BLOOD BORNE VIRUS INFECTION AND IMMUNE MODULATION IN BOYS WITH HAEMOPHILIA A

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By

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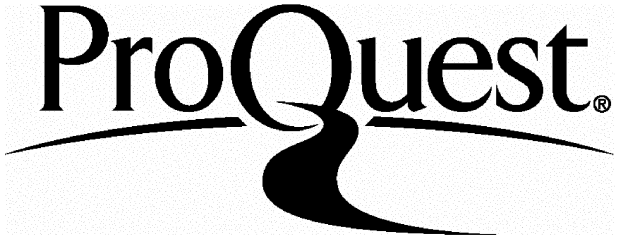

Abstract

A major complication of the treatment of haemophilia with factor concentrates was the transmission of viral infections particularly, hepatitis B, HIV and non-A non-B hepatitis (NANBH). There was also evidence of immune dysfunction occurring even in the absence of HIV infection and it remained unclear, whether this was due to other repeated viral infections or to the concentrates themselves.

The first aim of this study was to ascertain the safety of an intermediate purity factor VIII concentrate, BPL 8Y, which was dry heated at 80°C for 72 hours. Twenty five previously untreated patients, followed for up to eleven years have shown no evidence of infection with HIV, hepatitis B or NANBH.

As it became apparent that this group of boys was remaining free of significant viral infections it provided an opportunity to follow the group prospectively as regards immune function. To determine firstly, whether the previously described immune abnormalities occurred in a virus free population and secondly, if they did occur what was the relation of the immune dysfunction to concentrate treatment.

IgG levels remained stable over eleven years and no consistent changes in CD4 or CD8 levels were seen in twenty one of the patients followed over ten years.

Lymphocyte proliferation to mitogens and monocyte function were compared with a control group, and with two other groups of haemophiliacs, one HIV seropositive and a second group who, although remaining HIV seronegative had become infected with hepatitis viruses.

The responses of the BPL 8Y haemophiliacs were comparable to those of controls and better than those of the other haemophiliacs. However, looking at the data closely the responses at sub-optimal concentrations of mitogens were lower than those of controls although this was not statistically significant. A similar picture was also seen in the monocyte function assay. In conclusion, these patients, remaining free of significant viral infections are not demonstrating dramatic changes in immune function, but, at the same time they are not entirely normal. These subtle changes imply that patients must continue to be studied and that there is no room for complacency.

For my father,

Ieuan Thomas Evans

1928-1978

Acknowledgements

I was employed as a research fellow in the Department of Haematology at the Children's Hospital in Birmingham from 1989 until 1992, during which time much of the work described in this thesis was carried out. I was at this time funded by a grant from the Bio Products Laboratory, Elstree. I was also involved in the care and monitoring of some of the first children to enter the study when I was a senior house officer in the same department in 1987. During this time I was supervised by the Head of Department Dr. Frank Hill, to whom I am grateful for his advice and support both during and after my time at the Children's Hospital.

During the time of the laboratory studies I worked together with Dr. John Pasi, whose work at that time concentrated on the group 2 and 3 patients described in this thesis which formed the basis of his PhD. We enjoyed a productive partnership and I thank him for his support and friendship.

Dr. Susan Skidmore of East Birmingham Hospital was responsible for the hepatitis C antibody testing and Dr. Trudy Gentle also of East Birmingham Hospital performed the T cell subset analysis using the Facscan.

Although I left the department in 1992, it was important to ensure prolonged follow up of the children described in this study, in terms of monitoring both their immune function and the viral safety of the product they were receiving. This was continued up until 1996 and would not have been possible without the commitment of the sisters on the Haemophilia Unit at the Children's Hospital, Marion Gregory, Angela Westoby and Lynne Mathers. In addition to the sisters on the Haemophilia Unit, the secretary in the department, Pat Mann, was and has continued to be invaluable in enabling me to maintain strong links to the unit resulting in the successful long term follow up.

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Dr. Stephen Marriage of St. Mary's Hospital, London developed the software to calculate the CD4 and CD8 z scores. I am grateful to both him and Dr. Bertram Mueller-Myshok of the Bernhard Nocht Institute in Hamburg for statistical advice.

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List of Publications resulting from this work

Evans JA, Pasi KJ, Williams MD, Hill FGH
Consistently normal CD4+, CD8+ levels in haemophilic boys only treated with a virally safe factor VIII concentrate (BPL 8Y)
British Journal of Haematology 1991: 79: 457-461

Evans JA, Pasi KJ, Hill FGH
Evidence for the aetiological role of blood borne virus infections in causing reduced lectin induced T cell proliferation in haemophilic boys
British Journal of Haematology 1995: 91: 197-202

Pasi KJ, Evans JA, Wadhwa M, Thorpe R, Hill FGH
Association of changes in monocyte antigen presentation and cytokine production in haemophilic boys with treatment and blood borne virus infection
British Journal of Haematology 1995: 91: 191-196

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CHAPTER ONE

THE HISTORY OF HAEMOPHILIA AND ITS TREATMENT

"For it was taught: If she circumcised her first child and he died, and a second one also died, she must not circumcise her third child" was how haemophilia was first described in the second century in the Talmud (Rosner 1969). The first description in the medical literature appeared in 1793 (Bulloch & Fildes 1911), followed by more detailed descriptions of a bleeding disorder transmitted by a mother to her affected sons in 1803 (Otto 1803). Early family studies led to the suspicion that the condition was sex linked. Hay in 1813 stated "the children of bleeders are never subject to this disposition; but their grandsons, by their daughters, are" (Hay 1913). Sex linked inheritance was later confirmed by selective mating experiments in haemophilic dogs (Brinkhous & Graham 1950, Brinkhous 1951).

The condition had become known as 'haemophilia' in 1828 and by the beginning of this century it was established that samples of blood taken from people suffering from haemophilia clotted much more slowly than samples from normal individuals. Discovering what was actually wrong in haemophilia was made more difficult because the 'normal' coagulation process was not fully understood.

This process and the defect resulting in haemophilia were gradually elucidated during the first half of the 20th century.

It was known that the coagulation of blood occurred as a result of the formation of insoluble fibrin from the soluble fibrinogen, and that thrombin was the substance possessing the power to perform this conversion. Thrombin was generated as a result of the interaction of prothrombin, calcium and thrombokinase, the latter being derived from certain cellular elements of the body when they were broken up or destroyed. In 1911, Addis set out to isolate the individual components from haemophilic blood and compare the amount and activity of each with that isolated from normal blood. He concluded that the cause of the delay in the coagulation of haemophilic blood was due to a qualitative defect in the prothrombin resulting in a slow rate of thrombin formation. He also established that the addition of normal plasma to that from a haemophilic corrected the delay in coagulation, demonstrating that the defect was not due to the presence in haemophilic plasma of an inhibiting substance (Addis 1911).

During the 1930's and 1940's fractionation techniques were developed by which plasma and other body fluids could be separated into their various protein components. It was established that the portion of plasma responsible for the correction of the clotting defect in haemophilia was closely associated with the globulin fraction and that it was free from both prothrombin and fibrinogen (Patek & Taylor 1937, Minot & Taylor 1947). This component was called anti-haemophilic globulin (AHG) (Lewis et al 1946) and was later assigned the Roman numeral Factor VIII at the International Committee for the nomenclature of blood clotting factors in 1962 (Wright 1962).

It had also become apparent that AHG deficiency did not account for all cases of haemophilia, when it was demonstrated that mixing the blood of certain haemophiliacs in vitro with that of most others led to the correction of the clotting defect (Pavlovsky 1947). Studies of several patients in 1952 showed that the inheritance of this condition was also sex linked but that a different clotting factor was deficient (Biggs et al 1952). This factor was known as Christmas factor, and has subsequently been renamed factor IX. Christmas disease has now been renamed haemophilia B and factor VIII deficiency is haemophilia A.

It had become apparent that a number of different protein fractions were important in the process of coagulation. In 1964 the classic coagulation cascade, a series of proteolytic cleavages ultimately resulting in the formation of an insoluble fibrin clot, was described and has remained the basis of our understanding of coagulation today (MacFarlane 1964, Davie & Ratnoff 1964).

There are other disorders of coagulation resulting from deficiencies of the various other proteins involved in the process but haemophilia A remains the commonest occurring world-wide in approximately 5 in 100,000 of the whole population.

It later became clear that the plasma component termed anti-haemophilic globulin and renamed factor VIII was in fact a complex of two distinct proteins with different biochemical and immunological properties and coded for by entirely separate genes. The factor VIII procoagulant protein (FVIII:C), which is deficient in haemophilia is complexed in the plasma with von Willebrand factor (the factor VIII related protein,

FVIII:R). Von Willebrand factor is necessary for normal platelet adhesion and for primary haemostasis. Both quantitative and qualitative abnormalities of this protein result in a prolonged bleeding time and von Willebrand's disease, the inheritance pattern of which, in contrast to haemophilia, is autosomal.

The gene for FVIII:C has been cloned (Toole et al 1984) and the amino acid sequence and structure of the protein has been defined (Vehar et al 1984). The availability of the cDNA for human factor VIII has allowed the construction of plasmids that would direct the expression of FVIII protein in mammalian cell lines. This has resulted in the production of a highly purified factor VIII which may well eventually be used as the sole treatment for haemophilia A (Wood et al 1984).

Clinical features of haemophilia A

Haemophilia A is the commonest of the inherited bleeding disorders and has an incidence of 5 in 100,000 of the whole population (Biggs 1977) and occurs in all ethnic groups. It is a sex-linked condition resulting from a mutation of the factor VIII (FVIII) gene on the X chromosome leading to defective FVIII function. This may be due to a failure to synthesise FVIII, reduced synthesis or the synthesis of an abnormal FVIII variant.

Affected males carry the mutant allele on their single X chromosome and heterozygous females ('carriers') have the mutant allele on one X chromosome and the normal allele on the other. A homozygous haemophilic female is therefore theoretically possible but extremely rare.

Of the children born to an affected male, his sons will be normal and not transmit the disease and his daughters will be obligate carriers. There is a one in two chance of the affected gene passing from a carrier female to her children, therefore male children born to a carrier have a one in two chance of being affected and female children a one in two chance of being themselves carriers.

Approximately one third of newly diagnosed haemophiliacs appear 'de novo'. This may be due to a new mutation or it may be that the condition has been passed through

several generations of females and no affected family members are known.

In haemophilia, primary haemostasis, the formation of a platelet plug, is normal; so that the development of petechiae or purpura is not a feature of the condition. Clot formation is however impaired resulting in bleeding from large vessels and the development of haematomata together with bleeding into joints and muscles. Bleeding from mucous membranes is not common but haematuria and gastro-intestinal bleeding can occur.

The severity of symptoms in general parallels the degree of deficiency of FVIII, and the disease pattern tends to run true in families with the same inherited defect.

The level of FVIII is expressed in terms of the amount present in normal plasma. One unit of FVIII is defined as that amount present in 1ml of fresh normal plasma and the normal range is 50-200 u/dl (Denson & Biggs 1976). In the past FVIII levels were expressed as percentages of normal. Levels below 50 u/dl are therefore, by definition, abnormal but generally bleeding problems are not seen at levels above 30u/dl.

Haemophilic patients are divided into three categories on the basis of their FVIII levels. Severe haemophiliacs have levels of <2 u/dl and suffer from frequent and apparently spontaneous bleeds. A moderate haemophiliac has levels of between 2-5u/dl and has fewer joint and muscle bleeds which occur usually after trauma, and a mild haemophiliac (> 5u/dl) would bleed only after severe trauma or dental or general surgery. The division into categories is only a rough guide. There are severe haemophiliacs who rarely bleed whereas some of those with moderate levels behave clinically more like a severely affected individual. Variations of lifestyle of course play a part in disease presentation and it is also possible that FVIII activity measured *in vitro* (categorising someone as moderate) is not effective *in vivo*.

If there is a family history of haemophilia the diagnosis of an affected child may be made early on in life by performing a FVIII assay on a blood sample from the baby or on blood from the umbilical cord.

Severe haemophiliacs tend to present as young children once they have begun to walk and develop bruising and subcutaneous haematomata as a result of frequent falls. The

condition can occasionally present in the neonatal period, with for example, severe cephalohaematomata, prolonged bleeding from the umbilical cord or a large haematoma from an intra-muscular injection of vitamin K. However, it is more common for the children to present slightly later with multiple bruises and swellings and haemophilia is an important diagnosis to consider in a child where non-accidental injury is being considered.

As a severe haemophiliac begins to walk he becomes prone to developing haemarthroses which can become a recurrent and chronic problem. Bleeding can occur into most joints but the commonest to be involved are knees, elbows and ankles. The onset of a joint bleed is usually associated with some discomfort and it is important that children learn to recognise this so that treatment can be given early. If left untreated the joint will swell and become very painful. As with any other joint injury the surrounding muscles waste and once the bleed has subsided the joint may remain very unstable and as a result bleeding re-occurs as soon as mobilisation begins.

Recurrent bleeding into a joint results in inflammation and the gradual development of chronic damage and crippling deformity. The development of adequate treatment and increasing knowledge about the condition means that severe joint damage should occur much less frequently now and in the future.

The other major sites of bleeding include muscles, bleeding into which can result in nerve compression and ischaemic damage. Haematuria and gastro-intestinal bleeding may occur, the latter may be the first presenting sign in a mild haemophiliac who, in adult life develops a peptic ulcer.

Other individuals are diagnosed when they suffer severe trauma or undergo surgery or dental extractions. The incidence of intracranial bleeding is fortunately low, but it is extremely important that head injuries in children with haemophilia are treated with great caution and appropriate treatment with FVIII concentrate.

The Development of Treatment of Haemophilia

The clinical picture and the quality of life of people with haemophilia changed

dramatically with the development of blood products containing a large concentration of FVIII. In 1983, before the devastating effects of HIV transmission became apparent, the life expectancy of a severe haemophiliac was approaching that of a normal individual (Rizza & Spooner 1983) whereas the picture 50 years before had been entirely different. In 1937 Carroll Birch reviewed the course of disease in a large group of patients with haemophilia. The clinical picture was that of painful deformity in early childhood accompanied by fear of death after trivial injury and an almost certain early demise. Of 113 patients 82 died before their 15th year and only six survived for greater than forty years (Birch 1937).

Treatment

In 1840, a paper in the Lancet described how the life of an eleven year old boy whose life had been in danger on several occasions as a result of haemorrhage was saved by the transfusion of fresh blood from "a stout healthy young woman". The boy had had an operation to correct a squint six days before and had continued to bleed (Lane 1840). Other recommended treatment modalities at that time included those that are still of use today such as the application of ice and splinting and other less useful examples including the oral administration of lead, antimony, strychnine and turpentine.

Attempts at treatment by the transfusion of whole blood and plasma were hampered both by the limited supply and by problems of volume overload (MacFarlane 1972). It was one hundred years after Lane's original observation that it became generally understood that by transfusing whole blood one was temporarily replacing a missing clotting factor in the recipient thus enabling his own blood to clot.

The aim of treatment would be to replace the deficient fraction in pure, small and concentrated amounts.

As a result of the development of fractionation techniques, a plasma component which corrected the clotting defect in haemophilic plasma *in vitro* had been isolated. This was later assigned the name Factor VIII but was initially known as anti-haemophilic

globulin (AHG). The development of the thromboplastin generation test in 1953 enabled the amount of AHG to be assayed which was an extremely important development (Biggs & Douglas 1953). It then became possible to establish the amount of AHG required to achieve a haemostatic response, how long this response would last and also how much AHG was present in various preparations.

Early therapeutic preparations were made from ox and pig blood in an attempt to overcome the limited supply of human blood (Bidwell 1955a & b). However, these animal products proved to be pyrogenic and antigenic and also induced thrombocytopenia in some cases.

A major breakthrough in the development of therapeutic materials came with the discovery of cryoprecipitate. When frozen plasma was thawed in the cold (4°C) the cold insoluble precipitate recovered was found to contain 60-70 % of the plasma factor VIII (Pool 1964). The separated plasma could be further fractionated into other components and the cryoprecipitate used directly for treatment and also used as the basis for further fractionation and purification techniques in the development of the FVIII concentrates that are used today.

The discovery of cryoprecipitate revolutionised haemophilia care. It was found to be effective in controlling bleeding and it was prepared by a method available in many blood banks and which was relatively simple (Pool & Shannon 1965). Disadvantages were that it had to be stored frozen at -20°C or less and its reconstitution before use was time consuming. Storage problems meant that it was unsuitable for home therapy. The amount of factor VIII in each bag also varied making decisions about how much to give difficult.

An advantage,however, which became apparent after the initiation of the use of multi-donor concentrates was that a patient using cryoprecipitate was less likely to become infected with blood borne viruses by virtue of the fact that they were receiving blood products from fewer donors.

After cryoprecipitate, methods were developed for the large scale fractionation of plasma to prepare freeze dried (lyophilised) concentrates of factor VIII, which could be

reconstituted into small volumes and were convenient to use not only in the hospital setting but also for home therapy.

Different techniques were developed for fractionation using combinations of cryoprecipitation and various precipitation methods with for example, ethanol, polyethylene glycol and amino acids. Although the benefits of the large pool concentrates were enormous, it became apparent during the 1970's that haemophiliacs were at high risk of becoming infected with hepatitis and later with HIV. Further steps in the production process were added in an attempt to improve viral safety with varying results. These have included heating concentrates in the dry state or in solution and the addition of solvents or detergents.

Different preparation methods result in the development of concentrates of differing purity. "Purity" is defined as specific activity or units of factor VIII per milligram of total protein present in the concentrate. It is used as an index to divide concentrates into three very broad categories. High purity concentrates have a specific activity exceeding 0.5 iu/mg protein, intermediate purity products have a specific activity of 0.2-0.5 iu/mg protein and frozen or freeze dried cryoprecipitate 0.1-0.2 iu/mg protein. It was found that purifying the concentrates by passing them through a chromatography column lined with a monoclonal antibody with high affinity for factor VIII, removed nearly all the extraneous unnecessary proteins and produced products of extremely high specific activity (Addiego et al 1992). These monoclonally purified high purity products became available in the late 1980s.

The yield of factor VIII from the original source plasma drops as further purification stages are included in production.

A major factor determining which therapeutic material was received by a patient was the limited supply of donated blood and the various products derived from it. In 1967 cryoprecipitate was introduced for treatment purposes and in 1973 freeze dried intermediate purity concentrates were introduced. Since this time the demand for concentrates has steadily increased. In 1973 5.5% of haemophiliacs were receiving commercial concentrates(Biggs 1977). At this time, 60 million international units of

FVIII were used in the United Kingdom to treat haemophilia and von Willebrand's disease and this had risen to 160 million units by 1994 (UK Haemophilia Centre Directors Annual Returns 1994). In the 1970's, however, the supply of plasma obtained from British volunteer blood donors was limited and insufficient to cope with the demand for treatment. Therefore, factor VIII concentrates produced by commercial companies in the USA were imported. The first commercial lyophilised concentrate was licensed for use in the UK in 1973 and in that year 5% of British haemophiliacs were being treated with imported commercial concentrates (Biggs 1977). The development of home therapy programmes in the mid 1970's resulted in the increasing use of commercial concentrates and a corresponding decline in cryoprecipitate use. By 1976, 20.7% of haemophiliacs were being treated with commercial concentrates and this figure had increased to 60% by 1980 (Biggs & Spooner 1977, Rizza & Spooner 1983).The use of concentrates decreased slightly between 1982 and 1985 with the discovery of the increased susceptibility of haemophiliacs to AIDS. Virucidal steps in the production process were developed and the use of concentrates once again increased.

As already mentioned the increased use of concentrates resulted in haemophiliacs being exposed to very much larger numbers of donations. A single treatment of cryoprecipitate usually exposed a patient to approximately 6 donors, whereas a batch of the early British concentrate was prepared from 250-750 voluntary donations and commercial products from the USA contained plasma from as many as 5000 paid donors.

Physicians responsible for the care of haemophiliacs during the 1970's expressed concern at the dependance on imported products (Biggs 1977) and that blood from foreign, paid donors may be more likely to transmit infection. However, self-sufficiency did not occur until, in the late 1980's there was a major government investment in plasma fractionation and in 1990 73% of the 110 million units of FVIII used in the UK was derived from plasma of unpaid British donors.

The definitive way of providing a product free from " human viral contamination " is

to avoid blood donation altogether. The gene for factor VIII was cloned in 1984 (Toole et al 1984) and following this, extensive work resulted in the production of a recombinant factor VIII product which has been shown to be both theraputic and well tolerated (Schwartz et al 1990).

The next major step for the future will be the development of gene therapy enabling an individual haemophiliac to produce his own factor VIII but as yet haemophiliacs remain dependant on infusions.

It is important to note that there was one success in the search for a non plasma derived treatment for haemophilia A. In 1977 it was shown that the intravenous administration of 1- Deamino-8-D arginine vasopressin (DDAVP) to mild haemophiliacs resulted in a significant short term increase in their factor VIII levels (Mannucci et al 1977) and this surely reduced the exposure of some individuals to contaminated factor VIII products.

At the present time, severe haemophiliacs and some of those with slightly higher levels of FVIII are treated with FVIII concentrates. The plasma concentration of FVIII required for haemostasis ranges between 10 and 40 iu/dl. The half life of transfused FVIII concentrate is between 8 and 12 hours and between 60 and 80% of the FVIII transfused is recovered in the blood. Different plasma levels of FVIII are required for different bleeds and clinical situations. A plasma level of 15 to 20 iu/dl is required for minor haemarthroses and haematomas and 20 to 40 iu/dl for severe haemarthroses and muscle haematomas. For major surgery levels of 80 to 100 iu/dl are required and it is essential that in the case of a severe bleed or post surgery that plasma levels of FVIII are maintained and are not allowed to become sub-therapeutic.

Calculating the dose of factor VIII

A dose of 1 international unit (iu) of Factor VIII per kg body weight results in an increase of factor VIII concentration in the recipient's plasma of approximately 2iu of factor VIII /dl. Hence having established what the clinical problem is and the factor VIII level that is required the dose of factor VIII can be calculated from the simple

formula:

$$\text{Number of units required} = \frac{\text{weight (kg) x rise required (iu/dl)}}{2}$$

The rise achieved by giving 1 iu/kg may be slightly higher or lower than 2, because of the variation between patients. For an individual, transforming the above formula gives rise to that individual's recovery constant or K, as in;

$$\frac{\text{Rise observed (iu/dl)}}{\text{Dose (iu/kg)}} = K$$

It is important to assess what an individual's response is to a given dose of factor VIII by measuring the rise in factor VIII levels achieved. This does not need to be done after every dose, but should be done where it is imperative that high levels are achieved for example pre-operatively or if there is a very severe or life threatening bleed.

The administration of FVIII must be combined with measures to ensure that a bleed does minimal damage. This includes immobilisation and subsequent physiotherapy in the case of a severe joint or muscle bleed and the education of both the child and his parents concerning how to recognise bleeds and the importance of early treatment.

Home Therapy

As already mentioned the introduction of cryoprecipitate revolutionised the lives of many haemophiliacs. A treatment became available that would prevent the development of chronically damaged joints and a life of disability. However, as a result of having a treatment available the number of hospital visits increased together with an increasing amount of lost school and work time. It was also important to treat bleeds early and time was wasted getting to the hospital. Training patients to treat themselves at home would potentially lead to a better quality of life. The first home treament programme was set up in 1960 by Dr. Holden in Fort Worth, Texas, using fresh frozen plasma. With the introduction of lyophilised concentrates which were convenient to

use the number of home therapy programmes rapidly increased.

Criteria for starting home therapy varies from centre to centre, but it is usually only severely affected individuals who participate. Training can start while the child is still young, when he and his parents can learn how to recognize bleeds and which bleeds can be treated at home and which require hospital attendance. Ideally, both parents and then the children are taught how to perform venepuncture. It is of vital importance that patients on home therapy are kept under frequent outpatient review.

When home therapy was initially introduced the amount of FVIII used actually increased over the initial twelve months but then again decreased towards levels used before home therapy was started (Rizza, Biggs & Spooner 1978). The major advantages of home therapy were that treatment could be given early and that there was a major social and psychological benefit in that it resulted in much less disruption to everyday life.

Haemophilia is a rare condition, requiring specialist management. It is important that the administration of FVIII treatment is combined with counselling and education of the patient and his family. In 1954, Haemophilia Centres were first set up by a Medical Research Council committee. The role of these centres increased as treatment possibilities expanded and today within the UK there are 23 Comprehensive Care Haemophilia Centres and a further 109 smaller haemophilia centres (Ludlam 1998). The Comprehensive Care Haemophilia Centres provide multidisciplinary care with input from nurses, social services, physiotherapy and associated medical specialties, for example orthopaedics and those involved in the management of hepatitis and HIV. They also provide the facilities for carrier detection and genetic counselling. The activities of these units are co-ordinated by the Haemophilia Centre Directors who are responsible for the annual collection of statistics concerning the number of patients, treatment used and treatment complications, along with the development of management guidelines. All patients with coagulation disorders are registered with a particular centre and their clinical progress and FVIII usage closely monitored.

CHAPTER TWO

COMPLICATIONS OF HAEMOPHILIA TREATMENT WITH LARGE POOL FACTOR CONCENTRATES

The transmission of bloodborne viruses

An increased incidence of acute hepatitis in patients with haemophilia was reported soon after the introduction of clotting factor concentrates (Kasper 1972).

Serological studies for markers of hepatitis B infection demonstrated that greater than 50% and in some studies up to 90% of treated haemophiliacs had developed hepatitis B surface antibodies, indicating past exposure. 5-10% of these individuals were chronic carriers of hepatitis B, in that they were hepatitis B surface antigen (HBsAg) positive and were therefore infectious and at increased risk for developing chronic liver damage.

Since the introduction of screening blood donations for HBsAg, outbreaks of hepatitis B have still been reported. A safe and effective vaccine against hepatitis B was introduced in 1984 and this has further reduced the risk of infection. Vaccine should be given to all newly diagnosed haemophiliacs prior to them receiving concentrates.

Hepatitis, in the absence of serological markers of hepatitis A or B, known as non-A non-B hepatitis (NANBH) was found to occur in virtually 100% of patients receiving concentrates for the first time (Fletcher et al 1983, Kernoff et al 1985). The reported incidence of jaundice in haemophiliacs however, was only about 3% per year (Rizza & Spooner 1983). In the majority of individuals infection was not associated with any clinical signs or symptoms and was detected only by biochemical evidence of abnormal liver function. The long term significance of this widespread infection with both hepatitis B and particularly NANBH remained unclear.

During the late 1970's several units performed series of liver biopsies to assess the incidence and severity of liver disease. A study from Italy (Mannucci et al 1982) found the predominant lesion to be chronic persistent hepatitis (CPH) and little evidence of progression over a three year period. However other studies including one from Sheffield showed a significant progression of disease over a period of years from chronic persistent hepatitis to chronic active hepatitis and cirrhosis (Hay et al 1985). A significant number of haemophiliacs were presenting with serious and life-threatening complications of chronic liver disease such as oesophageal varices (Miller et al 1988).

It has become clear that liver disease is progressive in the haemophiliac population and will remain a significant cause of morbidity and mortality in future years (Eyster et al 1992).

Hepatitis C

Up until 1989, no aetiological agent or serological test for NANBH existed. However, in 1989 workers at the Chiron Corporation in California took large volumes of of highly infective chimpanzee plasma, which had been produced from hepatitis transmission experiments. From this they isolated a viral genomic clone encoding an antigen which bound to antibody in the serum of patients with chronic NANBH. The virus from which this clone was derived was named hepatitis C (HCV). The hepatitis C antibody test was developed using the recombinant polypeptide derived from the clone (Choo et al 1989, Kuo et al 1989).

Testing of blood from treated haemophiliacs who had biochemical evidence of NANBH revealed a high proportion of anti-HCV seropositivity, a study in the UK showing 59% of haemophiliacs exposed to concentrates were seropositive with 76% of those with an annual factor VIII usage of greater than 10,000 units.(Makris 1990).

The frequency of HCV antibodies in blood donors varies throughout the world, being 0.05% in one study in the UK (Irving et al 1994), 0.36% in the USA (Murphy et al 1996) and 0.98% in Japan (Yamaguchi et al 1994). These relatively high prevalences indicate why infection in haemophiliacs was so widespread; plasma pools contained donations from 1000's of donors so it was extremely likely that a pool would contain an infected donation.

HCV antibody is not detected until an average of 15 weeks (4-32 weeks) after the onset of hepatitis. Therefore HCV antibody assays will identify some but not all blood donors infected with the virus. The relationship between serological status and infectivity is not clear.

Not all patients treated with concentrates and with both biochemical and histological evidence of liver disease were HCV antibody positive, although the majority of cases

were. Retrospective testing on stored samples demonstrated in some cases loss of antibody. Other explanations possibly include a failure to seroconvert in a virus antigen positive individual or the possibility of a further infectious agent.

Human Immunodeficiency Virus

An acquired cellular immunodeficiency manifesting as Pneumocystis carinii pneumonia occurring in previously healthy homosexual men was first described in 1981 (Centers for Disease Control 1981a & 1981b,Gottlieb et al 1981). Concern about blood borne transmission of an infectious agent increased in 1982 when, following a platelet transfusion a baby developed unexplained cellular immunodeficiency and opportunistic infections, and the donor subsequently developed AIDS (Ammann et al 1983).

The first cases of AIDS occurring in haemophiliacs were reported in 1982 (Centers for Disease Control 1982).

Following the early reports and extensive epidemiological work it became obvious that there was an underlying infectious cause for the secondary immunodeficiency which was assigned the acronym AIDS (acquired immune deficiency syndrome). Virological studies showed conclusively that the virus was transmissable through blood cells and plasma and was associated with an asymptomatic but contagious carrier state (Groopman et al 1984). The discovery of the aetiological agent now known as the human immunodeficiency virus (HIV) (Barre–Sinoussi et al 1983, Gallo et al 1984) subsequently led to the development of a serological test for HIV in 1984. HIV antibody testing revealed that a large number of severe haemophiliacs treated with large pool factor VIII concentrates between 1979 and 1984 had seroconverted to HIV. By 1985 in the United Kingdom 44% of over 2000 haemophilia A patients were found to be HIV antibody positive with 59% of those with severe disease seropositive (UK Haemophilia Centre Directors 1986). Many individuals had also become severely immunocompromsed and developed clinical AIDS, whereas others remained asymptomatic although HIV seropositive.

As of June 2000 1351 (1339 men and 12 women) in the United Kingdom were known to have become infected with HIV through treatment with clotting factor concentrates (Communicable Disease Report 2000). 844 (62%) of these individuals have died.

It had been observed early on in the AIDS epidemic that progression to symptomatic AIDS was slower in those who acquired the infection during childhood and adolescence compared to those who became infected during adulthood (Goedert et al 1989). This is indeed the case for the cohort of haemophiliacs infected with HIV in the United Kingdom. By the year 2000, only 9% of those infected when over the age of 40 are still alive, compared with 56% of those aged less than 20 when diagnosed.

When comparing the HIV infected haemophiliac population with individuals infected by other routes there is an interesting difference in causes of death. Of the 844 individuals infected by blood products who have died, 242 (29%) did so without having developed an AIDS defining condition whereas in other groups only 5% are recorded as dying without having developed an AIDS defining condition. 181 of the 242 individuals who died had a recorded cause of death; 56 were liver disease, 41 cardiovascular disease and 38 cases of malignancy (Communicable Disease Report 2000). Liver disease, particularly hepatitis C is contributing significantly to morbidity and mortality in the haemophiliac group. Individuals co-infected with HIV and HCV have been shown to have more severe hepatic fibrosis and a higher frequency of cirrhosis (Dieterich et al 1999), together with higher HCV RNA levels in those infected with both HIV and HCV compared with those infected with HCV alone (Eyster et al 1994).

This observation has been confirmed by long term follow up studies of individuals treated with blood products before 1985, some of whom have both HIV and HCV infection and others HCV infection alone. One such study of 310 patients followed for twenty five years in a single haemophilia centre has shown a very bad outcome in those infected with both HIV and HCV. In this group progression rates to death twenty five years after exposure to HCV were 47% due to any cause and 19% due to liver disease. However, for those infected with HCV alone liver disease progressed much

more slowly, with only a 3% progression to a liver related death. Interestingly, four of the six liver related deaths that occurred in HIV negative patients were associated with an increased alcohol intake (Yee et al 2000).

The introduction of highly active combination anti-retroviral drug therapy (HAART) for the treatment of HIV infection during the mid 1990's has been associated with a decline in the number of deaths in all groups of individuals infected with HIV including those through clotting factor concentrates. Considering again the cohort of 1351 individuals infected with HIV through blood products in the United Kingdom; 993 were known to be alive at the beginning of 1992. Of these 36% died in the ensuing four years , whereas in the four years following 1996 after the introduction of treatment only 19% of the remaining 637 died(Communicable Disease Report 2000). There is much discussion as to the effect of HAART on hepatitis C infection. The use of certain protease inhibitors may increase the risk of hepatotoxicity (Sulkowski et al 2000) and it may well be that hepatitis occurs as a result of the restoration of anti HCV immune responses (John et al 1998).

Immunological consequences of HIV infection

After seroconversion to HIV there is an asymptomatic period of variable length prior to the development of symptoms. During this time there is a progressive development of immune abnormalities resulting eventually in a profound immunosuppression. Although the HIV virus is present at low titres in the peripheral blood during this asymptomatic period it is found to be actively replicating in lymph nodes.

Infection with HIV results in a progressive decline in the number of circulating CD4 positive (T4) lymphocytes. The CD4 cell has been found to be the principal target of HIV; the CD4 molecule present on the cell surface acting as a high affinity receptor for the envelope glycoprotein (gp 120) of the virus (Klatzman et al 1984, Rosenberg and Fauci 1989). Many CD4 cells are killed as a direct result of infection with HIV, whereas others survive with a low level of chronic viral infection acting as a reservoir for viral replication and further infection (Zagury et al 1986). HIV is also able to infect

other cells which express the CD4 molecule on their surfaces. This includes cells of the monocyte-macrophage line, which unlike the CD4 cells do not appear to be killed directly by the virus but become chronically infected. A number of monocyte and macrophage functions have been demonstrated as being compromised in HIV infection including monocyte chemotaxis, monocyte dependant T cell proliferation and C3 receptor mediated clearance of red blood cells by tissue macrophages (Rosenberg and Fauci 1989).

A large number of other cells have been infected with HIV in vitro and also in vivo, including B cell lines, glial cells, cervical cells and bone marrow progenitor cells (Rosenberg and Fauci 1989).

Infection and depletion of the CD4+ lymphocyte subpopulation, which has a pivotal role in the induction of the immune response, results in profound immunosuppression and the susceptibility of an individual to a wide range of opportunistic infections and neoplasms.

The Development of Safer Factor Concentrates

Since it became apparent that recipients of large pool plasma concentrates were at an extremely high risk of becoming infected with different viruses, huge efforts have been made to render these products "safe". Initial steps include donor selection and exclusion of donors perceived as being "at risk" for carrying such infections. It is commonly thought that the safest populations are the groups of volunteer donors who receive no payment, however to demonstrate that sometimes these perceptions are sometimes mistaken, one study has shown that repeated donations from selected paid donors are less likely to be contaminated by viruses (Taswell 1987).

Antibody screening tests of donations will also significantly reduce the risk of infection, although not entirely because these will fail to detect infected donations where the individual has not yet sero-converted; the so called "window period" in HIV and HCV infections.

Because infected donations will for several reasons therefore "slip through the net" products need to be treated during the manufacturing process to attempt to eliminate these viruses. The evaluation of the various treatment processes is done by means of prospective studies of patients, who previously have received no blood products, such as that group described in this thesis.

The various viral inactivation methods employed included heating in the dry state, heating in solution, the addition of organic solvents and detergents or heating in the presence of vapour.

In the early 1980's heating to between 60° and 68° for various time periods between 24 and 72 hours was shown to be inadequate to eliminate both HIV (Williams et al 1990) and hepatitis (Colombo et al 1985, Lush et al 1988).

Heating "wet" to 60° in the presence of the organic solvent n-heptane was found to reduce but not to eliminate the risk of hepatitis (Kernoff et al 1987). Pasteurization (heating in solution) has been shown in large studies of prospectively treated patients not to have transmitted HIV or hepatitis B (Schimpf et al 1989, Kreuz et al 1992). However cases of hepatitis have been reported in haemophiliacs treated with these products although not within the context of prospective safety studies (Brackmann et al 1988, Schulman 1992). Whether these seroconversions were indeed related to the product or not is not entirely clear but the reports raise sufficient concern that one must always remain vigilant and that no product is 100% safe. The concentrates treated with a combination of an organic solvent (tri n-butyl phosphate) and a detergent (sodium cholate, Tween 80 or Triton X-100) have shown no seroconversions to hepatitis B or C or to HIV in several large studies (Horowitz et al 1988, Di Paolantonio et al 1992) . These viruses have a lipid envelope, rendering them susceptible to this treatment, whereas non- enveloped viruses remain resistant as has been shown by the fact that these products are capable of transmitting both parvovirus B19 (Azzi et al 1992) and hepatitis A (Mannucci 1992).

Although dramatic improvements have been made in the elimination of viruses, it is important not to become complacent. The possibility of the emergence of a new virus resistant to presently used treatment methods must always be born in mind.

Immune Modulation in Haemophilia

Large pool factor VIII concentrates have been considered as a potential cause of abnormalities of immune function described in haemophiliacs in the absence of infection with HIV.

A clinical observation made at the Birmingham Children's Hospital in 1981 gave rise to concern that haemophiliacs were in some way immunocompromised (Beddall et al 1985).

An outbreak of tuberculosis occurred on a children's ward, where a child subsequently found to have spinal tuberculosis, had been admitted. On screening contacts the child's mother, who had been resident on the ward was found to have open pulmonary tuberculosis and a number of patients had been exposed. The children admitted to this ward included those with coagulation disorders, children with leukaemia and other tumours and other children with a variety of general paediatric problems. Following exposure, 10 out of 21 (48%) of the children who had been receiving cytotoxic chemotherapy developed clinical tuberculosis, in comparison with 3 out of 75 (4%) of the general paediatric patients who were exposed. Surprisingly, 6 of the 16 (38%) boys with haemophilia who had been inpatients and had been treated with commercial FVIII concentrates also developed evidence of infection with tuberculosis, implying that their ability to handle pathogens was impaired in a comparable way to children being treated with immunosuppressive chemotherapy. At that time HIV testing was unavailable and the clinical syndrome subsequently known as AIDS was only first being described. Testing of retrospective stored serum samples however, revealed that only 2 of the 6 boys who contracted tuberculosis would have been HIV seropositive at that time.

With the onset of so called AIDS various groups of haemophiliacs were immunologically investigated revealing a wide spectrum of abnormalities.

Reports in 1983 (Lederman et al 1983, Menitove et al 1983) described cases of men with haemophilia A, who had been treated with large pool factor concentrates, developing a spectrum of opportunistic infections. The pattern of disease closely resembled the acquired immunodeficiency syndrome that had recently been described in homosexual men and intravenous drug users. These haemophiliacs also had immune abnormalities similar to these other groups including abnormal lymphocyte subpopulations and reduced lymphocyte responses to mitogens.

Among the abnormalities described were a relative increase in the number of circulating T suppressor (CD8 cells and a reduced number of T helper (CD4) relative to the number of CD8 cells; (the CD4/CD8 ratio).

When a commercial assay became available to detect IgG antibodies to what was then known as HTLV-III and is now HIV, it became possible to test the sera of all haemophiliacs and to determine if they had been exposed to the virus. It then also became possible to see whether seroconversion to HTLV-III correlated with the development of abnormal immune parameters.

Shannon in 1986 looked at a population of paediatric haemophilia A patients and found that lymphocyte subsets in children with haemophilia were similar regardless of their seroconversion status. Of children who had been treated with commercial factor VIII concentrates, there were some who had seroconverted to HTLV-III and some who had remained seronegative. However, both groups had significantly higher numbers of CD8 lymphocytes and significantly decreased CD4/CD8 ratios when compared with age-matched non-transfused control children (Shannon et al 1986a & b).

In one study the degree of immunosuppression seen, reflected by reduced lymphocyte responses to mitogens, appeared to be related to the amount of FVIII concentrate received (Sullivan et al 1986). However, because those individuals who had seroconverted to HIV were also those who tended to have received larger amounts of concentrate, it was difficult to assess the relative contribution of the HIV infection and

the treatment concentrates themselves to the development of the immune abnormalities. Another study suggested that lymphocyte mitogen responses were depressed to the same extent in both HIV positive and negative individuals. (Mahir et al 1988)

Evidence of dysregulation of the cellular immune response was increasing, and abnormalities of the humoral response were also detectable. Total levels of IgG were found to be significantly raised in large cohorts (Lee et al 1985, Moffat et al 1985), and an Italian study demonstrated that peripheral blood mononuclear cells (PBMC) from haemophiliacs when grown in culture had higher spontaneous production of IgG than cells from controls. However, pokeweed mitogen induced IgG and IgM production by PBMC was reduced in haemophiliacs when compared with controls, all implying that there was some underlying dysregulation of B cell function and antibody production (Biagiotti et al 1986).

Similar B cell abnormalities had been well described in patients with AIDS (Lane et al 1983) and in the Italian study of Biagotti the individuals who were HIV positive had more marked abnormalities than the seronegative individuals. However the seronegative haemophiliacs had significantly greater spontaneous IgG production than controls suggesting a B cell abnormality independant of HIV infection.

Phenotypic and functional abnormalities of monocytes, including adherence ability and chemotactic responses were also described in a group of haemophiliacs, three out of fourteen of whom were HIV seronegative. No reference was made however, as to whether the abnormalities were as severe or less so in the negative individuals (Roy et al 1988).

It was therefore clear that there was widespread immune dysfunction occurring in cohorts of haemophilia patients. It was very difficult however to evaluate the relative contributions of the putative causes of the immune dysregulation. What was evident however was that there were clearly defined abnormalities occurring in HIV seronegative as well as seropositive individuals.

A wide range of functional immune abnormalities having been described in patients with haemophilia, attention focused on the possible immune-modulating effects of the concentrates themselves. A number of studies showed that lymphocytes from normal healthy donors had reduced proliferative responses to phytohaemagglutinin (PHA) when cultured in vitro in the presence of a variety of FVIII concentrates in a dose dependant fashion (Lederman et al 1986). The reduced proliferative responses were subsequently shown to be due to reduced production of interleukin-2 (IL-2) in vitro (Thorpe et al 1989).

A number of defects in monocyte function were found to be induced in vitro by the addition of concentrates including reduced Fc receptor expression, impaired antigen presentation, bacterial killing and oxygen radical production (Eibl et al 1987, Mannhalter et al 1988). T lymphocyte function, dependant on monocytes such as PHA proliferation may well have resulted from the down regulation of monocytes by FVIII concentrates but it was also shown that monocyte independant lymphocyte function was down regulated by FVIII concentrates (Hay et al 1990).

Similar findings were demonstrated in vivo, in that it was shown that monocyte phagocytic function was significantly down regulated following infusion of two different FVIII concentrates and also intravenous pooled immunoglobulin. (Pasi et al 1990). As treatment frequency was reduced the monocyte function returned to baseline.

Having established that the immune modulating effects of FVIII concentrates were at least in part independent of HIV infection, the question remained as to what was the underlying cause.

Early studies (Lederman et al 1986) showed that the inhibitory effect was not solely due to increasing protein concentration. These results were confirmed by later studies, which showed that the inhibitory in vitro effect of various FVIII concentrates was independent of product purity and was also not related to the mode of purification.

However, a large number of studies had clearly demonstrated that FVIII concentrates were potent inhibitors of both lymphocyte proliferation and IL-2 secretion in vitro.

Thorpe again showed that the degree of inhibition was unrelated to protein concentration and that the inhibitory activity of various intermediate purity products was extremely varied (Thorpe et al 1989).

Eibl looking at the inhibition of monocyte functions in vitro found by fractionation that the immune-modulating activity was due to a high molecular weight fraction present within the concentrates, which had no FVIII activity per se (Eibl et al 1987). This was assumed to be immunoglobulin aggregates or immune complexes containing IgG. The fractions containing IgG monomers had no inhibitory activity and polymeric IgG itself had been shown to have similar down modulating effects on monocyte function (Mannhalter 1988). The role of circulating immune complexes in down regulating the early immune response in vivo had already been demonstrated in animal experiments. Animals previously immunised with an antigen, when rechallenged with that antigen and simultaneously exposed to an intracellular pathogen had dramatically reduced resistance to that infection when compared to being exposed to the pathogen in the absence of antigenic rechallenge (Virgin & Unanue 1984).

The down regulation of immune function in vitro was not confined to products of intermediate purity (Wadhwa et al 1992). High purity products (although not those manufactured by recombinant technology), had similar levels of inhibitory activity. However, a significant proportion of the inhibitory activity of the high purity products could be removed by dialysis suggesting that some of the stabilising solutions in these products, such as citrate were responsible for the inhibition. One of the formulation buffers used in both an intermediate and high purity product had strong inhibitory activity when used alone (Wadhwa et al 1992).

Some inhibitory activity remained in the high purity products following dialysis, and in the intermediate purity products dialysis had little effect on reducing the inhibition. The residual inhibition was again found to be due to a component of the high molecular weight fraction, of around 200 KDs. Fibrinogen and fibronectin are present in large amounts in this fraction but they are unlikely to be the inhibitory component as

in vitro experiments in the presence of purified fibrinogen and fibronectin do not demonstrate any down regulation.

The concentrates inhibit at an early stage of the immune response and it has also been demonstrated that cells re-acquire their ability to respond if the FVIII concentrate is removed from the system (Wadhwa et al 1992). This implies that the way the concentrates function is not simply by "blocking" cell surface receptors and also that the "inhibitory component" is not adsorbed into the cell rendering it down regulated when it meets the mitogen. This is an important observation when considering the wide range of immune abnormalities described in haemophiliacs. The in vitro experiments are an extrapolation of what occurs in vivo at the time of an infusion of concentrate. It has been documented that cessation of infusions results in a return of immune function to baseline normality (Pasi et al 1990).

Some of the later in vitro studies made direct comparisons of different factor VIII concentrates including BPL 8Y (Thorpe et al 1989, Pasi et al 1990 & Wadhwa et al 1992). Six different concentrates were shown by Thorpe to inhibit IL-2 secretion by between 8 and 97%. The wet heated products (both pasteurised and treated with a solvent-detergent method) were most inhibitory with the dry heated products less so. In this study the identity of the products were not stated but our group has been informed that BPL 8Y demonstrated 32% inhibition (R. Thorpe, personal communication). These results were later confirmed by Wadhwa's study, where BPL 8Y showed 25% inhibition of IL-2 secretion. Again the wet treated products were more inhibitory but it is interesting to note that another product also heated at 80^0C for 72 hours (produced by the Scottish National Blood Transfusion Service) showed 82% inhibition.

In vitro monocyte function was also down regulated by BPL 8Y, the degree of inhibition being comparable to that seen with pasteurised and solvent detergent treated products (Pasi et al 1990). These studies highlight the relevance of in vivo studies of individuals exposed to BPL 8Y.

The question remains as to what is the cause of the chronic immune abnormalities described in the various cohorts. It has been postulated that repeated exposures to the large alloantigen load and possibly the high molecular weight "inhibitory component" in particular results in an increasing burden on the reticulo-endothelial system and results in the chronic immune abnormalities (Schulman 1991).

Madhok repeated the observation that intermediate purity concentrates down regulated IL-2 secretion and lymphocyte proliferation, whereas a high purity monoclonally purified concentrate did not have an inhibitory effect. However the peripheral blood mononuclear cells (PBMC) of previously treated haemophiliacs had increased IL-2 production in the presence of the high purity concentrate, an effect that was not seen in cultures of PBMCs from normal donors. This suggested the presence of a clone of T cells primed to factor VIII, or to a component in the concentrate, as a result of repeated exposure (Madhok et al 1991). BPL 8Y itself contains the substances which have been proposed as being responsible for the immune down-regulation. These include fibrinogen and fibronectin, which are present albeit in lower concentrations than other intermediate purity products due to the purification process (Winkelman et al 1989). There are also measurable levels of immunoglobulin and therefore the potential for the development of immune complexes.

Attention later focused on the presence in concentrates of the cytokine transforming growth factor (TGF-ß). This comprises a family of multifunctional peptides that regulate cellular growth and differentiation. Looking at the range of effects of concentrates on cytokine activity, it was proposed that the pattern was reminiscent of that produced by TGF-ß (Wadhwa et al 1994). BPL 8Y, together with other intermediate purity and ion-exchange purified products were shown to contain active TGF-ß, whereas concentrates purified by immunoaffinity and recombinant techniques contained none (Pearson et al 1999). There was also a broad correlation between levels of active TGF-ß and the ability of concentrates to inhibit lymphocyte proliferation or IL-2 secretion.

BPL 8Y was demonstrated as down regulating IL-5 induced proliferation of TF-1 cells (a human erythroleukaemic cell line) in a manner similar to TGF-ß and the response was reversed by the addition of a monoclonal antibody to TGF-ß. However, the inhibitory effects of other concentrates were only partially or not at all reversed by the addition of specific antibody (Wadhwa et al 1994). In addition, in the later study the addition of TGF-ß antibody did not reverse the effect on lymphocyte proliferation at all by all products including BPL 8Y implying that other substances must be responsible (Pearson et al 1999). This study also showed that there were large discrepancies in the relative potencies of purified TGF-ß and coagulation factors in different bioassays.

It remains unclear what might be responsible for the immune modulating effects seen in vitro produced by BPL 8Y and other concentrates.

The role of possible immune modulators present within the concentrates has been extensively investigated, without as yet a clear answer. As a result of repeated exposures to concentrates however, patients with haemophilia are also exposed to, and become infected by a number of viruses.

Immune abnormalities have been described in a number of acute and chronic viral infections (White & Lesesne 1983). Abnormal lymphocyte subpopulations and reduced response to mitogens have been described in both cytomegalovirus and hepatitis B infections, with the abnormalities persisting in those developing chronic active hepatitis (Carney et al 1981, Carella et al 1982, Thomas 1981). It could be postulated therefore that repeated exposure to hepatitis viruses, and maybe others may lead to more permanent immune abnormalities.

CHAPTER THREE

STRATEGY OF CLINICAL STUDY AND PURPOSES OF THE PROJECT

As described in the previous chapter the major complication of the treatment of haemophilia with large pool FVIII concentrates has been the transmission of viral infections, particularly HIV, hepatitis B and non-A non-B hepatitis. It also became apparent that there was evidence of immune abnormalities in haemophiliacs even in the absence of HIV infection. There was also some clinical evidence of a predisposition to infection (Beddall et al 1985) and the possibility of a greater susceptibility to malignancy (Schulman 1991), related to underlying immune-dysregulation.

Although of course HIV was and remains the major cause of immunosuppression, the relative contributions of other potential causes remained unclear.

The groups of patients in whom immune abnormalities had been described would have received a variety of concentrates and cryoprecipitate, the concentrates having been of differing purities and prepared by a number of different methods, as different virus inactivation strategies were introduced. Although many of the studies showed that the immune abnormalities appeared to be more severe in the patients who had received larger amounts of concentrate, the actual concentrates received were seldom mentioned and it was not evident as to whether the patients were infected with other viruses in the absence of HIV. The question remained, were other viral infections or some additional component of the concentrate responsible for the immune dysregulation?

Was the effect a result of repeated concentrate infusions and repeated and chronic viral infections, only becoming apparent after a long period of treatment, or would the abnormalities appear early on in treatment, giving rise to concern about the effect of immunosuppression on the developing immune system.

A further question was the clinical relevance of the increasing reports of the in vitro experiments showing that a variety of factor VIII products could down regulate the function of both lymphocytes and monocytes from normal donors. (Lederman et al 1986, Eibl et al 1987). Whether this short term down regulation early in the immune response was relevant to the clinical situation and had a contributing role to the more chronic immune abnormalities remained to be elucidated.

The first aim of this study was therefore to establish a cohort of patients with haemophilia A and to treat them with only one factor VIII concentrate. The product to be studied, BPL 8Y was prepared from British blood donations which were screened for both HIV antibodies and hepatitis B surface antigen. The manufacturing process included dry heating at 80°C for 72 hours. This product was introduced in 1985, when the first patients included in this study were enrolled. They were to be followed up extremely closely to detect any evidence of viral infection, including HIV, hepatitis B and non A non B hepatitis by monitoring liver function tests. In 1984 the International Committee on Thrombosis and Hemostasis (ISTH) had drawn up recommendations for uniform criteria for the design and conduction of safety studies of new concentrates used in the treatment of haemophilia (Schimpf et al 1987). These guidelines were updated in 1989 (Mannucci & Colombo 1989). As will be described later in this thesis it was difficult to adhere to the strict guidelines for testing in the early phase of this study. These problems were largely overcome by the recruitment of research fellows, dedicated to the running of the haemophilia service and the coordination of follow up.

During the course of the safety study, in 1989, it became apparent that this group of boys was remaining free of significant viral infections, unlike any historical groups. This provided an opportunity therefore, to follow the group prospectively as regards immune function. The aims were to determine firstly, whether the previously described immune abnormalities occurred in a virus free population and secondly, if they did occur what was the relation of the immune dysfunction to concentrate treatment in terms of amount and the time interval since treatment received.

It was initially planned to prospectively monitor the T lymphocyte subsets, which was started in 1989 and continued through to 1995, together with regular immunoglobulin measurements.

During 1990 and 1991 the lymphocyte proliferation and monocyte function of the patients was also assessed and investigated in relation to the treatment received.

The cohort would be followed in parallel with two other groups, one a group of HIV infected haemophiliac boys and a second most important control group, who had received a variety of concentrates and cryoprecipitate in the past, but had remained HIV negative. This second group were comparable to the previous HIV negative cohorts where immune abnormalities had been described.

One obvious problem conducting long term prospective studies in children requiring blood tests will be compliance. This problem will be addressed. Another is the interpretation of results in that the immune system of children develops and established 'normal ranges' of immune parameters do not apply. This was a particular problem when it came to analysing the T lymphocyte subset results. It had to be determined whether any changes were occurring independent of 'normal' age- related changes.

In summary, the purposes of the project were firstly, to establish a cohort of boys with haemophilia A, treated with a single factor VIII concentrate, to ensure they remained free of significant bloodborne viral infection, and secondly, to investigate whether these boys would develop evidence of immune dysfunction.

CHAPTER FOUR

PATIENTS

The patients enrolled in the following studies were all boys with haemophilia A who were attending the regional haemophilia centre at the Children's Hospital, Birmingham. For the purposes of the studies the boys were divided into three groups. Informed parental consent was gained before the boys were studied.

Group 1

Group 1 consisted of 25 boys with haemophilia A (patient numbers 1 to 25). The baseline clinical details of these boys are described in table 4.1. These boys were recruited between July 1985 and August 1990. They were all treated with a single commercial factor VIII concentrate, BPL 8Y produced by the Bioproducts Laboratory, Elstree UK.

All boys were immunised against hepatitis B receiving at least the first dose prior to the first infusion of FVIII concentrate.

Of these boys, eighteen were classified as being severe haemophiliacs on the basis of laboratory factor VIII levels (<0.02u/ml). three were moderate ($\geq$ 0.02 and $\leq$ 0.05u/ml) and four mild (>0.05u/ml). They received their first dose of treatment between the ages of 1 and 108 months.

Group 2

Group 2 consisted of twenty one boys with haemophilia A (patient numbers 26 to 46), who had been treated with both cryoprecipitate and a variety of FVIII concentrates, both heated and unheated. Clinical details are described in table 4.2 and viral status in table 4.3.All twenty one boys remained HIV seronegative on regular three monthly testing. Six boys had evidence of previous infection with hepatitis B and fourteen were found to be hepatitis C seropositive once testing was introduced. It is possible that the seven who were tested HCV negative, had also been infected at one time and had since lost antibody.

All the boys in this group were receiving BPL 8Y and had been doing so since 1988.

Group 3

Group 3 consisted of twenty four boys (patient numbers 47 to 70), who were HIV seropositive. They had all been treated with a variety of FVIII concentrates prior to 1985. Clinical details, including date of seroconversion to HIV and CDC status are as in tables 4.4, 4.5 and 4.6. All had serological evidence of infection with both hepatitis B and C, four of them remaining hepatitis B surface antigen positive. The stage of HIV disease is classified according to the classification system used at that time (WHO 1986). Fourteen patients were asymptomatic (stage II), seven had generalised lymphadenopathy and three had clinical diagnoses consistent with clinical AIDS.

Controls

This group consisted of healthy volunteers, both children and young adults at low risk for both HIV infection and NANBH infection. Serological testing for viral infections was not performed on the control subjects. It was necessary to include young adults in certain control groups because of the difficulty in obtaining blood samples from healthy age-matched children.

During the course of the study certain immunological assays were performed and comparisons were made between the three patient groups and the controls. It is clear that the patient groups differ not only in respect to the viral infections they have acquired. They have all been treated with different blood products and very importantly at the time the assays were carried out they were of different ages. In an ideal study immunological comparisons would be made between the groups of boys when they were of the same age and at the same time points starting treatment. However in this study this was not possible as no historical immunological data was available on the groups two and three.

Both T cell subsets and serum immunoglobulins change during childhood and age was taken into account when looking at the results of the group one patients.

There is no published data as to whether age has an effect on the lymphocyte and monocyte responses described here. In an attempt to look at whether there was an age

related effect in the functional immunological assays all the data from the control groups was analysed for any relation to age prior to making any comparisons with the study groups. Ideally however an age matched healthy control group should have been used.

patient number	date of birth	factor VIII level	presentation	date of first treatment	age at first treatment (months)	reason for first treatment
1	14.6.84	0.00u/ml	finger bleed	19.7.85	13	finger bleed
2	8.12.84	0.00u/ml	left knee bleed	28.12.85	12	left knee bleed
3	15.4.85	0.00u/ml	head injury	3.1.86	9	head injury
4	26.7.84	0.00u/ml	buttock bleed	20.1.86	18	buttock bleed
5	6.9.85	0.00u/ml	Arm muscle bleed	31.5.86	8	Arm muscle bleed
6	20.9.85	0.01u/ml	cord blood	6.6.86	9	Cut ear
7	25.7.83	0.00u/ml	cord blood	1.9.86	38	cover for hepatitis B vaccine
8	20.6.86	0.00u/ml	cord blood	28.9.86	3	hand injury
9	25.11.77	0.08u/ml		1.12.86	108	surgery- toe nail avulsion
10	9.10.85	0.00u/ml	right knee bleed	27.1.87	15	right knee bleed
11	30.7.86	0.00u/ml	cord blood	4.2.87	7	cover for hepatitis B vaccine
12	28.4.85	0.00u/ml	right ankle bleed	6.2.87	22	right ankle bleed
13	22.11.80	0.10u/ml	mother known carrier	31.3.87	76	head injury
14	19.11.85	0.00u/ml	mouth bleed	12.4.87	17	mouth bleed
15	18.9.87	0.00u/ml	cord blood	5.10.87	1	bleeding umbilicus
16	16.11.82	0.20u/ml	mother known carrier	6.5.88	66	Dental treatment
17	17.5.86	0.04u/ml	mother known carrier	19.9.89	40	chin injury
18	20.8.86	0.01u/ml	severe bruising	8.10.89	38	left thigh bleed
19	19.11.88	0.00u/ml	torn frenulum	18.10.89	11	torn frenulum
20	2.6.83	0.08u/ml	mother known carrier	20.11.89	80	tonsillectomy
21	10.9.87	0.00u/ml	bruising	22.11.89	26	Abdominal muscle bleed
22	11.12.88	0.02u/ml	cord blood	16.1.90	13	head injury
23	16.4.88	0.00u/ml	mother known carrier	24.3.90	23	head injury
24	28.12.83	0.01u/ml	brother known haemophiliac	17.5.90	77	right ankle bleed
25	14.4.89	0.02u/ml	mother known carrier	1.8.90	16	cut lip

Table 4.1: Clinical details of group 1 boys – those treated solely with BPL 8Y

patient number	date of birth	factor VIII level	date of diagnosis	date of first treatment
26	28-11-80	0.00u/ml	1987	1987
27	1-12-81	0.00u/ml	1982	1983
28	6-6-82	0.01u/ml	1983	1983
29	30-12-76	0.02u/ml	1977	1983
30	31-12-81	0.01u/ml	1983	1985
31	17-8-82	0.00u/ml	1983	1985
32	5-11-82	0.00u/ml	1983	1985
33	22-8-82	0.00u/ml	1982	1982
34	2-6-74	0.06u/ml	1977	1978
35	13-7-76	0.08u/ml	1978	1979
36	27-9-75	0.02u/ml	1977	1979
37	4-11-74	0.03u/ml	1977	1982
38	3-7-80	0.01u/ml	1981	1982
39	30-5-75	0.17u/ml	1979	1980
40	14-2-81	0.01u/ml	1981	1982
41	15-1-83	0.01u/ml	1983	1983
42	30-6-76	0.08u/ml	1978	1983
43	13-7-76	0.04u/ml	1976	1978
44	26-2-75	0.10u/ml	1979	1979
45	13-5-71	0.14u/ml	1977	1986
46	7-6-74	0.00u/ml	1979	1980

Table 4.2: Clinical details of Group 2 boys

patient number	HIV antibody status	hepatitis B status	hepatitis C antibody status
26	NEG	Vaccinated	POS
27	NEG	Vaccinated	POS
28	NEG	Vaccinated	NEG
29	NEG	Vaccinated	NEG
30	NEG	Vaccinated	NEG
31	NEG	HBcAb POS	POS
32	NEG	HBsAb POS	POS
33	NEG	Vaccinated	POS
34	NEG	Vaccinated	NEG
35	NEG	Vaccinated	POS
36	NEG	HbsAb POS	POS
37	NEG	HBsAb POS	POS
38	NEG	Vaccinated	POS
39	NEG	Vaccinated	NEG
40	NEG	HBcAb POS	POS
41	NEG	HBsAb POS	POS
42	NEG	Vaccinated	POS
43	NEG	Vaccinated	POS
44	NEG	HBsAg POS	NEG
45	NEG	Vaccinated	NEG
46	NEG	HBcAb POS	POS

Table 4.3: Virological status of Group 2 boys.
Note: HbsAb and HbcAb denote naturally acquired infection and seroconversion.

patient number	date of birth	factor VIII level	date of diagnosis	date of first treatment
47	1-1-77	0.00u/ml	1977	1978
48	13-3-80	0.01u/ml	1981	1981
49	14-3-82	0.00u/ml	1983	1983
50	20-3-77	0.00u/ml	1978	1978
51	17-11-80	0.01u/ml	1981	1981
52	11-4-75	0.04u/ml	1976	1979
53	21-8-72	0.00u/ml	1973	1978
54	1-5-80	0.01u/ml	1980	1980
55	14-12-74	0.05u/ml	1975	1978
56	30-12-75	0.02u/ml	1976	1980
57	14-4-72	0.00u/ml	1972	1974
58	6-12-76	0.00u/ml	1977	1978
59	18-9-75	0.00u/ml	1976	1978
60	3-5-78	0.00u/ml	1978	1978
61	24-7-72	0.00u/ml	1972	1978
62	2-10-78	0.00u/ml	1978	1978
63	27-11-75	0.00u/ml	1975	1978
64	19-8-75	0.00u/ml	1975	1978
65	27-3-80	0.00u/ml	1980	1981
66	5-4-72	0.00u/ml	1972	1976
67	30-9-77	0.00u/ml	1978	1978
68	11-3-73	0.00u/ml	1977	1978
69	24-1-71	0.00u/ml	1971	1974
70	27-3-69	0.02u/ml	1976	1977

Table 4.4: Clinical details of group 3 boys.

patient number	HIV antibody status	Hepatitis B status	Hepatitis C antibody status
47	POS	HBcAb POS	POS
48	POS	HBcAb POS	POS
49	POS	HBsAb POS	POS
50	POS	HBcAb POS	POS
51	POS	HBcAb POS	POS
52	POS	HBsAg POS	POS
53	POS	HBcAb POS	POS
54	POS	HBcAb POS	POS
55	POS	HBsAg POS	POS
56	POS	HBcAb POS	POS
57	POS	HBcAb POS	POS
58	POS	HBcAb POS	POS
59	POS	HBsAg POS	POS
60	POS	HBcAb POS	POS
61	POS	HBcAb POS	POS
62	POS	HBcAb POS	POS
63	POS	HBcAb POS	POS
64	POS	HBcAb POS	POS
65	POS	HBsAg POS	POS
66	POS	HBcAb POS	POS
67	POS	HBcAb POS	POS
68	POS	HBcAb POS	POS
69	POS	HBcAb POS	POS
70	POS	HBsAb POS	POS

Table 4.5: Virological status of group 3 boys.

patient number	date 1st HIV-ab positive	seroconversion accuracy	CDC stage at start of study	CDC stage at end of study	change in CDC stage	date AZT started	date of death
47	6/83	unknown	IVE	IVE		5/89	
48	6/83	2 years	III	III			
49	4/84	unknown	III	III			
50	6/83	3 years	II	II		7/90	
51	4/84	8 months	III	III			
52	4/84	3 months	II	IVE	6/90		
53	6/83	4 years	II	II			
54	10/86	2 months	III	III			
55	4/86	3 months	IVE	IVE		11/89	
56	12/82	1 month	II	IVE	10/89	7/90	
57	1/82	2 years	IVC	IVC		10/87	11/90
58	11/83	5 months	II	IVE	1/90		
59	11/82	unknown	II	II		3/91	
60	10/86	3 months	II	II			
61	6/83	1 year	II	IVC2	1/89		
62	4/84	3 months	III	III			
63	6/83	unknown	II	II			
64	6/83	3 years	II	II			
65	4/84	unknown	III	III		9/90	
66	11/83	3 months	II	II			
67	4/84	1 year	III	III			
68	6/83	4 months	II	IVD	5/89	5/89	7/89
69	11/83	4 months	II	II			
70	12/83	4 months	II	II			

Table 4.6: Group 3 – details of HIV infection. Note: Seroconversion accuracy denotes availability of last HIV negative sample.

CHAPTER FIVE

METHODOLOGY

Routine Testing

General Haematology and Coagulation

Haemoglobin estimation, total and differential white cell and platelet counts were made on venous blood samples anticoagulated with Ethylene Diamine Tetra acetic Acid (EDTA) using a Coulter S Plus Automated Counter.

Age related normal ranges were obtained from Nelson's Textbook of Pediatrics (WB Saunders & Co.)

VIII:C was measured using a modified two stage assay (Diagnostic Reagents Ltd, UK) based on the thromboplastin generation test (Denson 1966).

Screening Test for Factor VIII inhibitors

The principle of this test is that if normal plasma is mixed with an equal volume of patient plasma containing an inhibitor to factor VIII, the APTT will be significantly prolonged during the incubation phase. The reaction between the coagulation factors and its inhibitor is both time and potency dependent. A minimum incubation time of one hour at 37°C is necessary for the detection of weak inhibitors.

Citrated plasma is required for the assay.

1.The following are incubated at 37°C for 60 minutes:

450 µl of test plasma

450 µl of normal plasma (Sigma Diagnostics)

150 µl test plasma and 150 µl normal plasma together (MIXTURE INCUBATED)

2. At sixty minutes a mixture of the incubated test and control plasmas is prepared using 150µl of each. (MIXTURE STAT)

3. A PTT (partial thromboplastin time) is performed on the following:

a. The original 50:50 mixture (MIXTURE INCUBATED)
b. The freshly prepared 50:50 mixture (MIXTURE STAT)
c. The incubated control plasma

d. The incubated test plasma

A difference of greater than four seconds between the MIXTURE STAT and the MIXTURE INCUBATED indicates the presence of an inhibitor. A very high concentration of inhibitor is present if both the MIXTURE STAT and the MIXTURE INCUBATED give a prolonged APTT.

If an inhibitor screening test was found to be positive then a confirmatory assay using the Bethesda method described below would be performed.

Measurement of factor VIII:C inhibitors; Bethesda Method (Kasper et al 1975)

The principle of this test is that normal plasma is added to dilutions of test plasma and incubated at 37°C for two hours. As factor VIII:C inhibitors are time dependent, the added factor VIII:C from the normal plasma will be progressively neutralised. If the concentration of the added factor VIII:C (normal plasma) and the incubation time is standardised, the strength of the inhibitor may be defined in units according to the amount of factor VIII:C neutralised. A Bethesda Unit is defined as that which will destroy 50% of factor VIII:C in 2 hours at 37°C.

The following procedure is carried out.

1. Doubling dilutions of the test plasma are prepared in 200µl volumes using Owren's buffer.
2. 200µl of Owren's buffer is pipetted into a separate tube as the control.
3. 200µl of normal plasma (Sigma Diagnostics) is added to all tubes including the control. These are capped mixed and incubated at 37°C for two hours.
4. After two hours incubation a one stage factor VIII assay is carried out using the Sysmex CA1500 on all the incubation mixtures. The control tube is used as the 100% (1u/ml) factor VIII:C reference plasma.
5. An MDA analysis is performed for the control tube to obtain a reference curve and single point analysis on each of the test dilutions.

6. The dilution of test plasma that gives a residual factor VIII:C nearest to 50% (0.5u/ml) but within the range of 30-60% (0.3-0.6u/ml) is chosen for the calculation of the inhibitor.
7. From the standard graph of residual factor VIII:C vs inhibitor units (log/log graph paper), the inhibitor level corresponding to the residual factor VIII:C for the chosen test dilution with the residual factor VIII:C nearest to 50% is read off. The inhibitor value form the graph is mulitiplied by the test dilution to give the final Bethesda inhibitor value (U/ml).

If the assay is being performed on a patient with either residual factor VIII levels either from treatment or if they are mild or moderate, this must be removed prior to the assay. This is done by incubating the plasma at 56° for 30 minutes which will destroy the factor VIII in the specimen. 100µl of aluminium hydroxide suspension is added to 900µl of the heated plasma, which removes any other factors precipitated at 56°. The supernatant plasma can be removed and then the inhibitor assay performed.

Liver Function Tests

Serum alanine aminotransferase (ALT), bilirubin and alkaline phosphatase were measured using standard laboratory methods by the department of Clinical Chemistry, Birmingham Children's Hospital, using a COBAS BIO centrifugal analyzer.

(Normal ranges used at this hospital; ALT <40 iu/l, alkaline phosphatase 250-750 iu/l and total bilirubin 0-15 µmol/l).

Immunoglobulins

Total IgG, IgA and IgM were measured on serum samples by a routine nephelometric technique by the Department of Immunology, East Birmingham Hospital.

Age related normal ranges were produced by the Department of Clinical Immunology at Birmingham University Medical School. (See appendix 10.1).

Virology

anti-HIV antibody:

This was measured by a particle agglutination test (Serodia-HIV, Fujirebio Inc, Japan) according to the manufacturer's instructions by the Department of Virology, Birmingham Children's Hospital.

Hepatitis B serology:

This was carried out at the Department of Virology, East Birmingham Hospital. Hepatitis B surface antigen and antibody were detected using a radioimmunoassay (RIA) (BioProducts Laboratory).

