliacs this abnormality could be due to the continued infusion of excessive amounts of foreign protein in the form of the Factor VIII concentrates.

The subset ratio therefore, although not diagnostic of AIDS on its own, can be used to examine immune alterations in patients at risk from AIDS. Perhaps of more use in HIV positive patients is the enumeration of absolute number of T_4^+ cells.

This study has examined the T cell subset ratios in our haemophiliac population. The absolute numbers of T_4 cells have also been enumerated in an attempt to correlate differences with the previously mentioned parameters.

4.2 MATERIALS AND METHODS

4.2.1 Cell Preparation

Heparinised blood was obtained by venepuncture and peripheral blood mononuclear cells were isolated by a modification of the method by Boyum (1968). Briefly, blood was diluted 1:1 with Minimum Essential Medium (MEM) and layered on to lymphoprep gradients (Nycomed, Birmingham, U.K.). After centrifugation at 400 g for 30 minutes, the interface containing the peripheral blood mononuclear cells (lymphocytes and monocytes) were removed and washed twice in MEM by centrifugation and then resuspended for counting.

4.2.2 Staining with Monoclonal Antibodies

The method used was indirect staining using a monoclonal antibody and a FITC-conjugated secondary antibody to the monoclonal. Monoclonal antibodies used were purchased from commercial suppliers. In this study, we used the following monoclonal antibodies as shown:-

Specificity	Mab	Equivalent
T Total	Leu 4*	OKT_3
T Helper	Leu 3a	OKT_4
T cytotoxic/suppressor	Leu 2a	OKT_8

*Monoclonals were purchased from Becton Dickinson (Lab. Impex, Richmond, U.K.)

Cells to be stained were counted and adjusted to $5x10^6$/ml in Hanks/10% FCS. 0.2 ml aliquot ($1x10^6$ cells) were used for each monoclonal being tested. Monoclonal antibody was added to appropriate tube as per Manufacturer's recommended use.

Leu 4	-	20µl	
Leu 3a	-	20µl	Per 0.2 ml (10^6 cells)
Leu 2a	-	20µl	

Cells were incubated on ice for 45 minutes. After incubation cells were washed twice in Dulbecco's PBS/10% FCS by centrifugation at 400 g for 10 minutes at 4°C.

For the second incubation a commercially obtained FITC-conjugated Goat anti-Mouse Immunoglobulin from Becton Dickinson was added to each tube. 200 µl at 1:250 dilution was used. The tubes were then once again incubated on ice for 30 minutes after which they were washed as previously described. After the final wash the cells were fixed by addition of 1 ml of 1% Paraformaldehyde fixative solution and stored at 4°C until enumeration. As a negative control for non-specific staining by the secondary antibody, one tube received 5µl of mouse IgG instead of the monoclonal antibody and then the secondary antibody as described.

The percentage positive cells staining for each monoclonal were enumerated on a Becton Dickinson Flourescein Activated Cell Sorter (FACS).

All blood samples were taken from patients who were clinically well, showing no obvious signs of infection or illness (other than HIV infection where applicable).

Factor VIII concentrate consumption was assessed from the patients' case notes and records and mean consumption of factor concentrate for previous years calculated. Patients who had used more than 20,000 units of concentrate annually for the previous three years were considered to be "heavy users".

4.3 RESULTS

There is no significant difference in the absolute number of lymphocytes when comparing haemophiliacs with an age and sex-matched control population, the medians and ranges being 1.8 (0.8-5.51) and 2.05 (1.4-5) x 10^9/L cells respectively. Also, there is no difference when comparing the various groups of haemophiliacs either with themselves or with the controls. The HIV^+ haemophiliacs had a had a median lymphocyte count of 1.84 (range 1.02-3.7) x 10^9/L, the "heavy users" 1.63 (range 0.8-3.68) x 10^9/L and the "light users" a median of 1.75 (range 0.93-5.51) x 10^9/L. The use of monoclonal antibody staining allowed enumeration of absolute numbers of T cells, T helper cells and T suppressor cells by multiplying the percentage positive staining cells by the total lymphocyte count obtained from routine haematology differential white cell counts. The results are shown in Table 4.1.

The only significant differences were in the T_4 (T helper) numbers. When all treated haemophiliacs were taken together and compared to controls there was a significant difference ($p<0.05$). The HIV^+ T_4 numbers also differed significantly from controls, ($p<0.001$), from HIV negative haemophiliacs ($p<0.05$) and from HIV negative heavy users ($p<0.05$). No differences were seen in T_3 (total T cell) or T_8 (cytotoxic/suppressor T cell) when comparing any group combinations. There was, however, perhaps a trend that a few HIV^+ haemophiliacs had increased T_8 numbers. Although not significant, this would be in keeping with some other workers' findings that, on occasion, haemophiliacs, especially those who are HIV^+, can have increased T_8 numbers.

A number of the seropositive haemophiliacs were studied

	ALL HAEMOPHILIACS (n=54)	HIV+ (n=18)	HIV- HEAVY USER (n=19)	HIV- LIGHT USER (n=17)	CONTROL (n=12)
T_3	1.14† $ (0.52 - 3.58)	1.14 (0.77 - 2.44)	1.07 (0.52 - 2.80)	1.32 (0.54 - 3.58)	1.38 (1.05 - 2.4)
T_4	0.62 (0.22 - 2.42)	0.53 (0.3 - 0.81)	0.62 (0.3 - 1.63)	0.64 (0.22 - 2.42)	0.84 (0.57 - 1.52)
T_8	0.47 (0.12 - 1.63)	0.48 (0.3 - 1.63)	0.45 (0.17 - 1.18)	0.42 (0.12 - 1.43)	0.52 (0.36 - 0.81)
T_4/T_8 RATIO	1.38 (0.25 - 4.98)	0.9 (0.25 - 1.75)	1.53 (0.76 - 3.72)	1.6 (0.62 - 4.98)	1.56 (1.16 - 2.47)

†Median (range)

$ ($x10^9$Cells/L).

Table 4.1: Absolute T Cell Numbers and Ratios in Haemophiliacs.

previously, i.e. from mid-1984 to early 1985. Data was available for absolute cell counts on total lymphocytes and T cell subsets. The cohort studied earlier differed only in the absolute numbers of T helper cells when compared to the HIV^+ patients in this study ($p<0.05$). The medians and ranges being 0.69 (0.4-1.74) x 10^9cell/L for the 1984-85 cohort compared to 0.53 (0.3-0.81) x 10^9 cells/L for this present study. Eight of the patients in this study had been examined earlier and therefore it was possible to do a comparison on absolute numbers in 1984-85 compared to 1986-87. However as Table 4.2 shows there were no significant differences in either total lymphocyte count or in any of the T cell subset counts for these eight patients.

	TOTAL LYMPHOCYTES	T TOTAL	T HELPER	T CYTOTOXIC/SUPPRESSOR
1984-85	1.83 † # (0.9 - 2.45)	1.25 (0.67 - 1.62)	0.6 (0.4 - 1.08)	0.57 (0.19 - 1.07)
1986-87	1.95 (1.73 - 2.99)	1.24 (0.89 - 1.97)	0.65 (0.32 - 0.73)	0.72 (0.26 - 0.95)

† Median (range) n=8.

$x10^9$/L.

Table 4.2: Lymphocyte Counts of Paired Samples from HIV^+ Patients.

4.4 DISCUSSION

Lymphopenia has been described as one of the major laboratory findings amongst AIDS patients. Carr et al (1984) have also reported lymphopenia in a cohort of their haemophilia A populaton. This study has not found lymphopenia either in the haemophiliac population as a whole or in those who are HIV antibody positive. Many workers have reported inverted T cell subset ratios (T_4/T_8) in both AIDS patients and also in the haemophiliac population (Moffat et al, 1985). This study too has found abnormalities in the subset ratios. However, this difference is only significant in the group of patients who are HIV^+. The major contributing factor to this abnormality is a marked decrease in T_4 numbers ($p<0.01$: vs control). Some workers (Stein et al, 1985) have shown an increase in T_8 numbers as a cause for inverted ratios, while others (Carr et al, 1984) have found normal T suppressor (T_8) numbers in their studies. This study showed no significant difference in T_8 numbers although there may be a slight trend for HIV^+ haemophiliacs to have higher numbers. If the cause for inversion of the ratio is studied for each individual then the vast majority are due to a decrease in absolute numbers of T_4.

As mentioned, many reports have suggested that inverted ratios in haemophiliacs are caused by an absolute increase in T_8 numbers. An inversion of this type is often referred to as "immune augmentation" and is known to occur as a response to a variety of stimuli, especially viruses. Haemophiliacs are subjected to repeated antigenic stimulation from the plasma proteins and viruses found in clotting factor concentrate (Gjerset, Martin, Counts et al, 1984). It is of interest that most of the reports of increased T_8 numbers come from the United States and countries heavily dependent on their commercially produced

factor concentrates, whereas reports from Scotland (Carr et al, 1984) and this work where the patients are treated with "home produced" concentrate do not find this increased T_8 effect. Perhaps there is something different in the manufacturing process resulting in different protein constituents in the concentrate or perhaps the commercial concentrate has more contaminating viruses which could cause this "immune augmentation" effect. As mentioned in Chapter 2, the commercial concentrate is made from paid donations rather than volunteer donation as is the case in Scotland. It is well known that this encourages undesirable groups to donate, e.g. drug addicts who need money to pay for their drug requirements. Homosexuals have also been shown to represent a substantial percentage of all donors and, of course, it has been shown that such groups very often have a high rate of viral and other infections which could carry over into blood donations and therefore into blood products.

The amount of factor concentrate consumption does not seem to be an important factor as there was no differences between "heavy" and "light users". All of the HIV^+ patients are heavy users of concentrate. As their T_4 numbers differed significantly from HIV^- "heavy users" ($p<0.05$), then the inference is that the abnormality is not due simply to the use of concentrate but rather to exposure to HIV.

These findings are in keeping with what is known about HIV, i.e. that it is selectively tropic for the T_4 subpopulation of T cells. The results from the comparison of the eight HIV^+ patients in 1984-85 to 1986-87 are interesting. It may have been expected that there would have been a decrease over time of absolute number of lymphocytes and more specifically of T_4 cells in the HIV^+ population as has been shown to be the case in AIDS patients. Perhaps it is simply that the time span between the two samples is too short to see any significant

decrease in cell numbers, or perhaps the T helper cell count will only drop as clinical signs of infection develop. Whatever the reason, from a prognostic point of view it is obviously good to have a steady T helper cell count with no apparent drop in cell numbers. The fact that there was a significant difference in T_4 numbers in an unmatched population of HIV^+ patients at the two different time points may suggest that as the length of the time of infection increases there is a trend in the population towards a decrease in T_4 numbers. However, it would certainly be of more value to follow the same cohort over time thus allowing comparisons on T_4 cell numbers within individuals which would better reflect what will be happening in the HIV^+ population as time progresses.

The implications of having a decrease in T_4 numbers is, of course, very worrying from an immunological standpoint. As described earlier, the T_4 cell is probably the most important cell in the immune response having such an immunoregulatory role.

In summary, there is a decrease in absolute numbers of T_4 cells only in the HIV^+ population. Therefore, abnormalities seem to be associated with HIV infecton and not specifically with amount of Factor VIII usage. The more concentrate a patient uses, however, the greater the risk of receiving a batch contaminated with HIV. In a recent study, Gupta (1987) has shown that double monoclonal antibody staining allows subdivision of the T_4 population into inducers of help and inducers of suppression. Both supopulations are equally depleted in early HIV infection. This is currently being studied in our haemophiliac population.

Investigations on other arms of the immune response are needed to see what effect either factor consumption or HIV infection with consequent depletion of T_4 cells has had on the immune status of the haemophiliacs.

CHAPTER FIVE

RESPONSE TO MITOGEN AND SOLUBLE ANTIGEN

5.1 INTRODUCTION

One of the easiest and therefore usually one of the first laboratory investigations to be carried out when studying the immune response is to measure the ability of the patients' cells to respond to *in vitro* stimulation. There are many agents generally termed mitogens which can be used. As the name suggests these agents switch on mitotic divisions, stimulating lymphocytes into DNA synthesis, blast transformation, and ultimately division of the lymphocytes. Lectins which are non-enzymatic, non-antibody sugar binding proteins are the most commonly used mitogens. It is now known that lectin induced proliferation reflects IL-2 dependent expansion of IL-2 reactive T cells. The mechanism involved, although not completely understood, is more complex than the simple binding of lectin to the cell surface. There are probably complex cellular interactions involving presentation of lectin and also the requirement of IL-2. Many mitogens are specific for either T cells, B cells or both and this study has used Phytohaemagglutinin (PHA) and Conconavalin A (Con A) both T cell mitogens and Pokeweed Mitogen (PWM) which is known to be a T cell dependent B cell activator.

Another approach to study *in vitro* proliferation is to use a soluble antigen. Whereas mitogen stimulates large numbers of lymphocytes, antigens stimulate far fewer cells which are specifically sensitised to the antigen in question. In this study the soluble recall antigen, Tuberculin PPD, which is a purified preparation from Mycobacterium tuberculosis was used. Most people will have been vaccinated against T.B. and should recognise and respond to PPD.

The T helper cell as mentioned previously is the central cell type in the immune system. One of its primary unique immune functions among

those of the T cell repertoire is its ability to recognise and proliferate in response to soluble protein antigens, which must be processed and presented to it by antigen presenting cells such as blood monocytes. This recognition capability coupled with the ability of the helper T cell to recruit other immune cells into action is thought to be the essence of the specificity of the immune system. One of the most constantly reported immunologic abnormality in AIDS patients is decreased blast transformation to mitogens and antigens (Gottleib et al, 1981) (Lane et al, 1983). Lane and colleagues (1985) have shown a decrease in the response to soluble antigen by lymphocytes from AIDS patients. Their study suggests that the defect is caused by an intrinsic defect in the ability of the lymphocytes to recognise and respond to soluble antigen. Reduced responses to mitogens have also been described in various studies on the haemophiliac population (Moffat, Bloom, Jones et al, 1985) (Stein et al, 1985).

This study has looked at the patient's response and tried to correlate any abnormality with the usage of concentrate or exposure to HIV.

5.2 MATERIALS AND METHODS

5.2.1 Patients

A cohort of 50 patients were studied. Patients were grouped according to their HIV antibody status and their mean annual concentrate consumption.

5.2.2 Cell Preparation

Cells were separated as described in Chapter 4. For assay cells were cultured in RPMI supplemented with 10% pooled human serum (RPMI/10% PUHS).

5.2.3 Mitogens and Soluble Antigen

The mitogens PHA, Con A and PWM were all purchased from Sigma (Poole, U.K.). PPD was obtained from The State Serum Institute, Copenhagen, Denmark.

The mitogens have been used for many years in this laboratory and have been standardised many times. A representative dose response curve is shown in the results section for each. The final concentrations used were PHA (5µg/ml), Con A (50µg/ml) and PWM (0.1µg/ml). The optimum cell concentrations had previously been determined to be 5×10^5/ml for PHA and Con A and 10^6/ml for PWM. It was necessary to optimise the concentration of both cells and PPD to be used in the antigen stimulation assay, the results of which are given in the results section. For the assays cells were used at 10^6/ml and PPD at 5 or 0.5µg/ml. All concentrations are final concentrations.

5.2.4 Assay

Cells were cultured in triplicate in 96 well 'U' shape micro-

titre plates (Gibco, Paisley, U.K.). To 100 µl of cell suspension was added, 100 µl of mitogen or antigen to give final concentration as stated above. Cells were cultured for 3 days for mitogen and 5 days for PPD at 37°C in a humidified atmosphere with 5% CO_2. Four hours to end of assay (19 hours for PPD) each well was pulsed with 1µCi of ^{3}H- Thymidine. The cultures were harvested on an automatic cell harvester (Skatron, Flow Labs, Irvine, U.K.) by suction through discs of glass fibre paper, the paper was dried and the discs were transferred to scintillation vials. Following addition of scintillation fluid, samples were counted on a Beta counter (LKB Croydon, U.K.). and thus, an estimate of the mitotic activity in the cultures as determined by incorporation of ^{3}H-Thymidine into DNA was obtained.

5.2.5 Expression of Results

Results are expressed as a corrected count per minute. The mean of triplicate of results obtained in the absence of any mitogen (or antigen) ie. spontaneous incorporation of ^{3}H-Thymidine is subtracted from the mean of triplicate from result in the presence of stimulus.

5.3 RESULTS

Figures 5.1 to 5.5 show the results of standardisation of the various mitogens and antigen.

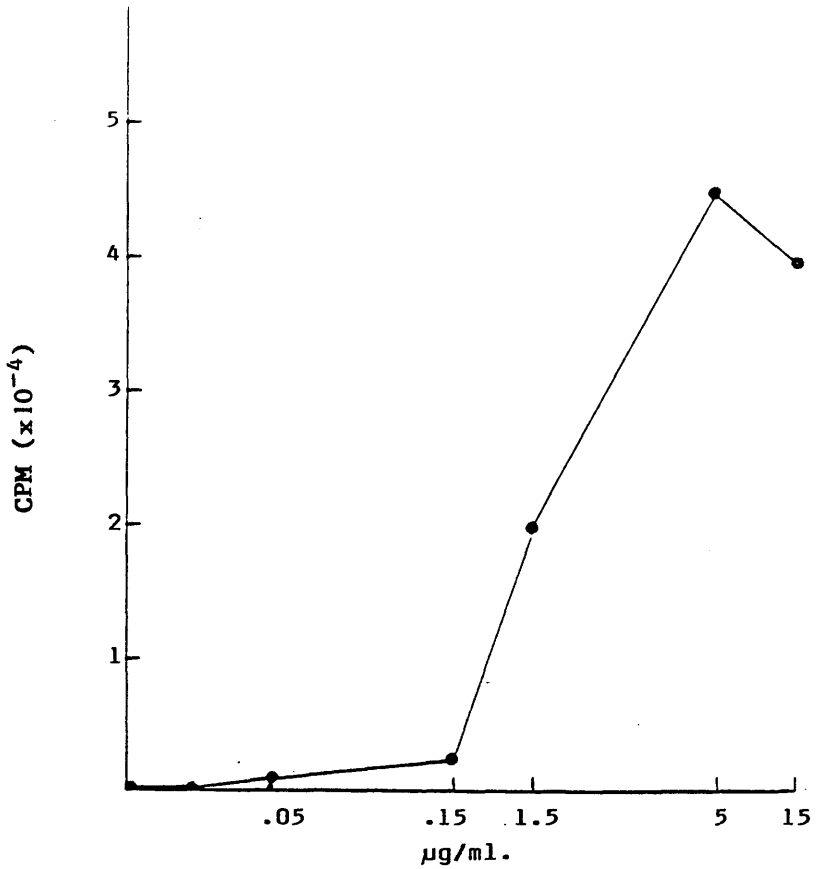
The patients are divided into groups as for other tests. These groups are denoted by the following:-

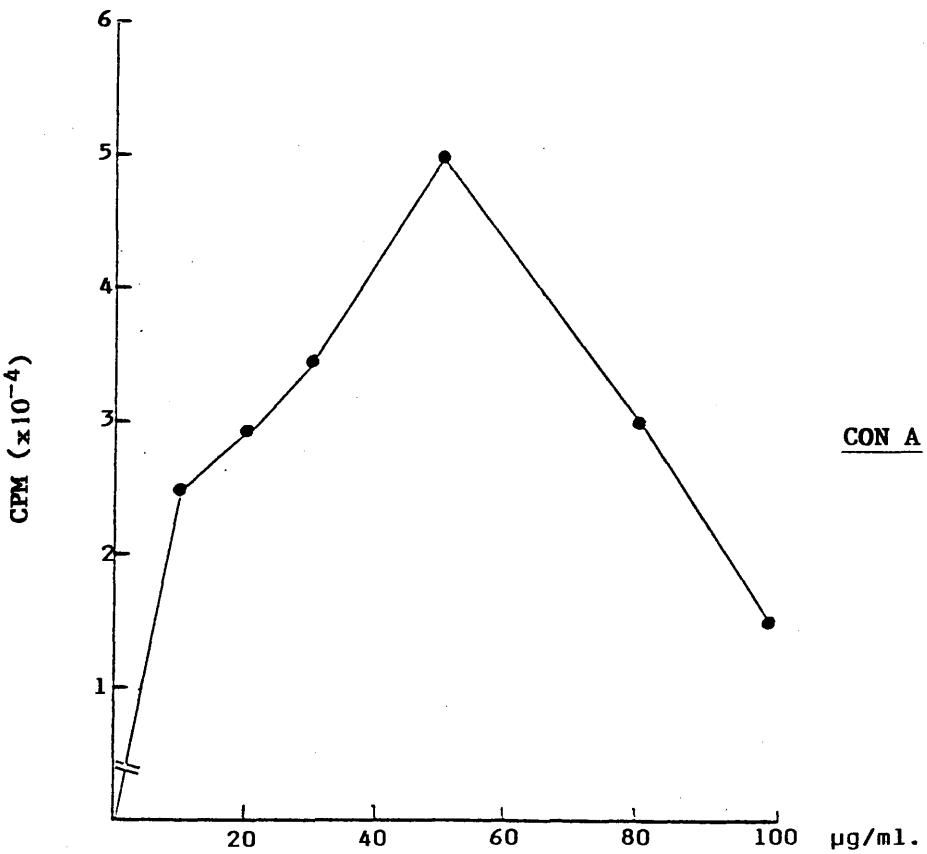
"Heavy users" - (H)
"Light users" - (L)
Not treated - (N)
Controls - (Con)
HIV^+ haemophiliacs - (+)

This nomenclature is used in all subsequent scatter plots.

A scatter plot for response to PHA is shown in Figure 5.6. When treated haemophiliacs are considered as one group, irrespective of HIV status, their response to PHA is not significantly different from controls. However, when patients are divided either on grounds of HIV status or factor usage, then differences are seen.

HIV^+ patients have a reduced response compared to treated negative haemophiliacs ($p<0.005$) and compared to controls ($p<0.05$). Treated HIV negative haemophiliacs do not differ significantly from controls. When looking at another T cell mitogen, Con A, no significant differences are seen between any of the groups of patients or between patients and controls as shown in Figure 5.7. The T cell dependent B cell mitogen PWM was also studied and results are shown in Figure 5.8. All treated haemophiliacs taken as a single group have a reduced response compared to the control population ($p<0.005$). HIV^+ haemophiliacs also have a reduced response compared to treated HIV^- haemophiliacs taken together ($p<0.005$) and also negatives when split into "heavy users" ($p<0.001$) and "light users" ($p<0.005$). The HIV^- treated haemophiliacs do not differ from controls.





Figures 5.1 and 5.2: Dose Response Curves of Mitogens.

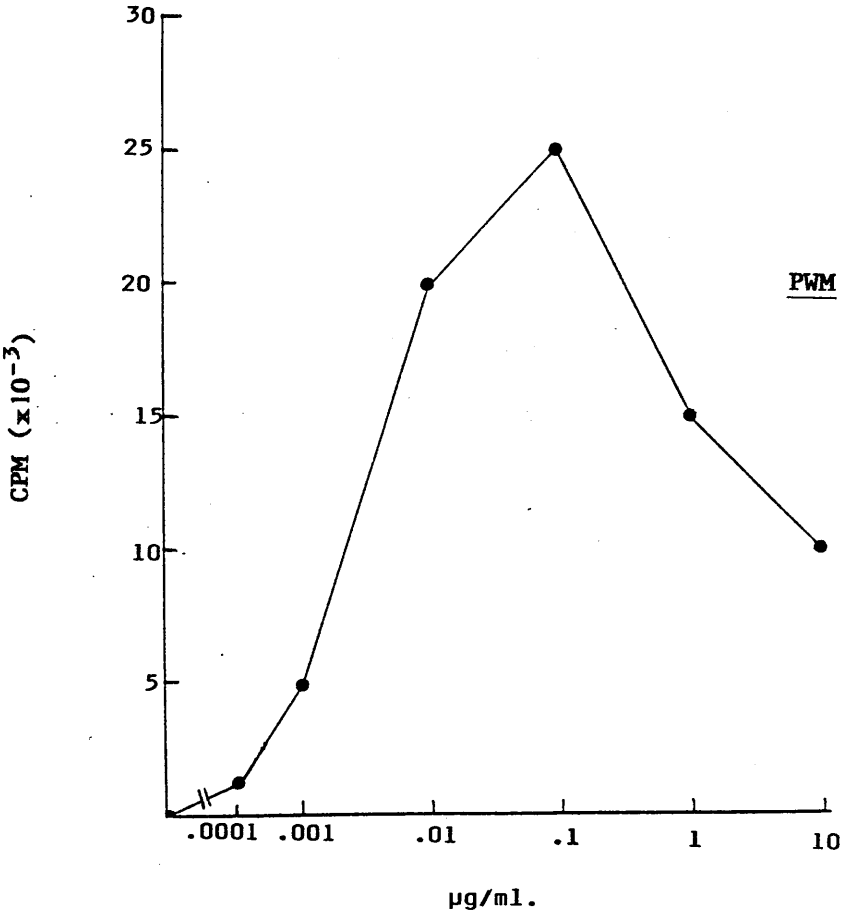
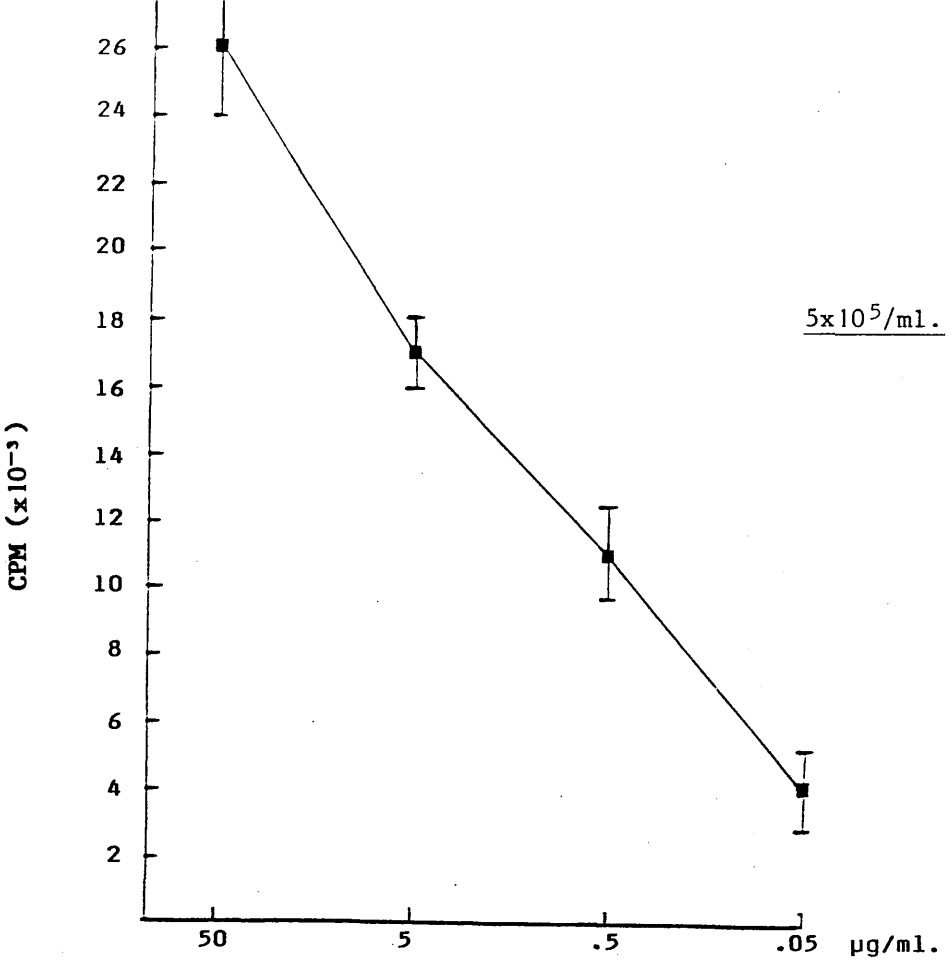
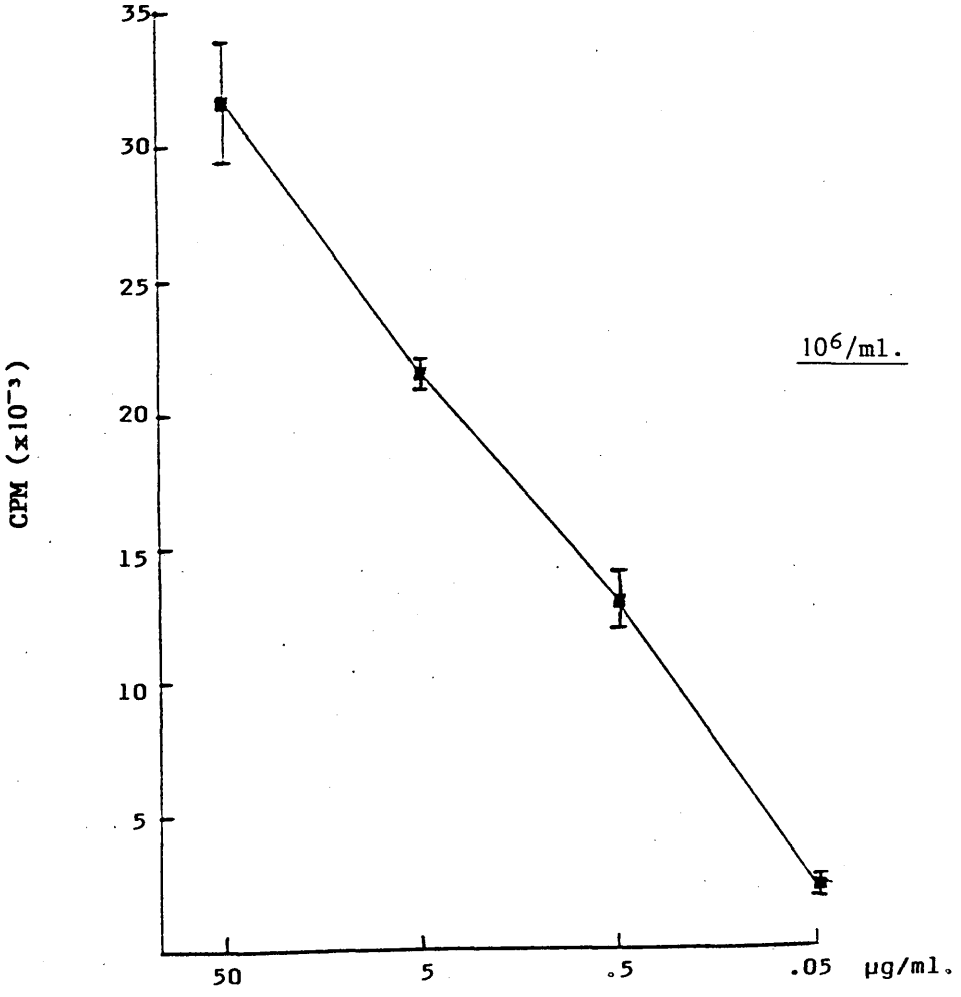


Figure 5.3: Dose Response Curve of PWM.





Figures 5.4 and 5.5: Dose Response Curves to PPD Cells at $5x10^5$/ml. and 10^6/ml.

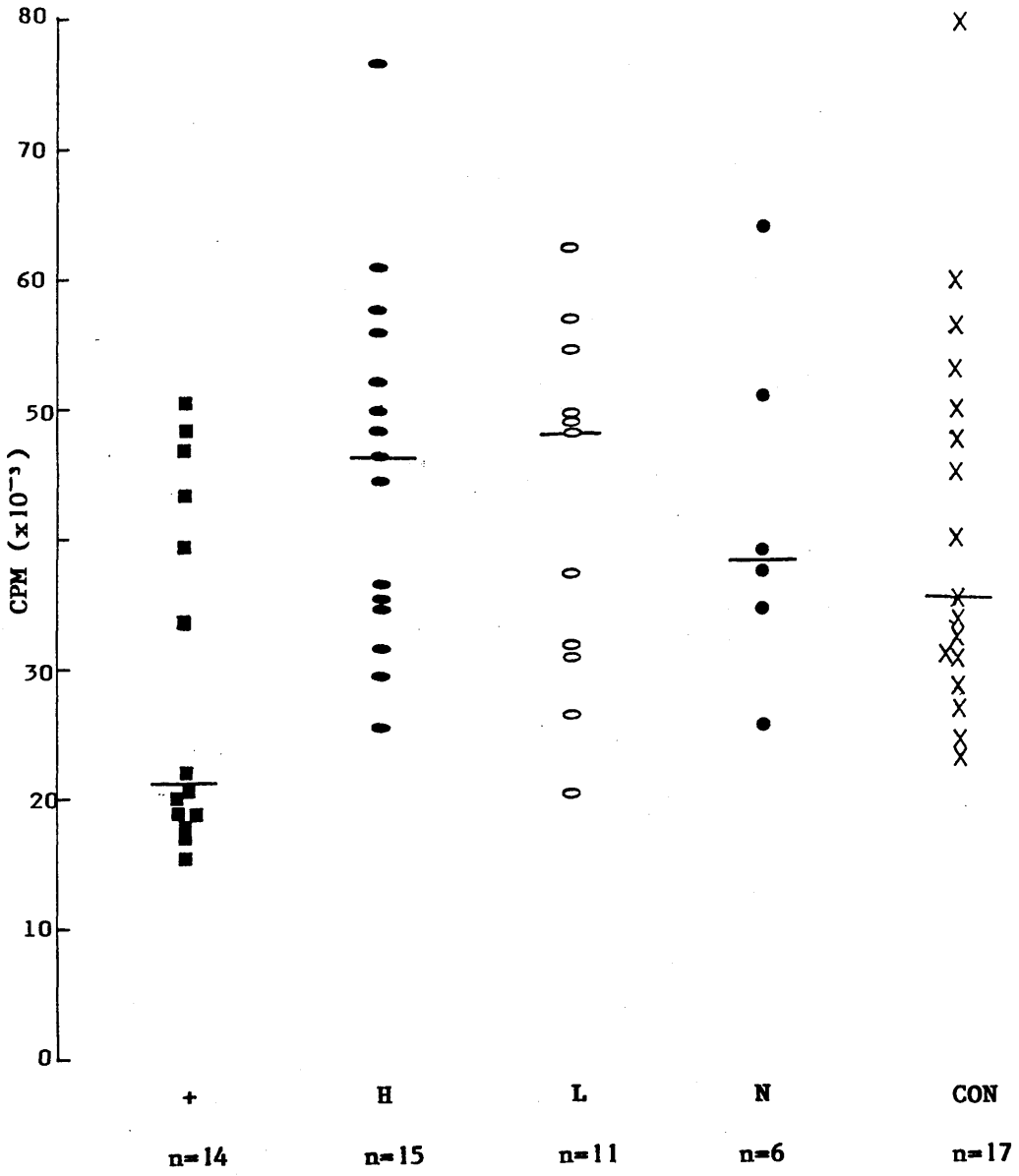


Figure 5.6: Response to PHA (5µg/ml).

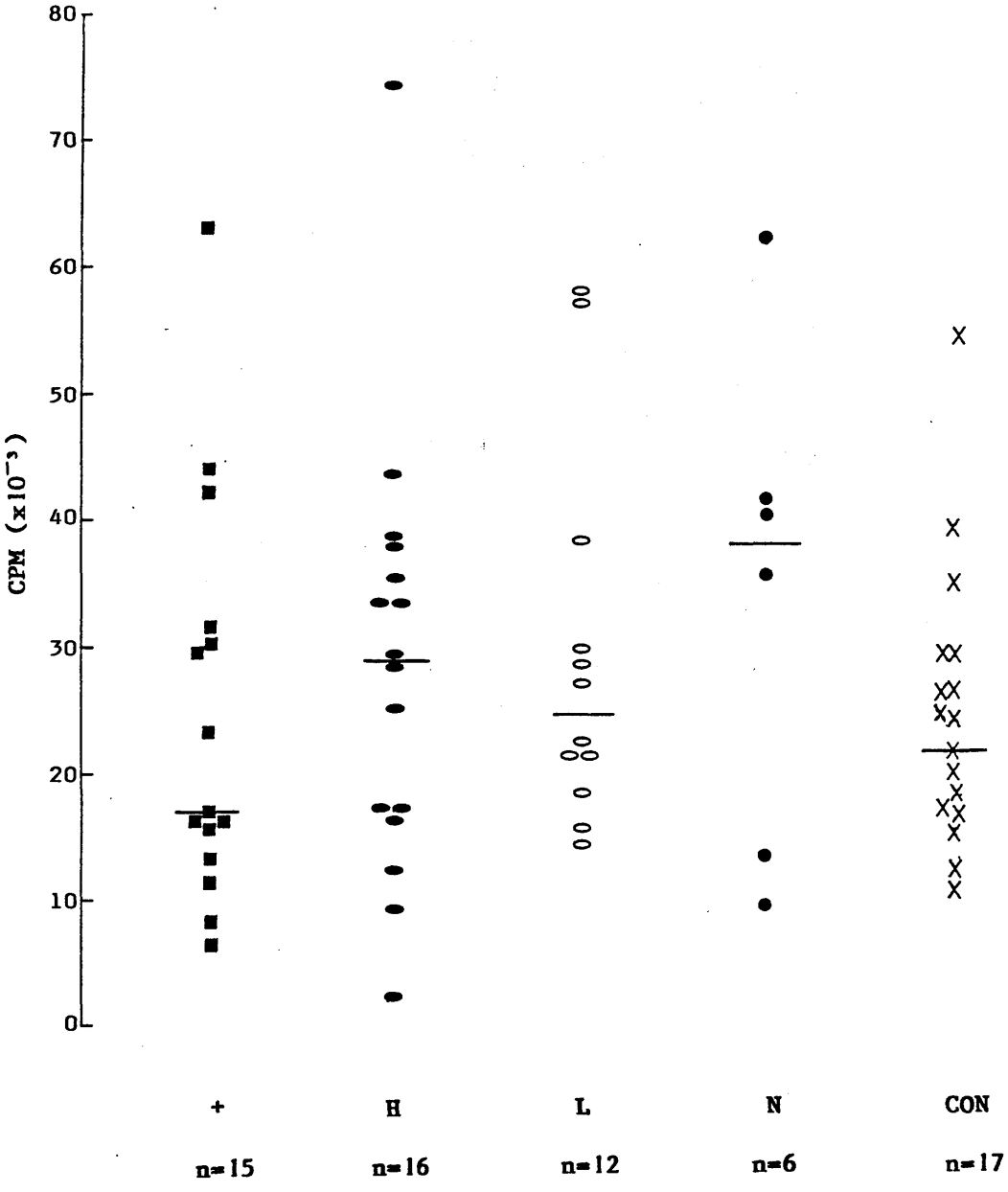


Figure 5.7: Response to CON A (50µg/ml)

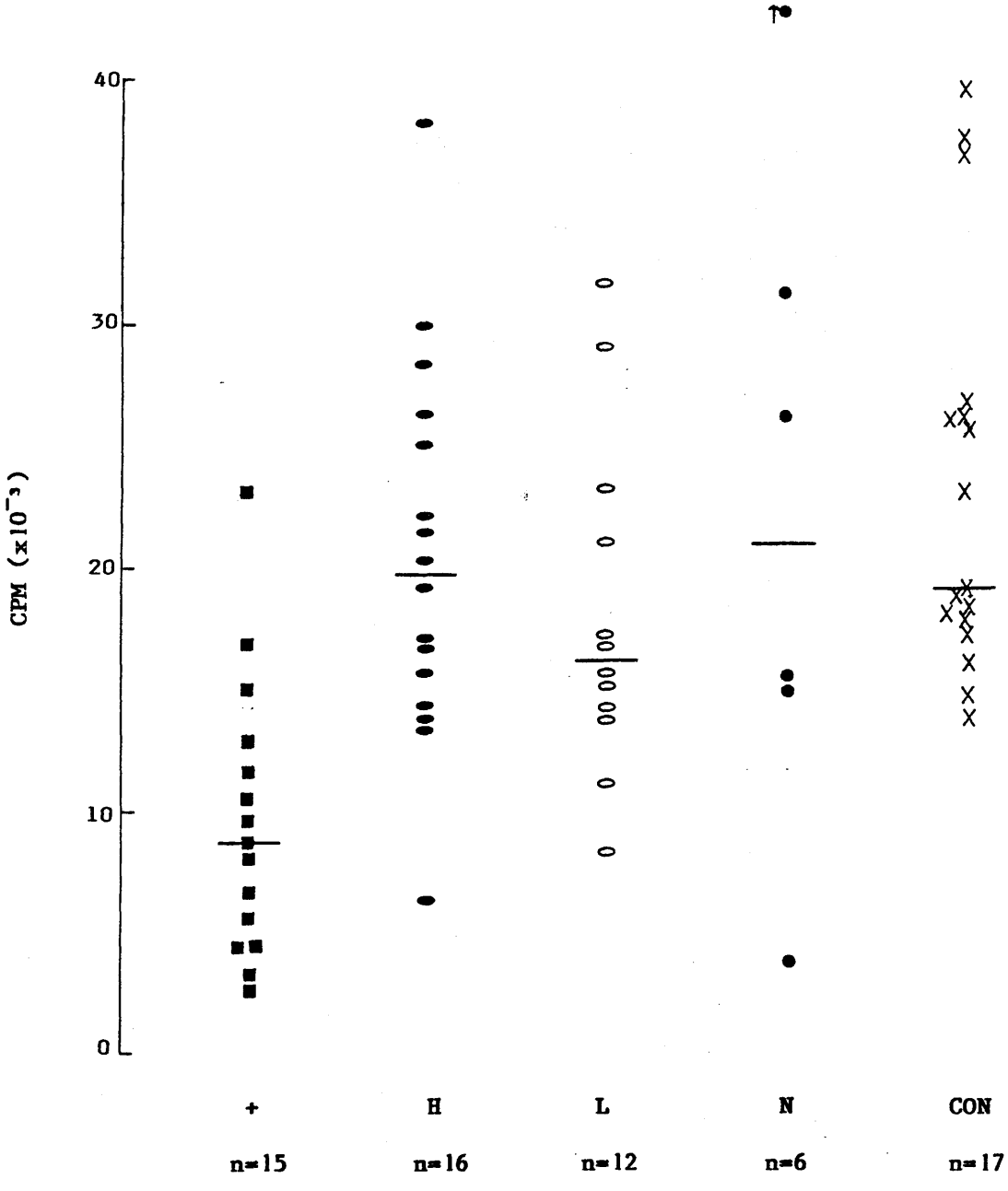


Figure 5.8: Response to PWM (0.1 μg/ml)

The results obtained for the PPD stimulation at both concentrations are essentially the same, as shown in Figures 5.9 and 5.10. All treated haemophiliacs as a group had a reduced response compared to controls, $p<0.02$ at 5 µg/ml and $p<0.01$ at 0.5 µg/ml. Comparing HIV^+ with treated HIV^- the former had a reduced response at both PPD concentrations, $p<0.005$ at 5 µg/ml and $p<0.002$ at 0.5 µg/ml. HIV^+ haemophiliacs also had a significantly reduced response to PPD at both concentrations when compared to controls, $p<0.001$ at both PPD concentrations. HIV^- treated haemophiliacs collectively or split according to concentrate usage did not differ from controls.

Finally the positive haemophiliacs showed a reduced response compared to both "heavy" and "light" seronegative users; $p<0.005$ and $p<0.05$ respectively when 5 µg/ml PPD was used and $p<0.01$ and $p<0.01$ respectively at 0.5 µg/ml PPD concentration.