Hepatitis C serology:

Dr. S. Skidmore at the Department of Virology, East Birmingham Hospital, carried out all the hepatitis C antibody assays.

The first generation assay, an ELISA, used plates where the wells were coated with a non-structural recombinant hepatitis C viral protein, C-100 (Ortho Diagnostics, UK). The second generation assays used antigens from the nucleocapsid and other non-structural proteins (Wellcome Diagnostics, Beckenham, UK). The results of all the first generation assays were confirmed by the second generation tests.

Cellular Studies

Media

RPMI 1640 (Gibco Ltd, UK), with penicillin 200u/ml (Britannia Pharmaceuticals Ltd, UK), streptomycin 100u/ml (Evans Medical Ltd, UK) and glutamine (2mM) (Gibco Ltd) was used in all instances and will be referred to as RPMI 1640 unless otherwise stated. Heat inactivated human serum was prepared from healthy volunteer donors who were known to be blood group A Rh(D) Positive. 100ml of blood was taken into a sterile bottle containing glass beads and defibrinated by gentle inversion for at least 10

minutes. The defibrinated blood was drawn from the beads and centrifuged at 3000 rpm for 10 minutes. Serum was then removed and heated at 56°C for 30 minutes to heat inactivate complement. Sterile aliquots of heat inactivated serum were frozen at -20°C until use. The same donor serum was used for all monocyte antigen presentation assays.

Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from anticoagulated whole blood by density gradient centrifugation on Ficoll Hypaque (Lymphoprep, Nygaard Ltd, UK). Buffy coats (prepared from centrifuged whole blood at 3000rpm for 10 minutes) or whole blood, were diluted in RPMI 1640, and layered over Ficoll Hypaque and spun at 400g for 25 minutes at room temperature. The PBMCs were then removed from the interface and washed three times in RPMI 1640.

T lymphocyte subsets

Manual method:

T lymphocyte subsets were identified by indirect immunofluorescence using fluoresene isothiocyanate conjugated (FITC) rabbit anti mouse immunoglobulin (Williams et al 1988).

Venepuncture was performed on patients between 9 and 11am, (prior to the infusion of concentrate). 10mls of blood was taken directly into lithium heparin containers. PBMCs were separated as described above on the same day, resuspended in PBSAA (0.1% sodium azide/PBS/1% bovine serum albumin) and incubated with a 1/25 dilution of each monoclonal antibody.

The monoclonal antibodies used throughout the study were CD11 (pan T lymphocytes), CD4 (includes T helper lymphocytes) and CD8 (includes T suppressor lymphocytes), (Coulter Electronics Ltd, UK)

The monoclonal antibodies were stored at -20°C in plastic torpedo tubes. Before use they were diluted with 150 µl of PBSAA. 50 µl of cell suspension was added to each torpedo tube. The cell suspension and monoclonal antibodies were mixed well and left at room temperature for 15 minutes, being agitated twice during this period. The cells were then washed three times with PBSAA and after the final wash the supernatant was completely removed and the cell pellet resuspended in 200 µl of rabbit anti-mouse immunoglobulin -FITC (Dako Ltd, UK; diluted 1/20 with PBSAA). The samples were agitated and left at room temperature as before for 15 minutes. The washing procedure with PBSAA was repeated three times, leaving 100µl of PBSAA on the pellet after the final wash. One drop of 8% formalin was added to each tube to prevent clumping and the pellet resuspended. One drop of cell suspension was placed on a glass slide, covered with a glass slip and examined immediately, using a Leitz Ploem fluorescent (mercury vapour) microscope system. 200 cells were counted and the percentage showing fluorescence determined. Having determined the percentage of lymphocytes expressing the cell surface markers, the absolute T cell subset counts were calculated

using the absolute lymphocyte count measured on a simultaneous full blood count. The T4:T8 ratio was then obtained from these absolute counts.

FACSCAN Method:

Flow cytometric analysis was introduced in August 1989 and performed by the Department of Immunology, East Birmingham Hospital. This method used two colour combinations of fluorescent labelled monoclonal antibodies to label T cells in whole blood. After labelling, a hypotonic lysing buffer was added to lyse red cells whilst leaving white cells intact. These were then fixed and analysed on a fluorescence activated counter, gated to lymphocytes.

Briefly, samples of 2mls EDTA anticoagulated whole blood were drawn before 10am and transported at ambient temperature to East Birmingham Hospital within 2 hours. A sample was also taken from a normal donor to control for transport conditions.

100µl EDTA anticoagulated whole blood was incubated with CD3, CD4 and CD8 monoclonal antibodies at room temperature for 10 minutes in reduced light (in combinations CD3-CD4 and CD3-CD8). CD3 and CD4/CD8 were labelled with differing fluorochromes. 2ml of FACSlyse (Becton Dickinson Ltd, UK) was added to each tube and incubated for a further 10 minutes at room temperature. Tubes were then centrifuged at 675rpm for 5 minutes and the supernatant discarded. Cells were resuspended in 2ml PBS and 0.5ml 2% formaldehyde. Cell suspensions were then analysed on a Becton-Dickinson FACScan. Control samples and control monoclonal antibodies were included with all runs.

Lymphocyte Proliferative Studies

All assays were performed in the morning prior to any treatment being given to the patients. The minimum time interval between the assay and the last infusion of factor VIII concentrate was 24 hours.

20mls of citrated blood was taken from each individual and PBMCs separated as described above. After washing PBMCs were resuspended at a concentration of 1 x 10^6 cells/ml in RPMI 1640 and 10% heat inactivated human serum.

1 x 10^5 cells in a final volume of 200 µl of medium were plated in U wells in 96 well microtitre plates. The cells were incubated with either, phytohaemagglutinin (PHA), Concanavalin A (Con A) (Sigma Chemical Co.), or heat inactivated *Escherichia Coli* 089 H16 (E. Coli) (NCTC).

Assays were performed in triplicate and unstimulated background control cultures were included with every assay.

Cells were incubated at 37°C / 5% CO_2 for 72 hours (PHA and Con A) or 7 days (E. Coli) in a humidified atmosphere. 18 hours before the end of incubation each well was pulsed with 0.3 µCi of tritiated (3H) thymidine (Amersham, UK). Cells were harvested onto glass fibre filters using an 8 channel cell harvestor (Nunc GIBCO) washing each well 6 times with distilled water. The filters were air dried and 3H-thymidine content and hence proliferation was determined by liquid scintillation counting (Optiphase scintillant, Packard Tri-Carb Counter). and the results expressed as mean counts per minute (CPM), following deduction of background proliferation measured by the unstimulated control.

$$\text{Proliferation (CPM)} = \text{CPM}_{\text{assay}} - \text{CPM}_{\text{control}}$$

The proliferative responses could also be expressed as stimulation indices.

$$\text{Stimulation index} = \frac{(\text{CPM}_{\text{assay}} - \text{CPM}_{\text{control}})}{\text{CPM}_{\text{control}}}$$

PHA and Con A were reconstituted in RPMI 1640 and stored in aliquots at -20°C until use. E. Coli was grown overnight in L-broth at 37°C with agitation. Cultures were then centrifuged at 30,000 rpm for 10 minutes at 4°C. The pellets were then washed in RPMI 1640 and recentrifuged. The final pellet was resuspended in RPMI 1640 and the

concentration of bacteria then adjusted to 20 x 10^8/ml and checked by optical density at 600nm. The suspension was heat inactivated by heating at 80°C for three hours. Aliquots were stored at 4°C until use. The original NCTC E. Coli slope was cultured to check for contamination prior to each batch of antigen being produced.

Optimization of Mitogen Concentrations

Lymphocyte proliferative assays were performed using PHA, Con A and heat inactivated E. Coli on peripheral blood mononuclear cells from four healthy volunteer donors (in the case of E. coli three donors were used).. The concentrations of both PHA and Con A used ranged between 0.625 µg/ml and 100 µg/ml and for E. Coli 2 x 10^5 to 2 x 10^7 /ml. The proliferative responses are shown in tables 5.1 and 5.2. The dose response curves (graphs 5.1, 5.2 and 5.3) were examined to establish the optimum concentrations of lectins and bacteria to use in the assays.

As can be seen from the graphs each of the four donors had different dose response curves, indicating that probably for a population their would be a range of concentrations at which the best proliferative response would be seen. It was therefore decided to use three different concentrations of PHA and Con A in the patient proliferation assays (table 5.3), ranging from sub optimal to above optimal.
Heat inactivated E. Coli was only used at optimal concentration (2 x 10^6/ml).

concentration	Con A				PHA			
µg/ml	Donor 1	Donor 2	Donor 3	Donor 4	Donor 1	Donor 2	Donor 3	Donor 4
0.625	695	324	1382	2745	481	13885	4229	1132
1.25	4594	7846	3990	2893	7197	29913	16371	4254
2.5	8197	30055	10083	5032	25532	37958	26117	33229
5.0	11461	56365	12114	9131	32607	45306	27654	31842
10	20282	39424	19262	19778	27472	40814	25415	36028
25	21645	46569	22480	21361	24328	39314	18489	37942
50	7811	15174	12407	40228	18686	32113	12499	26277
100	857	3393	4977	10978	10049	20677	7130	1277

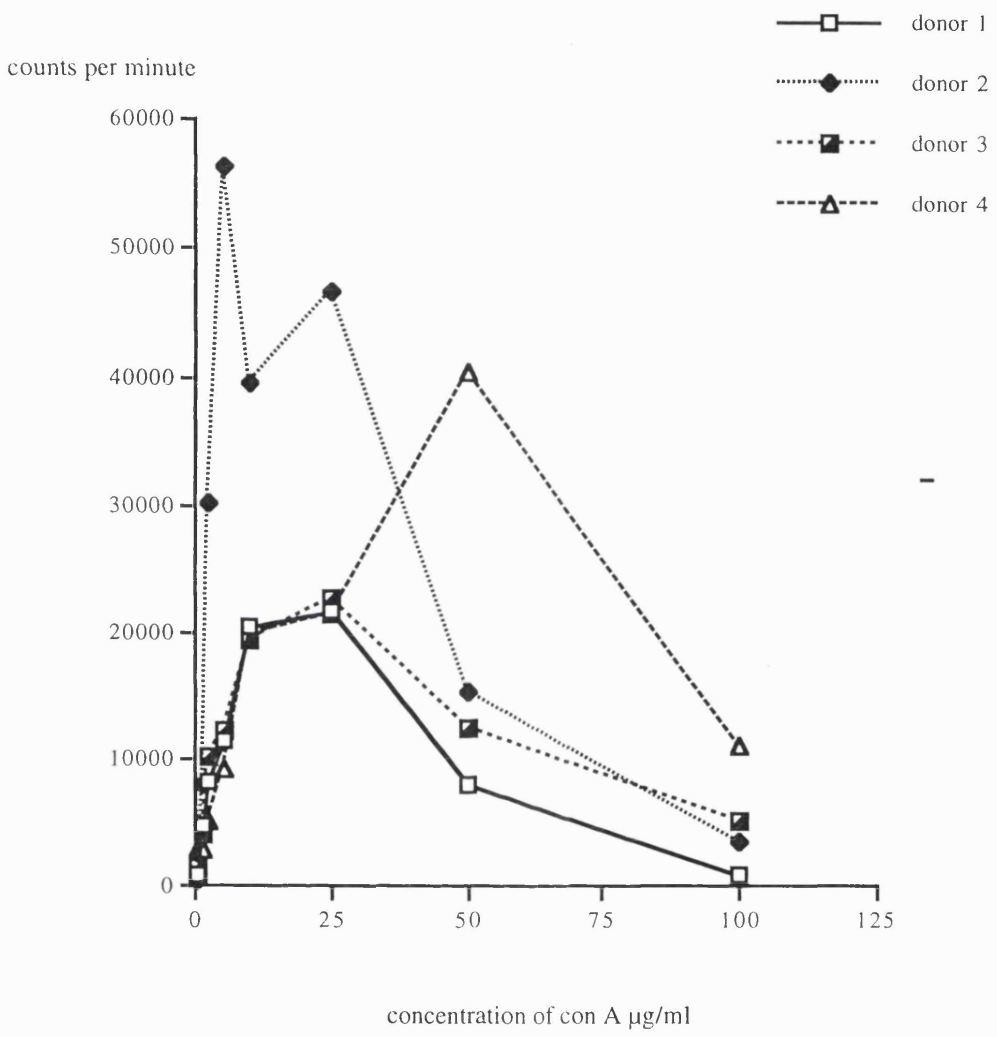
table 5.1: results of lymphocyte proliferation assays to determine optimal concentrations of lectins. (proliferation expressed as counts per minute, CPM)

Concentration Bacteria / ml	Donor 1	Donor 2	Donor 3	Donor 4
2×10^5	5366	6768	4352	1129
2×10^6	13833	18045	10443	7262
2×10^7	20430	13562	9877	6891

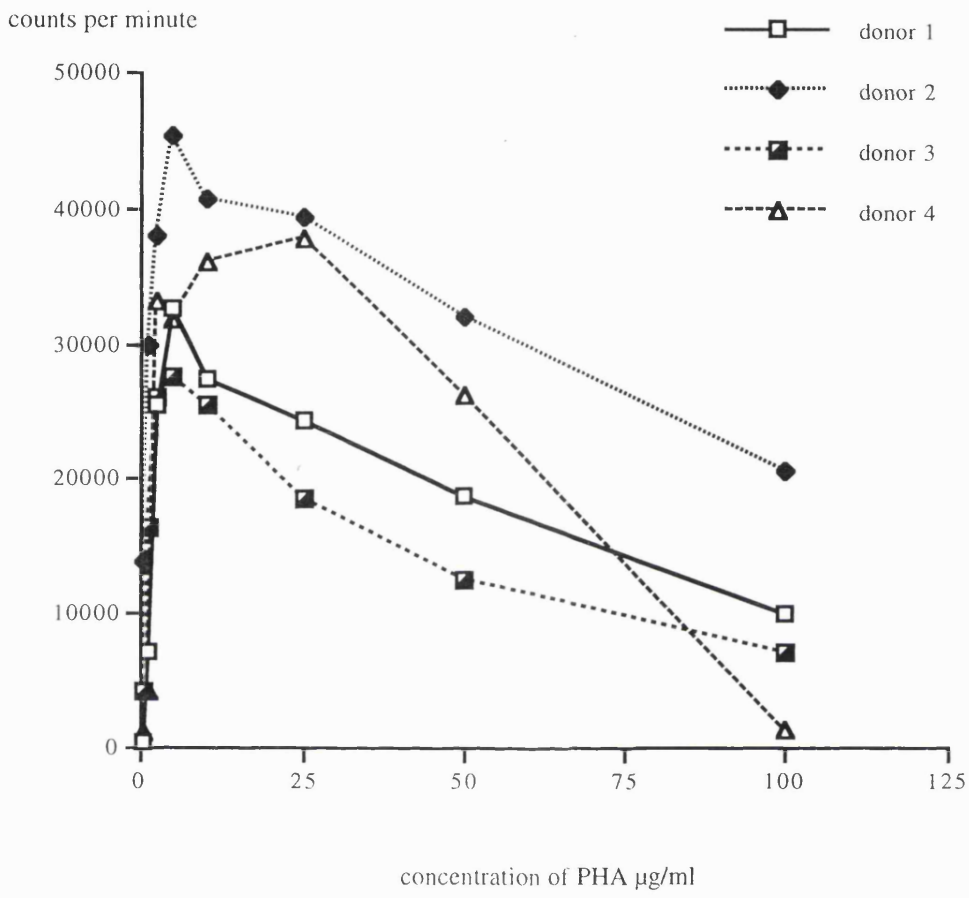
table 5.2: results of lymphocyte proliferation expressed as counts per minute (CPM) to heat inactivated E. Coli..

Mitogen	Final Concentration	Response
PHA	50 µg / ml	above optimal
	25 µg / ml	above optimal
	5 µg / ml	optimal
Con A	50 µg / ml	above optimal
	25 µg / ml	optimal
	5 µg / ml	sub optimal
E. Coli	20 x 10^5/ml	optimal

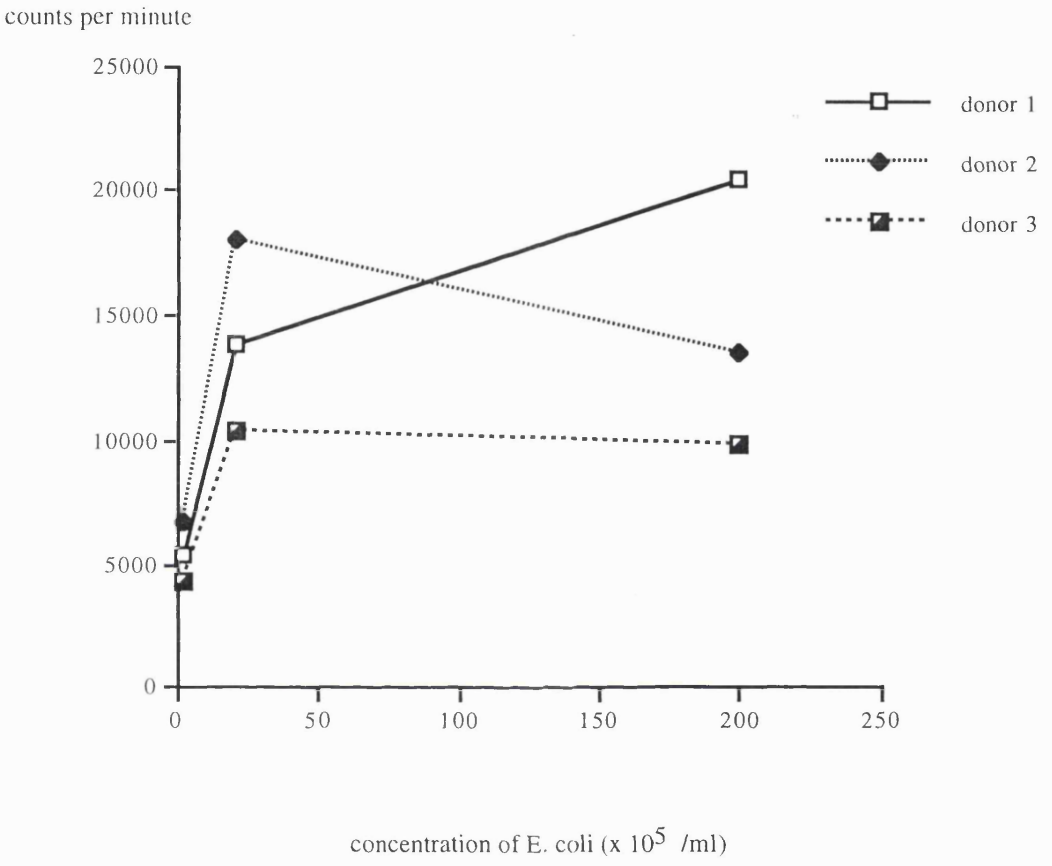
table 5.3: Concentrations of mitogens used in the final assays (E.coli was used only at one final concentration of 20 x 10^5/ml)



graph 5.1: Dose response curves for lymphocyte proliferation to Con A. The proliferative response as expressed as counts per minute of lymphocytes from four healthy normal donors. Optimal response seen at 25µg/ml Con A



graph 5.2: Dose response curves for lymphocyte proliferation toPHA. The proliferative response as expressed as counts per minute of lymphocytes from four healthy normal donors. Optimal response seen at 5 µg/mlPHA



graph 5.3: Dose response curves for heat inactivated E. coli. The proliferative response as expressed as counts per minute of lymphocytes from three healthy donors. Optimal response seen at a concentration of 20 x 10^5/ml of E. coli.

Monocyte T cell interaction using E. Coli

Blood for these assays was again drawn in the morning prior to any infusions of factor VIII concentrate. The minimum time interval since the last infusion was at least 24 hours. 20 ml of blood was taken into preservative free heparin and peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll-Hypaque as described above. The PBMCs were washed and resuspended in RPMI 1640 and 10% heat inactivated human serum at a concentration of 2.5 x 10^6 cells / ml.

Monocyte antigen presentation was measured by the method of Mannhalter (Mannhalter et al 1986). Monocytes were prepared by adherence; 2ml aliquots of suspended mononuclear cells were incubated at 37°C / 5% CO_2 for 24 hours in a humidified atmosphere. The supernatant was discarded and the remaining adherent monolayers were washed three times with RPMI 1640 prewarmed at 37°C.

The monolayers were then incubated with a suspension of heat inactivated E. Coli 089 H16 at a concentration of 1 x 10^8/ml for three hours at 37°C / 5% CO_2. The supernatant was then discarded and monocyte layers washed three times and then harvested by gentle scraping with a sterile plastic pasteur pipette and washed three times in RPMI 1640 / 10% heat inactivated serum.

Cytospin preparations of washed monolayers from random experiments were stained using a modified Wright's stain and a non-specific esterase (alpha naphthyl acetate esterase) to assess the percentage of monocytes obtained. Morphologically, greater than 90% were monocytes. Cytochemical identification showed a mean of 94.5± 1.04% (mean ± SEM) positively staining monocytoid cells.

The monocytes, acting as a sole source of antigen were then added at two different concentrations, either 1 x 10^5 or 5 x 10^4 cells, to 1 x 10^5 lymphocytes in a total volume of 200 µl in 96 well plates. The cultures were incubated at 37°C / 5% CO_2 for seven days and pulsed with 0.3µCi ^{3}H-thymidine (tritiated thymidine) 18 hours before harvesting onto glass fibre filter mats. ^{3}H-thymidine incorporation was determined by

liquid scintillation counting as described above. All assays were performed in triplicate and compared with control cultures devoid of any antigen.

Results were expressed as either mean counts per minute (CPM) or stimulation index (SI) as defined above.

Statistical methods

Chapter 7

In the analysis of T cell subsets in chapter 7, in order to eliminate age as a confounding factor in the analysis, z scores for each measurement of the percentage of CD4 and CD8 cells were calculated. The z score is a measure of how different an individual is from the average of all children of the same age. Scores larger than plus or minus 1.88 occur outside the 3rd or the 97th centile, and would therefore be said to be outside the normal range for a child of that age. By examining serial z scores it is possible to determine whether there is a progressive change in CD4 and CD8 counts which are occurring independent of age. The calculations were made based upon the centile curves that had been generated by the follow up of children born to HIV infected women who themselves did not get the infection (The European Collaborative Study 1992).

The so called LMS method which was used in the construction of the centiles in the European Collaborative Study allows the centile lines to be expressed mathematically (Cole 1990) and provides a method of calculating z-scores.

M represents the median CD4 percentage for a particular age

S represents the skewness of the distribution around that median

L is a power coefficient which will transform the spread of reference CD4 percentages at that age to a normal distribution.

These three coefficients can be combined to convert a measured CD4 or CD8 percentage at a particular age to a z-score.

For CD4 Percentages

For a child aged x years, we need to calculate values for the coefficients L, M and S.

The value for L is simply given 0.939, at all ages.

The value for S is given as 0.230, again at all ages.

The value for M is calculated as $((0.155 \times e^{(-0.574 \times x)}) - 0.6662)$.

For an observed CD4 percentage, y, at age x, the z score is calculated by substituting the calculated values for L, M and S into the formula:-

$$\text{z-score} = \{[1/(L \times S)] \times [((y^L)/(1 + (L \times M))) - 1]\}.$$

For CD8 Percentages

For a child aged x years, we need to calculate values for the coefficients L, M and S.

The value for L is simply given 0.031, at all ages.

The value for S is given by the calculation $(0.344 - (0.01986 \times x))$

The value for M is calculated as $(((-0.243 \times x) - 0.284) \times e^{(-0.836 \times x)} - 1.3023)$.

For an observed CD8 percentage, y, at age x, the z score is calculated by substituting the calculated values for L, M and S into the formula:-

$$\text{z-score} = \{[1/(L \times S)] \times [((y^L)/(1 + (L \times M))) - 1]\}.$$

In order to calculate z-scores for the study, novel software was developed (by Dr. Stephen Marriage of St. Mary's Hospital, London) which calculated the exact z-scores directly, only requiring the percentage of CD4 and CD8 positive cells and the age to be put in to the programme.

Each patient was assessed initially as an individual to see whether a significant change in CD4 or CD8 count occurred over time. This was done using simple linear regression (Statview version 5.0, SAS Institute Inc.). Multiple regression was then used to investigate the relationship between the CD4 and CD8 counts and the FVIII treatment received, both in terms of the cumulative treatment received and in an attempt to assess the short term acute effect of FVIII treatment, the treatment received in the week , month and three months prior to the T cell subset assay.

The assumptions made in employing the regression models include;

1. that there is a linear relationship between the CD4, CD8 z scores and the age of the children and also between the values and the amount of treatment received. Although in both cases, the plots show a lot of scatter, a straight line, indicating a linear relationship, as opposed to a curve best describes the patterns. This would not have been the case if the raw data for the CD4 and CD8 values had been used.
2. The analyses were all carried out on an individual-patient basis, rather than on the group as a whole. It was felt reasonable to assume that repeated assays on the same individual would be independent of each other in this analysis, although obviously this would not have been a reasonable assumption to make had the analysis included all of the patients as a whole.
3. It is also assumed that the residual values (the predicted value from the regression line minus the observed value) are normally distributed with a mean of 0. This was checked and in the majority of cases the values were normally distributed. In six cases there was one outlying value, which may reflect an unusual result occurring by, chance, by laboratory error or another confounding factor.

Chapters 8 & 9

The lymphocyte proliferative responses were expressed as counts per minute (cpm). The responses of each of the four groups in both assays were not normally distributed. Therefore in order to compare the responses of the four groups the Kruskal-Wallis one way analysis of variance for non parametric data was used. This established that there was a highly significant difference between the four groups.

Pairwise comparisons were then performed using the Mann Whitney U test for non parametric data. This involves multiple tests which may lead to a false number of significant results. The resultant p values were therefore adjusted to take account of the number of tests performed. This was done using a simple form of the Bonferroni correction by multiplying the p-values obtained by the number of tests performed, in

this case six. Only those tests which had p-values <0.05 after adjustment were significant.

The relationship between the proliferative responses of the group 1 patients were then investigated with regard to the total treatment received at the time of the assay and the number of days since the last infusion of factor VIII concentrate. In order to do this using simple regression the data should be normally distributed. This was not however the case. The data was transformed using several methods including log transformation, square root and reciprocal transformation, none of which transformed all the data sets to a normal distribution.

Therefore in order to investigate this Spearmans rank correlation test for non parametric data was used. A disadvantage of using such a test is that it may not be as stringent as when using tests for parametric data. Spearmans rank correlation test was also used to investigate the relationship between proliferative response and age within group 4, the control group.

Chapter 10

The levels of serum IgG measured on the patients were grouped together according to the year after treatment began. The levels of IgG were compared by the Kruskal-Wallis one way analysis of variance for non parametric data to investigate whether there was a significant increase or decrease in mean IgG level over eleven years of follow up.

A comparison was made with two other groups of haemophiliacs within the unit on whom serum IgG levels were available. The year in which these patients had started treatment with either concentrate or cryoprecipitate was recorded and IgG levels were taken from if possible the seventh year after treatment began in order to make a direct comparison. The levels of IgG were again compared by the Kruskal-Wallis one way analysis of variance for non parametric data and having established that there was a significant difference between the three groups, pairwise comparisons were made using the Mann Whitney U test.

CHAPTER SIX

SAFETY OF A VIRUS INACTIVATED FVIII CONCENTRATE AND DEFINING A NON-A NON-B HEPATITIS AND HIV FREE COHORT

The primary aim of this study was to ascertain the virological safety of the factor VIII concentrate BPL 8Y.

In 1984 the International Committee on Thrombosis and Hemostasis (ISTH) drew up recommendations for uniform criteria for the design and execution of safety studies of new concentrates used in the treatment of haemophilia (Schimpf et al 1987).

It was decided that safety studies should be prospective but that there would be no control group; for obvious reasons in that it would be unethical to give people untreated products knowing they were "unsafe". Groups of patients treated previously with untreated concentrates in whom the attack rate of hepatitis was 100% would act as historical controls (Fletcher et al 1983, Kernoff et al 1985).

It was recommended that patients entered into safety studies should be those who had never received blood or blood products in the past, known as 'virgins' or 'previously untreated patients' (PUPs). There had been some discussion as to whether to include patients who had only been infrequently treated with blood or single donor products in the past but it was decided not to recommend this (Mannucci & Colombo 1989).

The recommendations for patients to be entered into safety studies of new concentrates are as follows:

i. they had received no previous transfusion with blood or any blood product

ii they had normal baseline serum levels of alanine aminotransferase (ALT)

iii. they had no history or current evidence of liver disease

iv. they were taking no medication likely to raise ALT levels

v. they had no serum marker for hepatitis B infection - except antibody to hepatitis B surface antigen having received vaccination

All the patients included in this study were immunised against hepatitis B. The accelerated dosing regimen was used, giving three doses; the first at diagnosis or in the case of an emergency before the first treatment, the second at one month and the third at two months. These were given subcutaneously in the deltoid or upper thigh region.

Children aged between 0 and 12 years received half the adult dose of vaccine (10 μg in 0.5ml) (Department of Health 1996).

Follow up

At the time of the ISTH recommendations and at the start of this safety study of BPL 8Y, the gene for hepatitis C had not been cloned and hepatitis C antibody testing was not available. Therefore the criteria for follow up was measuring liver transaminase levels to detect the development of non-A non-B hepatitis (NANBH). It was assumed, as the NANBH attack rate was so high in those given untreated concentrates, that hepatitis would occur as a result of the first infusion of concentrate if it was going to occur. It was recommended therefore that serum levels of alanine aminotransferase (ALT) should be measured prior to the first infusion of concentrate and then at two weekly intervals for the first four months, and then monthly for a total follow up period of six months.

The patients entered into the BPL 8Y study would continue to be sampled at monthly intervals to one year and thereafter two monthly until the end of the study.

The frequent sampling over the first four months caused some concern particularly as the majority of subjects entering safety studies would be children. However, it was necessary to sample so frequently to avoid missing a transient rise in transaminases, as NANBH may be a biochemically short lived disease (Kernoff et al 1987, Carnelli et al 1987). This was well demonstrated in a safety study of a dry heated concentrate, which did transmit NANBH, where three out of eleven episodes of hepatitis would have been missed if blood samples had been taken more than fifteen days apart (Colombo et al 1985).

NANBH is defined as the presence of ALT levels 2.5 times higher than the upper limit of normal, on at least two consecutive occasions fifteen days apart.

Patients who received any blood product other than BPL 8Y during the follow up period would be excluded from the final analysis. At the same time as the liver function tests were measured serum was also sent for markers of hepatitis B infection;

(hepatitis B surface antigen, hepatitis B surface antibodies and hepatitis B core antibodies) and HIV antibodies.

A full blood count and white cell differential were also performed at each venepuncture.

Informed consent detailing both the frequency and the type of blood tests that were to be performed was obtained from the parents prior to the first infusion of concentrate.

The concentrate: BPL 8Y

8Y, which became available in 1985 is prepared from large pool fresh frozen plasma from unpaid donors of the National Blood Transfusion Service in England and Wales. Each unit of blood is screened for hepatitis B surface antigen by third generation tests, but at the time of this study they were not screened for surrogate markers of NANBH or hepatitis B core antibody. Since 1985 donated blood has also been screened for HIV antibodies.

Following fractionation of the plasma and freeze drying of the concentrate it is heated in the dry state at 80^{o} C for 72 hours in its final container.

8Y has a VIII:C specific activity of approximately 2 IU / mg protein and contains most of the intermediate/high molecular weight forms of von Willebrand factor antigen (Lawrie et al 1989). Preliminary clinical studies showed that its biological half life, the recovery of factor VIII:C and its effectiveness in stopping bleeding episodes in patients with haemophilia were satisfactory (Winkelman et al 1989).

Each batch of 8Y was prepared from between 15,000 and 25,000 blood donations.

Results

Twenty five boys with Haemophilia A were enrolled into the study between July 1985 and August 1990.

Patient details are described in chapter 4. Eighteen boys with severe haemophilia (FVIII:C < 0.02u/ml) were enrolled together with three boys with moderate disease (FVIII:C 0.02-0.05 u/ml) and four with mild haemophilia (FVIII:C > 0.05 u/ml).

The age at entry to the study ranged between 1 month and 108 months (mean 29.8 months, median 17 months).

The safety study was completed in December 1991, the duration of follow up ranged from 16 to 77 months, (mean 48 months, median 57 months).

Treatment received

Details of the quantities of treatment received in terms of total units of FVIII received per year and total units FVIII per kilogram body weight received per year are summarized in appendices 6.1.1 to 6.1.25.

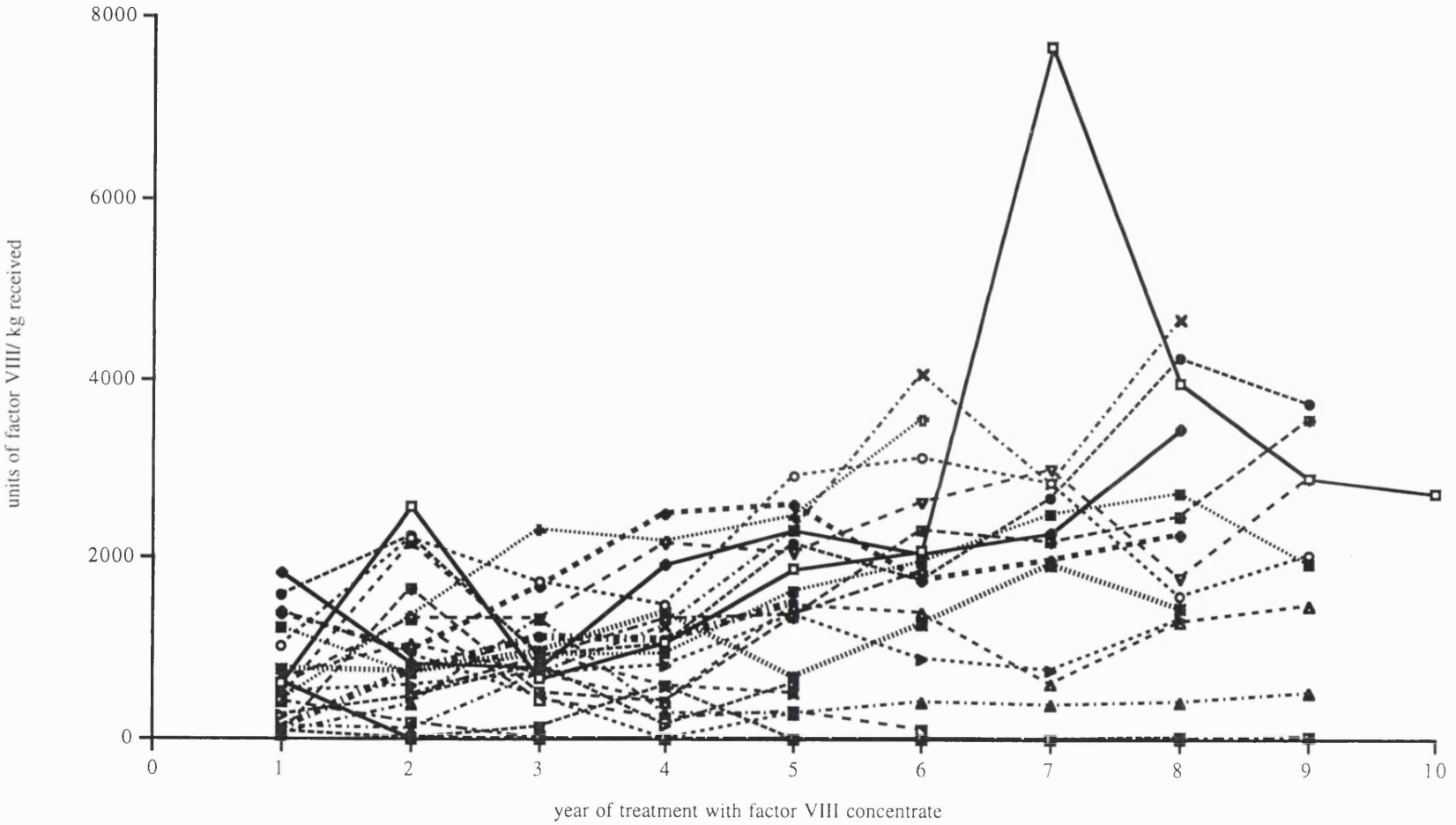
The boys were all weighed whenever they were given an infusion of FVIII, and for those receiving infrequent treatment, they were weighed as part of their regular three monthly outpatient review. Hence, a mean weight for each year was calculated in order to derive the total units of FVIII received per kilogram body weight per year. The mean treatment expressed as units of Factor VIII per kg body weight per year received by each boy is shown in graph 6.1.

The amount of factor VIII given to the eighteen boys with severe haemophilia is shown in graph 6.2.

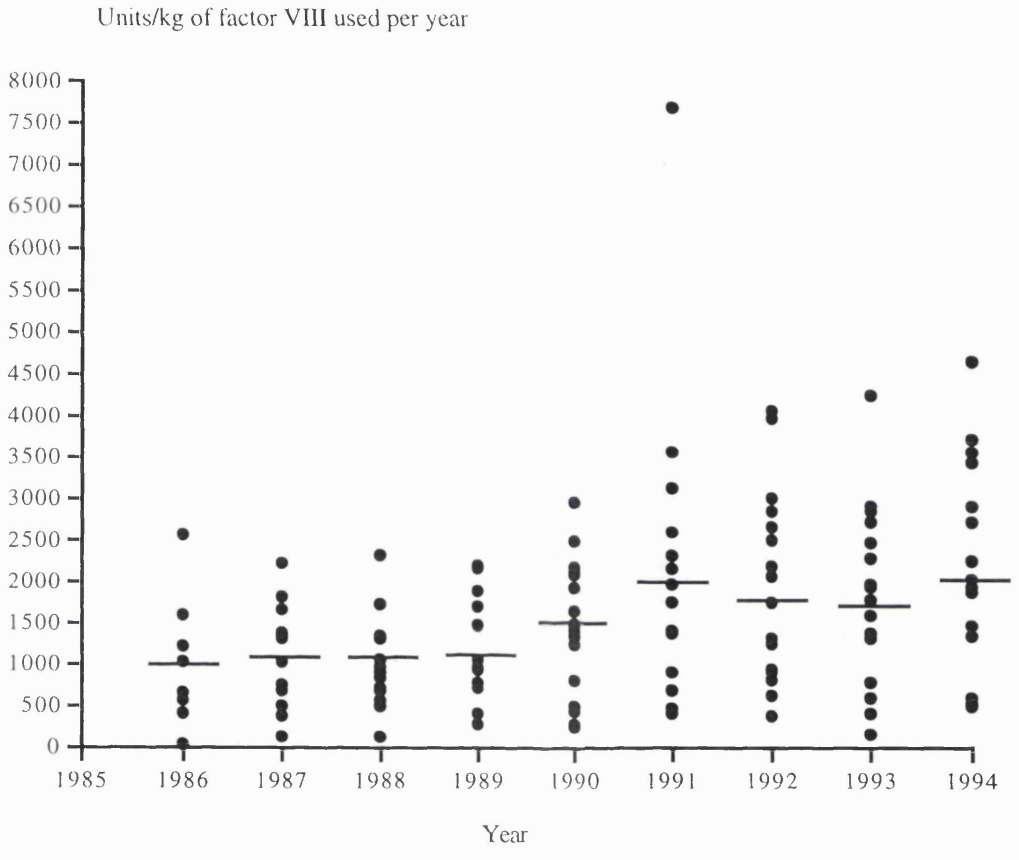
It can be seen from the graphs that there was a gradual increase in use of factor VIII with increasing age, particularly after 1990. This is occurring independently of increasing weight. The reasons for this increasing use are not entirely clear. It is probably accounted for by increasing activity of the children and increasing numbers of bleeding incidents. It may also reflect growing confidence in the factor VIII product with the result that parents were presenting with their children more readily.

Increasing concentrate usage over the same time period has also been documented in other haemophilia treatment centres. This was accounted for by the introduction of improved products, but mainly by the introduction of prophylaxis regimes (Miners et al 1998). During the BPL 8Y study, primary prophylaxis regimes were not used routinely, however a number of patients had intermittent periods of prophylaxis for example to interrupt a succession of joint bleeds.

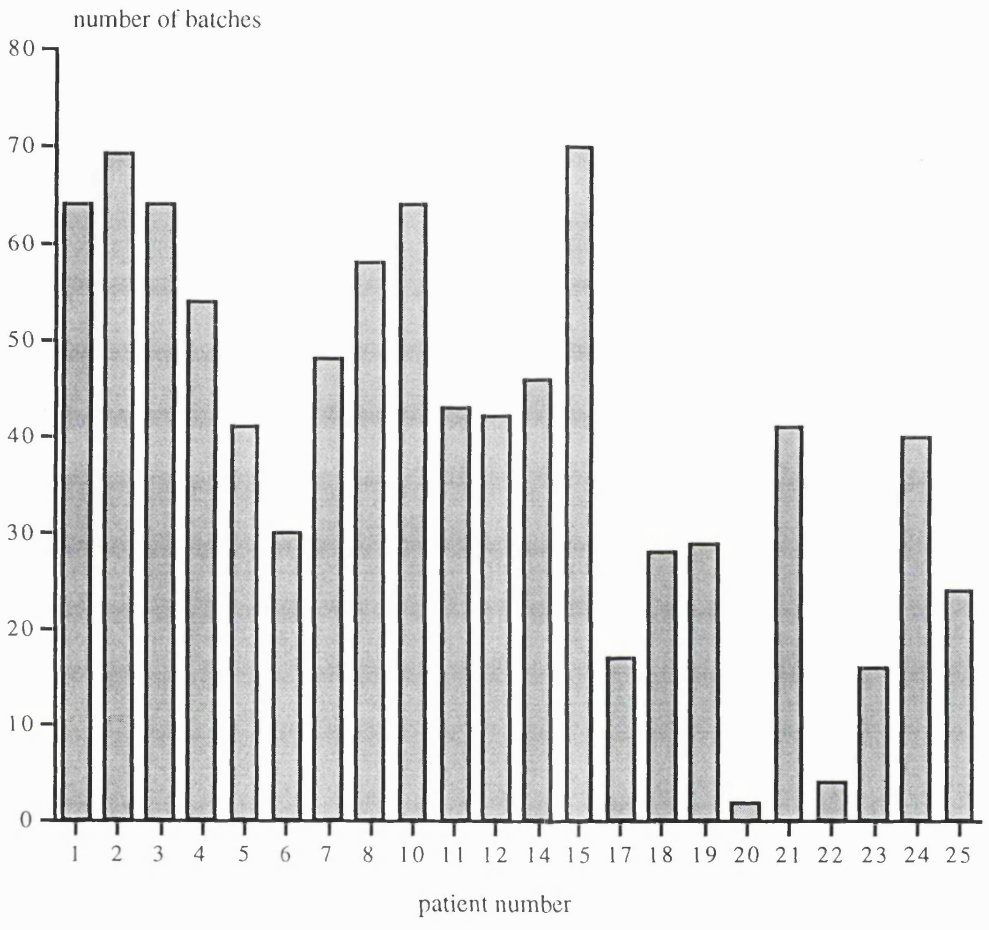
Thousands of plasma donations are included in the production of factor VIII and therefore efforts are made to limit the number of batches received by each patient. For severely affected haemophiliacs, they will ultimately receive many different batches, however for those who receive less treatment it is important to try and use the minimum number of batches possible. The total number of different batches received by each patient are shown in graph 6.3. Over a period of eight years between 1986 and 1994 twenty two of the patients studied received between 2 and 70 different batches of factor VIII. The mean number of batches received was 40. This is important information to document, as all donations included in each batch are recorded by the manufacturer. Therefore if it should become apparent that a plasma donor has developed an infection such as hepatitis for example, individuals who have received a particular batch can be traced and tested.



graph 6.1: quantity of factor VIII concentrate received by each patient in each year of treatment (expressed as units / kg body weight).



graph 6.2: Units of factor VIII/kg body weight used by severe haemophilia A patients treated solely with BPL 8Y between 1986 and 1994. Horizontal bars represent mean values



Graph 6.3: Total number of batches of factor VIII concentrate BPL 8Y received by patients between 1986 and 1994

Blood test results

Results of blood tests (hepatitis B serology, HIV serology, alanine aminotransferase, alkaline phosphatase and bilirubin levels) are detailed in appendices 6.2.1- 6.2.25.

Adherence to protocol for the detection of NANBH

One of the major concerns of the ISTH when the recommendations for studies of safety of clotting factor concentrates were made was that patient compliance would be difficult to achieve. This problem is well demonstrated in figure 6.1 where the adherence to the protocol is depicted.

The patients in this study were all children, with a median age at entry of only 17 months. Parents of such children are naturally anxious that their children undergo as few procedures as possible both to minimise traumatic experiences and because of anxiety that repeated venepunctures may cause damage to their veins. Children of this age may well be difficult to venepuncture. Parents may also be reluctant to bring children to the hospital for testing when treatment per se is not required because of travelling to and from the hospital. Some families lived a significant distance from the hospital because it offers a regional service.

Referring to figure 6.1, the initial patients admitted into the study with BPL 8Y (patients 1-8) during 1985 and 1986 did not adhere to the testing protocol. Following this time staffing levels in the Haemophilia Unit were increased in the form of research fellows and specialist nurses and as a result compliance improved greatly. Patients 9 to 14 were tested mainly on a monthly basis, with extra samples being taken if a patient required treatment with factor VIII. Full compliance with this regime was sought and was aided by active involvement of parents in learning to perform venepunctures and giving treatment. A parent discussion group was set up to provide increased information about treatment and safety studies (Westoby et al 1992). Regular reminders about attending for blood tests were given by both letter and telephone. At this time it had been argued that monthly testing was adequate to identify most patients

with NANBH and at the Proceedings of the World Federation of Haemophilia in 1988 monthly testing was acknowledged to be acceptable.

Doubts however remained that brief increases in transaminases would be missed , therefore from late 1987 until the end of the study (patients 15-25) it was attempted to adhere fully to the recommendations and to perform 2 weekly transaminase levels for the first four months after the first treatment episode. This was achieved in eight of the subsequent eleven patients admitted to the study. Of the remaining three (patients 16, 19 and 25), only one of the blood tests was omitted during the first four months. These three patients still comply with the recommendations however because only one test was missed and the results on the occasions on either side were within the normal range.

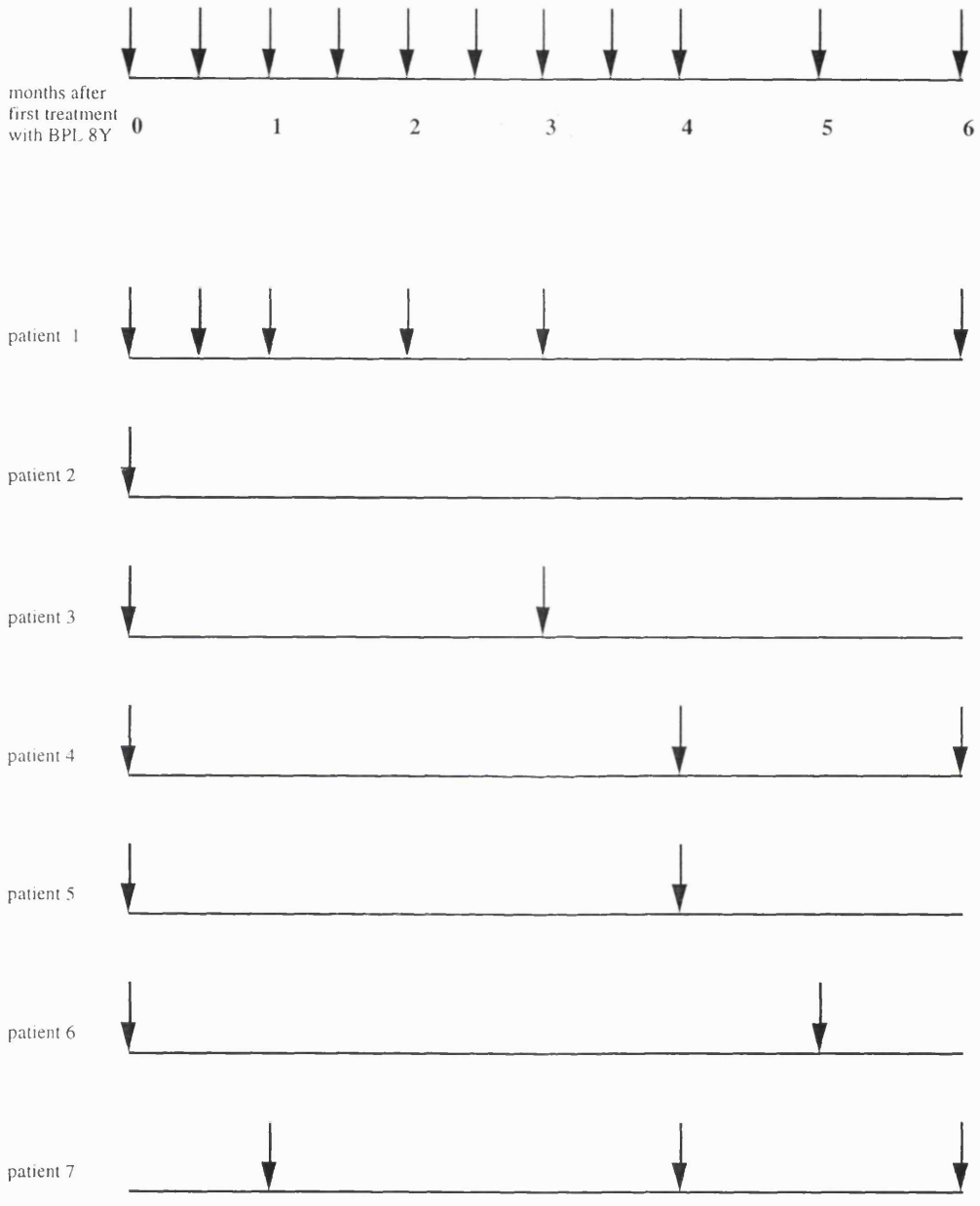


Figure 6.1 Follow up of previously untreated patients treated with BPL 8Y. The top bar in the figure represents the ICTH recommended blood testing regime for patients treated with a new product.. Each pateint is represented by a horizontal bar with the arrows above the bar depicting when the blood tests were performed.

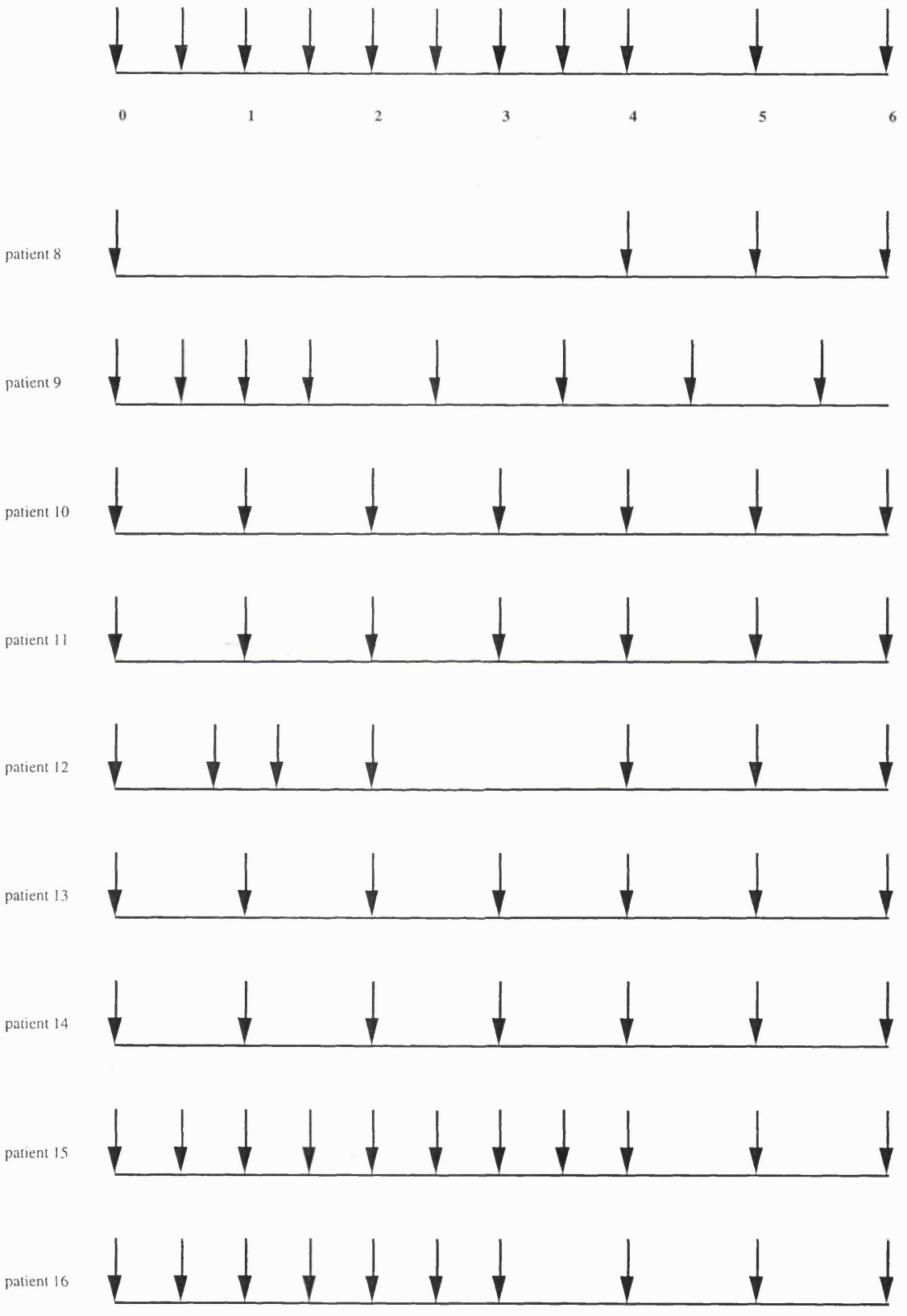


Figure 6.1 (ii) Follow up of previously untreated patients 8-16. The top bar represents hte ISTH recommended blood testing regime for patients treated with a new product.

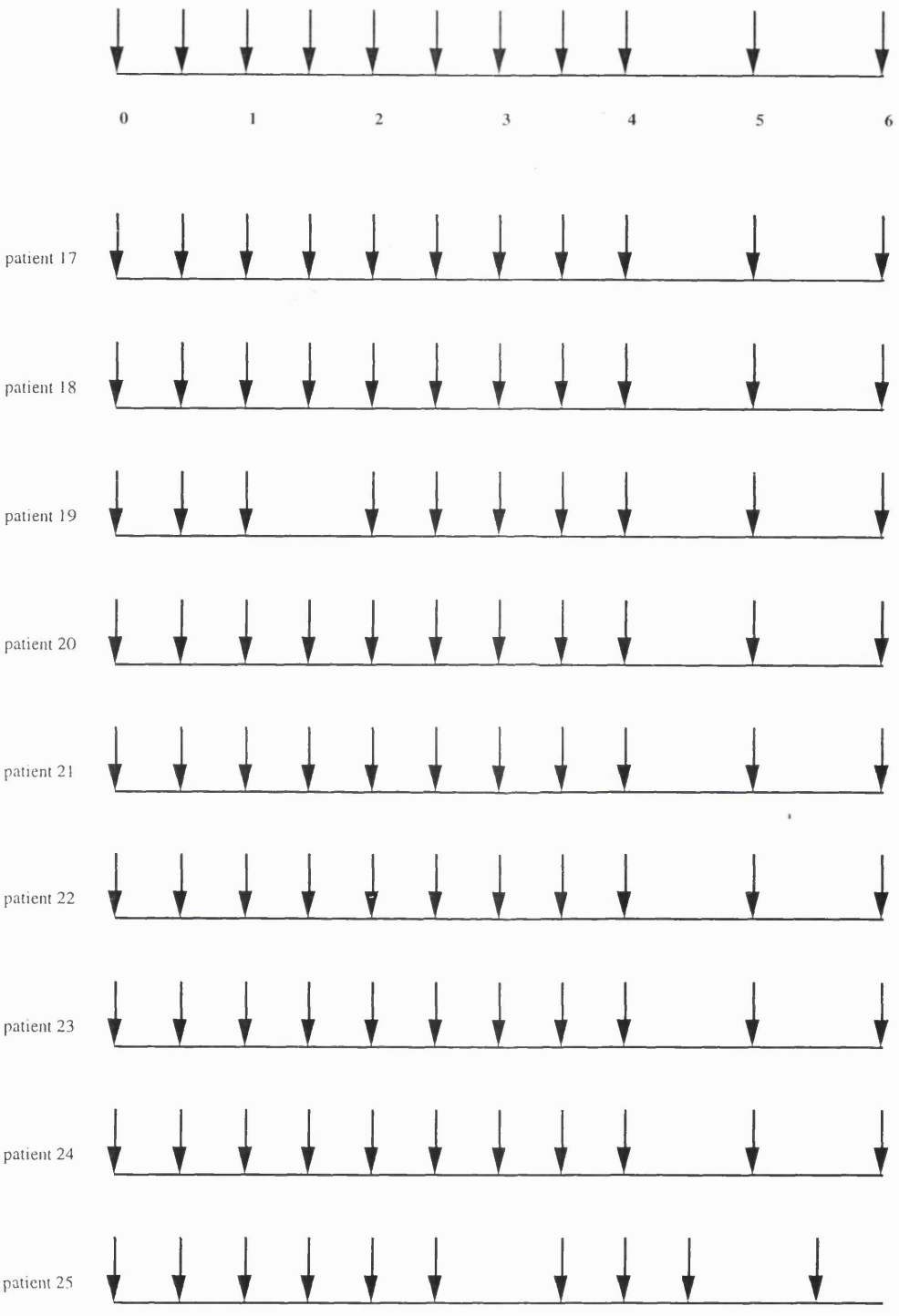


Figure 6.1 (iii) Follow up of previously untreated patients 17-25. The top bar represents the ISTH recommended blood testing regime for patients treated with a new product.

Incidence of raised hepatic transaminases during the follow up period

Referring to appendices 6.2.1 – 6.2.25, during the first six months follow up, no patient had raised ALT levels. For the patients 2 to 8 this result is meaningless as the patients were tested so infrequently. However it indicated that patients 1,9,10,11,12,13 and 14 were unlikely to have contracted NANBH following the first treatment episode and that patients 15 – 25 were highly unlikely to have done so.

All patients continued to be followed up with monthly tests in the first year after the start of treatment and two monthly thereafter. Four patients had raised ALT levels on at least one occasion during this follow up period as detailed in table 6.1.

Patient	Date of first treatment	Date of raised ALT	Value (upper limit of normal 40 IU/l)
2	28.12.85	18.11.93	160
3	3.1.86	9.12.94	58
6	6.6.86	15.7.91	119
20 *	20.11.89	13.3.91	67
		13.8.91	105
		13.1.92	73

Table 6.1 Incidence of raised ALT during follow up period.

All values returned to normal and remained so.

*Patient 20 had received no treatment since December 1989 (14 months) prior to the first abnormal value and had had regular blood tests. He was otherwise completely well and no other cause for the raised transaminases have been found and his liver function remains entirely normal.

It is likely that throughout the course of a prospective study that occasional transient changes in laboratory values will be seen, that occur as a result of laboratory or machine error or just by chance. It may be considered surprising that so few slightly abnormal values were seen when so many individual tests were carried out. Only one

patient had more than one raised value (none of which were 2.5 times normal) and no underlying cause was found. Of the other three patients no further investigations were carried out and the transaminases returned to normal and remained so.

Testing for hepatitis C antibodies

The majority of cases of blood or blood product transfusion associated hepatitis were found not to be associated with a positive serology for hepatitis A or B and these cases came to be known as non A non B hepatitis (NANBH) (Feinstone et al 1978). Further evidence was gathered confirming that a transmissable agent was responsible by experiments infecting chimpanzees from man (Feinstone et al 1981).

A virus specific antigen associated with non A non B hepatitis was discovered in the late 1989. This was done through the isolation of a viral genomic clone from large volumes of highly infective chimpanzee plasma derived through the transmission experiments. This clone coded for an antigen which bound to antibodies in the serum of patients with chronic NANBH (Kuo et al 1989). This led to the development of a specific antibody test for the hepatitis virus which came to be known as hepatitis C. Using this original clone as a base further clones were detected and the complete genome was sequenced (figure 6.2) (Choo et al 1991), and the virus was found to be closely related to the flaviviruses.

The early diagnostic tests were based on the detection of antibodies reactive with recombinant proteins produced from the clones. The so called first generation assays, using a non-structural recombinant protein C-100, were positive in between 80 and 90% of blood donors suspected of transmitting HCV infection (Alter et al 1989). These assays had a relatively high rate of false positives particularly in patients with autoimmune disease and if used on old stored serum samples.

The increasingly sensitive and specific second generation assays used antigens from the nucleocapsid and other non-structural proteins and were found to become positive earlier on in the course of the infection in comparison with the antibodies detected by

the first generation assays. 98% of individuals with so called NANBH were seropositive for hepatitis C using the second generation assays (Nakatsuji et al 1992).

It later become possible to detect the virus itself by PCR, a highly conserved region of the genome being used as the target region for the primers as it was later shown that there was considerable variation between different isolates of hepatitis.

It is currently believed that approximately 50% of HCV infections become chronic, 70% of which have abnormal histology on liver biopsy, with a spectrum of abnormalities ranging from chronic active hepatitis to cirrhosis.

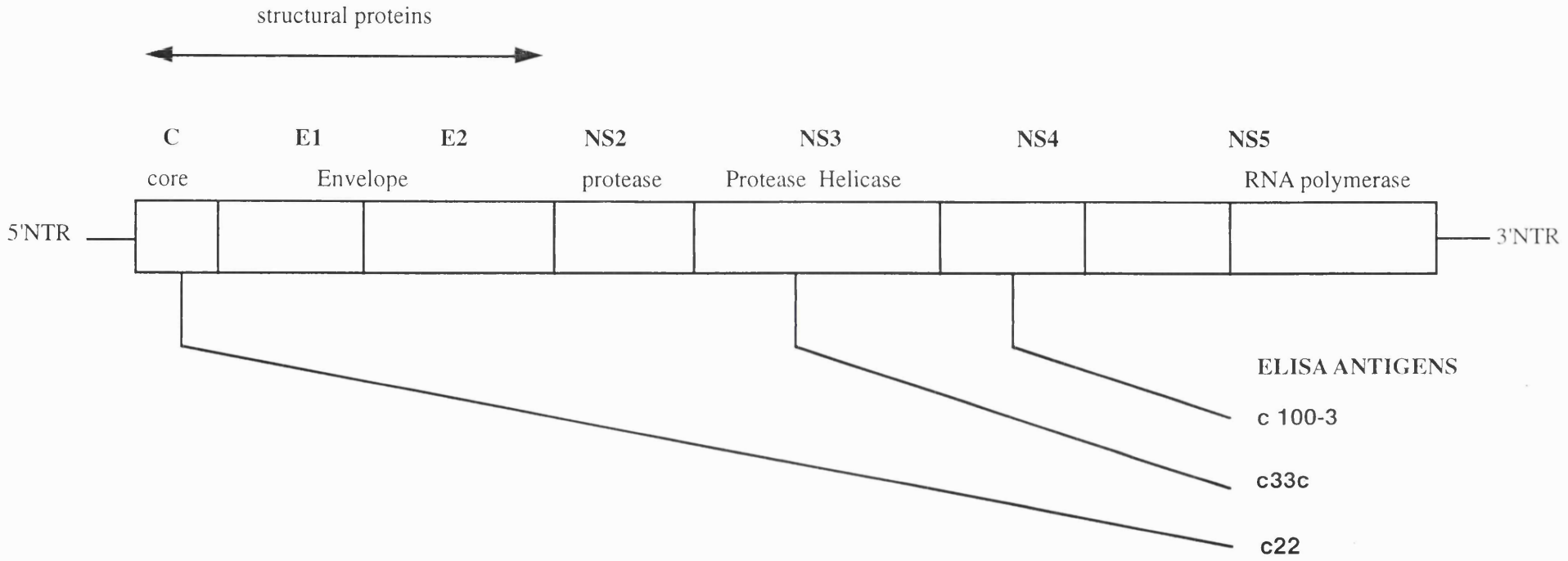


figure 6.2 Organisation of the RNA genome of HCV

Hepatitis C antibody testing of the BPL 8Y cohort

When the first generation assays became available in 1989 the most recent serum samples from the patients (numbers 1-15) admitted into the safety study up to that time point were tested. The time from the first infusion of factor VIII to the time of the assay was between 17 and 44 months (table 6.2).

At the same time serum from patients in treated in the same unit suspected as having NANBH were also tested (Skidmore et al 1990) and they were all found to be positive by the first generation antibody test.

The initial first generation tests for hepatitis C antibodies on patients 1 to 15 were all negative.

Since this time the patients have been regularly tested and all remain consistently negative for hepatitis C antibodies (appendix 6.3).

Patient	Date of first treatment with FVIII	Date of first assay for hepatitis C	Time since first treatment (months)
1	19.7.85	10.3.89	44
2	28.12.85	16.1.89	37
3	3.1.86	2.3.89	37
4	20.1.86	20.1.89	36
5	31.5.86	21.10.88	29
6	6.6.86	3.1.89	31
7	1.9.86	14.10.88	25
8	28.9.86	25.10.88	25
9	1.12.86	20.1.89	25
10	27.1.87	18.1.89	24
11	4.2.87	13.3.89	25
12	6.2.87	21.2.89	24
13	31.3.87	24.2.89	23
14	12.4.87	15.2.89	22
15	5.10.87	2.3.89	17

Table 6.2 Timing of first hepatitis C antibody test in relation to first treatment episode with the factor VIII concentrate BPL 8Y

Discussion

Soon after the introduction of large pool concentrates an increase in the number of cases of acute hepatitis in recipients were seen (Kasper 1972). These cases occurred in relatively few of the patients, but what was later to become apparent was that many more had had subclinical hepatitis infections, detectable only through raised transaminases. The significance of these infections was only to become apparent later in the form of chronic hepatitis, cirrhosis and liver failure.

Before the introduction of the screening of donated blood for hepatitis B antigen and the development of a hepatitis B vaccine, many haemophiliacs became infected and studies showed that over 50% of patients had previous evidence of infection (Cederbaum et al 1982, Rickard et al 1982). As concern increased over the possible transmission of viruses and patients receiving large pool concentrates were closely monitored, it was observed that the majority had transiently raised transaminases following the first infusion. In the absence of the development of hepatitis A or B antibodies this was considered to be due to a third or possibly more transmissable agents and until the cloning of the hepatitis C genome and the development of specific antibody tests, this hepatitis was described as non A non B hepatitis (NANBH). Its prevalence in the blood donor population was relatively high. It was estimated as being 0.3% in the UK in the 1980's (Collins 1983), but more recent studies have found it to be lower at 0.05% (Irving et al 1994). However seroprevalence is significantly higher in other countries such as the USA and Japan (Murphy et al 1996, Yamaguchi et al 1994). The high prevalence explains why haemophiliacs were so at risk of acquiring NANBH. Each batch of large pool concentrates contained thousands of donations and was therefore highly likely to contain at least one that was infectious.

Experience increased as to the significance of infection with hepatitis in this population, through a number of studies of liver biopsies in haemophiliac populations (Hay et al 1985, Makris et al 1996). Over time the results of these studies have become increasingly disturbing. The initial studies showed that although a minority of patients

had evidence of active hepatitis and cirrhosis, the majority had what was initially thought to be the more quiescent chronic persistent hepatitis.

However it was found that in the haemophiliac population this form was more aggressive and that the tendency was towards progression, a fear that was confirmed by the increasing number of patients presenting with the complications of chronic liver disease (Makris et al 1996).

Following the introduction of the large pool concentrates it then became clear that measures would have to be taken to make the products safer. The pressure to do this increased dramatically with the advent of the Acquired Immune Deficiency Syndrome (AIDS) epidemic in the early 1980's.

Many treatment methods were investigated and introduced, involving treatment of the concentrates with solvents and detergents, wet heating and dry heating at a series of different temperatures. The treatment processes all reduced the yield of products, which were already extraordinarily expensive to produce, resulting in pressure to make a safe and economically viable product.

Hepatitis B screening of donated blood was already available and HIV antibody testing was introduced in 1986. However treatment processes had to be adequate to destroy viruses, which despite screening entered the pool, either as a result of error, or in the case of a donation from an antibody negative , although infected person.

The elimination of the as yet uncharacterised NANBH remained a challenge. In the absence of antibody tests surrogate markers of infection were relied upon and then with the increasing number of "treated" products appearing on the market the need for a standardised testing protocol was paramount. The first of these was produced in 1984 (Schimpf et al 1987) and revised in 1989 (Mannucci et al 1989), the details of which are described in detail earlier in this chapter.

It was recommended that only patients, who had never before received concentrates, should be included in safety studies of new products. Historically such patients had an 100% attack rate with NANBH when receiving untreated products and a historical control group therefore already existed (Fletcher et al 1983, Kernoff et al 1985). It was

argued that patients who had received single donor products in the past or who had been infrequently treated could also be included, but it was decided that this should not be so. Firstly these patients may appear not to become infected because they may before have contracted subclinical NANBH and then have developed an "immunity" to it, therefore being unreliable candidates. Also when relying on surrogate markers of infection, the exact pattern of the raised transaminases in the presence of both acute and chronic NANBH infections was not clear. For example a raised transaminase occurring after the infusion of a new product may be due to an acute infection from that new product, or the manifestation of a chronic NANBH acquired from a previously infused product. Such doubts in interpreting safety studies would have resulted in lack of confidence in the results.

The stringency of the protocol was such that compliance was a major problem. The majority of patients enrolled into safety studies were small children, for whom two weekly blood testing was considered by many parents to be too much. It was considered by some that monthly blood levels of transaminases would be sufficient,which was more acceptable to parents and our experience was that this protocol could be well adhered to. However, the risk of missing a short lived transaminitis by only monthly testing was underlined by a prospective safety study of a dry heated factor VIII concentrate (Colombo 1985) when 3 of 11 episodes of hepatitis would have been missed if blood samples had been obtained more than fifteen days apart.

It proved to be possible for even children to adhere to the stricter protocol in this study as demonstrated by the latter 11 patients admitted. This was aided by increasing the information available to the parents. A parent information and discussion group was set up where the reasons behind safety studies were fully explored and as a result compliance reached almost 100%.

None of the patients in this study, whose blood was tested either according to the full protocol or monthly had raised levels of ALT during the first six months after the first treatment episode. There were thereafter only four patients who had at some time point

raised transaminase levels. The most significant of these occurred in a mild haemophiliac who only had one period of FVIII treament, during a tonsillectomy. He was followed exactly according to the ISTH protocol and the raised transaminases occurred sixteen months after he received factor VIII and in the face of normal transaminases in the immediate post treatment phase were unlikely to be due to NANBH acquired through the concentrate.