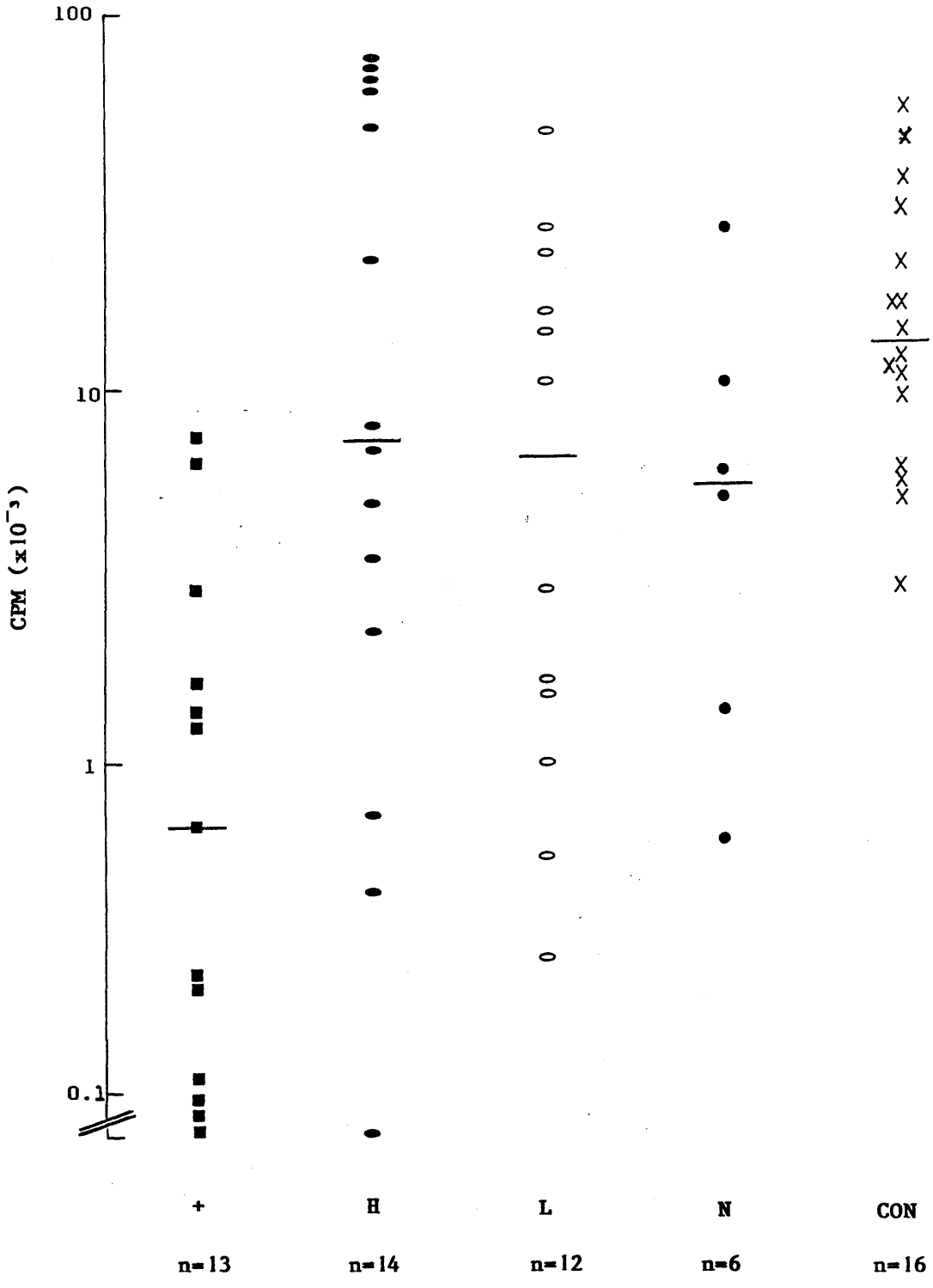


Figure 5.10: Response to PPD (0.5µg/ml).

5.4 DISCUSSION

The findings of this study differ somewhat from other studies in that significant differences in patients' mitogen responses compared to those of controls were only seen in haemophiliacs who were HIV^+. No mitogen or PPD gave a significantly decreased response when HIV^- treated haemophiliacs were compared to a control population. Therefore, the use of Factor VIII concentrate, irrespective of the amount, does not seem to cause an impairment of mitogenic response. Other groups (Lee et al, 1985) (Moffat et al, 1985) correlated a reduced response with severity of haemophilia in those asymptomatic for HIV infection. This study, however, only showed impaired responses in those who are HIV^+. Our HIV^+ haemophiliacs had a reduced response to PHA, PWM and PPD but not Con A. The reduced responses are most probably due to the observed drop in T_4 numbers in this group. It is known that PWM is a T cell dependent B cell mitogen (i.e. requires T cell help [from T_4^+ cells]). Also, the PPD response requires antigen recognition by T_4^+ cells and therefore if T_4^+ numbers are low it would be expected that the response will also be diminished. This is the cause here, and is in agreement with other studies on AIDS patients and those who are HIV^+ (Lane et al, 1985).

PHA and Con A are both T cell mitogens yet the response to only PHA is reduced in the HIV^+ group. It is possible that these two mitogens stimulate different subpopulations of T cells and this could account for the difference if, for instance, the population which responds to Con A is normal in this group of patients while that population which responds to PHA is in some way affected or perhaps reduced in number.

Most of the reported studies on haemophiliacs find a reduced number of T_4 cells as well as a decrease in proliferative response to mitogen, irrespective of HIV status. In this study, however, T_4 numbers were reduced only in the HIV^+ patients. This could account for the discrepancy between this and other studies, i.e. no abnormality was found in the absence of HIV infection.

In summary, therefore, as proliferation to stimulus was normal in the HIV^- groups, factor consumption does not seem to impair the response. In the HIV^+ group, there was a significant decrease in response which correlates with a drop in T_4 numbers in this group. Continued monitoring using these tests may be useful in determining if asymptomatic haemophiliacs continue to have a normal response and also to determine if the <u>in vitro</u> response to mitogen or antigen declines in the HIV^+ group as T_4 cell numbers drop or as their clinical condition deteriorates.

CHAPTER SIX

INTERLEUKIN-2

6.1 INTRODUCTION

Interleukin-2 (IL-2), originally termed T cell growth factor, represents one element in a cascade of lymphokines released during an immune response as can be shown in Figure 6.1. The original reports of mitogenic factors present in mixed leucocyte cultures came in 1965 (Gordon and McLean, 1965). It was thought then that many of the factors present in culture supernate were of T cell origin. In 1976 there was a major advance with the discovery that it was possible to select and maintain the continuous proliferation of T cells (Morgan, Ruscetti and Gallo, 1976). This discovery meant that it was possible to construct a specific, quantitative assay to measure the mitogenic factor responsible for T cell growth (Gillis, Ferm, Ou, et al, 1978). Thus, for the first time, it was possible to demonstrate the existence of an activity separate from Lymphocyte Activity Factor (LAF) that functioned in promoting continued T cell growth (Smith, Gillis and Baker, 1979). As a result of these findings, it was decided to use common terms for putative macrophage-derived factors and lymphocyte derived factors. Interleukin-1 was chosen for macrophage-derived factors and Interleukin-2 was chosen was lymphocyte-derived factors. In recent years much work has concentrated on the role of IL-2 on the immune system.

Primarily associated with T cell proliferation, IL-2 also directly or indirectly plays a role in the proliferation and maturation of other cell types. Such responses depend quantitatively on a source of IL-2 and expression of receptors for the factor (Smith, 1980). It is now known that IL-2 is released by T cells in response to two signals supplied by antigen pulsed accessory cells such as monocytes. The first signal is antigen, presented in the context of proteins of

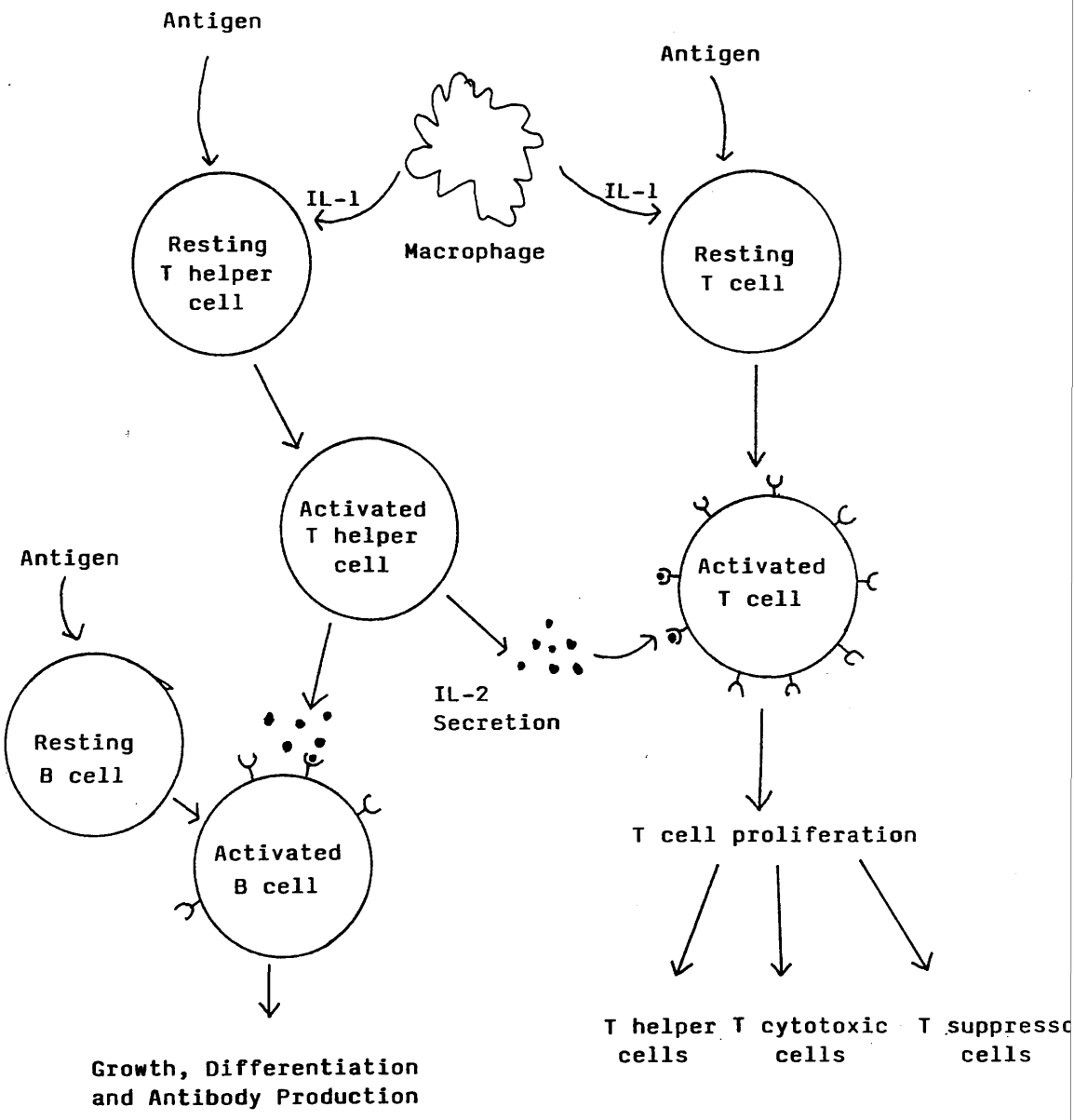


Figure 6.1: Role of IL-2 on T Cell and B Cell Growth.

the Major Histocompatibility Complex (MHC) and the second signal is Interleukin-1 (formerly LAF). Although all subclasses of T cells have been shown to release IL-2 under the appropriate conditions, helper T cells appear to be the major source (Pfizenmaier, Scheurich, Daubener et al, 1984). This, therefore, could have important consequences in AIDS where the T helper cell, the major producer of IL-2 is the very cell which HIV attacks and destroys. Much work has looked at IL-2 production in AIDS patients (Hauser et al, 1984) and those who are infected by HIV (Borzy, 1987).

Although some of the early studies suggested that the production of IL-2 by cells from AIDS patients was normal (Lane et al, 1985), it is now generally accepted that there is a deficiency of IL-2 production in response to mitogen when whole cell populations from AIDS patients are examined (Murray, Welte, Jacobs et al, 1985). This deficiency has been found to be proportionate to the extent of T cell depletion in the cell preparations studied (Gluckman et al, 1985). However, surviving T cells respond normally to the addition of exogenous IL-2 (i.e. proliferation, IL-2 receptor expression, enhancement of cytotoxicity and NK cell activity) (Gluckman et al, 1985 ; Hauser et al, 1984 ; Reddy, Pinyarat and Grecco, 1984).

To examine the haemophiliac population an assay to measure IL-2 production by patients lymphocytes <u>in vitro</u> has been set up in this laboratory. Most of the assay systems for measuring IL-2 are biological assays which make use of cell lines or primed cells which require the presence of IL-2 for continued growth. Other systems have been devised to quantitate IL-2 in supernate samples by ELISA.

6.2 MATERIALS AND METHODS

6.2.1 Technical Aspects of Assay

The assay made use of the IL-2 dependent cell line HT_2A (Cetus Corp., California, USA). This cell line was maintained in our laboratory by feeding with exogenous human recombinant IL-2 (BCL,Lewes, U.K.) every 48 hours and cutting back and re-seeding as necessary. To prepare for an assay, cells were seeded at a density of $4x10^4$/ml and fed with 20 U/ml IL-2. They were incubated at 37°C/5% CO_2, pulsed at 48 hours with more IL-2 and then used for assay the next day. Cells had usually reached a density of 10^6/ml on the day of assay.

For the assay, enough cells were removed from the flask, washed twice in complete RPMI/20% FCS and resuspended at $1.5x10^5$/ml. 100 µ/l aliquots were dispensed into flat bottomed 96 well plates (Nunc. Gibco Ltd., Paisley, Scotland, U.K) to which were also added 100 µl of dilutions of supernate or standard being tested (in triplicate). After approximately 24 hours incubation, the plates were pulsed with 1µCi ^{3}H-Thymidine (20 µl of 50µ Ci/ml per well) and incubated for a further 4 hours. Plates were then harvested on a cell harvester (Skatron, Flow Labs., Irvine, U.K.). Cells were washed onto filter paper discs and incorporated ^{3}H-Thymidine measured on a Beta counter.

6.2.2 IL-2

The IL-2 used for maintaining the cell line and for use as a standard in the IL-2 assay was human recombinant IL-2 purchased from BCL. Although it was recommended to grow cells at 100 U/ml, we found that cells grew better at 20 U/ml and this concentration was used to maintain the cell line.

6.2.3 Cell Preparations

Peripheral blood lymphocytes were obtained as described in Chapter 4 and cultured in complete RPMI/2% FCS. It was decided to use 2% FCS as most published methods use low serum concentrations and if FCS was used then there could be no carry over of human IL-2 which could occur if using human serum.

6.2.4 IL-2 Supernate Production

It was first of all necessary to determine the optimum conditions for producing IL-2. Variables investigated were cell concentration and mitogen concentration. PHA obtained from Wellcome (London, U.K.) was used throughout.

Lymphocytes from normal controls were used to optimise IL-2 production. Cells were used at 5 x 10^6/ml, 2 x 10^6/ml, 1 x 10^6/ml and 0.5 x 10^6/ml in the presence of PHA varying in concentration from 5 µg/ml to 0.5 µg/ml. It was decided to incubate cells for 48 hours as most published methods on IL-2 production have found peak production at this time. After incubating cells for 48 hours supernates were recovered by centrifugation and stored at -20°C until assayed.

Results for calibration of assay are shown in Results Section. For production for supernates by patients it was decided to use cells at 10^6/ml. Most other published methods also used cells at 10^6 /ml. Patients' cells were cultured for 48 hours in the presence of PHA at 2 or 1 µg/ml. As a control for spontaneous production of IL-2, cells were also cultured in RPMI/2% FCS without any PHA.

6.2.5 Expression of Results

Results are expressed as units/ml IL-2 (U/ml) calculated by probit analysis of mean net counts per minute of test supernate

dilutions. Results were determined at the 50% of maximal ^{3}H-Thymidine incoration end point of our laboratory standard. As a standard, human recombinant IL-2 purchased from BCL was used. A standard curve was obtained by serial two-fold dilutions of the laboratory standard, starting at 100 U/ml final concentration.

6.3 RESULTS

Results for standardisation of the assay are shown in Figures 6.2 and 6.3. Results shown represent the mean ± SE for 8 normals at each point. As can be seen from the Figures there is little difference between 0.5 x 10^6 cells/ml and 1 x 10^6 cells/ml at either 2 or 1 µg/ml. However, 2 x 10^6 or 5 x 10^6 cells/ml gave much reduced responses. Other concentrations were tried, i.e. 5 µg and 0.5 µg/ml. At 0.5 µg/ml there was no good response at any cell concentration and at 5 µg/ml the response was very varied. It was decided, therefore, to use cells at 10^6/ml and use the PHA at 2 µg/ml and 1 µg/ml.

Figures 6.4 and 6.5 show the response of the different patient groups at 1 and 2 µg/ml respectively. If all treated haemophiliacs were taken as one group, irrespective of HIV status and compared to normals, then there was no significant difference in IL-2 production when 1 µg/ml PHA was used, but patients produced less than normals at 2 µg/ml ($p < 0.02$). There was a significant decrease in IL-2 production between HIV^+ haemophiliacs and all treated HIV^- haemophiliacs at both PHA stimulation concentration. $p<0.01$ for 1 µg/ml and $p<0.05$ at 2 µg/ml. HIV^- treated haemophiliacs, as one group, did not differ from controls at either concentration. As the numbers in the "never treated group" were small and showed a large range of levels, it was questionable whether or not to include them in the statistical analysis. At 1 µg/ml PHA the "never treated group" did not differ from any other group combination. At 2 µg/ml PHA, however, the "never treated group" showed increased production compared to HIV^+ ($p<0.005$),
"light treated" HIV^- ($p<0.02$), controls ($p<0.05$), all HIV^- treated haemophiliacs ($p<0.02$) and all treated haemophiliacs irrespective of

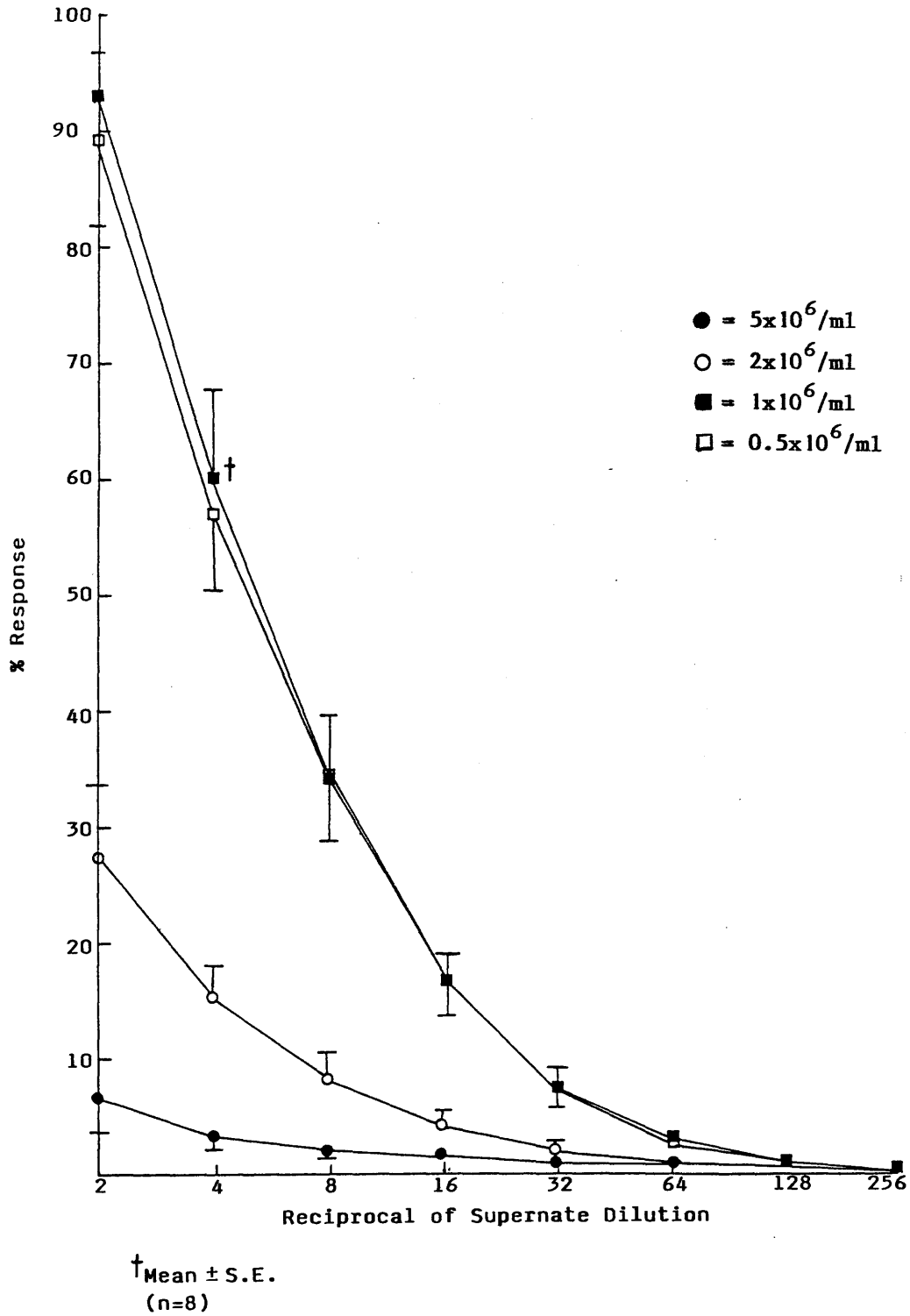


Figure 6.2: IL-2 Production in Normals (PHA at 1μg/ml).