The development and introduction of hepatitis C antibody tests, leading to the confirmation that the RNA flavivirus was responsible for the majority of cases of post transfusion NANBH, enabled the confirmation that these patients treated solely with BPL 8Y had remained free of infection. This, together with consistently negative antibody tests for HIV and the absence of markers for hepatitis B infection confirmed that this product was not capable of transmitting the three viruses which had so long been a problem in the treatment of haemophiliacs with large pool concentrates.

One other long term follow up of the use of BPL 8Y has been published (Brown et al 1998), describing the follow up of 33 patients over a median period of 96 months. They also documented no evidence of transmission of hepatitis C, hepatitis B or HIV. BPL 8Y was widely used in the United Kingdom during the late 1980's and 1990's. There is good evidence that from the point of view of hepatitis B and C and HIV that it is safe. However that does not mean that it is totally free from the risk of viral transmission. As is well demonstrated by the AIDS epidemic, viruses can suddenly appear and some may have characteristics rendering them less susceptible to the viral inactivation processes in use. For example, viruses which lack a lipid envelope are not eliminated in the production process of BPL 8Y. One such virus, parvovirus B19 was not routinely tested for in the course of this study, but in the study of Brown et al the patients studied had a 100% prevalence of parvovirus B19 antibody (Brown et al 1998). This prevalence is significantly higher than that which would be expected, for example in those sixteen patients under the age of sixteen years, in whom an antibody prevalence of <52% would be expected (Cohen & Buckley 1988). There have been well documented outbreaks of hepatitis A, although not specifically related to 8Y,

which also lacks a lipid coat, in groups of haemophiliacs in recent years (Mannucci et al 1994). This virus was previously considered not to be a transfusion risk, being spread by the faeco-oral route but it evidently can be and was transmitted.

Hepatitis G, a flavivirus like hepatitis C is present in 3% of the donor population (Ludlam 1997) and has been demonstrated as being transmissable by plasma products, in haemophiliacs the prevalence being between 12 and 15% ((Jarvis et al 1996). It appears as yet to have no serious clinical consequences and may not be hepatotoxic and it is not recommended that haemophiliacs be routinely tested for it (Makris et al 2001). Although these other viruses do not have the fatal consequences of HIV and the other hepatitis viruses, the implication remains that plasma derived products including BPL 8Y are still capable of transmitting viruses and there will always remain the possibility of new viruses or of pre-existing ones changing to become more dangerous. It would be far from correct to say that any plasma derived product is virally safe.

CHAPTER SEVEN

STUDIES OF T LYMPHOCYTE SUBSETS IN HAEMOPHILIACS TREATED SOLELY WITH BPL 8Y

Background

T lymphocytes are responsible for cell mediated immunity and are also essential for the development of antigen specific antibody responses. B cells produce antibody but are dependent in doing so on intact T cell function. The T lymphocytes undergo maturation, differentiation and selection in the thymus gland.

One of the initial steps in the assessment of immune function of a host is to count the total number of T lymphocytes and sub-populations of T cells responsible for mediating different functions (Gelfand & Finkel 1996). The assessment is done by detecting the presence of cell surface protein markers specific for the different populations. All mature T cells have CD3 (Reinherz et al 1979), an antigen which is associated with the T cell receptor (TCR) and is required for the latter's expression and function (Borst et al 1983, Meuer et al 1983). The CD3+, or total T cell population can be further subdivided into CD4+ and CD8+ populations, which, in turn can also be subdivided (Evans et al 1978, Kung et al 1979).

The CD4 and CD8 molecules are involved in antigen presentation and are necessary for the initiation of T cell activation. On resting T cells, the CD4 or CD8 molecules are not linked directly to the T cell receptor but become associated with it when the receptor recognises the antigen/major histocompatibility complex (MHC) on the surface of an antigen presenting cell (Glaichenhaus et al 1991). In general the CD4+ cells, the T helper and inducer cells, recognise peptides bound to class II MHC molecules and the CD8+, or suppressor and cytotoxic T cells recognise those bound to class I MHC molecules. (Konig et al 1992).

The number of CD4+ and CD8+ cells can be expressed as a percentage, as an absolute count and also as a ratio of the number of CD4+ to CD8+ cells. The absolute count, which is of course dependent on the total white blood cell count, is a good indicator of the degree of T cell deficiency. In adults a CD4 count of less than 500 x 10^6/l is associated with impaired cell mediated immunity and counts below 200 x 10^6/l result in profound suppression (Lang et al 1989). The ratio of CD4 to CD8 cells should be

greater than one. Ratios below 0.3 are associated with severe T cell deficiency. Both the percentage of the T cell subsets and the CD4/CD8 ratio are good ways of following long term trends as they are independent of the total white blood cell count.

Lymphocyte counts and subsets vary with age in childhood. They are higher in younger age groups and gradually decline normally with age towards adult values. The spread of the normal ranges of both the counts and the CD4/CD8 ratio is also wider in infants and young children. Several attempts have been made to establish normal ranges for children. (Falcao 1980, Hicks et al 1983). More recently the European Collaborative Study of infants born to women with HIV-1 infection published centile charts for age related standards for T lymphocyte subsets based on the follow up of HIV uninfected children born to HIV infected women (The European Collaborative Study 1992). These were the first standards based on smoothly changing centiles as opposed to the previously published age-grouped standards, where difficulties arose because of age-break points.

Reduced percentages and absolute CD4 counts and reversed CD4/CD8 ratios are characteristic of HIV infection and are used as a means of monitoring the degree of immune dysfunction. Abnormalities may also be seen in other viral infections, autoimmune diseases and in some haematological malignancies.

T cell subsets in the context of haemophilia

Reduced numbers of CD4+ cells, and relative increases in CD8+ cells were reported as occurring in the first haemophiliacs to be diagnosed as having AIDS (Lederman et al 1983). Studies at that time of healthy haemophiliacs who had been treated with large pool concentrates also revealed similar quantitative T cell abnormalities, the more severe of which occurring in those who had received larger quantities of blood products (Lee et al 1985). In 1983, the virus that causes AIDS, HIV initially known as HTLV-III was isolated (Barre-Sinoussi et al 1983) and a serological test to detect IgG

to the virus was developed (Samgadharan et al 1984). It therefore became possible to determine which of the haemophiliacs had been exposed to the virus. A number of studies showed that T lymphocyte abnormalities occurred even in the absence of HIV antibodies (Shannon et al 1986b, Carr et al 1984) In the cohort of haemophiliacs followed in Edinburgh, absence of HIV infection has been confirmed in some of these individuals by the polymerase chain reaction, (Peutherer et al 1990) despite which this group of patients show continued abnormalities of T cell subsets.

Aim of the T cell subset studies

T cell subset abnormalities have therefore been documented both in the presence and absence of HIV infection in haemophiliacs, who were treated with a variety of blood products including untreated and treated factor concentrates and cryoprecipitate. These patients were also infected with other viruses. The aim of this study was to follow prospectively a group of previously untreated patients, performing regular assessments of CD4+ and CD8+ cell counts and ratios, and to determine if any immunological changes occurred, and if they did so their relationship to the amount of treatment received.

Methods

Patients

Twenty one of the twenty five patients included in the original BPL 8Y safety study were included in this part of the study. Patients 9,13, 16 and 19 were excluded because fewer than five assays were performed, either because their care was transferred (patient 19) or because they lived too far away from the hospital to be able to come up to the hospital with a fresh blood sample. Details of the patients are as in chapter 4. Blood was taken for T cell subsets where possible before the first infusion of factor VIII and at approximately three to six monthly intervals thereafter.

T lymphocyte subset analysis

T lymphocyte subset analysis was performed manually prior to 1991, after which analysis was done by flow cytometric analysis (FACSCAN) at the Department of Immunology, East Birmingham Hospital.

Manual method

T lymphocyte subsets were identified by indirect immunofluorescence using fluoresene isothiocyanate conjugated (FITC) rabbit anti mouse immunoglobulin (Williams et al 1988).

Venepuncture was performed on patients between 9 and 11am, (prior to the infusion of concentrate). 10mls of blood was taken directly into lithium heparin containers.

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on Ficoll Hypaque (Lymphoprep, Nygaard Ltd, UK). The PBMCs were then washed three times in RPMI 1640 and resuspended in PBSAA (0.1% sodium azide/PBS/1% bovine serum albumin) and incubated with a 1/25 dilution of each monoclonal antibody.

The monoclonal antibodies used throughout the study were CD11 (pan T lymphocytes), CD4 (includes T helper lymphocytes) and CD8 (includes T suppressor lymphocytes), (Coulter Electronics Ltd, UK)

The cell suspension and monoclonal antibodies were mixed well and left at room temperature for 15 minutes, being agitated twice during this period. The cells were then washed three times with PBSAA and after the final wash the supernatant was completely removed and the cell pellet resuspended in 200 µl of rabbit anti-mouse immunoglobulin -FITC (Dako Ltd, UK; diluted 1/20 with PBSAA).The samples were agitated and left at room temperature as before for 15 minutes. The washing procedure with PBSAA was repeated three times, leaving 100 µl of PBSAA on the pellet after the final wash. One drop of 8% formalin was added to each tube to prevent clumping and the pellet resuspended. One drop of cell suspension was placed on a glass slide, covered with a glass slip and examined immediately, using a Leitz Ploem fluorescent

(mercury vapour) microscope system. 200 cells were counted and the percentage showing fluorescence determined. Having determined the percentage of lymphocytes expressing the cell surface markers, the absolute T cell subset counts were calculated using the absolute lymphocyte count measured on a simultaneous full blood count. The T4:T8 ratio was then obtained from these absolute counts.

FACSCAN Method

Flow cytometric analysis was performed by the Department of Immunology, East Birmingham Hospital. This method used two colour combinations of fluorescent labelled monoclonal antibodies to label T cells in whole blood. After labelling, a hypotonic lysing buffer was added to lyse red cells whilst leaving white cells intact. These were then fixed and analysed on a fluorescence activated counter, gated to lymphocytes.

Briefly, samples of 2mls EDTA anticoagulated whole blood were drawn before 10am and transported at ambient temperature to East Birmingham Hospital within 2 hours. A sample was also taken from a normal donor to control for transport conditions.

100µl EDTA anticoagulated whole blood was incubated with CD3, CD4 and CD8 monoclonal antibodies at room temperature for 10 minutes in reduced light (in combinations CD3-CD4 and CD3-CD8). CD3 and CD4/CD8 were labelled with differing fluorochromes. 2ml of FACSlyse (Becton Dickinson Ltd, UK) was added to each tube and incubated for a further 10 minutes at room temperature. Tubes were then centrifuged at 675rpm for 5 minutes and the supernatant discarded. Cells were resuspended in 2ml PBS and 0.5ml 2% formaldehyde. Cell suspensions were then analysed on a Becton-Dickinson FACScan. Control samples and control monoclonal antibodies were included with all runs.

Treatment

Complete treatment records were available on every patient. The treatment received (expressed as units of FVIII per kilogram body weight) in the time periods one week,

one month and three months before the assay were calculated for each assay for each patient. Serial CD4 and CD8 counts were therefore available on each patient and the relationship between these and treatment received was investigated.

Statistics

The statistical methods used are described in detail in chapter 5. In order to eliminate age as a confounding factor in the analysis, which was important as these patients were being investigated during the time period when the CD4 and CD8 counts change the most, z scores for each measurement of the percentage of CD4 and CD8 cells were calculated and used in the analysis. The z score is a measure of how different an individual is from the average of all children of the same age. The changes in CD4 and CD8 over time were assessed by simple linear regression, while the effect of treatment on the CD4 and CD8 levels was investigated using multiple regression.

Results

The results of the serial T lymphocyte subsets are shown in appendices 7.1.1 -7.1.25.
The serial z scores for CD4 and CD8 percentages plotted against the age of the patient are shown and graphs 7.1.1 to 7.1.25 with the data in appendices 7.2.1-7.2.25.
Serial T cell subset measurements were performed on each of the twenty one patients as detailed in table 7.1. Patients recruited later into the study had CD4 and CD8 cell counts measured from the time at which they were first treated with factor VIII, whereas in other cases T cell analysis started up to 25 months after the first treatment episode (median 11 months after the first treatment episode). A median of 14 assays were performed on each patient (range 6-17) over 32 to 96 months (median 88months), with a total time of follow up since the patients first received FVIII treatment of between 54 and 121 months (median 97 months).
Each patient was studied individually to assess the change of CD4 and CD8 over time, using z scores to eliminate age related change. The regression coefficients and p values

for each individual patient are shown in table 7.2, with significant changes highlighted in bold print.

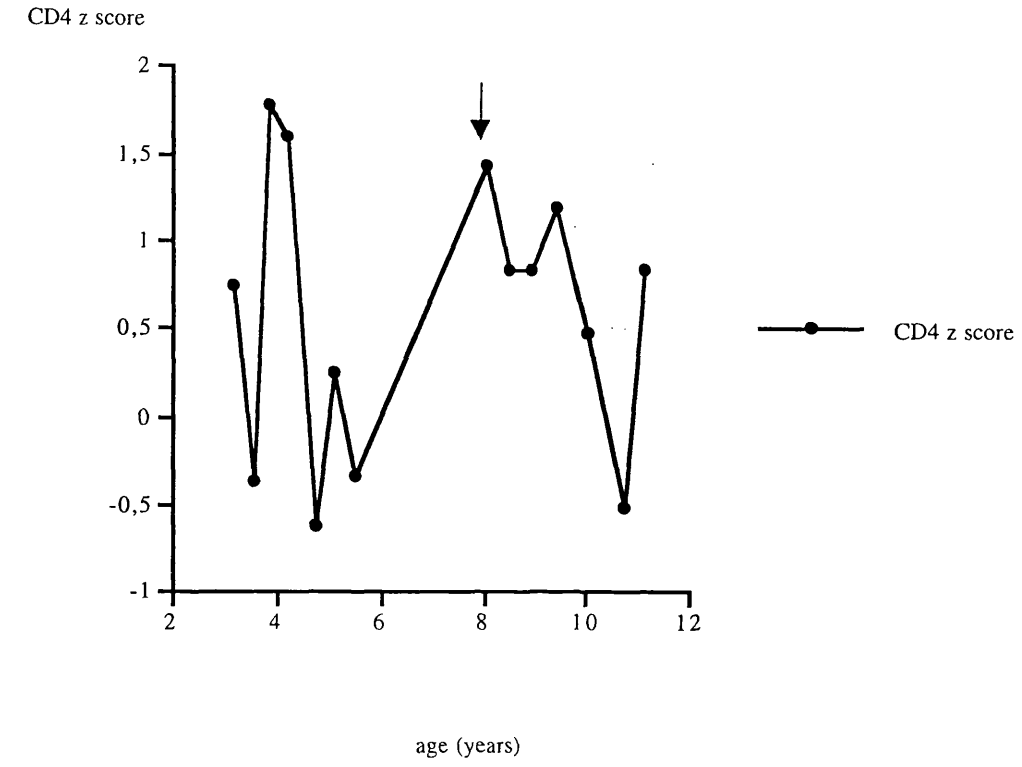
There were only two significant changes in CD4 values, one increasing (patient 25), and one decreasing (patient 5). Both of these patients demonstrated no significant CD8 changes.

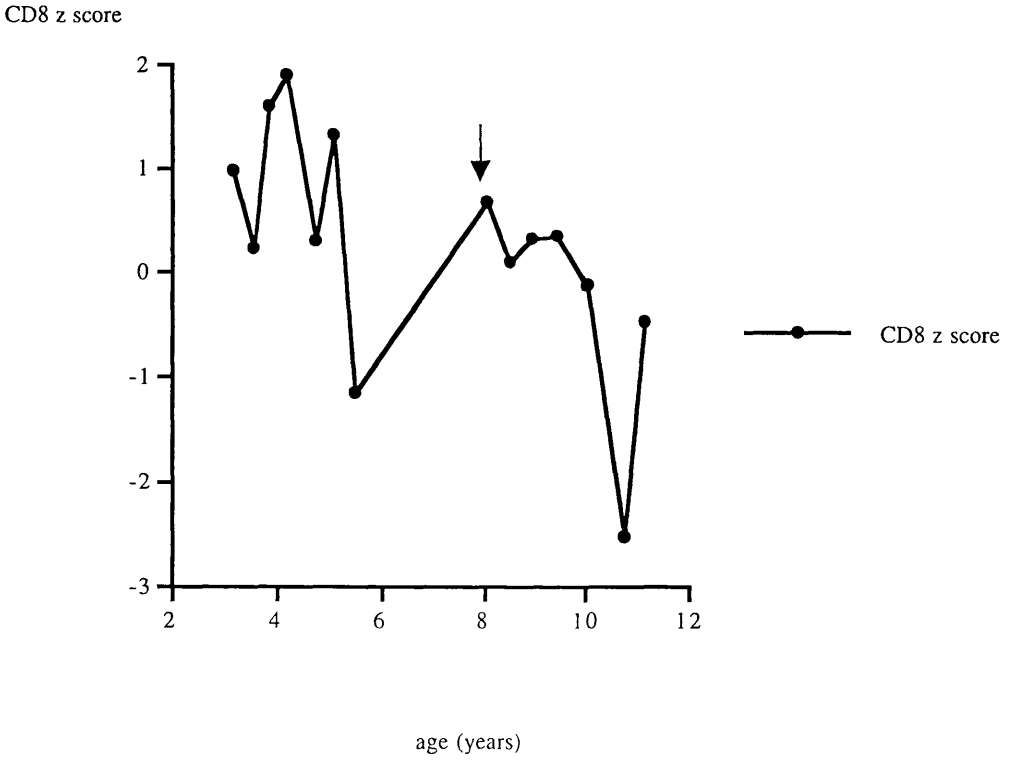
Of the 21 patients three had a significant increase in CD8 values (patients 12, 15 and 22), all z score values stayed within the normal range (between + 1.88 and – 1.88), while three other patients (1, 3 and 4) had decreasing CD8 values. All six patients had stable CD4 levels.

In summary, 19 of the 21 patients had stable CD4 cell values and 15 of 21 stable CD8 values throughout the study period. Of those who did have changes in CD8 values, equal numbers of patients had increasing and decreasing values.

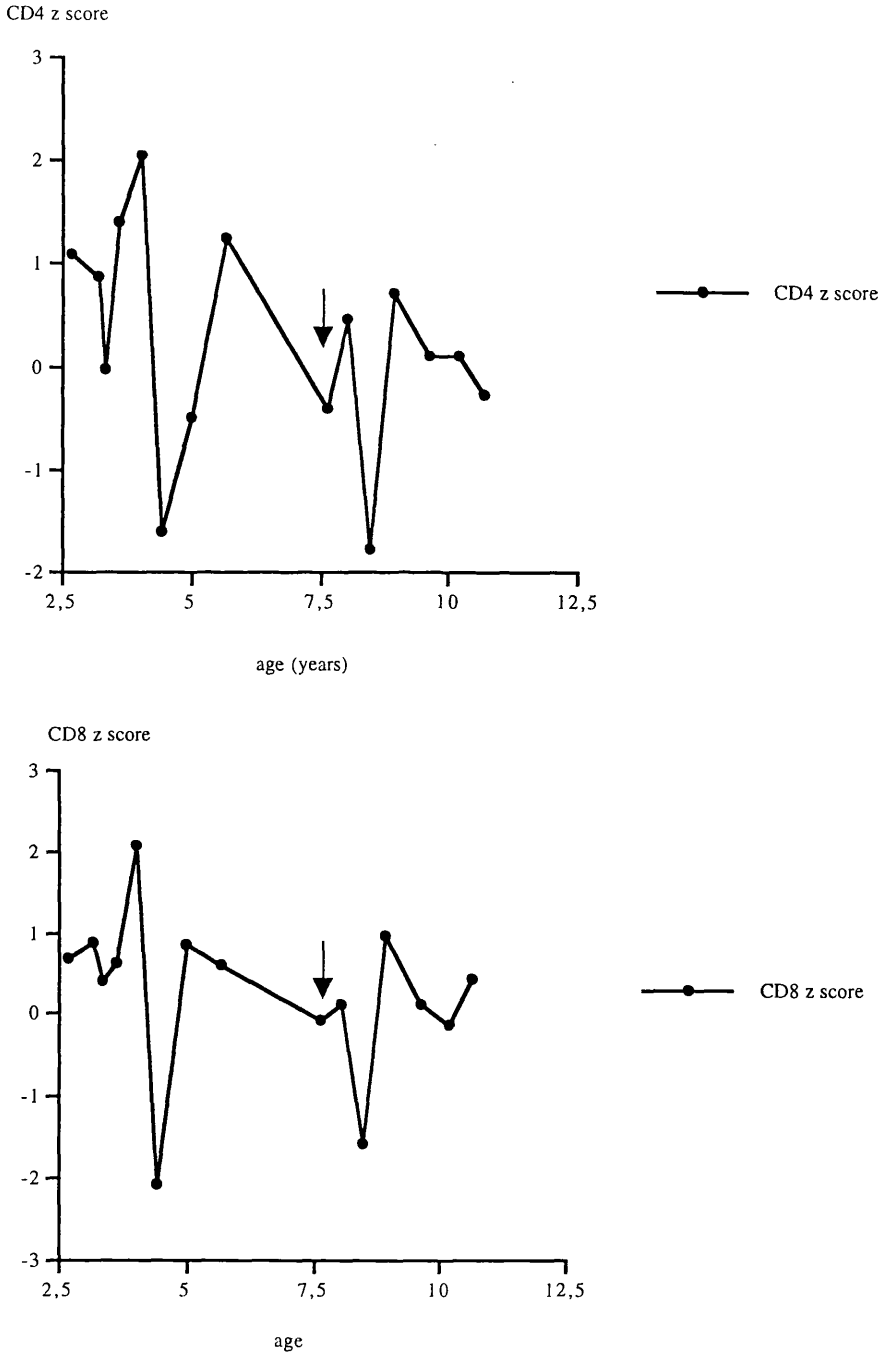
Patient	Total duration of follow up (months)	Number of assays performed	Time between first FVIII treatment and first T cell subset assay (months)	Duration of T cell subset follow up (months)
1	121	14	25	96
2	116	15	20	96
3	110	14	18	92
4	115	15	19	96
5	110	14	15	95
6	110	15	15	95
7	102	14	13	89
8	107	14	11	96
10	97	14	11	86
11	97	15	9	88
12	101	17	6	95
14	99	15	4	95
15	87	15	0	87
17	70	10	0	70
18	70	9	2	68
20	71	9	0	71
21	69	10	0	69
22	66	9	0.5	65.5
23	51	6	11	40
24	63	7	0	63
25	54	7	22	32
	MEAN 89.8	**MEAN 12.2**	**MEAN 9.6**	**MEAN 80.2**
	MEDIAN 97	**MEDIAN 14**	**MEDIAN 11**	**MEDIAN 88**

Table 7.1 Details of T cell subset assays performed on 21 patients

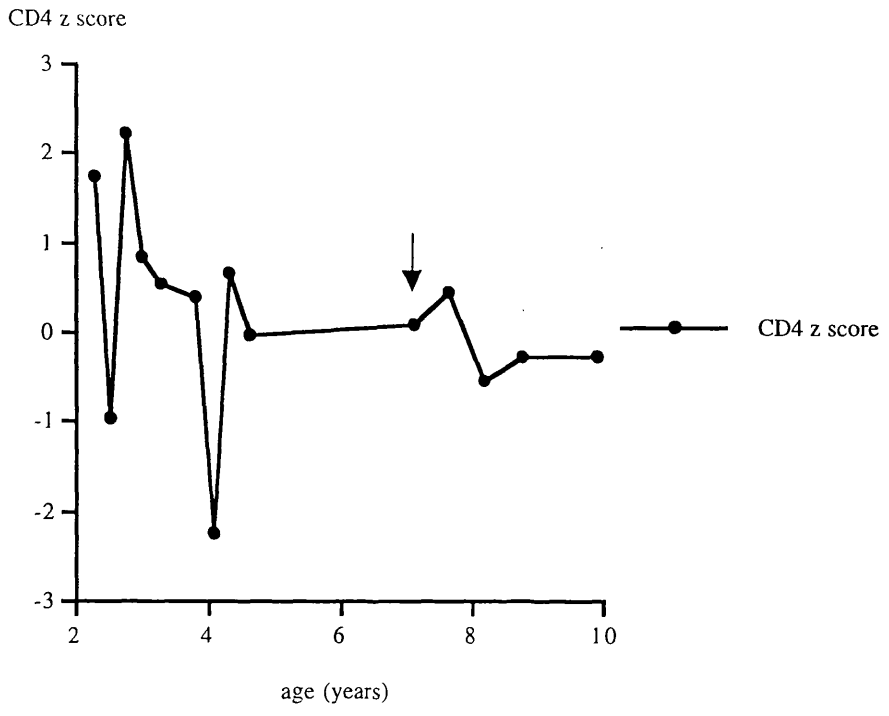


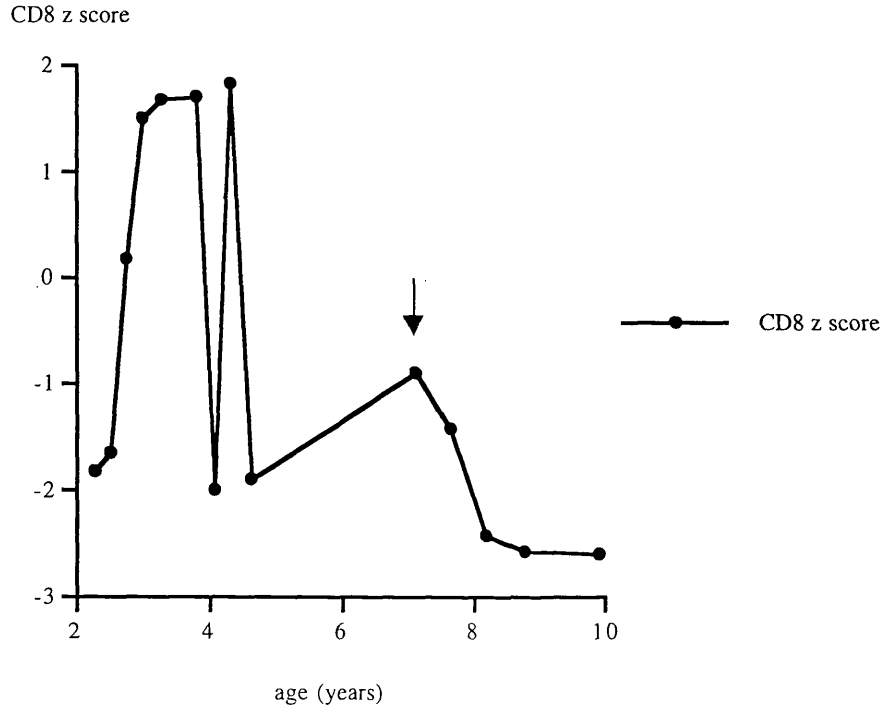


graph 7.1.1 patient 1: Change in CD4 % and CD8% z score with age
arrow denotes first assay performed by FACSCAN method

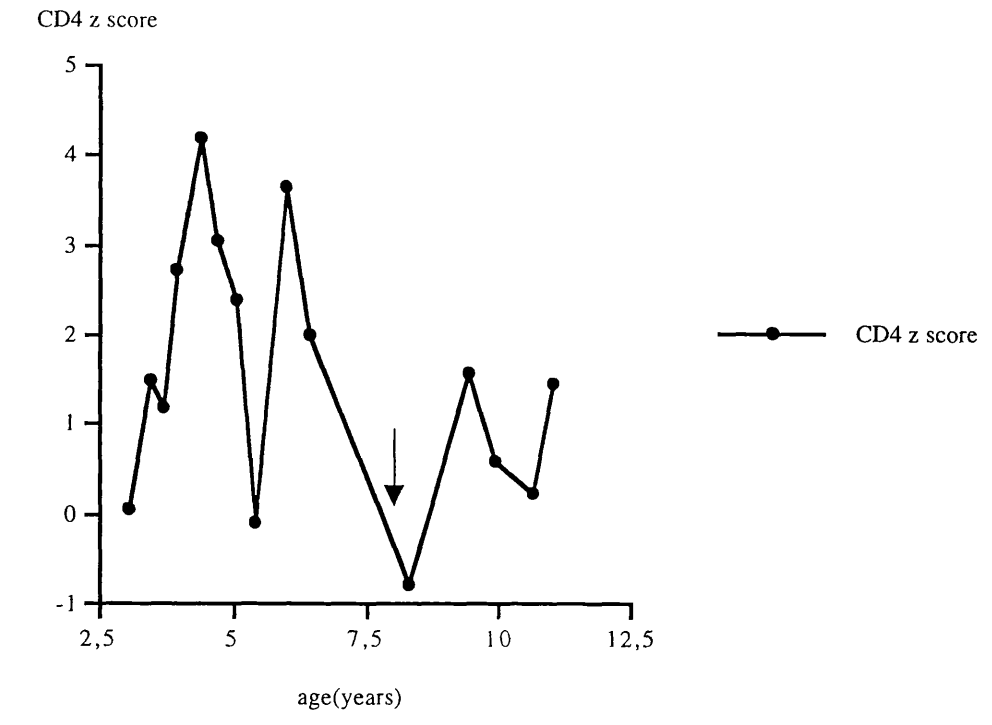


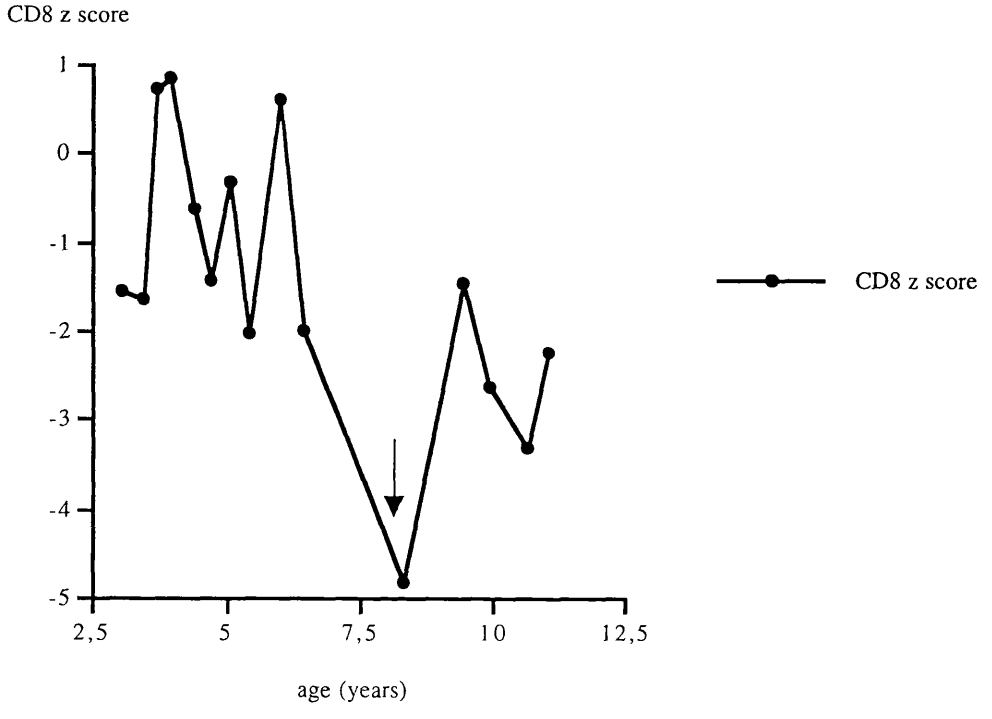
graph 7.1.2 patient 2: Change in CD4% and CD8% z score with age
arrows denote first assay performed by FACSCAN method



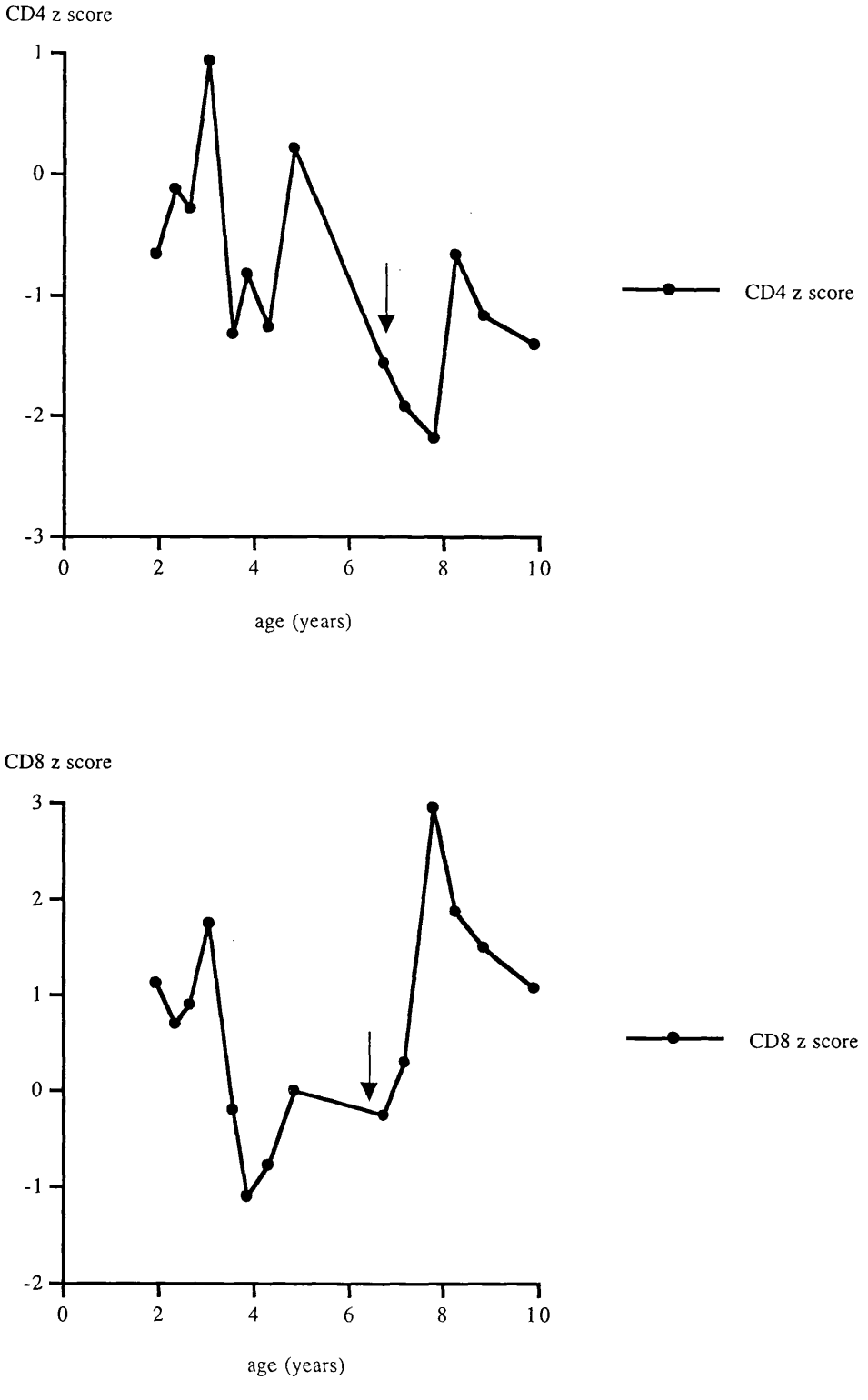


graph 7.1.3 patient 3: Change in CD4% and CD8% z score with age
arrows indicatewhen assay first performed with FACSCAN method

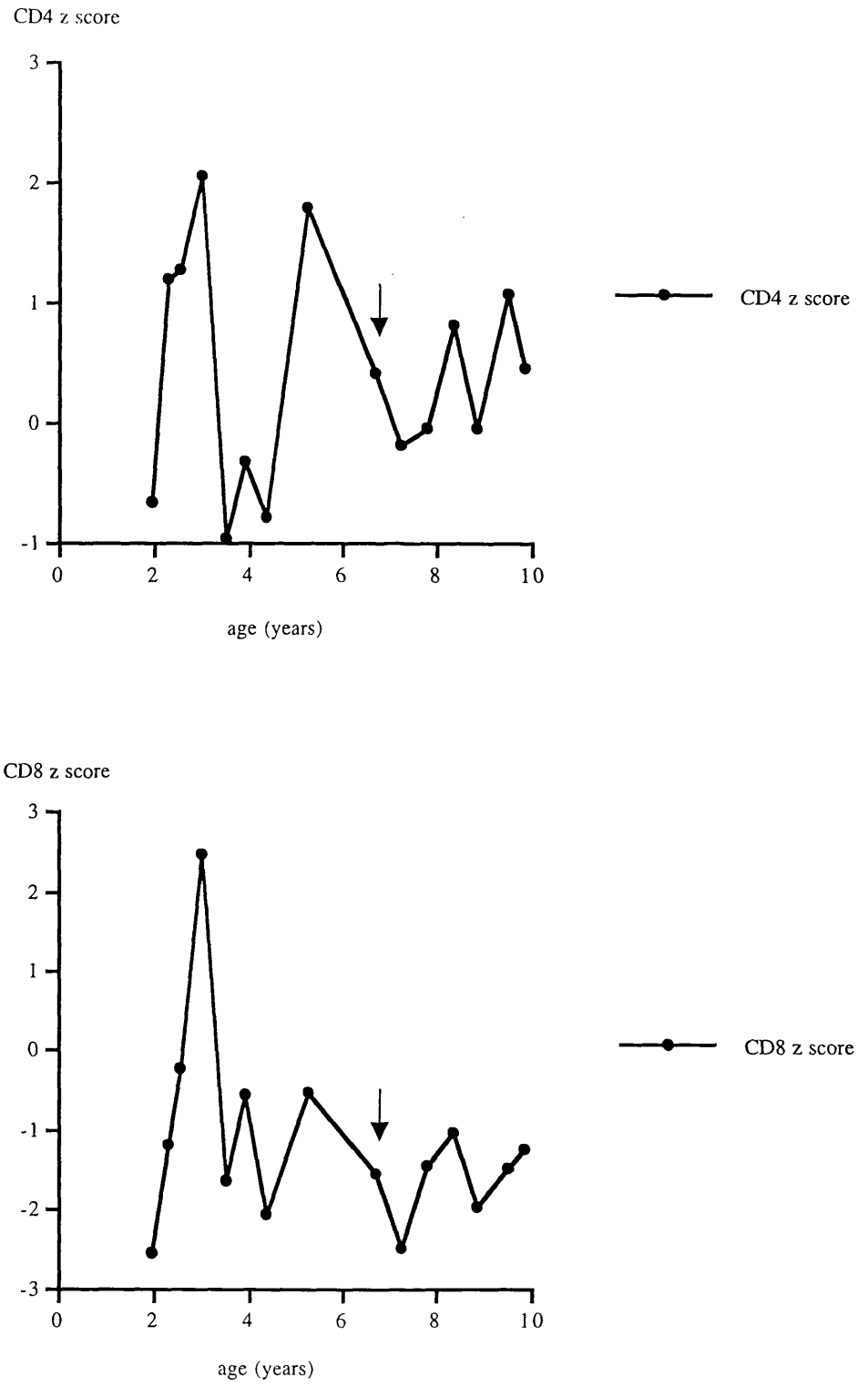




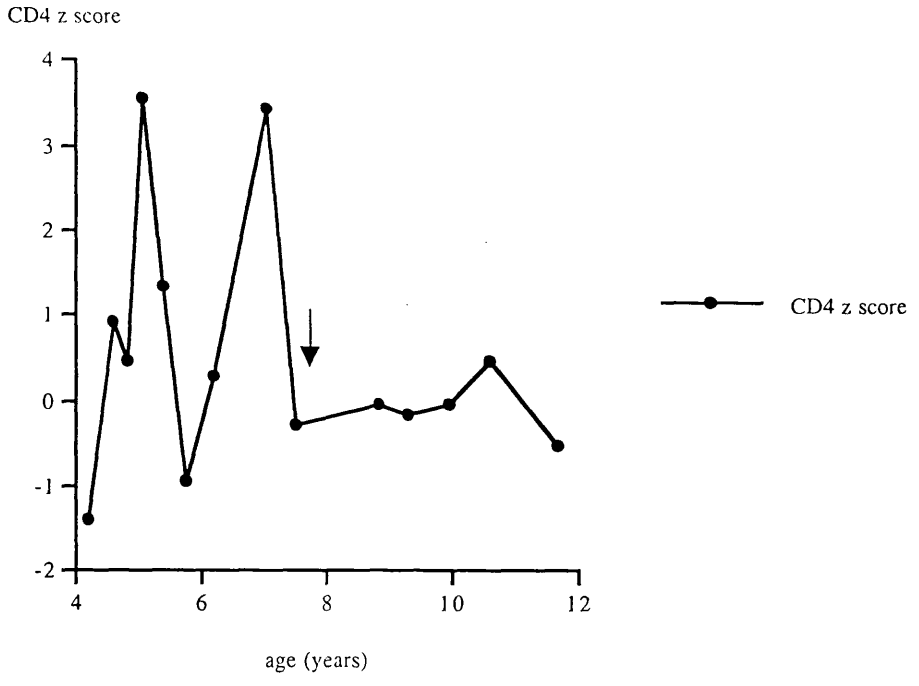
graph 7.1.4 patient 4: Change in CD4% and CD8% z score with age
arrows indicate when assay first performed with FACSCAN method

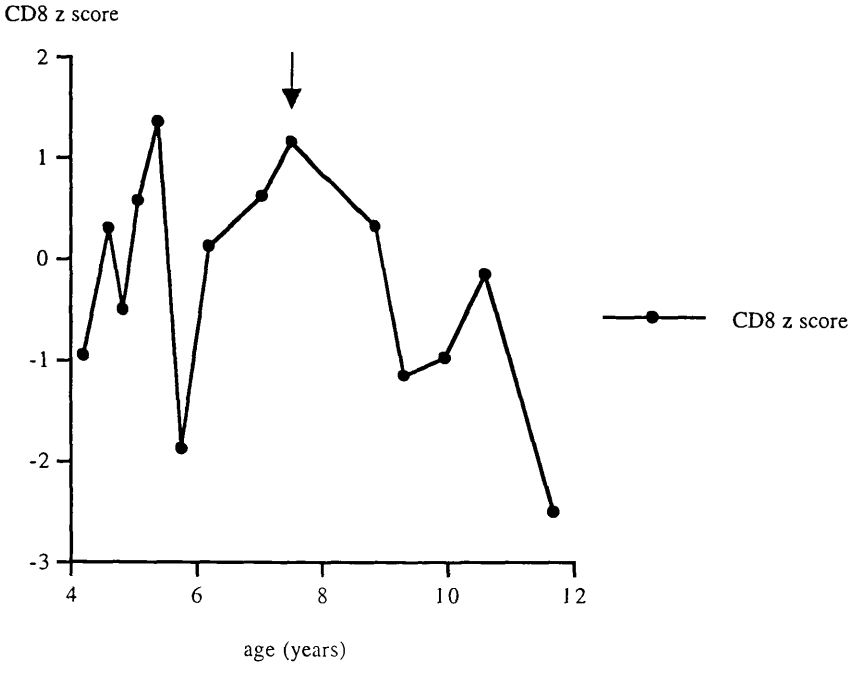


graph 7.1.5 patient 5: Change in CD4% and CD8% z score with age
arrows indicate when assays first performed with FACSCAN method

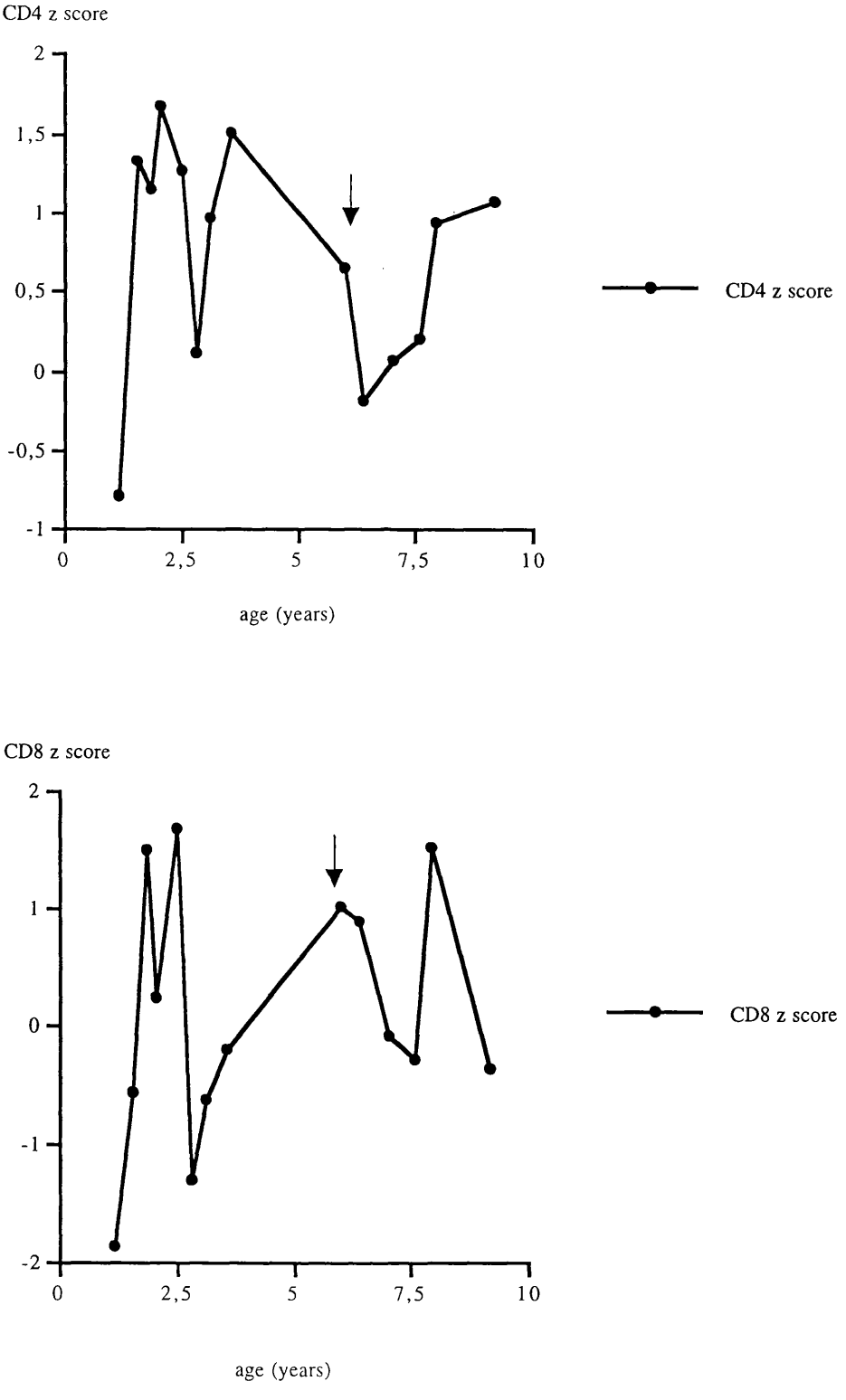


graph 7.1.6 patient 6: Change in CD4% and CD8% z score with age
arrows indicate when assays first performed with FACSCAN method

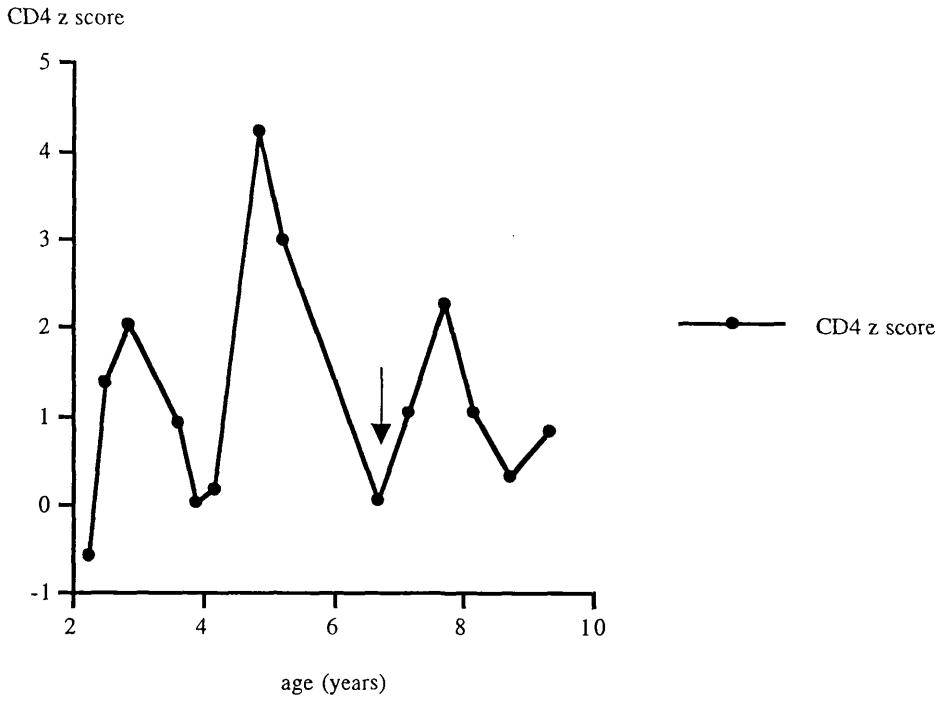


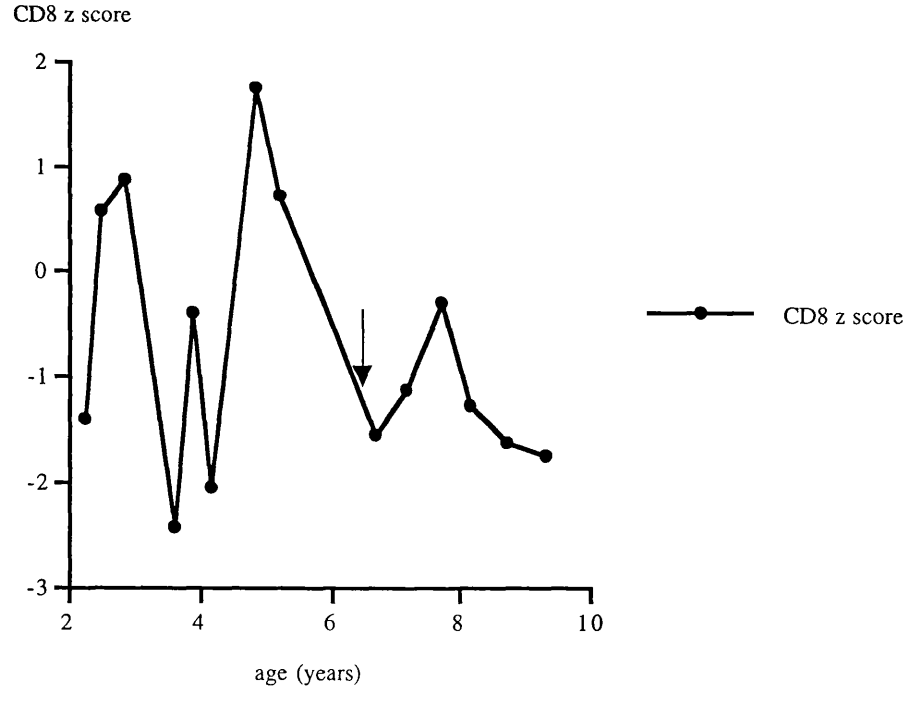


graph 7.1.7 patient 7: Change in CD4% and CD8% z score with age
arrows indicate when assays first performed with FACSCAN method

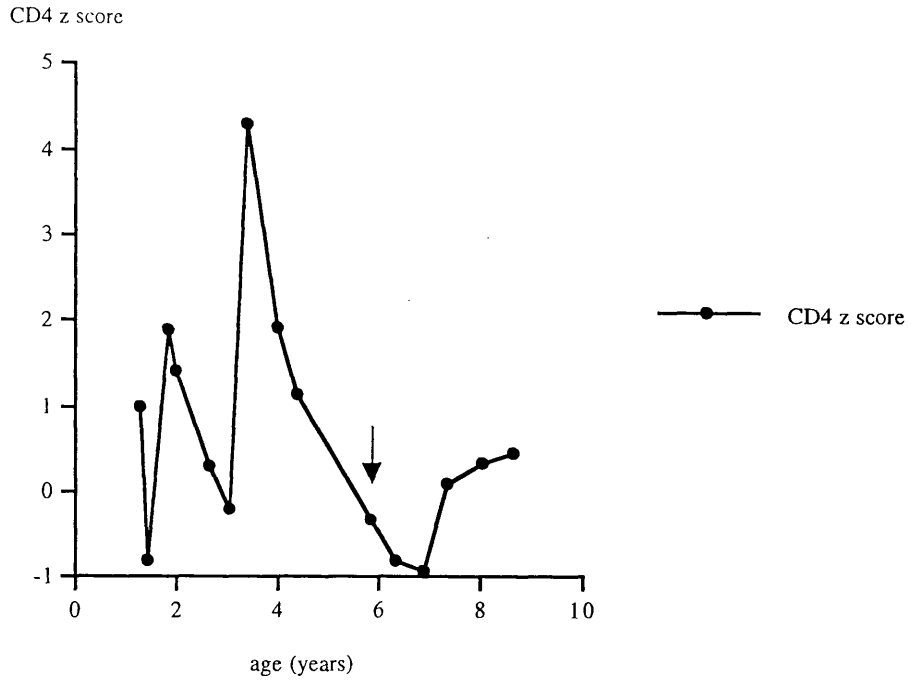


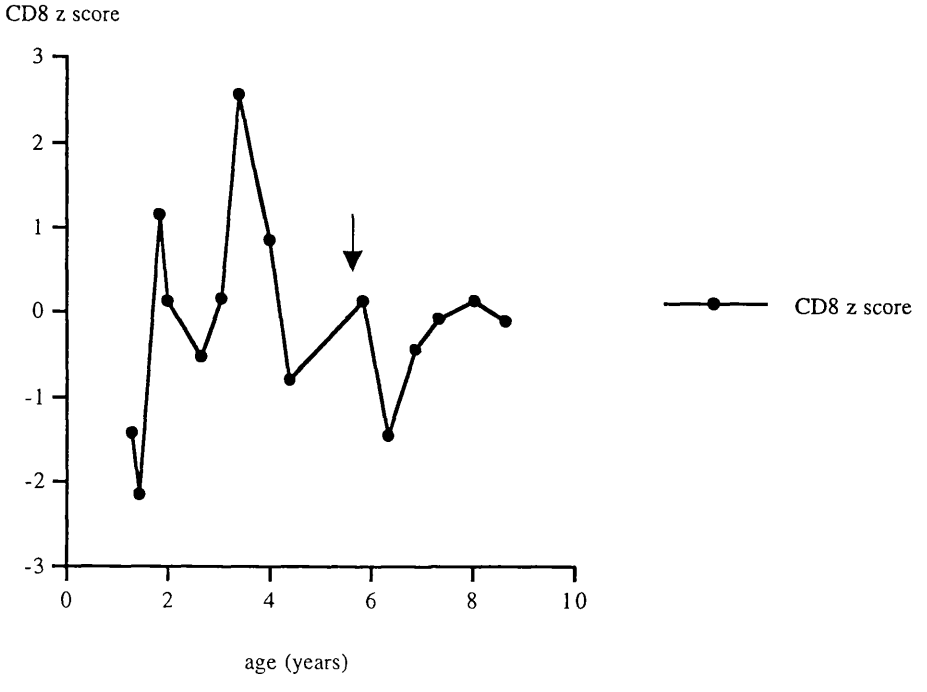
graph 7.1.8 patient 8: Change in CD4% and CD8% z score with age
arrows indicate when assays first performed by FACSCAN method



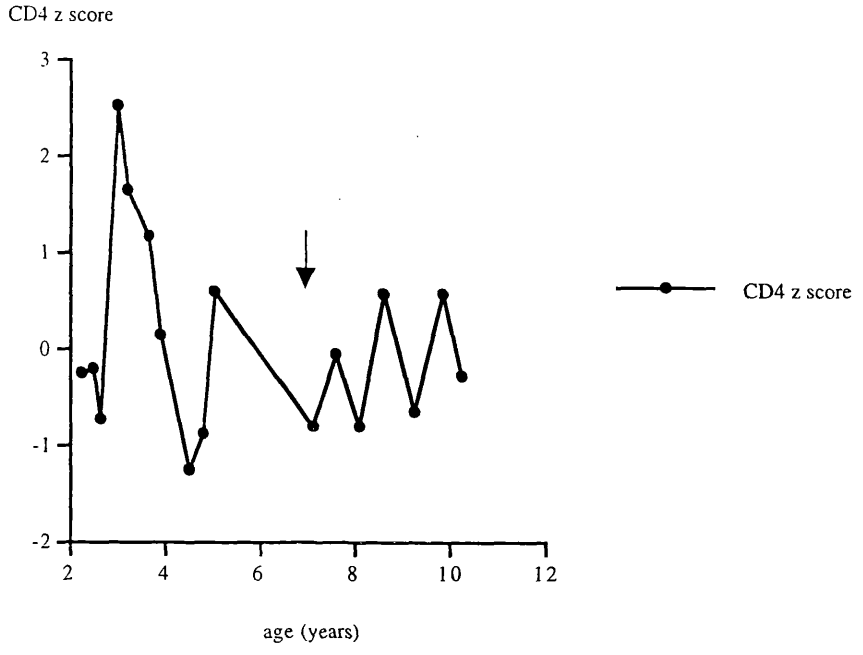


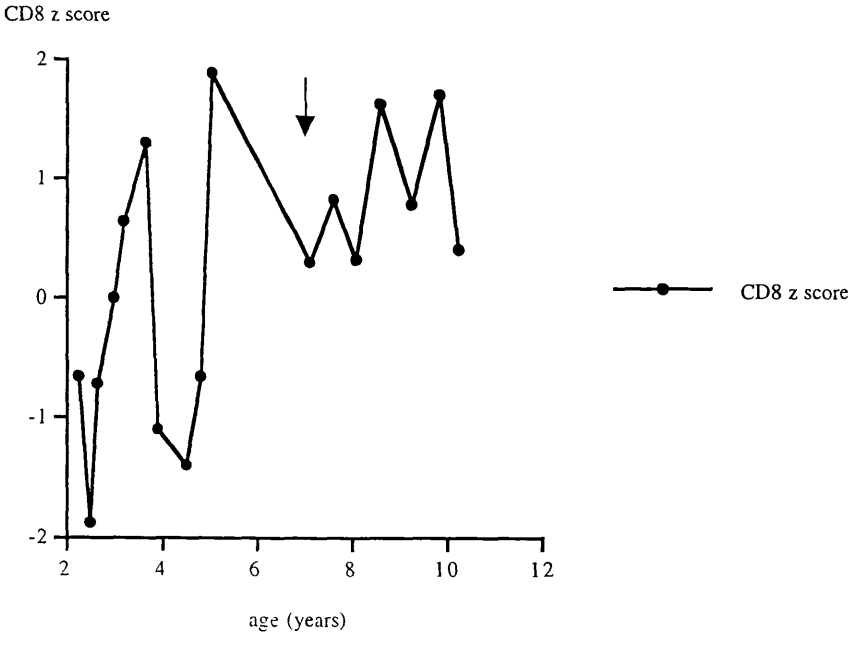
graph 7.1.10 patient 10: Change in CD4% and CD8% z score with age
arrows indicate when assay first performed by FACSCAN method



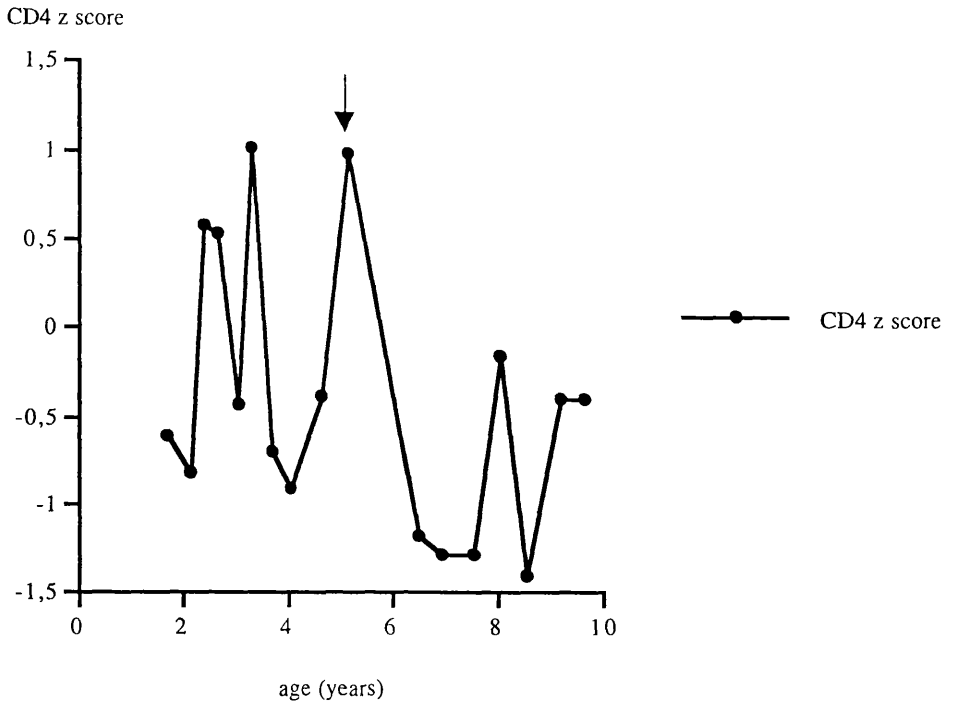


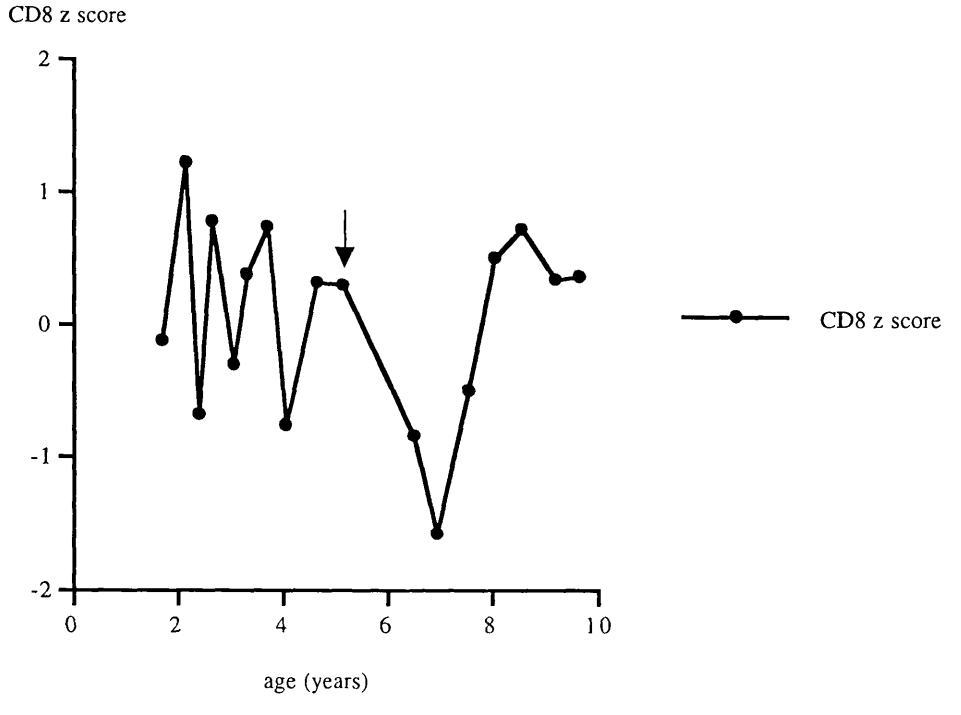
graph 7.1.11 patient 11: Change in CD4% and CD8% z score with age
arrows indicate when assay first performed with FACSCAN method



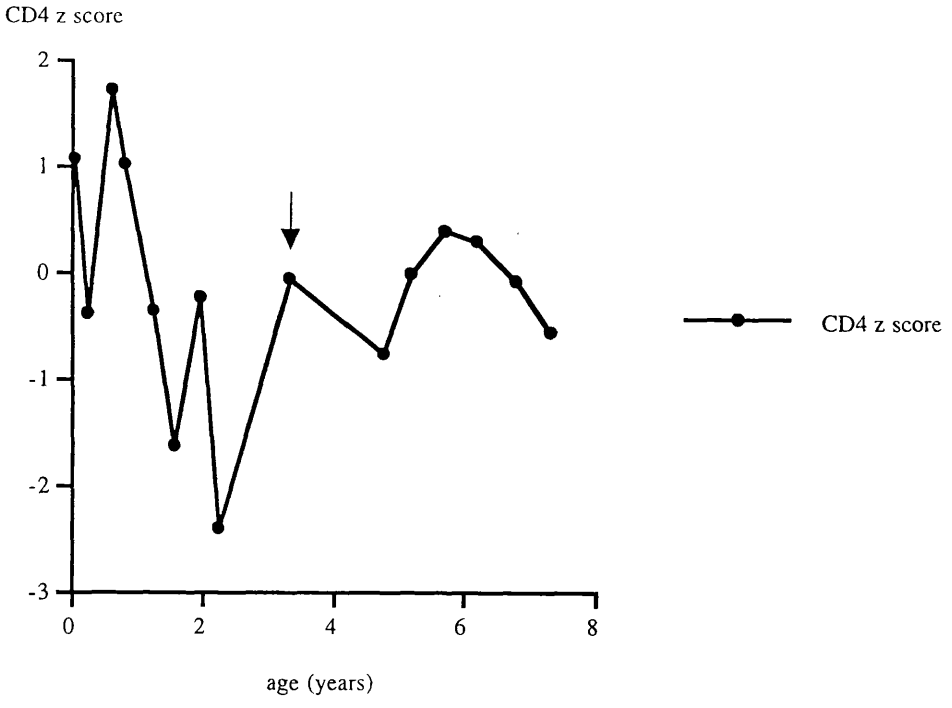


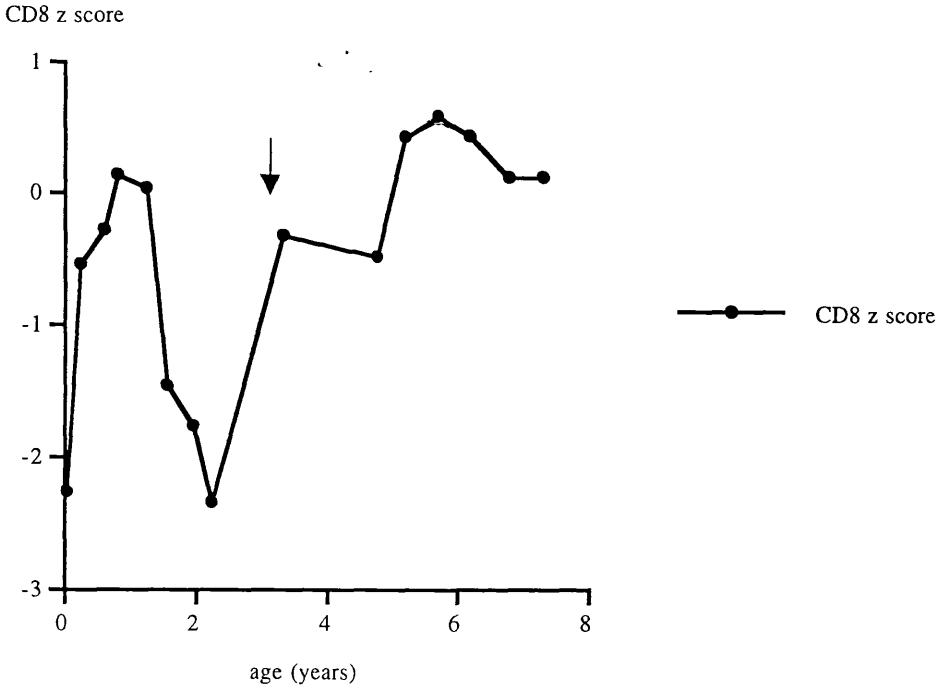
graph 7.1.12 patient 12: Change in CD4% and CD8% z score with age
arrows indicate when assay first performed with FACSCAN method



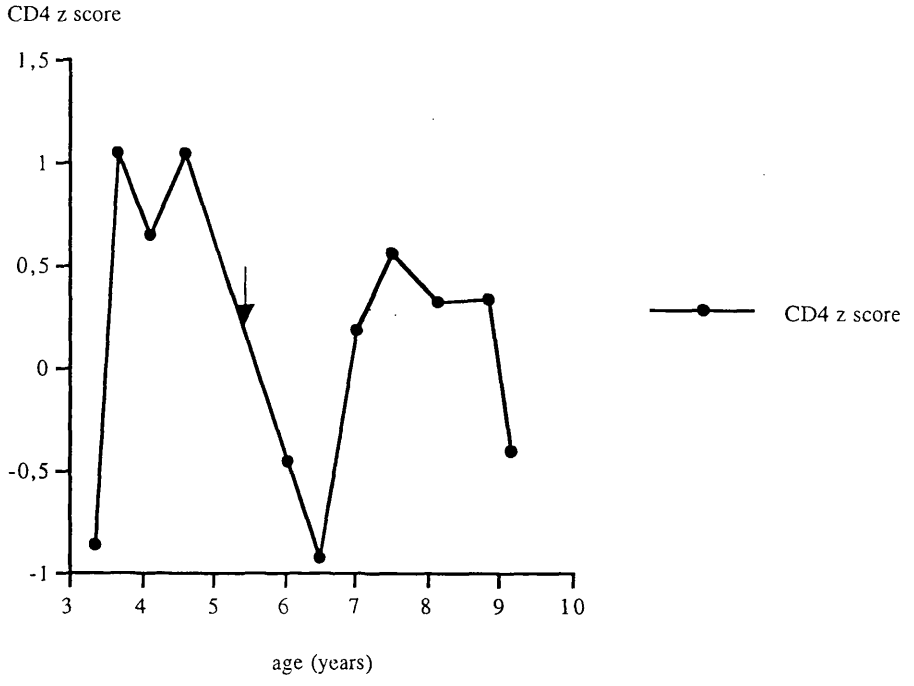


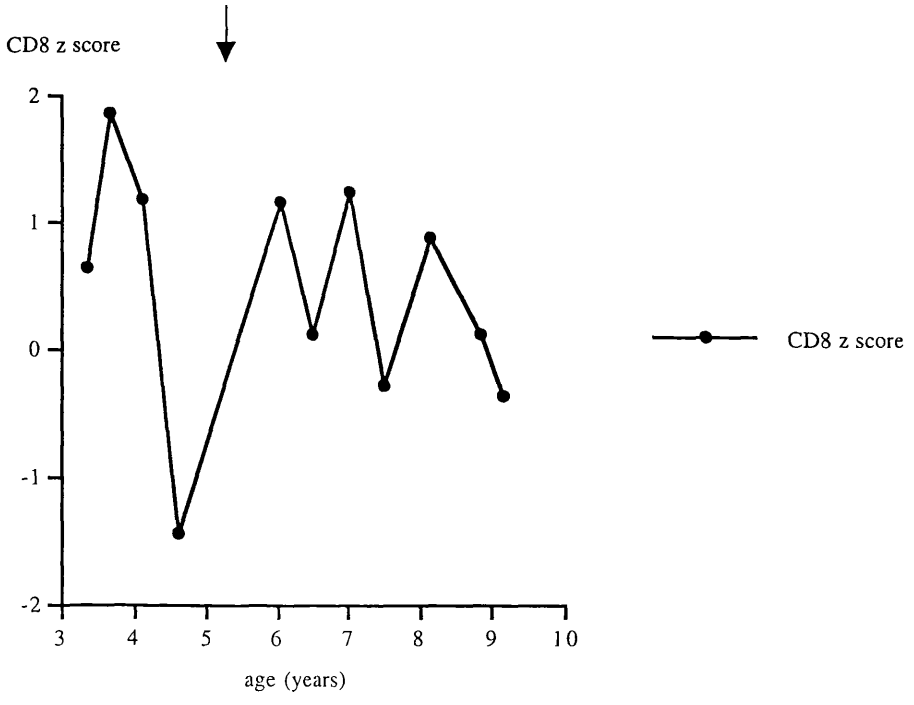
graph 7.1.14 patient 14: Change in CD4% and CD8% z score with age
arrows indicate when assay first performed with FACSCAN method