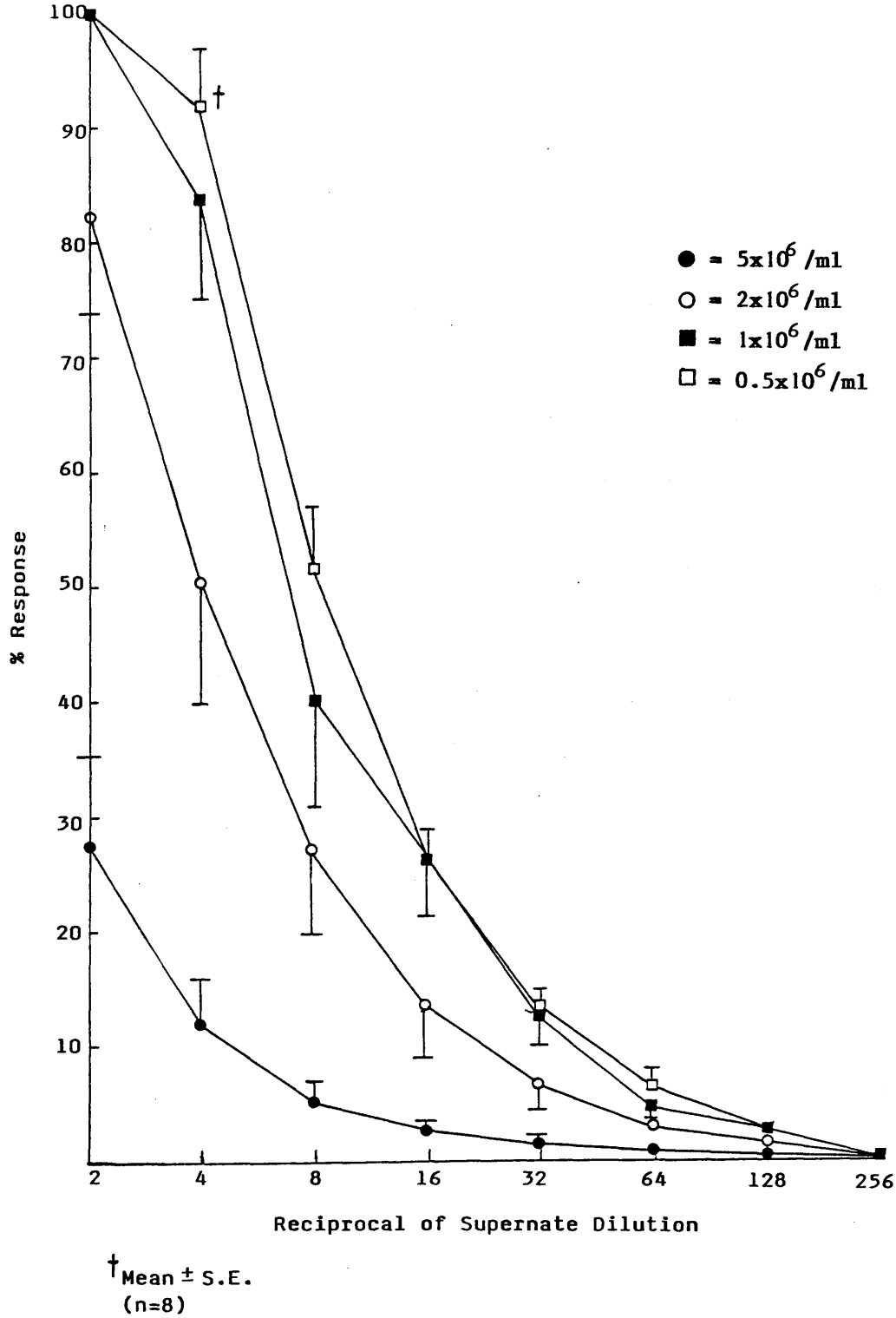


Figure 6.3: IL-2 Production in Normals (PHA at 2µg/ml).

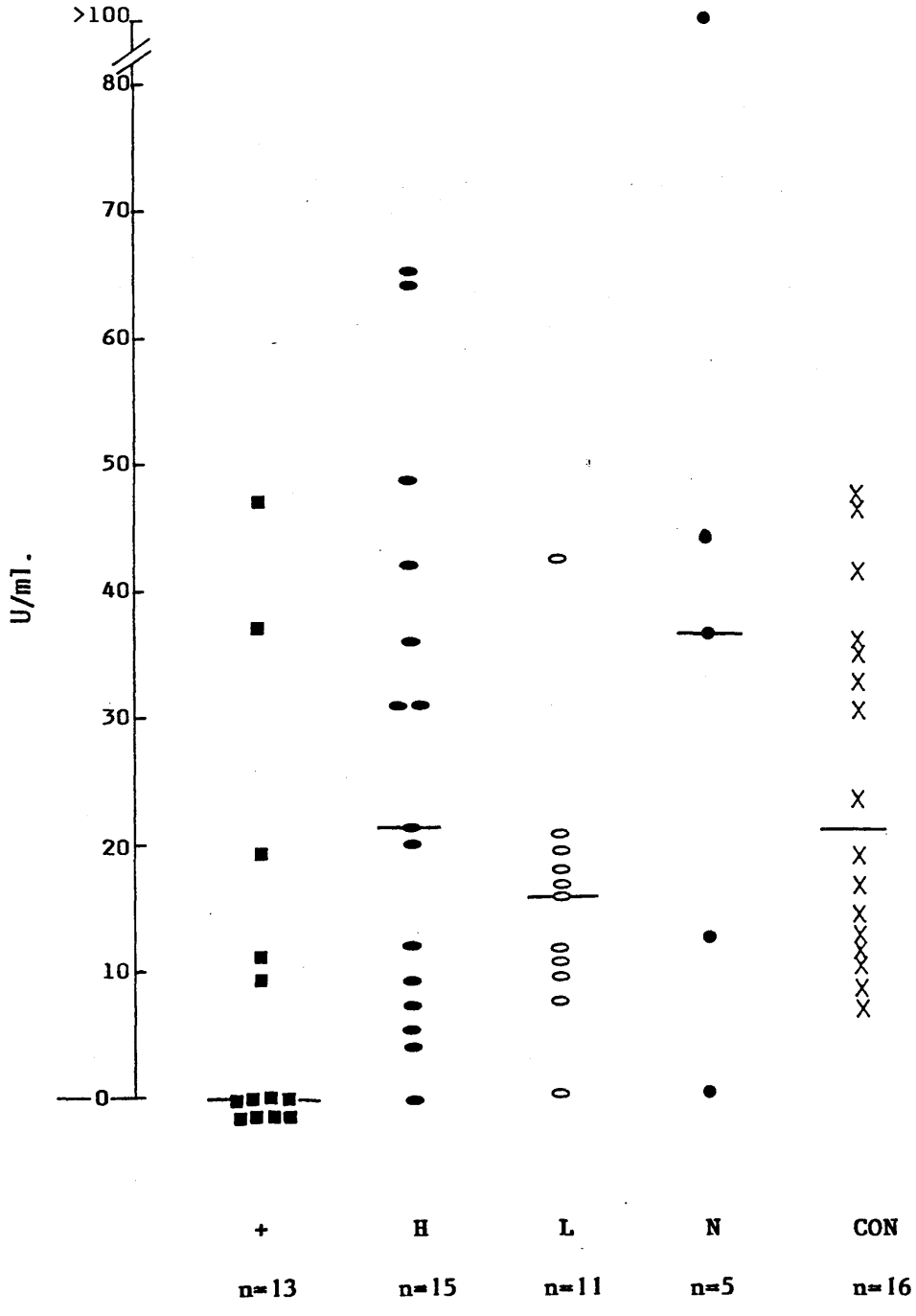


Figure 6.4: IL-2 Production (PHA at 1μg/ml).

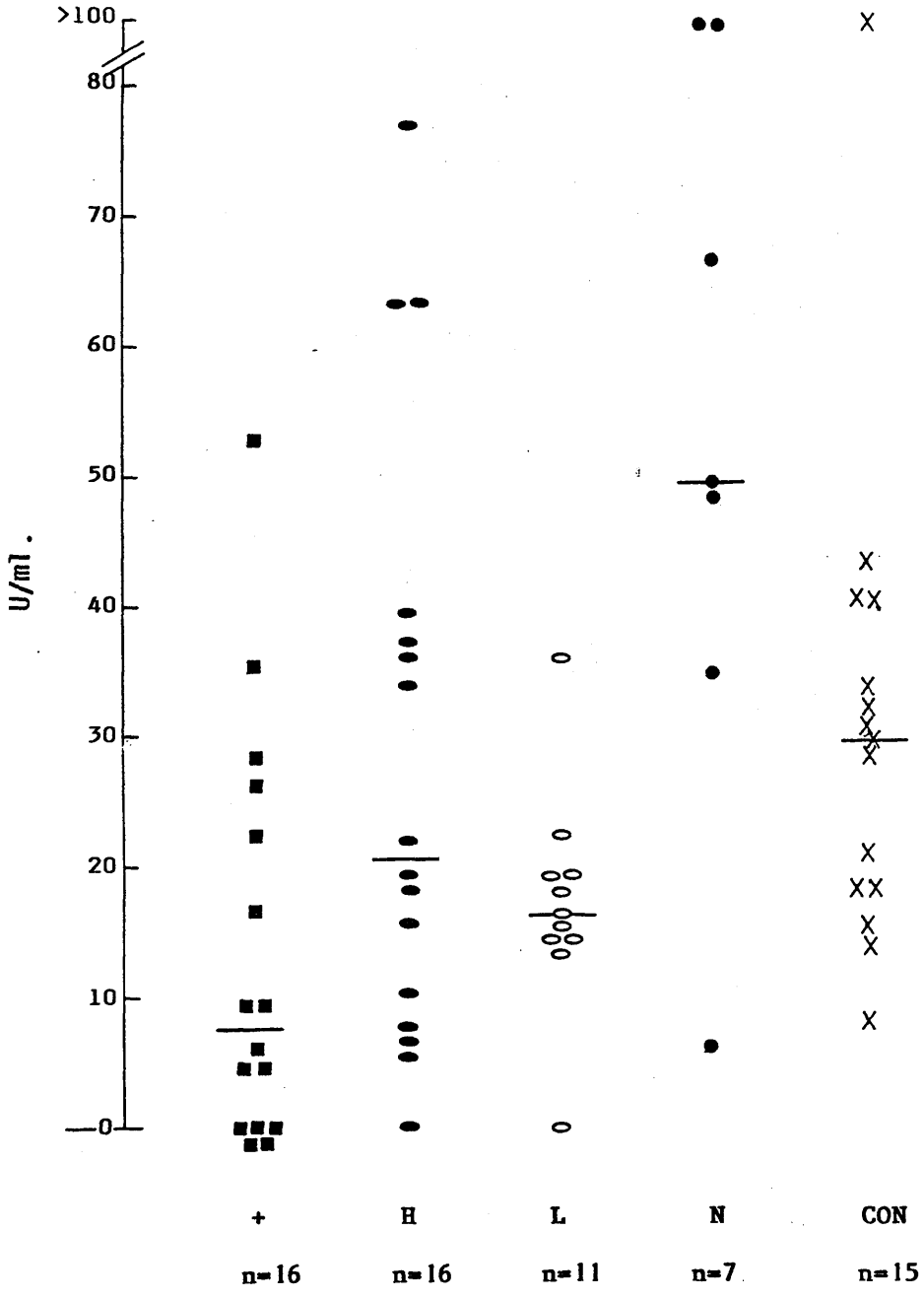


Figure 6.5: IL-2 Production (PHA at 2µg/ml).

HIV status ($p<0.01$).

When the treated haemophiliacs were split according to HIV status and factor usage as shown in Figures 6.4 and 6.5 then the results were more or less as expected. HIV^+ haemophiliacs produced significantly less IL-2 in response to both 1 µg/ml and 2 µg/ml when compared to controls, $p<0.01$ and $p<0.005$ respectively. They also produced less than the "heavy treated" group. $p < 0.02$ for 1 µg/ml and $p < 0.05$ for 2 µg/ml. Rather surprisingly, they did not produce significantly less than the "light treated" group at either PHA concentration. The "heavy users" produced similar levels of IL-2 at both concentrations when compared to the light users and controls. Another suprising finding was that at 2 µg/ml PHA, but not at 1 µg/ml, the "light users" produced less IL-2 than controls $p < 0.05$. No patient or control had detectable spontaneous production of IL-2, i.e. when the cells were cultured in the absence of PHA. The possibility of residual PHA in the supernate being carried over into the assay was also investigated and, when PHA was added at varying concentrations in the absence of IL-2, no stimulation of the cell line occurred. Also, when PHA at varying concentrations were added to supernates containing a known amount of IL-2, the response was equivalent to that expected for the IL-2 concentration alone, i.e. PHA and IL-2 do not have a synergistic effect on the response of the cell line.

6.4 DISCUSSION

Since the discovery of IL-2 much work has gone into studying its role in the immune response. It is now well established that this lymphokine is very important for an intact immune system. T cells have been shown to have an IL-2 requirement for sustained growth, and they die very quickly in culture if medium is not supplemented with it. T helper cells (T_4) are known to be the main producers of IL-2 on stimulation with antigen and IL-1 and of course the T_4 cell which is the cell infected by HIV.

Studies on the effect of IL-2 on the proliferative ability of purified T cells of AIDS patients (Borzy, 1987) have shown a decreased response compared to controls. When T_4^+ cells were further purified they too had a decreased response. Borzy also found that the production of IL-2 was reduced. This present study has shown that the HIV^+ haemophiliacs have impaired production of IL-2 when compared to HIV^- treated haemophiliacs and a control population. HIV^- "heavy users" did not differ from controls which suggests that the use of large quantities of factor concentrate does not impair their cells ability to produce the lymphokine. A strange result was that at 2 µg/ml PHA, "light users" produced significantly less IL-2 than controls. This raises the possibility that being a haemophiliac means impaired production of IL-2 but through the use of large quantities of concentrate the cells are stimulated by antigenic challenge from the concentrate to produce levels of IL-2 comparable to controls. This would account for the near normal levels in "heavy users" but reduced levels in "light users". However, this possibility seems very unlikely because at 2 µg/ml PHA, haemophiliacs who had never been treated showed increased levels compared to normals ($p<0.05$).

Therefore, simply being a haemophiliac obviously does not infer low production of IL-2. Also, production of IL-2 in the "light users" was not impaired at 1 μg/ml stimulation. "Heavy" and "light" users are arbitrary terms and there is no concrete evidence that treated HIV^- haemophiliacs differ in IL-2 production in accordance with the amount of concentrate they consume. When taken as one group, HIV^- treated haemophiliacs do not differ from controls. Therefore, the most probable conclusion is that HIV^- treated haemophiliacs as a whole do not differ from controls and when divided into groups, i.e."heavy" and "light", the unexpected result seen with the "light users" at 2 μg/ml PHA is one of those unexplained anomalies.

Of much more interest is the result of IL-2 production in the HIV^+ haemophiliacs, i.e. impaired production at both stimulation concentrations compared to controls and HIV^- "heavy users". The HIV^+ are all "heavy users" yet they differ significantly from the HIV^- "heavy users". Therefore, HIV is inferred as the cause for this decreased production of IL-2.

The reason for this reduced production remains to be explained. There was no correlation between absolute lymphocyte count, or absolute T_4 count, and the level of IL-2 produced in the HIV^+ haemophiliacs. In fact, some of the highest production levels occurred in those patients who had lowest numbers of T_4 cells on staining with monoclonal antibodies. Other studies have shown (Borzy et al, 1987) a lack of correlation with absolute numbers of lymphocytes and have suggested that variations in T cell subsets are not a major cause of the observed decreased IL-2 production. Several workers have found a decreased ability of T_4 cells from AIDS patients to proliferate to exogenously added IL-2 and Borzy (1987) has suggested that there are both quantitative and qualitative

abnormalities in the IL-2 - T lymphocyte system.

Other possibilities for the apparent decrease in production are decreased expression on the cells surface of receptors for IL-2, an abnormal function or ratio of the high to low affinity IL-2 receptors or even metabolic defects within the cell, distal to the receptor-ligand binding stage. Prince (1984) has shown decreased expression of IL-2 receptors on cells from AIDS patients but Lane and colleagues (1985) have shown that cells from AIDS patients can be induced to express normal receptor numbers by mitogen stimulation. Arya and Gallo (1985) have shown that in cultured HIV infected cells the level of transcription of the IL-2 gene is unimpaired. Thus, as there is no evidence suggesting abnormal regulation of the IL-2 gene, the functional deficiency of the IL-2 producing cells is most probably at a later stage in IL-2 production or may be the result of a more generalised virus-induced metabolic derangement of protein (IL-2) synthesis.

Another possibility for decreased production in the HIV^+ population is the findings by Donnelly (1986) that there is a serum factor in AIDS patients which inhibits proliferation to IL-2. Siegel and colleagues (1985) have also shown that this factor in the serum of AIDS patients, which seems to be difficult to wash off once cells are incubated in the serum, can also inhibit the production by normal lymphocytes of IL-2. Preliminary experiments exploring this possibility did not find similar results when studying haemophiliac sera (see Chapter 9).

Different groups have conflicting results on IL-2 production in AIDS patients. The differences between different groups could be explained by a variety of reasons. An important factor must be the clinical condition of patients being studied and whether or not they

are on different drug regimes. Also, technical aspects of the assay may differ; such as choice of lectin for stimulation, length of incubations, target cells used in assay and cell concentrations etc.

In this study, 48 hours incubation was used as this time has been shown to be optimum for production of IL-2 in studies of normal cells (Gillis, 1983). Another important factor could be the use of target cell. Some workers use 14 day old lectin-stimulated blast cells from normal donors and others use IL-2 dependent cell line. In my opinion, there is less likely to be variation if you use a cell line, which after all is originated from a single cell, rather than having to produce blast cells from normals which will be a mixture of different cell types.

There are more intricate experiments on the role of IL-2 in the haemophiliac population, which could be done, if time permitted. The number of cells expressing the IL-2 receptor could be determined. Other experiments outwith the capabilities of this current study, but which could be interesting, would be cell separation experiments. If the various T cell subsets could be separated then it would be possible to better investigate the mechanisms involved in IL-2 production. Such experiments could include cell mixing with autologous cell populations to see if, for example, T_8^+ suppressor cells from haemophiliacs inhibited proliferation of autologous T_4^+ cells to exogenously supplied IL-2. The effects of factor concentrate or HIV administration could also be studied <u>in vitro</u> looking at its effect or expression on IL-2 receptors and subsequent production of and response to IL-2. Preliminary studies along these lines are presented later in the Thesis (Chapter 9).

Another recently available assay which would be interesting to do is that for the quantitative determination of IL-2 receptors in

serum. Elevated serum levels of free IL-2 receptor have been reported in various lymphoproliferative disorders, including HTLV-1 associated adult T cell leukaemia (Nelson, 1986). Organ transplant patients as well as those with AIDS have also demonstrated elevated serum IL-2 receptor levels. The level of receptor in the serum or plasma may be important in identifying or characterising disease processes and may serve an important role in the immunoregulation of cell growth and differentiation. These free receptors must somehow be released from the cell surface of activated T cells. It would be interesting to measure levels of free receptors in the haemophiliacs to see if the levels correlated with disease state, severity of haemophilia or usage of factor concentrate.

In summary, therefore, this current study has shown that HIV^+ haemophiliacs produce significantly less IL-2 than either controls or HIV^- treated haemophiliacs. No significant difference was seen between "heavy" and "light" users suggesting that amount of factor consumption is not critical in the ability to produce IL-2 in response to mitogenic stimulation.

Finally, treated HIV^- haemophiliacs produced less IL-2 at 2 µg/ml PHA stimulation than haemophiliacs never treated ($p<0.02$). This effect, apparently due to the "light users", was not seen at 1 µg/ml stimulation.

CHAPTER SEVEN

IMMUNOGLOBULINS AND B CELL FUNCTION

7.1 INTRODUCTION

The B lymphocyte is the white cell responsible for immunoglobulin production. B cells once mature are programmed to produce antibodies of one specificity, i.e. against one antigen. The huge reservoir of B cells in the body gives a huge repertoire of possible antibodies to be produced. Once stimulated by antigen the B cell will mature into the antibody secreting plasma cells. B cells recognise antigen by means of the B cell receptor. This is in fact antibody on the cell surface, of the same specificity as that which the cell will go on to produce once stimulated. Cross linking of the receptors by antigen triggers the B cell. Like most of the immune functions, B cells can be controlled by T cells. However, some of the B cell responses to certain mitogens or antigens, e.g. lipopolysaccharide can occur independent of T cell help. The immunoglobulins produced can be one of 5 classes. These are IgG, IgM, IgA, IgD and IgE. The first three are the most common. IgG is the main circulating antibody. IgM is the primary immunoglobulin usually produced first in the humoral immune response. IgA is found especially in mucosal and gut related responses. IgE seems to be important in allergic and hypersensitivity responses such as hay fever. The exact biological function of IgD is unknown.

Before AIDS the B cells had been little studied in haemophiliac populations. As already discussed the virus causing AIDS shows tropism for T helper cells and as a result most the immunological research on AIDS has centred on T cell responses. However, some workers have studied the B cell. Hypergammaglobulinaemia is a characteristic finding in HIV^+ infected subjects (Schroff et al, 1983) (Fauci, 1984).

Despite the polyclonal immunoglobulin elevation, HIV infected subjects, and particularly AIDS patients, mount a poor antigen-specific antibody response after *in vivo* immunisation. Primary responses seem to be affected to a greater degree than secondary responses (Lane et al, 1983). Patients mount and sustain an ongoing antibody response to HIV that can be of considerable titre, waning only with end stage disease (Sarngadharan et al, 1984) (Biggar, Melbye, Ebbeson, et al, 1985). The normal sequence of events that occur when B cells become antibody secreting plasma cells can be dissected *in vitro* into discrete stages of activation, proliferation and differentiation. Largely as a result of the work by Lane, Fauci, and co-workers, B cell responses in AIDS patients have been extremely well characterised. Lymphocytes from AIDS patients produce a poor immunoglobulin response *in vitro* to PWM stimulation (Lane et al, 1983). This phenomenon is not due entirely to abnormalities caused by defective T cell help because purified B cells from patients are poorly responsive when mixed with normal T cells and the B cells are not stimulated by T cell independent activators such as formalin treated Staphylococci (Lane et al, 1983) (Pahwa et al, 1984). Thus, a high degree of apparently spontaneous, polyclonal B cell stimulation occurs in HIV-infected subjects rendering them poorly responsive to normal early activation signals. Diffuse elevations of all three major immunoglobulin classes, though chiefly IgG and IgA seem to be raised in HIV infected patients. (Siegal, 1984). Serum IgD has also been shown to be raised as much as tenfold and some workers have considered it a good marker for this disease (Chess, Daniels, North et al, 1984) (Mizuma, Zolla-Pazner, Litwin et al, 1987).