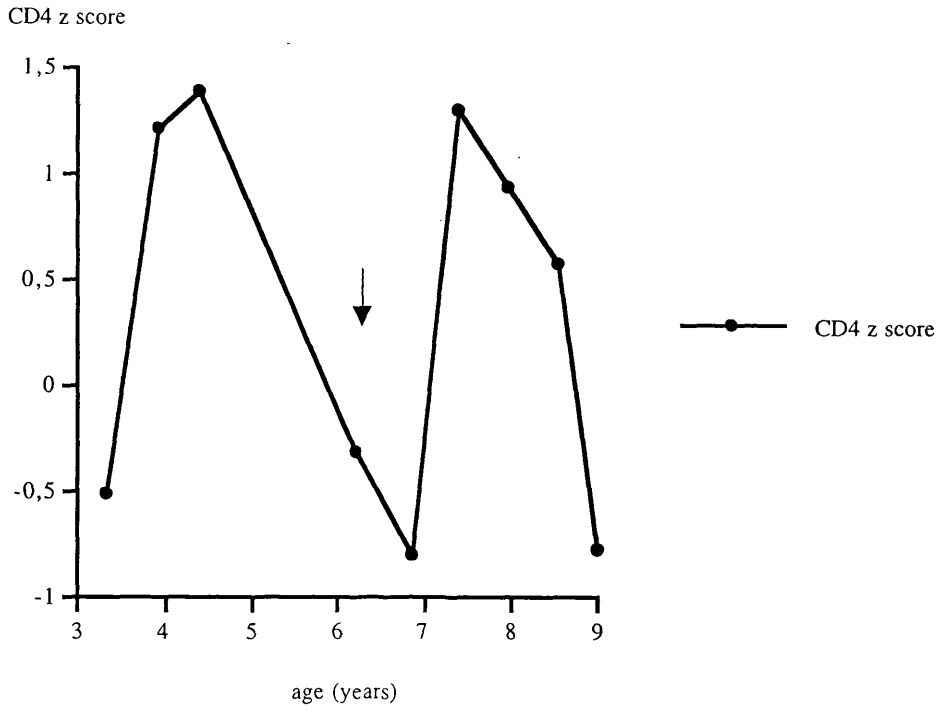


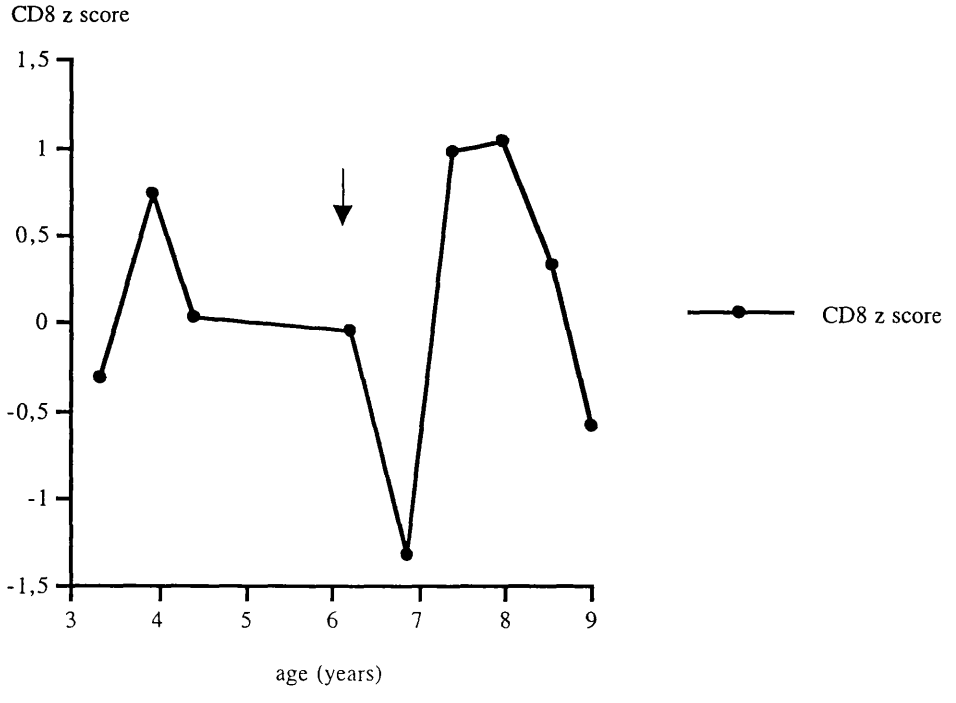
graph 7.1.15 patient 15: Change in CD4% and CD8% z score with age
arrows indicate when assay first performed with FACSCAN method



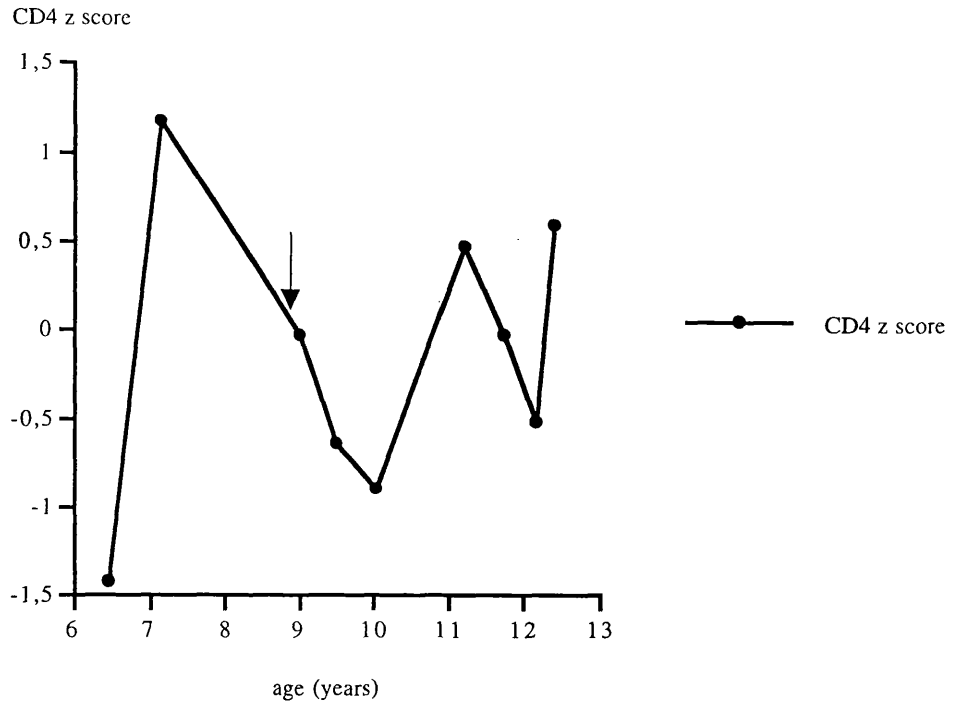


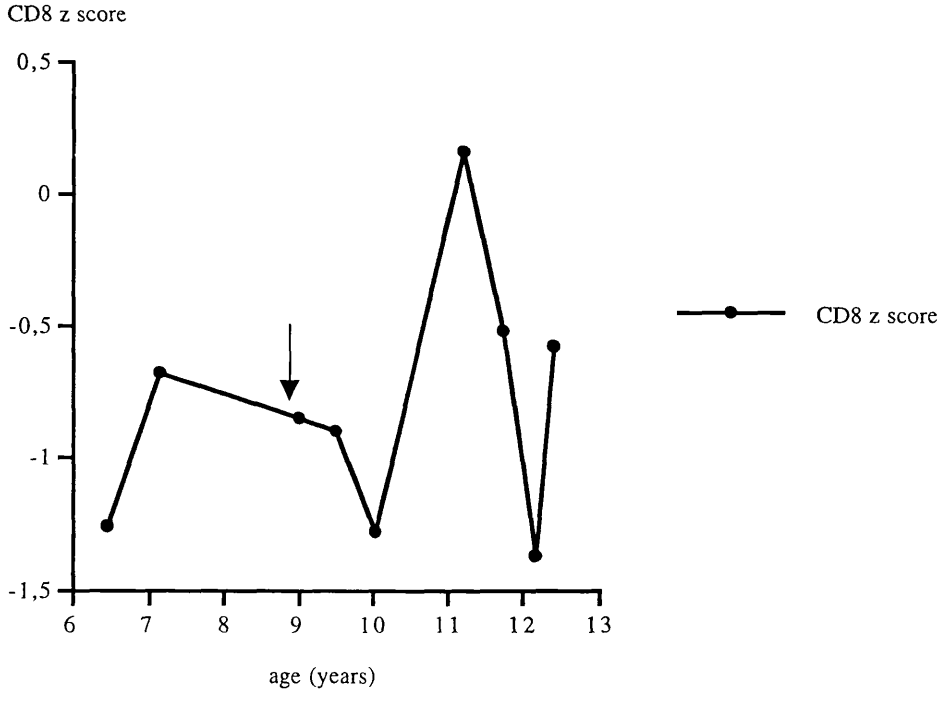
graph 7.1.17 patient 17: Change in CD4% and CD8% z score with age
arrows indicate when assays first performed with FACSCAN method



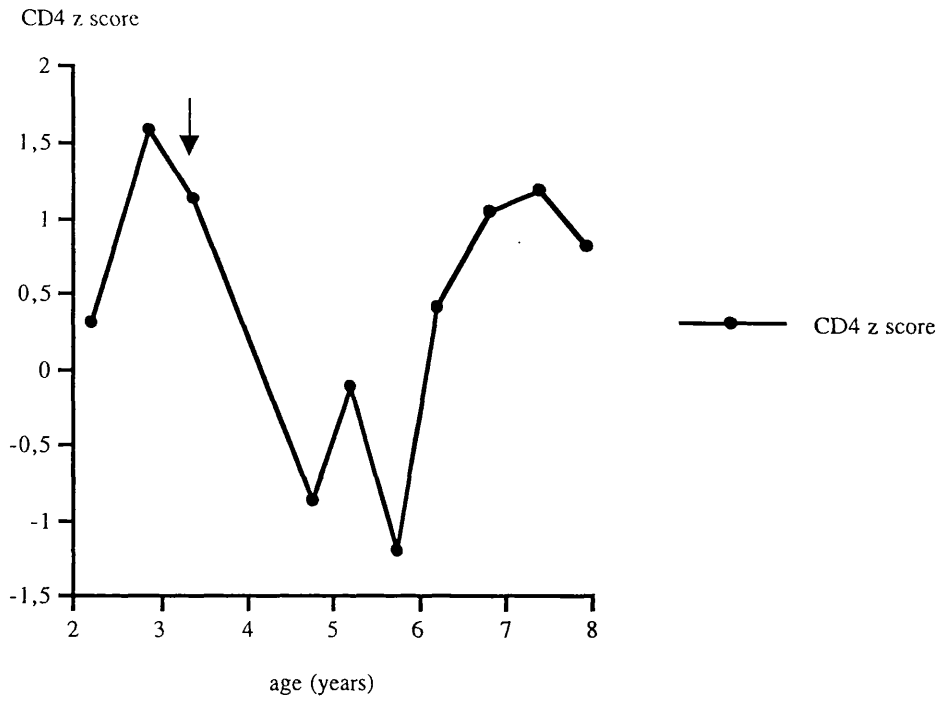


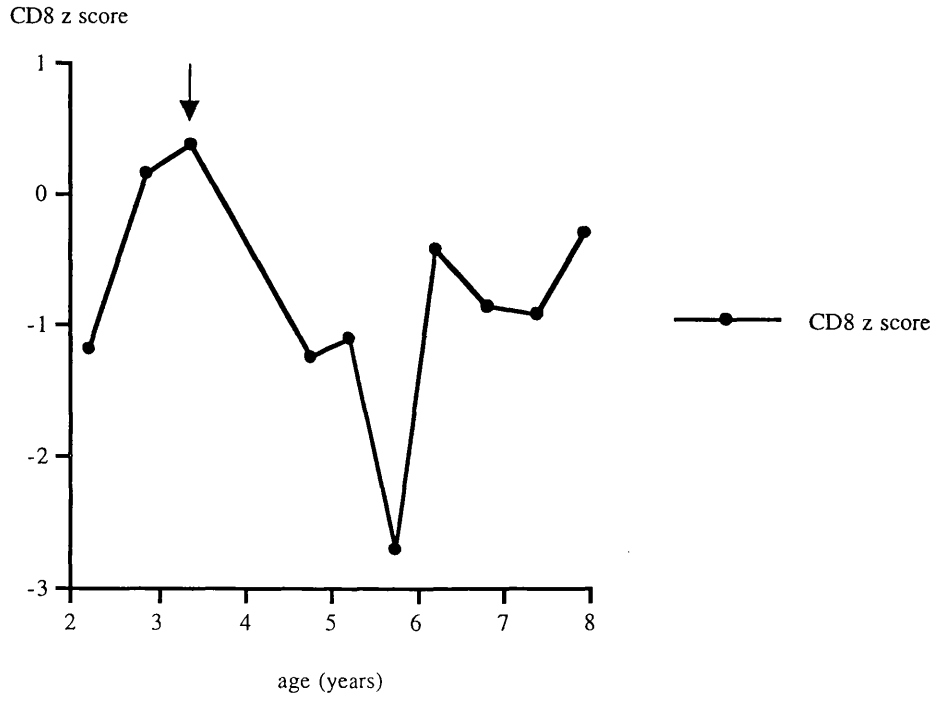
graph 7.1.18 patient 18: Change in CD4% and CD8% z score with age
arrows indicate when assays first performed with FACSCAN method



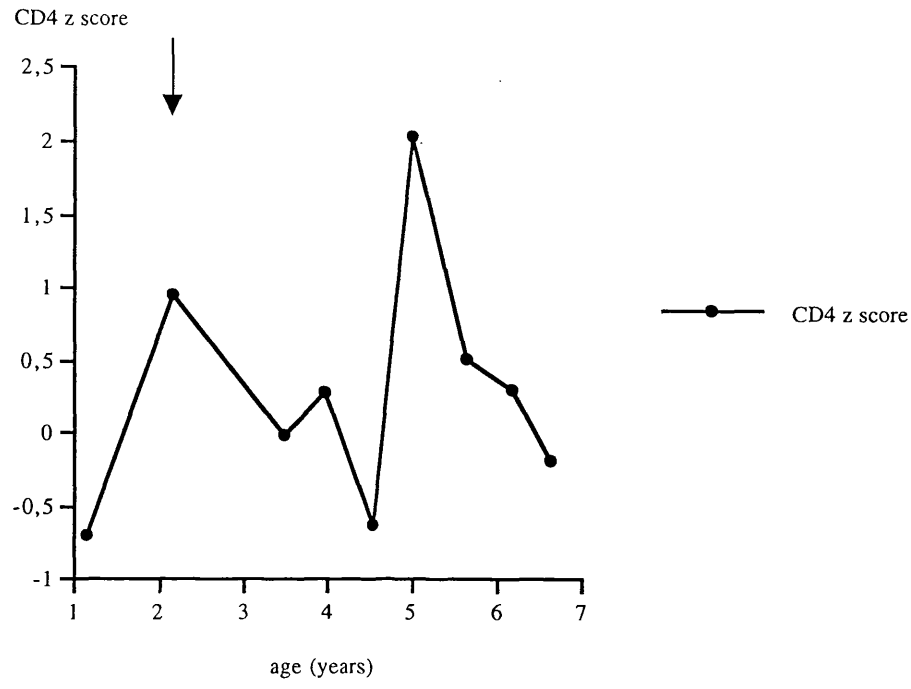


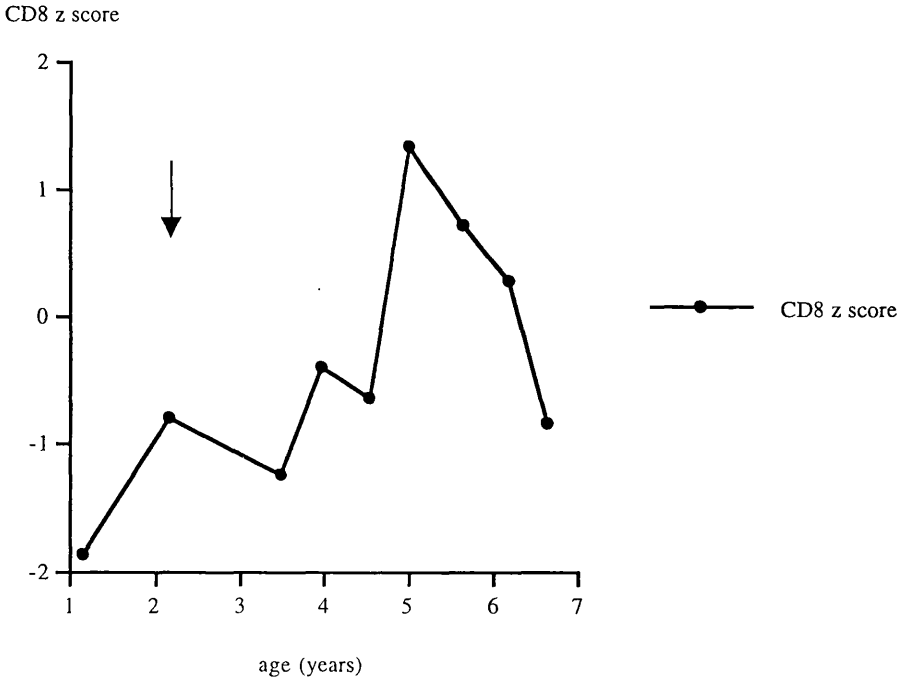
graph 7.1.20 patient 20: Change in CD4% and CD8% z score with age
arrows indicate when assays first performed with FACSCAN method



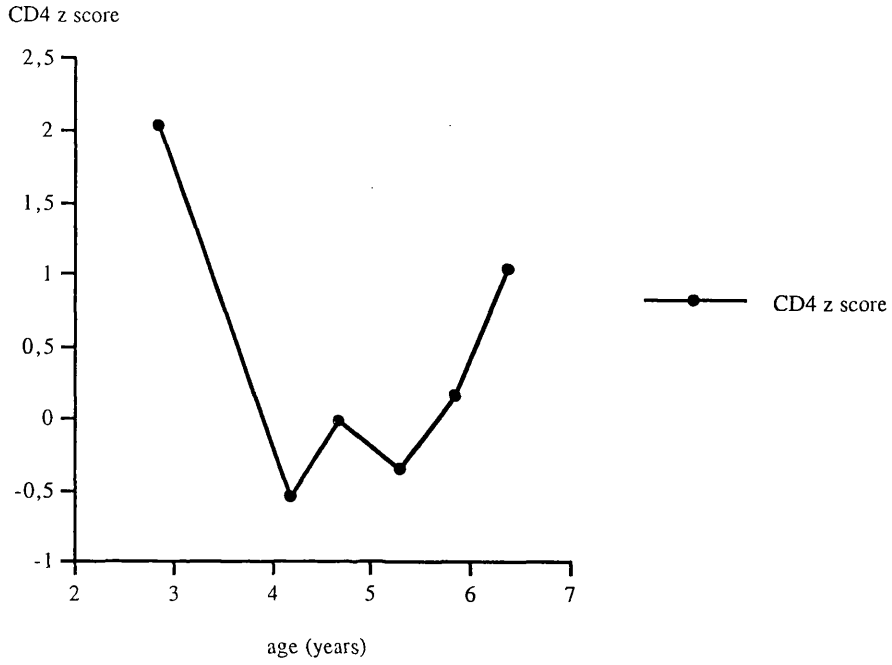


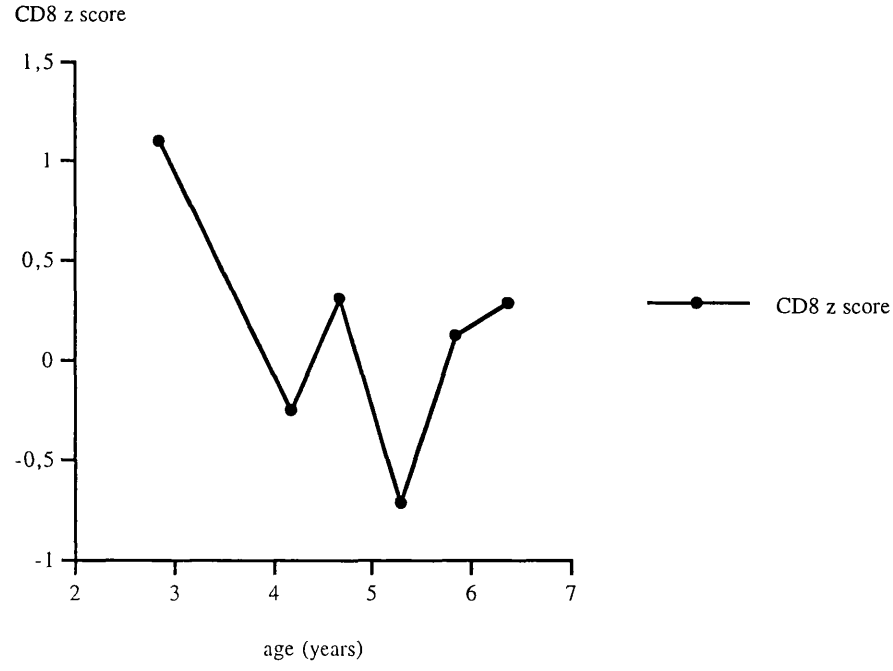
graph 7.1.21 patient 21: Change in CD4% and CD8% z score with age
arrows indicate when assays performed first with FACSCAN method



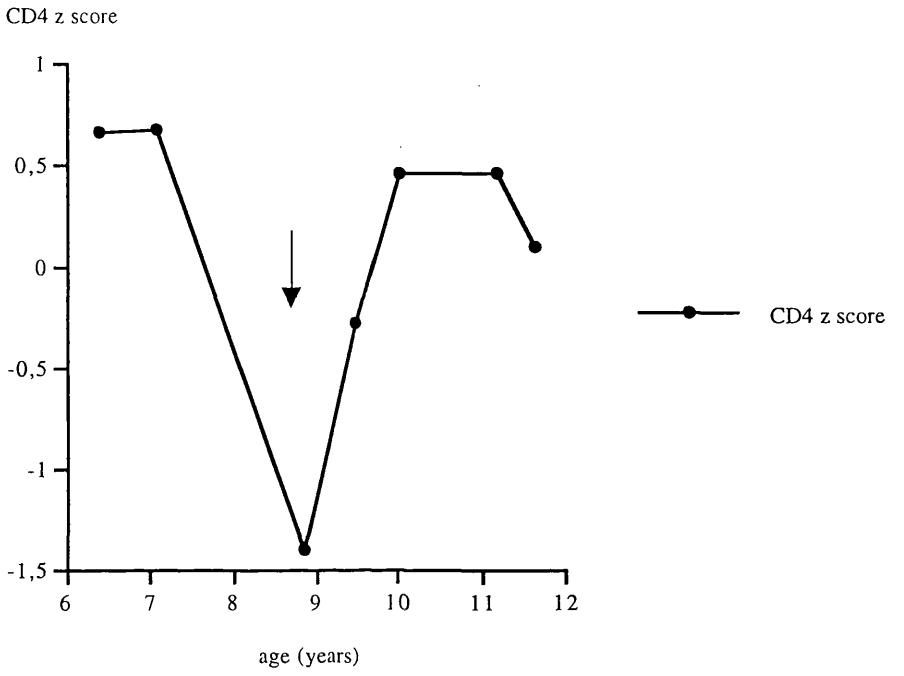


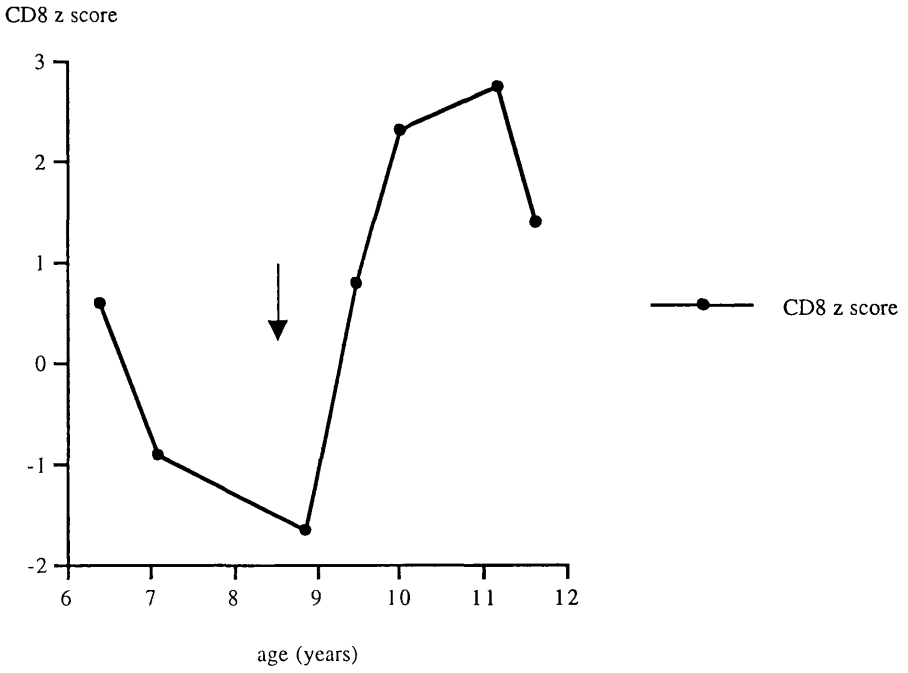
graph 7.1.22 patient 22: Change in CD4% and CD8% z score with age
arrows indicate when assays performed first by FACSCAN method



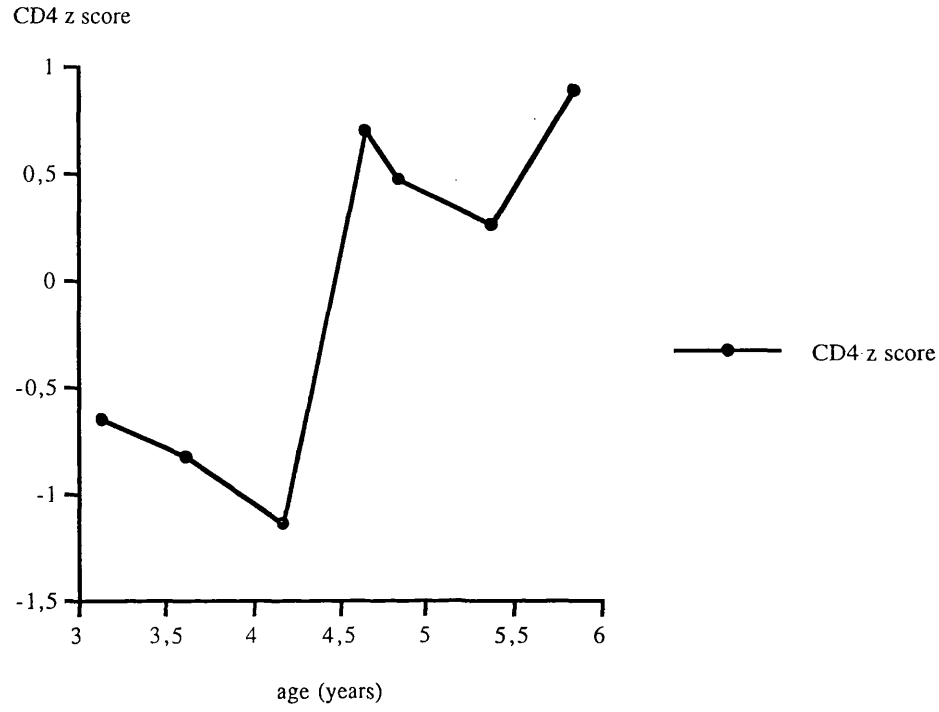


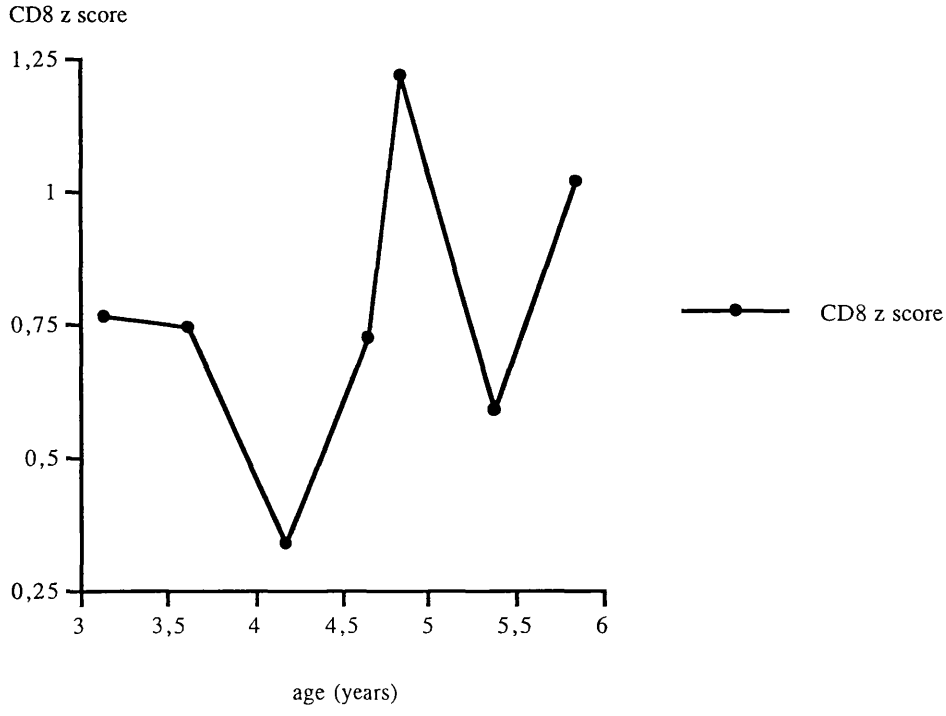
graph 7.1.23 patient 23: Change in CD4% and CD8% z score with age
all assays performed using FACSCAN method





graph 7.1.24 patient 24: Change in CD4% and CD8% z score with age
arrows indicate when assay first performed with FACSCAN method





graph 7.1.25 patient 25: Change in CD4% and CD8% z score with age
all assays performed using FACSCAN method

patient	CD4 and CD8 percentage Z scores measured against time		
		Regression coefficient (r)	P value
1	CD4	0.018	0.95
	CD8	**-0.599**	**0.02**
2	CD4	-0.348	0.2
	CD8	-0.24	0.39
3	CD4	-0.319	0.27
	CD8	**-0.548**	**0.04**
4	CD4	-0.332	0.23
	CD8	**-0.6**	**0.018**
5	CD4	**-0.58**	**0.03**
	CD8	+0.337	0.24
6	CD4	0.001	0.99
	CD8	-0.281	0.31
7	CD4	-0.222	0.45
	CD8	-0.384	0.18
8	CD4	-0.158	0.59
	CD8	+0.231	0.43
10	CD4	0.013	0.96
	CD8	-0.315	0.27
11	CD4	-0.332	0.23
	CD8	0.037	0.89
12	CD4	-0.232	0.37
	CD8	**+0.567**	**0.017**
14	CD4	-0.317	0.25
	CD8	0.1	0.72
15	CD4	-0.168	0.57
	CD8	**+0.589**	**0.027**
17	CD4	-0.162	0.64
	CD8	-0.29	0.38
18	CD4	-0.162	0.68
	CD8	0.001	0.95
20	CD4	+0.203	0.59
	CD8	+0.282	0.46
21	CD4	0.012	0.97
	CD8	-0.175	0.63
22	CD4	+0.178	0.65
	CD8	**+0.621**	**0.07**
23	CD4	-0.35	0.49
	CD8	-0.48	0.34
24	CD4	-0.155	0.74
	CD8	+0.615	0.14
25	CD4	**+0.793**	**0.03**
	CD8	+0.302	0.51

Table 7.2 All patients; CD4 and CD8 % z scores against age – regression coefficients and p values
Significant values are in bold

Relationship of serial T lymphocyte subsets to FVIII treatment received

Total treatment received at the time of each assay and the treatment received (expressed in units FVIII / kg body weight) in the week, month and three months prior to the assay are shown in appendices 7.3.1 to 7.3.25.

Multiple regression analysis was used to investigate the effect of treatment on the serial CD4 and CD8 z scores of each individual patient. Five independent variables were included in the analysis, total FVIII treatment received at the time of the assay (units FVIII), time since the first treatment episode, and treatment received in the week, one month and three months prior to the assay (units per kg FVIII). Nineteen of the twenty one patients were included in the analysis. Patients 20 and 23 were excluded because they had received insufficient treatment over the study period resulting in insufficient data to be included in the model.

Firstly, considering those patients whose CD4 and CD8 z scores were demonstrated to change significantly over time.

Patient 5 had a decreasing CD4 z score but on multiple regression these scores showed no significant correlation with either, total cumulative treatment received, treatment received in the previous week, month or three months or to the time since treatment began.

Of those three patients who had decreasing CD8 z scores, the scores of patients 1 and 3 showed no significant correlation on regression against the five variables, whereas in the case of patient 4, treatment received in the previous month ($p=0.02$) and three months ($p=0.05$) showed significant correlation. However, when the relationship of each of these two variables was investigated using simple regression neither was found to be significant. (Graph 7.2.1)

Of the three patients who had increasing CD8 z scores, the scores of two (patients 12 and 22) showed no significant correlation on multiple regression against the five variables. The CD8 scores of the third patient (15) showed however significant correlation with FVIII treatment received in the month ($p=0.09$) and three months

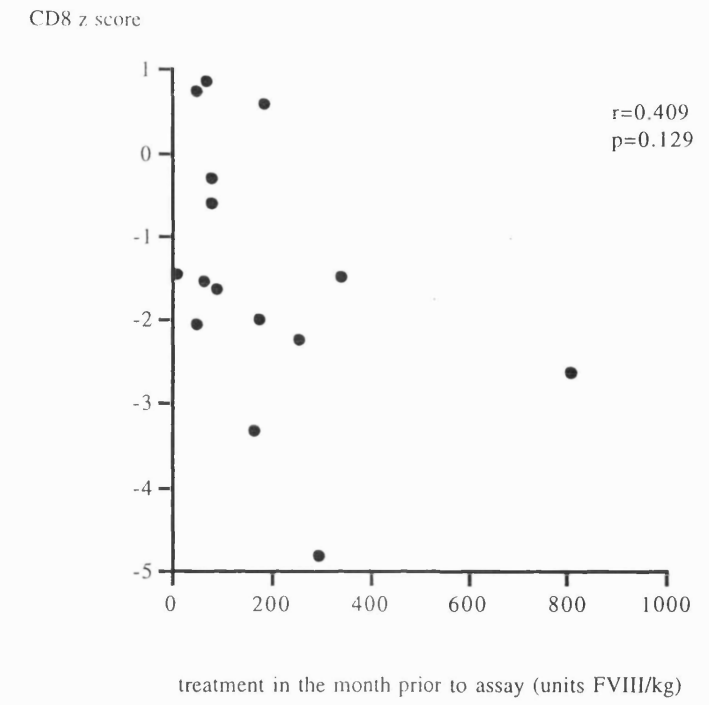
(p=0.09) prior to the assay. The association was also significant when each variable was plotted against CD8 z scores on simple regression. (Graph 7.2.2)

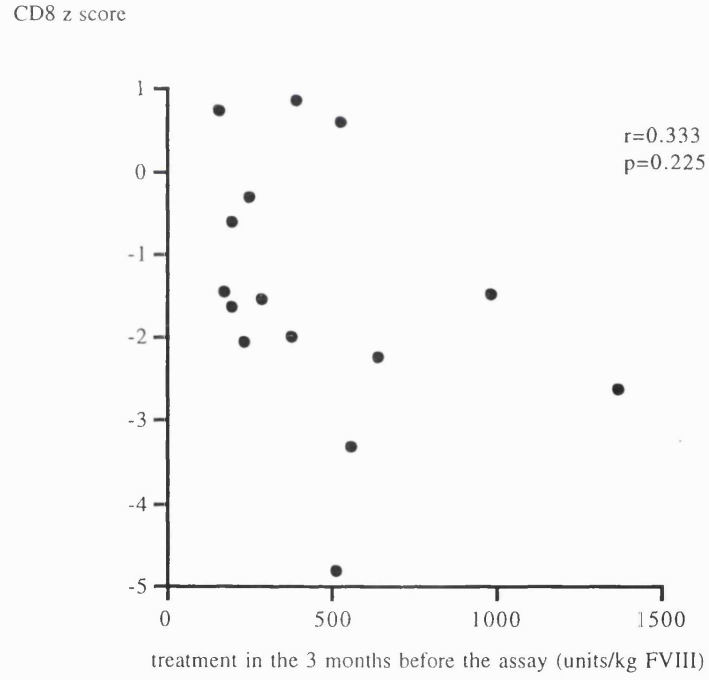
Of the other patients investigated, significant correlations between either CD4 or CD8 z scores and the five treatment variables were found in only two cases.

The CD4 and CD8 z scores of patient 6 did not change significantly over time but on multiple regression CD8 was found to be associated with the total treatment received (p=0.04) and to the time since treatment started (p=0.03). Neither of these associations were found to be significant using simple regression (graphs 7.2.3).

The CD4 z scores of only one patient (patient 11) were shown to be correlated on multiple regression to the FVIII treatment received in the week (p=0.01), one month (p=0.02) and three months (0.03) prior to the assay. Using simple regression only the treatment received in the previous three months was found to be associated (graph 7.2.4)

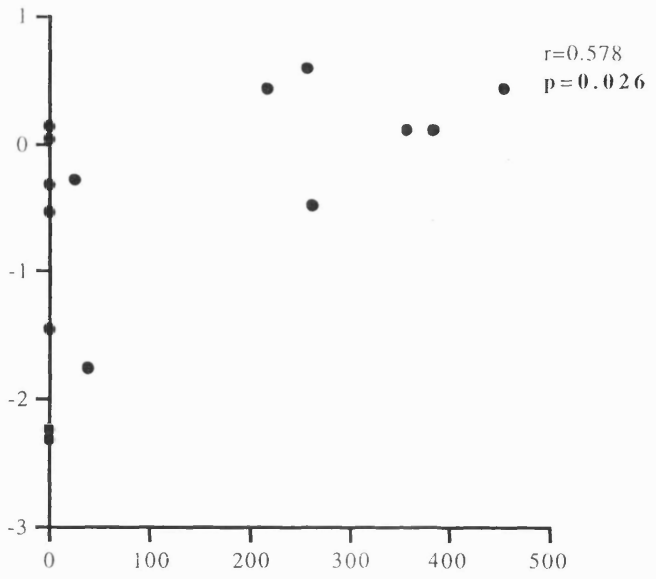
A summary of the T cell subset analysis of the patients investigated as individuals is shown in table 7.3.





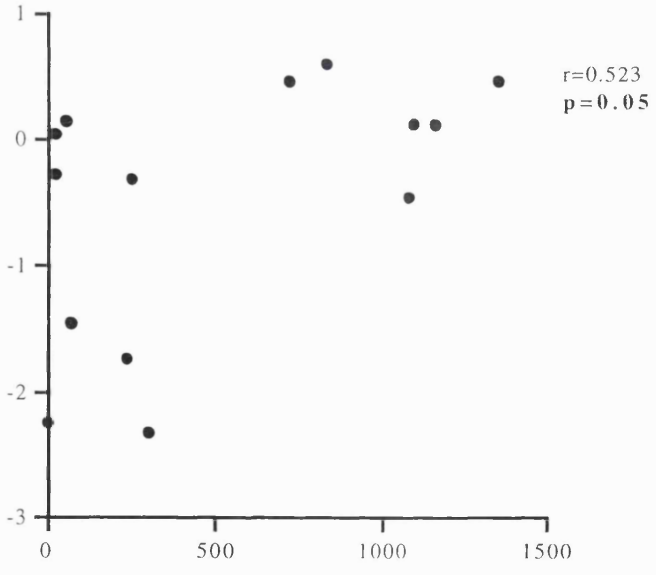
graph 7.2.1 patient 4
Plot of CD8 z score against factor FVIII treatment received in the month before (top) and three months before the assay, showing no significant correlation

CD8 z score



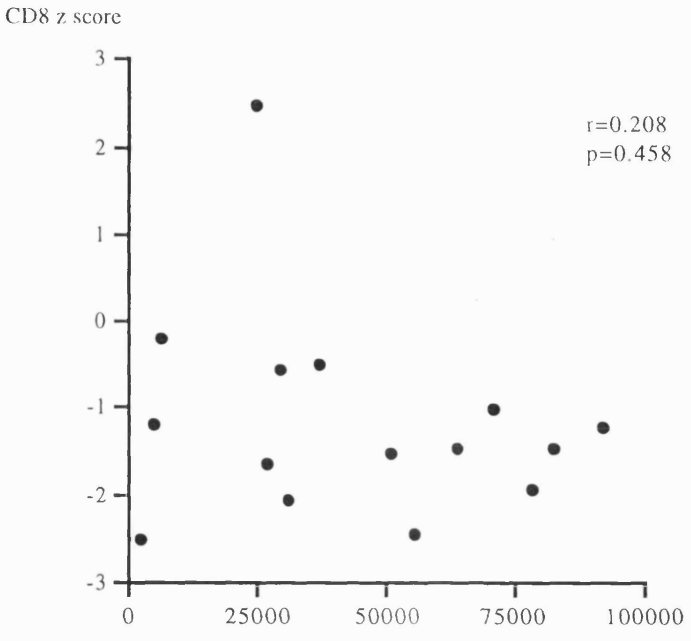
treatment (units FVIII/kg) in the month befroe the assay

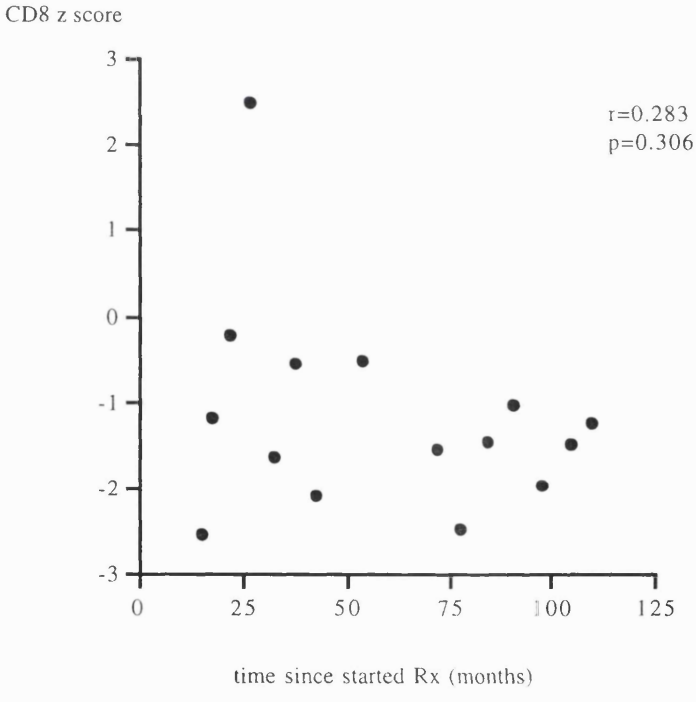
CD8 z score



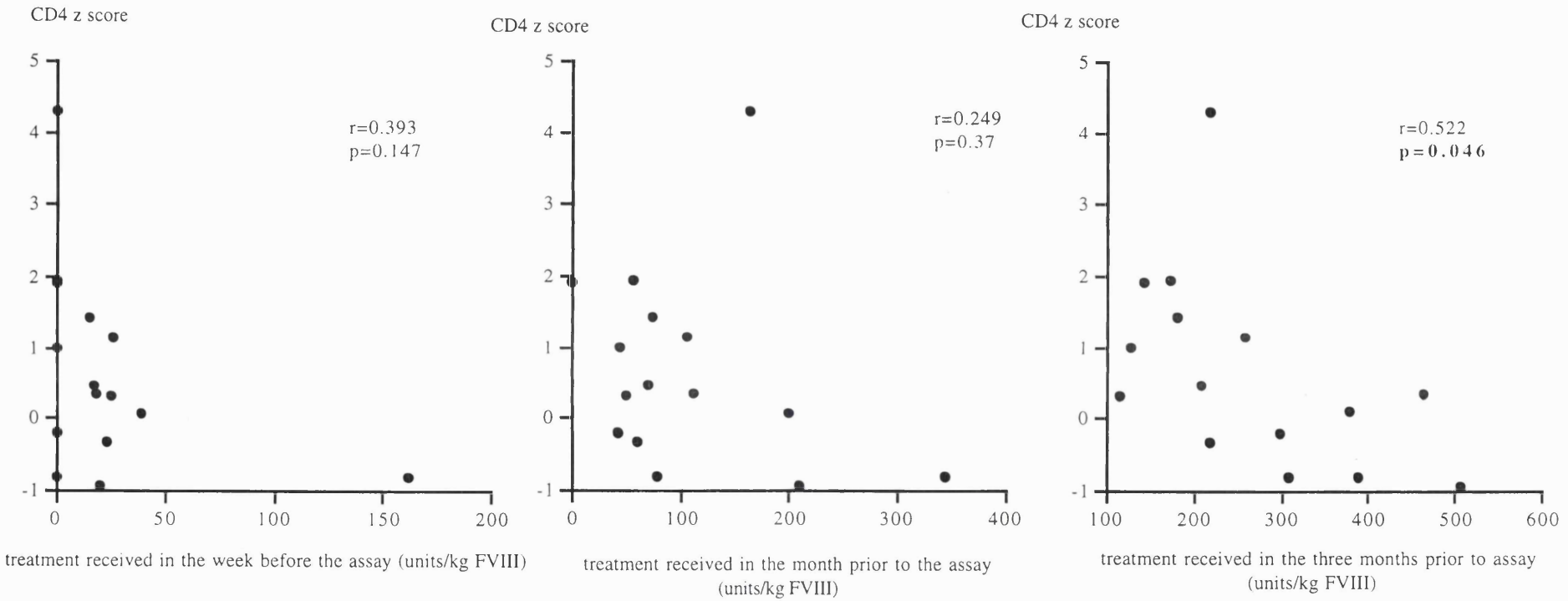
treatment received in the 3 months before the assay

graph 7.2.2 patient 15
Plot of CD8 z score against factor VIII treatment showing significant correlation between treatment received in the month (top) and three months prior to the assay





graph 7.2.3 patient 6
Plot of CD8 z score against total cumulative treatment received (top) and time since treatment started showing no significant correlation



graph 7.2.4 patient 11
Relationship of CD4 z score to FVIII treatment received in the week, month and three months prior to the assay, showing only a significant correlation between decreasing CD4 z score and the amount of treatment received in the previous three months

Patient	CD4 z scores	CD8 z scores	Multiple regression; relationship of CD4 & CD8 to treatment variables	Confirmation of significant correlation between CD4 and CD8 z scores and treatment variables using simple regression
5	Decreasing (p=0.03)		No significant correlation	
1		Decreasing (p=0.02)	No significant correlation	
3		Decreasing (p=0.04)	No significant correlation	
4		Decreasing (p=0.018)	CD8 z scores related to: FVIII received in the previous one month (p=0.02) FVIII received in the previous three months(p=0.05)	No significant correlation between either variable and the CD8 z scores
12		Increasing (p=0.017) within the normal range	No significant correlation	
15		Increasing (p=0.027) within the normal range	CD8 z scores related to: FVIII received in the previous one month (p=0.09) FVIII received in the previous three months(p=0.09)	Both variables have significant correlation with CD8 z scores One month p=0.02 Three months p=0.09
22		Increasing (p=0.07) within the normal range	No significant correlation	
6			CD8 z scores related to: total treatment received (p=0.04) time since first treatment (p=0.03)	No significant correlation between either variable and CD8 z scores
11			CD4 z scores related to: FVIII received in the week (p=0.01), one month (p=0.02) and three months (p=0.03) prior to the assays	Significant correlation only between CD4 z scores and FVIII received in the three months before the assay (p=0.04)

Table 7.3 Summary of T cell subset analysis

Discussion

In this study of 21 patients studied over a period of up to ten years after the first treatment episode there are no clear changes in CD4 or CD8 counts occurring over time. The CD4 counts of 20 individuals are stable and all but 4 individual CD4 measurements (from a total of 258 assays) are within or above the normal range. The CD8 counts show more changes but three patients have increasing counts and three decreasing. There is therefore no clear pattern of changes within this group and they are, as a group not demonstrating the previously described changes in CD4 and CD8 counts seen in HIV seronegative haemophiliacs (Sullivan et al 1986, Shannon et al 1986b, Cuthbert et al 1992).

There are however a number of criticisms to be made about this study.

Of the original group of 25 patients in the viral safety study 21 were included in the T cell subset assay. Patient 19 was excluded because he moved away, however the exclusion of patients 9, 13 and 16 was unfortunate. They were three of the four mild haemophiliacs in the study and were excluded from the analysis because they lived a distance away and did not come to the Regional Haemophilia Centre on a regular basis. It would be important to have studied them because they all received very little treatment and their patterns of serial T cell subsets might have provided important information, providing a small 'internal control' group.

A second criticism on this point is the lack of a control group. The calculation of z scores is based on serial T cell subset values from a cohort of healthy children. It was stated in the publication of this data that as children grow older they do not track a particular centile line and 'within child' variation is seen (European Collaborative Study 1992). However there is a lot of variation seen in the individuals studied here and the use of a control group of age matched children followed over a similar time period would have provided useful information as to whether this degree of variation is always seen. As always in paediatric studies it is difficult to obtain samples from healthy children particularly on a regular basis. Another useful control group would have been the haemophiliacs who remain free of HIV but have been treated with a

variety of concentrates and have contracted hepatitis. There was no long term serial data available on these patients but this would have been extremely valuable. The lack of this information underlines the importance of collecting the data now on the group of BPL 8Y treated patients. Whatever the results show, if the data is well collected this group can serve as a control for future groups of patients perhaps treated in different ways. In addition to the use of control groups it would have been preferable to have studied the patients from the start of treatment on a regular basis, for example at exactly four monthly intervals. This would have made comparisons between individuals and controls easier. In some cases there are long intervals of more than one year between assays.

A problem in this study of serial data is that in 1991 the assay for the T cell counts was changed from a manual method to using an automated FACSCAN. Such a major change in method during a study of serial values is obviously far from ideal. The manual method was probably more prone to laboratory error and the production of extreme results and overall less reliable. However, some extreme results were also seen with the automated method. Dividing the data and looking at the results from the two periods separately, only one individual had any significant change in CD4 or CD8 over time using simple regression. However the time periods covered were probably too short to draw any major conclusions from this. One individual (15) had decreasing CD4 z scores during the manual period and subsequently completely stable scores during the latter half of the study. No other patient showed such dramatic differences between the two halves of the study which does increase confidence in looking at the data together. Looking at some individuals the results seem to show less fluctuation during the FACSCAN period (for example individuals 3, 12 and 15) however this is not a general observation, the converse is true in cases 8 and 18. Ideally the study should have used the same method throughout and would have done so if the FACSCAN had been available earlier. The decision to change was viewed in the long term, eventually a series of data acquired in a standardised way would be built up.

The use of z scores was a novel way of investigating haemophiliac children. Although normal ranges for the percentage and total count of CD4 and CD8 lymphocytes had been established in adults, in children these values are very much age dependant, increasing in the first year of life and then gradually falling towards adult values. Therefore, when prospectively following a group of children, normal 'age-related' changes need to be accounted for. In this study this was done by calculating the z score of each individual CD4 and CD8 measurement, the z score being the measure of how different an individual is from the average of all children of the same age, the standard deviation. Scores larger than plus or minus 1.88 occur outside the 3rd or the 97th centile, and would therefore be said to be outside the normal range for a child of that age.(The European Collaborative Study 1992) By examining serial z scores it was possible to determine whether there was a progressive change in CD4 and CD8 counts, occurring independent of age. Absolute lymphocyte counts during childhood vary widely and therefore the percentage of CD4 and CD8 cells were used in the prospective follow up.

Several of the patients have at least one extreme value outside of the normal range, possibly occurring by chance or as a result of laboratory error, or due to other external influences, for example viral infections which are known to increase CD8 levels.

These extreme values may strongly influence the analysis when looking for changes over time and must be borne in mind when drawing conclusions. The assessment of the relationship of treatment given to the z scores might have shown whether the extreme values were due to unusual treatment situations but the results of the analysis did not support this.

Looking more closely at the patients who had changes in the CD4 and CD8 counts over time. Only one of the 21 patients analysed had a significantly decreasing CD4 z score (patient 5). In this case 14 serial CD4 assays were done over a 95 month period starting 15 months after the patient first received FVIII treatment. Of the fourteen measurements two fell below the normal range (the tenth and eleventh in the series) but the subsequent three values, measured over a 20 month period were all in the

normal range. The CD8 z scores of this individual showed a slight but insignificant increase over time.

One patient had significantly increasing CD4 z scores during the study. This was the last patient to be recruited and had only seven assays over 32 months. All values were well within the normal range, the first three being below the mean for age and the latter four above. These four measurements taken over a two and a half year period have been stable.

In summary therefore, with one exception the CD4 counts of this population of haemophilic boys are remaining stable over a period of up to ten years.

When the CD8 z scores are considered, more patients demonstrated significant changes, however three had increasing CD8 z scores while three others had decreasing values. Looking closely at the increasing CD8 values, because this is one of the immune abnormalities previously most commonly described in groups of haemophiliacs. Two of the three patients had all values within the normal range (patients 12 and 22), whereas the third (patient 15) had two of fifteen values outside of the normal range of which one was taken on the day he was born and may be therefore difficult to interpret and the other was the seventh in the series. Of these three patients all had stable CD4 values.

In addition to three patients with increasing CD8 values, three had decreasing values, also in the presence of stable CD4 values. Patient 1 had decreasing CD8 z scores, all of which except one was in the normal range. The latter measurements of patients 3 and 4 are below the normal range. The meaning of low levels of CD8 cells in the presence of a normal total lymphocyte and CD4 count is unclear. Low CD8 cell counts have not been previously described in haemophilia but this observation should be noted particularly as it has been observed in two of the patients who have been under treatment for the longest period of time.

In order to look at the changes in the serial CD4 and CD8 values more closely the values were investigated in relation to the FVIII treatment that the patients were receiving. The patients in this study had received vastly different amounts of treatment

by the end of the study period (33,245 – 714,570 units FVIII, mean 297554). It was investigated to see whether there was any association between the change in CD4 and CD8 levels, measured in terms of the r values determined for each patient in the simple regression and the total treatment received. This would demonstrate whether, in the absence of statistically significant CD4 and CD8 changes, there was a trend towards changes in those who had received more FVIII concentrate. For CD4 there was no association, whereas in the case of CD8 there was a negative association in that higher amounts of treatment were significantly associated with more negative r values ($p=0.02$) reflecting falling CD8 levels. This effect could be accounted for by the three patients who had been longest in the study who have already been described.

The pattern and amount of treatment given before the individual assays however did not account for fluctuations in the CD4 and CD8 z count.

To assess the effect of treatment on the CD4 and CD8 values a multiple regression model was used on the individual patients. At the time these studies were being performed treatment was being given on demand, as it was required as opposed to regular prophylaxis. There was, therefore often a big variation in the way treatment was given both in terms of the amount of treatment and the pattern in which it was given both of which may have had an effect on the T lymphocyte subsets. Before some assays an individual may have received a lot of treatment in the days leading up to the assay whereas at other times he may have received very little. There will also have been occasions when he received a lot of treatment in the time period six weeks before the assay and little or none immediately before.

To try and assess whether treatment given at these different time periods had an effect on the CD4 and CD8 counts a multiple regression model was used, assessing the relationship between CD4, CD8 and the following three variables; treatment in the week before the assay, the month before the assay and three months before. However, the value of the results obtained from this analysis may be limited because these three variables are not independent of each other. The treatment in the three months before includes that of both the month before and the week before and likewise that of the

month before includes that of the week before. These variables are therefore likely to be highly correlated.

Of those eight patients who had changes in either the CD4 or CD8 levels over the time period, the values of six showed no correlation with either the total treatment received at the time of the assay or the treatment received in the various time periods before the assay. Two patients showed a correlation between their CD8 z scores (patient 4 where they were decreasing and patient 15 where they were increasing) and treatment received in the previous month and three month time periods. Only one other patient had CD4 levels which were related to their previous treatment (patient 11).

In summary only three patients of nineteen patients showed a relationship between the treatment given expressed as the three variables and the either the CD4 or CD8 values, implying that treatment given is having little effect in the short term.

The three treatment variables were altered slightly to make them independent of each other and the multiple regressions repeated. The following three variables were used.

i. Treatment in the week before the assay
ii. (Treatment in the month before the assay) – (Treatment in the week before the assay)
iii. (Treatment in the 3 months before the assay)-(Treatment in the week before the assay)

Repeating the multiple regression showed little change from the original analysis, the same three patients (4, 11 and 15) showing some relationship; in patient 4 the decreasing CD8 was associated with treatment only in the month before and interestingly patient 15 where previously increasing CD8 had been associated with treatment received, CD4 was associated with treatment received in the week and three months before the assay whereas the relationship between CD8 and treatment was no longer significant. Clearly the lack of consistency in the results would suggest that there is no large effect of treatment received on either the CD4 or CD8 counts.

Two patients were excluded from the multiple regression analysis (20 and 23) because they had received very little treatment and this resulted in insufficient data to fit into the model (appendices 7.3.20 and 7.3.23). These two exclusions are unfortunate as these patients represent one of the extremes, individuals receiving little or no treatment being an important 'internal control'. It is important to note however that although

these two individuals received little treatment their CD4 and CD8 values also showed quite a lot of variation.

In summary, after up to ten years of follow up, the CD4 and CD8 counts of these individuals are not demonstrating any marked trends either over time or in relation to treatment received. Only longer follow up at regular intervals, ideally with comparisons to a control group will provide more evidence that this group of treated haemophiliacs remaining free of HIV and hepatitis B and C do not develop changes in T lymphocyte subsets.

CHAPTER EIGHT

STUDIES OF T LYMPHOCYTE PROLIFERATION IN HAEMOPHILIC BOYS

Background

T lymphocytes

A sub-population of lymphocytes, thymus dependant or T-lymphocytes are responsible for the immune response against micro-organisms which are intracellular and are also essential for the development of antigen specific antibody responses. T cells derive from haemopoetic stem cells in the bone marrow but mature and differentiate in the thymus.

The cells have a surface receptor (the T cell receptor) with a single type of specificity or combining site for a specific antigen. Once the cell is activated it is able to express its latent functional properties and undergo cell division with the resultant daughter cells expressing the same recognition or combining site.

A subset of T lymphocytes, the T-helper (CD4) cells respond to stimulation by the appropriate antigen by proliferating and producing a number of soluble factors known as cytokines which are involved in stimulating other cells including macrophages to kill the intracellular organisms. They are also essential for immunoglobulin production by B lymphocytes (Reinharz et al 1979, Yarchoan et al 1982). Another subset, the cytotoxic T cells (CD8), upon recognising appropriate antigen on the surface of an infected cell respond by directly lysing that target cell. They are also responsible for the suppression of a number of immune responses including immunoglobulin production (Reinherz et al 1980).

Both the T helper cells and the cytotoxic T cells only recognise antigen on the surface of infected cells and also only when the antigen is in association with a surface "marker". The surface markers are members of a group of molecules known as the major histocompatibility complex (MHC) (Benacerraf & McDevitt 1972, Bjorkman et al 1987). Involved in the evolution of the "cell-mediated immune response" mediated by T-lymphocytes, therefore, are three important recognition components; the receptor on the surface of the T cell itself which recognises a specific antigen, which in itself must be in association with a major histocompatibility complex molecule.

The Major Histocompatibility Complex (MHC)

This group of molecules was first identified through its ability to evoke powerful transplant rejection and are coded for by a group of closely related loci on chromosome 6. The group are subdivided into three distinct types, two of which, class I and class II have structural similarities and are vital in the process of antigen recognition (Sachs 1984).

Class I molecules are present on the surface of all nucleated cells. They are most abundant on lymphoid cells and are membrane bound heterodimers consisting of a heavy peptide chain of 43 kDa which is non-covalently bound to a smaller 11 kDa peptide known as Beta-2-microglobulin (ß2M). This protein being necessary for the expression of the class I molecule (Zylstra 1990). The study of the structure of these molecules has provided the model for how the MHC molecules are vital in the T cell antigen recognition process (Sette et al 1989). The class I trans-membrane protein is folded into three globular domains, $\alpha 1$ and $\alpha 2$ which are distal to the cell membrane and $\alpha 3$ which together with ß2M is closest to the cell membrane. The $\alpha 1$ and $\alpha 2$ domains are folded to form a "cavity" or "cleft" like structure consisting of a beta pleated sheet floor with two alpha helices above it. The MHC system is highly polymorphic and the amino acid changes responsible for this polymorphism are restricted to the a1 and a2 domains (Shimojo et al 1990). The antigenic determinant that is to be presented to the T cell is positioned in association with the class I MHC molecule at the site of the "floor" of the cavity, whereas the part of the MHC molecule most distal to the surface membrane (the alpha helices) are involved in binding to or associating with the receptor on the surface of the T cell.

Class I MHC molecules in association with antigen are responsible for signalling to cytotoxic (CD8) T cells.

Class II molecules are not present on so many different cell types, being found on B cells, macrophages and other antigen presenting cells, such as Langerhans cells (Dezutter & Dambuyant 1984). T helper (CD4) cells recognise antigen in association with class II MHC molecules. Also some cytotoxic T cells are class II restricted (ie

only recognise antigen in association with class II) but the importance and how widespread this is has yet to be established.

Class II molecules are also transmembrane heterodimers with considerable sequence homology with class I. The two polypeptide chains, alpha and beta (34 and 28 kDa) each fold into two globular domains (α1 & α2, ß1 & ß2) and have a very similar structure to that of class I, with the 2 domains distal to the cell surface membrane (α1 and ß1) forming the "cavity" consisting of the beta pleated sheet floor and the two extending alpha helices. α1 and ß1 are also the site of the amino acid changes responsible for the many polymorphisms of the molecule.

Cytotoxic T cells taken from an individual recovering from a viral infection will only kill virally infected cells with the same MHC haplotype as themselves. For example, influenza nucleoprotein specific T cells from an HLA A2 (class I) donor are only able to kill HLA-A2 influenza infected target cells. Similarly, T helper cells are only able to respond to class II MHC haplotypes identical to those on the cells which originally primed them.

The Antigen and the T cell receptor (TCR)

B cells recognise epitopes on native or original antigen, whereas T cells recognise antigen on the surface of cells in association with an MHC molecule. The antigen which the T cell actually "sees" has been processed by the antigen presenting cell (Germain 1993). Experiments have demonstrated that if the protein ovalbumin is added to macrophages at $0^{o}C$, the macrophages then washed and warmed to $37^{o}C$, then left for one hour and then added to a target cell population of T cells, then the T cells will proliferate in response to the antigen. However, if the macrophages are fixed with glutaraldehyde as soon as they reach $37^{o}C$ then they are unable to stimulate a proliferative response. This demonstrates that some sort of processing occurs within the macrophage prior to them being able to initiate a proliferative response.

Exogenous soluble protein antigens are endocytosed into the antigen presenting cell (Lanzavecchia 1990). They are then unfolded and digested by proteases within the cell

to form small peptides which are then released on to the surface of the cell with class II MHC molecules where they are recognised by specific T helper cells.

Proteins which are produced endogenously as a result of infection of the cell by a micro-organism are also processed by the cell prior to their presentation to cytotoxic T cells. They are not processed by the same method as the exogenous proteins but by a cytoplasmic pathway, again being digested into a series of short peptides (9-16 amino acids long).The peptides become associated with usually class I MHC molecules which have been synthesised by the cell and are presented on the cell surface to cytotoxic T cells. Each T cell clone (both helper and cytotoxic) will only respond to one short peptide. Native proteins will be processed into several peptides each with their individual T cell specificity.

As previously mentioned some cytotoxic T cell responses are in fact class II restricted, for example those that kill measles and rabies infected cells.

The antigens are still processed by the cytoplasmic pathway but become bound to class II molecules as opposed to class I.

Each T cell has a specific receptor for antigen recognition on its surface. The T cell receptor (TCR) is again a transmembrane heterodimer consisting of two peptide chains of 40-50 kDa. There are two types of TCR, TCR1 which is composed of gamma and delta chains (γ & ∂) and TCR2 which is predominant in adult life and consists of alpha and beta chains (α & ß). The chains are bound by a di-sulphide bond and each is folded into 2 domains. The domains of each of the chains which are closest to the cell surface are of a relatively invariant structure, whilst the two distal domains show a high degree of variability, producing a similar pattern to the constant and variable regions (Fab fragment) of the immunoglobulin molecule (Williams & Barclay 1988). It has been demonstrated that both the α and ß chains are necessary for antigen recognition. The TCR recognises antigen in association with MHC molecules on the surface of antigen presenting cells. A second molecule in the membrane of T cells is intimately linked with the TCR heterodimer. This is the CD3 molecule, composed of seven peptide

chains four of which are bound in pairs by di-sulphide bridges (Weissmann 1988). The function of the CD3 molecule is to transduce the signal of the TCR recognising an antigen to the inside of the cell.

Stimulation of the T cell receptor by antigen in association with MHC molecules is insufficient to produce proliferative responses in purified populations of resting T cells (in G_0 phase of the cell cycle). T cells will not respond in the absence of Interleukin-1 (IL-1), which is produced by mononuclear cells (ie. by the cell which is presenting the antigen to the T cell). The involvement of other surface proteins such as CD4 and CD8 are vital (Springer 1990).

The interaction leads to a large number of biochemical changes both in the T cell membrane and within the cell, including membrane lipid changes and increased intracellular calcium ions (Ca^{2+}), both as a result of mobilisation within the cell and the opening of membrane ion channels. There is activation of protein kinases, cyclic nucleotide changes resulting in synthesis of RNA and proteins and eventually DNA synthesis. Ultimately this leads to progression through the cell cycle and proliferation of a clone of T cells specific for the antigen which initiated the response.

Studies of T cell activation; The use of lectins

T cell activation occurs as a result of the interaction between the antigen specific T cell receptor and the corresponding antigen in association with an MHC molecule on the surface of an antigen presenting cell. The presence of soluble factors including IL-1 and accessory molecules on the surfaces of the interacting cells are also important. This specific response is difficult to study as T cells with a given antigen specificity are present only at a very low frequency in a non immune population. A number of different reagents have been used to substitute for the MHC/Antigen complex, some of which can polyclonally activate T cells, (ie: stimulate a sizeable proportion of the lymphocytes of all normal individuals). In 1960 Nowell discovered that an aqueous extract of the kidney bean *Phaseolus Vulgaris* known as phytohaemagglutinin (PHA)

was able to produce large, dividing blast-like cells in cultures of human peripheral blood (Nowell 1960). It was demonstrated that the precursors of these blasts were small lymphocytes. Other plant derived proteins, known as lectins have since been discovered. Concanavalin A (CON A) like PHA has a selective proliferative effect on T cells whereas pokeweed mitogen (PWM) is both a T and B cell mitogen. When compared to the lymphocyte response to specific antigen, the response to lectins of course occurs in a greater proportion of lymphocytes and the detectable biochemical and physiological changes occur more rapidly. There is no evidence however that there is any qualitative difference in the changes induced by specific and non-specific activators and the latter have been widely used in the study of lymphocyte proliferation and differentiation.

The lectin molecules have different carbohydrate specificities and there is a wide variety of cell surface glycoproteins to which they can bind. Recent studies with PHA and CON A suggest that they can bind to component chains of the T cell receptor (TCR) and that their ability to activate T cells is dependant on the expression and function of the TCR (Kanellopoulus et al 1985, Weiss et al 1987). It has been demonstrated that mutants of the human T cell leukaemia line of Jurkat cells which do not express the TCR do not proliferate in response to a combination of PHA and phorbol myristate acetate (PMA). However, if as a result of DNA transfection of this cell line TCR expression is restored this results in the cells developing the ability to respond to this combination (Ohashi et al 1985).

It is important to note that the lectins bind to other cell surface receptors in addition to the TCR/CD3 complex and it has also been demonstrated that monoclonal antibodies directed against cell surface molecules not known to be associated with the TCR complex are also capable of activating T cells.

Lectin induced actvation has provided a means of assessment of the ability of lymphocytes from patients suffering from a diversity of diseases to proliferate and is of clinical relevance.

Studies of lectin induced T cell proliferation in patients with haemophilia

Reduced T lymphocyte proliferative responses to lectins have been reported in both HIV seropositive and seronegative haemophiliacs treated with large pool FVIII concentrates (Lederman et al 1983, Moffat et al 1985, Mahir et al 1988). Mahir demonstrated reduced PHA responses in 9 haemophiliacs and 2 patients with Von Willebrands disease. Five were HIV seropositive but there was no difference between those who were seropositive or negative. The possibility that the reduced proliferation might be in part due to excessive T suppressor cell function was negated by repeating the assays with CD4 cell enriched populations, which also showed reduced responses.

In vitro assays have shown that lectin induced proliferation of peripheral blood mononuclear cells from normal donors is reduced in a dose dependant fashion by the presence of FVIII concentrates (Hay 1990).

Studies of lectin induced T cell proliferation in the cohort of haemophiliacs treated solely with BPL 8Y

Patients:

Twenty three of the original cohort of twenty five boys were studied (group 1). All, at the time of the study were seronegative for anti-HIV antibody and anti-HCV antibody and had been immunised against hepatitis B.

All had been treated with only one factor VIII product, namely BPL 8Y.

Patient details including age, period of follow up (time since first infusion), total units of FVIII received and the number of days since the last infusion of concentrate are detailed in table 8.1.

Comparison groups

The T cell proliferative responses of three other groups of individuals were studied and compared to the responses of group 1. Clinical details of groups 2, 3 and 4 are shown in table 8.2.

Group 2 comprised 17 haemophilic boys, treated with a variety of concentrates who remain HIV seronegative. 12 were seropositive for hepatitis C and five had evidence of having been infected with hepatitis B.