The reason for B cell activation has been a matter of speculation. HIV can infect B cell lines which express the CD4

molecule but it is not clear what proportion of B cells express CD4, and no one has shown spontaneous immunoglobulin production after inoculation of purified normal B cells with HIV. It is possible that the phenomenon is a secondary rather than primary consequence of infecton with HIV, though recent work by Schmitten and colleagues (1986) have shown that HIV can cause purified B cells to proliferate to as great a degree as that produced by T cell independent B cell mitogens. Immunoregulatory abnormalities within the CD4 T cell compartment are compatible with the notion that relative preservation of the more potent CD4 helper subset in concert with a preferential loss of the CD4 subset that induces CD8 T cell suppressors, allows unrestrained B cell activation. However, a recent publication (Gupta, 1986) has shown that in early HIV infection both the inducers of help and the inducers of suppression are equally depleted. There is no evidence that loss of suppressor-inducers necessarily results in spontaneous B cell activation, nor has it been demonstrated that CD4 suppressor-inducer function is abnormal in HIV infected subjects. Another explanation, may be that the abnormalities in T cell regulation allow infection or reactivation of other viral infections such as EBV or CMV that, in turn, are B cell activators. In support of this idea is a nearly universal finding of exposure to EBV and CMV in AIDS patients (Quinnan, Masur, Rook et al, 1984). No one has reported a definitive correlation between this exposure and immunoglobulin or B cell abnormalities in HIV infected subjects.

Finally, it has been proposed that HIV infected T cells elaborate B cell stimulating factors. This followed the finding that cell lines transformed by HTLV-1 show elaboration of lymphokines (Salahuddin, Marham, Lindner et al, 1984). However, it has not been found with the predominantly cytopathic HIV.

In this study, we have measured the circulating immunoglobulin levels and also investigated the ability of HIV positive and negative haemophiliac patients' cells to produce immunoglobulin in response to stimulus.

7.2 MATERIALS AND METHODS

7.2.1 Serum Collection

Serum was collected from clotted blood by centrifugation and stored at -20°C until assayed. Serum immunoglobulins were measured by radial immunodiffusion and laser nephelometry.

7.2.2 Immunoglobulin Production In Vitro

For immunoglobulin production, peripheral blood mononuclear cells were collected as described in Chapter 4. After counting cells were adjusted to 10^6/ml in RPMI supplemented with 20% FCS. 1 ml of cells were dispensed in wells of a 24 well plate (Flow Labs., Irvine, U.K.). To this was added 1 ml of RPMI (as a negative control) or 1 ml of PWM (Gibco) or 1 ml of formalin treated Staphylococcus A. (Staph.A).

Cells were incubated in RPMI/10% FCS for 7 days at 37°C/5% CO_2. Thereafter, supernate was removed and stored at -70°C until assayed for presence of IgM and IgG by ELISA. Optimum concentrations of mitogen and antigen were determined on normal subjects prior to study on patients - see Results.

7.2.3 ELISA for Determination of IgG and IgM

IgG and IgM produced spontaneously or as a result of stimulation were measured by an ELISA technique. The basis of an ELISA is that the substance being measured, in this case immunoglobulin, is trapped on a microtitre plate which has been previously coated with antibody against that particular substance. Then a secondary antibody to the substance is added and this will in turn bind. The second antibody is conjugated with the enzyme alkaline phosphatase and by addition of the substrate OPD there is a development of colour which is

	HIV+ n=19	HIV- HIGH USER n=25	HIV- LOW USER n=16	NO TREATMENT n=13	CONTROL n=14
UNSTIM.	76 #† (11 - 355)	34 (10 - 178)	41 (10 - 400)	30 (10 - 135)	52 (10 - 190)
STIMULATED	111 (36 - 400)	304 (39 - 400)	230 (50 - 400)	160 (27 - 400)	400 (230 - 400)
S.I.	4* (-40 - 1190)	443 (105 - 3900)	525 (0 - 3233)	281 (33 - 2700)	662 (53 - 3900)

ng/ml.
† Median (range)

Table 7.3: Supernate Levels of IgM (PWM Stimulation).

* $\frac{\text{Stim.} - \text{Unstim.}}{\text{Unstim.}} \times 100$

	HIV+ n=19	HIV- HIGH USER n=25	HIV- LOW USER n=16	NO TREATMENT n=13	CONTROL n=14
UNSTIM.	180 #† (33 - 400)	85 (23 - 400)	115 (52 - 400)	90 (16 - 400)	71 (12 - 150)
STIMULATED	132 (29 - 400)	85 (45 - 400)	151 (50 - 400)	110 (21 - 400)	100 (36 - 228)
S.I.	-14* (-61 - 17)	5.8 (-61 - 150)	-2 (-29 - 639)	28 (-31 - 185)	23 (-21 - 200)

† Median (range)

Table 7.4: Supernate Levels of IgG (PWM Stimulation).

* $\frac{\text{Stim.} - \text{Unstim.}}{\text{Unstim.}} \times 100$

ng/ml.

proportional to the amount of alkaline phosphatase present which, of course, depends on the amount of immunoglobulin which bound from the sample. The reaction is then stopped by the addition of 3M sodium hydroxide and the optical density (OD) at 405 nm read on a Skatron spectraphotometer (Flow Labs.). The OD obtained is the end product of the assay and is directly related to the amount of immunoglobulin which was present in the initial sample.

By incorporating samples of purified immunoglobulin of known concentration it is possible to construct a standard curve of OD vs Immunoglobulin concentration from which the unknown concentration of immunoglobulin in the samples can be determined.

The buffers used in the assay are described in the Appendix.

Both the goat anti-human IgG (orIgM) and the second antibody, the alkaline phosphatase conjugated goat anti-human IgG (or IgM) were obtained from Sigma. Both these antibodies were used at 1:1000 dilution as recommended by the supplier.

Purified IgG and IgM were obtained from Sigma and stored in aliquots at -70°C until use.

The OPD substrate was obtained from Sigma and used as recommended.

7.2.4 Problems Associated With Assay

It was necessary to first of all optimise the concentrations of mitogen or antigen used for production of immunoglobulins. It was decided to use 10^6 cells per culture (final concentration of 0.5×10^6/ml) on logistic grounds. Most published methods use 7 day cultures for optimum production of immunoglobulin and this time was used for production of supernate. It was also necessary to grow cells in fetal calf serum as human serum contains immunoglobulins which would

interfere with and be detected by the assay. The assay itself is very straightforward and uses commercially available immuno-reagents as per manufacturer's specifications.

Staph. A. was prepared for use as an antigen by growing up the bacteria in Meullar Hinton broth and then treating for 18 hours with 10% buffered formalin at 37°C. Bacteria were then washed and stored in saline at 4°C until use. Concentration of Staph. A. were were determined by plate pouring analysis. Briefly, tenfold dilutions of Staphylococci preparations were plated on blood agar and incubated overnight. The colonies were then counted and it was possible to calculate what OD of bacterial suspension was equivalent to what number of Colony Forming Units (CFU). It is assumed that one colony originates from one bacterium, therefore, 10^6 CFU = 10^6 bacteria/ml. When different concentrations of PWM were used there was no significant differences in amount of IgG or IgM produced over a wide concentration range using normals. From a 1:10 dilution of PWM right down to a 1:1000 dilution IgM and IgG, production did not drop. It was decided, therefore, that a working concentration of PWM at1:250 (1:500 final) would be used in the production of immunoglobulin containing supernates.

Similarly with Staph. A. as stimulus, no noticeable difference was seen in a range from 10^9/ml down to 10^6/ml. Only from 10^5/ml down did the levels of IgM start to drop. It was decided, therefore, to use Staph.A at 10^7/ml (working concentrations).

There seemed to be a problem with IgG production when using Staph A. It is known that soluble protein A (SPA) which can be released by Staph. A. will bind IgG and form immune complexes. This would therefore take out the IgG and none would be detected by ELISA. Formalin treatment should prevent leakage of SPA, but it was decided only to assay IgM when using the T cell independent Staph. A.

7.3 RESULTS

7.3.1 Serum Levels

Serum levels are shown in Table 7.1. IgA was raised in all groups ($p<0.001$) compared to control group. IgM was raised in the HIV^+ group ($p<0.02$) and HIV^- but treated group ($p<0.05$) when compared to controls. IgG was raised in both the HIV^+ group and HIV^- but treated group when compared to controls ($p<0.001$). Patients who had received treatment within the last year had increased IgG levels compared to those who were untreated ($p<0.001$). In HIV^- patients current raised IgG levels correlated with amount of treatment ($r=0.36$ $p<0.02$).

Serum samples were available for 87 patients from whom we knew IgG levels in 1981. Results of this are shown in Table 7.2. As can be seen, there is a significant increase in the HIV^+ patients in 1986 compared to 1981 ($p<0.005$) but no increase in the HIV^- patients. Serum IgD was not raised in any patient group.

7.3.2 Supernate Immunoglobulin Production by PWM and Staph. A. Stimulation

The levels of IgM and IgG produced in response to 7 day stimulus is shown in Tables 7.3 and 7.4. Results are shown as ng/ml produced and also as a stimulation index (SI). The SI is worked out as:

$$\text{Stimulation Index (SI)} = \frac{\text{Stimulated - Unstimulated}}{\text{Unstimulated}} \times 100$$

A high SI will mean that cells were stimulated in presence of mitogen and a low SI will mean cells could not be stimulated so much above the unstimulated level, i.e. could not be stimulated to produce more immunoglobulin than they already spontaneously produce.

	CONTROLS n=33	HIV+ n=12	HIV− TREATED n=55	HIV− NO TREATMENT n=33
IgA	1.4*† (0.8 - 3.0)	2.7 (1.2 - 5.7)	2.1 (0.4 - 5.7	2.2 (0.9 - 6.1)
IgM	1.5 (0.3 - 2.8)	1.7 (0.6 - 12.6)	1.9 (0.2 - 4.9)	1.5 (0.8 - 5.0)
IgG	10.2 (4.1 - 18.5)	20 (15.2 - 39)	14 (9.1 - 21.8)	11.8 (7.6 - 17.9)
IgD	1.2 (0.6 - 9.6)	2.1 (0.6 - 7.9)	1.5 (0.6 - 1.3)	

* g/L
†Median (range)

Table 7.1: Serum Immunoglobulin Levels 1985-86.

	HIV+ n=12	HIV− n=75
1981	14.7*† (7.8 - 21)	14.9 (8.4 - 22)
1986	22.2 (12.1 - 39)	16 (8.7 - 21.8)

* g/L
†Median (range)

Table 7.2: IgG Levels in Patients with Paired Samples.

IgM

For IgM production in response to PWM stimulation all treated haemophiliacs when taken as one group had a significantly reduced SI ($p<0.05$) compared to controls. When the groups were taken separately only the HIV^+ had a reduced SI compared to control ($p<0.001$). This group also had a reduced SI ($p<0.001$) compared to both HIV^- "heavy" "light" users.

By looking at either the simulated or unstimulated levels, it should be possible to detect whether the patients have a high spontaneous production or an inability to be stimulated to produce more immunoglobulin.

The HIV^+ group has higher unstimulated levels of IgM compared to the "heavy users" ($p<0.005$) and "light users" ($p<0.01$) and those never treated ($p<0.005$). No other group has increased unstimulated levels. On stimulation, treated haemophiliacs as a group have a significantly lower level of stimulation ($p<0.001$) as compared to controls. Taken as separate groups the HIV^+ patients produce significantly less on stimulation compared to the others: $p<0.005$ compared to "heavy users", $p<0.05$ compared to "light", and $p<0.001$ compared to controls. Both "heavy" and "light" users also produce less than controls ($p<0.01$) although they do not differ from each other in levels of IgM for spontaneous or stimilated samples. Paired Wilcoxons comparing stimulated (with PWM) and unstimulated within a group show significance in all groups ($p<0.01$) except the HIV^+ group. Therefore all of the patient groups except the HIV^+ can be stimulated to produce more IgM.

IgG

The results for IgG are essentially similar. When taking all

treated haemophiliacs as a single group they have a reduced SI compared to both controls ($p<0.01$) and haemophiliacs who had not received treatment ($p<0.05$). Again on dividing patients into their respective groups it is only the HIV^+ patients which differ from controls ($p<0.001$). They also differ significantly from HIV^- "heavy users" ($p<0.002$), "light users" ($p<0.05$) and those never treated ($p<0.001$).

Looking at the stimulated levels produced there is no difference between any group. There are, however, differences if unstimulated levels are examined. HIV^+ patients seem to spontaneously produce more IgG than controls ($p<0.001$), HIV^- "heavy users" ($p<0.005$) and those never treated ($p<0.02$). HIV^+ also spontaneously produce more than treated HIV^- haemophiliacs as one group ($p<0.01$) and all treated treated haemophiliacs, irrespective of their HIV status or factor consumption, have a higher spontaneous production than normals ($p<0.02$). "Heavy users" do not spontaneously produce more immunoglobulin than controls but rather surprisingly "light users" marginally do ($p<0.05$). Paired Wilcoxons show no difference between spontaneous and stimulated levels for HIV^- "heavy" and "light" users or those never treated. As expected controls show a difference in stimulated giving higher levels than controls ($p<0.01$). Unexpectedly, however, HIV^+ patients seem to produce more spontaneously than they do when stimulated ($p<0.02$).

IgM Production to Staph. A. Stimulation

The results of IgM production are shown in Table 7.5.

Treated haemophiliacs have a smaller SI than controls ($p<0.01$). HIV^+ patients are lower than controls ($p<0.005$) as are HIV^- "heavy users" ($p<0.02$). Treated HIV^- haemophiliacs, irrespective of amount,

	HIV^+ n=12	HIV^- HIGH USER n=13	HIV^- LOW USER n=8	CONTROL n=10
UNSTIM.	76 # † (11 - 355)	34 (10 - 178)	41 (10 - 400)	50 (10 - 190)
STIMULATED	89 (16 - 275)	51 (12 - 188)	117 (32 - 400)	166 (25 - 400)
S.I.	-5 * (-41 - 132)	15 (-41 - 452)	77 (-52 - 556)	156 (-17 - 2700)

† Median (range)

* $\frac{\text{Stim.} - \text{Unstim.}}{\text{Unstim.}} \times 100$

ng/ml.

Table 7.5: Supernate Levels of IgM (Staph.A Stimulation).

also had a lower SI than controls ($p<0.05$). "Heavy users" appear to produce less IgM than controls ($p<0.05$), whereas "light users" do not, when levels in the stimulated supernates are analysed. This suggests that heavy treatment impairs the ability to respond to antigen *in vitro* and produce IgM in amounts comparable to controls.

Paired Wilcoxons were again looked at to determine if within individual groups there were differences between stimulated and spontaneous production. As expected the HIV^+ group showed no differences but the control population produced more IgM on stimulation ($p<0.01$) as did the HIV^- treated patients taken as a single group ($p<0.05$). "Heavy users" produced more on stimulation than they did spontaneously ($p<0.02$) but surprising "light users" did not. This latter group was small ($n=8$) and six patients produced more on stimulation while two did not.

At the same time as T cell subsets were being performed the numbers of circulating B cells were determined. These did not differ for the patient groups: HIV^+ (median 0.1 [range 0.04-0.42] x 10^9/L), HIV^- "heavy" (median 0.1 [range 0.03-0.46] x 10^9/L), and HIV^- "light user" (median 0.12 [range 0.05-0.33] x 10^9/L), nor did they differ significantly from the normal range (0.07-0.53 x 10^9/L).

In summary, all treated haemophiliacs had increased serum levels of IgA, IgG and IgM compared to controls, irrespective of their HIV status. This would suggest an intrinsic B cell defect in some way caused by the use of concentrate. In HIV^+ patients there is a progressive polyclonal rise in IgG levels related to HIV infection. IgD was not raised in any patient group. From the *in vitro* work, it can be seen that HIV^+ patients have a higher spontaneous production of IgM and IgG compared to the others and they cannot be stimulated to produce more. Treated HIV^- haemophiliacs, although not producing more

IgM or IgG spontaneously compared to controls, cannot be stimulated to produce immunoglobulins to the same degree as controls. Therefore, the use of Factor VIII concentrate in some way seems to be inhibiting the ability to produce immunoglobulin *in vitro*.

7.4 DISCUSSION

AIDS has generally been regarded as a disease affecting the T cell arm of the immune response. Hypergammaglobulinaemia and elevated levels of immune complexes are near universal findings in HIV infected individuals. Despite the polyclonal immunoglobulin elevation, HIV infected subjects, particularly AIDS patients, mount a poor antigen-specific antibody response after *in vivo* immunisation. Largely through the work of Lane, Fauci and colleagues, B cell responses in AIDS patients have been extremely well characterised. *In vitro* studies have shown that these patients produce a poor immunoglobulin response to PWM stimulation. Co-culture experiments have shown that it is not simply a T cell defect as normal T cells cannot restore the response. Similar work by Brieva (1985) looking at the abnormal B cell function in haemophiliacs found comparable results. His study showed that spontaneous production of IgM, IgG and IgA in culture was three times higher than controls but on stimulation with PWM, the levels produced were significantly lower than normals. The amount of clotting factor used correlated with increased spontaneous imumnoglobulin production, decreased PWM-driven production of immunoglobulins and increased serum IgG levels.

As yet the cause for the polyclonal B cell activation is unknown but could be caused by a variety of reasons including:
(i) T cell independent activation and transformation of B cells by EBV or CMV in the absence of regulatory T cells; (2) production of B cell activating factors from HIV infected T cells, and (3) direct activation of B cells by HIV. Recent work by Schmittman and colleagues (1986) has tried to address the problem of polyclonal B cell

activation. They have found that different HIV isolates can cause as great a degree of proliferation of B cells as any of the known T cell independent B cell mitogens used. This activation is due to direct B cell-virus contact and the magnitude of response in their studies suggests that this type of activation may be the major mechanism responsible for the hypergammaglobulinaemia in AIDS patients. If this is so, then there may be an alternative receptor site for the virus on B cells, as most B cells do not express the CD4 molecule thought to be part of the HIV receptor on T helper cells.

This study has looked at B cell function and immunoglobulin levels for any abnormality. As described in Chapter 5 , the proliferative response to PWM, a T cell dependent B cell mitogen, was reduced when treated haemophiliacs were compared to controls. On dividing the patients according to HIV status and amount of concentrate used in therapy, only HIV^+ patients differed from controls. Serum levels of IgG and IgM were raised in both the HIV^+ and HIV^- treated group and IgA was raised in all groups studied compared to controls. These results agree with other studies on both HIV^+ patients and haemophiliacs. It is interesting that the study on paired samples shows that although in 1981 HIV^+ and HIV^- patients had similar IgG levels, in 1986 they differed from each other and more striking, the HIV^+ patients had increased levels compared to their 1981 levels ($p<0.005$). This is probably a consequence of infection with HIV. No association was found with current transaminase levels or other viral infection and IgG levels. It would seem from these findings that concentrate usage results in B cell activation. This causes an increase of certain immunoglobulin isotypes which does not significantly change over time unless HIV infection supervenes, which results in sustained and progressive rise. It is unclear but a

possibility that the defect in HIV^- patients is due to concentrate related immunoregulatory defects such as specific antigenic stimulus with foreign antigens in the Factor VIII and future in vitro studies may help elucidate the reason for the observed hypergammaglobulinaemia in asymptomatic HIV^- haemophiliacs. Earlier studies (Chess et al, 1984) and more recently one by Mizuma and colleagues (1987) suggested that IgD could be a useful marker for AIDS or HIV related disorders. The later study found up to ninefold increase in serum IgD levels in AIDS-related complex patients compared to controls. This elevated level started to decrease in AIDS but was still higher than control levels. They found that increased IgD correlated with increased IgG or IgA, increased percentage of B cells and decreased percentage of T helper cells. This current study has not been able to show a significant increase in IgD levels in the HIV^+ haemophiliacs but it is interesting to note that the highest level of all patients was from a serum sample taken from the only haemophiliac to die from AIDS in our centre.

The results from the in vitro stimulation are in keeping with those of others (Lane et al, 1983). HIV^+ patients spontaneously produce more IgG and IgM than the other groups tests. Also, on stimulation, the HIV^+ patients could not be stimulated to the same degree as other patients or controls to produce IgG or IgG. However, the other patients (all HIV^-) produced less IgM on stimulation with PWM than controls. This suggests that while HIV^+ patients have polyclonal activation of B cells resulting in spontaneous production of IgG or IgM and an inability to be stimulated further to produce more there is also some intrinsic defect in the HIV^- patients. There was no difference between HIV^- "heavy" and "light" users either in spontaneous or stimulated production of IgM or IgG suggesting that

amount of usage is not crucial. The fact, however, that HIV^- asymptomatic patients were incapable of producing comparable levels to controls on stimulation suggests that there is, indeed, some defect in the B cells from treated haemophiliacs. The cause of this defect is most likely due to the use of clotting factors, but the mechanism by which the clotting factor usage induces these B cell alterations is still unknown. Allogeneic co-culture experiments (Brieva et al, 1985) have shown that the failure of haemophiliacs' B cells to produce immunoglobulin in response to PWM is not due to altered T cell function. This suggests that either a defect of the PWM responsive B cell subset or an excessive suppressor activation of monocytes could be invoked as the cause of this observed alteration.

Future work could look at the specificity of the immunoglobulin produced *in vivo*. It would also be interesting to determine if a large percentage of B cells are producing antibody causing the observed hypergammaglobulinaemia. Lane (1983) has shown that around 0.1-1% of B cells from AIDS patients are responsible for the hypergammaglobulinaemia. A high proportion of the total immunoglobulin is anti-HIV, but in *in vitro* culture cells cannot be induced to produce higher levels of anti-HIV antibody.

Co-culture experiments, especially in the HIV^- patients may help determine what is causing this abnormal production of immunoglobulin *in vivo* coupled with a decreased ability to produce immunoglobulin *in vitro* after stimulation.

CHAPTER EIGHT

β_2-MICROGLOBULIN LEVELS

8.1 INTRODUCTION

β_2-Microglobulin (β_2M) is a small single chain polypeptide (Mr=11,600) that is found on the surface of most nucleated cells. It may play an important role in the immune system of the body as it is found on the cells surface as the constant subunit, noncovalently linked to the classic transplantation antigens encoded by the major histocompatability complex (MHC) (Poulik, Ferrone, Pellegrino et al, 1974). It may also normally be present in trace amounts in both serum and urine (Woo, Floyd, Longley et al, 1980). Measurements on the concentration of β_2M have been used as a reliable indicator of glomerular and tubular functions (Ervin and Wibell, 1973). Abnormally high serum concentrations have been reported in many disease states including rheumatoid arthritis (Talal, Grey, Zvaifler et al, 1975), malignant tumours (Kither, Cejka, Belamaric et al, 1974) and more striking increases were shown in lymphoproliferative disorders (Poulik, Farrah and Smithies, 1972) and acute viral infections (Forman, 1982). Francioli (1982) first showed increased β_2M levels in an immunodeficient homosexual man. More recently there have been reports of increased serum levels of β_2M in AIDS cases (Bhalla, Safai, Mertelsmann et al, 1983). Serum β_2M is thought to originate mainly as a result of being released from the surface of cells and increases therefore, with cell turnover. Therefore, increased values in AIDS patients are most probably indicative of increased cell turnover or cell death.