Group 3 comprised 22 haemophilic boys all of whom were HIV seropositive, all had serological evidence of infection with hepatitis B and C and four remained hepatitis B surface antigen positive. Three boys were receiving the antiretroviral drug zidovudine.

All group 2 and 3 boys had received treatment with a variety of different FVIII products but for at least 18 months prior to the assay they had all been receiving only BPL 8Y.

Group 4 (control group) comprised 19 healthy volunteer donors (age range 2 - 47 years, median 12 years 1 month). None had any history of liver disease or risk factors for HIV infection.

patient number	age (months)	Time since first treatment (months)	total FVIII received (units)	days since last infusion
1	74	61	135125	2
2	63	51	52715	1
3	57	48	109235	7
4	67	49	82820	1
5	53	45	37750	60
7	78	40	37465	1
8	44	41	60965	7
9	155	46	16200	120
10	58	43	66320	55
11	48	41	47530	19
12	61	39	35640	5
13	111	35	5265	125
14	56	39	77900	1
15	29	29	10705	23
16	92	26	10870	184
17	45	5	6210	1
18	41	3	2875	19
19	23	12	23440	1
21	34	8	6645	1
22	22	9	21960	32
23	29	6	3280	21
24	80	3	3640	32
25	19	3	1530	67

Table 8.1 Group 1 details (patients treated only with BPL 8Y)

Group	number in group	age (months)	median age (months)	FVIII exposure	Viral Status					
					HIV		HBV		HCV	
					positive	negative	positive	negative	positive	negative
1	23	19-153	56	BPL 8Y	0	23	0	23	0	23
2	17	86-184	115	various	0	17	1	16	12	5
3	22	105-224	168	various	22	0	22	0	22	0
4 (controls)	19	24-564	154	none	not tested (normal controls)					

Table 8.2 clinical details of comparison groups 2, 3 and 4

Laboratory methods

All assays were performed in the morning prior to any treatment being given to the patients. The assays were performed as described in chapter 5.

Peripheral blood mononuclear cells (PBMC's) were isolated from 20mls of anticoagulated whole blood by density gradient centrifugation on Ficoll Hypaque (Lymphoprep, Nygaard Ltd, UK). After washing the cells were resuspended at a concentration of 1 x 10^6/ml in RPMI and 10% heat inactivated serum.

1 x 10^5 cells in a final volume of 200µl were incubated with phytohaemagglutinin (PHA) and concanavalin A (Con A) at concentrations of 50, 25 and 5 µg/ml. The cells were incubated at 37°C in 5% carbon dioxide for 72 hours, and were pulsed 18 hours before the end of the culture with 0.3 µCi of tritiated thymidine (Amersham, UK). The cells were harvested on to glass fibre filters which were washed and dried and the thymidine content and hence proliferation was determined by liquid scintillation counting. All assays were performed in triplicate and the results were expressed as mean counts per minute (cpm) following deduction of background proliferation measured by the unstimulated control that was included in every assay.

Statistical analysis

The statistical methods used are described in detail in chapter 5. Briefly, the Kruskal Wallis one way analysis of variance was used to compare the responses of the four groups and the Mann Whitney U test to make pairwise comparisons between the groups.

Spearman's rank correlation coefficients were used to investigate within group 1 the relationship between the responses and both treatment received and the time since the last treatment episode before the assay.

Results

The control group (group 4)

This group consisted of 11 children and 8 adults. The results of the proliferative responses, expressed as counts per minute are given in table 8.3.1. There was no significant association between the proliferative response and age at any concentration of PHA and Con A. (table 8.3.2).

Comparison of the four groups

The proliferative responses to PHA and Con A at each concentration, (50, 25 and 5 μg/ml) of each of the four groups are shown in tables 8.4 to 8.6 and graphs 8.1 to 8.6. Using one-way analysis of variance there was a highly significant difference between the four groups in terms of their proliferative responses to PHA and Con A at all concentrations. Multiple comparisons were then used to determine which individual groups significantly differed from each other, the results being adjusted to take account of multiple testing. The results are summarised in table 8.7.

To summarise the results;

Comparison of groups 1,2 and 3 with the control group 4

- There was no significant difference between the proliferative responses of group 1 and those of the healthy controls (group 4) at all concentrations of PHA and Con A.
- The responses of group 2 were generally lower than group 4, however the difference only reached statistical significance with Con A at concentrations of 25 and 5μg/ml.
- The responses of group 3 were significantly lower than those of group 4 at all concentrations except PHA 25μg/ml

Comparison of group 1 with groups 2 and 3

- The responses of group 1 were significantly better than group 3 (HIV positive group) at all concentrations.

- The responses of group 1 were higher than group 2, however the difference only reached statistical significance with Con A at concentrations of 25 and 5 μg/ml. (graphs 8.2 and 8.3)

Comparison of groups 2 and 3

- There was no significant difference between the responses of groups 2 and 3 at any concentration of PHA or CON A

Patient number	age (months)	Proliferation (counts per minute)					
		Con A 50 µg/ml	Con A 25 µg/ml	Con A 5 µg/ml	PHA 50 µg/ml	PHA 25 µg/ml	PHA 5 µg/ml
1	552	32078	30430	24622	32369	42945	43810
2	360	22585	19521	19448	38829	40093	38638
3	312	37079	29729	22014	41308	12654	7775
4	77	28010	27246	19603	46739	48400	48692
5	420	22438	15562	8420	23066	25292	31938
6	564	14180	17155	9194	17009	15220	18135
7	77	11536	7511	1264	7202	27382	24296
8	312	22192	18082	5556	30789	33165	28254
9	31	13603	7511	2670	25371	26370	16276
10	106	6744	3005	68	18495	19342	14722
11	63	9048	5905	2398	21440	30990	17581
12	456	15724	18047	7006	19198	20123	24041
13	24	6799	28061	21476	17033	21183	37517
14	153	18865	15274	6065	23057	27339	26517
15	126	20576	17865	8922	20385	21199	26435
16	480	29883	59302	29103	65491	72656	82945
17	145	36546	34080	12627	67880	65274	59424
18	125	31635	28888	6390	33706	52672	48411
19	103	47567	34631	16225	62981	38256	52953
Population means		22478	21990	11741	32229	33713	34124

Table 8.3.1 Proliferative responses of group 4 (normal controls) to PHA and CON A

Lectin concentration (expressed as μg/ml)	Spearman's rank correlation coefficient	p-value	significance
CON A 50	0.37	0.11	NS
CON A 25	0.29	0.22	NS
CON A 5	0.38	0.11	NS
PHA 50	0.14	0.54	NS
PHA 25	-0.004	0.98	NS
PHA 5	0.13	0.59	NS

Table 8.3.2 Control group (group 4): correlation of lymphocyte proliferative responses with age.
NS = not significant at the 5% level

patient number	PROLIFERATION (cpm)					
	Con A 50 µg/ml	Con A 25 µg/ml	Con A 5 µg/ml	PHA 50 µg/ml	PHA 25 µg/ml	PHA 5 µg/ml
1	26280	18905	6023	22523	45237	34070
2	21528	22548	6565	18671	26533	35493
3	43516	34256	7326	40879	56859	47367
4	20675	31182	8398	34625	26898	41047
5	10016	6759	497	12839	18841	24202
7	35438	27362	9526	22771	38509	43108
8	20774	13907	4412	26662	58225	42389
9	14162	44043	26931	34802	47125	72547
10	29702	9521	2031	86278	30090	8415
11	32850	35999	9323	54494	55455	50827
12	9399	7626	1537	15455	15963	14645
13	16655	12798	2739	18558	23784	22993
14	22803	11285	2404	61992	84302	62748
15	15607	13337	5245	27375	31746	27439
16	10465	5459	1734	21740	6095	12621
17	27973	21066	9829	26509	28602	28723
18	14007	7599	981	30494	41333	34644
19	26437	21767	8596	49029	48815	42123
21	58008	53507	19695	76993	96796	85125
22	60582	35894	4576	64633	66716	73180
23	23041	13292	6382	78487	40979	21734
24	10924	8413	2441	13956	17633	16014
25	13748	30904	28637	34480	52552	137407
Population means	**24547**	**21192**	**7645**	**38010**	**41699**	**42559**

Table 8.4 group 1 lymphocyte proliferative responses to Con A and PHA

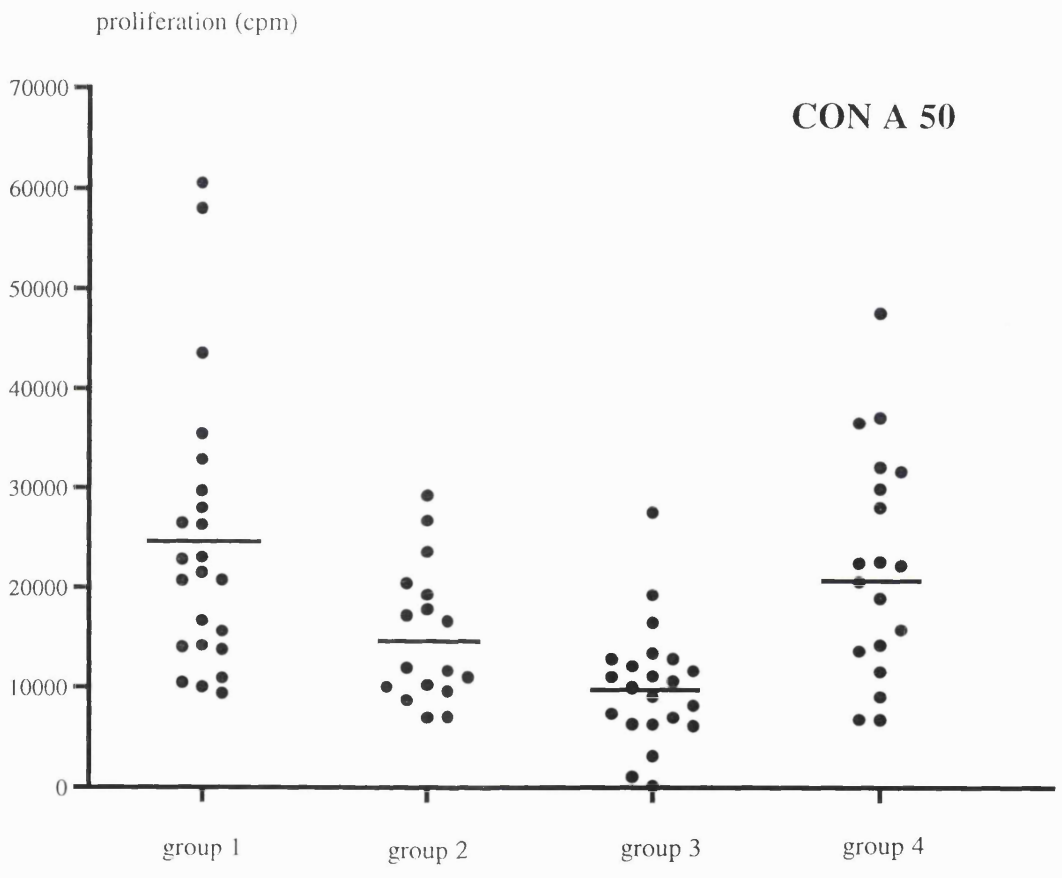
NB. From the original group of 25 patients, 2 (numbers 6 and 20) were omitted from this study

patient number	Proliferation (counts per minute)					
	Con A 50 µg/ml	Con A 25 µg/ml	Con A 5 µg/ml	PHA 50 µg/ml	PHA 25 µg/ml	PHA 5 µg/ml
26	17177	9206	1960	22250	25745	26705
27	10223	2574	511	16088	19391	22186
28	10042	4218	1181	19483	22489	21979
29	6978	4407	1946	15380	17822	15452
30	9619	7113	2718	20607	31478	18014
31	20464	11982	2650	35630	34417	37510
32	11000	6675	627	25785	31679	28016
33	17824	12987	1826	22106	30272	37802
34	8721	4482	274	25293	34823	23696
36	19297	13598	2682	20206	21948	34396
37	26725	18799	5645	24597	29502	24617
38	11673	7172	822	27069	26975	19935
39	7075	5900	1499	20430	24003	20091
40	29241	13362	2529	23912	29318	30092
41	23603	13370	3889	27538	32725	39929
42	16637	14038	3325	18717	24656	27073
43	11950	5593	529	37456	49546	28420
Population means	**15191**	**9146**	**2036**	**23679**	**28634**	**26818**

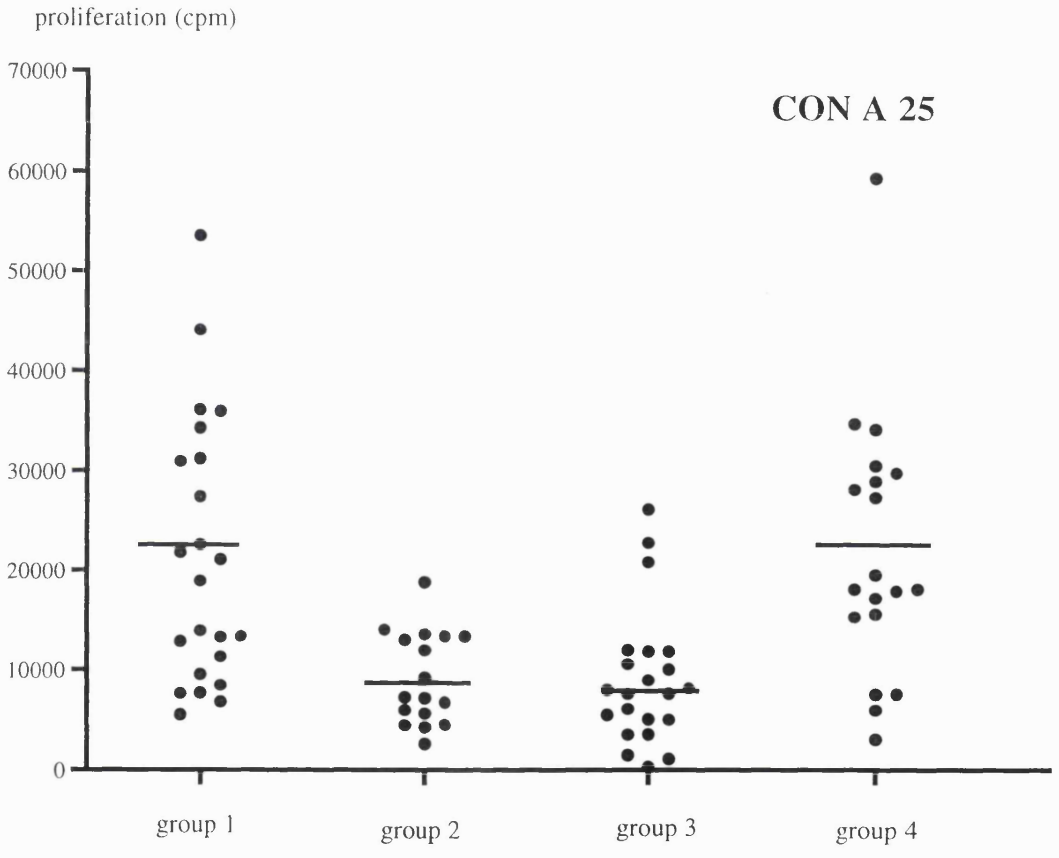
Table 8.5 Group 2: lymphocyte proliferative responses to Con A and PHA

Patient number	Proliferation (counts per minute)					
	Con A 50 µg/ml	Con A 25 µg/ml	Con A 5 µg/ml	PHA 50 µg/ml	PHA 25 µg/ml	PHA 5 µg/ml
47	6295	3493	650	4903	10194	102
48	27489	22717	11417	37235	37691	27614
49	19235	20784	4363	17986	22840	23400
50	9859	11833	1800	12852	16662	18798
51	11108	5023	569	21592	26673	23643
52	3117	1065	395	6962	8712	7049
53	6982	3469	2613	21402	23114	22550
54	16435	11812	1995	33748	43826	32297
55	10595	26071	10925	15721	27895	30041
56	9045	8906	1524	20474	23337	16372
57	7352	7578	1160	17120	21726	14939
58	127	268	78	951	2286	12347
59	13402	4978	164	17342	25155	15659
60	11599	6026	720	24168	28677	28150
61	11039	7531	1081	28231	36961	28907
62	12100	10532	2158	11480	14818	16077
63	10005	9989	4566	7431	8936	10717
64	6317	7958	2918	7945	11246	12384
65	6115	11932	6414	7068	16299	26981
66	12832	5442	770	24005	28405	24577
67	1089	1431	0	8050	12774	6749
68	8162	8101	2025	4685	2269	4900
Population means	**10013**	**8952**	**2650**	**15970**	**20477**	**18375**

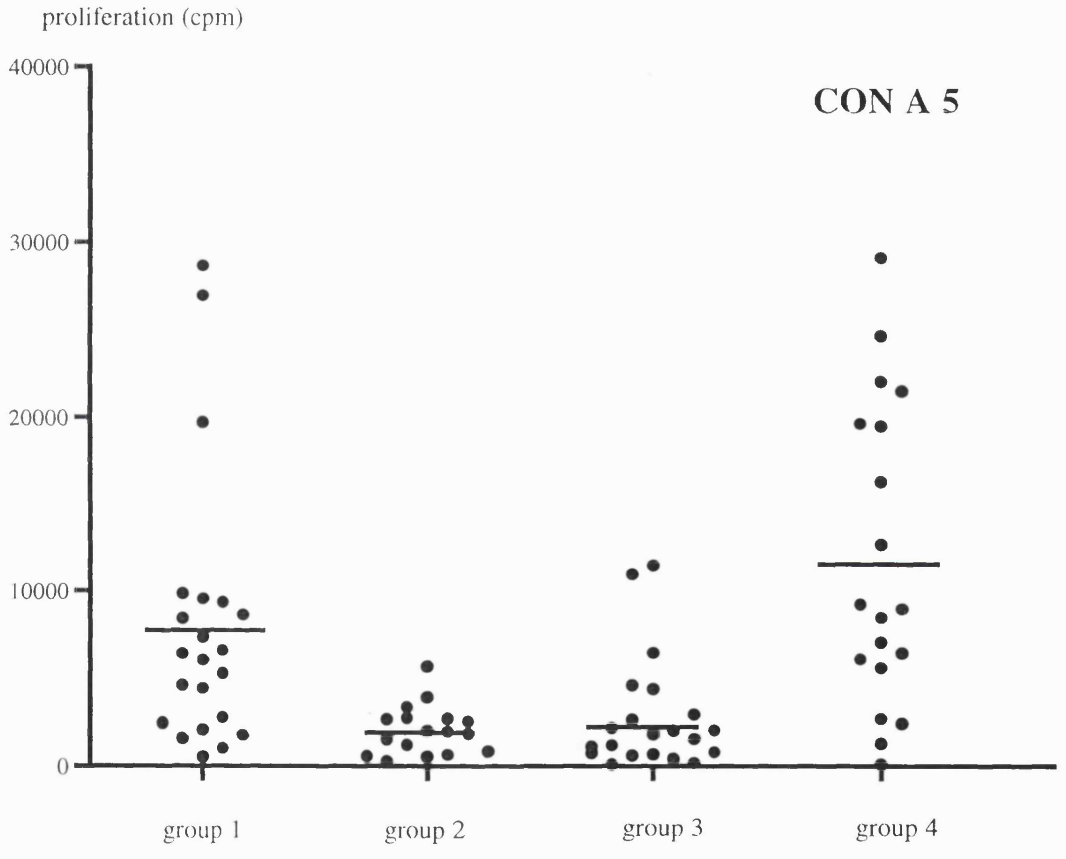
Table 8.6 Group 3 lymphocyte proliferative responses to Con A and PHA



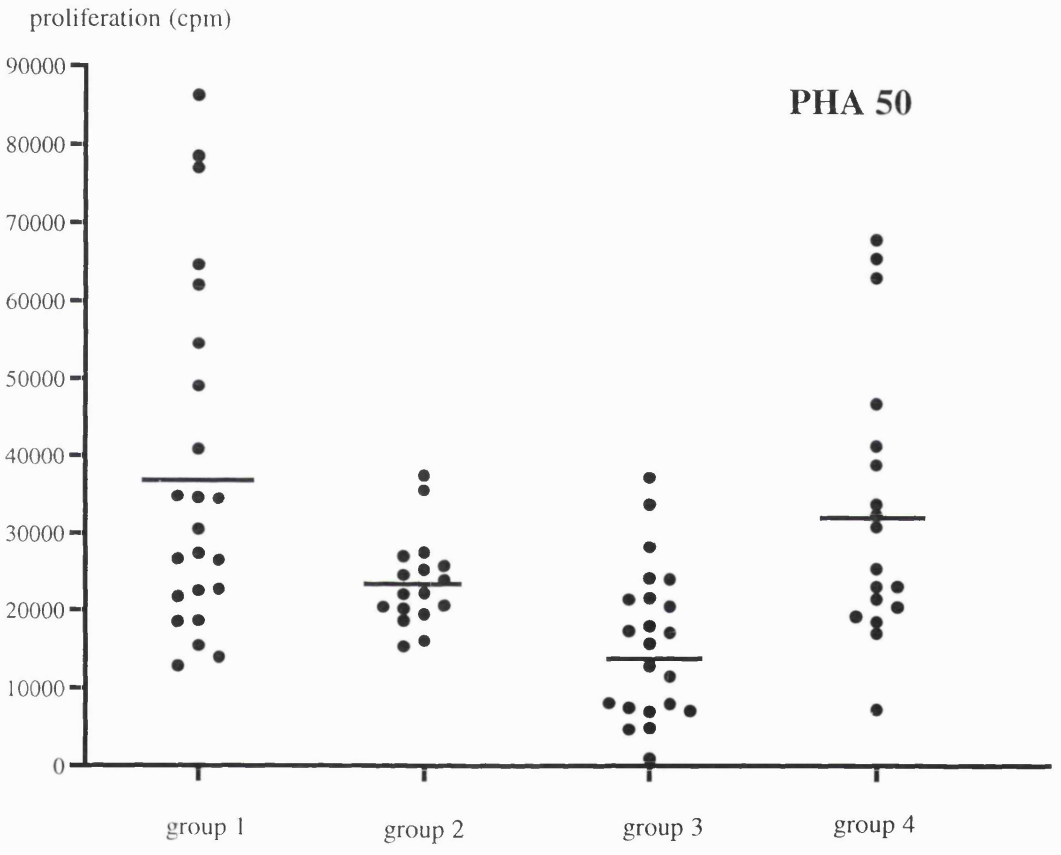
graph 8.1
lymphocyte proliferation (cpm) to CON A50 µg/ml
the bars represent the population means



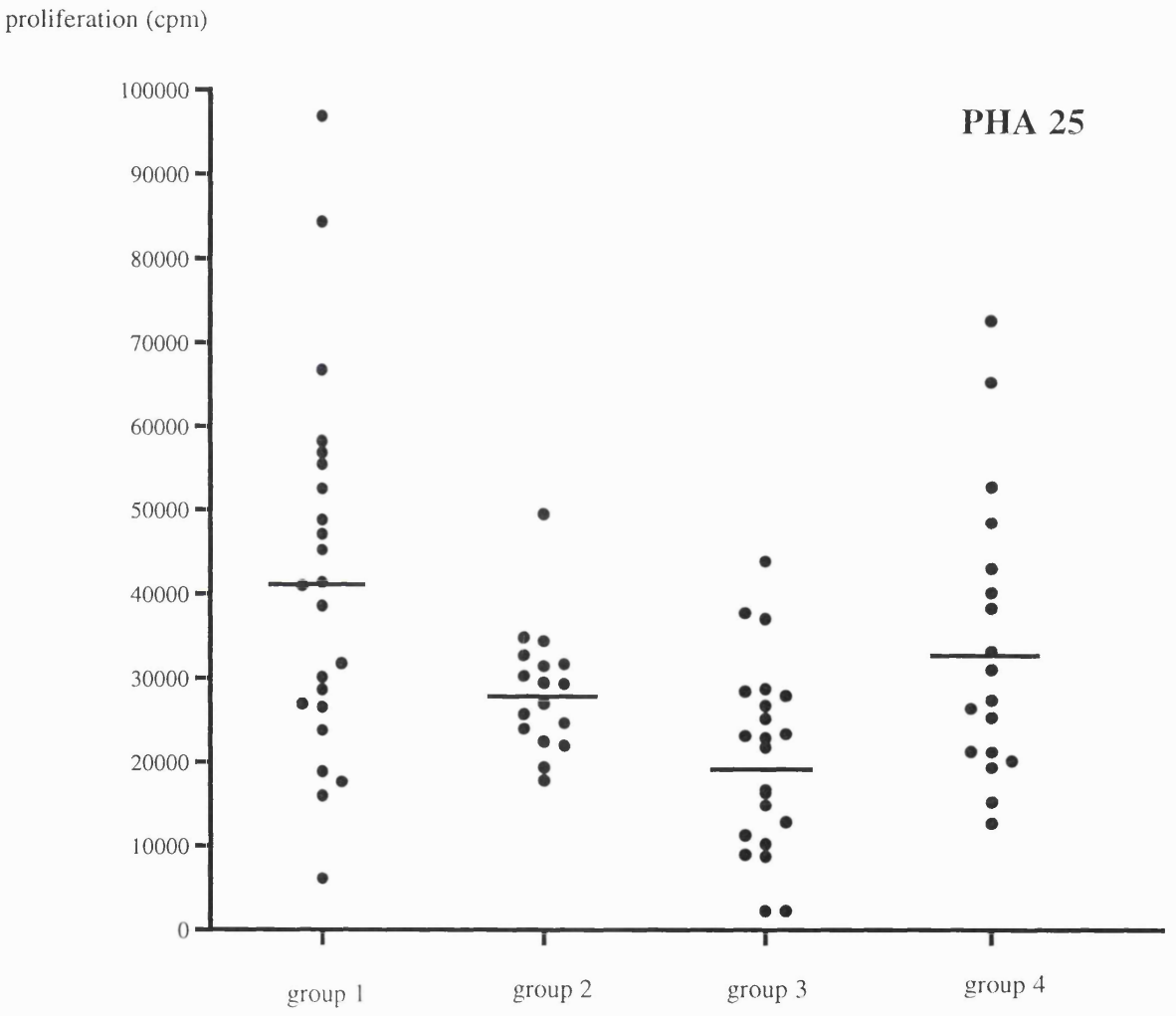
graph 8.2
lymphocyte proliferation (cpm) to CON A25 µg/ml
the bars represent the population means



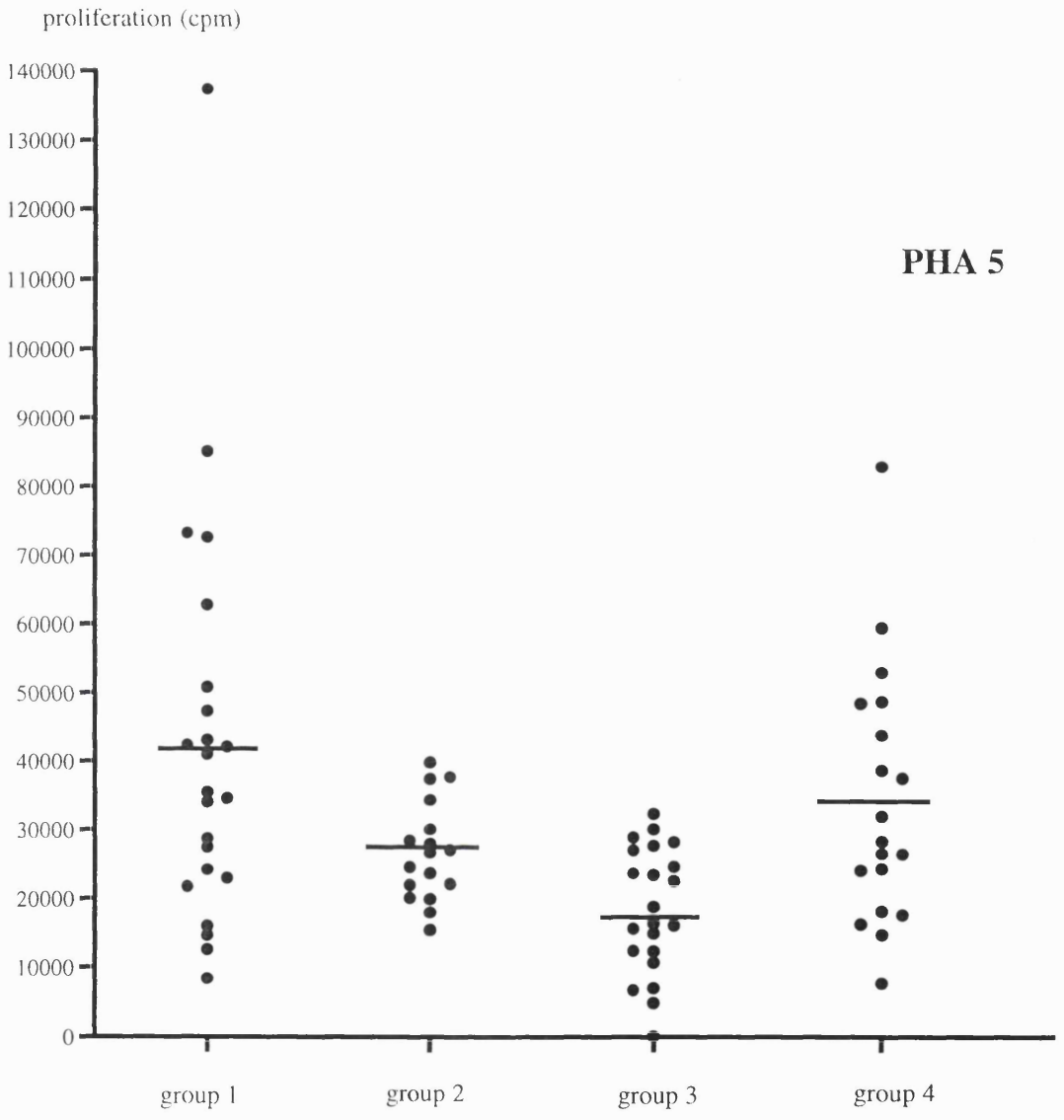
graph 8.3
lymphocyte proliferation (cpm) to CON A 5 µg/ml
the bars represent the population means



graph 8..4
lymphocyte proliferation (cpm) to PHA 50 µg/ml
the bars represent the population means



graph 8.5
lymphocyte proliferation (cpm) to PHA25 µg/ml
the bars represent the population means



graph 8.6
lymphocyte proliferation (cpm) to PHA5 µg/ml
the bars represent the population means

Concentration of lectin	Groups to be compared	Adjusted p value
Con A 50μg/ml	Group 1 v group 4	0.8
	Group 2 v group 4	0.3
	Group 3 < group 4	**0.0012**
	Group 1 v group 2	0.12
	Group 1 > group 3	**<0.0001**
	Group 2 v group 3	0.12
Con A 25 μ/ml	Group 1 v group 4	0.93
	Group 2 < group 4	**0.002**
	Group 3 < group 4	**0.004**
	Group 1 > group 2	**0.006**
	Group 1 > group 3	**0.002**
	Group 2 v group 3	0.5
Con A 5 μg/ml	Group 1 v group 4	0.7
	Group 2 < group 4	**0.0006**
	Group 3 < group 4	**0.0006**
	Group 1 > group 2	**0.006**
	Group 1 > group 3	**0.006**
	Group 2 v group 3	0.79

Table 8.7.1 Summary of significant differences between lymphocyte proliferative responses of the four groups to CON A

Group 1 = haemophiliac patients treated with only BPL 8Y
Group 2 = haemophiliac patients previously treated with a variety of products but remain HIV seronegative
Group 3 = haemophiliac patients who are HIV seropositive
Group 4 = healthy controls
Significant results are in bold

Concentration of lectin	Groups to be compared	Adjusted p value
PHA 50µg/ml	Group 1 v group 4	0.5
	Group 2 v group 4	0.3
	Group 3 < group 4	**0.01**
	Group 1 v group 2	0.3
	Group 1 > group 3	**0.0006**
	Group 2 v group 3	0.06
PHA 25 µ/ml	Group 1 v group 4	0.2
	Group 2 v group 4	0.7
	Group 3 v group 4	0.06
	Group 1 v group 2	0.3
	Group 1 > group 3	**0.002**
	Group 2 v group 3	0.06
PHA 5 µg/ml	Group 1 v group 4	0.47
	Group 2 v group 4	0.4
	Group 3 < group 4	**0.02**
	Group 1 v group 2	0.3
	Group 1 > group 3	**0.002**
	Group 2 v group 3	0.06

Table 8.7.2 Summary of significant differences between lymphocyte proliferative responses of the four groups to PHA

Group 1 = haemophiliac patients treated with only BPL 8Y
Group 2 = haemophiliac patients previously treated with a variety of products but remain HIV seronegative
Group 3 = haemophiliac patients who are HIV seropositive
Group 4 = healthy controls
Significant results are in bold

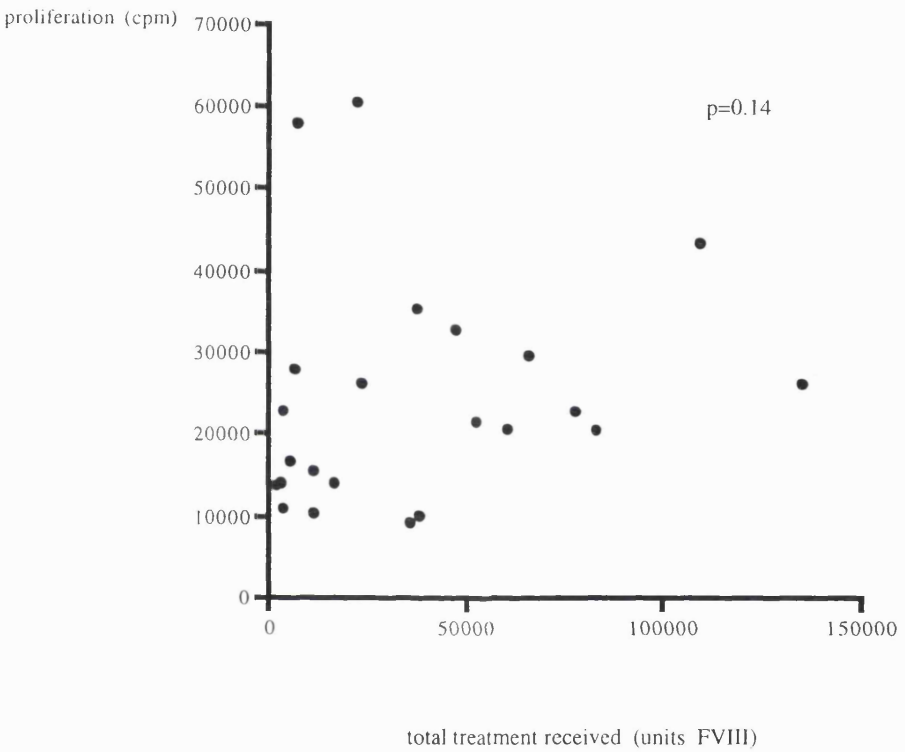
Relationship to treatment received

The total units of FVIII received by the group 1 boys ranged between 1530 and 135125 units. (mean, 37221 units; median 35640 units) (table 8.1).

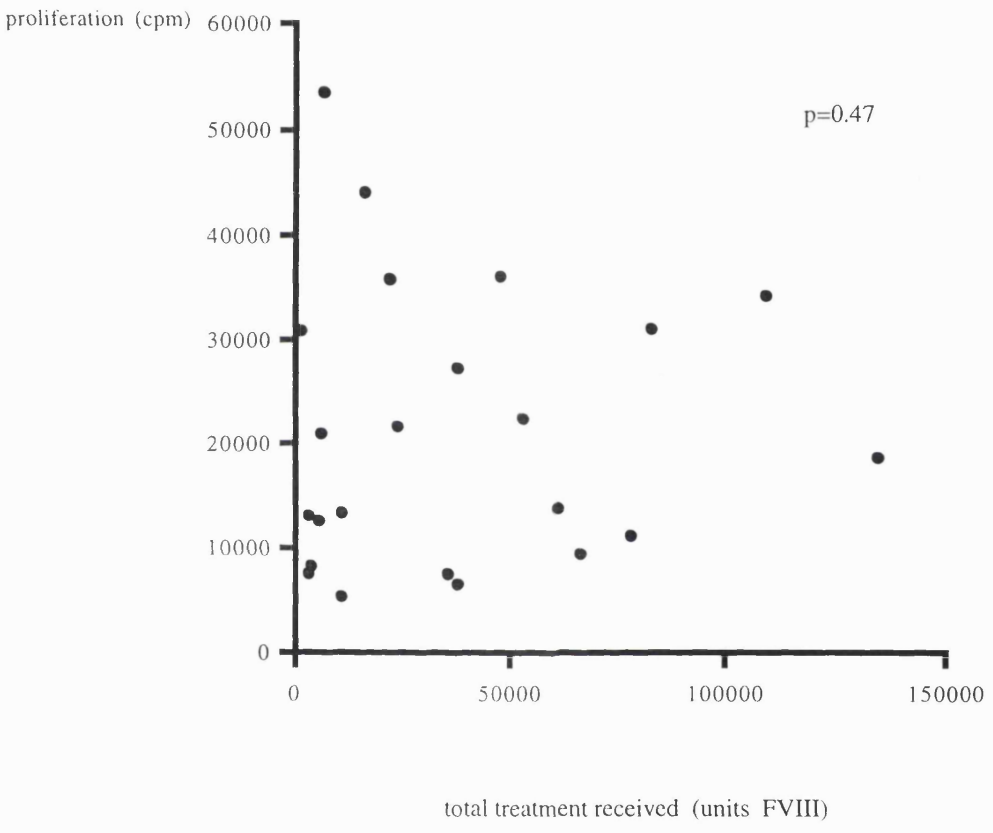
There was no significant association between the proliferative responses at any concentration and the total treatment that had been received at the time of the assay (graphs 8.7.1 – 8.7.6).

All the assays were performed a minimum of 24 hours after the last infusion of FVIII. Within group 1 the time interval between the last infusion of FVIII and the proliferation assays varied between 1 and 184 days, (median 19 days) as shown in table 8.1

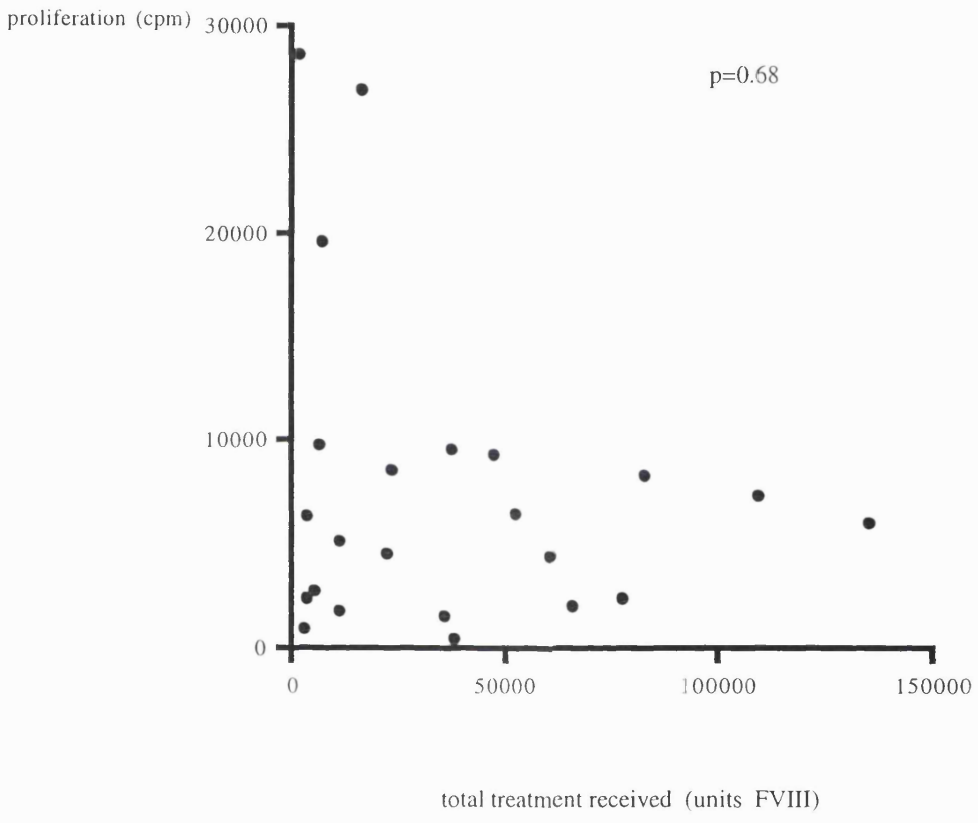
At the concentration of CON A 50μ/ml there was a significant relationship between increasing length of time since the last infusion of factor VIII and reduced proliferative response ($p=0.04$) (graph 8.8.1). At all other concentrations of CON A and PHA there was no significant association between the proliferative responses and the time interval between the assay and the last FVIII infusion (graphs 8.8.2 – 8.8.6).



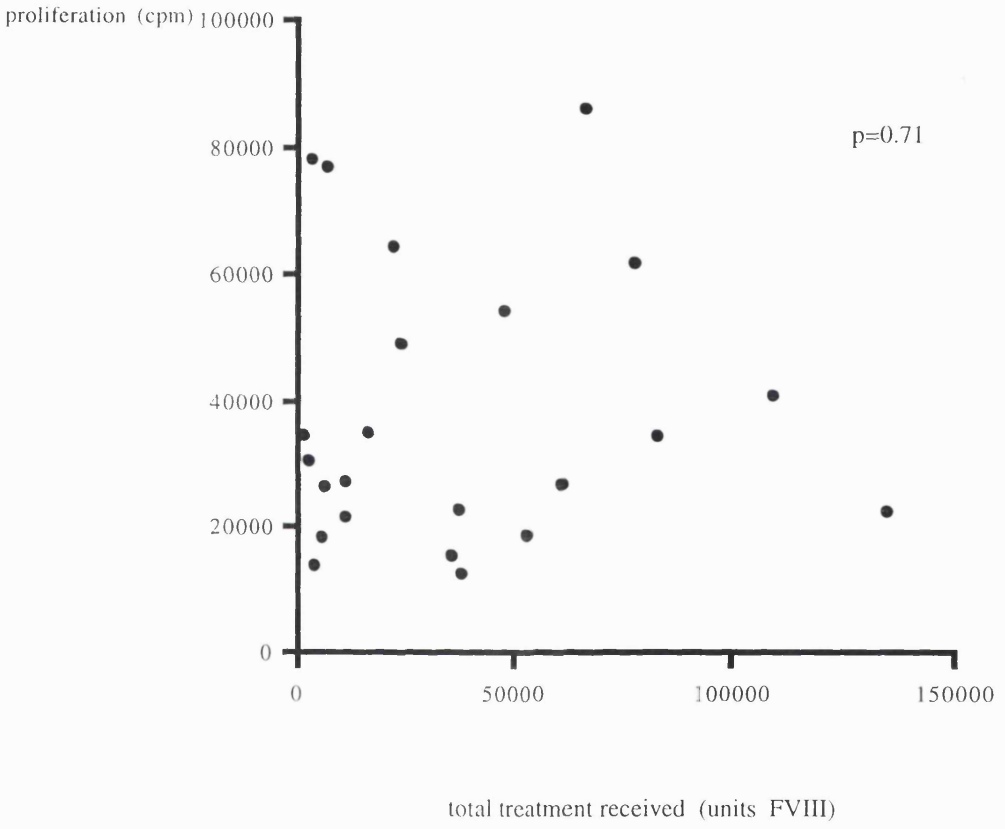
graph 8.7.1:
group 1: relationship of proliferation (cpm) to total treatment received (units FVIII) (CON A 50)
There is no significant correlation between proliferative response and total treatment received



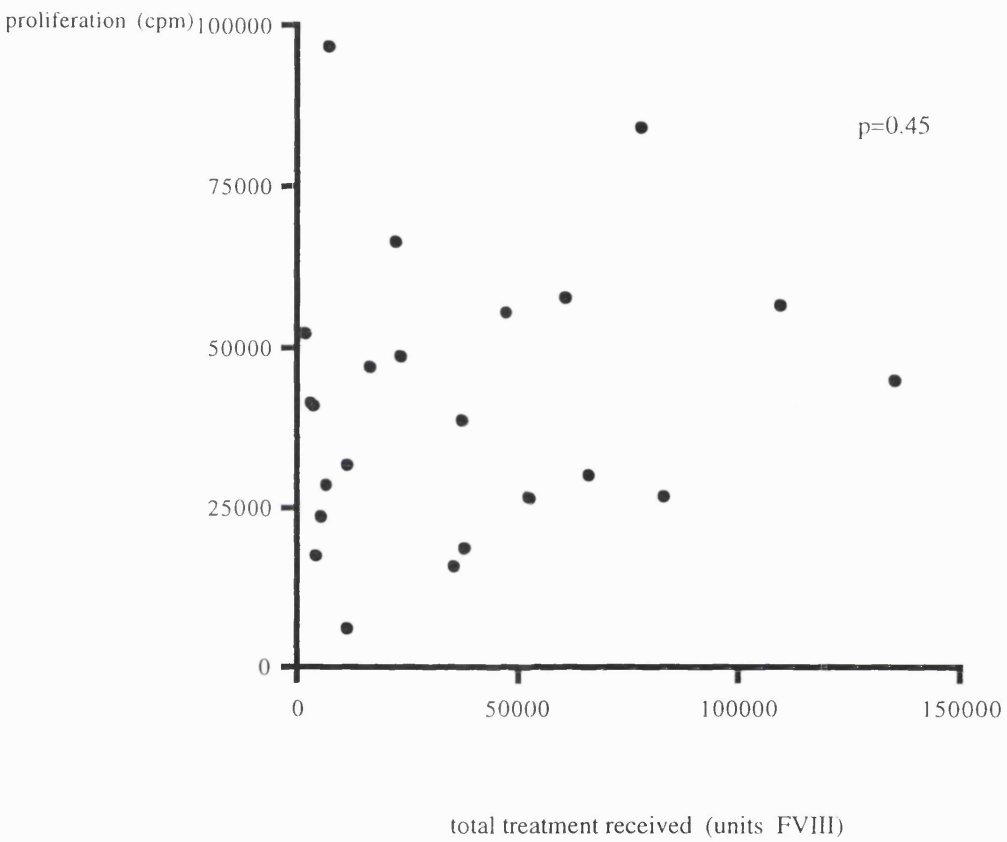
graph 8.7.2:
group 1: relationship of proliferation (cpm) to total treatment received (units FVIII) (CON A 25)
There is no significant correlation between proliferative response and total treatment received



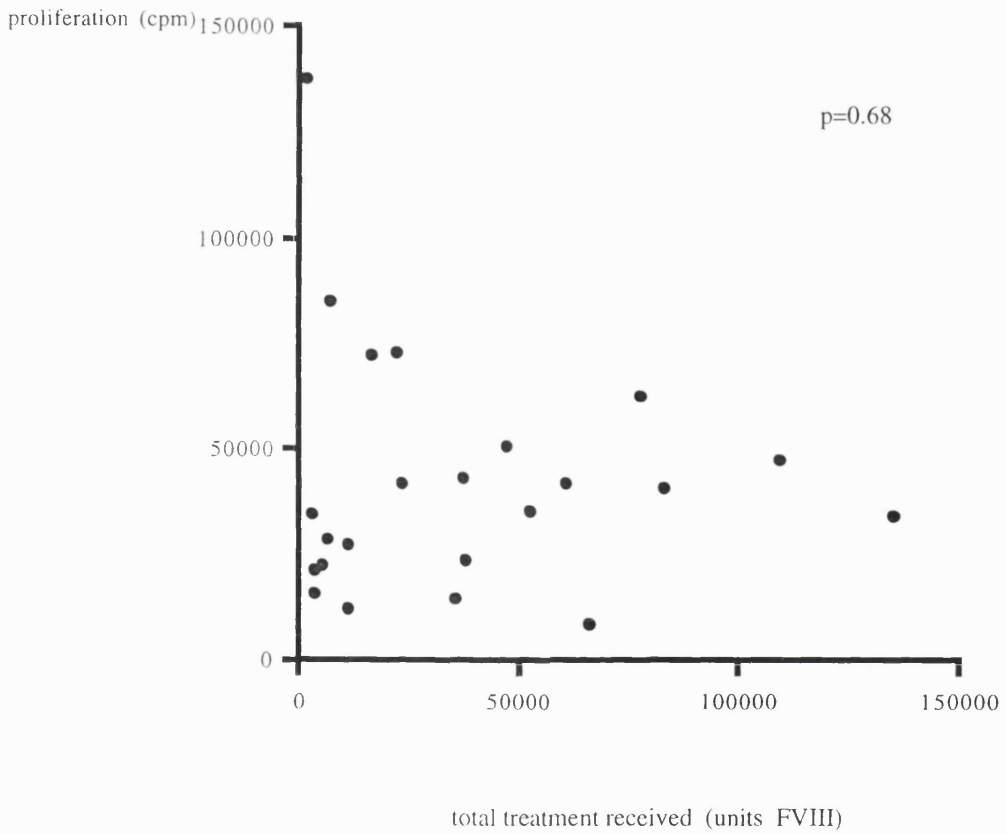
graph 8.7.3:
group 1: relationship of proliferation (cpm) to total treatment received (units FVIII) (CON A 5)
There is no significant correlation between proliferative response and total treatment received



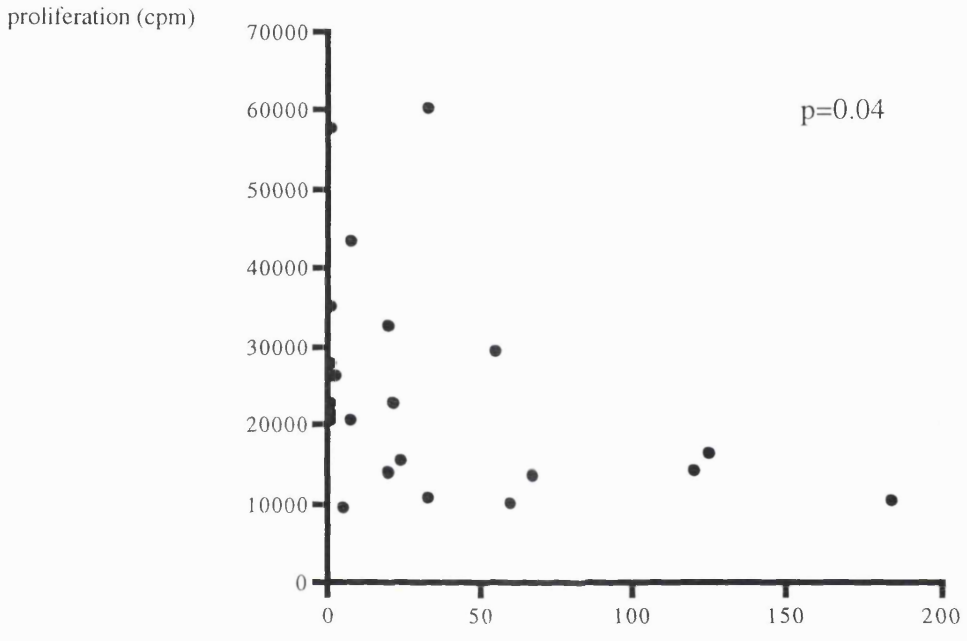
graph 8.7.4
group 1: relationship of proliferation (cpm) to total treatment received (units FVIII) (PHA 50)
There is no significant correlation between proliferative response and total treatment received



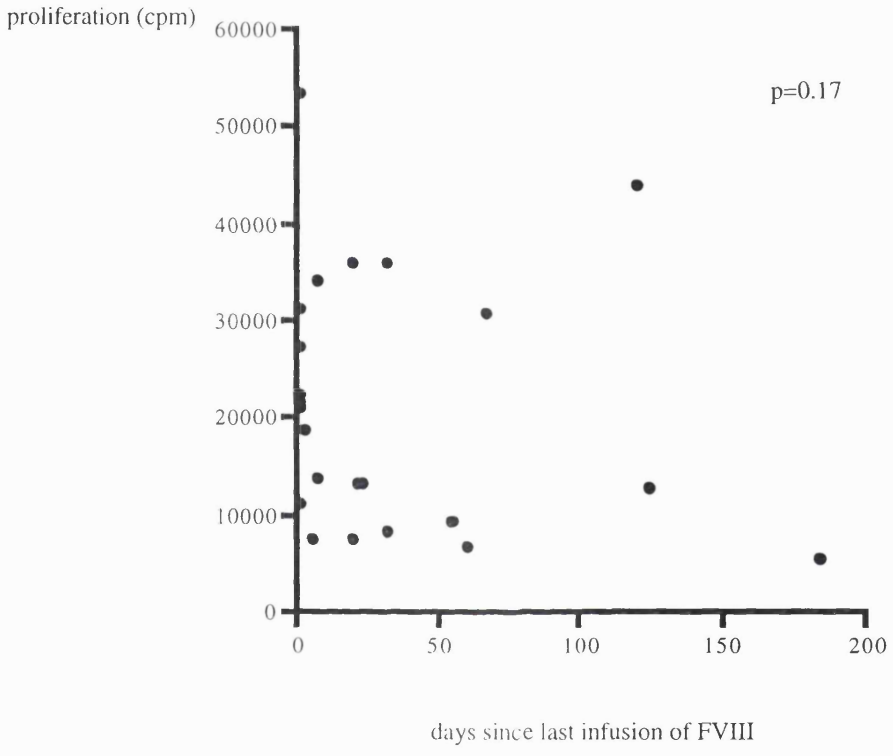
graph 8.7.5
group 1: relationship of proliferation (cpm) to total treatment received (units FVIII) (PHA 25)
There is no significant correlation between proliferative response and total treatment received



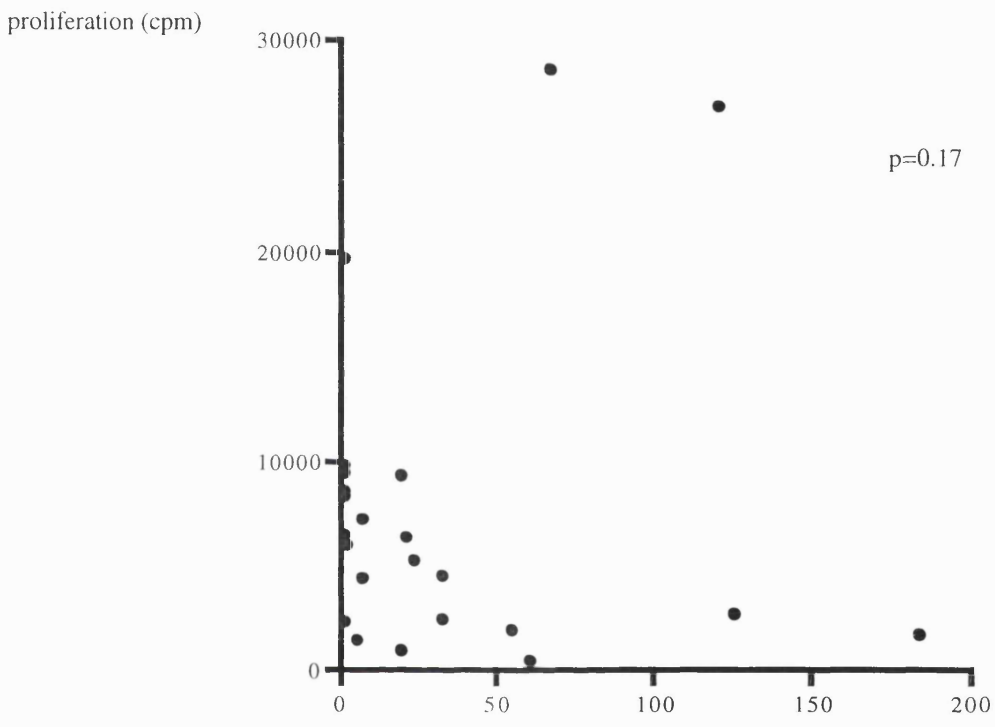
graph 8.7.6
group 1: relationship of proliferation (cpm) to total treatment received (units FVIII) (PHA 5)
There is no significant correlation between proliferative response and total treatment received



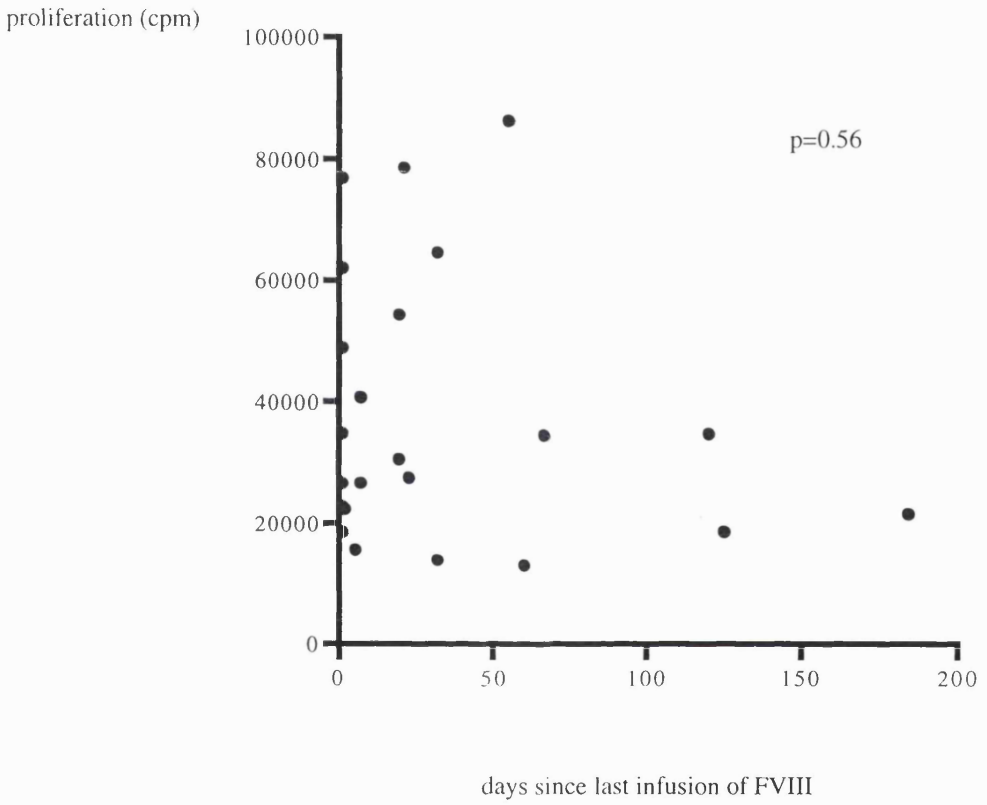
graph 8.8.1
group 1: relationship of proliferation (cpm) to time since last infusion of FVIII concentrate (CON A 50)
There is a negative correlation between the proliferative response and the time since the last infusion of factor VIII



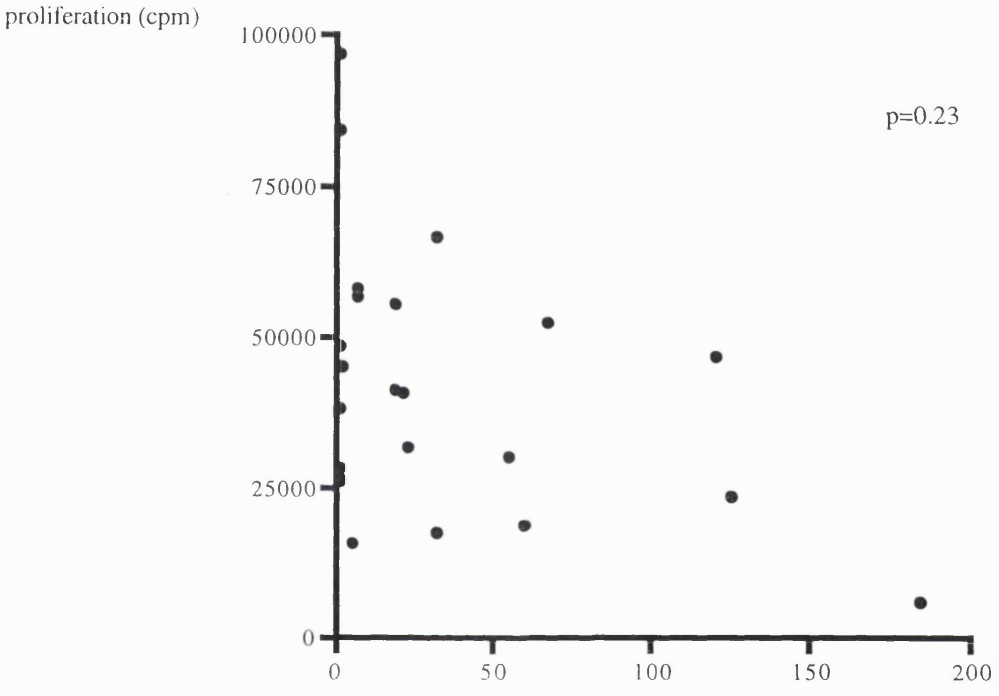
graph 8.8.2
group 1: relationship of proliferation (cpm) to time since last infusion of FVIII concentrate (CON A 25)
There is no significant correlation between proliferative response and the time since
the last infusion of factor VIII



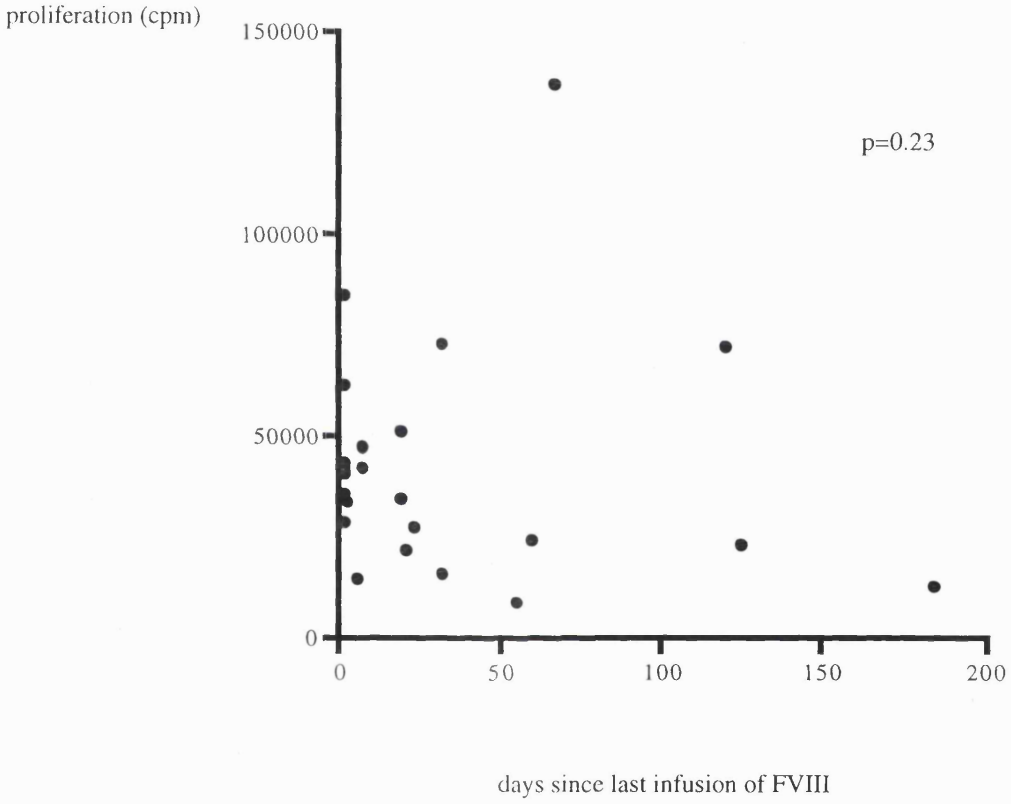
graph 8.8.3:
group 1: relationship of proliferation (cpm) to time since last infusion of FVIII concentrate (CON 5)
There is no significant correlation between proliferative response and the time since the last infusion of factor VIII



graph 8.8.4
group 1: relationship of proliferation (cpm) to time since last infusion of FVIII concentrate (PHA 50)
There is no significant correlation between proliferative response and the time since the last infusion of factor VIII



graph 8.8.5:
group 1:relationship of proliferation (cpm) to time since last infusion of FVIII concentrate (PHA 25)
There is no significant correlation between proliferative response and the time since the last infusion of factor VIII



graph 8.8.6
group 1: relationship of proliferation (cpm) to time since last infusion of FVIII concentrate (PHA 5)
There is no significant correlation between proliferative response and the time since the last infusion of factor VIII

Discussion

The assay described in these studies is used as a standard test in the assessment of cell mediated immunity . It had also been widely used in earlier studies on haemophiliacs showing reduced T-lymphocyte responses to lectins in both HIV seropositive and seronegative haemophiliacs treated with large pool FVIII concentrates. It was therefore decided to use it in this group of patients.

At all concentrations of lectins used in these assays there was no significant difference between the lymphocyte proliferative responses of the BPL 8Y patients (group 1) and the control group 4. The responses of group 1 were significantly better than those of group 3 at all concentrations and significantly better than those of group 2 at concentrations of CON A of 25 and 5μg/ml. This group of patients appear to be behaving differently from previously reported groups of haemophiliacs.

This assay does, however have its limitations. The responses of healthy immunocompetent controls are known to vary from day to day. Ideally one should look at a series of values on any one individual, for example over a series of days. It may be that 'one off' measurements pick up either a very high or a very low measurement. The assays here were also performed on different days under as far as possible the same laboratory conditions, however slight changes in external conditions cannot always be accounted for. Performing a series of assessments would be difficult, because of the need to take several samples from children over a short period of time. The children are also outpatients making it inconvenient to attend.

The control group 4 in this study is far from ideal, containing only seven children under the age of ten years. Although there was shown to be no correlation between age and lymphocyte proliferation in this group it is possible that there are differences between immune responses in children and adults. Ideally a group of age matched control children should have been used, but it is always difficult to take samples from normal healthy children.

Groups 2 and 3 are similar to the previously studied groups of haemophiliacs in whom lymphocyte proliferation abnormalities were described. It is therefore not surprising to

see broadly reduced lymphocyte proliferative responses in these two groups, with those boys infected with HIV being the most severely affected, and at all lectin concentrations the proliferative response of the HIV positive group (group 3) were significantly worse than both the control group and group 1. It can also be clearly seen from the graphs that the lymphocyte responses of group 2 are reduced at all concentrations and are approaching those of the HIV seropositive group. However the difference in responses between group 2 and both group 1 and controls only reached statistical significance with Con A at concentrations of 25 and 5 μg/ml.

When comparing the three treatment groups however, in an ideal situation, the groups should have been matched for age, length of time on and amount of treatment received. Detailed immune function was not part of the follow up of group 2 and group 3 boys in the early years after they started treatment, this only becoming an issue later when it became apparent they were at risk of acquiring HIV. Information taken at time points comparable to the group 1 boys is therefore not available.

It must be said that at the time of the study the group 1 patients had only been on treatment for between 3 and 61 months and the possibility that they will go on to develop reduced responses cannot be ruled out. It would be important to repeat the assays in the future to enable further comparisons particularly with group 2 and an age matched healthy control group to be made. An important observation in this respect, however is that there was no relationship within group 1 between proliferative response and total treatment received. In future prospective studies of factor concentrates, an immune function assessment arm could be incorporated, for example testing lymphocyte proliferation (and comparing to age matched controls) on a yearly basis. Incorporating expensive and time consuming tests into routine patient care is however difficult. One purpose of studies such as those described here is to help to decide what would be beneficial in becoming part of 'normal' follow up.

The results of group 2 correspond to the previously observed immune abnormalities seen in HIV seronegative haemophiliacs, the reason for which requires an explanation. Both recurrent and chronic viral infections and repeated exposure to foreign proteins

have been postulated as contributing to the immune abnormalities described in haemophilic patients. The majority of these patients, irrespective of their HIV status, would have been infected with blood-borne viruses including hepatitis C and in some cases hepatitis B.

Within group 2 the boys who were hepatitis C seropositive had significantly reduced responses at low concentrations of Con A (5μg/ml) compared to those who were hepatitis C seronegative, implicating hepatitis C infection as a causative factor; however it may also imply that those patients had been in the past treated with greater quantities of FVIII. It would be important to investigate this group of boys in more detail to investigate whether the HCV positive subgroup show other evidence of being more immunosuppressed than the HCV antibody negative group. Their hepatitis C infection should be more closely examined to see if there was a relationship between hepatitis C viral loads or between virus genotype and immune function. If as implied by this study HCV is playing an important role in immune dysfunction, this needs to be more thoroughly investigated.

Looking again at the results in the presence of the lowest concentration of CON A. At low, sub-optimal concentrations of Con A (5μg/ml) the response of group 1 was lower than that of the control group 4 although not reaching statistical significance. It may be that the use of suboptimal concentrations of mitogen or stringent test conditions reveal abnormalities of lymphocyte function that would otherwise remain inapparent. In other published studies lower concentrations of lectins were indeed used including one where patients treated predominantly or exclusively with monoclonally-purified or recombinant factor VIII were found to have reduced lymphocyte proliferation and interferon-γ production when compared with controls (Newton-Nash et al 1996). It would have been a useful extension of the study described here to have gone on to investigate this observation further by using lower concentrations of both lectins, for example 1 and 2μg/ml. This may have clarified whether at lower concentrations of lectins the group 1 boys do show differences when compared to a control group.

Other possible factors were explored to investigate whether within the group FVIII treatment was influencing the lymphocyte responses which were not apparent when looking at the group as a whole.

The patients in group 1 had been treated with FVIII for between 3 and 61 months (median 39 months) and had therefore received vastly differing amounts of FVIII (between 1530 and 135125 units). It could be postulated that those patients having received only small amounts of treatment would have normal lymphocyte function whereas those having received a lot of FVIII concentrate would have had lower responses. This was found definitely not to be the case as there was no correlation between lymphocyte response at any concentration of lectin and the total amount of treatment received. Indeed when all the patients were looked at there appeared to be no relationship between lymphocyte response in any of the groups and treatment received in the last year, again suggesting that recent treatment received is not a major influence.

Within group 1 there was also a wide variation in the time between the last infusion of FVIII and the proliferation assay (1 – 184 days, median 19 days). This was important to consider in light of in vitro experiments which had demonstrated the down regulation of lymphocyte function when FVIII concentrates were present in the assay, (Hay 1990, Lederman 1986) implying the possibility of acute down-regulation of immune function following infusion of treatment. In these in vitro studies however the concentrations of FVIII used was substantially greater than would be achieved in vivo after treatment. BPL 8Y has not been studied in a lymphocyte proliferation setting but has been studied in relation to IL-2 production (Wadhwa et al 1992) where it was seen to reduce IL-2 production from stimulated T lymphocytes by 25% although this was less than other products of similar purity. However in the studies described here all patients studied had received no treatment within the previous 24 hours. There was no relationship seen between the time since the last infusion and the lymphocyte response in five of the assays. At a concentration of 50μg/ml of CON A the responses were reduced in those who had not received treatment for a long period of time. This was

not seen in the other assays and is a surprising result, which must be interpreted with caution.

The fact that there was in general no relationship seen between proliferative response and time since the last infusion does not of course discount the possibility of acute down-regulation of immune function following an infusion. It would be important to look at serial measurements of lymphocyte proliferation taken after an infusion of factor VIII perhaps on an hourly basis in these patients to better investigate the clinical relevance of the in vitro observations. If indeed acute changes are seen to occur however, there is no residual effect seen at 24 hours together with no cumulative effect as a result of increasing amounts of treatment.

The group 1 patients are unlike any other group previously investigated. They were treated with a single type of concentrate and were closely followed and were shown to remain free of hepatitis B, hepatitis C and HIV. They therefore represent a group from which one of the suggested causes of immune modulation - chronic blood borne virus infection has been eliminated and at this point in time do not have reduced lymphocyte proliferation to lectins.

Despite the limitations of this study – in that more assays, under more stringent conditions, with more suitable controls could have been carried out, the results imply that the presence of chronic blood borne viral infections other than HIV, such as hepatitis C contribute significantly to previously documented immune abnormalities. The concentrates themselves appear to be playing a more minor role. More detailed investigation of those individuals infected with HCV alone would be useful together with continued monitoring of groups of patients such as group 1, allowing comparisons to be made with future groups of haemophiliacs treated with different products.

CHAPTER NINE

STUDIES OF MONOCYTE FUNCTION IN HAEMOPHILIC BOYS

Background

Down regulation of the monocyte-macrophage system in the context of the treatment of haemophilia with large pool concentrates has been demonstrated both in the laboratory and by clinical observation. Tests of specific monocyte function on patients with haemophilia have been shown to be abnormal along with reduced function when experiments are performed in vitro in the presence of FVIII concentrates. An outbreak of tuberculosis in a group of haemophilic patients whose susceptibility to the infection was comparable to that of a group of children receiving immunosuppressive therapy for cancer, implied that the observed clinical abnormalities have clinical implications.

The reason for the immune dysfunction in the absence of HIV infection remains unclear and it has been suggested that they may result from the prolonged exposure to proteins or to another component in the concentrates or, alternatively occur as a result of repeated and chronic blood borne virus infections.

A cohort of patients has been established who were treated with only a single FVIII product, BPL 8Y and through prospective study were shown to remain free of infection with HIV, hepatitis B and hepatitis C infection. This group were therefore shown to be free of one of the potential causes of the previously described immune modulation and therefore a series of immune function tests were performed to ascertain as to whether they remained completely immunocompetent. This included looking at the interaction between monocytes and T cells , which had in other groups of haemophiliacs been shown to be abnormal.

The mononuclear phagocyte system

This system which plays a pivotal role in the immune response, consists of bone marrow promonocytes, circulating blood monocytes and both mobile and tissue macrophages. The blood monocytes being the precursors of most, although not all tissue macrophages. (Van Furth et al 1975). Initially thought to be primarily phagocytes, capable of ingesting and digesting exogenous material , it has become clear that these cells are also responsible for the presentation of antigen to other cells,

notably T and B lymphocytes and they also secrete cytokines such as IL-1 and IL-6 which are involved in the activation of both T and B cells and the propagation of the immune response (Auger & Ross 1992).

Monocyte function in the context of Haemophilia

Laboratory evidence of immune dysfunction has been widely reported in haemophilia in both the presence and absence of HIV infection. The clinical consequences of HIV infection have become startlingly clear, whereas the implication of and possible clinical consequences of non-HIV related immune dysfunction remain to be seen.

There have been as yet few reports of increased susceptibility to infections in these groups of patients, however one such report concerned a group of children, regularly treated with clotting factor concentrates, exposed to a case of open tuberculosis who were shown to be almost equally as susceptible to mycobacterium tuberculosis (MTB) as children receiving treatment for cancer. 38% of the clotting disorder patients developed evidence of infection, that was not related to underlying HIV infection, compared with 48% of children with leukaemia and solid tumours who were exposed. Both groups significantly greater than children with other disorders who were exposed, among whom only 4% developed evidence of MTB infection. (Bedall et al 1985a)

The implication of this clinical observation was that there was in these patients an underlying defect in the monocyte macrophage system, known to be of central importance in the immune response against intracellular pathogens.

There have been a number of in vitro studies showing the reduction in monocyte function that occurs in the presence in the assays of FVIII concentrates (Eibl 1987, Mannhalter 1988) and also one in vivo study demonstrating acute down regulation of monocyte dependant Fc phagocytosis following FVIII infusion (Pasi 1990).

Specific monocyte dysfunction including, reduced expression of MHC class II antigens, decreased phagocytosis, adherence and chemotaxis have been demonstrated in a group of haemophiliacs, the majority of whom had HIV infection (Roy et al 1988),

although reduced monocyte T cell interaction has been described in patients also without HIV (Mannhalter et al 1986).

Having established a group of patients remaining free of chronic blood borne virus infections despite treatment with a large pool product, monocyte T cell interaction was studied and compared to that of three other groups of patients; a healthy control group, a group of HIV infected haemophiliacs and a group of HIV negative haemophiliacs who had been treated with a variety of concentrates and were comparable to the historical groups of haemophiliacs in whom immune dysfunction had been demonstrated.

The Assay to assess monocyte T cell interaction

Monocyte antigen presentation was determined according to the method of Mannhalter et al (1986). As previously described in chapter 8, a central process in the immune response is the processing of foreign antigen by cells of the mononuclear phagocyte system followed by the presentation of the processed antigen in association with class II MHC antigens on the surface of the mononuclear cell to T lymphocytes. Only in this context will the T cell recognise the 'foreign antigen' and respond by proliferation and the production of cytokines leading to the cascade of the immune response.

This assay looked specifically at the ability of mononuclear cells to process and present a specific antigen, in this case heat inactivated *Escherichia Coli* 089 (*E. coli* 089). Proliferation of lymphocytes in response to the antigen presented by the monocytes (acting as a sole source of antigen) was measured.

Methods

Patients:

Eighteen of the original cohort of twenty five boys were studied (group 1). All, at the time of the study were seronegative for anti-HIV antibody and anti-HCV antibody and had been immunised against hepatitis B.

All had been treated with only one factor VIII product, namely BPL 8Y.

Patient details including age, period of follow up (time since first infusion), total units of FVIII received and the number of days since the last infusion of concentrate are detailed in table 9.1.

Comparison groups

Monocyte T cell interaction was assessed in three other groups of individuals and compared to the responses of group 1. Clinical details of groups 2, 3 and 4 are shown in table 9.2.

Group 2 comprised 13 haemophilic boys, treated with a variety of concentrates who remain HIV seronegative. 10 were seropositive for hepatitis C all and six had evidence of having contracted hepatitis B, one of whom remained surface antigen positive.

Group 3 comprised 18 haemophilic boys all of whom were HIV seropositive, all had serological evidence of infection with hepatitis B and C and four remained hepatitis B surface antigen positive. Three boys were receiving the antiretroviral drug zidovudine. All group 2 and 3 boys had received treatment with a variety of different FVIII products but for at least 18 months prior to the assay they had all been receiving only BPL 8Y.

Group 4 (control group) comprised 15 healthy volunteer donors (age range 2 - 47 years, median 29 years). None had any history of liver disease or risk factors for HIV infection.

patient number	age (months)	Time since first treatment (months)	total FVIII received (units)	Days since last infusion
1	74	61	135125	2
2	69	57	67345	2
3	66	57	173440	2
4	72	54	106730	1
5	58	50	51335	1
9	155	46	16200	120
10	58	43	66320	55
11	48	41	47530	19
12	61	39	35640	5
13	111	35	5265	125
15	29	29	10705	23
16	92	26	10870	184
17	50	10	20810	2
18	47	9	4190	19
19	23	12	23440	1
20	80	3	15060	80
21	34	8	6645	1
22	22	9	21960	32
24	80	3	3640	32

Table 9.1 Group 1 details (patients treated only with BPL 8Y)

Group	number in group	age (months)	median age (months)	FVIII exposure	Viral Status					
					HIV		HBV		HCV	
					positive	negative	positive	negative	positive	negative
1	19	22-155	61	BPL 8Y	0	19	0	19	0	19
2	13	87-181	112	various	0	13	6	7	10	3
3	18	105-224	168	various	18	0	18	0	18	0
4 (controls)	15	31-564	348	none	not tested (normal controls)					

Table 9.2 clinical details of comparison groups 2, 3 and 4

Laboratory methods

All assays were performed in the morning prior to any treatment being given to the patients.

Briefly, peripheral blood mononuclear cells (PBMC's) were isolated from 20mls of anticoagulated whole blood by density gradient centrifugation on Ficoll Hypaque (Lymphoprep, Nygaard Ltd, UK). After washing the cells were resuspended at a concentration of 2.5 x 10^6/l in RPMI and 10% heat inactivated serum. Monocytes were then obtained by adherence. Following washing the monolayers were incubated with a suspension of heat inactivated *E. coli* 089 H16 at a concentration of $1x10^8$/ml for three hours at 37°C and in 5% CO_2, after which the monocytes were washed and incubated with $1x10^5$ lymphocytes, using two concentrations of monocytes ($1x10^5$ and $5x10^4$ cells) to a final volume of 200µl in 96 well microtitre plates. The monocytes were therefore acting as the sole source of antigen to stimulate the lymphocytes.

At the same time $1x10^5$ PBMCs from each of the patients were incubated with 20 x 10^5/ ml heat-inactivated *E. coli* 089 H16 in 96 well plates under the same conditions for 7 days.

All cells were incubated at 37 °C in 5% carbon dioxide for 7 days, and were pulsed 18 hours before the end of the culture with 0.3 µCi of tritiated thymidine (Amersham, UK). The cells were harvested on to glass fibre filters which were washed and dried and the thymidine content and hence proliferation was determined by liquid scintillation counting. All assays were performed in triplicate and the results were expressed as mean counts per minute (cpm) following deduction of background proliferation measured by the unstimulated control that was included in every assay.

Statistical Analysis

The statistical methods used are described in detail in chapter 5. Briefly, the Kruskal Wallis one way analysis of variance was used to compare the responses of the four groups and the Mann Whitney U test to make pairwise comparisons between the groups.

Spearman's rank correlation coefficients were used to investigate within group 1 the relationship between the responses and both treatment received and the time since the last treatment episode before the assay.

Results

The lymphocyte proliferative responses of each of the four groups are shown in tables 9.3 (group 1), 9.4 (group 2), 9.5 (group 3) and 9.6 (control group).

There was no correlation within the control group between proliferative responses and age.

Patient number	proliferation					
	Expressed as counts per minute			Expressed as stimulation index		
	$1x10^5$/l added monocytes	$5x10^4$/l added monocytes	E. coli	$1x10^5$/l added monocytes	$5x10^4$/l added monocytes	E. coli
1	5162	984	5617	6.91	2.10	14.04
2	5055	10895	4817	7.23	17.66	11.00
3	1954	1655	13813	2.06	3.17	18.03
4	633	43	4177	2.85	0.19	12.32
5	150	92	37690	0.29	0.21	110.85
9	17096	14311	10732	9.42	6.73	7.19
10	3441	1524	510	6.67	4.60	3.23
11	3208	3079	11455	5.79	10.30	44.23
12	829	211	719	3.20	0.37	4.04
13	253	116	310	0.38	0.34	1.29
15	1099	1880	3262	2.69	4.21	9.85
16	8437	2880	787	12.96	6.26	2.24
17	184	1906	5349	0.47	5.81	19.31
18	91	92	3224	0.16	0.23	8.76
19	4882	361	5476	0.84	0.26	7.19
20	192	463	715	0.45	1.35	1.81
21	130	104	1930	0.36	0.5	5.44
22	1970	159	2136	0.84	0.26	7.19
24	1689	4298	13760	0.96	5.11	10.38
mean	**2971**	**2371**	**6657**	**3.4**	**3.7**	**15.7**

Table 9.3 Group 1 proliferative responses to heat inactivated E. coli and to monocytes presenting antigen

Patient number	proliferation					
	Expressed as counts per minute			Expressed as stimulation index		
	$1x10^5/l$ added monocytes	$5x10^4/l$ added monocytes	E. coli	$1x10^5/l$ added monocytes	$5x10^4/l$ added monocytes	E. coli
27	334	198	1027	0.94	0.53	4.63
40	4666	3203	4818	7.79	6.92	12.20
41	379	52	5436	3.08	0.21	19.01
33	192	573	3380	0.52	0.91	16.90
26	233	4216	4290	0.34	2.36	25.09
30	290	52	601	0.59	0.18	2.16
34	2805	1955	370	3.30	5.70	1.10
31	3713	4703	5886	4.91	4.84	18.45
39	1522	1946	1979	1.01	1.79	2.88
32	146	139	2190	0.29	0.59	12.88
38	72	148	468	0.30	0.46	1.98
37	1753	1429	4608	0.81	1.52	16.28
36	195	182	4045	0.81	0.73	10.59
Mean	**1239**	**1402**	**2749**	**1.89**	**2.05**	**11.1**

Table 9.4 Group 2 proliferative responses to heat inactivated E. coli and to monocytes presenting antigen

Patient number	proliferation					
	Expressed as counts per minute			Expressed as stimulation index		
	$1x10^5$/l added monocytes	$5x10^4$/l added monocytes	E. coli	$1x10^5$/l added monocytes	$5x10^4$/l added monocytes	E. coli
47	283	0	0	1.01	0	0
48	122	324	471	0.38	0.52	1.88
49	414	0	0	0.91	0	0
50	106	16	1100	0.45	0.06	4.74
51	2118	2560	2238	3.19	6.40	7.72
52	1625	2613	4588	6.63	11.36	24.15
53	384	581	1469	1.54	3.38	9.01
54	173	64	1228	0.15	0.13	1.98
55	103	21	925	0.33	0.09	2.84
56	139	1194	4051	0.45	6.06	6.53
57	2400	4400	5787	4.57	8.38	11.02
58	406	1071	936	0.29	1.35	1.86
59	892	903	3374	0.69	0.92	5.48
60	1246	1180	4373	0.79	1.78	7.04
61	51	411	323	0.09	2.58	1.86
62	277	194	0	2.69	1.64	0
63	290	69	7698	0.48	0.13	17.14
64	6292	2907	2720	25.37	8.48	8.14
mean	**962**	**1028**	**2293**	**2.8**	**2.95**	**6.2**

Table 9.5 Group 3 (HIV positive) proliferative responses to heat inactivated E. coli and to monocytes presenting antigen

control number	proliferation					
	Expressed as counts per minute			Expressed as stimulation index		
	$1x10^5$/l added monocytes	$5x10^4$/l added monocytes	E. coli	$1x10^5$/l added monocytes	$5x10^4$/l added monocytes	E. coli
1	4538	8353	9823	0.80	2.62	8.71
2	10767	3770	18045	5.48	1.92	9.18
3	1428	500	1906	1.62	1.37	6.98
4	4895	2270	11724	16.21	2.46	23.59
5	1274	1123	1578	2.82	3.74	6.89
6	2044	1022	3227	4.06	1.99	8.13
7	1154	1463	4763	3.48	2.91	12.91
8	853	908	3704	0.67	1.94	14.3
9	9091	5903	1225	39.19	25.44	5.21
10	2028	2588	3745	2.84	5.04	6.12
11	1667	1825	1340	1.31	1.07	1.18
12	11319	15321	10443	17.23	60.8	52.48
13	3989	4144	7427	11.30	15.35	24.43
14	5938	7120	1861	34.13	30.17	14.77
15	4689	2767	3013	9.10	16.67	8.10
mean	**4378**	**3938**	**5588**	**10.0**	**11.6**	**13.5**

Table 9.6 Group 4 (controls) proliferative responses to heat inactivated E. coli and to monocytes presenting antigen

Summary of results

A comparison of the proliferative responses of each of the four groups to heat inactivated *Escherichia coli* is shown in graph 9.1. Using Kruskal-Wallis one way analysis of variance, there were no differences in the proliferative response between the four groups.

In graphs 9.2 and 9.3 ($1x10^5$ and $5x10^4$ monocytes respectively) the proliferative responses of each of the groups to monocytes acting as the sole source of antigen are shown. Using one-way analysis of variance there was a highly significant difference between the four groups in terms of their proliferative responses ($1x10^5$.monocytes p=0.001 and $5x10^4$ monocytes p=0.01).

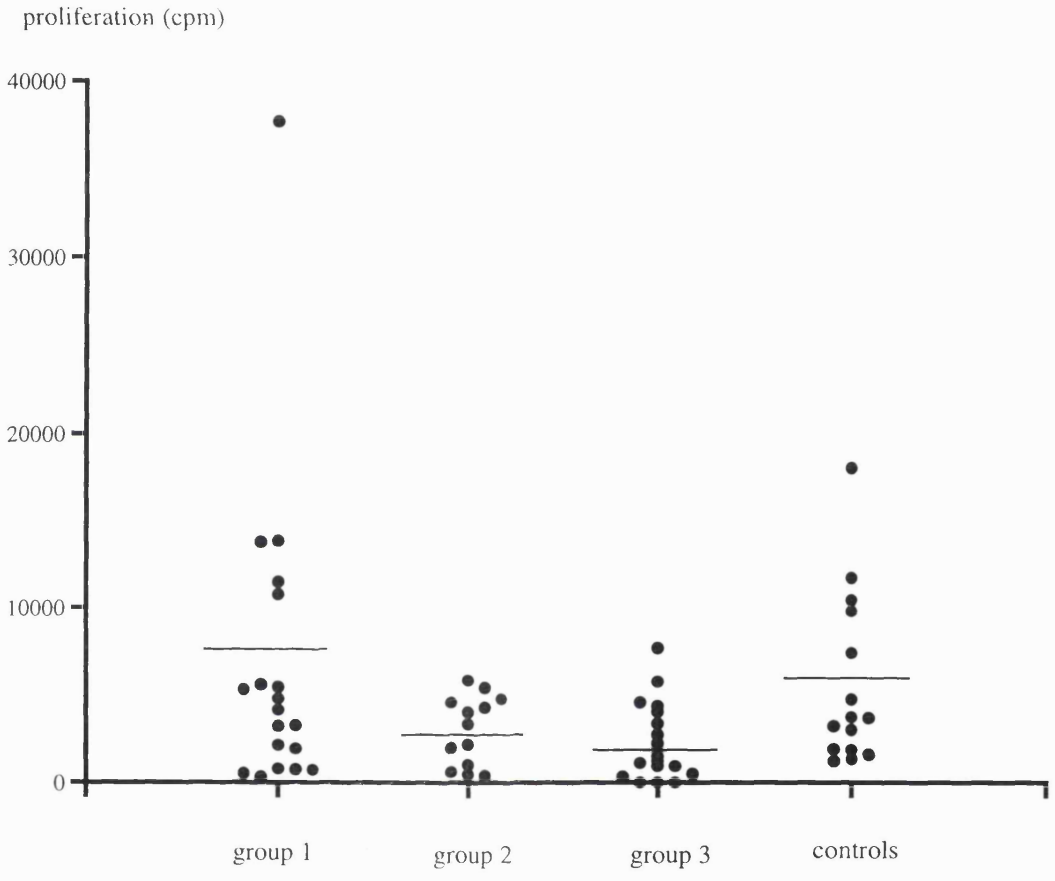
Multiple comparisons were then used to determine which individual groups significantly differed from each other, adjusting the resulting p values to compensate for multiple testing.

Comparison of the groups 1,.2 and 3 with the control group

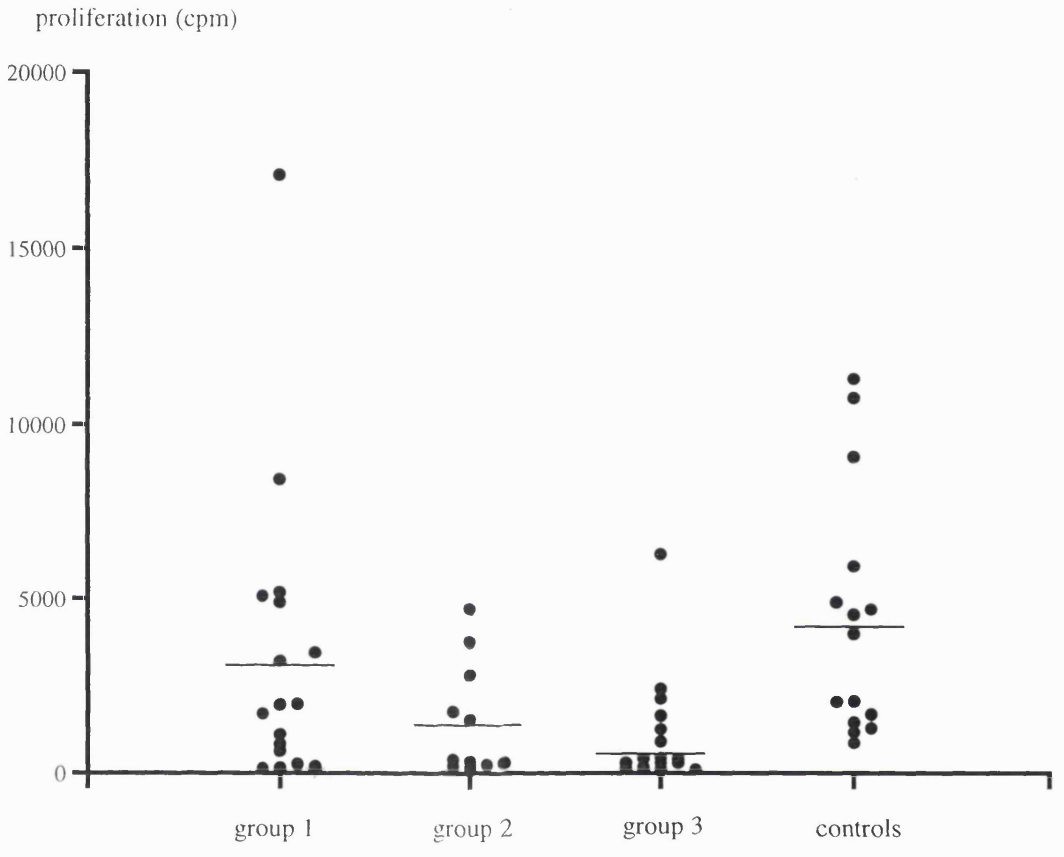
- There was no significant difference between the proliferative responses of group 1 and the control group at both concentrations of monocytes.
- The responses of group 3 were significantly decreased when compared with the control group.
- The responses of group 2 were lower than those of the control group but this reached statistical significance at the concentration of $1x10^5$.monocytes.

Comparison of the three patient groups

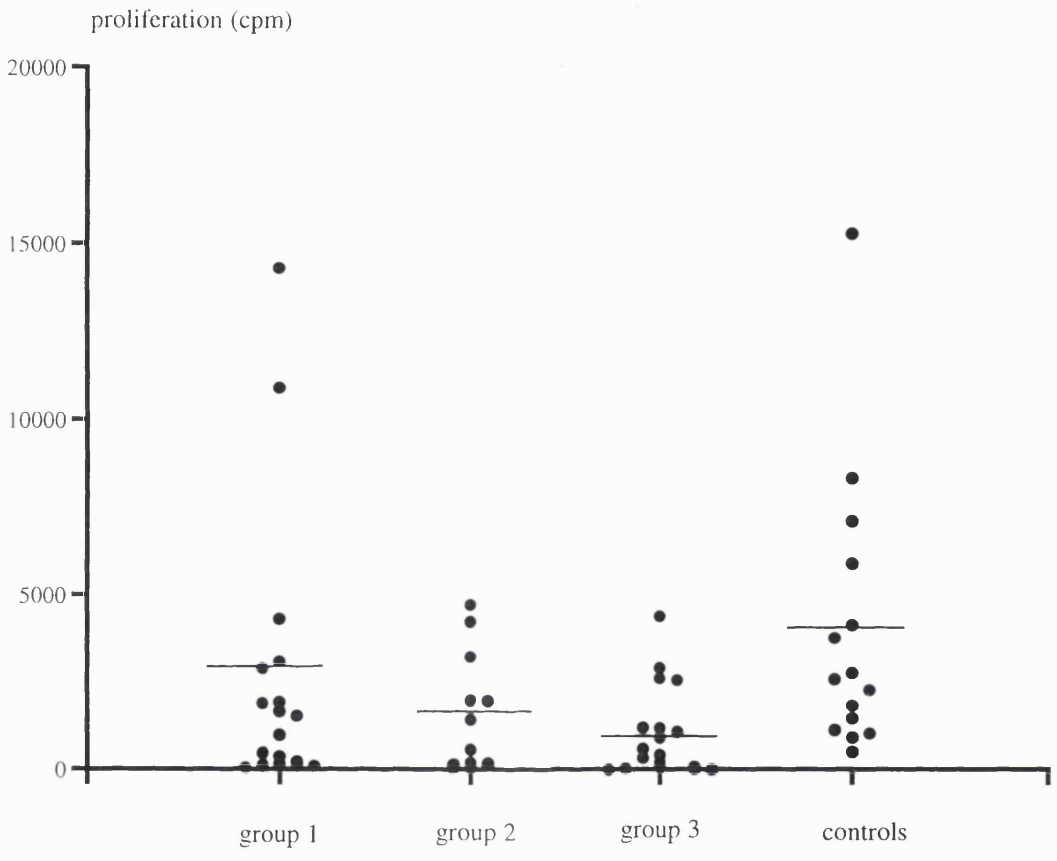
- At both concentrations of monocytes there was no significant difference between the responses of the groups 1,2 and 3.



graph 9.1 Lymphocyte proliferative response to heat inactivated E. coli
The horizontal bars represent the population means



graph 9.2 Monocyte-Antigen presentation
Proliferation of lymphocytes expressed as counts per minute in response to incubation with $1x10^5$ monocytes presenting antigen (heat inactivated E. coli)
The bars represent the population mean

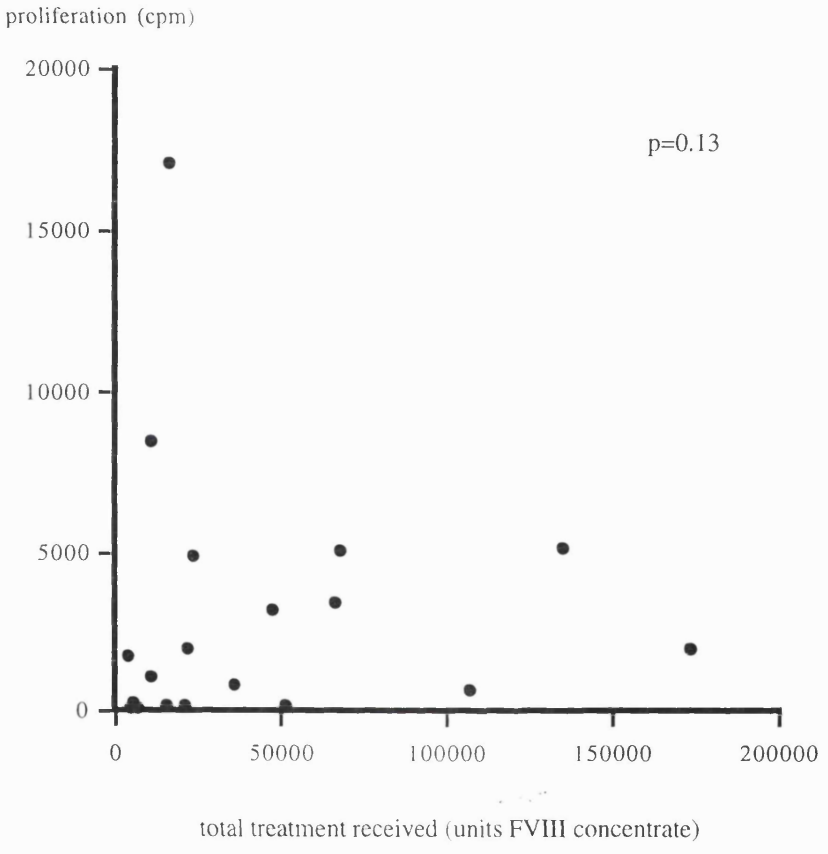


graph 9.3 Monocyte-Antigen presentation

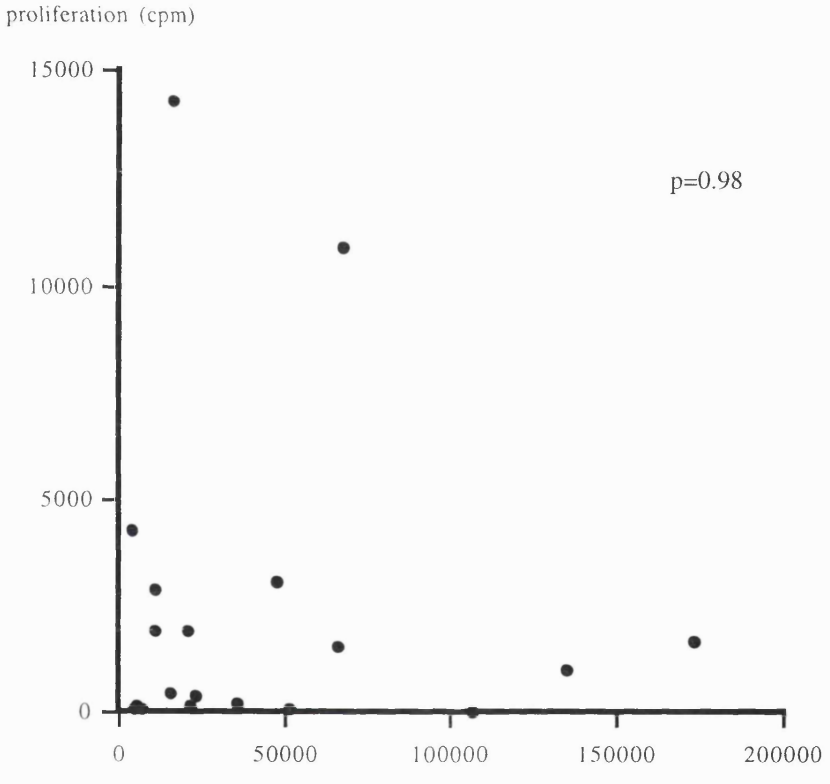
Proliferation of lymphocytes expressed as counts per minute in response to incubation with $5x10^4$ monocytes presenting antigen (heat inactivated E. coli)
The bars represent the population means

Relation to treatment received

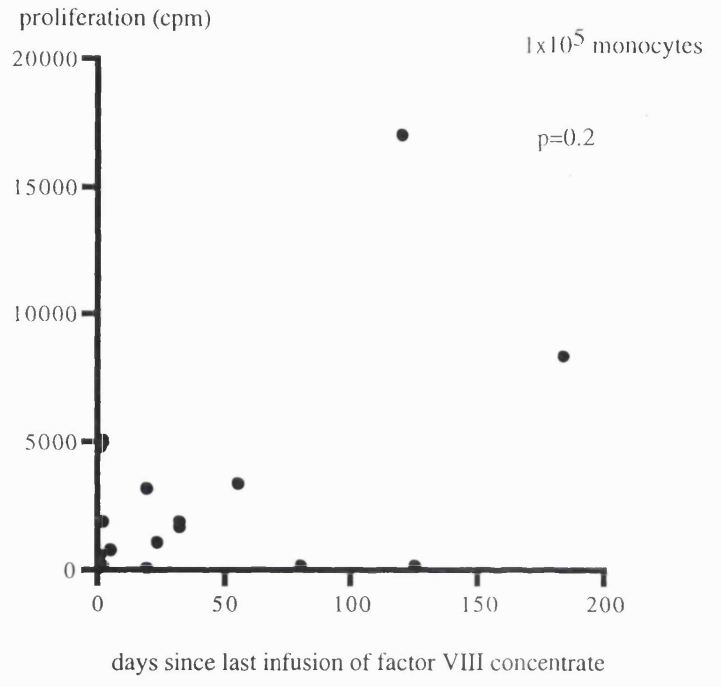
The total units of FVIII concentrate received by the haemophiliacs in group 1 at the time of the assay ranged between 3640 and 173440 units (median 21960 units). There was no correlation between the proliferative responses of the boys and the total amount of treatment received at either of the monocyte concentrations (graphs 9.4.1 and 9.4.2). All the assays were performed a minimum of twenty four hours after the last infusion of factor VIII. The time interval between the last infusion of FVIII and the assay varied between 1 and 184 days (median 19 days). There was no correlation between the proliferative responses of the boys and the length of time since the last treatment at either of the monocyte concentrations (graphs 9.5)

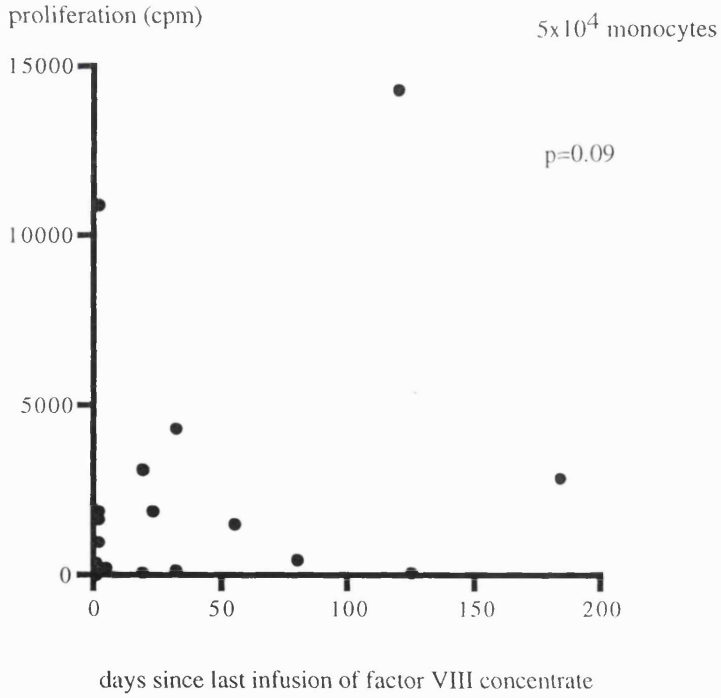


graph 9.4.1
group 1: relationship of proliferation (cpm) to total treatment received (units FVIII)
($1x10^5$monocytes)
There is no significant correlation between proliferative response and total treatment received



graph 9.4.2
group 1: relationship of proliferation (cpm) to total treatment received (units FVIII)
($5x10^4$monocytes)
There is no significant correlation between proliferative response and total treatment received





graph 9.5
relationship of proliferation (cpm) to the time since the last infusion of factor VIII (days) showing no significant correlation

Discussion

The presentation of antigen by monocytes to lymphocytes is a key event early in the immune response as a result of which both the cell mediated and humoral arms of the immune response are triggered.

This test of interaction between antigen presenting monocytes and T cells had previously been well described and had demonstrated down regulation of function in haemophiliacs treated with factor concentrates (Mannhalter et al 1986). It was therefore decided to use it to investigate the group of patients treated with BPL 8Y.

Further work from the same group attributed the down regulation to a component of factor VIII concentrates containing aggregates of immunoglobulin or immune complexes containing IgG (Eibl et al 1987).

The assay is however complex involving several steps in the procedure. All individual assays were performed in triplicate but on different patients on different days. It is therefore impossible to exclude minor variations in laboratory conditions which may have had an effect on the results. Ideally several assays should have been performed on healthy control individuals to assess what day to day variation occurs under normal circumstances. It would have then been possible to say whether performing one assay on each individual was sufficient, or what seems more likely whether a series of assays should have been performed to obtain a range of results.

The results obtained in the assays performed in this study expressed as counts per minute are in a range comparable to those described by the group who developed the assay. However, the cell responses in this assay are much lower than those obtained in the simpler assessment of lymphocyte response to lectins described in the previous chapter. When many of the results in the three comparison patient groups are low indicating poor proliferation it makes it more difficult to draw strong conclusions when comparing the groups with each other. Are the results low because the immune system is truly not functioning well or is it related to an error in the assay?

This is reflected by the fact that although the responses of group 1 appeared to be slightly higher than groups 2 and 3 there was no statistical difference found between

the groups. At the same time the responses of group 1 were not significantly different from the control group whereas those of group 2 and 3 were significantly worse than the control group, albeit group 2 at only one of the monocyte concentrations. These statistical results mean that one can interpret or emphasise the results as one wants, either stressing that group 1 did not differ from controls (ie behaving normally) or did not differ from the other two patient groups, implying a trend towards abnormal function. This study has therefore not provided a clear conclusion.

The study of the patient groups 2 and 3 in this study confirm the earlier observations of Mannhalter et al. The results of group 1 seem to fall somewhere in between. The selection of better control groups for group 1 may have been helpful in clarifying whether the responses of group 1 were normal or abnormal. Although within the control group 4 age was not related to response it would have been preferable to have used a group of age matched controls and to have performed several assays on each individual. The need for repeated venepunctures and the time available meant that this was not possible. It would also have been ideal to have performed the assay on the three patient groups at similar time points in terms of age and after they had received concentrates for a similar length of time. At the time the studies were done this was not possible as the assays had not been performed on the group 2 and 3 patients at earlier time points. Repeating the assays on the group 1 boys in the future and comparing the results with the present results on group 2 and 3 would remove 'time on treatment' as a variable. Performing serial assessments of immune function on the group 1 boys might give a better indication of whether immune dysfunction was developing. It may well be however that this assay is not suitable as a discriminator because of its complex nature and the absence of a broad 'normal' range.

All groups had no difference in response to a 7 day culture with heat inactivated E. coli, and it may well be that this is a less sensitive assay- in effect allowing enough exposure time to compensate for minor immune deficits. In contrast the short exposure time the monocytes had to the antigen in the other assay was not sufficient when the system is for some reason not functioning normally.

Looking in more detail at the responses within group 1 in relation to the treatment received, which may have revealed that those more heavily treated had poorer responses. This was in fact not the case as there was no significant association between proliferative response and total treatment received.

There was also no significant relationship between proliferative response and the time since the last infusion of factor VIII. This was important to look at in view of the in vitro data showing down regulation of monocyte function in vitro by the addition of concentrates (Eibl et al 1987). The fact that there was no relationship does not rule out the possibility that immune function is acutely down regulated following an infusion. It has indeed been demonstrated elsewhere (Pasi et al 1990). This could have been investigated more thoroughly by performing assays immediately after an infusion of factor VIII and during the subsequent twenty four hours. Carrying out such a study in a small child would however be difficult. The clinical implication is clear, if an individual is challenged with a potential pathogen at the same time as having an infusion of factor VIII, the response to that pathogen may not be adequate.

In summary this assay proved not to be ideal in the investigation of whether the patients remaining free of significant viral infection have evidence of immune modulation. The responses of the group 1 boys were no different from the control group but also not statistically different from the two other patient groups leaving the results open to ambiguous interpretation.

Repeating the assay at time points in the future and comparing results with a more suitable control group may provide clearer evidence, but it may be better to investigate the use of other simpler tests of monocyte function which are more discriminatory. .

The results do serve to emphasize the need for continued monitoring and surveillance of all patients receiving factor concentrates while also seeking the best tests with which to perform this assessment. This should also be considered for those patients treated only with newer high purity products, which have also been shown to down regulate monocyte function in vitro (Mannhalter et al 1990).

CHAPTER TEN

STUDIES OF SERUM IMMUNOGLOBULINS IN HAEMOPHILIACS TREATED SOLELY WITH BPL 8Y

Introduction

When it became apparent that haemophiliacs treated with large pool factor VIII concentrates were at risk for the acquired immunodeficiency syndrome, large groups of patients were studied and were found to have a range of abnormalities of both the cellular and humoral arms of the immune response. Total levels of IgG were found to be significantly raised in large cohorts of patients (Lee et al 1985, Moffat et al 1985). The higher IgG levels were found in older patients and in some studies in those who had received larger amounts of treatment (Lee et al 1985), while in other studies the changes occurred regardless of replacement therapy (Shannon et al 1986).

In vitro studies also demonstrated abnormalities of the humoral response. An Italian study demonstrated that peripheral blood mononuclear cells (PBMC) from haemophiliacs when grown in culture had higher spontaneous production of IgG than cells from controls. However pokeweed mitogen induced IgG and IgM production by PBMC was reduced in haemophiliacs when compared with controls, all implying that there was some underlying dysregulation of B cell function and antibody production (Biagiotti et al 1986).

Similar B cell abnormalities had been well described in patients with AIDS (Lane et al 1983) and in the Italian study of Biagiotti the individuals who were HIV positive had more marked abnormalities than the seronegative individuals. However the seronegative haemophiliacs had significantly greater spontaneous IgG production than controls suggesting a B cell abnormality independent of HIV infection.

It has been postulated that these abnormalities occur as a result of chronic antigenic stimulation, presumably from foreign proteins in the concentrates.

In this study total levels of serum IgG, IgA and IgM were measured in the group of patients, solely treated with BPL 8Y. These were measured prospectively over a period of years.

In healthy children, total immunoglobulin levels increase over the first three years of life therefore one would expect to see an initial increase in levels in these children

anyway. The normal values used by the laboratory in this study are seen in appendix 10.1.

The question remained as to whether this group of haemophiliacs, would have a gradual increase in levels of immunoglobulin as seen previously, although they were remaining free of significant viral infection.

Methods

Total IgG, IgA and IgM were measured prospectively on twenty four of the group of twenty five boys recruited into the study (group 1). Between two and eight assays (mean 5.3) assays were performed over a time period of between 3.5 and 11.2 (mean 7.6) years after commencing treatment with factor VIII concentrate.

IgG levels were also available on two further groups of patients which were used for comparison.

Group 2: 20 boys treated with cryoprecipitate and a variety of concentrates in the past. Fourteen of these boys were positive for hepatitis C antibodies, and 6 were negative. Seven of them had evidence of paast infection with hepatitis B.

Group 3: 22 boys also treated with a variety of concentrates in the past, all of whom were seropositive for HIV.

Results

The results of the total IgG, IgA and IgM levels of group 1 are shown in appendices 10.2.

One patient (18) had one IgG level above the normal range on one occasion, with subsequent values returning to within the normal range. All other IgG levels were within the normal range and looking at each patient individually there was no significant increase in IgG over the period of study.

Looking at the IgA results, six patients had levels above the normal range on ten occasions. Three of these results were from one patient (9) who was a mild haemophiliac, and had received minimal treatment. His IgG and IgM levels were within the normal range and were stable. One patient had two IgA levels below the normal range.

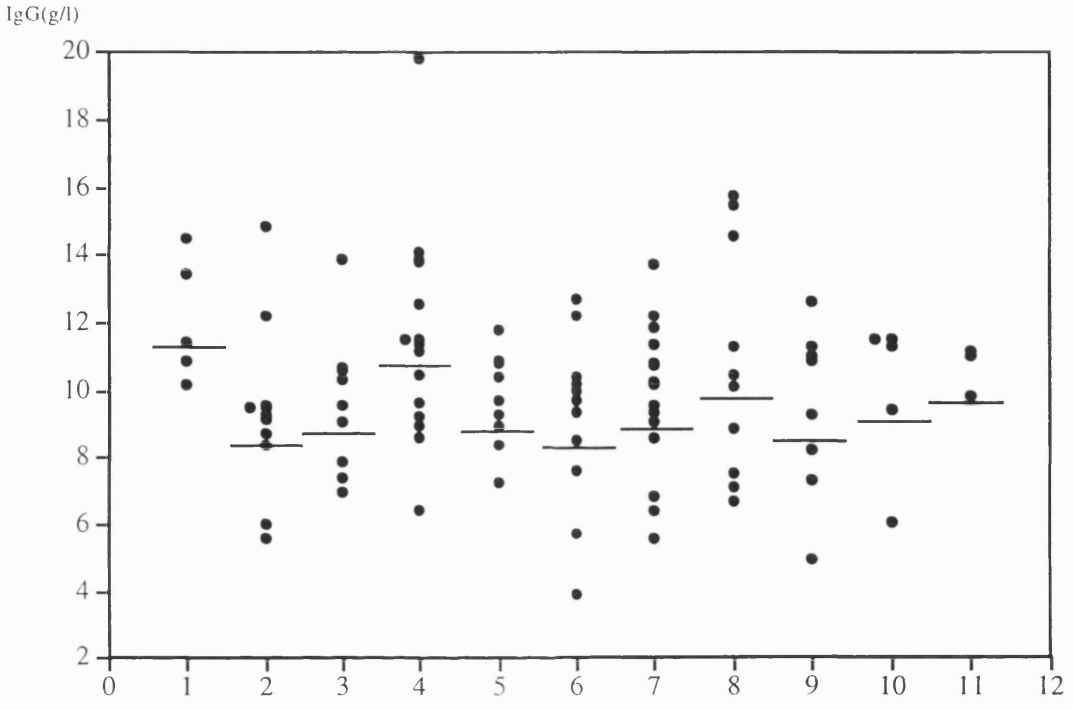
Six boys had nine IgM levels slightly above the normal range, all of which subsequently returned to normal.

Between birth and the age of three, the level of IgG in healthy children increases, reaching a steady 'normal range' above the age of three years (appendix 10.1). The levels of IgG were investigated in the twenty four boys in the time period when they were older than three years to avoid any changes being due to normal 'ageing'.

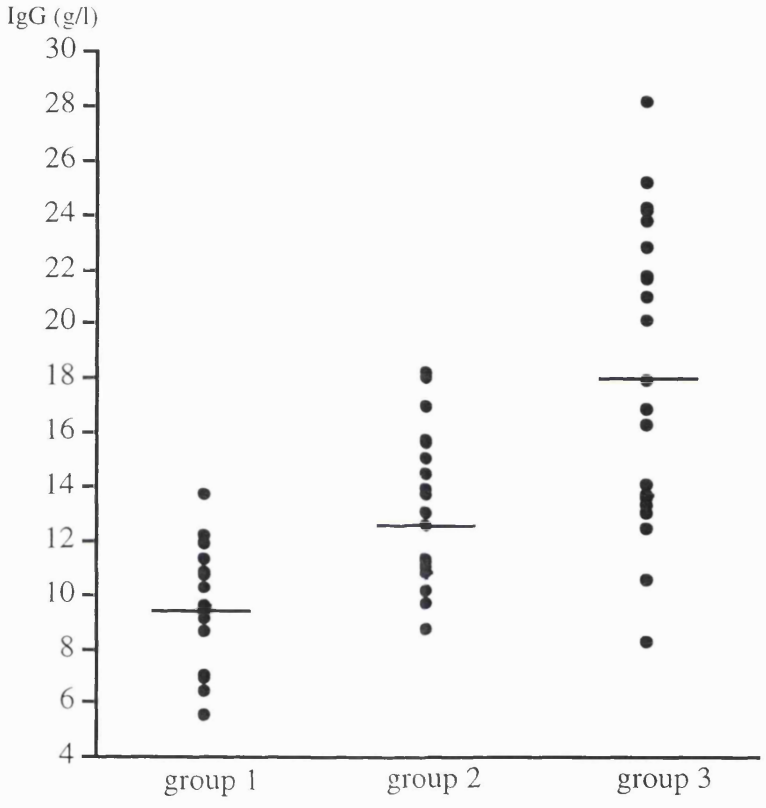
The implication in previously studied groups of haemophiliacs is that changes in Ig levels are related to treatment with concentrates. Therefore the IgG levels were looked at according to the number of years since treatment had started. The results are shown in graph 10.1.

The levels of IgG are grouped together according to the year after treatment began. The levels of IgG were compared by the Kruskal Wallis test for multiple comparisons which showed no significant difference between the levels measured in each year after starting treatment. There is no significant increase or decrease in mean IgG level over eleven years of follow up.

A comparison was made with two other groups of haemophiliacs within the unit on whom serum IgG levels were available. The year in which these patients had started treatment with either concentrate or cryoprecipitate was recorded and IgG levels were taken from if possible the seventh year after treatment began in order to make a direct comparison. The individual IgG levels and the time at which they were taken are shown in appendices 10.3 and 10.4. The comparison with group one is shown in graph 10.2.



graph 10.1: Total IgG levels in the years following the start of factor VIII treatment in patients aged over three years treated solely with BPL 8Y. The upper limit of normal for this age group is 16.1 g/l. Horizontal bars depict mean values, which are not increasing over time.



graph 10.2: Comparison of IgG levels of three groups of haemophiliacs.

group 1: treated solely with BPL 8Y
group 2: treated with cryoprecipitate & a variety of concentrates- HIV negative
group 3: treated with cryoprecipitate & a variety of concentrates- HIV positive

The patients in each of the three groups were of similar ages and had been on treatment for a similar length if time (table 10.1).

Group	Number in group	Age (range and mean)	Length of time on concentrate	Mean IgG level (g/l)
1	16	9.3 (7.3-14.1)	7.4 (7.2-7.9)	9.9
2	20	10.4 (6-15)	6.3 (2-9)	13.1
3	22	9.5 (7-13)	7 (6-8)	18.2

Table 10.1: Comparison of IgG levels in three groups of haemophiliac boys seven years after starting treatment. (Complete data in appendices 10.3 and 10.4).

As can be seen in the graph although the boys are of similar ages and have been on concentrate for similar lengths of time, the IgG level is higher in both group two and three when compared to group one. The levels of IgG in each of the three groups were compared by the Kruskal Wallace test which showed a highly significant difference between the three groups ($p<0.0001$).

Using the Mann Whitney U test to compare two groups, the IgG levels of both group 2 ($p=0.0016$) and group 3 ($p<0.0001$) were significantly higher than those of group 1.

Discussion

Repeated infusions of factor VIII concentrates had been proposed as being the cause of non-specific hypergammaglobulinaemia documented in haemophiliacs. The raised levels were most marked in older individuals who had received large quantities of treatment who were most likely to have been infected with HIV. There was also evidence however of B cell dysregulation in patients remaining HIV seronegative.

In this study, it can be seen that through a period of follow up of up to 11.2 years, twenty four haemophiliacs have stable levels of IgG. It may be said that the boys are as yet too young to draw any conclusions from this, as it was seen in previous studies that immunoglobulin levels were more commonly raised in adults as compared with children.

Therefore, a comparison was made with the two other groups of haemophiliacs in the same unit. The levels of IgG after approximately seven years of concentrate treatment were compared in the three groups, who at the time the samples were taken were of similar ages.

As might be expected, the group three patients infected with HIV have significantly raised IgG levels when compared with the other two groups. Interestingly group two who have been treated with different concentrates, and the majority of whom are positive for hepatitis C also have higher levels of IgG when compared with group one. This result implies that when looking at group one there has been a sufficient time period to allow for some abnormalities to occur. In an ideal situation one would have a group of healthy age matched controls to compare with group one, because although group two have higher levels, only three boys have levels outside of the normal range. It is possible that group one, although within the normal range also has higher baseline levels. This, however is less likely to be the case when it is considered that there has been no increase in IgG level over up to eleven years of follow up of group one. If this group did have a higher baseline than healthy boys of the same age, one would have expected to see an increase in levels over the follow up period.

In summary therefore, it appears that this group of patients treated with one intermediate purity concentrate, remaining free of blood borne viral infections are not demonstrating evidence of B cell dysregulation, seen in previous groups of haemophiiacs, both HIV seropositive and negative.

CHAPTER ELEVEN

FACTOR VIII INHIBITORS IN A COHORT OF HAEMOPHILIACS TREATED SOLELY WITH BPL 8Y

A serious complication of haemophilia is the development of antibodies or inhibitors to factor VIII. This has enormous clinical and economic implications and monitoring groups of patients for the development of inhibitors is a very important part of the follow up of patients being treated with a new factor VIII concentrate.

Methods

The patients recruited into the study had screening tests for the production of inhibitors. This was started in 1990-1991 and therefore the first patients admitted into the study were not from the outset regularly screened.

On each occasion an initial screening assay was performed and if this was shown to be positive then a Bethesda Inhibitor assay (Kasper et al 1975) would be performed as described in chapter 5.

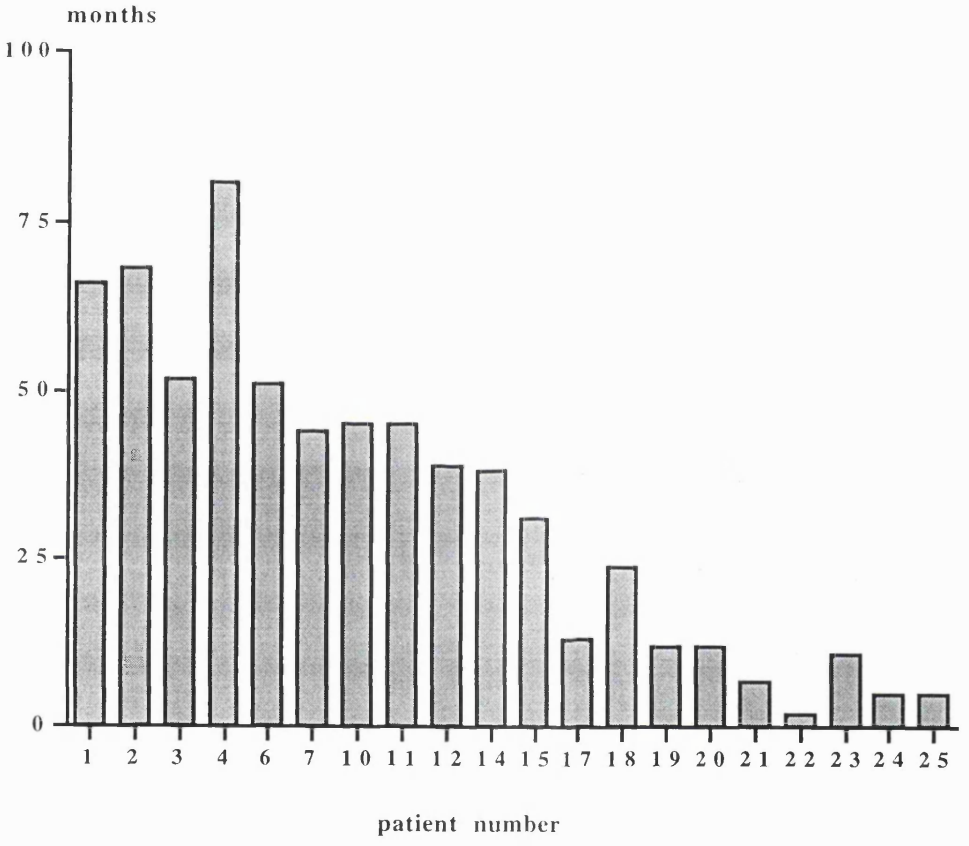
Results

The results of inhibitor screening tests are shown in appendix 11.1.

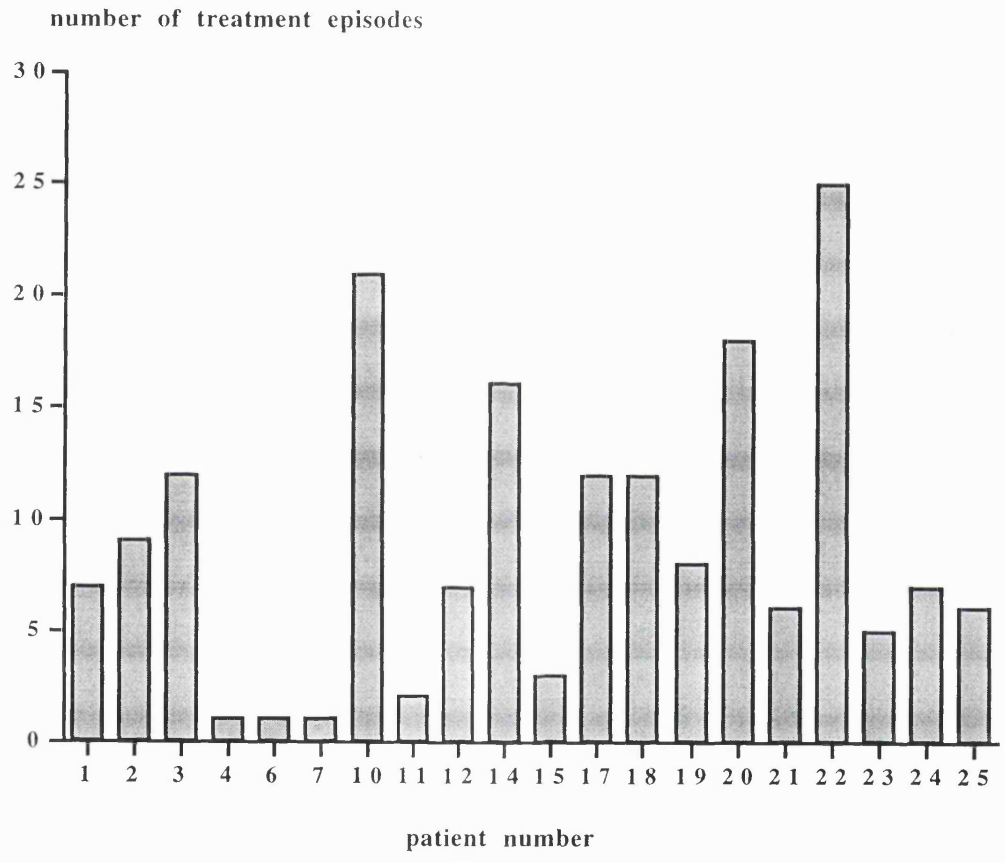
Twenty of the twenty five patients had regular inhibitor screens between 1990 and 1995. Only one patient on one occasion had an equivocal test result, which was subsequently consistently negative.

Unfortunately not all of the patients were screened from the outset of the study. Graph 11.1 shows the time between the start of factor VIII treatment and the introduction of regular screening assays, which ranged between two months and five years.

There has been some discussion as to whether the pattern in which factor VIII is administered has some bearing on the tendency to develop inhibitors. The number of treatment episodes received by each patient in the first three months of starting treatment is shown in graph 11.2. The number of treatment episodes received in this initial treatment period varied greatly from 1 to 25 episodes.



graph 11.1: time interval (months) between start of factor VIII treatment and regular inhibitor assays



graph 11.2: Number of treatment episodes received by each patient during the first three months of factor VIII treatment

Discussion

The development of antibodies to factor VIII or as they are known 'inhibitors', is a serious complication of haemophilia A. It results in the inability to raise factor VIII levels by conventional treatment and a clinical picture similar to that which was seen before treatment with factor concentrates became established (Rizza & Spooner 1983, McMillan et al 1988). The development of inhibitors in a mild or moderate haemophiliac, although rare, dramatically changes the pattern of disease effectively converting them into a severe haemophiliac (Hay et al 1998).

There has been much discussion as to the incidence of inhibitors. 6% of all UK registered haemophilia A patients between 1969 and 1980 developed inhibitors (Biggs 1977, Rizza and Spooner 1983). However the prevalence of transiently occurring inhibitors is much higher at around 40% (Bray et al 1994, Ehrenforth et al 1992) but between 5 and 10% persist and are clinically relevant.

It has long been clear that there is some sort of genetic predisposition to inhibitor formation as they occur more commonly in family members and there are also racial differences in incidence being more common for example in African Americans and individuals of Latin origin (Aledort & Dimichele 1998). With the rapidly increasing knowledge about the mutations within the factor VIII genome, why certain individuals have an increased tendency to develop inhibitors is becoming clearer. Patients with large deletions or inversions of the gene tend to produce no circulating factor VIII, and will therefore react to infused factor VIII concentrate as a 'foreign' protein. This group of patients have a high prevalence of inhibitors at between 35 and 40% (Schwaab et al 1995a, Tuddenham & McVey 1998). A high incidence is also seen in individuals with mutations resulting in a stop codon, for the same reason being that they produce no circulating factor VIII.

Individuals with mild and moderate haemophilia have a lower incidence of inhibitors although they do occur. They have circulating factor VIII, although the levels are low and therefore one would expect that infused factor VIII would not be recognised as being foreign. Again a genetic basis appears to provide the explanation for some of

these haemophiliacs having a greater tendency to produce inhibitors. The mutations in these individuals tend to be clustered around a certain point on the genome, which may well lead to the production of factor VIII antigenically different from the factor VIII that is infused in concentrates (Schwaab et al 1995b, Fijnvandraat K et al 1997, Thompson et al 1997).

Genetic predisposition does not provide the sole reason for inhibitor development and much concern has focused on the role of the products themselves triggering their production. The introduction of the higher purity and recombinant products gave rise to much concern that they were associated with a higher incidence of inhibitors (Bray et al 1994, Lusher et al 1993).

These inhibitors were often transient and of no clinical consequence (Zanon et al 1999). It is now generally accepted that rather than a true increase in incidence of inhibitors, these results reflect the fact that the patients are now being studied more closely and transient appearances of inhibitors are more likely to be picked up.

In this study of an intermediate purity product no patient developed factor VIII inhibitors over a five year period. One cannot, however, make strong conclusions about the development of inhibitors in patients treated with BPL 8Y based on this study. The patients were not regularly screened directly after starting treatment. It has been shown that inhibitors tend to develop early, during the first infusions of factor VIII (Schwarzinger et al 1987, Ehrenforth et al 1992), and more quickly in previously untreated patients rather than those changing to a new product. However although transient low titre inhibitors may have been missed no clinically significant inhibitor developed, This was also seen in another long term study of 33 patients treated with BPL 8Y (Brown et al 1998) although how stringent the testing regime was is not indicated in the paper.

Some of the patients described in the study described here were included in a review of inhibitor development in patients treated with BPL 8Y from the Royal Free Hospital (Yee et al 1997). In 37 severe haemophiliacs treated solely with BPL 8Y no one

developed a significant inhibitor over a ten year period, although based on historical data one might have expected between 5 and 10% of the patients to have done so.

Indeed in the time period between 1975 and 1985 in the same two large treatment centres in the UK, 22 of 145 (15%) severe haemophilia A patients developed inhibitors. (Yee et al 1997). It would be incorrect however to imply that no patient treated with BPL 8Y has ever developed an inhibitor; in a summary of inhibitor development reported between 1990 and 1993, 10 of 32 reported cases of inhibitors were treated with 8Y prior to development of the inhibitor but this could simply reflect the fact that at that time most UK patients were being treated with 8Y (Colvin et al 1995, Dr. E. Gascoigne, BPL; personal communication). There have been no studies of inhibitor development in patients treated with BPL 8Y comparable with the studies on the new high purity recombinant products, in terms of strict regular testing from the outset of treatment (Bray et al 1994, Lusher et al 1993).

Although the testing regime for inhibitors in this study was not as stringent as that which would now be recommended it does appear that the incidence of inhibitors in this group is lower than expected, the explanation for which is not clear. One possibility is that it is a result of an immunomodulatory effect of the factor VIII product itself, or that it is a direct effect of the well preserved von Willebrand factor multimers known to be present in this concentrate (Lawrie et al 1989). The multimers may be blocking the epitopes on the light chain of the factor VIII molecule, to which it is known most of the inhibitor antibodies react (Yee et al 1997). In support of this, in experiments looking at the effect of inhibitor antibodies on different concentrates, it has been documented that the antibodies are less inhibitory towards factor VIII in concentrates rich in von Willebrand factor (Berntorp et al 1996, Suzuki et al 1996). That the low inhibitor incidence is occurring as a result of immune modulation is unlikely because as has already been described this group of haemophiliacs are not demonstrating marked changes in immune function, in contrast to previous groups of haemophiliacs where immune changes and inhibitors were found frequently to co-exist.

Changing products may also be important, as was illustrated by an outbreak of inhibitors which occurred with the introduction of a pasteurised version of a previously dry heated intermediate purity concentrate (Rosendaal et al 1993). Such incidents may not only occur when the product is changed but also when different batches of the same product are introduced as was seen with ann outbreak of inhibitors in Germany and Belgium (Peerlinck et al 1997, Rosendaal et al 1997). With complex production and purification methods some slight alteration may lead to an antigenic change leading to inhibitor formation. Such observations underline the importance of continued monitoring of groups of patients and particular caution when a group of patients are switched to another product, which may well be the case for the group of patients described in this study.

The fact remains that the formation of inhibitors have dramatic consequences both clinically and economically. It appears that groups of patients such as the one described here have a low incidence of inhibitors. As new production methods are introduced it is important that all groups of patients are monitored extremely closely so that products appearing to predispose to inhibitor formation can be recognised and appropriate action can be taken.

CHAPTER TWELVE

SUMMARY OF RESULTS

Twenty five patients with haemophilia A were recruited into this study between 1985 and 1991. All were treated with the intermediate purity factor VIII concentrate BPL 8Y, which is prepared from United Kingdom blood donations and treated by heating at 80°C for 72 hours.

There was no evidence of transmission of hepatitis B, hepatitis C or the human immunodeficiency virus by this concentrate, all patients remaining HIV and hepatitis C seronegative. They were all vaccinated against hepatitis B.

A series of immune parameters were assessed in this group of patients. Serial T lymphocyte subsets were performed on twenty one of the patients. With one exception the CD4 counts remained stable over a period of ten years of study. Six patients had changes in CD8 counts, three increasing and three decreasing. No consistent association was found between CD4 and CD8 counts and the treatment received.

Serum IgG levels remained stable over eleven years of follow up. The level of IgG in this group of patients was lower when compared with two other groups of haemophiliacs, who had been treated for a similar time period but with earlier generations of concentrates. One group was of HIV seropositive patients and the second had been treated with a variety of concentrates and the majority of whom were infected with hepatitis C or B.

Lymphocyte proliferation to the T cell mitogens were assessed in twenty three of the patients and were compared with the two other groups of haemophiliacs and a group of healthy controls. There were no differences between the proliferative responses of the BPL 8Y group and those of the controls, and their responses were consistently better than those of the HIV seropositive haemophiliacs. Although the responses of the BPL 8Y patients were always better than those of the HIV seronegative, hepatitis C positive group this only reached statistical significance at lower concentrations of CON A.

It was observed that at a sub-optimal concentration of CON A the response of the BPL 8Y haemophiliacs was reduced compared to that of controls but this difference did not reach statistical significance.

The ability of monocytes to present antigen to lymphocytes was assessed in eighteen of the patients. The response was measured in terms of the ability of lymphocytes to proliferate in the presence of monocytes acting as the sole source of antigen. The antigen used was heat inactivated *Escherichia coli.* Although the responses of all three groups of haemophiliacs was equal to the control group to prolonged exposure to the antigen, marked differences were seen when the sole source of antigen was patient's monocytes exposed to E. coli for only three hours. There was no statistical difference between the responses of the controls and the BPL 8Y group, whereas the responses of the other two groups were significantly lower. However looking at the results, the responses of the BPL 8Y group were tending to be lower than those of the controls although still better than those of the other haemophiliacs.

In both the monocyte and lymphocyte proliferation experiments there was no association found between the cell responses and the amount of factor VIII treatment received.

CHAPTER THIRTEEN

CONCLUSIONS AND DISCUSSION

This study set out with two main aims, firstly to establish whether individuals treated with a new plasma derived product would remain free of significant viral infection and secondly if this was the case would they go on to develop the previously described immune abnormalities seen in haemophiliacs.

There is clear evidence from this and other studies that HIV, hepatitis B and hepatitis C are not transmitted by this product. However this does not mean that it is entirely free from risk. As was well demonstrated by the AIDS epidemic, viruses can suddenly appear and some may have characteristics rendering them less susceptible to the viral inactivation processes in use. For example, viruses which lack a lipid envelope are not eliminated in the production process of BPL 8Y. One such virus, parvovirus B19 was not routinely tested for in the course of this study, but in another cohort also solely treated with BPL 8Y the patients studied had a 100% prevalence of parvovirus B19 antibody (Brown et al 1998). This is significantly higher than that which would be expected in the healthy population. (Cohen & Buckley 1988). There have been well documented outbreaks of hepatitis A, although not specifically related to 8Y, which also lacks a lipid coat, in groups of haemophiliacs in recent years (Mannucci et al 1994). This virus was previously considered not to be a transfusion risk, being spread by the faeco-oral route but it evidently can be and was transmitted.

Hepatitis G, a flavivirus like hepatitis C is present in 3% of the donor population (Ludlam 1997) and has been demonstrated as being transmissable by plasma products, in haemophiliacs the prevalence being between 12 and 15% ((Jarvis et al 1996). It appears as yet to have no serious clinical consequences and may not be hepatotoxic and it is not recommended that haemophiliacs be routinely tested for it (Makris et al 2001). Although these other viruses do not have the fatal consequences of HIV and the other hepatitis viruses, the implication remains that plasma derived products including BPL 8Y are still capable of transmitting viruses and there will always remain the possibility of new viruses or of pre-existing ones changing to become more dangerous. It would be far from correct to say that any plasma derived product is virally safe.

Viruses are not the only cause of concern. During the 1990's there were increasing reports of the progressive and fatal neuro-degenerative disease Creutzfeld-Jakob Disease (CJD). This is caused by the transmission of a prion protein, which in some way is capable of modifying the structure of proteins in the nervous system of the invaded host and causing extensive damage. Transmission by neural-derived tissue, such as corneal transplants and growth hormone injections is well documented, but there have been to date no infections by blood transfusion (Heye et al 1994), although a possible link to human albumin has been proposed (Creange et al 1996). A study of 33 post-mortem brain specimens from haemophiliacs between 1962 and 1995 also showed no evidence of prions or classical histological changes (Lee et al 1998). However, concern was sufficient to lead to the recommendation that batches of plasma products contain donations from affected individuals should be withdrawn (World Federation of Hemophilia 1995).

Turning to the question of immune modulation. This study set out to ask whether the FVIII concentrate BPL 8Y caused immune modulation in vivo, taking note of the fact that it did down regulate function in vitro (Thorpe et al 1989, Pasi et al 1990 & Wadhwa et al 1992).

Only one other published study has looked at patients treated long term with only BPL 8Y (Brown et al 1998). In this study of 33 patients treated for up to 152 months with BPL 8Y the only immune function tests carried out were CD4 and CD8 cell subsets. There were borderline reductions in CD4 counts, five patients having had levels below the age related normal range on two occasions. However only one patient had persistently low CD4 counts. No comment was made as to what was happening to the counts of individuals over time.

These results provide no convincing evidence for developing immune dysfunction.

To summarise the findings during the course of the study described here:

Over ten years of follow up the patients have stable levels of CD4 and CD8 cells and serum IgG.

The peripheral blood lymphocytes of these patients have normal responses to T cell mitogens when compared to a healthy control group.

In a study of monocyte T cell interaction the proliferative responses of lymphocytes to antigen presented by autologous monocytes were comparable to responses of a healthy control group.

Have the studies described here clearly answered the question, 'does BPL 8Yinduce immune modulation in vivo'? It has to be said that this question remains unanswered. After ten years of follow up, this group of patients are not exhibiting the immune abnormalities described in previously described groups of HIV seronegative haemophiliacs, in that they have stable CD4 and CD8 counts and serum IgG levels.

Based just on these results, could one say that the concentrate does not cause immune modulation? It would be wrong to do so because although these tests give a broad reflection of the two arms of the immune system they are not sufficient on their own to rule out any form of immune dysfunction.