Zolla-Pazner (1984) has suggested that in conjunction with other laboratory tests, is a useful marker for the early stages of AIDS.

8.2 MATERIALS AND METHODS

8.2.1 Serum Collection and Patients

Serum samples were obtained by centrifugation of clotted blood samples and were stored at -20°C until assayed. All samples were assayed without knowledge of HIV antibody status. Two hundred and forty three samples were assayed. These included 46 age and sex matched healthy normals, 112 samples from HIV negative patients, 60 HIV positive samples and 25 samples from Factor IX deficient patients.

8.2.2 β_2-Microglobulin Assay

β_2M was measured using the commercially available Micro RIA from Pharmacia (Pharmacia, Milton Keynes, U.K.). Briefly, this is a double antibody radioimmunoassay method. β_2M in the sample competes with a fixed amount of ^{125}I-labelled β_2M for the binding sites of the specific antibody to β_2M. Bound and free β_2M are separated by addition of a second antibody immunoadsorbant (a Sepharose-anti-sheep IgG raised in horse) followed by centriguation and decanting. The radioactivity in the resulting pellet was then measured on a gamma counter. The amount of radioactivity is inversely proportional to the quantity of β_2M in the sample.

By using known concentrations of β_2M it is possible to construct a standard curve of radioactivity measured versus β_2M concentration enabling the determination of the levels in patients' samples.

8.3 RESULTS

Results are expressed as a percentage of the mean of triplicate counts of the "0-Standard", i.e. when no β_2M is present.

Therefore : % activity bound

$$= \frac{\text{mean count (of standard or unknown)}}{\text{count of 0-Standard}} \times 100$$

Figure 8.1 shows a standard curve for the % activity bound versus β_2M concentration. From this, it was possible to calculate the concentration of β_2M in unknown samples once % activity bound was determined. The concentration was simply calculated by reading the point on the x-axis corresponding to the point where the % activity bound cut the standard curve.

Figure 8.2 shows a scatter plot of concentration for the different groups studied. The HIV^+ haemophiliacs had a median level of 2.2 [range 0.49-4.55] mg/L. This was significantly more ($p<0.001$) than the levels obtained for both the HIV^- haemophiliacs (median 1.79 [range 0.72-3.3] mg/L and also in the controls (median 1.4 [range 0.98 - 5.4]). All Factor VIII patients taken together also had significantly greater levels than controls ($p<0.001$). Some FIX samples were also available and they gave a median level of 1.7 mg/L with a range of 1.2-3.45. This was significantly greater than controls ($p<0.02$).

In all patients there was a significant correlation between β_2M levels and hepatic dysfunction, as suggested by elevated ALT, ($r=0.35$, $p<0.02$) but no association was evident with β_2M levels and the amount of concentrate used in the preceeding year.

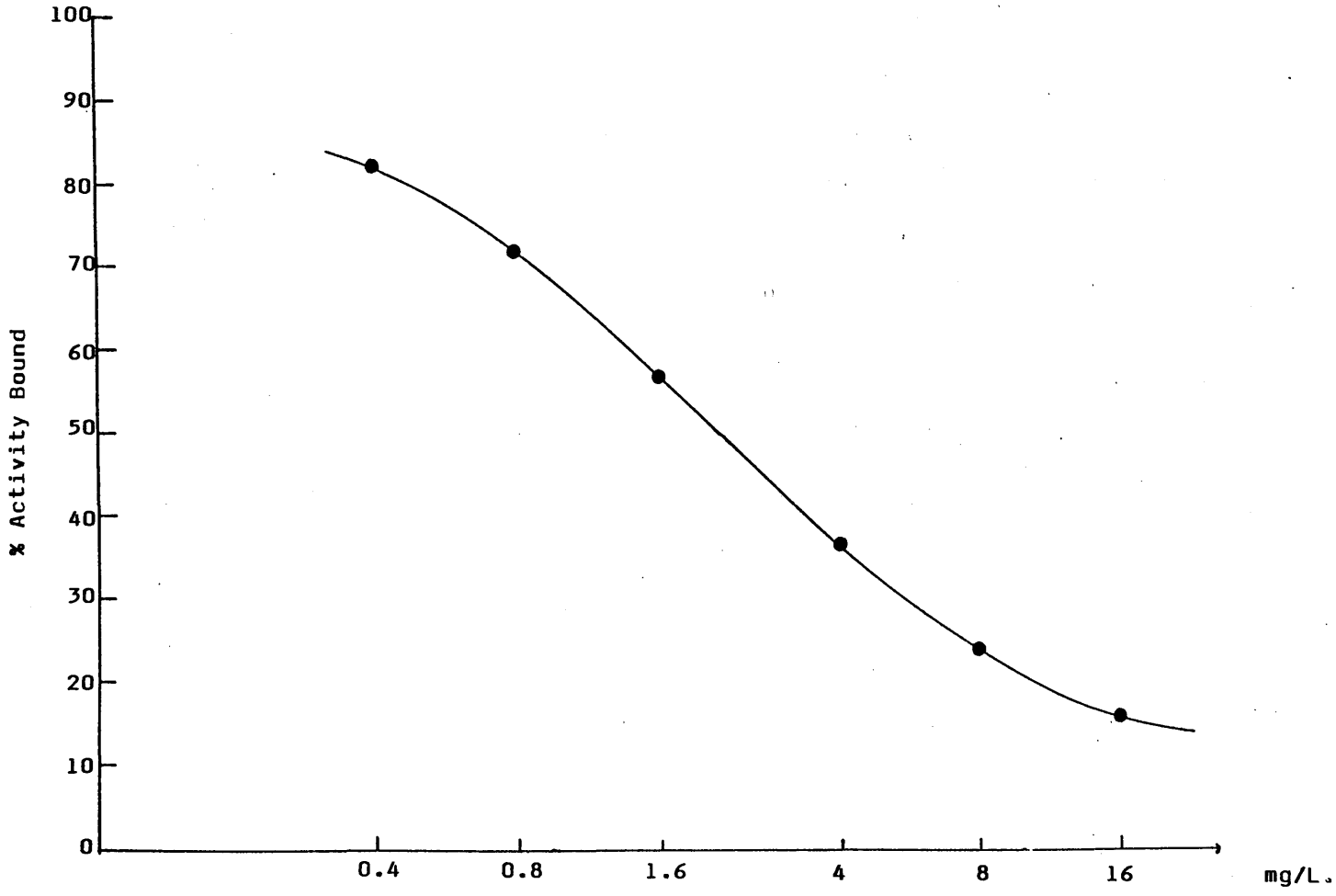


Figure 8.1: Standard Curve of % Activity Bound Vs. β_2M Concentration

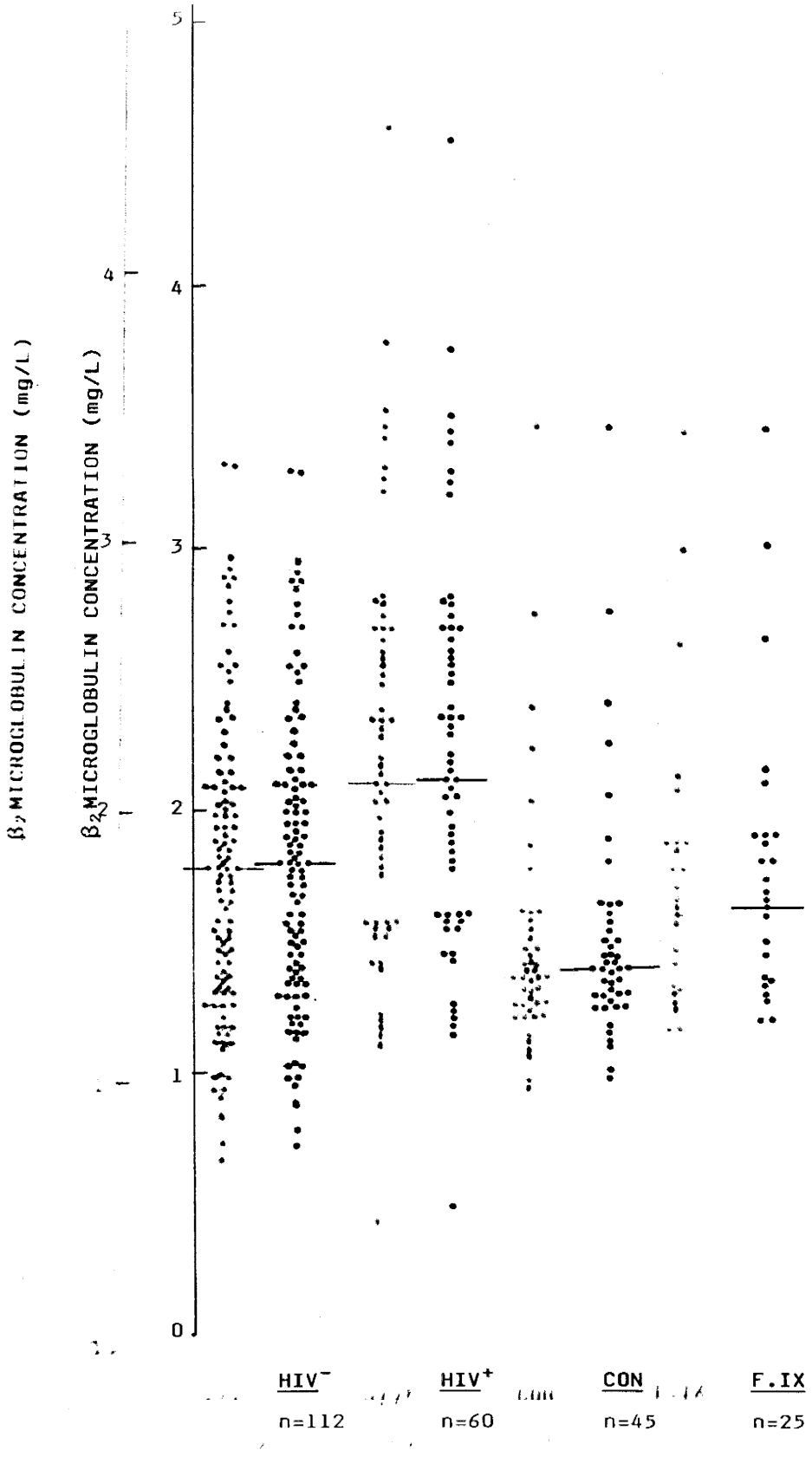


Figure 8.2: β_2Microglobulin Levels in Haemophilia.

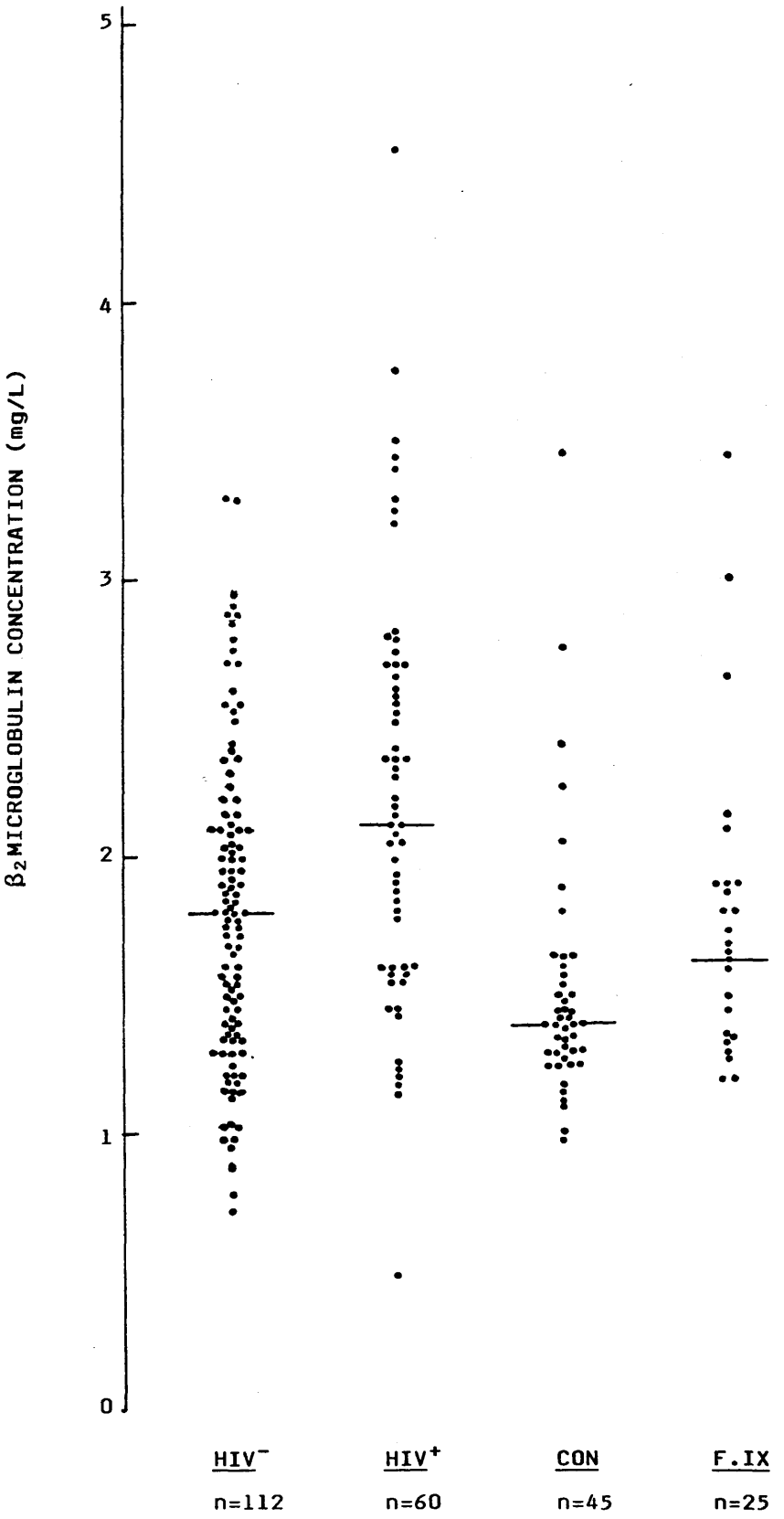


Figure 8.2: β_2Microglobulin Levels in Haemophilia.

8.4 DISCUSSION

There have been many attempts to find a surrogate marker indicative of AIDS. Zolla-Pazner and colleagues (1984) state that the use of the β_2M test, while not specific for AIDS, appears to be a highly useful marker of the disease. The sensitivity of the disease, i.e. the probability that concentrations above a certain level will correctly classify persons with AIDS was 100% for both patients in their study with confirmed AIDS and also patients with suspected AIDS. They conclude that β_2M quantitation, used in conjunction with other laboratory tests, is a useful marker for early stages of AIDS.

This study is in keeping with other reports (Stein et al, 1985) that increased β_2M levels are observed in haemophilia A patients. However, it differs from the findings of Lee and colleagues (1985) who did not find significant differences between haemophilia A and B patients and controls. There is a possibility that the increased levels observed in the haemophiliacs are as a consequence of carry over of β_2M in the concentrate. However, on checking batches of Factor VIII, no detectable β_2M levels were found. Elevated levels of β_2M are most probably due to cell turnover with β_2M being shed from the cells surface. Increased levels are also thought to be associated with viral and other antigenic exposures through the use of blood products.

In our group of patients there was no clear difference in levels associated with any clinical signs of disease. However, it is of interest that the highest β_2M level recorded in the HIV^+ group came from a patient who shortly afterwards died, the diagnosis being AIDS.

In summary, therefore, in this study haemophiliacs have been shown to have increased β_2M levels when compared to controls. This was not due to a carry over of β_2M in their treatment. Also, HIV^+

patients have significantly higher levels than HIV⁻ones. However, the levels in haemophilia appear to be a reflection of biochemical hepatic dysfunction as well as HIV related disease. It is unclear whether or not levels will be useful in indicating those who will develop symptomatic HIV disease but perhaps if the results are used in conjunction with other laboratory findings they will be of use in backing up clinical findings while trying to diagnose disease states.

SECTION II

CHAPTER NINE

MISCELLANEOUS STUDIES

9.1 INTRODUCTION

This section is a collection of various *in vitro* experiments which seem best put together as one. The *in vitro* effect of Factor VIII concentrate and its buffer, as well as disrupted HIV on various *in vitro* immunological tests already described in this Thesis, namely, mitogenesis, IL-2 production and immunoglobulin production has been studied.

This study has also looked at the effect of patients' serum on the inhibition of mitogenesis and IL-2 production.

Various workers including ourselves have tried to examine what role direct cell to Factor VIII concentrate contact has in various immunological tests. McDonald and colleagues (1985) have described experiments in which they found that a diffusable factor in Scottish Factor concentrate could inhibit lymphocyte transformation *in vitro*. Our previously unreported findings on similar work are presented here.

At the time of doing this work there had been few reports on the effect of Factor VIII concentrates on *in vitro* assays but recent publications (Lederman, Saunders, Toossi et al, 1986) (Wang, Beck, Furlan et al, 1985) have reported their findings on similar experiments. Also, very little had appeared in the literature on the effect of disrupted HIV on various *in vitro* assays. However, a recent paper by Pahwa et al (1985) has reported findings on similar work.

Finally, with the report by Siegal and colleagues (1985) that IL-2 production by normal lymphocytes could be inhibited by sera from AIDS patients, it was decided to look at the effect of haemophiliac sera on IL-2 production.

The work on these topics, therefore: (i) Factor VIII concentrate; (ii) HIV and, (iii) Haemophiliac sera - will be dealt with separately.

9.2 FACTOR VIII CONCENTRATE

9.2.1 Introduction

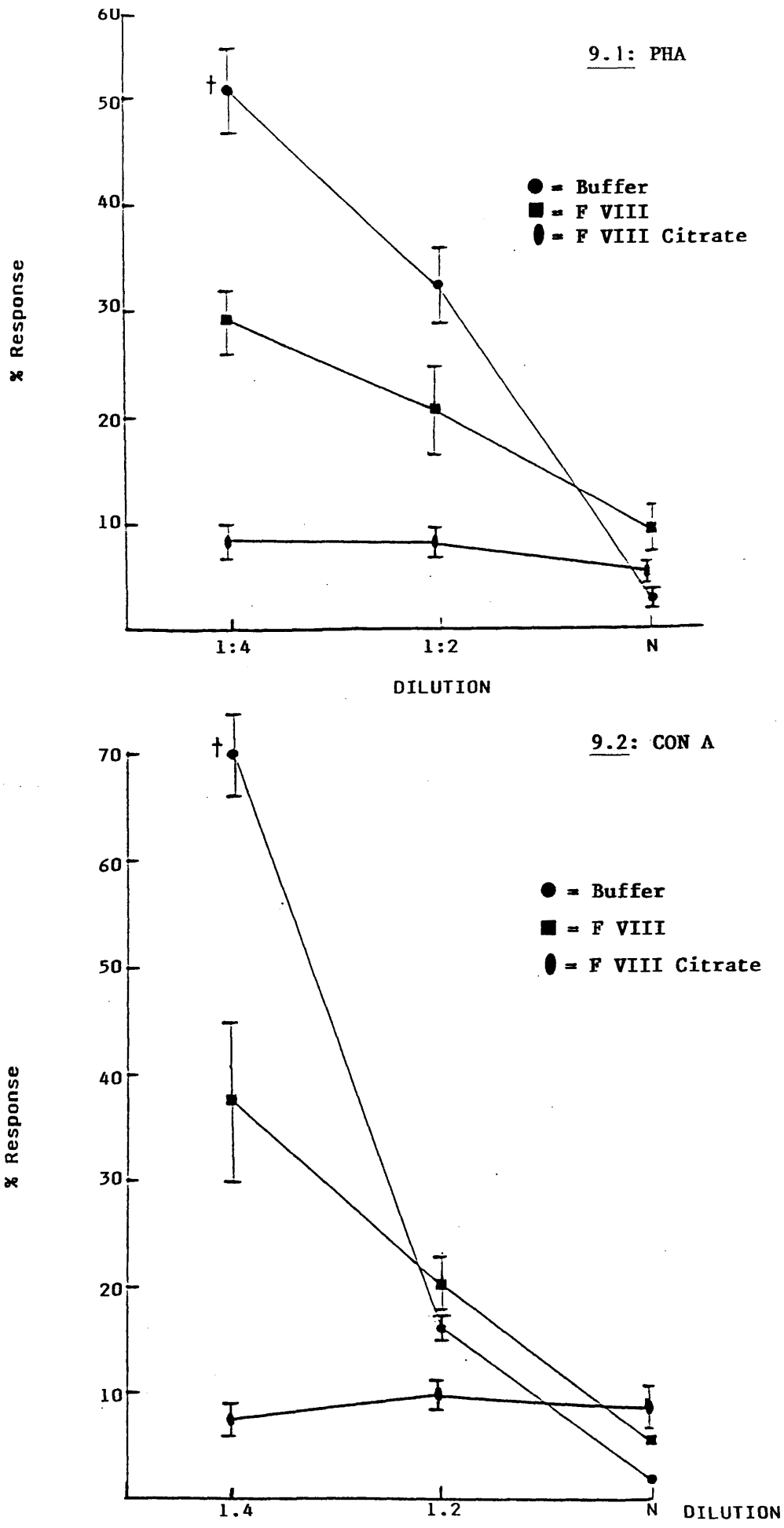
Published reports have shown the ability of Factor VIII concentrate to inhibit mitogenesis and IL-2 production. In the experiments described here, the effect of concentrate on IL-2 production and mitogenesis by normal lymphocytes has been studied. The assays for IL-2 production and mitogenesis have been described previously and only modifications will be mentioned here.