At the time when these studies were performed they provide no evidence that these patients have down regulation of the immune system as has been seen in previously reported groups of haemophiliacs. There are, however significant limitations to the interpretation of these results. Firstly, looking at the functional assays. These were performed between 3 and 61 months after starting treatment (average 30 months) and this may be insufficient time or the patients may have received too little treatment to have developed the previously reported abnormalities. The responses of the BPL 8Y group were better than the two other patient groups included in the study. This is unsurprising in the case of group 3 who have HIV infection. Group 2 is a more interesting comparison group, similar to the 'historical' groups of HIV seronegative haemophiliacs in whom immune abnormalities have been described. Their lymphocyte responses were generally poorer than those of group 1 and the healthy controls but this reached statistical significance at only two concentrations of one mitogen. They would have comprised a better control group if they had been on treatment for a similar

amount of time and had received similar amounts of treatment as those in group 1. Group 2 patients had been receiving treatment for an average of 94 months at the time of the assay. Unfortunately data was not available on them at an equivalent time point to group 1 as immune function testing had not been performed in the past. A true comparison between the two groups can only be made at a point in the future when the group 1 boys have been treated for a similar amount of time. It is important to note however that within group 1 where patients had been on treatment for very different lengths of time there was no relationship between lymphocyte responses and total treatment received which implies that immune function is being maintained in this group despite treatment.

It was only possible in the studies of IgG levels to make a direct comparison between the three groups using historical data and comparing IgG levels when the patients had been treated for comparable time periods. Here it can be clearly seen that after seven years on treatment group 2 and 3 patients have significantly higher levels of IgG than the group one patients. The results of the other studies described here would have greater weight if such direct comparisons had been possible.

All the studies would have benefited from the use of an age matched control group for the group 1 patients. The control group used in the functional assays contained seven children under ten years and on testing there appeared to be no relationship between response and age however it would have been better to make direct comparisons with age matched control children. It would also have been useful to have such a control group for the serial T cell subset studies although the use of z scores tried to eliminate the problem of normal age related changes. Normal childhood ranges for T cell subsets have only recently been reliably established and not much is known about what the normal day to day fluctuation is. The patients described here although having overall stable levels of CD4 and CD8 showed considerable variation in levels and a comparison with age matched healthy children would have been useful. Comment has already been made about the problems of frequent blood testing in children with regard

to the diagnosis of non A non B hepatitis and it is obviously more difficult to obtain samples on healthy children for comparison, especially on more than one occasion.

Looking closely at the results from the functional assays, where as already said there was no statistical difference between the responses of group 1 and controls. However simply looking at the responses to low, sub-optimal concentrations of Con A the response of the group 1 patients was lower than that of the control group although not reaching statistical significance. Similarly in the study of monocyte function, the responses of group 1 although lower, were not significantly different from the control group but they were also not statistically different from groups 2 and 3 whose responses were significantly poorer than controls leaving the result open to different interpretations. These studies would have benefited from further investigations using lower concentrations of mitogens as have been used in other studies and making direct comparisons with appropriate control groups. Although the statistics point towards there being no difference between group 1 and controls, simply looking at the data gives rise to some doubt as to whether the results of group 1 are entirely normal.

Other studies have used lower concentrations of mitogens for example, in a study of haemophilia A patients treated predominantly or exclusively with monoclonally-purified or recombinant factor VIII, they were found to have reduced lymphocyte proliferation and interferon-γ production when compared with controls (Newton-Nash et al 1996).

The relevance of in vitro observations that the addition of concentrates in cell culture can down regulate immune function was not well tested by the studies described here. All functional assays were performed at least twenty four hours after the last infusion of factor VIII and there was no relationship between lymphocyte response and time since last treatment. However acute down regulation of function in the hours following an infusion was not investigated. If there is such an effect as the results of the in vitro studies imply then the clinical implication is clear; if an individual is challenged with a potential pathogen at the same time as having an infusion of factor VIII, the response to that pathogen may not be adequate. Such studies would obviously be difficult to

perform, taking frequent relatively large samples from children but the feasibility of doing such studies should be investigated.

In summary the BPL 8Y patients are not demonstrating previously described abnormalities of immune function described in HIV seronegative haemophiliacs. This is within the limitations of the studies described here. The functional studies should be repeated at future points in time when more accurate comparisons in terms of amount of treatment and time on treatment can be made with groups such as group 2.

The lymphocyte proliferation studies should also be extended to see whether when lower concentrations of mitogens are used, abnormalities of lymphocyte function are revealed or not.

Dynamic studies following infusions of factor VIII should also be undertaken to investigate the relevance of the in vitro studies.

The use of complex studies such as the monocyte T cell interaction is questionable. It is a complex assay with therefore difficult to reproduce reliably and prone to laboratory error. Also the range of results are such that it becomes difficult to establish differences between different groups. The immune function tests described here were chosen because they had been used before to investigate previous groups of haemophiliacs and the measurement of T cell subsets, immunoglobulins and lymphocyte proliferation to mitogens form part of the routine investigation of immune problems. The use of other easily reproducible tests should be considered for future longitudinal studies.

Although the lymphocyte proliferation assays were performed after a relatively short time on treatment in the group as a whole, the monitoring of T cell subsets and IgG levels cover a much longer time period. The fact that these are both remaining stable implies that this group of patients is behaving differently to previously reported groups such as the group 2 patients. One of the differences between these groups and group 1 is the presence of chronic viral infections such as hepatitis C and the results of this study imply that hepatitis C could indeed be responsible for some of the changes.

The question remains as to whether hepatitis C infection itself results in immunosuppression. HCV is commonly detected in lymph nodes, the pancreas and less

frequently in adrenal glands, bone marrow, thyroid tissue and spleen (Laskus et al 1998). The presence of replicating HCV has also been described in peripheral blood mononuclear cells (Makris et al 1994, Okuda et al 1999) although this remains controversial (Lanford et al 1995). The presence of active replicating virus in these cells could explain some of the immunological abnormalities observed in these patients.

It appears that in those individuals with chronic hepatitis C there is a weakened HCV specific cytotoxic T cell (CTL) response compared with those who have a self limiting infection. (Rehermann et al 2000). HCV specific CD8+ cells were found to be dysfunctional in that they were unable to synthesise inflammatory cytokines or to lyse infected cells. However responses in the same individuals against other chronic or latent viruses such as CMV or EBV were relatively unaffected (Gruener et al 2001). These studies make the hypothesis that HCV infection has a general immunosuppressive effect unlikely.

However another study looking at over 1000 patients coinfected with HIV and HCV treated with potent antiretroviral therapy implied that direct HCV pathogenicity on lymphocytes should be considered (Greub et al 2000). Those patients coinfected with HIV and HCV showed impaired CD4 cell recovery after starting antiretroviral therapy compared to those infected with HIV alone. This proposed impact of HCV might prove clinically relevant as an increase of 50 CD4 cells / μl during the first six months after the start of treatment is associated with a 68% decrease in any AIDS related opportunistic illness.

HCV has also been associated with a wide range of autoimmune phenomena (Horcajada et al 1999) and with an increase of B cell lymphomas (Zuckerman et al 1997).

This all indicates a complex interaction between HCV and the immune system, which is not yet fully understood.

In terms of the study described here hepatitis C is implicated as accounting for the differences in immune response seen between the study group and the group 2

controls. A more extensive investigation of the group 2 individuals would perhaps have helped clarify the issue. A detailed investigation of the group 2 patients in terms of their HCV genotypes, viral loads and immune responses both HCV related and against other infections would have enabled a stronger conclusion as to the role of hepatitis C in immunosuppression to be made.

The question remains is it important to continue to monitor these patients to see if they develop evidence of immune modulation? It remains unclear as to what the clinical relevance of such observations are and that will perhaps only become clear when the problems of HIV and chronic hepatitis are past. Only long term surveillance and reporting of morbidity and mortality of treated haemophiliacs will reveal whether any changes however slight are important. Extending the studies described on the group 1 patients here may increase confidence that no significant changes are occurring and it may be that moving on to non plasma derived products will remove doubt altogether. However note must be taken of the fact that recent studies have shown subtle immune changes in individuals treated only with recombinant products (Newton-Nash et al 1996).

The close follow up of these patients remains of paramount importance because the pattern of treatment is changing dramatically. Repeated bleeds into joints, particularly ankle joints, resulting in chronic arthropathy are a major cause of morbidity in haemophiliacs. This continued to be the case despite the introduction of concentrates. Joints damaged by bleeds were more prone to re-bleeding and further damage. Such serious problems were seen in those individuals with severe haemophilia as opposed to those with moderate or mild disease. Raising the level of factor VIII above 0.01u/ml by the use of regular infusions was proposed as being a way of preventing repeated bleeds and the development of arthropathy.

Such prophylactic regimes were introduced in 1968 in Sweden (Nilsson et al 1970)and the use of doses of factor VIII 25-40 units per kg body weight three times a week have been shown to be successful in the prevention of joint disease. (Liesner et al 1992, Aledort et al 1994). This form of treatment is now widely recommended in countries

where it is economically feasible (Lusher 1997), because it is of course very expensive. It has been estimated as costing £2400 per kg body weight per year in children in the UK (Liesner et al 1997).

It is also now recommended that previously untreated patients and patients remaining HIV seronegative be treated with recombinant products (UK Haemophilia Centre Directors). Although not derived from human plasma, these products are also potentially contaminated with traces of animal proteins and non-human viruses found in media used in the production process. The potential increased risk of factor VIII inhibitors in patients treated with such products has already been discussed. This is compared with groups such as that described here treated with an intermediate purity product where inhibitor incidence is very low. The concern remains that such high purity products may also cause immune modulation.

In 1980, the life expectancy of haemophiliacs was approaching that of a healthy person (Rizza & Spooner 1983). This was cruelly shattered by the advent of HIV infection. At the end of the 1990's a similar point has again been reached, with the elimination of infection with HIV, hepatitis B and hepatitis C from concentrates. Several levels of intervention are necessary to ensure that this seemingly 'safe' treatment remains so. Where plasma derived products are used donor selection and screening of donations are vital in addition to any virucidal treatment process, as is indeed active immunisation of recipients where possible. The administration of all products must be followed by active surveillance for both infectious complications, inhibitor formation and immune changes. Only detailed long term studies will reassure physicians and there patients that these products are safe and if indeed problems do develop they must be detected and acted upon as soon as possible.

The aim of this study was firstly to determine the viral safety of a new factor VIII product which it successfully did. Secondly it tried to address the problem of immune modulation. It is fair to say that these patients within the limits of the study are not demonstrating dramatic changes in immune function. It would be wrong to imply that they will not go on to develop immune dysfunction and further studies should be

carried out to clarify this issue. It is extremely important not to over-interpret observations and non-significant results or trends. At the same time subtle changes imply that patients must continue to be studied and that there is no room for complacency.

APPENDICES

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
1	19.7.85	1985	11.1	6155	600
		1986	14.0	36035	2574
		1987	16.1	10825	672
		1988	18.7	19725	1055
		1989	20.0	37680	1884
		1990	22.4	46525	2077
		1991	25.0	192335	7693
		1992	27.6	109580	3970
		1993	28.8	83680	2905
		1994	32.1	87120	2714

appendix 6.1.1

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
2	28.12.85	1986	10.0	12155	1215
		1987	13.6	9505	699
		1988	15.2	13715	902
		1989	16.7	15510	929
		1990	19.0	30820	1622
		1991	21.5	42025	1955
		1992	23.6	58995	2500
		1993	26.0	70660	2718
		1994	29.4	56585	1925

appendix 6.1.2

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
3	3.1.86	1986	11.3	11465	1015
		1987	15.0	33055	2204
		1988	18.0	31080	1727
		1989	22.8	33635	1475
		1990	27.3	80075	2933
		1991	30.4	95180	3131
		1992	38.7	110570	2857
		1993	44.5	70445	1583
		1994	53.7	108500	2020

Appendix 6.1.3

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
4	20.1.86	1986	11.1	17590	1585
		1987	14.1	31410	2228
		1988	16.2	14880	918
		1989	17.0	17870	1051
		1990	18.0	38705	2150
		1991	21.8	38245	1754
		1992	24.0	63800	2658
		1993	27.8	117720	4234
		1994	34.0	126900	3732

Appendix 6.1.4

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
5	31.5.86	1986	10.7	7030	657
		1987	13.0	13465	1036
		1988	15.6	10935	700
		1989	16.0	6320	395
		1990	18.2	26795	1472
		1991	21.2	29595	1396
		1992	23.2	14155	610
		1993	26.9	34935	1299
		1994	34.4	50890	1479

appendix 6.1.5

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
6	6.6.86	1986	11.1	455	41
		1987	13.2	4955	375
		1988	15.6	20755	1330
		1989	17.9	4855	271
		1990	20.8	5765	277
		1991	22.9	9080	396
		1992	24.2	9365	387
		1993	27.1	11085	409
		1994	31.5	15970	507

Appendix 6.1.6

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
7	1.9.86	1986	12.2	255	21
		1987	13.3	21810	1640
		1988	15.8	7980	505
		1989	17.4	6900	396
		1990	19.1	25695	1345
		1991	21.9	50360	2300
		1992	24.0	52240	2177
		1993	29.5	72330	2452
		1994	32.3	115250	3568

appendix 6.1.7

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
8	28.9.86	1986	3.8	2105	554
		1987	8.9	11715	1316
		1988	11.7	15320	1309
		1989	13.0	28020	2155
		1990	14.2	29225	2058
		1991	16.1	41945	2605
		1992	16.6	49590	2987
		1993	18.5	33090	1789
		1994	20.5	59410	2898

appendix 6.1.8

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
9	1.12.86	1986	25.6	10710	418
		1987	29.1	5490	189
		1988	31.5	0	0
		1989	34.0	0	0
		1990		0	0
		1991		0	0
		1992		0	0
		1993		0	0
		1994	57.0	1275	22

appendix 6.1.9

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
10	27.1.87	1987	11.8	21515	1823
		1988	15.0	12415	828
		1989	16.8	13345	794
		1990	20.4	39140	1919
		1991	23.5	54385	2314
		1992	26.8	55360	2066
		1993	32.0	73095	2284
		1994	35.1	121080	3449

Appendix 6.1.10

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
11	4.2.87	1987	10.6	8015	756
		1988	13.3	9690	728
		1989	16.5	16045	972
		1990	18.4	25590	1391
		1991	20.4	14175	695
		1992	22.1	27805	1258
		1993	24.6	47765	1942
		1994	28.2	41090	1457

appendix 6.1.11

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
12	6.2.87	1987	14.8	7350	496
		1988	16.8	9690	576
		1989	19.3	13695	709
		1990	21.6	17395	805
		1991	25.0	34490	1380
		1992	27.3	24480	897
		1993	33.1	25330	765
		1994	36.1	48025	1330

Appendix 6.1.12

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
13	31.3.87	1987	23.0	1920	83
		1988	26.0	0	0
		1989	28.0	3345	120
		1990	29.2	16975	581
		1991	-	0	0
		1992	-	0	0
		1993	-	0	0
		1994	40.1	765	19

appendix 6.1.13

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
14	12.4.87	1987	11.9	16360	1375
		1988	13.2	12680	961
		1989	15.9	26760	1683
		1990	18.2	45150	2481
		1991	22.5	58300	2591
		1992	28.9	50975	1764
		1993	31.3	61755	1973
		1994	36.3	82025	2260

appendix 6.1.14

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
15	5.10.87	1987	4.9	665	136
		1988	8.5	920	108
		1989	11.8	8295	703
		1990	14.0	17530	1252
		1991	15.7	36215	2307
		1992	18.1	73465	4059
		1993	19.2	54770	2853
		1994	21.3	99415	4667

appendix 6.1.15

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
16	6.5.88	1988	17.5	10870	621
		1989	-	0	
		1990	-	0	
		1991	-	0	
		1992	-	0	
		1993	-	0	
		1994	-	0	

appendix 6.1.16

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
17	19.9.89	1989	-	5660	-
		1990	19.0	25310	1332
		1991	20.2	8805	436
		1992	23.4	5260	225
		1993	25.5	7725	303
		1994	30.6	3285	107

appendix 6.1.17

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
18	8.10.89	1989		275	-
		1990	15.2	7485	492
		1991	16.9	15055	891
		1992	19.3	25320	1312
		1993	22.0	30375	1381
		1994	24.5	45725	1866

Appendix 6.1.18

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
19	18.10.89	1989		2060	
		1990		30860	
		1991			
		1992			

appendix 6.1.19

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
20	20.11.89	1989	23.7	15060	635
		1990	24.2	0	0
		1991	27.0	0	0
		1992	31.6	0	0
		1993	34.1	0	0
		1994	35.5	0	0

appendix 6.1.20

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
21	22.11.89	1989	15.0	3150	396
		1990	16.1	20980	1345
		1991	17.4	23860	2300
		1992	19.1	36490	2177
		1993	21.8	47305	2452
		1994	24.5	96245	3568

appendix 6.1..21

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
22	16.1.90	1990	16.5	22980	1393
		1991	-	500	-
		1992	-	305	-
		1993	19.7	0	0
		1994	23.4	6500	278

appendix 6.1.22

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
23	24.3.90	1990	13.0	3280	252
		1991	14.9	6940	466
		1992	14.8	11890	803
		1993	17.0	2735	161
		1994	18.9	11290	597

appendix 6.1.23

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
24	17.5.90	1990	20.7	8855	428
		1991	23.4	50590	2162
		1992	26.2	24540	937
		1993	28.2	16410	582
		1994	31.0	15940	514

appendix 6.1.24

patient	date of first treatment	year	mean weight (kg)	total treatment received (units FVIII)	treatment received per kg
25	1.8.90	1990	14.5	1530	106
		1991	15.3	11120	727
		1992	18.5	20865	1128
		1993	20.5	22155	1081
		1994	22.2	33180	1495

appendix 6.1.25

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV ab
31.7.85	16	511	5	-	-	-	-
14.8.85	0	532	5				
2.9.85	0	395	5				
2.10 85	0	411	5				
13.11.85	0	1277	5	-	-	-	-
17.2.86	24	259	5	-	+	-	-
3.3.86	0	0	0				
20.5.86	15	390	5				
6.8.86	9	355	5				
19.9.86	24	328	5	-	+	-	-
21.10.86	13	373	5	-	+	-	-
19.1.87	8	329	5	-	+	-	-
26.2.87	9	332	5	-	+	-	-
10.4.87	10	370	5	-	+	-	-
6.5.87	8	351	5	-	+	-	-
10.6.87	9	364	5	-	+	-	-
9.7.87	10	348	5	-	+	-	-
11.8.87	9	338	5	-	+	-	-
19.8.87	10	348	5	-	+	-	-
17.9.87	8	335	5	-	+	-	-
30.10.87	5	379	0				
27.11.87	5	341	5				
4.1.88	9	313	5	-	+	-	-
11.2.88	11	348	5	-	+	-	-
7.3.88	15	397	8	-	+	-	-
22.4.88	11	322	5	-	+	-	-
10.5.88	11	336	5				
16.5.88	11	378	5	-	+	-	-
26.7.88	12	390	5	-	+	-	-
19.8.88	12	399	5	-	+	-	-
6.9.88	10	336	5	-	+	-	-

Appendix 6.2.1: patient 1, first treated 31.7.85

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV ab
24.10.88	17	348	5				
6.12.88	24	319	5	-	+	-	-
29.12.88	14	274	5				
25.1.89				-	+	-	-
10.3.89	11	373	5	-	+	-	-
22.5.89	11	325	5	-	+	-	-
19.7.89	9	315	5	-	+	-	-
11.9.89	13	395	3	-	+	-	-
17.11.89	9	338	3	-	+	-	-
6.2.90	16	325	2	-	+	-	-
15.8.90	16	342	4	-	+	-	-
3.1.91	6	353	6	-	+	-	-
23.8.91	9	325	8	-	+	-	-
9.7.92				-	+	-	-
17.12.92				-	+	-	-
20.5.93				-	+	-	-
27.8.93				-	+	-	-
25.11.93	18			-	+	-	-
7.7.94	16			-	+	-	-
16.12.94	21			-	+	-	-
16.3.95	24			-	+	-	-
7.8.95	20			-	+	-	-
30.10.95	26			-	+	-	-
25.1.96	28			-	+	-	-
12.4.96	14			-	+	-	-

Appendix 6.2.1 cont'd: patient 1, first treated 31.7.85

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV ab
28.12.85	25	513	5	-	-	-	-
14.8.86	15	580	5	-	+	-	-
28.10.86	11	518	5	-	+	-	-
8.12.86	10	479	5	-	+	-	-
27.1.87	8	444	5	-	+	-	-
26.2.87	18	486	5	-	+	-	-
13.4.87	9	505	5	-	+	-	-
11.6.87	9	509	5	-	+	-	-
13.7.87	9	513	5	-	+	-	-
14.8.87	6	400	5	-	+	-	-
15.9.87	12	447	5	-	+	-	-
12.10.87	14	409	5	-	+	-	-
14.12.87	10	399	6	-	+	-	-
2.2.88	10	407	5	-	+	-	-
8.3.88	11	365	5				
7.4.88	11	443	5	-	+	-	-
13.6.88	12	390	5	-	+	-	-
13.7.88	10	405	5	-	+	-	-
24.9.88	17	299	5	-	+	-	-
1.12.88	6	368	5	-	+	-	-
16.1.89	12	263	5	-	+	-	-
2.5.89	16	362	5	-	+	-	-
15.5.89	5	401	5	-	+	-	-
24.7.89	15	477	5	-	+	-	-
15.11.89	8	378	2	-	+	-	-
15.2.90	9	402		-	+	-	-
23.3.90	8	1584	3	-	+	-	-
3.5.90	12	531	4	-	+	-	-
6.8.90	9	468	3	-	+	-	-
18.12.90	5	424	2	-	+	-	-
19.8.91	6	353	6	-	+	-	-

Appendix 6.2.2:patient 2, first treated 28.12.85

Date	ALT	Alk Phos	Bilirubin	HbsAg	HbsAb	HbcAb	HIV ab
18.11.93	**160**			-	+	-	-
12.4.94	20			-	+	-	-
4.8.94	15			-	+	-	-
23.2.95	15			-	+	-	-
21.4.95	12			-	+	-	-
17.8.95	12			-	+	-	-
28.12.95	37			-	+	-	-
11.4.96	12			-	+	-	-

Appendix 6.2.2 cont'd :patient 2, first treated 28.12.85

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV ab
25.4.86	19	415	9				
15.5.86				-	+	-	-
20.8.86	15	409	15	-	+	-	-
10.11.86	11	366	5	-	+	-	
16.1.87	6	344	11	-	+	-	-
10.2.87	10	461	17	-	+	-	-
5.3.87	6	445	13	-	+	-	-
24.3.87	9	467	14	-	+	-	-
17.4.87	9	375	9	-	+	-	-
29.5.87	6	360	13	-	+	-	-
3.7.87	7	316	13	-	+	-	-
22.7.87	10	360	15	-	+	-	-
20.8.87	7	360	17	-	+	-	-
6.10.87	6	359	11	-	+	-	-
23.10.87	7	382	9	-	+	-	-
20.11.87	5	377	16	-	+	-	-
11.12.87	5	345	17	-	+	-	-
14.1.88	10	367	17	-	+	-	-
1.2.88	6	376	16	-	+	-	-
3.3.88	6	355	9	-	+	-	-
10.4.88	5	340	15	-	+	-	-
27.4.88	5	418	15	-	+	-	-
16.5.88	5	419	19	-	+	-	-
6.6.88	7	402	5				
10.6.88	5	414	5	-	+	-	-
19.7.88	11	444	5	-	+	-	-
1.9.88	5	384	10	-	+	-	-
11.11.88	5	398	9	-	+	-	-
9.1.89	10	357	14	-	+	-	-

Appendix 6.2.3: patient 3, first treated 3.1.86

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV ab
1.3.89	12	941	9	-	+	-	-
8.5.89	8	374	5	-	+	-	-
2.8.89	8	360	10	-	+	-	-
10.10.89	14	380	11	-	+	-	-
1.12.89	19	347	15	-	+	-	-
23.3.90	15	299	17	-	+	-	-
11.5.90	12	426	12	-	+	-	-
20.7.90	18	403	15	-	+	-	-
10.10.90	11	350	10				
22.10.90				-	+	-	-
15.2.91	19	304	6	-	+	-	-
8.5.91	16	323	9	-	+	-	-
4.6.92				-	+	-	-
21.8.92				-	+	-	-
10.12.92				-	+	-	-
7.4.93				-	+	-	-
27.1.94	29			-	+	-	-
5.5.94	28			-	+	-	-
18.8.94				-	+	-	-
9.12.94	58			-	+	-	-
13.1.95	34			-	+	-	-
23.3.95	32			-	+	-	-
2.6.95	32			-	+	-	-
24.8.95	29			-	+	-	-
24.10.95	23			-	+	-	-
26.3.96	32			-	+	-	-
11.7.96				-	+	-	-

Appendix 6.2.3 cont'd: patient 3, first treated 3.1.86

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
20.1.86	10	447	13				
19.2.86				-	-	-	-
8.5.86	9	575	5	-	+	-	-
29.7.86	8		5	-	+	-	-
12.8.86	11	1220	5	-	+	-	-
5.9.86	13	1443	5				
20.10.86	17	589	5	-	+	-	-
22.12.86	10	491	7	-	+	-	-
9.1.87	7	415	5	-	+	-	-
5.2.87	12	515	5	-	+	-	-
5.3.87	9	343	5	-	+	-	-
2.4.87	9	422	5	-	+	-	-
30.4.87	9	484	6	-	+	-	-
2.6.87	12	514	5	-	+	-	-
31.7.87	10	428	5	-	+	-	-
6.8.87	9	504	5	-	+	-	-
16.9.87	15	392	5	-	+	-	-
1.10.87	11	425	5	-	+	-	-
7.11.87	12	521	5	-	+	-	-
11.12.87	19	540	5	-	+	-	-
28.1.88	13	622	5	-	+	-	-
5.3.88	13	632	5	-	+	-	-
24.3.88	9	618	5	-	+	-	-
7.4.88	11	600	5	-	+	-	-
11.5.88	11	535	7	-	+	-	-
8.7.88	17	482	5	-	+	-	-
16.8.88	27	453	5				
9.9.88	13	461	6	-	+	-	-
20.10.88	11	442	5	-	+	-	-
23.12.88	21	435	5	-	+	-	-
20.1.89	6	468	10	-	+	-	-

Appendix 6.2.4: patient 4 first treated 20.1.86

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV ab
13.4.89	7	363	5	-	+	-	-
15.6.89	6	354	5	-	+	-	-
7.8.89	16	416	5	-	+	-	-
27.10.89	7	397	7	-	+	-	-
5.12.89	7	410	4	-	+	-	-
25.7.90	2	482	6	-	+	-	-
8.11.90	18	417	5	-	+	-	-
4.1.91	11	517	6	-	+	-	-
9.5.91	4	440	6	-	+	-	-
31.1.92	16	425	11	-	+	-	-
1.3.93				-	+	-	-
29.7.93				-	+	-	-
1.10.93				-	+	-	-
6.1.94	22			-	+	-	-
5.5.94	15			-	+	-	-
14.7.94	5						
25.11.94				-	+	-	-
17.8.95	19			-	+	-	-
27.2.96				-	+	-	-
5.6.96	10			-	+	-	-

Appendix 6.2.4 cont'd: patient 4 first treated 20.1.86

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
2.6.86	13	623	5	-	-	-	-
24.10.86	18	734	5	-	-	-	-
28.11.86				-	+	-	-
3.12.86				-	+	-	-
19.1.87	14	630	5	-	+	+	-
4.2.87	15	820	5	-	+	+	-
20.2.87	11	829	6	-	+	-	-
12.3.87	12	767	5	-	+	+	-
15.4.87	14	811	5	-			-
15.5.87	15	778	5	-	+	-	-
26.6.87	7	733	7	-	+	-	-
20.7.87	8	698	7	-	+	-	-
19.8.87	12	733	6	-	+	-	-
7.9.87	10	720	5	-	+	-	-
19.10.87	16	809	5				
8.1.88	6	581	5	-	+	-	-
15.4.88	5	670	5	-	+	-	-
10.5.88	6	397	5	-	+	-	-
22.6.88	8	590	5	-	+	-	-
16.9.88	5	746	5	-	+	-	-
21.10.88	6	681	5	-	+	-	-
24.11.88	9	562	5	-	+	-	-
17.3.89	5	627	5	-	+	-	-
3.5.89	8	502	5	-	+	-	-
4.7.89	5	567	5	-	+	-	-
8.9.89	7	629	5	-	+	-	-
26.10.89	13	653	6	-	+	-	-
8.12.89	8	561	2	-	+	-	-
12.1.90	8	471	4	-	+	-	-
22.3.90	7	427	10	-	+	-	-
11.5.90	9	648	6	-	+	-	-
15.6.90	26	492	1	-	+	-	-

Appendix 6.2.5: patient 5 first treated 31.5.86

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV ab
31.10.90	12	622	2	-	+	-	-
18.1.91	10	511	6	-	+	-	-
7.3.91	12	602	7	-	+	-	-
27.9.91	9	573	9	-	+	-	-
15.1.92	9	483	11	-	+	-	-
28.5.92				-	+	-	-
12.11.92				-	+	-	-
17.3.93				-	+	-	-
24.6.93				-	+	-	-
5.10.93				-	+	-	-
2.12.93	21			-	+	-	-
1.3.94	19			-	+	-	-
7.7.94	16			-	+	-	-
15.10.94	22			-	+	-	-
9.2.95	22			-	+	-	-
20.7.95	21			-	+	-	-
10.11.95	24			-	+	-	-
21.3.96	26			-	+	-	-
13.6.96	19			-	+	-	-

Appendix 6.2.5 cont'd: patient 5 first treated 31.5.86

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
14.11.86	12	538	5	-	-	-	-
26.2.87	8	567	5	-	-	-	-
10.4.87	8	517	5	-	-	-	-
11.5.87	16	591	5	-	-	-	-
8.6.87	18	546	5	-	+	-	-
6.7.87	20	540	5	-	+	-	-
3.8.87	11	560	5	-	+	-	-
2.9.87	10	390	6	-	+	-	-
12.10.87	12	508	5	-	+	-	-
9.11.87	18	452	7	-	+	-	-
14.12.87	15	427	5	-	+	-	-
31.12.87	13	484	5	-	+	-	-
1.2.88	19	435	5	-	+	-	-
26.2.88	18	408	5	-	+	-	-
18.3.88	19	395	5	-	+	-	-
7.4.88	16	513	5	-	+	-	-
17.6.88	7	431	5	-	+	-	-
14.7.88	11	323	5	-	+	-	-
15.8.88	14	615	5				
19.9.88	11	514	5	-	+	-	-
17.10.88	10	583	5	-	+	-	-
15.11.88	9	539	5	-	+	-	-
16.12.88	13	467	5	-	+	-	-
13.1.89	11	438	5	-	+	-	-
3.2.89	16	443	5	-	+	-	-
17.3.89	9	553	5	-	+	-	-
10.5.89				-	+	-	-
16.6.89	14	508	5	-	+	-	-
18.8.89				-	+	-	-
25.9.89	17	448	2	-	+	-	-
15.11.89	16	449	2	-	+	-	-
29.1.90	10	554	7	-	+	-	-

Appendix 6.2.6: patient 6 first treated 6.6.86

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
9.3.90	12	460	3	-	+	-	-
24.5.90	17	432	2	-	+	-	-
25.6.90	12	433	5	-	+	-	-
14.9.90	14	485	6	-	+	-	-
28.12.90	9	436	4	-	+	-	-
22.2.91	8	381	6	-	+	-	-
19.4.91	15	370	7	-	+	-	-
15.7.91	**119**	421	7	-	+	-	-
14.8.91	15	403	8				
23.9.91	9	365	8	-	+	-	-
3.1.92	8	311	8	-	+	-	-
5.5.92				-	+	-	-
17.12.92				-	+	-	-
23.4.93				-	+	-	-
7.10.93				-	+	-	-
27.1.94	19			-	+	-	-
25.3.94	20			-	+	-	-
4.8.94	26			-	+	-	-
21.11.94	26			-	+	-	-
20.3.95				-	+	-	-
5.6.95	26			-	+	-	-
3.8.95				-	+	-	-
11.12.95				-	+	-	-
11.4.96	19			-	+	-	-

Appendix 6.2.6 cont'd: patient 6 first treated 6.6.86

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
24.10.86	10	400	9	-	-	-	-
9.1.87	10	456	5	-	-	-	-
20.3.87	12	473	11	-	+	-	-
24.4.87	8	411	5	-	+	-	-
23.6.87	15	413	5	-	+	-	-
14.8.87	10	413	5	-	+	-	-
16.9.87	26	404	13				
30.9.87	11	417	7	-	+	-	-
27.10.87	13	465	5	-	+	-	-
27.11.87	11	457	5	-	+	-	-
29.12.87	17	477	13	-	+	-	-
15.2.88	14	423	10	-	+	-	-
28.3.88	11	471	8	-	+	-	-
29.4.88	16	461	5	-	+	-	-
16.5.88	12	502	9	-	+	-	-
5.7.88	10	383	5	-	+	-	-
11.8.88	14	467	6	-	+	-	-
10.9.88	9	400	9	-	+	-	-
14.10.88	13	370	6	-	+	-	-
14.11.88	17	389	8	-	+	-	-
13.1.89	16	431	5	-	+	-	-
10.3.89	14	415	6	-	+	-	-
24.4.89	8	416	5	-	+	-	-
14.7.89	16	315	5	-	+	-	-
26.9.89				-	+	-	-
9.10.89	19	435	13	-	+	-	-
15.12.89	16	407	10	-	+	-	-
4.1.90	15	406	8	-	+	-	-
24.4.90	13	410	8	-	+	-	-
1.8.90	11	443	9	-	+	-	-
6.11.90	12	379	9	-	+	-	-

Appendix 6.2.7: patient 7 first treated 1.9.86

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
18.1.91	19	285	11	-	+	-	-
24.6.91				-	+	-	-
9.10.91	19	386	16	-	+	-	-
14.5.92				-	+	-	-
21.8.92				-	+	-	-
30.10.92				-	+	-	-
22.1.93				-	+	-	-
1.7.93				-	+	-	-
17.2.94	18			-	+	-	-
11.5.94	16			-	+	-	-
23.6.94	20			-	+	-	-
12.9.94	17			-	+	-	-
23.3.95	27			-	+	-	-
22.8.95	16			-	+	-	-
2.11.95	17			-	+	-	-
28.3.96	35			-	+	-	-

Appendix 6.2.7 cont'd: patient 7 first treated 1.9.86

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
28.9.86	5	657	5	-	-	-	
12.1.87	14	438	5	-	+	-	-
16.2.87	12	401	5	-	+	-	-
10.3.87	11	434	5	-	+	-	-
6.4.87	10	443	6	-	+	-	-
22.5.87	9	392	5	-	+	-	-
19.6.87	6	452	5	-	+	-	-
4.8.87	14	399	5	-	+	-	-
3.9.87	17	392	5	-	+	-	-
12.10.87	11	444	5				
22.10.87	10	480	5	-	+	-	-
1.12.87	9	315	8	-	+	-	-
16.12.87	5	355	5	-	+	-	-
30.12.87	8	370	5	-	+	-	-
3.2.88	12	421	5	-	+	-	-
15.3.88	25	394	5	-	+	-	-
22.4.88	13	376	5	-	+	-	-
24.5.88	22	355	5	-	+	-	-
11.7.88	8	368	5				
18.7.88	5	810	5				
22.8.88	6	368	5				
24.10.88	13	461	5				
10.4.89	9	345	5	-	+	-	-
3.6.89	16	395	5	-	+	-	-
3.8.89	7	368	5	-	+	-	-
2.10.89	9	342	5	-	+	-	-
10.11.89	8	316	6	-	+	-	-
3.1.90	4	419	3	-	+	-	-
19.3.90	2	337	1	-	+	-	-
11.1.91	8	253	6	-	+	-	-
8.7.93				-	+	-	-

Appendix 6.2.8: patient 8 first treated 28.9.86

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
3.2.94				-	+	-	-
13.4.94	48			-	+	-	-
7.6.94	8			-	+	-	-
30.6.94	34			-	+	-	-
6.9.94	19			-	+	-	-
3.4.95	14			-	+	-	-
11.5.95	12			-	+	-	-
24.8.95				-	+	-	-
6.11.95				-	+	-	-
26.4.95	17			-	+	-	-

Appendix 6.2.8 cont'd: patient 8 first treated 28.9.86

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
16.12.86	17	523	5				-
22.12.86	12	407	5	-	-	-	-
6.1.87	16	516	5	-	-	-	-
2.2.87	13	651	5	-	-	-	-
3.3.87	8	422	12	-	+	-	-
30.3.87	19	498	5	-	+	-	-
28.4.87	9	473	5	-	+	-	-
27.5.87	6	612	5	-	+	-	-
29.6.87	6	585	5	-	+	-	-
29.7.87	5	507	5	-	+	-	-
28.8.87	9	568	8	-	+	-	-
29.9.87	11	581	5	-	+	-	-
27.10.87	10	572	6	-	+	-	-
24.11.87	16	648	8	-	+	-	-
31.12.87	12	583	7	-	+	-	-
2.2.88	16	624	5	-	+	-	-
19.2.88	12	605	5	-	+	-	-
23.3.88	11	639	5	-	+	-	-
19.4.88	11	603	5	-	+	-	-
20.5.88	16	629	6	-	+	-	-
15.6.88	6	591	5	-	+	-	-
13.7.88	8	603	5	-	+	-	-
15.8.88	5	633	5	-	+	-	-
2.9.88				-	+	-	-
5.10.88	9	598	6	-	+	-	-
27.10.88	10	577	5	-	+	-	-
19.12.88	13	622	6	-	+	-	-
17.2.89	9	498	5	-	+	-	-
31.3.89	6	527	6	-	+	-	-
24.5.89	7	511	7	-	+	-	-
24.7.89	8	543	6	-	+	-	-

Appendix 6.2.9: patient 9 first treated 1.12.86

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
8.9.89	19	550	5	-	+	-	-
27.10.89	19	603	7	-	+	-	-
22.12.89	12	482	2	-	+	-	-
21.2.90	5	481	1	-	+	-	-
23.4.90	7	639	8	-	+	-	-
31.8.90	11	542	7	-	+	-	-
22.10.90	12	495	2	-	+	-	-
27.12.90	13	560	2	-	+	-	-
1.3.91	6	474	9	-	+	-	-
25.4.91	6	591	6	-	+	-	-
24.6.91	3	654	9	-	+	-	-
30.8.91	9	626	9	-	+	-	-
27.12.91	12	598	9	-	+	-	-
1.7.92				-	+	-	-
7.1.93				-	+	-	-
16.4.93				-	+	-	-
16.7.93				-	+	-	-
6.1.94	16			-	+	-	-
15.4.94	14			-	+	-	-
25.8.94				-	+	-	-

Appendix 6.2.9 cont'd: patient 9 first treated 1.12.86

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
27.1.87	9	499	5	-	-	-	-
26.2.87	16	573	5				
20.3.87	11	443	5	-	+	-	-
24.4.87	20	517	6	-	+	-	-
22.5.87	16	501	9	-	+	-	-
24.6.87	22	597	15	-	+	-	-
24.7.87	26	526	6	-	+	-	-
28.8.87	19	490	9	-	+	-	-
21.9.87	12	2970	8	-	+	-	-
6.10.87	24	828	7	-	+	-	-
31.10.87	15	436	10	-	+	-	-
27.11.87	17	518	10	-	+	-	-
30.12.87	18	449	5	-	+	-	-
21.1.88	18	457	6				
17.2.88	17	489	7				
24.2.88	14	471	5	-	+	-	-
6.3.88	17	472	7	-	+	-	-
11.3.88	12	480	10	-	+	-	-
8.4.88	17	539	6	-	+	-	-
6.5.88	15	560	8	-	+	-	-
3.6.88	21	461	9	-	+	-	-
24.6.88	14	505	8				
22.7.88	15	347	5	-	+	-	-
12.8.88	16	450	5	-	+	-	-
23.9.88	14	418	8	-	+	-	-
21.10.88	15	465	5	-	+	-	-
25.11.88	18	478	7	-	+	-	-
23.12.88	22	349	5	-	+	-	-
18.1.89	16	501	8	-	+	-	-
17.3.89	13	458	6	-	+	-	-
12.5.89	12	485	5	-	+	-	-

Appendix 6.2.10: patient 10 first treated 27.1.87

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
30.6.89	19	394	5	-	+	-	-
23.8.89	8	447	9	-	+	-	-
13.10.89	26	360	7	-	+	-	-
11.12.89	6	444	5	-	+	-	-
2.2.90	7	498	14	-	+	-	-
21.3.90	14	427	6	-	+	-	-
25.5.90	21	493	12	-	+	-	-
8.8.90	14	420	7	-	+	-	-
15.10.90	11	442	7	-	+	-	-
27.12.90	7	442	11	-	+	-	-
22.2.91	12	363	7	-	+	-	-
10.4.91	25	152	8	-	+	-	-
4.6.91	10	409	12	-	+	-	-
30.8.91	13	386	17	-	+	-	-
29.11.91	18	340	13	-	+	-	-
11.6.92				-	+	-	-
3.12.92				-	+	-	-
2.3.93				-	+	-	-
2.12.93	22			-	+	-	-
11.3.94	28			-	+	-	-
30.6.94	21			-	+	-	-
30.9.94	17			-	+	-	-
2.2.95				-	+	-	-
19.5.95	33			-	+	-	-
14.8.95	27			-	+	-	-
27.10.95	18			-	+	-	-
3.7.96				-	+	-	-

Appendix 6.2.10 cont'd: patient 10 first treated 27.1.87

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
4.2.87	16	457	5	-	-	-	-
6.3.87	28	458	5	-			
10.4.87	15	458	5	-	-	-	-
8.5.87	16	448	5	-	-	-	-
12.6.87	14	510	5	-	+	-	-
10.7.87	7	427	5				
14.8.87	15	424	5	-	+	-	-
16.9.87	9	470	5	-	+	-	-
9.10.87	9	458	5	-	+	-	-
6.11.87	13	455	5	-	+	-	-
3.12.87	12	390	5	-	+	-	-
30.12.87	20	324	5	-	+	-	-
1.2.88	11	399	5				
6.3.88	13	414	5				
6.4.88	9	413	5	-	+	-	-
29.4.88	13	392	5	-	+	-	-
23.5.88	10	417	5				
9.6.88	12	391	5				
23.6.88	11	330	5	-	+	-	-
20.7.88	12	368	5	-	+	-	-
5.8.88	12	303	5	-	+	-	-
8.9.88	15	380	5	-	+	-	-
21.10.88	24	391	5	-	+	-	-
21.11.88	11	457	5				
9.1.89				-	+	-	-
8.2.89	12	456	5	-	+	-	-
13.3.89	7	409	5	-	+	-	-
12.4.89	10	406	5	-	+	-	-
8.6.89	10	370	5	-	+	-	-
10.8.89				-	+	-	-
16.10.89	19	415	4	-	+	-	-

Appendix 6.2.11: patient 11 first treated 4.2.87

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
5.12.89	9	376	3	-	+	-	-
6.2.90	12	388		-	+	-	-
9.7.90	10	422	6	-	+	-	-
7.11.90	6	362	3	-	+	-	-
8.3.91	8	273	9	-	+	-	-
11.10.91	13	385	5	-	+	-	-
4.6.92				-	+	-	-
19.11.92				-	+	-	-
10.6.93				-	+	-	-
25.11.93	17			-	+	-	-
4.3.94	16			-	+	-	-
4.8.94	23			-	+	-	-
7.12.94	13			-	+	-	-
16.3.95	21			-	+	-	-
16.6.95	19			-	+	-	-
31.8.95	14			-	+	-	-
11.4.96	14			-	+	-	-

Appendix 6.2.11 cont'd: patient 11 first treated 4.2.87

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
6.2.87	21	432	5	-	-	-	-
26.2.87	14	428	5	-	-	-	-
12.3.87	19	490	5	-	+	-	-
3.4.87	18	426	5	-	+	-	-
9.6.87	16	380	5	-	+	-	-
7.7.87	10	471	5	-	+	-	-
5.8.87	13	475	5	-	+	-	-
7.9.87	14	431	5	-	+	-	-
5.10.87	14	426	5	-	+	-	-
22.10.87	14	421	8	-	+	-	-
20.11.87	11	481	5	-	+	-	-
16.12.87	11	312	5	-	+	-	-
18.1.88	18	385	6	-	+	-	-
11.2.88	18	384	5	-	+	-	-
24.3.88	12	337	5	-	+	-	-
6.5.88	17	412	5	-	+	-	-
10.6.88	13	406	5	-	+	-	-
12.7.88	15	423	5	-	+	-	-
23.7.88	5	450	5				
2.8.88	9	530	5	-	+	-	-
31.8.88	20	1586	5				
29.9.88	15	452	7	-	+	-	-
5.10.88	17	422	5				
25.10.88	12	351	5				
14.11.88	12	389	6	-	+	-	-
19.12.88	12	367	5	-	+	-	-
26.1.89	11	407	5	-	+	-	-
21.2.89	7	430	12	-	+	-	-
31.3.89	42	134	5	-	+	-	-
28.4.89	12	402	5	-	+	-	-
20.6.89	14	417	5	-	+	-	-

Appendix 6.2.12: patient 12 first treated 6.2.87

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
18.8.89	22	342	5	-	+	-	-
20.10.89	7	279	6	-	+	-	-
2.1.90	11	332	5	-	+	-	-
6.2.90	9	386	3	-	+	-	-
26.4.90	16	368	6	-	+	-	-
23.5.90	10	320	3	-	+	-	-
21.8.90	7	398	3	-	+	-	-
30.10.90	13	366	6	-	+	-	-
8.5.91	13	370	5	-	+	-	-
20.8.91	15	353	12	-	+	-	-
5.11.91	22	355	10	-	+	-	-
3.4.92				-	+	-	-
4.6.92				-	+	-	-
8.10.92				-	+	-	-
10.3.93				-	+	-	-
9.12.93	24			-	+	-	-
29.3.94	17			-	+	-	-
21.7.94	21			-	+	-	-
31.10.94	25			-	+	-	-
9.3.95	27			-	+	-	-
24.7.95	21			-	+	-	-
23.10.95	21			-	+	-	-
22.2.96	29			-	+	-	-
4.6.96	14			-	+	-	-

Appendix 6.2.12 cont'd: patient 12 first treated 6.2.87

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
31.3.87	19	227	13	-	-	-	-
30.4.87	6	245	26	-	-	-	-
15.6.87	7	276	9	-	-	-	-
14.7.87	5	268	12	-	+	-	-
18.8.87	10	268	28	-	+	-	-
22.9.87	8	288	15	-	+	-	-
21.10.87	8	215	12	-	+	-	-
17.11.87	10	276	10	-	+	-	-
22.12.87	7	274	12	-	+	-	-
25.1.88	9	278	12	-	+	-	-
23.2.88	12	320	19	-	+	-	-
20.4.88	10	320	6	-	+	-	-
8.6.88	11	280	5	-	+	-	-
19.8.88	5	275	5				
4.10.88	6	301	17	-	+	-	-
11.10.88				-	+	-	-
28.11.88	8	257	13	-	+	-	-
23.12.88	11	221	14	-	+	-	-
24.2.89	6	357	15	-	+	-	-
9.6.89	17	227	5				
17.7.89	21	245	3	-	+	-	-
2.10.89	11	372	16	-	+	-	-
4.12.89	7	284	13	-	+	-	-
13.2.90	7	365	21	-	+	-	-
8.5.90	2	409	13	-	+	-	-
13.8.90	5	342	21	-	+	-	-
8.10.90	9	259	20	-	+	-	-
20.12.90	4	248	21	-	+	-	-
8.3.91	6	256	16	-	+	-	-
17.5.91	3	282	37	-	+	-	-
2.8.91	8	281	30	-	+	-	-

Appendix 6.2.13: patient 13 first treated 31.3.87

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
3.10.91	10	263	29	-	+	-	-
31.1.92	14	252	24	-	+	-	-
2.7.92				-	+	-	-
5.11.92				-	+	-	-
20.5.93				-	+	-	-
9.12.93	18			-	+	-	-
21.7.94	15			-	+	-	-
5.1.95	19			-	+	-	-
23.6.95	10			-	+	-	-

Appendix 6.2.13 cont'd: patient 13 first treated 31.3.87

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
12.4.87	13	419	5	-	-	-	-
21.5.87	10	376	5				
16.6.87	12	470	5				
13.7.87	11	458	5	-	+	-	-
4.8.87	18	694	5	-	+	-	-
3.9.87	11	451	5	-	+	-	-
7.10.87	14	330	5	-	+	-	-
16.10.87	12	346	5	-	+	-	-
4.11.87	15	313	5	-	+	-	-
30.11.87	14	342	5	-	+	-	-
8.1.88	14	367	5	-	+	-	-
9.2.88	15	342	5	-	+	-	-
6.3.88	14	463	5				
12.4.88	17	456	5	-	+	-	-
12.5.88	13	444	5	-	+	-	-
6.6.88	21	436	5	-	+	-	-
19.7.88	16	466	5	-	+	-	-
7.9.88	13	432	5	-	+	-	-
19.10.88	13	304	5	-	+	-	-
12.12.88	11	330	5	-	+	-	-
16.1.89	16	411	5				
15.2.89	12	407	5	-	+	-	-
13.3.89	13	442	5	-	+	-	-
20.4.89	11	243	5	-	+	-	-
13.6.89	12	318	5	-	+	-	-
2.8.89	15	366	5	-	+	-	-
9.10.89	9	331	4	-	+	-	-
11.12.89	11	343	2	-	+	-	-
6.2.90	10	352	2	-	+	-	-
10.4.90	3	454	3	-	+	-	-
13.6.90	13	445	5	-	+	-	-

Appendix 6.2.14: patient 14 first treated 12.4.87

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
16.7.90	12	362	2	-	+	-	-
21.8.90	15	461	5	-	+	-	-
14.1.91	20	391	7	-	+	-	-
2.7.91	18	337	7	-	+	-	-
2.12.93	29			-	+	-	-
19.3.94	31			-	+	-	-
30.6.94	26			-	+	-	-
1.10.94	20			-	+	-	-
26.1.95	30			-	+	-	-
20.7.95	29			-	+	-	-
2.11.95	25			-	+	-	-
1.2.96	38			-	+	-	-
20.5.96	29			-	+	-	-

Appendix 6.2.14 cont'd: patient 14 first treated 12.4.87

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
5.10.87	30	604	51	-	-	-	-
21.10.87	27	778	22	-	-	-	-
3.11.87	33	899	18				
17.11.87	40	998	13	-	-	-	-
1.12.87	37	925	7	-	-	-	-
16.12.87	36	932	8				
30.12.87	34	815	7				
13.1.88	42	745	5	-	+	-	-
3.2.88	40	521	5	-	+	-	-
2.3.88	31	498	5	-	+	-	-
6.4.88	27	479	5				
25.4.88	23	550	5	-	+	-	-
23.5.88	24	576	6	-	+	-	-
10.6.88	24	548	5	-	+	-	-
6.7.88	19	441	5	-	+	-	-
3.8.88	29	498	5	-	+	-	-
9.9.88	22	631	5	-	+	-	-
4.10.88	21	525	5	-	+	-	-
2.11.88	18	514	5	-	+	-	-
6.12.88	14	424	5	-	+	-	-
4.1.89	19	494	5	-	+	-	-
1.3.89	19	538	5	-	+	-	-
6.4.89	12	526	5	-	+	-	-
3.5.89	18	522	5	-	+	-	-
25.5.89	16	494	7	-	+	-	-
30.6.89	9	433	5	-	+	-	-
21.7.89	19	536	5	-	+	-	-
30.8.89	34	653	5	-	+	-	-
27.9.89	27	507	4	-	+	-	-
20.10.89	14	510	3	-	+	-	-
14.12.89	16	511	3	-	+	-	-

Appendix 6.2.15: patient 15 first treated 5.10.87

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
14.2.90	20	547	5	-	+	-	-
25.4.90	9	451	4	-	+	-	-
11.6.90	13	462	5	-	+	-	-
15.8.90	14	507	3	-	+	-	-
10.10.90	11	481	5	-	+	-	-
7.12.90	15	339	3	-	+	-	-
27.2.91	13	330	4	-	+	-	-
30.4.91	15	346	5	-	+	-	-
11.6.91	11	424	6	-	+	-	-
9.8.91	11	412	7	-	+	-	-
21.11.91	30	352	9	-	+	-	-
18.6.92	11	370	11	-	+	-	-
2.8.92	21	341	12	-	+	-	-
3.12.92	14	317	8	-	+	-	-
26.2.93	9	323	7	-	+	-	-
10.6.93	11	359	10	-	+	-	-
28.9.93	14	347	10	-	+	-	-
2.12.93	15	287	9	-	+	-	-
4.3.94	12	364	9	-	+	-	-
7.7.94	5	304	12				
12.10.94	13	381	7	-	+	-	-
12.1.95	13	388	9	-	+	-	-
24.7.95				-	+	-	-
27.10.95	13	389	10	-	+	-	-
25.1.96	22	359	10	-	+	-	-
12.4.96	11	349	11	-	+	-	-
3.7.96	7	434	9	-	+	-	-

Appendix 6.2.15 cont'd: patient 15 first treated 5.10.87

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
5.5.88	17	288	5	-	-	-	-
17.5.88	12	286	7	-	-	-	-
26.5.88	11	304	9				
10.6.88	17	375	5	-	-	-	-
20.6.88	16	370	7	-	-	-	-
4.7.88	19	343	5	-	+	-	-
18.7.88	5	358	5	-	+	-	-
2.8.88	16	426	5	-	+	-	-
31.8.88	13	412	11	-	+	-	-
29.9.88	23	309	5	-	+	-	-
27.10.88	13	335	7	-	+	-	-
29.10.88	13	341	8	-	+	-	-
24.11.88	15	393	8	-	+	-	-
22.12.88	18	325	5	-	+	-	-
19.1.89	12	388	7	-	+	-	-
7.2.89	18	386	7	-	+	-	-
16.3.89	18	378	5	-	+	-	-
28.6.89	28	293	5	-	+	-	-
16.11.89				-	+	-	-
9.7.90	15	285	18	-	+	-	-
26.2.91				-	+	-	-

Appendix 6.2.16: patient 16 first treated 6.5.88

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
19.9.89	23	608	4	-	+	-	-
5.10.89	10	428	4	-	+	-	-
19.10.89	15	374	3	-	+	-	-
2.11.89	14	427	7	-	+	-	-
17.11.89	11	388	5	-	+	-	-
30.11.89	6	423	7	-	+	-	-
14.12.89	9	279	2	-	+	-	-
28.12.89	4	364	1	-	+	-	-
11.1.90	7	359	4	-	+	-	-
8.2.90	9	396	4	-	+	-	-
9.3.90	13	376	4	-	+	-	-
12.4.90	1	330	1	-	+	-	-
10.5.90	12	499	5	-	+	-	-
7.6.90	13	389	3	-	+	-	-
2.7.90	12	277	5	-	+	-	-
6.8.90	18	394	6	-	+	-	-
30.8.90	18	402	4				
20.9.90	13	448	6	-	+	-	-
31.10.90	17	413	7	-	+	-	-
21.11.90	12	335	2	-	+	-	-
28.12.90	11	335	3	-	+	-	-
25.2.91	15	307	8	-	+	-	-
12.4.91	19	364	8	-	+	-	-
3.7.91	15	320	7	-	+	-	-
1.10.91	11	346	13	-	+	-	-
28.5.92				-	+	-	-
19.11.92				-	+	-	-
20.5.93				-	+	-	-
18.11.93	24			-	+	-	-
17.2.94	22			-	+	-	-
7.7.94	19			-	+	-	-

Appendix 6.2.17: patient 17 first treated 19.9.89

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
16.3.95	28			-	+	-	-
20.7.95	24			-	+	-	-
24.11.95	37			-	+	-	-
4.4.96	19			-	+	-	-

Appendix 6.2.17 cont'd: patient 17 first treated 19.9.89

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
8.10.89	22	303	3	-	+	-	-
20.10.89	13	303	3	-	+	-	-
1.11.89	11	282	9	-	+	-	-
15.11.89	14	271	2	-	+	-	-
29.11.89	23	299	5	-	+	-	-
13.12.89	17	263	1	-	+	-	-
27.12.89	16	262	1	-	+	-	-
5.1.90	13	246	3	-	+	-	-
24.1.90	12	324	5	-	+	-	-
23.2.90	15	382	6	-	+	-	-
21.3.90	11	259	1	-	+	-	-
11.4.90	33	269	3	-	+	-	-
9.5.90	14	358	3	-	+	-	-
30.5.90	15	310	3	-	+	-	-
28.6.90	17	283	3	-	+	-	-
24.7.90	11	286	2	-	+	-	-
24.8.90	17	278	4	-	+	-	-
5.9.90	14	300	5	-	+	-	-
3.10.90	9	311	3	-	+	-	-
31.10.90	11	275	3	-	+	-	-
28.11.90	11	241	3	-	+	-	-
21.12.90	8	190	6	-	+	-	-
23.1.91	8	204	4	-	+	-	-
20.2.91	3	221	7	-	+	-	-
19.3.91	8	199	5	-	+	-	-
18.4.91	14	241	3	-	+	-	-
31.7.91	23	220	6	-	+	-	-
18.10.91	19	289	5	-	+	-	-
19.3.92				-	+	-	-
5.8.92				-	+	-	-
29.10.92				-	+	-	-

Appendix 6.2.18: patient 18 first treated 8.10.89

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
24.6.93				-	+	-	-
6.1.94	17			-	+	-	-
15.4.94	21			-	+	-	-
11.8.94	26			-	+	-	-
22.12.94	28			-	+	-	-
2.3.95	27			-	+	-	-
17.8.95	22			-	+	-	-
13.10.95	21			-	+	-	-
20.2.96	43			-	+	-	-
29.8.96	28			-	+	-	-

Appendix 6.2.18 cont'd: patient 18 first treated 8.10.89

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
18.10.89	11	341	3				
31.10.89	11	362	6				
17.11.89	7	343	2				
14.12.89	8	356	1				
28.12.89	7	380	1	-	+	-	-
11.1.90	5	342	2	-	+	-	-
26.1.90	14	285	5	-	+	-	-
9.2.90	8	359	3	-	+	-	-
6.3.90	9	320	4	-	+	-	-
3.4.90	11	324	1	-	+	-	-
3.5.90	11	440	10	-	+	-	-
8.6.90	12	292	1	-	+	-	-
29.6.90	1	330	4	-	+	-	-
1.8.90	1	2025	7	-	+	-	-
6.9.90	12	344	5	-	+	-	-
5.10.90	6	288	3	-	+	-	-
5.11.90	5	276	1	-	+	-	-
10.12.90	7	317	2				
17.1.91	3	261	14	-	+	-	-
14.2.91	7	311	2	-	+	-	-
25.3.91	3	289	22	-	+	-	-
21.9.91	3	258	10	-	+	-	-
4.2.92	26	246	8	-	+	-	-

Appendix 6.2.19: patient 19 first treated 18.10.89

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
20.11.89	34	442	5	-	+	-	-
4.12.89	9	322	6	-	+	-	-
18.12.89	22	384	3	-	+	-	-
3.1.90	18	422	3	-	+	-	-
19.1.90	12	372	4	-	+	-	-
2.2.90	14	459	2	-	+	-	-
19.2.90	24	485	4	-	+	-	-
2.3.90	32	406	1	-	+	-	-
16.3.90	22	606	3	-	+	-	-
30.3.90	29	497	3				
13.4.90	21	510	3	-	+	-	-
15.5.90	28	454	3	-	+	-	-
5.6.90	35	465	1	-	+	-	-
9.7.90	16	327	11	-	+	-	-
31.7.90	18	439	2	-	+	-	-
14.9.90	27	553	5	-	+	-	-
25.10.90	26	420	4	-	+	-	-
16.11.90	25	410	6	-	+	-	-
24.1.91	43	551	11	-	+	-	-
13.3.91	**67**	280	17	-	+	-	-
13.8.91	**105**	427	10	-	+	-	-
30.10.91				-	+	-	-
13.1.92	**73**	378	11	-	+	-	-
11.6.92				-	+	-	-
3.12.92				-	+	-	-
17.6.93				-	+	-	-
17.2.94	31						
13.5.94	41						
11.11.94	29			-	+	-	-
2.3.95	37			-	+	-	-
23.10.95	45			-	+	-	-

Appendix 6.2.20: patient 20 first treated 20.11.89

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
22.2.96	41			-	+	-	-
13.5.96	41			-	+	-	-

Appendix 6.2.20 cont'd: patient 20 first treated 20.11.89

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
22.11.89	14	503	2	-	-	-	-
8.12.89	24	419	3	-	+	-	-
22.12.89	14	396	1	-	+	-	-
4.1.90	15	414	5	-	+	-	-
18.1.90	19	391	5	-	+	-	-
6.2.90	14	416	3	-	+	-	-
20.2.90	14	415	5	-	+	-	-
2.3.90	15	432	2	-	+	-	-
16.3.90	14	432	3	-	+	-	-
21.4.90	17	740	8	-	+	-	-
18.5.90	15	402	1	-	+	-	-
12.6.90	12	467	6	-	+	-	-
13.7.90	34	441	6	-	+	-	-
9.8.90	12	412	4	-	+	-	-
6.9.90	15	369	5	-	+	-	-
4.10.90	10	406	4	-	+	-	-
1.11.90	19	378	5	-	+	-	-
30.11.90	12	384	3	-	+	-	-
23.1.91				-	+	-	-
14.6.91	10	384	5	-	+	-	-
20.9.91	4	330	6	-	+	-	-
14.11.91	26	273	6	-	+	-	-
2.12.93	24			-	+	-	-
18.3.94	22			-	+	-	-
30.6.94	15			-	+	-	-
23.9.94	16			-	+	-	-
26.1.95	14			-	+	-	-
31.5.95	11			-	+	-	-
19.10.95	18			-	+	-	-
25.1.96	24			-	+	-	-
19.4.96	20			-	+	-	-
18.7.96	29			-	+	-	-

Appendix 6.2.21: patient 21 first treated 22.11.89

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
16.1.90	13	353	3	-	-	-	-
29.1.90	16			-	-	-	-
12.2.90	16	396	3	-	-	-	-
26.2.90	11	391	1	-	-	-	-
9.3.90	12	414	2	-	-	-	-
26.3.90	12	361	2	-	+	-	-
9.4.90	10	490	3	-	+	-	-
26.4.90	1	343	3	-	+	-	-
11.5.90	14	450	4	-	+	-	-
8.6.90	11	364	2	-	+	-	-
28.6.90	16	413	6	-	+	-	-
3.8.90	17	470	4	-	+	-	-
4.9.90	9	272	1	-	+	-	-
1.10.90	10	362	3	-	+	-	-
7.11.90	11	365	4	-	+	-	-
6.12.90	10	283	3	-	+	-	-
4.1.91	9	304	4				
6.2.91	17	314	2	-	+	-	-
4.4.91	6	336	6	-	+	-	-
4.6.91	11	308	4	-	+	-	-
4.6.92				-	+	-	-
26.11.92				-	+	-	-
17.6.93				-	+	-	-
9.12.93	21			-	+	-	-
30.3.94	32			-	+	-	-
15.9.94	14			-	+	-	-
16.2.95	13			-	+	-	-
31.7.95	17			-	+	-	-
23.10.95	20			-	+	-	-
18.1.96	22			-	+	-	-
7.6.96	11			-	+	-	-

Appendix 6.2.22: patient 22 first treated 16.1.90

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
24.3.90	8	498	3	-	-	-	-
6.4.90	13	496	3	-	-	-	-
23.4.90	16	372	3	-	-	-	-
4.5.90	11	506	4	-	+	-	-
21.5.90	13	342	1	-	+	-	-
4.6.90	12	357	2	-	+	-	-
19.6.90	13	328	3	-	+	-	-
30.6.90	26	243	2	-	+	-	-
13.7.90	7	298	5	-	+	-	-
13.8.90	12	317	3	-	+	-	-
3.9.90	7	267	1	-	+	-	-
8.10.90	15	295	3	-	+	-	-
5.11.90	12	326	3	-	+	-	-
3.12.90	7	311	5	-	+	-	-
31.12.90	3	248	2	-	+	-	-
18.2.91		819	15	-	+	-	-
25.3.91				-	+	-	-
19.4.91	15	334	4	-	+	-	-
14.6.91	32	331	5				
5.7.91				-	+	-	-
10.10.91	21	400	5	-	+	-	-
11.6.92				-	+	-	-
10.12.92				-	+	-	-
29.7.93				-	+	-	-
1.11.93	18						
17.2.94	11			-	+	-	-
6.5.94	15			-	+	-	-
25.8.94	22			-	+	-	-
10.11.94	19			-	+	-	-
23.10.95				-	+	-	-
5.4.96				-	+	-	-

Appendix 6.2.23: patient 23 first treated 24.3.90

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
17.5.90	8	321	4				
30.5.90	12	368	3	-	+	-	-
14.6.90	8	358	6	-	+	-	-
27.6.90	11	388	6				
11.7.90	13	455	4	-	+	-	-
24.7.90	8	375	5	-	+	-	-
8.8.90	10	415	6	-	+	-	-
24.8.90	35	366	5				
5.9.90	26	375	6	-	+	-	-
3.10.90	9	396	3	-	+	-	-
31.10.90	9	353	4	-	+	-	-
28.11.90	15	332	6	-	+	-	-
21.12.90	3	287	21	-	+	-	-
23.1.91	11	347	3	-	+	-	-
20.2.91	3	271	7	-	+	-	-
19.3.91	3	306	10	-	+	-	-
19.4.91	8	252	5	-	+	-	-
31.7.91	15	280	7	-	+	-	-
18.10.91	18	397	6	-	+	-	-
19.3.92				-	+	-	-
5.8.92				-	+	-	-
29.10.92				-	+	-	-
6.1.94	19			-	+	-	-
15.4.94	18			-	+	-	-
11.8.94	21			-	+	-	-
22.12.94	26			-	+	-	-
2.3.95	22			-	+	-	-
17.8.95	16			-	+	-	-
20.2.96	16			-	+	-	-
4.6.96				-	+	-	-
29.8.96	23						

Appendix 6.2.24: patient 24 first treated 17.5.90

Date	ALT	Alk Phos	Bilirubin	HBsAg	HBsAb	HBcAb	HIV Ab
1.8.90	14	619	6	-	+	-	-
15.8.90	12	663	2	-	+	-	-
29.8.90	8	617	1	-	+	-	-
14.9.90	8	575	4	-	+	-	-
26.9.90	12	60	4				
12.10.90	26	548	4	-	+	-	-
7.11.90	15	505	4	-	+	-	-
21.11.90	8	524	3	-	+	-	-
6.12.90	10	427	4				
4.1.91	9			-	+	-	-
6.2.91	9	406	4				
27.2.91	9	443	7	-	+	-	-
4.4.91				-	+	-	-
4.6.91	11	548	4	-	+	-	-
9.9.91	14	471	6	-	+	-	-
9.12.93	13			-	+	-	-
17.2.94	23			-	+	-	-
1.9.94	21			-	+	-	-
11.11.94	22			-	+	-	-
16.2.95	20			-	+	-	-
13.7.95	18			-	+	-	-
20.10.95	16			-	+	-	-
18.1.96	23			-	+	-	-
5.6.96	19			-	+	-	-

Appendix 6.2.25: patient 25 first treated 1.8.90

patient	date	hepatitis C antibody
1	10.3.89	negative
	25.3.94	negative
	7.7.94	negative
	16.12.94	negative
	16.3.95	negative
	7.8.95	negative
	30.10.95	negative
	25.1.96	negative
	12.4.96	negative
2	16.1.89	negative
	30.5.91	negative
	12.4.94	negative
	4.8.94	negative
	23.2.95	negative
	21.4.95	negative
	17.8.95	negative
	28.12.95	negative
	11.4.96	negative
3	2.3.89	negative
	8.5.91	negative
	18.8.94	negative
	9.12.94	negative
	13.1.95	negative
	23.3.95	negative
	24.8.95	negative
	24.10.95	negative
	26.3.96	negative
4	20.1.89	negative
	9.5.91	negative
	5.5.94	negative
	25.11.94	negative
	17.8.95	negative
	27.2.96	negative
5	21.10.88	negative
	1.3.94	negative
	7.7.94	negative
	15.10.94	negative
	20.7.95	negative
	10.11.95	negative
	21.3.96	negative

Appendix 6.3. results of hepatitis C antibody tests

patient	date	hepatitis C antibody
6	3.1.89	negative
	14.8.91	negative
	27.1.94	negative
	25.3.94	negative
	4.8.94	negative
	21.11.94	negative
	20.3.95	negative
	3.8.95	negative
	11.12.95	negative
	11.4.96	negative
	5.8.96	negative
7	14.10.88	negative
	10.10.91	negative
	11.5.94	negative
	23.6.94	negative
	12.9.94	negative
	23.3.95	negative
	22.8.95	negative
	2.11.95	negative
	28.3.96	negative
8	25.10.88	negative
	3.2.94	negative
	13.4.94	negative
	30.6.94	negative
	5.9.94	negative
	3.4.95	negative
	24.8.95	negative
	6.11.95	negative
9	20.1.89	negative
	15.4.94	negative
	25.8.94	negative
10	18.1.89	negative
	10.5.91	negative
	11.3.94	negative
	30.9.94	negative
	2.2.95	negative
	19.5.95	negative
	14.8.95	negative
	27.10.95	negative
	8.2.96	negative
	3.7.96	negative

Appendix 6.3.continued results of hepatitis C antibody tests

patient	date	hepatitis C antibody
11	13.3.89	negative
	11.10.91	negative
	4.8.94	negative
	7.12.94	negative
	16.3.95	negative
	16.6.95	negative
	31.8.95	negative
	11.4.96	negative
12	21.2.89	negative
	9.5.91	negative
	29.3.94	negative
	21.7.94	negative
	31.10.94	negative
	24.7.95	negative
	23.10.95	negative
	22.2.96	negative
	4.6.96	negative
13	24.2.89	negative
	17.5.91	negative
	18.3.94	negative
	21.7.94	negative
	5.1.95	negative
	23.6.95	negative
14	15.2.89	negative
	19.3.94	negative
	30.6.94	negative
	1.10.94	negative
	26.1.95	negative
	20.7.95	negative
	2.11.95	negative
	1.2.96	negative
	20.5.96	negative
15	2.3.89	negative
	11.6.91	negative
	7.7.94	negative
	12.10.94	negative
	12.1.95	negative
	1.5.95	negative
	24.7.95	negative
	27.10.95	negative
	25.1.96	negative
	12.4.96	negative
	3.7.96	negative

Appendix 6.3.continued results of hepatitis C antibody tests

patient	date	hepatitis C antibody
17.	7.5.91	negative
	17.2.94	negative
	7.7.94	negative
	16.3.95	negative
	20.7.95	negative
	24.11.95	negative
	4.4.96	negative
18.	18.10.91	negative
	11.8.94	negative
	22.12.94	negative
	2.3.95	negative
	17.8.95	negative
	13.10.95	negative
	20.2.96	negative
	4.6.96	negative
19.	23.3.91	negative
20.	13.1.92	negative
	11.11.94	negative
	2.3.95	negative
	31.7.95	negative
	23.10.95	negative
	22.2.96	negative
	13.5.96	negative
21.	7.5.91	negative
	23.9.94	negative
	26.1.95	negative
	31.5.95	negative
	16.8.95	negative
	19.10.95	negative
	25.1.96	negative
	19.4.96	negative
	18.7.96	negative