9.2.2 Methods and Results

Factor VIII concentrate was obtained from the Protein Fractionation Unit, Edinburgh. For mitogenesis 50 µl of both cells and mitogen or PPD were added to the wells. To this was added 100 µl of either neat Factor VIII (6 U/ml) or twofold dilutions of the concentrate which were made up in RPMI. Factor VIII was reconstituted in RPMI rather than distilled water so that in culture the cells still had their normal medium, other than the salts which are in the lysophilised concentrate. The buffer for Factor VIII is 0.02M Tris/0.02M Trisodium Citrate adjusted to pH 7.3. This was made up in RPMI and used in the same way as the concentrate. Factor VIII was also diluted in RPMI containing the same concentration of Tris and Trisodium Citrate as buffer. This would allow dilution of the Factor VIII while keeping the buffer concentration the same. These three preparations were tested in mitogenesis and proliferative response to PPD.

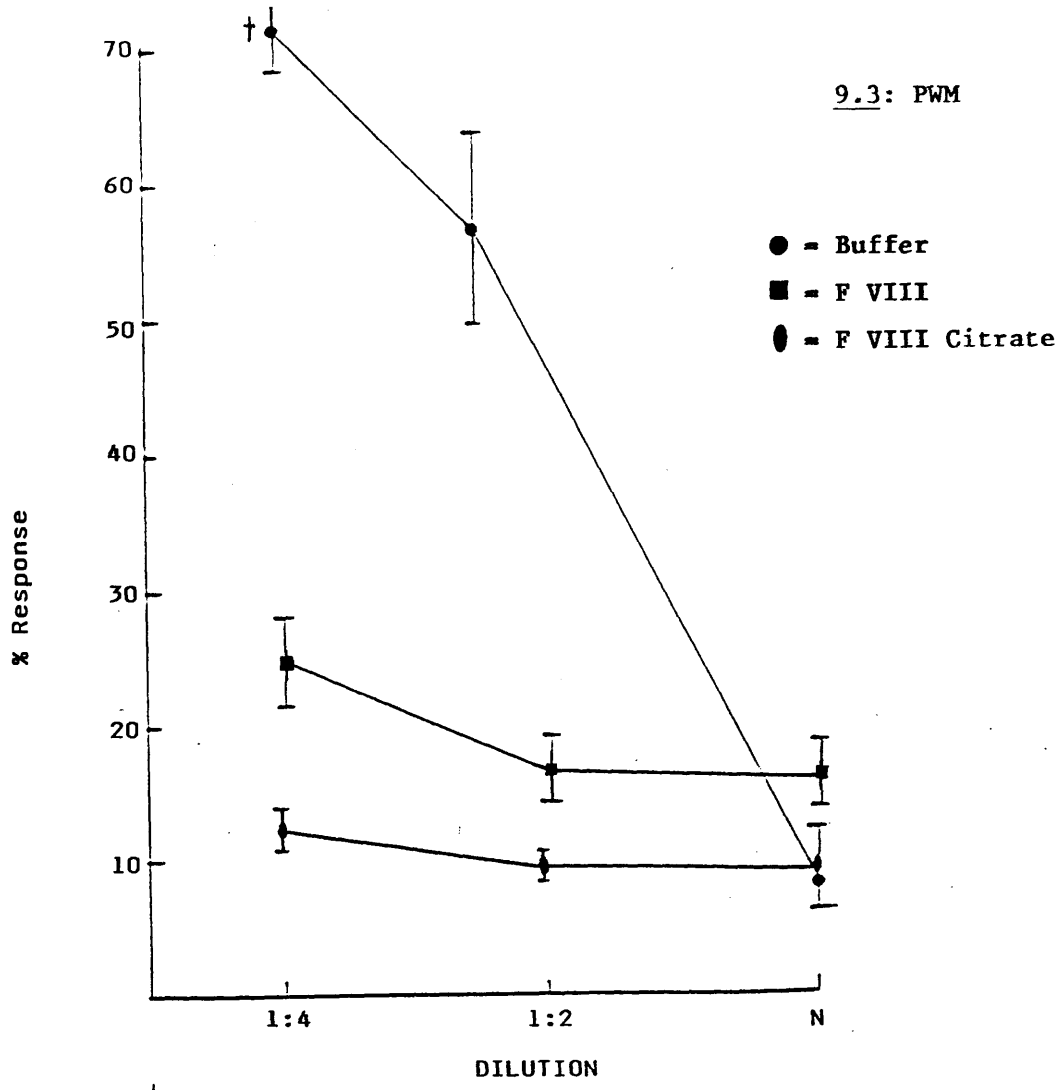
The results are shown in Figures 9.1 to 9.4.

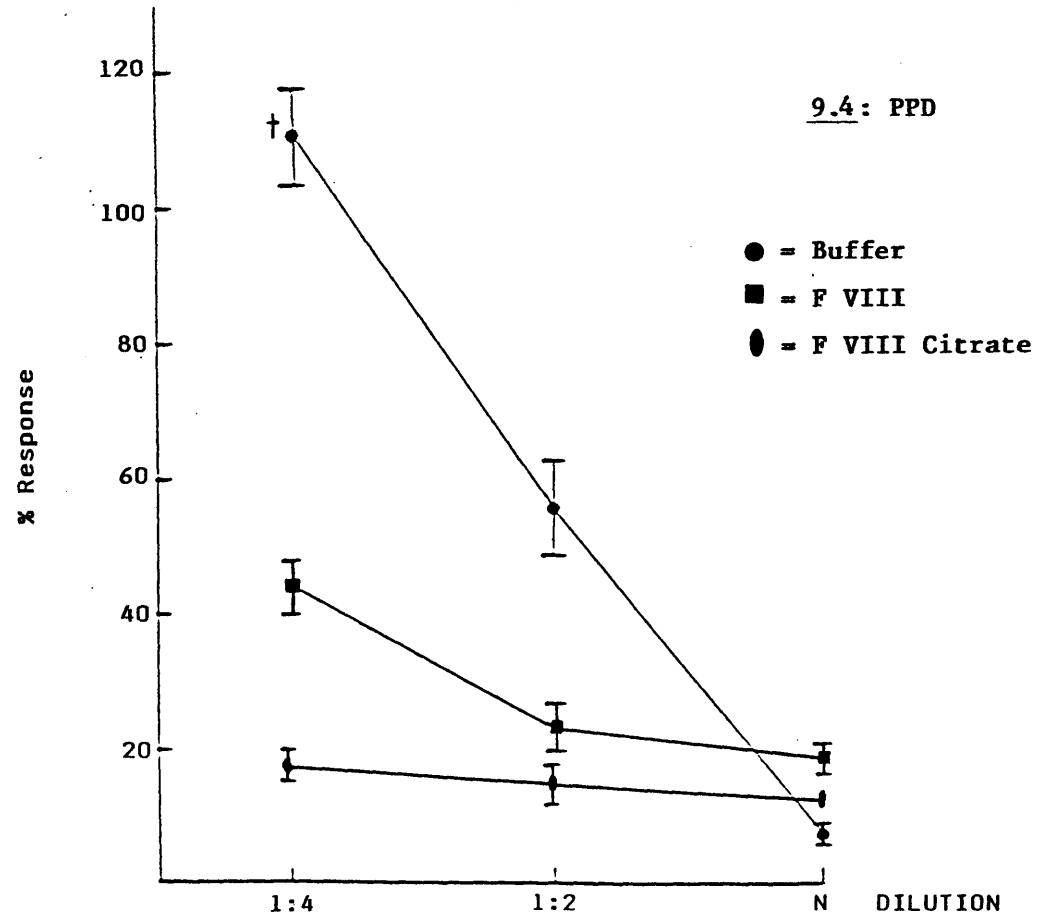
Results are mean of eight normals and are expressed as a percentage response of maximum response in the absence of any concentrate and



Figures 9.1 and 9.2: Mitogen Responses in Presence of Concentrates and Buffer.

†Mean ± S.E. (n=8).





Figures 9.3 and 9.4: Response to Mitogen and Antigen in Presence of Concentrate and Buffer.

† Mean ± S.E. (n=8).

buffer. As can be seen from the graphs there is a dose dependent inhibition for all three mitogens and PPD. The response when buffer only is present recovers most by serial dilution. When Factor VIII is diluted in medium containing buffer, there is near constant inhibition at all three concentrations. Therefore, the buffer is probably causing this effect as it is kept constant while the concentrate is diluted. Inhibition caused by Factor VIII reduces as serial dilutions are used but does not reach the level of "buffer alone" suggesting that the concentrate itself plays a major role in this observed inhibitiion.

In summary, addition of concentrate causes inhibition of mitogenesis which is dose dependent. The addition of buffer alone can also cause inhibition but on dilution it is less marked than that caused by the concentrate itself, and response tends towards normal as buffer concentrate is reduced.

McDonald (1985) suggested that this inhibiting factor was a low molecular weight substance which could be removed by prior dialysis of the concentrate. However, on several occasions I was unable to remove the inhibition observed on mitogenesis if concentrate was dialysed overnight against 500 vols. of medium prior to addition into assay. Citrate is a known constituent of the concentrate buffer and added to mitogenesis over a concentration range 5-40 mM it totally inhibited the response.

One final small experiment carried out on the effect of concentrate on mitogenesis was to look at the effect of pre-incubating cells in concentrate or buffer. If cells were incubated overnight at 37°C/5% CO_2 and then they were washed and resuspended at appropriate concentration, the responses to PHA, Con A and PWM were still totally inhibited. However, if cells were incubated in medium containing

buffer, washed and added to mitogens, then there was a differential return of response depending on the mitogen. PHA returned to 71%, Con A to 80% and PWM to 86% of their respective normal mitogenic responses. This would suggest that the main cause of inhibition was not the cells being in buffer, although this did have some effect, but rather the concentrate itself. Perhaps concentrate adheres to the cells duing this pre-incubation and cannot be washed off. Then on addition to mitogenic assay, cells are either incapable of binding the mitogen due to concentrate coating the cell or concentrate in some other way inhibits the cells response, perhaps by binding to the mitogen.

The effect of concentrate on the cells ability to produce IL-2 was also studied. Eight normals were used and their cells were set up to produce IL-2 as described in Chapter 6. To the cultures, however, were added Factor VIII at varying concentration or buffer equivalent which was made up in medium. After incubation the supernates were removed as normal and assayed in a standard IL-2 assay as described previously. For each normal, a supernate was produced using PHA at 2 µg/ml. The response of the IL-2 dependent cell line to this supernate, (as determined by incorporation of ^{3}H-Thymidine) was taken as 100% and the response produced by other supernates were expressed as a percentage of the maximum.

The responses obtained are shown in Table 9.1.

Results are mean ± S.E. of eight normals and are expressed as % response.

Supernate		% Response
Medium only		10.1 ± 0.8
Buffer only		8.5 ± 0.8
FVIII		5.3 ± 0.5
PHA + Buffer		7.4 ± 0.8
PHA + FVIII	6 U/ml	3.9 ± 0.5
" "	3 U/ml	37.7 ± 4.4
" "	1.5 U/ml	53.8 ± 8.3

Mean ± S.E.

(n=8)

Table 9.1: Effect of Concentrate and Buffer on IL-2 Production by Normals.

The results show that the addition of Factor VIII can inhibit the production of IL-2 in response to PHA. This effect is dependent on the concentration of Factor VIII used and at high concentrations (6U/ml) the response obtained is even less than that obtained for the spontaneous production of IL-2 in the absence of PHA. Lederman (1985) has shown that the inhibition observed is not caused by the Factor VIII inhibiting the IL-2 cell line from proliferating to IL-2 because on addition of concentrate to a supernate containing IL-2 the response of the IL-2 cell line was similar to response obtained when supernate without concentrate was added.

The effect of incubation of cells in concentrate in the production of immunoglobulin could not be studied by the method previously described because the concentrates themselves contain large amounts of human immunoglobulin which carried over into the ELISA assay.

9.2.3 DISCUSSION

The results described here represent some preliminary work on the effect of concentrate on various *in vitro* assays. They are by no means complete. Those experiments, however, were not the main theme of the project and were done as some additional work. The question of the effect of concentrate on lymphcyte responses could easily produce enough work for at least one complete thesis.

These studies, however, have demonstrated that Factor VIII concentrate can profoundly suppress lymphocyte proliferation to mitogen and antigen. The response was inhibited by concentrate in a dose dependent fashion. Lymphocyte proliferation is dependent on the production by T cells of IL-2 and this work has shown that IL-2 production can also be inhibited by IL-2 in a dose dependent fashion. The reason for the observed findings is unknown. It could be simple lectin-concentrate binding thus preventing cell stimulation. However, when cells were pre-incubated in concentrate, without any obvious toxic effect (confirmed by trypan blue exclusion which was comparable to that for cells incubated in medium), then washed, the inhibitory effect remained. It is possible, however, that concentrate coated the cells which could not easily be washed off. Another possibility is the citrate content in the concentrate buffer. The work has shown that buffer can produce a similar dose dependent inhibition. Citrate could be reducing the availability of calcium and possibly other cations required for lymphocyte activation. Whatever the reason, the fact remains that concentrate has inhibitory effects on mitogenesis and IL-2 production. Future experiments could look at the kinetics of this inhibition and perhaps separation techniques could be used, e.g. column gel filtration in order to separate the concentrate into its various components which could be analysed separately.

The clinical significance of the *in vitro* immunosuppressive effects of the concentrate is uncertain. Several workers have reported an observed subclinical immunodeficiency in haemophiliacs in the absence of HIV infection (Lederman, Ratnoff, Evatt et al, 1985). It is always possible that these cases reflect infection of HIV in the absence of serological evidence of infection but this is unlikely. Administration of preparations containing Factor VIII may result in a subclinical immunosuppressed state that may serve as a co-factor for the development of clinical immunodeficiency or AIDS after exposure to HIV. Ludlam and colleagues (1985) have shown that the risk of seroconversion after exposure to a presumed batch of HIV infected concentrate was correlated with the annual consumption of concentrate and also pre-exposure T helper/T suppressor subset ratios.

9.3 EFFECT of HIV on IN VITRO ASSAYS

9.3.1 Introduction

HIV is known to be the causative agent of AIDS. It is selectively tropic for the T_4^+ lymphocytes (helper T cell). The CD4 molecule on T_4 cells serves as the receptor for HIV, binding the gp110 envelope glycoprotein of the virus.

Most *in vitro* work on AIDS has involved looking at the responses of patients own cells in various assays. This study, however, has examined the effect of HIV on the response of normal lymphocytes in some of the assays previously described for the patient study.

9.3.2 Methods and Results

Again, most of the methods have been described earlier and only alterations to methodology will be described here. This study has used a disrupted HIV viral preparation (a kind gift from Dr. E. Follett). The crude extract was at 9 mg/ml and was further diluted in medium to 1 mg/ml to serve as a reference stock solution. The preparation was examined for its ability to inhibit proliferative response to mitogens and antigen. IL-2 production was also examined in the presence of HIV. Dilutions of the stock solution (1 mg/ml) were made in medium to give varying final concentrations in the assays. The HIV was used at 1/50, 1/100, 1/250, 1/500 and 1/1000 dilution of the stock. For mitogenesis 100 µl of appropriate dilution was added to 50 µl of cells and 50 µl of mitogen. Cell and mitogen concentrations were as before. For IL-2 production, 0.5 ml of HIV was added to wells with 1 ml of cells and 0.5 mls of PHA. Once again all the other conditions were as before with PHA at 2 µg/ml and cells at 10^6/ml. The toxic effect of HIV was determined by looking

at viability of cells after 48 hour culture for IL-2 production. The viabilities were similar for the wells with PHA and those with HIV added as well as PHA (95±3%). The results of the response to various mitogens or antigen are shown in Figure 9.5 Each point represents mean of eight normals ±S.E. Results are expressed as a percentage response of that obtained for each normal in the presence of mitogen but absence of HIV.

The results show that proliferative response to mitogen or antigen is inhibited in a dose dependent fashion by HIV. HIV on its own (in absence of mitogen) gave stimulation values equal or less than those obtained for negative controls, i.e. when medium was used instead of mitogen. Therefore, on its own it is not stimulatory.

The production of IL-2 in the presence of HIV is shown in Figure 9.6. Each point represents mean ±S.E. of seven normals. Results are expressed as a % response of that obtained for each normal in the absence of HIV. Again, IL-2 production is inhibited in a dose dependent fashion by HIV. HIV alone did not induce normal lymphocytes to produce detectable levels of IL-2.

9.3.3 Discussion

The small study agrees with and expands on the findings of a recent publication (Pahwa et al, 1985) which looked at the influence of HIV on polyclonal B cell activation. Disrupted HIV viral preparations had inhibitory biologic effects on normal lymphoid cells. HIV was capable of inhibiting proliferative response to mitogen and antigen in a dose dependent fashion. This inhibitory effect was extended to the ability of normal cells to produce IL-2 in response to stimulation with PHA. At the present time it is not clear how the influences of killed virus are to be explained, but further studies

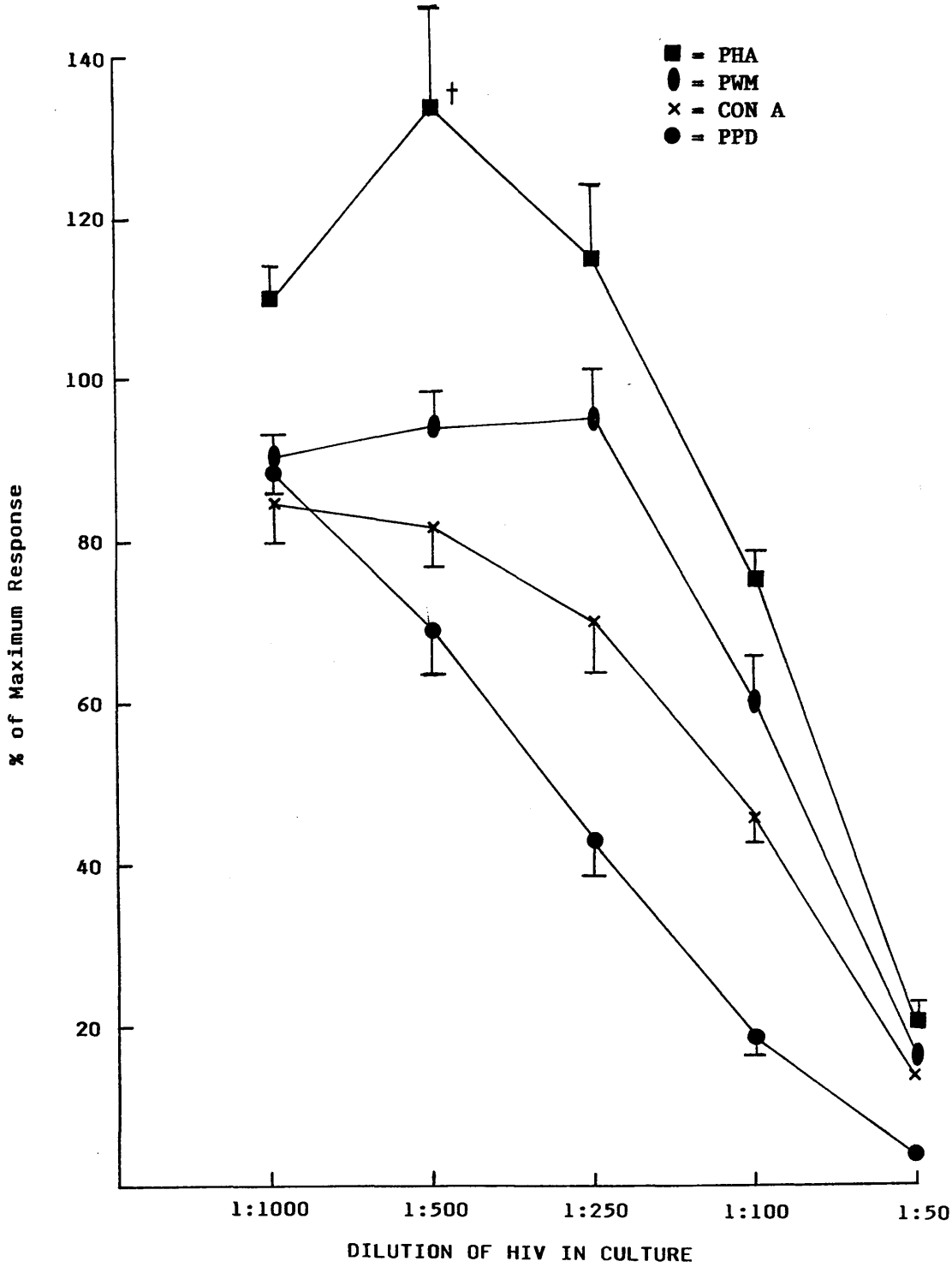


Figure 9.5: Response of Normals in the Presence of HIV

†Mean ± S.E.
(n=8).