Appendix 6.3.continued results of hepatitis C antibody tests

patient	date	hepatitis C antibody
22.	4.6.91	negative
	30.3.94	negative
	15.9.94	negative
	16.2.95	negative
	31.7.95	negative
	23.10.95	negative
	18.1.96	negative
	7.6.96	negative
23.	10.10.91	negative
	17.2.94	negative
	6.5.94	negative
	25.8.94	negative
	10.11.94	negative
	23.10.95	negative
	5.4.96	negative
24.	18.10.91	negative
	15.4.94	negative
	11.8.94	negative
	22.12.94	negative
	2.3.95	negative
	17.8.95	negative
	10.11.95	negative
	20.2.96	negative
25.	4.6.91	negative
	17.2.94	negative
	1.9.94	negative
	11.11.94	negative
	13.7.95	negative
	20.10.95	negative
	18.1.96	negative
	5.6.96	negative

Appendix 6.3.continued results of hepatitis C antibody tests

date of assay	lymphocyte count	CD4 percentage	absolute CD4 count (x 10^6/l)	CD8 percentage	absolute CD8 count (x 10^6/l)	CD4/CD8 ratio
17.8.87		44		33		1.3
4.1.88	3.9	34	1326	27	1053	1.3
22.4.88	4.4	52	2288	39	1716	1.3
19.8.88	3.5	50	1750	42	1470	1.2
10.3.89	3.1	31	961	28	868	1.1
19.7.89	3.2	38	1216	36	1152	1.0
14.12.89		33		20		1.6
9.7.92	2.0	47	950	30	600	1.6
17.12.92	2.1	42	890	27	570	1.6
20.5.93	1.6	42	680	28	450	1.5
25.11.93	1.7	45	770	28	470	1.6
7.7.94	2.1	39	820	26	560	1.5
16.3.95	2.0	31	630	19	380	1.7
7.8.95	1.3	42	550	25	330	1.7

appendix 7.1.1 Patient 1, first treated 19.7.85;
serial CD4 and CD8 absolute values and percentages

date of assay	lymphocyte count	CD4 percentage	absolute CD4 count (x 10^6/l)	CD8 percentage	absolute CD8 count (x 10^6/l)	CD4/CD8 ratio
14.8.87	3.7	48	1776	30	1110	1.6
16.2.88		45		32		1.4
8.4.88	5.4	37	1998	28	1512	1.3
15.7.88	5.6	49	2744	30	1680	1.6
16.12.88		54		44		1.2
15.5.89	4.4	23	1012	15	660	1.5
15.12.89		32		32		1.0
6.8.90	3.4	46	1560	30	1000	1.5
23.7.92	2.3	32	740	26	600	1.2
17.12.92	2.7	39	1060	27	740	1.4
3.6.93	2.7	21	570	20	540	1.1
18.11.93	2.1	41	860	31	660	1.3
4.8.94	2.9	36	1060	27	780	1.4
23.2.95	2.4	36	860	26	620	1.4
17.8.95	1.9	33	630	28	540	1.2

appendix 7.1.2 Patient 2, first treated 28.12.85;
serial CD4 and CD8 absolute values and percentages

date of assay	lymphocyte count	CD4 percentage	absolute CD4 count (x 10^6/l)	CD8 percentage	absolute CD8 count (x 10^6/l)	CD4/CD8 ratio
22.7.87	3.7	55	2035	14	518	3.9
23.10.87	3.4	30	1020	15	510	2.0
15.1.88	4.7	58	2726	26	1222	2.2
11.4.88		45		38		1.2
21.7.88	3.3	42	1386	40	1320	1.0
31.1.89		40		40		1.0
8.5.89	4.4	18	792	15	660	1.2
3.8.89	3.6	42	1512	41	1476	1.0
6.12.89	2.5	36	900	16	400	2.2
4.6.92	2.9	36	1050	22	630	1.7
10.12 .92	2.4	39	950	20	490	1.9
1.7.93	3.0	31	920	17	510	1.8
27.1.94	2.4	33	790	17	400	2.0
18.8.94			1040		630	1.7
23.3.95	2.1	33	690	18	370	1.9

appendix 7.1.3 Patient 3, first treated 3.1.86;
serial CD4 and CD8 absolute values and percentages

date of assay	lymphocyte count	CD4 percentage	absolute CD4 count (x 10^6/l)	CD8 percentage	absolute CD8 count (x 10^6/l)	CD4/CD8 ratio
6.8.87	4.3	38	1634	16	688	2.4
15.1.88		50		16		3.1
8.4.88	2.8	47	1316	31	868	1.5
10.7.88	3.8	60	2280	32	1216	1.9
13.12.88		72		22		3.3
14.4.89	4.1	62	2542	18	738	3.4
15.8.89		56		24		2.3
14.12.89	3.6	35	1260	16	576	2.2
25.7.90	4.1	66	2700	30	1230	2.2
4.1.91	3.8	52	1980	17	650	3.0
30.7.92			1170		510	2.3
12.11.92	2.9	29	840	11	310	2.7
6.1.94	2.5	48	1210	21	530	2.3
14.7.94	2.0	40	800	18	370	2.2
23.3.95	2.3	37	860	17	400	2.2
17.8.95	2.4	47	1130	20	480	2.4

appendix 7.1.4 Patient 4, first treated 20.1.86;
serial CD4 and CD8 absolute values and percentages

date of assay	lymphocyte count	CD4 percentage	absolute CD4 count (x 10^6/l)	CD8 percentage	absolute CD8 count (x 10^6/l)	CD4/CD8 ratio
19.8.87	6.8	34	2312	34	2312	1.0
12.1.88	6.2	38	2356	30	1860	1.3
19.4.88	5.4	36	1944	32	1728	1.1
20.9.88	4.8	46	2208	41	1968	1.1
17.3.89	4.9	26	1274	24	1176	1.1
4.7.89		30		19		1.6
11.12.89	5.5	26	1430	21	1155	1.2
5.7.90	5.2	38	1976	26	1352	1.5
28.5.92	3.8	23	860	25	960	0.9
12.11.92	1.3	20	260	28	360	0.7
24.6.93	3.3	18	580	46	1510	0.4
2.12.93	3.3	30	990	37	1220	0.8
7.7.94	3.1	26	820	34	1050	0.8
9.2.95	3.2	25	800	39	1240	0.6
20.7.95	3.4	24	820	31	1060	0.8

appendix 7.1.5 Patient 5, first treated 31.5.86;
serial CD4 and CD8 absolute values and percentages

date of assay	lymphocyte count	CD4 percentage	absolute CD4 count (x 10^6/l)	CD8 percentage	absolute CD8 count (x 10^6/l)	CD4/CD8 ratio
2.9.87	2.7	34	918	11	297	3.1
31.12.87	4.7	50	2350	17	799	2.9
8.4.88		50		23		2.2
20.9.88	4.1	56	2296	50	2050	1.1
17.3.89	3.7	29	1073	16	592	1.8
18.8.89	5.1	34	1734	22	1122	1.5
17.1.90	3.1	30	930	15	460	2.0
28.12.90	3.0	51	1530	23	690	2.2
11.6.92	2.3	39	890	19	440	2.0
17.12.92	2.1	34	710	16	340	2.1
8.7.93	2.2	35	770	20	430	1.8
27.1.94	2.2	42	920	22	480	1.9
4.8.94	2.2	35	770	19	420	1.8
20.3.95	1.8	44	790	21	380	2.1
3.8.95	1.7	39	660	22	370	1.8

appendix 7.1.6 Patient 6, first treated 6.6.86;
serial CD4 and CD8 absolute values and percentages

date of assay	lymphocyte count	CD4 percentage	absolute CD4 count (x 10^6/l)	CD8 percentage	absolute CD8 count (x 10^6/l)	CD4/CD8 ratio
1.10.87		25		20		1.3
18.2.88	3.3	44	1452	28	924	1.6
18.5.88	2.6	40	1040	23	598	1.7
12.8.88	2.4	66	1584	30	720	2.2
16.12.88		47		36		1.3
24.4.89	2.6	28	728	17	442	1.6
29.9.89	2.3	38	874	27	621	1.4
6.2.90		32		30		1.1
1.8.90	2.4	64	1530	30	720	2.1
28.1.91	1.9	33	620	33	620	1.0
14.5.92	2.1	35	740	28	580	1.3
5.11.92	1.7	34	570	22	371	1.5
1.7.93	1.6	35	560	23	360	1.6
17.2.94	1.4	39	550	26	360	1.5
23.3.95	2.2	31	690	20	440	1.6

appendix 7.1.7 Patient 7, first treated 1.9.86;
serial CD4 and CD8 absolute values and percentages

date of assay	Lymphocyte count	CD4 percentage	absolute CD4 count (x 10^6/l)	CD8 percentage	absolute CD8 count (x 10^6/l)	CD4/CD8 ratio
17.8.87		35		13		2.7
30.12.87	6.8	54	3672	20	1360	2.7
22.4.88	6.7	51	3417	38	2546	1.3
15.7.88	6.7	55	3685	26	1742	2.1
16.12.88		50		40		1.2
11.4.89	6.9	39	2691	17	1173	2.3
3.8.89	4.5	46	2070	21	945	2.2
3.1.90	5.6	50	2800	24	1340	2.1
11.6.92	3.5	41	1430	33	1170	1.2
19.11.92	2.1	34	720	32	670	1.1
8.7.93	3.2	36	1150	26	850	1.4
3.2.94	2.6	37	960	25	640	1.5
3.6.94	2.8	43	1210	35	970	1.2
24.8.95	3.2	44	1400	25	810	1.7

appendix 7.1.8 Patient 8, first treated 28.9.86;
serial CD4 and CD8 absolute values and percentages

Date of assay	lymphocyte count	CD4 percentage	absolute CD4 count (x 10^6/l)	CD8 percentage	absolute CD8 count (x 10^6/l)	CD4/CD8 ratio
31.12.87	4.2	34	1428	16	672	2.1
8.4.88	5.5	51	2805	29	1595	1.8
12.8.88	6.8	56	3808	32	2176	1.7
30.12.88		61		44		1.4
12.5.89	5.5	45	2475	13	715	3.5
24.8.89	5.7	37	2109	23	1311	1.6
11.12.89	2.8	38	1064	15	420	2.5
8.8.90	4.0	72	2880	40	1600	1.8
27.12.90	4.5	61	2700	31	1390	2.0
11.6.92	2.8	36	1010	19	540	1.9
3.12.92	2.4	44	1060	21	510	2.1
24.6.93	2.6	54	1400	25	650	2.2
2.12.93	2.2	44	960	21	470	2.0
30.6.94	2.8	38	1060	20	550	1.9
2.2.95	2.6	42	1090	20	530	2.1
14.8.95			1070		510	2.1

appendix 7.1.10 Patient 10, first treated 27.1.87;
serial CD4 and CD8 absolute values and percentages

Date of Assay	lymphocyte count	CD4 percentage	absolute CD4 count (x 10^6/l)	CD8 percentage	absolute CD8 count (x 10^6/l)	CD4/CD8 ratio
6.11.87	6.9	52	3588	15	1035	3.5
30.12.87	6.8	34	2312	12	816	2.8
23.5.88	5.3	58	3074	34	1802	1.7
21.7.88	5.0	53	2650	25	1250	2.1
11.12.88		38		26		1.5
13.3.89	4.5	41	1845	21	945	1.9
10.8.89	5.3	36	1908	26	1378	1.4
5.12.89	0.9	75	675	51	46	1.5
26.7.90	2.8	53	1480	32	890	1.7
18.12.90	3.2	46	1500	21	700	2.2
4.6.92	2.6	33	870	27	720	1.2
19.11.92	2.4	29	700	19	470	1.5
10.6.93	2.2	28	610	24	520	1.2
25.11.93	2.0	36	720	26	520	1.4
4.8.94	1.7	38	640	27	460	1.4
16.3.95	1.3	39	510	26	340	1.5

appendix 7.1.11 Patient 11, first treated 4.2.87;
serial CD4 and CD8 absolute values and percentages

Date of assay	lymphocyte count	CD4 percentage	absolute CD4 count (x 10^6/l)	CD8 percentage	absolute CD8 count (x 10^6/l)	CD4/CD8 ratio
6.8.87	6.0	37	2220	20	1200	1.9
23.10.87	6.6	37	2442	14	924	2.6
18.12.87	1.7	32	544	20	340	1.6
5.5.88	3.9	60	2340	25	975	2.4
15.7.88	4.3	52	2236	30	1290	1.7
20.12.88	4.2	47	1974	36	1512	1.3
31.3.89	4.2	38	1596	19	798	2.0
3.7.89		35		31		1.1
20.10.89	2.1	26	546	18	378	1.4
6.2.90	3.6	29	1040	22	790	1.3
23.5.90	3.1	41	1270	41	1270	1.0
4.6.92	2.7	29	790	28	750	1.1
3.12.92	2.3	35	800	31	710	1.1
3.6.93	2.1	29	610	28	590	1.0
9.12.93	2.1	40	840	35	730	1.2
21.7.94	1.9	30	580	30	570	1.0
9.3.95	2.4	40	960	34	816	1.2
24.7.95	2.4	33	790	28	670	1.2

appendix 7.1.12, patient 12, first treated 6.2.87;
Serial CD4 and CD8 absolute values and percentages

Date of assay	lymphocyte count	CD4 percentage	absolute CD4 count (x 10^6/l)	CD8 percentage	absolute CD8 count (x 10^6/l)	CD4/CD8 ratio
6.8.87	6.1	35	2135	23	1403	1.5
18.1.88	6.0	32	1920	35	2100	0.9
13.4.88	5.7	44	2508	20	1140	2.2
21.7.88	5.0	43	2150	31	1550	1.4
13.12.88	6.0	34	2040	23	1380	1.5
13.3.89	7.1	46	3266	28	1988	1.6
3.8.89	5.7	31	1767	31	1767	1.0
12.12.89	3.5	29	1015	21	735	1.4
17.7.90	3.1	33	1020	28	900	1.2
14.1.91	4.2	44	1840	28	1180	1.6
28.5.92	2.6	26	670	22	580	1.2
5.11.92	2.4	25	611	19	467	1.3
10.6.93	2.4	25	610	24	570	1.1
2.12.93	2.4	34	820	29	690	1.2
3.6.94	2.0	24	470	30	590	0.8
26.1.95	1.8	32	570	28	510	1.1
20.7.95	1.8	32	570	28	510	1.1

appendix 7.1.14, patient 14, first treated 12.4.87;
Serial CD4 and CD8 absolute values and percentages

Date of assay	lymphocyte count	CD4 percentage	absolute CD4 count (x 10^6/l)	CD8 percentage	absolute CD8 count (x 10^6/l)	CD4/CD8 ratio
5.10.87		62		12		5.1
18.12.87		44		21		2.1
22.4.88		64		22		2.9
10.7.88	5.3	55	2915	25	1325	2.2
13.12.88	5.4	39	2106	24	1296	1.6
5.4.89	4.5	26	1170	15	675	1.7
30.8.89	4.9	38	1862	14	686	2.7
14.12.89	4.3	18	774	12	516	1.5
16.1.91	4.6	37	1700	23	1060	1.6
18.6.92	3.9	30	1190	23	910	1.3
3.12.92	1.6	36	570	29	470	1.2
10.6.93	2.0	39	790	30	600	1.3
2.12.93	2.2	38	830	29	630	1.3
7.7.94	2.7	35	940	27	740	1.3
12.1.95	2.6	31	820	27	700	1.2
24.7.95			740		590	1.3

Appendix 7.1.15 Patient 15, first treated 5.10.87;
Serial CD4 and CD8 absolute counts and percentages

Date of assay	lymphocyte count	CD4 percentage	absolute CD4 count (x 10^6/l)	CD8 percentage	absolute CD8 count (x 10^6/l)	CD4/CD8 ratio
19.9.89	6.8	30	2000	30	2000	1.0
11.1.90	3.3	46	1520	42	1380	1.1
2.7.90	3.1	42	1300	35	1080	1.2
28.12.90	3.0	45	1350	18	540	2.5
28.5.92	2.0	32	640	34	690	0.9
19.11.92	1.5	28	420	27	410	1.0
20.5.93	1.4	37	520	34	480	1.1
18.11.93	0.9	40	360	25	230	1.6
7.7.94	1.8	38	680	31	560	1.2
16.3.95	1.7	38	650	27	460	1.4
20.7.95	1.6	32	510	25	400	1.3

appendix 7.1.17 patient 17, first treated 19.9.89;
Serial CD4 and CD8 absolute values and percentages

Date of assay	lymphocyte count	CD4 percentage	absolute CD4 count (x 10^6/l)	CD8 percentage	absolute CD8 count (x 10^6/l)	CD4/CD8 ratio
13.12.89	7.0	33	2300	23	1600	1.4
25.7.90	3.6	47	1700	31	1100	1.5
14.1.91	5.3	48	2500	26	1400	1.8
29.10.92	3.8	33	1280	26	970	1.3
24.6.93	3.2	29	940	20	660	1.4
6.1.94	3.0	46	1370	32	970	1.4
11.8.94	3.4	43	1462	32	1088	1.3
2.3.95	3.6	40	1440	28	1020	1.4
17.8.95	2.6	29	760	24	620	1.2

appendix 7.1.18 patient 18, first treated 8.10.89;
Serial CD4 and CD8 absolute counts and percentages

Date of assay	lymphocyte count	CD4 percentage	absolute CD4 count (x 10^6/l)	CD8 percentage	absolute CD8 count (x 10^6/l)	CD4/CD8 ratio
20.11.89	4.5	24	1080	20	900	1.2
31.7.90	3.9	45	1750	23	900	1.95
11.6.92	3.1	35	1100	23	730	1.5
3.12.92	3.0	30	890	23	690	1.3
17.6.93	2.7	28	770	22	610	1.3
25.8.94	3.1	39	1220	27	830	1.5
2.3.95	3.4	35	1180	25	860	1.4
31.7.95	3.1	31	970	23	720	1.3
23.10.95	2.4	40	960	25	610	1.6

appendix 7.1.20 patient 20 first treated 20.11.89;
Serial CD4 and CD8 absolute values and percentages

Date of assay	lymphocyte count	CD4 percentage	absolute CD4 count (x 10^6/l)	CD8 percentage	absolute CD8 count (x 10^6/l)	CD4/CD8 ratio
22.11.89	6.8	42	2860	17	1150	2.5
18.7.90	3.5	52	1820	26	910	2.0
24.1.91	2.8	47	1320	28	780	1.7
4.6.92	2.9	29	840	19	550	1.5
19.11.92	2.0	35	700	20	390	1.8
3.6.93	2.6	26	680	14	370	1.8
2.12.93	2.4	39	930	24	590	1.6
30.6.94	2.2	44	970	22	490	2.0
26.1.95	1.7	45	770	22	380	2.0
17.8.95	1.8	42	756	25	450	1.7

appendix 7.1.21 patient 21, first treated 22.11.89
Serial CD4 and CD8 absolute values and percentages

Date of assay	lymphocyte count	CD4 percentage	absolute CD4 count (x 10^6/l)	CD8 percentage	absolute CD8 count (x 10^6/l)	CD4/CD8 ratio
29.1.90	5.1	36	1840	13	660	2.8
6.2.91	5.1	48	2500	19	970	2.5
4.6.92	4.0	37	1490	18	730	2.0
26.11.92	2.4	39	930	23	570	1.6
17.6.93	2.4	31	740	22	540	1.4
9.12.93	2.3	53	1230	36	820	1.5
28.7.94	2.3	40	930	31	710	1.3
16.2.95	2.0	38	760	28	560	1.4
31.7.95	2.1	34	720	22	470	1.5

appendix 7.1.22 patient 22, first treated 16.1.90
Serial CD4 and CD8 absolute values and percentages

Date of assay	lymphocyte count	CD4 percentage	absolute CD4 count (x 10^6/l)	CD8 percentage	absolute CD8 count (x 10^6/l)	CD4/CD8 ratio
18.2.91	3.8	56	2100	34	1290	1.6
11.6.92	4.4	32	1400	24	1060	1.3
10.12.92	3.8	36	1360	28	1060	1.3
29.7.93	2.4	33	800	22	540	1.5
17.2.94	2.8	37	1050	27	750	1.4
25.8.94	2.0	44	890	28	560	1.6

appendix 7.1.23 patient 23, first treated 24.3.90;
Serial CD4 and CD8 absolute counts and percentages

Date of assay	lymphocyte count	CD4 percentage	absolute CD4 count (x 10^6/l)	CD8 percentage	absolute CD8 count (x 10^6/l)	CD4/CD8 ratio
17.5.90	4.3	41	1760	30	1290	1.4
24.1.91	4.5	41	1850	22	990	1.9
29.10.92	3.7	24	890	20	750	1.1
24.6.93	2.3	33	760	30	700	1.1
6.1.94	3.0	39	1170	37	1116	1.0
11.8.94			1110		1050	1.1
2.3.95	3.6	39	1428	37	1357	1.1
17.8.95	2.5	36	900	31	780	1.2

appendix 7.1.24 patient 24, first treated 17.5.90
Serial CD4 and CD8 absolute values and percentages

Date of assay	lymphocyte count	CD4 percentage	absolute CD4 count (x 10^6/l)	CD8 percentage	absolute CD8 count (x 10^6/l)	CD4/CD8 ratio
4.6.92	2.5	32	810	31	780	1.0
26.11.92	2.2	30	660	31	680	1.0
17.6.93	2.3	27	630	28	660	1.0
9.12.93	2.8	42	1170	31	880	1.3
17.2.94	1.8	40	730	35	630	1.2
1.9.94	2.1	38	810	30	640	1.3
16.2.95	1.7	43	730	33	570	1.3

appendix 7.1.25 patient 25, first treated 1.8.90
Serial CD4 and CD8 absolute counts and percentages

Date of T cell assay	age (years)	% CD4 z score	% CD8 z score
17.8.87	3.173	0.752	0.991
4.1.88	3.556	-0.356	0.242
22.4.88	3.855	1.789	1.606
19.8.88	4.181	1.6	1.907
10.3.89	4.736	-0.616	0.317
19.7.89	5.095	0.253	1.349
14.12.89	5.5	-0.335	-1.129
9.7.92	8.068	1.433	0.697
17.12.92	8.509	0.83	0.124
20.5.93	8.931	0.833	0.346
25.11.93	9.448	1.202	0.366
7.7.94	10.062	0.471	-0.119
16.3.95	10.752	-0.516	-2.525
7.8.95	11.146	0.842	-0.462

Appendix 7.2.1 patient 1
Z scores of serial CD4 and CD8 percentages

Date of T cell assay	age (years)	% CD4 z score	% CD8 z score
14.8.87	2.68	1.099	0.682
16.2.88	3.19	0.869	0.88
8.4.88	3.332	-0.033	0.391
15.7.88	3.6	1.401	0.627
16.12.88	4.022	2.045	2.076
15.5.89	4.433	-1.611	-2.093
15.12.89	5.018	-0.479	0.858
6.8.90	5.659	1.245	0.597
23.7.92	7.622	-0.409	-0.075
17.12.92	8.025	0.458	0.122
3.6.93	8.485	-1.782	-1.579
18.11.93	8.945	0.711	0.961
4.8.94	9.654	0.101	0.136
23.2.95	10.209	0.102	-0.122
17.8.95	10.689	-0.268	0.431

Appendix 7.2.2 patient 2
Z scores of serial CD4 and CD8 percentages

Date of T cell assay	age (years)	% CD4 z score	% CD8 z score
22.7.87	2.267	1.749	-1.825
23.10.87	2.522	-0.972	-1.655
15.1.88	2.752	2.234	0.18
11.4.88	2.99	0.831	1.499
21.7.88	3.266	0.537	1.685
31.1.89	3.797	0.38	1.699
8.5.89	4.063	-2.249	-2.009
3.8.89	4.301	0.671	1.822
6.12.89	4.643	-0.017	-1.888
4.6.92	7.138	0.077	-0.888
10.12 .92	7.655	0.453	-1.436
1.7.93	8.211	-0.527	-2.418
27.1.94	8.786	-0.275	-2.585
23.3.95	9.936	-0.27	-2.608

Appendix 7.2.3 patient 3
Z scores of serial CD4 and CD8 percentages

Date of T cell assay	age (years)	% CD4 z score	% CD8 z score
6.8.87	3.028	0.035	-1.547
15.1.88	3.472	1.495	-1.643
8.4.88	3.702	1.186	0.744
10.7.88	3.956	2.729	0.857
13.12.88	4.383	4.176	-0.607
14.4.89	4.717	3.064	-1.439
15.8.89	5.054	2.394	-0.324
14.12.89	5.385	-0.096	-2.044
25.7.90	5.996	3.638	0.603
4.1.91	6.442	1.998	-2.001
12.11.92	8.298	-0.776	-4.822
6.1.94	9.448	1.566	-1.469
14.7.94	9.966	0.594	-2.619
23.3.95	10.656	0.227	-3.318
17.8.95	11.058	1.451	-2.24

Appendix 7.2.4 patient 4
Z scores of serial CD4 and CD8 percentages

Date of T cell assay	age (years)	% CD4 z score	% CD8 z score
19.8.87	1.949	-0.646	1.144
12.1.88	2.349	-0.103	0.705
19.4.88	2.617	-0.27	0.909
20.9.88	3.039	0.954	1.771
17.3.89	3.526	-1.31	-0.186
4.7.89	3.825	-0.803	-1.084
11.12.89	4.263	-1.253	-0.772
5.7.90	4.827	0.236	0.016
28.5.92	6.724	-1.545	-0.241
12.11.92	7.184	-1.919	0.302
24.6.93	7.797	-2.17	2.963
2.12.93	8.238	-0.651	1.879
7.7.94	8.832	-1.148	1.5
20.7.95	9.867	-1.397	1.075

Appendix 7.2.5 patient 5
Z scores of serial CD4 and CD8 percentages

Date of T cell assay	age (years)	% CD4 z score	% CD8 z score
2.9.87	1.949	-0.646	-2.528
31.12.87	2.278	1.206	-1.187
8.4.88	2.549	1.287	-0.217
20.9.88	3.001	2.078	2.481
17.3.89	3.488	-0.956	-1.646
18.8.89	3.91	-0.318	-0.551
17.1.90	4.326	-0.763	-2.069
28.12.90	5.27	1.819	-0.517
11.6.92	6.724	0.436	-1.538
17.12.92	7.242	-0.167	-2.473
8.7.93	7.797	-0.036	-1.459
27.1.94	8.353	0.828	-1.024
4.8.94	8.871	-0.027	-1.957
20.3.95	9.495	1.081	-1.478
3.8.95	9.867	0.471	-1.24

Appendix 7.2.6 patient 6
Z scores of serial CD4 and CD8 percentages

Date of T cell assay	age (years)	% CD4 z score	% CD8 z score
1.10.87	4.186	-1.38	-0.947
18.2.88	4.569	0.933	0.323
18.5.88	4.816	0.475	-0.477
12.8.88	5.051	3.565	0.593
16.12.88	5.396	1.351	1.369
24.4.89	5.749	-0.941	-1.862
29.9.89	6.182	0.299	0.131
1.8.90	7.02	3.446	0.639
28.1.91	7.513	-0.287	1.155
14.5.92	8.805	-0.027	0.342
5.11.92	9.284	-0.148	-1.148
1.7.93	9.936	-0.022	-0.951
17.2.94	10.568	0.473	-0.129
23.3.95	11.661	-0.515	-2.48

Appendix 7.2.7 patient 7
Z scores of serial CD4 and CD8 percentages

Date of T cell assay	age (years)	% CD4 z score	% CD8 z score
17.8.87	1.158	-0.785	-1.85
30.12.87	1.528	1.338	-0.547
22.4.88	1.84	1.158	1.513
15.7.88	2.07	1.676	0.249
16.12.88	2.491	1.271	1.682
11.4.89	2.809	0.11	-1.292
3.8.89	3.121	0.971	-0.612
3.1.90	3.54	1.507	-0.187
11.6.92	5.977	0.657	1.028
19.11.92	6.418	-0.183	0.915
8.7.93	7.05	0.076	-0.063
3.2.94	7.625	0.208	-0.279
3.6.94	7.953	0.945	1.523
24.8.95	9.177	1.079	-0.345

Table 7.2.8 patient 8
Z scores of serial CD4 and CD8 percentages

Date of T cell assay	age (years)	% CD4 z score	% CD8 z score
31.12.87	2.226	-0.579	-1.377
8.4.88	2.497	1.383	0.579
12.8.88	2.842	2.037	0.897
12.5.89	3.589	0.936	-2.416
24.8.89	3.874	0.035	-0.381
11.12.89	4.172	0.184	-2.034
8.8.90	4.83	4.238	1.764
27.12.90	5.216	2.995	0.73
11.6.92	6.672	0.068	-1.529
3.12.92	7.151	1.054	-1.119
24.6.93	7.707	2.271	-0.282
2.12.93	8.148	1.069	-1.253
30.6.94	8.723	0.342	-1.625
2.2.95	9.317	0.835	-1.748

Appendix 7.2.10 patient 10
Z scores of serial CD4 and CD8 percentages

Date of T cell assay	age (years)	% CD4 z score	% CD8 z score
6.11.87	1.27	1.005	-1.422
30.12.87	1.418	-0.8	-2.134
23.5.88	1.815	1.89	1.147
21.7.88	1.977	1.426	0.128
13.3.89	2.62	0.297	-0.536
10.8.89	3.031	-0.195	0.152
5.12.89	3.351	4.301	2.578
26.7.90	3.989	1.924	0.856
18.12.90	4.386	1.153	-0.787
4.6.92	5.848	-0.322	0.137
19.11.92	6.308	-0.803	-1.47
10.6.93	6.864	-0.916	-0.443
25.11.93	7.324	0.08	-0.069
4.8.94	8.014	0.335	0.122
16.3.95	8.627	0.464	-0.093

Appendix 7.2.11 patient 11
Z scores of serial CD4 and CD8 percentages

Date of T cell assay	age (years)	% CD4 z score	% CD8 z score
6.8.87	2.272	-0.233	-0.647
23.10.87	2.486	-0.184	-1.877
18.12.87	2.639	-0.722	-0.706
5.5.88	3.02	2.532	0.015
15.7.88	3.214	1.674	0.647
20.12.88	3.647	1.177	1.301
31.3.89	3.923	0.159	-1.1
20.10.89	4.479	-1.24	-1.398
6.2.90	4.778	-0.857	-0.651
23.5.90	5.068	0.612	1.888
4.6.92	7.102	-0.788	0.301
3.12.92	7.6	-0.039	0.839
3.6.93	8.099	-0.777	0.321
9.12.93	8.616	0.586	1.633
21.7.94	9.229	-0.646	0.788
9.3.95	9.862	0.593	1.702
24.7.95	10.237	-0.269	0.404

Appendix 7.2.12 patient 12
Z scores of serial CD4 and CD8 percentages

Date of T cell assay	age (years)	% CD4 z score	% CD8 z score
6.8.87	1.711	-0.602	-0.118
18.1.88	2.163	-0.817	1.234
13.4.88	2.398	0.579	-0.667
21.7.88	2.669	0.534	0.796
13.12.88	3.066	-0.421	-0.284
13.3.89	3.313	1.007	0.392
3.8.89	3.704	-0.695	0.744
12.12.89	4.063	-0.903	-0.745
17.7.90	4.657	-0.378	0.32
14.1.91	5.153	0.977	0.306
28.5.92	6.522	-1.172	-0.826
5.11.92	6.962	-1.29	-1.577
10.6.93	7.556	-1.283	-0.486
2.12.93	8.036	-0.157	0.51
3.6.94	8.537	-1.402	0.73
26.1.95	9.185	-0.397	0.356
20.7.95	9.665	-0.395	0.376

Appendix 7.2.14 patient 14
Z scores of serial CD4 and CD8 percentages

Date of T cell assay	age (years)	% CD4 z score	% CD8 z score
5.10.87	0.047	1.097	-2.245
18.12.87	0.249	-0.352	-0.53
22.4.88	0.594	1.741	-0.28
10.7.88	0.81	1.039	0.145
13.12.88	1.238	-0.342	0.043
5.4.89	1.547	-1.621	-1.458
30.8.89	1.949	-0.207	-1.754
14.12.89	2.24	-2.399	-2.324
16.1.91	3.329	-0.033	-0.317
18.6.92	4.75	-0.737	-0.47
3.12.92	5.21	0.017	0.451
10.6.93	5.728	0.404	0.598
2.12.93	6.207	0.3	0.455
7.7.94	6.801	-0.052	0.124
12.1.95	7.318	-0.537	0.121

Appendix 7.2.15 patient 15
Z scores of serial CD4 and CD8 percentages

Date of assay	age (years)	% CD4 z score	% CD8 z score
19.9.89	3.343	-0.853	0.64
11.1.90	3.655	1.062	1.877
2.7.90	4.126	0.653	1.199
28.12.90	4.616	1.056	-1.422
28.5.92	6.031	-0.439	1.166
19.11.92	6.511	-0.923	0.126
20.5.93	7.009	0.198	1.253
18.11.93	7.507	0.573	-0.274
7.7.94	8.14	0.336	0.882
16.3.95	8.83	0.342	0.127
20.7.95	9.175	-0.397	-0.345

Appendix 7.2.17 patient 17
Z scores of serial CD4 and CD8 percentages

Date of assay	age (years)	% CD4 z score	% CD8 z score
13.12.89	3.316	-0.503	-0.315
25.7.90	3.929	1.217	0.737
14.1.91	4.402	1.39	0.04
29.10.92	6.193	-0.312	-0.04
24.6.93	6.845	-0.792	-1.314
6.1.94	7.381	1.301	0.985
11.8.94	7.975	0.946	1.04
2.3.95	8.531	0.585	0.333
17.8.95	8.991	-0.771	-0.583

Appendix 7.2.18 patient 18
Z scores of serial CD4 and CD8 percentages

Date of T cell assay	age (years)	% CD4 z score	% CD8 z score
20.11.89	6.47	-1.423	-1.26
31.7.90	7.162	1.175	-0.672
11.6.92	9.027	-0.026	-0.843
3.12.92	9.506	-0.644	-0.897
17.6.93	10.042	-0.893	-1.271
25.8.94	11.231	0.474	0.167
2.3.95	11.748	-0.018	-0.513
31.7.95	12.162	-0.514	-1.366
23.10.95	12.392	0.599	-0.58

Appendix 7.2.20 patient 20
Z scores of serial CD4 and CD8 percentages

Date of T cell assay	age (years)	% CD4 z score	% CD8 z score
22.11.89	2.201	0.303	-1.172
18.7.90	2.853	1.591	0.17
24.1.91	3.373	1.132	0.388
4.6.92	4.734	-0.86	-1.228
19.11.92	5.194	-0.105	-1.088
3.6.93	5.73	-1.19	-2.688
2.12.93	6.229	0.423	-0.404
30.6.94	6.804	1.046	-0.854
26.1.95	7.379	1.179	-0.914
17.8.95	7.934	0.823	-0.291

Appendix 7.2.21 patient 21
Z scores of serial CD4 and CD8 percentages

Date of T cell assay	age (years)	% CD4 z score	% CD8 z score
29.1.90	1.133	-0.691	-1.847
6.2.91	2.155	0.948	-0.798
4.6.92	3.48	-0.012	-1.221
26.11.92	3.959	0.281	-0.39
17.6.93	4.515	-0.629	-0.622
9.12.93	4.994	2.035	1.343
28.7.94	5.626	0.521	0.738
16.2.95	6.182	0.299	0.295
31.7.95	6.634	-0.178	-0.838

Appendix 7.2.22 patient 22
Z scores of serial CD4 and CD8 percentages

Date of T cell assay	age (years)	% CD4 z score	% CD8 z score
18.2.91	2.842	2.037	1.109
11.6.92	4.153	-0.535	-0.249
10.12.92	4.652	-0.016	0.32
29.7.93	5.284	-0.344	-0.704
17.2.94	5.84	0.166	0.137
25.8.94	6.357	1.033	0.295

Appendix 7.2.23 patient 23
Z scores of serial CD4 and CD8 percentages

Date of T cell assay	age (years)	% CD4 z score	% CD8 z score
17.5.90	6.385	0.67	0.614
24.1.91	7.075	0.687	-0.882
29.10.92	8.838	-1.4	-1.647
24.6.93	9.489	-0.271	0.813
6.1.94	10.026	0.471	2.329
2.3.95	11.176	0.474	2.762
17.8.95	11.636	0.105	1.406

Appendix 7.2.24 patient 24
Z scores of serial CD4 and CD8 percentages

Date of T cell assay	age (years)	% CD4 z score	% CD8 z score
4.6.92	3.14	-0.644	0.769
26.11.92	3.619	-0.823	0.747
17.6.93	4.175	-1.137	0.34
9.12.93	4.654	0.702	0.726
17.2.94	4.846	0.477	1.22
1.9.94	5.383	0.268	0.594
16.2.95	5.843	0.893	1.021

Appendix 7.2.25 patient 25
Z scores of serial CD4 and CD8 percentages

date	time since first treatment (months)	treatment received prior to T lymphocyte subset assay (units FVIII / kg)			total treatment received (units FVIII)
		one week	one month	three months	
17.8.87	25	0	56	252	59859
4.1.88	30	0	0	23	65637
22.4.88	33	0	260	440	77832
19.8.88	37	0	77	203	87160
10.3.89	44	21	126	368	106300
19.7.89	48	0	150	308	119180
14.12.89	53	0	113	484	137180
9.7.92	84	63	304	1033	405790
17.12.92	89	165	309	1053	455560
20.5.93	94	34	173	893	495880
25.11.93	100	76	255	616	537885
7.7.94	108	32	214	476	572065
16.3.95	116	132	423	940	659900
7.8.95	121	121	348	813	714570

Appendix 7.3.1 patient 1
T cell subset assays; time since first treatment with FVIII and details of treatment received at the time of each assay

date	time since first treatment (months)	treatment received prior to T lymphocyte subset assay (units FVIII / kg)			total treatment received (units FVIII)
		one week	one month	three months	
14.8.87	20	0	50	281	16220
16.2.88	26	28	28	71	22090
8.4.88	28	57	87	175	24745
15.7.88	31	54	100	283	30310
16.12.88	36	0	57	127	35385
15.5.89	41	13	50	177	42130
15.12.89	48	0	40	67	50545
6.8.90	56	0	163	496	67090
23.7.92	79	0	0	97	130200
17.12.92	84	36	84	362	182725
3.6.93	90	56	238	612	200050
18.11.93	95	37	433	1185	244770
4.8.94	104	161	277	542	290650
23.2.95	110	31	103	411	317070
17.8.95	116	31	140	654	348480

Appendix 7.3.2 patient 2
T cell subset assays; time since first treatment with FVIII and details of treatment received at the time of each assay

date	time since first treatment (months)	treatment received prior to T lymphocyte subset assay (units FVIII / kg)			total treatment received (units FVIII)
		one week	one month	three months	
22.7.87	18	45	135	351	30315
23.10.87	21	154	184	514	38475
15.1.88	24	48	119	487	44925
11.4.88	27	24	113	574	54830
21.7.88	30	73	202	554	65640
31.1.89	36	37	92	234	77185
8.5.89	40	0	55	267	84535
3.8.89	43	69	174	478	95440
6.12.89	47	49	89	266	105340
4.6.92	77	27	274	715	327950
10.12 .92	83	44	184	573	389985
1.7.93	90	34	125	337	426645
27.1.94	96	47	161	367	474175
23.3.95	110	42	129	262	587990

Appendix 7.3.3 patient 3
T cell subset assays; time since first treatment with FVIII and details of treatment received at the time of each assay

date	time since first treatment (months)	treatment received prior to T lymphocyte subset assay (units FVIII / kg)			total treatment received (units FVIII)
		one week	one month	three months	
6.8.87	19	0	64	287	44160
15.1.88	24	0	91	198	49430
8.4.88	27	27	53	162	52050
10.7.88	30	56	73	396	58640
13.12.88	35	27	80	198	63020
14.4.89	39	12	12	173	67245
15.8.89	43	26	79	253	73020
14.12.89	47	0	53	238	80190
25.7.90	54	127	185	526	107495
4.1.91	60	66	176	380	121895
12.11.92	82	0	297	511	199130
6.1.94	96	52	344	985	341495
14.7.94	102	597	808	1369	412545
23.3.95	110	26	165	556	486560
17.8.95	115	38	258	640	523965

Appendix 7.3.4 patient 4
T cell subset assays; time since first treatment with FVIII and details of treatment received at the time of each assay

date	time since first treatment (months)	treatment received prior to T lymphocyte subset assay (units FVIII / kg)			total treatment received (units FVIII)
		one week	one month	three months	
19.8.87	15	0	17	121	16000
12.1.88	20	0	0	138	20495
19.4.88	23	0	0	0	20495
20.9.88	28	0	0	26	28470
17.3.89	34	0	0	27	31430
4.7.89	38	0	14	92	33110
11.12.89	43	65	150	205	37750
5.7.90	50	57	114	220	51335
28.5.92	72	45	102	151	99345
12.11.92	78	0	25	73	107255
24.6.93	85	0	0	184	115085
2.12.93	91	127	155	346	133945
7.7.94	98	7	83	154	169405
20.7.95	110	52	227	665	244005

Appendix 7.3.5 patient 5
T cell subset assays; time since first treatment with FVIII and details of treatment received at the time of each assay

date	time since first treatment (months)	treatment received prior to T lymphocyte subset assay (units FVIII / kg)			total treatment received (units FVIII)
		one week	one month	three months	
2.9.87	15	0	0	34	2585
31.12.87	18	0	0	200	5030
8.4.88	22	55	55	55	6320
20.9.88	27	0	0	102	25020
17.3.89	33	12	12	12	26735
18.8.89	38	0	38	49	29510
17.1.90	43	0	25	68	31020
28.12.90	54	0	0	12	36785
11.6.92	72	0	0	209	50945
17.12.92	78	0	23	82	55250
8.7.93	85	0	37	218	64035
27.1.94	91	24	129	145	71160
4.8.94	98	0	16	98	78320
20.3.95	105	0	0	29	82775
3.8.95	110	15	105	275	92115

Appendix 7.3.6 patient 6
T cell subset assays; time since first treatment with FVIII and details of treatment received at the time of each assay

date	time since first treatment (months)	treatment received prior to T lymphocyte subset assay (units FVIII / kg)			total treatment received (units FVIII)
		one week	one month	three months	
1.10.87	13	0	0	68	3260
18.2.88	17	109	109	136	23785
18.5.88	20	180	193	290	28370
12.8.88	23	0	0	193	28575
16.12.88	27	0	0	13	28780
24.4.89	31	0	0	60	30045
29.9.89	36	78	78	78	34545
1.8.90	47	0	0	466	55775
28.1.91	52	32	65	214	64080
14.5.92	68	43	195	533	130585
5.11.92	74	23	380	760	155835
1.7.93	82	285	361	487	198335
17.2.94	89	0	23	268	239610
23.3.95	102	47	195	901	370540

Appendix 7.3.7 patient 7
T cell subset assays; time since first treatment with FVIII and details of treatment received at the time of each assay

date	time since first treatment (months)	treatment received prior to T lymphocyte subset assay (units FVIII / kg)			total treatment received (units FVIII)
		one week	one month	three months	
17.8.87	11	24	121	568	14495
30.12.87	15	18	74	495	21575
22.4.88	19	18	77	329	25210
15.7.88	22	18	37	129	29250
16.12.88	27	48	145	420	35350
11.4.89	31	17	206	755	45805
3.8.89	35	40	180	692	57700
3.1.90	40	51	51	354	
11.6.92	69	17	86	1210	155870
19.11.92	74	34	154	418	159130
8.7.93	82	13	150	418	194660
3.2.94	89	25	99	376	213305
3.6.94	93	37	112	278	222080
24.8.95	107	44	319	919	326720

Appendix 7.3.8 patient 8
T cell subset assays; time since first treatment with FVIII and details of treatment received at the time of each assay

date	time since first treatment (months)	treatment received prior to T lymphocyte subset assay (units FVIII / kg)			total treatment received (units FVIII)
		one week	one month	three months	
31.12.87	11	0	0	73	21120
8.4.88	15	115	357	563	29565
12.8.88	19	14	28	41	30180
12.5.89	28	0	0	100	35900
24.8.89	31	27	94	169	38735
11.12.89	35	31	31	291	44305
8.8.90	43	0	127	307	117265
27.12.90	47	0	23	398	133450
11.6.92	65	97	180	430	156930
3.12.92	71	0	127	660	194165
24.6.93	77	24	158	670	226975
2.12.93	83	23	275	782	261905
30.6.94	89	200	456	1019	325510
2.2.95	97	57	299	962	419185

Appendix 7.3.10 patient 10
T cell subset assays; time since first treatment with FVIII and details of treatment received at the time of each assay

date	time since first treatment (months)	treatment received prior to T lymphocyte subset assay (units FVIII / kg)			total treatment received (units FVIII)
		one week	one month	three months	
6.11.87	9	0	44	129	4145
30.12.87	10	162	345	389	7800
23.5.88	15	0	0	144	11440
21.7.88	17	15	74	181	13845
13.3.89	25	25	50	115	18545
10.8.89	30	0	41	298	25995
5.12.89	34	0	164	218	31465
26.7.90	41	0	56	174	47530
18.12.90	46	26	106	258	57660
4.6.92	64	23	60	218	82835
19.11.92	69	0	77	309	95625
10.6.93	76	20	209	507	121875
25.11.93	81	39	199	378	138755
4.8.94	90	18	111	465	176855
16.3.95	97	17	69	208	195375

Appendix 7.3.11 patient 11
T cell subset assays; time since first treatment with FVIII and details of treatment received at the time of each assay

date	time since first treatment (months)	treatment received prior to T lymphocyte subset assay (units FVIII / kg)			total treatment received (units FVIII)
		one week	one month	three months	
6.8.87	4	151	321	501	8845
18.1.88	9	16	65	212	16425
13.4.88	12	33	203	321	20660
21.7.88	15	15	133	309	25375
13.12.88	20	16	33	65	28690
13.3.89	23	0	119	186	31645
3.8.89	28	57	99	390	41410
12.12.89	32	98	395	622	53545
17.7.90	39	84	170	688	78665
14.1.91	45	0	106	285	101430
10.6.93	74	23	117	631	236885
2.12.93	80	54	172	469	272720
3.6.94	86	21	150	444	303035
26.1.95	93	37	168	574	364150
20.7.95	99	11	126	563	415535

Appendix 7.3.14 patient 14
T cell subset assays; time since first treatment with FVIII and details of treatment received at the time of each assay

date	time since first treatment (months)	treatment received prior to T lymphocyte subset assay (units FVIII / kg)			total treatment received (units FVIII)
		one week	one month	three months	
5.10.87	0	0	0	0	0
18.12.87	2		0		
22.4.88	6	0	25	25	880
10.7.88	9	0	0	58	1370
13.12.88	14	0	0	25	1585
5.4.89	18	0	0	71	2425
30.8.89	22	0	38	236	6055
14.12.89	26	0	0	305	9880
16.1.91	39	0	0	252	27410
18.6.92	56	43	262	1085	98565
3.12.92	62	94	456	1354	138120
10.6.93	68	76	258	839	164360
2.12.93	74	38	217	723	190685
7.7.94	81	146	386	1099	238995
12.1.95	87	89	357	1163	298025

Appendix 7.3.15 patient 15
T cell subset assays; time since first treatment with FVIII and details of treatment received at the time of each assay

date	time since first treatment (months)	treatment received prior to T lymphocyte subset assay (units FVIII / kg)			total treatment received (units FVIII)
		one week	one month	three months	
19.9.89	0	0	0	0	0
2.7.90	10	27	410	555	20260
28.12.90	15	12	293	319	30970
28.5.92	32	0	66	78	41615
19.11.92	38	0	0	146	45035
20.5.93	44	0	0	22	45595
18.11.93	50	0	0	0	50740
7.7.94	58	0	0	0	52760
16.3.95	66	0	0	23	56780
20.7.95	70	0	0	0	56780

Appendix 7.3.17 patient 17
T cell subset assays; time since first treatment with FVIII and details of treatment received at the time of each assay

date	time since first treatment (months)	treatment received prior to T lymphocyte subset assay (units FVIII / kg)			total treatment received (units FVIII)
		one week	one month	three months	
13.12.89	2				
25.7.90	9				
14.1.91	15	57			8720
29.10.92	36	29	90	270	47350
24.6.93	44	0	125	315	64060
6.1.94	51	20	20	291	79000
11.8.94	58	0	127	348	99140
2.3.95	65	88	359	948	139775
17.8.95	70	94	407	1108	195780

Appendix 7.3.18 patient 18
T cell subset assays; time since first treatment with FVIII and details of treatment received at the time of each assay

date	time since first treatment (months)	treatment received prior to T lymphocyte subset assay (units FVIII / kg)			total treatment received (units FVIII)
		one week	one month	three months	
20.11.89	0	0	0	0	0
31.7.90	8	0	0	0	15060
11.6.92	31	0	0	0	15060
3.12.92	37	0	0	0	15060
17.6.93	43	0	0	0	15060
25.8.94	57	0	0	0	15060
2.3.95	64	122	450	450	33535
31.7.95	68	0	0	0	33535
23.10.95	71	0	0	0	33535

Appendix 7.3.20 patient 20
T cell subset assays; time since first treatment with FVIII and details of treatment received at the time of each assay

date	time since first treatment (months)	treatment received prior to T lymphocyte subset assay (units FVIII / kg)			total treatment received (units FVIII)
		one week	one month	three months	
22.11.89	0	0	0	0	0
18.7.90	8				
24.1.91	14	55	55	383	25090
4.6.92	31	29	206	631	65930
19.11.92	36	0	119	523	80530
3.6.93	43	90	191	396	105880
2.12.93	49	33	180	485	129830
30.6.94	55	85	377	900	169820
26.1.95	62	108	368	1192	238250
17.8.95	69	74	330	859	291720

Appendix 7.3.21 patient 21

date	time since first treatment (months)	treatment received prior to T lymphocyte subset assay (units FVIII / kg)			total treatment received (units FVIII)
		one week	one month	three months	
29.1.90	0.5	16	16	16	275
6.2.91	13	0	0	0	22980
4.6.92	29	0	0	0	23785
26.11.92	34	0	0	0	23785
17.6.93	41	0	0	0	23785
9.12.93	47	0	0	0	23785
28.7.94	54	0	11	11	24040
16.2.95	61	0	0	0	30335
31.7.95	66	0	31	103	33245

Appendix 7.3.22 patient 22
T cell subset assays; time since first treatment with FVIII and details of treatment received at the time of each assay

date	time since first treatment (months)	treatment received prior to T lymphocyte subset assay (units FVIII / kg)			total treatment received (units FVIII)
		one week	one month	three months	
18.2.91	11	0	16	16	4060
11.6.92	25	0	39	39	13040
10.12.92	31	0	0	577	22110
29.7.93	38	0	0	43	24595
17.2.94	45	0	0	0	24845
25.8.94	51	0	0	93	34215

Appendix 7.3.23 patient 23
T cell subset assays; time since first treatment with FVIII and details of treatment received at the time of each assay

date	time since first treatment (months)	treatment received prior to T lymphocyte subset assay (units FVIII / kg)			total treatment received (units FVIII)
		one week	one month	three months	
17.5.90	0	0	0	0	0
24.1.91	8	71			10535
29.10.92	29	0	0	0	79995
24.6.93	37	0	9	84	91100
6.1.94	44	0	71	176	100395
2.3.95	58	14	31	192	120085
17.8.95	63	0	30	129	126010

Appendix 7.3.24 patient 24
T cell subset assays; time since first treatment with FVIII and details of treatment received at the time of each assay

date	Time since first treatment (months)	treatment received prior to T lymphocyte subset assay (units FVIII / kg)			total treatment received (units FVIII)
		one week	one month	three months	
4.6.92	22	0	42	178	17350
17.6.93	34	12	36	97	41165
9.12.93	40	0	155	239	48835
17.2.94	42	0	161	549	59800
1.9.94	49	0	86	173	77245
16.2.95	54	42	88	518	95180

Appendix 7.3.25 patient 25
T cell subset assays; time since first treatment with FVIII and details of treatment received at the time of each assay

Age	IgG (g/l)	IgA (g/l)	IgM (g/l)
0-2 weeks	5.2-18.0	0.01-0.08	0.05-0.2
2-6 weeks	3.9-13.0	0.02-0.15	0.08-0.4
6-12 weeks	2.1-7.7	0.05-0.4	0.15-0.7
3-6 months	2.4-8.8	0.1-0.5	0.2-1.0
6-9 months	3.0-9.0	0.15-0.7	0.4-1.6
9-12 months	3.0-10.9	0.2-0.7	0.6-2.1
1-2 years	3.1-13.8	0.3-1.2	0.5-2.2
2-3 years	3.7-15.8	0.3-1.3	0.5-2.2
3-6 years	4.9-16.1	0.4-2.0	0.5-2.0
6-9 years	5.4-16.1	0.5-2.4	0.5-1.8
9-12 years	5.4-16.1	0.7-2.5	0.5-1.9
12-15 years	5.4-16.1	0.8-2.8	0.5-1.9
15-45 years	5.4-16.1	0.8-2.8	0.5-2.0

Appendix 10.1; Normal values for IgG, IgA and IgM.
Source: Clinical Immunology, University of Birmingham Medical School

Patient	Age	Time from first treatment (years)	IgG	IgA	IgM
1 19.7.85	3.2	2.1	10.4	**2.26**	0.79
	5.1	4	9.25	1.69	1.1
	8.1	7	11.9	2.34	**2.1**
	9.4	8.3	11.3	2.28	1.36
	10.0	8.9	11.56	2.2	1.3
	10.7	9.6	9.3	2.04	0.75
	11.5	10.4	11.33	2.51	0.95
	12.3	11.2	11.19	2.59	1.22
2 28.12.85	2.7	1.7	8.72	0.84	1.13
	3.4	2.4	8.74	0.84	1.13
	6.6	5.6	9.32	1.77	1.72
	8.9	7.9	14.6	2.1	1.47
	9.7	8.7	11.34	2.18	1.52
	10.2	9.2	11.5	2.46	1.31
	11.3	10.3	11.04	1.77	1.03
3 3.1.86	2.2	1.5	9.85	0.86	1.6
	4.0	2.3	8.42	0.81	0.88
	7.1	6.4	9.7	1.16	1.45
	8.7	8.0	15.5	**2.67**	**2.12**
	9.3	8.6	12.66	1.79	**2.04**
	10.9	10.2	13.95	2.15	1.13
	11.5	10.8	11.5	1.95	1.5
4 20.1.86	3.0	1.5	3.99	0.34	0.75
	5.0	3.5	10.3	0.98	1.65
	6.5	5.0	10.8	0.85	1.55
	9.5	8.0	15.8	1.13	**2.15**
	10.0	8.5	7.13	1.66	**3.87**
	10.7	9.2	8.23	1.22	1.36
	11.5	10.0	9.86	1.21	1.47
	12.1	10.6	10.48	1.89	1.84
5 31.5.86	2.0	1.4	11.3	0.83	1.22
	3.8	3.2	9.6	0.68	1.49
	6.8	6.2	9.39	1.38	1.77
	10.5	9.9	11.05	1.39	1.45
	11.0	10.4	9.45	1.53	1.49

Appendix 10.2 total IgG, Ig A and IgM levels in boys treated solely with BPL 8Y

Patient	Age	Time from first treatment (years)	IgG	IgA	IgM
6 6.6.86	2.0	1.3	5.33	0.84	0.59
	4.0	3.3	7.02	0.86	1.03
	6.7	6.0	7.61	1.2	1.16
	8.3	7.6	10.22	**2.67**	1.45
	10.0	9.3	**4.96**	1.95	0.98
	10.6	9.9	7.32	1.67	0.8
	11.0	10.3	6.09	1.79	0.67
7 1.9.86	4.2	1.0	14.5	**2.41**	1.04
	6.1	2.9	9.31	1.08	0.68
	7.5	4.3	12.6	1.9	0.91
	10.5	7.3	12.2	**2.78**	1.26
	11.0	7.8	10.8	**2.94**	1.37
	11.7	8.5	10.5	1.96	0.9
	12.6	9.4	10.89	2.15	0.77
8 28.9.86	1.1	0.9	7.45	0.39	0.43
	3.1	2.9	12.2	1.11	0.47
	6.0	5.8	11.8	1.64	0.78
	8.0	7.8	6.41	2.28	1.2
	8.8	8.6	6.69	2.81	0.96
9 1.12.86	10.0	1.0	13.5	**3.31**	1.31
	11.8	2.8	14.9	**3.30**	0.79
	13.5	4.5	14.1	**4.0**	1.67
10 27.1.87	2.2	1.0	7.57	0.62	1.25
	3.8	2.6	9.17	1.1	1.35
	5.2	4.0	9.62	1.15	1.45
	8.1	6.9	9.99	1.22	1.93
	8.7	7.5	9.59	0.97	**2.04**
	9.3	8.1	7.56	0.64	1.52
11 4.2.87	1.3	0.8	4.81	0.54	0.85
	3.0	2.5	5.61	0.68	0.88
	4.4	3.9	7.88	1.11	1.3
	7.3	6.8	10.2	2.40	1.67
	7.7	7.2	11.36	**2.52**	1.71
	8.0	7.5	10.25	1.83	1.52
	8.6	8.1	8.84	2.49	1.22

Appendix 10.2 continued; total IgG, Ig A and IgM levels in boys treated solely with BPL 8Y

Patient	Age	Time from first treatment (years)	IgG	IgA	IgM
12 6.2.87	2.5	0.7	5.41	0.37	0.6
	4.5	2.7	6.0	0.57	1.0
	7.1	5.3	7.3	0.98	0.91
	8.6	6.8	**3.9**	1.39	1.13
	9.2	7.4	5.6	0.9	1.2
13 31.3.87	13.0	6.7	8.7	0.79	0.87
	13.6	7.3	8.6	**0.66**	1.1
	14.1	7.8	9.1	**0.44**	1.13
14 12.4.87	1.7	0.3	6.74	0.37	0.76
	3.7	2.3	9.48	0.68	0.71
	5.1	3.7	10.6	1.01	0.94
	8.0	6.6	12.25	1.13	1.28
	8.5	7.1	10.73	1.77	0.46
	9.2	7.8	11.9	1.33	1.02
15 5.10.87	0.2	0.1	6.3	0.2	0.35
	1.9	1.8	4.9	0.35	0.51
	3.3	3.2	9.6	0.69	1.53
	6.2	6.1	10.0	1.12	1.51
	6.8	6.7	12.68	1.88	**2.31**
	7.3	7.2	13.73	1.78	**2.03**
16 6.5.88	6.2	0.7	10.2	0.91	0.89
	9.0	3.5	9.1	0.9	0.86
17 19.9.89	3.3	0	6.15	0.81	1.13
	6.0	2.7	9.48	1.5	1.81
	7.7	4.4	13.9	1.89	1.19
	8.1	4.8	8.94	2.53	1.51
18 8.10.89	4.4	1.3	10.9	1.13	1.27
	7.4	4.3	**19.8**	1.22	**2.05**
	8.0	4.9	11.54	0.98	1.44
	8.5	5.4	10.4	1.09	1.74
20 20.11.89	8.0	1.4	11.4	1.78	1.33
	10.7	4.1	11.15	2.26	1.64
	11.2	4.6	10.5	1.92	1.58
	11.7	5.1	10.9	2.46	2.43

Appendix 10.2 continued; total IgG, Ig A and IgM levels in boys treated solely with BPL 8Y

Patient	Age	Time from first treatment (years)	IgG	IgA	IgM
21 22.11.89	2.2	0.1	4.93	0.35	0.51
	4.7	2.6	9.58	0.69	1.59
	6.2	4.1	9.57	1.55	0.94
	6.8	4.7	7.42	1.19	1.05
	7.4	5.3	8.37	1.33	0.88
	10.2	8.1	10.1	1.46	0.65
22 16.1.90	5.0	4.0	3.5	1.27	0.78
	5.8	4.8	8.6	1.09	1.01
	8.0	7.0	6.87	0.99	0.71
23 24.3.90	2.8	0.8	6.64	0.31	0.59
	5.8	3.8	13.89	1.02	0.91
	6.4	4.4	11.5	0.64	0.83
	8.0	6.0	10.4	0.66	0.41
	9.5	7.5	9.36	1.08	0.56
24 17.5.90	7.0	0.5	8.79	0.75	1.44
	10.7	4.2	13.8	1.62	1.27
	11.1	4.6	11.4	1.73	1.36
	12.1	5.6	8.94	0.97	0.99
25 1.8.90	3.1	1.8	5.31	0.58	0.84
	4.6	3.3	9.2	1.5	2.04
	4.8	3.5	10.63	1.22	1.48
	5.3	4.0	6.4	1.2	1.74
	6.7	5.4	9.7	1.09	1.18
	7.3	6.0	8.49	0.83	1.00
	8.0	6.7	8.73	1.03	1.03

Appendix 10.2 continued; total IgG, Ig A and IgM levels in boys treated solely with BPL 8Y

Patient	Age (years)	Time since started treatment (years)	IgG (g/l)
26	9	2	8.72
27	8	6	12.6
28	7	6	17.0
29	13	6	8.76
30	8	4	9.66
31	7	4	14.5
32	7	4	10.8
33	7	7	13.7
34	13	9	11.2
35	11	8	13.0
36	12	8	15.1
37	15	7	13.7
38	9	7	15.6
39	12	7	15.7
40	8	7	10.2
41	6	6	11.3
42	11	4	13.9
43	11	9	18.0
44	12	8	11.0
46	13	7	18.2

Appendix 10.3: IgG levels in group two boys, who were previously treated with cryoprecipitate and a variety of concentrates, but remain HIV seronegative.

Patient	Age	Time since started treatment (years)	IgG (g/l)
47	8	7	16.3
48	7	6	24.3
49	8	7	20.1
50	8	7	21.8
51	9	8	16.9
52	12	8	17.9
53	13	7	13.7
54	7	7	12.5
55	11	7	13.6
56	12	7	28.2
57	9	7	10.5
58	9	7	8.2
59	10	7	24.2
60	7	7	13.0
61	13	7	16.9
62	7	7	25.2
63	10	7	23.8
64	10	7	21.7
65	9	8	13.3
66	11	7	22.8
67	8	7	14.1
68	12	7	21.0

Appendix 10.4: IgG levels in group 3 boys- previously treated haemophiliacs who are HIV seropositive.

Date	Inhibitor screen
15.1.91	Negative
1.2.91	Negative
17.12.92	Negative
20.5.93	Negative
27.8.93	Negative
25.11.93	Negative
25.3.94	Negative
7.7.94	Negative
16.12.94	Negative
16.3.95	Negative
7.8.95	Negative
30.10.95	Negative

Appendix 11.1.1: Patient 1 inhibitor screens
First treated 31.7.85

Date	Inhibitor screen
20.8.91	Negative
17.12.92	Negative
3.6.93	Negative
25.8.93	Negative
18.11.93	Negative
12.4.94	Negative
4.8.94	Negative
9.12.94	Negative
23.2.95	Negative
17.8.95	Negative
28.12.95	Negative

Appendix 11.1.2: Patient 2 inhibitor screens
First treated 28.12.85

Date	Inhibitor screen
24.5.90	Negative
21.8.92	Negative
10.12.92	Negative
1.7.93	Negative
29.10.93	Negative
27.1.94	Negative
5.5.94	Negative
18.8.94	Negative
9.12.94	Negative
13.1.95	Negative
23.3.95	Negative
2.6.95	Negative
24.8.95	Negative
24.10.95	Negative

Appendix 11.1.3: Patient 3 inhibitor screens
First treated 3.1.86

Date	Inhibitor screen
2.10.92	Negative
13.11.92	Negative
1.3.92	Negative
29.4.93	Negative
29.7.93	Negative
1.10.93	Negative
6.1.94	Negative
5.5.94	Negative
14.7.94	Negative
25.11.94	Negative
23.3.95	Negative
17.8.95	Negative

Appendix 11.1.4: patient 4 inhibitor screens
First treated 20.1.86

Date	Inhibitor screen
14.9.90	Negative
23.9.91	Negative
17.12.92	Negative
23.4.93	Negative
8.7.93	Negative
7.10.93	Negative
27.1.94	Negative
25.3.94	Negative
4.8.94	Negative
20.11.94	Negative
20.3.95	Negative
5.6.95	Negative
3.8.95	Negative
11.12.95	Negative

Appendix 11.1.6: patient 6 inhibitor screens
First treated 6.6.86

Date	Inhibitor screen
18.5.90	Negative
14.9.92	Negative
21.8.92	Negative
30.10.92	Negative
22.1.93	Negative
1.7.93	Negative
25.10.93	Negative
17.2.94	Negative
11.5.94	Negative
23.6.94	Negative
12.9.94	Negative
23.3.95	Negative
22.8.95	Negative
2.11.95	Negative

Appendix 11.1.7: patient 7 inhibitor screens
First treated 1.9.86

Date	Inhibitor screen
15.10.90	Negative
30.8.91	Negative
29.11.91	Negative
8.9.92	Negative
3.12.92	Negative
24.6.93	Negative
22.9.93	Negative
2.12.93	Negative
11.3.94	Negative
30.6.94	Negative
30.9.94	Negative
2.2.95	Negative
19.5.95	Negative
14.8.95	Negative
27.10.95	Negative

Appendix 11.1.10: patient 10 inhibitor screens
First treated 27.1.87

Date	Inhibitor screen
7.11.90	Negative
11.10.91	Negative
19.11.92	Negative
16.2.93	Negative
10.6.93	Negative
1.11.93	Negative
25.11.93	Negative
4.3.94	Negative
4.8.94	Negative
7.12.94	Negative
16.3.95	Negative
16.6.95	Negative
31.8.95	Negative

Appendix 11.1.11: patient 11 inhibitor screens
First treated 4.2.87

Date	Inhibitor screen
18.5.90	Negative
29.11.90	Negative
15.10.92	Negative
3.12.93	Negative
3.6.93	Negative
8.9.93	Negative
9.12.93	Negative
29.3.94	Negative
21.7.94	Negative
31.10.94	Negative
9.3.95	Negative
24.7.95	Negative
23.10.95	Negative

Appendix 11.1.12: patient 12 inhibitor screens
First treated 6.2.87

Date	Inhibitor screen
13.6.90	Negative
8.9.92	Negative
5.11.92	Negative
9.2.93	Negative
10.6.93	Negative
24.9.93	Negative
2.12.93	Negative
21.3.94	Negative
30.6.94	Negative
1.10.94	Negative
26.1.95	Negative
20.7.95	Negative
2.11.95	Negative

Appendix 11.1.14: patient 14 inhibitor screens
First treated 12.4.87

Date	Inhibitor screen
22.5.90	Negative
9.8.91	Negative
21.11.91	Negative
21.8.92	Negative
3.12.92	Negative
26.2.93	Negative
10.6.93	Negative
27.8.93	Negative
2.12.93	Negative
4.3.94	Negative
7.7.94	Negative
12.10.94	Negative
12.1.95	Negative
1.5.95	Negative
24.7.95	Negative
27.10.95	Negative

Appendix 11.1.15: patient 15 inhibitor screens
First treated 5.10.87

Date	Inhibitor screen
26.6.96	negative

Appendix 11.1.16: patient 16 inhibitor screens
First treated 6.5.88

Date	Inhibitor screen
31.10.90	Negative
1.10.91	Negative
14.8.92	Negative
19.11.92	Negative
16.2.93	Negative
20.5.93	Negative
27.8.93	Negative
18.11.93	Negative
17.2.94	Negative
7.7.94	Negative
17.10.94	Negative
16.3.95	Negative
20.7.95	Negative
24.11.95	Negative

Appendix 11.1.17: patient 17 inhibitor screens
First treated 19.9.89

Date	Inhibitor screen
18.10.91	Negative
5.8.92	Negative
29.10.92	Negative
19.2.93	Negative
24.6.93	Negative
15.10.93	Negative
6.1.94	Negative
15.4.94	Negative
11.8.94	Negative
22.12.94	Negative
2.3.95	Negative
17.8.95	Negative
13.10.95	Negative

Appendix 11.1.18: patient 18 inhibitor screens
First treated 8.10.89

Date	Inhibitor screen
10.10.90	Negative
19.4.91	Negative
24.4.91	Negative

Appendix 11.1.19: patient 19 inhibitor screens
First treated 18.10.89

Date	Inhibitor screen
16.11.90	Negative
30.10.91	Negative
3.12.92	Negative
17.6.93	Negative
24.8.93	Negative
18.11.93	Negative
13.5.94	Negative
25.8.94	Negative
11.11.94	Negative
2.3.95	Negative
31.7.95	Negative
23.10.95	Negative

Appendix 11.1.20: patient 20 inhibitor screens
First treated 20.11.89

Date	Inhibitor screen
12.6.90	Negative
20.9.91	Negative
6.10.92	Negative
19.11.92	Negative
12.2.93	Negative
3.6.93	Negative
15.9.93	Negative
2.12.93	Negative
30.6.94	Negative
23.9.94	Negative
26.1.95	Negative
31.5.95	Negative
16.8.95	Negative
19.10.95	Negative

Appendix 11.1.21: patient 21 inhibitor screens
First treated 22.11.89

Date	Inhibitor screen
22.3.90	Negative
26.11.92	Negative
17.6.93	Negative
8.10.93	Negative
9.12.93	Negative
30.3.94	Equivocal result
29.4.94	Negative
29.7.94	Negative
16.2.95	Negative
31.7.95	Negative
23.10.95	Negative

Appendix 11.1.22: patient 22 inhibitor screens
First treated 16.1.90

Date	Inhibitor screen
18.2.91	Negative
10.10.91	Negative
24.9.92	Negative
29.7.93	Negative
1.11.93	Negative
17.2.94	Negative
6.5.94	Negative
25.8.94	Negative
10.11.94	Negative
23.10.95	Negative

Appendix 11.1.23: patient 23 inhibitor screens
First treated 24.3.90

date	Inhibitor screen
31.10.90	Negative
18.10.91	Negative
5.8.92	Negative
29.10.92	Negative
19.2.93	Negative
24.6.93	Negative
15.10.93	Negative
6.1.94	Negative
15.4.94	Negative
11.8.94	Negative
22.12.94	Negative
2.3.95	Negative
17.8.95	Negative
10.11.95	Negative

Appendix 11.1.24: patient 24 inhibitor screens
First treated 17.5.90

date	Inhibitor screen
4.1.91	Negative
15.10.92	Negative
26.11.92	Negative
17.6.93	Negative
22.9.93	Negative
9.12.93	Negative
17.2.94	Negative
31.3.94	Negative
16.5.94	Negative
1.9.94	Negative
11.11.94	Negative
16.2.95	Negative
13.7.95	Negative
20.10.95	Negative
30.11.95	Negative

Appendix 11.1.25: patient 25 inhibitor screens
First treated 1.8.90

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