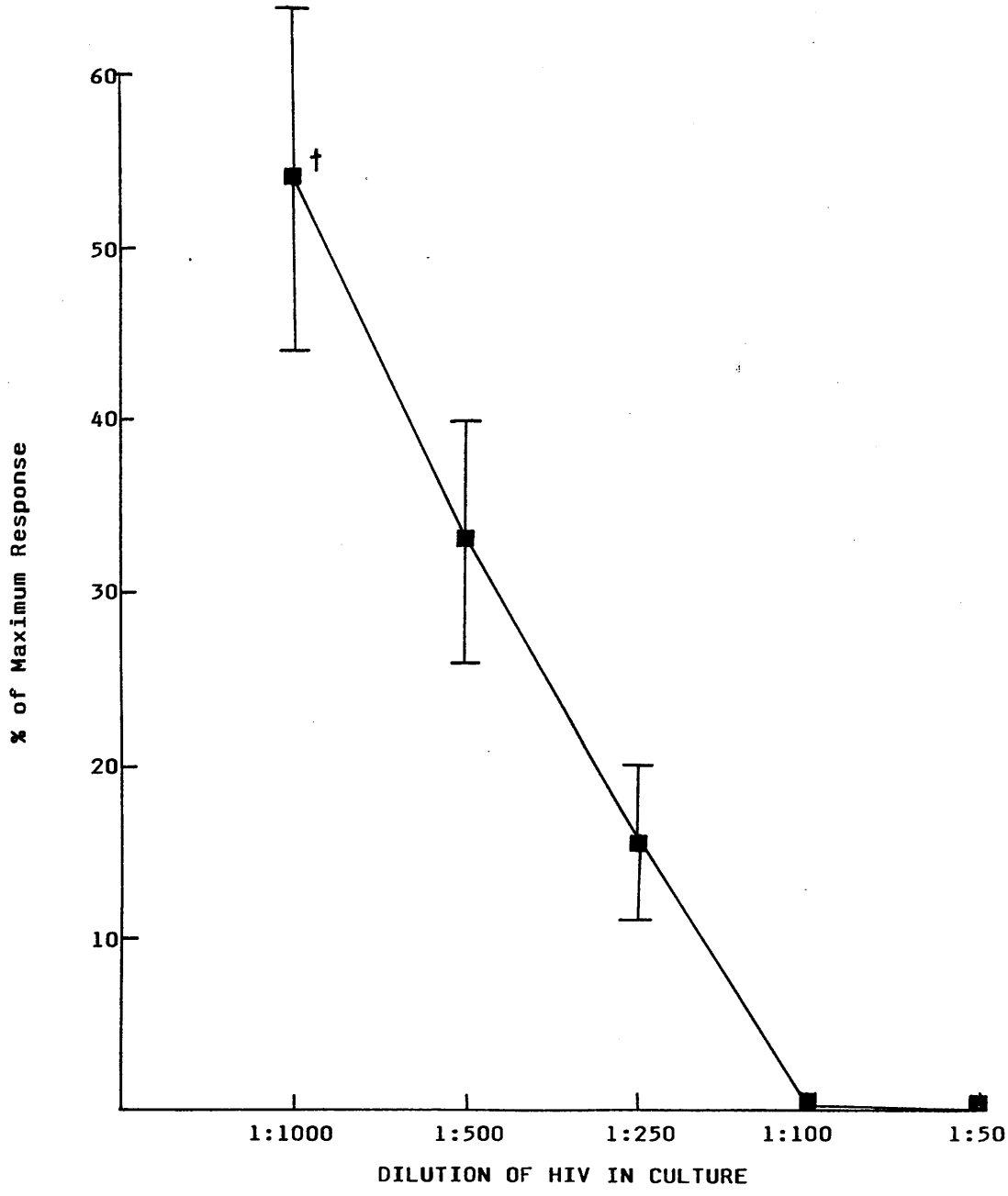


Figure 9.6: IL-2 Production in the Presence of HIV

† Mean ± S.E. (n=7).

examining the influences of different concentrations of virus, or different individual viral components in various *in vitro* assays, could help to explain the effects seen here. Pawha also found that proliferative responses to T and B cell mitogens could be inhibited. They also studied stimulation of immunoglobulin production by HIV preparations and these preparations could also influence the expression of certain membrane antigens on normal peripheral blood lymphocytes.

It is known that only a very small number of lymphocytes, perhaps as few as 1:1,000, or even 1:100,000 cells, appear to be infected by virus, in amounts detectable, yet AIDS patients show profound immunodeficiency. It is not only T cells which are affected but also other cells of the immune response as seen by the hypergammaglobulinaemia nearly always seen associated with HIV infection.

It is possible that further *in vitro* experiments using individual components of the virus may contribute towards a better understanding of the mechanisms of HIV infection with subsequent development of an immunocompromised state.

9.4 EFFECT OF HAEMOPHILIAC SERA ON IN VITRO ASSAY

9.4.1 Introduction

Siegel and colleagues (1985) have shown that sera from patients with AIDS can inhibit the production of IL-2 by normal lymphocytes. This small study has looked at the effect of haemophiliac sera on both IL-2 production and also the proliferative response to mitogens of normal peripheral blood lymphocytes.

9.4.2 Methods and Results

The mitogen assays were carried out as described before but instead of adding pooled human serum at 10% final concentration, this was substituted with haemophilic sera. In the IL-2 production assay different haemophilic sera were added at a final concentration of 10% instead of the FCS which is usually used when producing IL-2. Cells were cultured at 10^6/ml with 2 µg/ml PHA. All responses were compared and expressed as a percentage of that obtained when a standard pooled human serum was used. The three mitogens used before were tested with over thirty sera from haemophiliacs who were either HIV^+ or HIV^- "heavy" or "light" users or haemophiliacs who had received no treatment in the previous year. No significant differences were obtained with any mitogen tested when the different patient groups were compared with a group of individual controls. A similar result was obtained when the ability of sera to inhibit IL-2 production was tested as shown in Figure 9.7.

9.4.3 Discussion

These results suggest that whatever is causing the inhibition as seen by Siegel is not present in haemophiliac sera, even those who

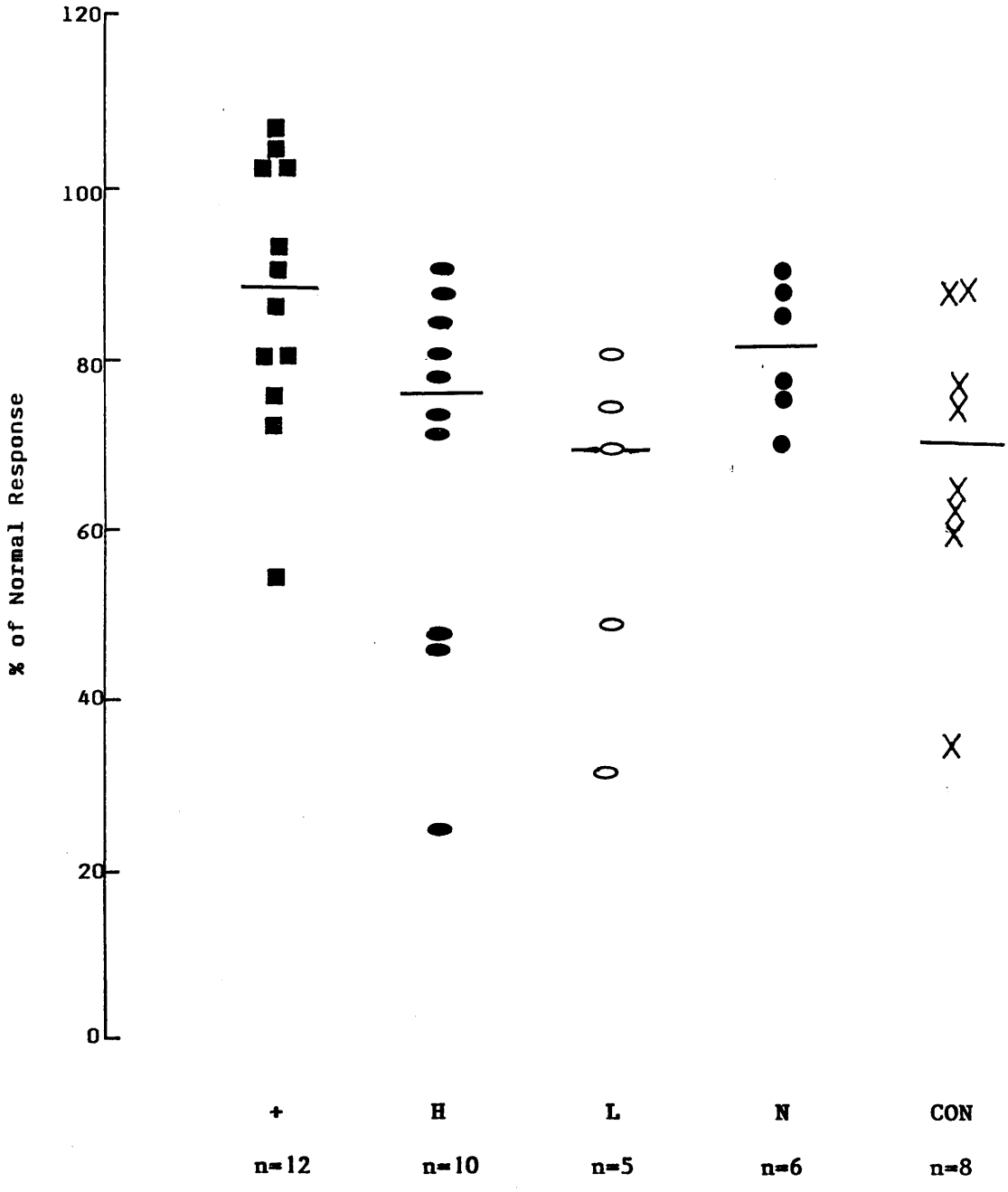


Figure 9.7: Effect of Different Sera on IL-2 Production by Normals.

were HIV^+. Of course, this was only a preliminary experiment and as with the question of the effect of factor concentration on *in vitro* assays there is still scope for further research in this area.

9.5 SUMMARY

This section of the Thesis was made up of various preliminary experiments which alone could form the basis for a complete thesis. Results, however, have shown that enough questions could be raised for a more complete study.

In summary, Factor VIII concentrate can inhibit various *in vitro* assays including mitogenesis and production of IL-2. The exact nature of this inhibition is unknown but involvement of various constituents of the concentrate buffer as well as some of the individual components of the concentrate themselves are suspected. Disrupted preparations of HIV can also inhibit mitogenesis and IL-2 production in a dose dependent fashion. The mechanism for this is as yet unknown.

Finally, sera from haemophiliacs, including those who are HIV^+ do not seem to inhibit mitogenesis or IL-2 production by normal peripheral blood lymphocytes.

CHAPTER TEN

A FINAL DISCUSSION

Since early in the AIDS epidemic it has been recognised that haemophiliacs through the use of clotting factor concentrates, produced from blood donations, are a high risk group for contracting AIDS. Most countries in the world have now reported cases of AIDS or at least signs of HIV infection in their haemophiliac populations. Cases of AIDS have been highest amongst haemophiliacs in countries which rely heavily on the commercially produced concentrate, largely from the United States.

Within the last few years concentrate has all been heat treated in an attempt to inactivate HIV, but unfortunately many thousands of patients have most probably been infected with contaminated products used before the introduction of heat treating. Hopefully, however, the numbers of new HIV seroconversions should slow down and eventually stop as all patients receive the new generation of concentrates. The incubation time of AIDS, however, will mean that new cases of full blown AIDS or related clinical conditions will continue to be reported for perhaps many years to come. The problem is still great and much work still has to be done.

Prior to AIDS, there had been reports in the medical literature to suggest that the use of clotting factor concentrates, even in the absence of HIV infection, could, in some way, produce an immunocompromised state for treated haemophiliacs. With this, as well as the desire to know more about the extent of, and effect of, HIV infection within the haemophiliac population, this study set out to examine the immune status of a cohort of the haemophiliacs who attend the Haemophilia Centre at Glasgow Royal Infirmary.

The fact that there were many regular clinics in the Royal

Infirmary for haemophiliacs, meant that it was possible to obtain samples as required for the various studies carried out.

The first investigation which we thought ought to be done was to determine the extent of HIV infection in our population. With the availability of commercially produced screening kits it was possible to test the patients for the presence of antibodies to HIV. Most of the patients have now been screened and 21 have proven to be positive. If the total number of patients is taken to be around 250, then the incidence of infection is around 8%. This figure is relatively low and is in contrast to many other reports on the incidence of infection in different haemophiliac populations. The most probable cause for this is that around the same time that the initial cases of AIDS were being reported in the United States, here in Scotland we were becoming self-sufficient in home produced concentrates. Therefore, the patients were no longer having to be treated with the commercial concentrate which has largely been blamed for spreading AIDS in the haemophiliacs. All our HIV antibody positive patients have at some time received commercially available concentrates or batches known to be contaminated with HIV. The one patient who has died, from our centre, received large quantities of imported concentrate while being treated when he lived in England. Scottish Factor VIII has not been entirely free from HIV contamination. Ludlam and colleagues (1985) have reported that 15 of their patients seroconverted after they all received concentrate from a single batch of home produced concentrate. An interesting observation from their study was that at least another 18 patients received this same implicated batch yet they did not seroconvert. There are several possible explanations for this as they discuss. It is possible that the existing immunological status of a patient, which may be influenced by the use of large quantities of

concentrate, may determine whether or not he will become infected with HIV after exposure to it, i.e. perhaps only those patients who are already immunocompromised in some way through the use of infusion of concentrates can readily be infected with HIV. Previous studies have shown that various parameters of the patients immune response can be influenced by factor consumption, e.g. inverted T cell ratios and diminished in vitro response to mitogens. (Stein et al, 1985).

It was decided to try to assess both in vivo and in vitro responses to our patients. Twenty-nine patients were tested for their response to skin testing with DNCB. This in vivo test is considered one of the best ways of examining both the afferent and efferent arms of the cell mediated immune response. It is, therefore, a relatively accurate measure of their current cell mediated immunological status. Results from this study showed that all haemophiliacs tested, irrespective of their HIV status, had a reduced response compared to a control population. This impaired response was present both in the HIV^+ patients and more interestingly also the seronegative patients. This is the first such report of depressed cell mediated immune response in the absence of infection with HIV. The impairment was related to the amount of concentrate used in therapy, lending more support to the suggestion that concentrate usage results in impaired immune function.

In vitro tests performed, included Tcell subset analysis and proliferative responses to mitogen and soluble antigen. T cell subset ratios were abnormal only in those patients who were HIV^+. In most cases the inverted ratio was due to a decrease in absolute numbers of T_4 cells. Some reports have suggested increased T_8 numbers in haemophiliacs (Stein et al, 1985) but like Carr and colleagues (1985) we found normal numbers of T_8 cells. Lymphopenia is a characteristic

finding in AIDS patients. We did not find this in any of our patient groups. However, we did find a drop in T_4 numbers in the HIV^+ group. This is in keeping with others and is probably as a result of T_4 depletion by HIV, which is known to be tropic for the T helper cell subset.

The in vitro response to mitogens amongst the HIV^- patients was comparable to controls. Amongst the HIV^+ patients, however, there was a significant decrease in response to PHA, a T cell mitogen, and PWM, a T cell dependent B cell mitogen. Both these findings are consistent with reports by others on HIV infected individuals. The in vitro response to the recall antigen PPD was normal in HIV^- patients but reduced in HIV^+ patients. Again, probably as a consequence of decreased numbers and/or a defective response of the T_4 cell.

Although AIDS produces an immunodeficient state, hypergammaglobulinaemia is another characteristic finding amongst HIV infected persons. This study has found increased serum levels of IgA, IgM and IgG in our population. These increased levels were highest in the HIV^+ patients but still significantly raised in the HIV^- ones. Raised IgG levels in the HIV^- patients correlated with the amount of concentrate therapy they received. Paired samples from 1981 and 1986 were available for 87 of the patients. Levels of IgG in 1986 were raised compared to 1981 only in the HIV^+ patients. This rise is assumed to be caused by ongoing HIV infection. Raised IgD levels have been suggested as a marker for AIDS or HIV related disease (Mizuma et al, 1987). This study, however, has not been able to detect raised IgD levels in any of our patient groups.

An assay was set up to measure the in vitro production of IgM and IgG in response to stimulation with mitogen. All patients seem to have an impaired ability to produce immunoglobulin on stimulation

compared to controls. Spontaneous production of immunoglobulins M and G, in the absence of mitogen, was raised in the HIV^+ group. The conclusions from this part of the study were that, in vitro, HIV^+ patients are switched on to produce immunoglobulin spontaneously, probably as a result of HIV infection but also possibly due to the use of concentrate. Also, all patients seem to have an intrinsic defect in their B cells as they cannot be stimulated to produce levels of immunoglobulin comparable with controls. As this occurs in the apparent absence of HIV infection, and the response of haemophiliacs who were not treated was normal, it is probably due in some way to the use of concentrate. Further studies, such as co-culturing various lymphocyte subsets with normal allogeneic cells may help elucidate this apparent defect in the patients' cells.

Many workers have tried to find surrogate markers which could indicate AIDS or progression towards AIDS, e.g. raised IgD levels. Another such marker suggested has been serum levels of β_2Microglobulin (Zolla-Pazner et al, 1984). In conjunction with other laboratory findings, levels of β_2M were successful in diagnosing AIDS cases. This present study has found increased levels in all patient groups but especially in the HIV^+ patients. This effect was not caused by carry over of β_2M in the concentrate as these preparations contained no detectable levels of the protein. Increased serum levels are thought to occur as a result of increased cell turnover and probably in our patients, increased levels are caused by the antigenic stimulation provided by the use of concentrate and also the HIV (in the HIV^+ group).

The T_4 cell is considered the central cell in the immune response. This cell also happens to be the one which HIV infects and depletes. These T helper cells have many functions as shown in

Figure 1.3. Amongst these is the production of IL-2, a lymphokine, required for T cell growth. The results on IL-2 production by AIDS patients have provided conflicting reports but it is now generally thought that IL-2 production is impaired in these patients (Murray et al, 1985). To study the production of IL-2 by the haemophiliacs, an IL-2 assay has been developed in our laboratory. The assay makes use of the IL-2 dependent cell line HT_2A. The assay had to be standardised as described in Chapter 6. The results from this study show that the cells from HIV^+ patients produce significantly less IL-2 than both other haemophiliacs and controls. In general, the HIV^- treated patients produced levels comparable to controls. Future work on IL-2 could look at purified T cell subsets to see if the abnormality in the HIV^+ patients was simply due to a decrease in T_4 numbers in this group or if there are other more complex functional defects in this group, such as over suppression.

Summarising the studies on the patients, this work has shown several defects in the patient's immune response. Some of these defects, e.g. reduced T_4 numbers, reduced IL-2 production, and reduced response to mitogen and antigen seem to be due largely to HIV infection. Other abnormalities such as increased serum immunoglobulin levels, increased β_2M levels, an ability to produce high levels of immunoglobulin *in vitro* after stimulation, and a reduced *in vivo* response to DNCB, although occurring in the HIV^+ patients, were also observed in the apparent absence of HIV infection. Therefore, although HIV can be blamed for some of the observed abnormalities, the infusion of concentrate, in the absence of HIV, seems capable of producing several abnormalities in the haemophiliacs.

In an attempt to look at the effect of concentrate on various responses, some preliminary experiments were carried out *in vitro*

using normal cells. These experiments involved looking at the effect of concentrate or its buffer on the response of normal cells in various assays such as IL-2 production and mitogenesis. The take home message from these experiments is that concentrate can inhibit mitogenesis and IL-2 production by normal lymphocytes in a dose dependent manner. Buffer used in the preparation of the concentrates can also cause a degree of inhibition but its effect is not as marked as the concentrate. Therefore, in some way, concentrate and perhaps its buffer inhibits responses of normal lymphocytes. This is an area which could be further investigated, as mentioned in Chapter 9.

The effect of HIV was also looked at in a similar way to the effect of concentrate. Again, the responses of normal cells were inhibited in a dose dependent fashion and this is another area of research which could be further expanded in the future.

The final topic examined was the effect of patients' sera on mitogenesis and IL-2 production of normals. Siegal et al (1985) have shown that sera from AIDS patients can inhibit the production of IL-2 by normal lymphocytes. This study, however, did not find inhibition of mitogenesis or IL-2 production by normals when the serum source in the assay was substituted for haemophiliac sera.

What work could be done in the future?

We are currently carrying out a study in collaboration with Professor Swanson Beck at Ninewells, Dundee. Mantoux reactions are being done on the patients and reaction sites are being biopsied. Then, by sectioning the biopsies, it will be possible to stain them using monoclonal antibodies and standard immunocytochemical techniques. It is hoped to quantitate the infiltrating cells.

Another study which is planned and is currently being developed is to assay IL-1 production by patients' monocytes to look for

abnormalities which could be associated with HIV infection or concentrate usage.

Some further experiments, such as co-culture experiments and an expansion of the in vitro work which concentrate and HIV preparations, have already been discussed. There are others though that would probably be worth following. Amongst the HIV^- patients further monitoring should be carried out to examine the effect of continued exposure to concentrates. It is also important to detect any new sero-conversions which may occur, although hopefully the incidence of new cases should now be very low.

The HIV^+ patients must also be closely followed looking for any deterioration in responses which may herald the progression of disease especially now that HIV antigen has been detected in the serum of several of the HIV^+ patients.

The haemophiliac population are a good group to study in an attempt to better understand the mechanisms and functions of HIV. Most likely, these patients have been infected with relatively low numbers of genetic variants of HIV. As viral antigens have now been isolated from some of our patients, it is an ideal opportunity to study the virus. Patients' cells could be cultured in an attempt to make isolates of the virus. Some basic molecular biology could then be done on isolated virus. It is important to determine whether or not isolates from different people are identical, similar, or even whether they show marked differences. Within individuals it would be very interesting to determine if only one isolate is found or if individuals show isolates which differ genetically. By studying individuals over a period of time, it should be possible to detect whether or not there are genetic variations in the sequential isolates of HIV obtained from an individual indicating genetic drift

of the virus. The answer to this question could have important implications in trying to develop both a successful vaccine against AIDS and also treatment regimes which will be effective in the treatment of those already infected.

Finally, this thesis has set out to make a study on the immunological status of the haemophiliac population. Hopefully, from both the findings, and from the discussions and points raised, it will be possible to continue with this line of research in an attempt to better understand the effect of both HIV infection and Factor VIII concentrate usage on the immune system of the haemophiliac patient.

APPENDIX

Mediums Used:

For Mitogens and B Cell Assay

RPMI (1640)

+

2mM L-Glutamine

+

Penicillin (50 U/ml)/Streptomycin (50 µg/ml)

+

Sodium Bicarbonate (13.5 mls of 7.5%/500 mls medium)

RPMI (Complete)

For IL-2 Work

RPMI (1640)

+

2 mM L-Glutamine

+

Penicillin (50 U/ml)/Streptomycin (50 µg/ml)

+

Indomethacin (1 mg/ml)

+

β-Mercaptoethanol ($5x10^{-5}$M)

+

Hepes (20mM)

+

Sodium Bicarbonate (5.67 mls of 7%/500 mls medium)

All Tissue Culture Mediums and Additives from Gibco (Paisley, Scotland, U.K.).

Buffers used in ELISA

Solutions

(1) 0.2 M Na_2CO_3

(2) 0.2 M $NaHCO_3$

(3) 0.01M $MgCl_2$

Coating Buffer:

1.1 ml of (1) + 1.4 ml of (2) + 7.5 mls distilled water.

Substrate Buffer:

1.1 ml of (1) + 1.4 ml of (2) + 1 ml of (3) + 6.5 mls distilled water.

Diluting Buffer:

PBS/0.5% BSA/0.05% Sodium Azide.

Washing Buffer:

PBS/0.5% BSA/0.05% Sodium Azide/0.05% Tween 20.

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