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Twenty-one years of haemophilia

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Introduction

Much has happened in the world of haemophilia since 1976, when the *Journal of Clinical Pathology* published Ingram's short history [1]. At that time, no-one thought that the genes for the different clotting factors and their structure would be determined, nor that the knowledge would lead to the production of an artificial factor VIII (FVIII) or factor IX (FIX). There have been notable advances in treatment too, as well as the terrible irony that some therapeutic materials have unwittingly conveyed serious infections, with the result that the survival rates of affected individuals, which became normal, would shorten again.

Advances in management

Haemophilia Centres

Increasing sophistication of the management of treatment has developed with the increasing sophistication of the materials. In the UK, Haemophilia Centres were originally set up to meet the demand of the Haemophilia Society that bleeders should be tested, so that surgery could be avoided. This initial diagnostic function has matured, so that the Haemophilia Centre now provides comprehensive care. Changes in the provision of health services in the UK may cause problems for the future provision of such care; but the introduction of audit has meant that the services provided by each hospital can now be checked objectively, if not quite precisely.

In accordance with the recommendations of the World Federation of Hemophilia [2], the UK has regulated Haemophilia Centres. In 1976 the Government published a document, HC(76)4, '*Arrangements for the Care of Persons Suffering from Haemophilia and Related Conditions*', recommending three tiers comprising Reference Centres (which later became Regional Centres), Haemophilia Centres and Associate Haemophilia Centres. In 1993 the Department of Health published a new set of Health Services Guidelines HSG (93)30 as '*The Provision of Haemophilia Treatment and Care*'. The former system which corresponded to the three-tier system recommended by the World Federation of Hemophilia was replaced by a two-tier system comprising Comprehensive Care Centres and Haemophilia Centres. The former will supply specialist care on all fronts with carrier testing and genetic advice, trained nursing, physiotherapy, social services, orthopaedic, paediatric and adult medical care including home treatment and prophylaxis, and regular review. It has recently become clear that care must also include the availability of advice and treatment, not only for HIV and AIDS, but also for liver disease. The latter will provide a range of treatment, but not a complete service, and will rely on the former for support. There are at present 21 Comprehensive Care Centres and 79 Haemophilia Centres. Some of the latter, which were formerly Associate Centres, see very few patients.

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The Haemophilia Centre Directors' Organization

The doctors responsible for haemophilia centres have held regular meetings since the 1960s. From time to time they made recommendations about management, but these assumed greater importance than originally intended when claims for compensation for HIV infection were first made, and patients and their lawyers wanted to know exactly what was considered good practice. In 1994 the United Kingdom Haemophilia Centre Directors' Organization (UKHCDO) was set up and registered as a charity. It acts as a forum for discussion makes recommendations about management which are increasingly likely to be taken as representing good practice.

Home treatment

Home treatment was well under way by 1976, when 60% of haemophiliacs in the UK were reported to be on home treatment or suitable for it [3]. It has now become the accepted means of administering treatment for every patient whose bleeds occur often enough for him or his family to maintain the necessary skills. The patients acknowledge that prompt treatment leads to quicker recovery. Less time is lost from work or school, and there have been no major disadvantages [4]. Figure 1 shows the great improvement of school attendance for a haemophiliac boy after starting home treatment. The details of each hospital's home treatment programme vary substantially: no uniform system has evolved.

Home treatment has revolutionized the approach to the management of a chronic disease. Quite apart from the obvious benefits of prompt treatment, there is an enormous boost to confidence with the knowledge that a patient and his family can take direct responsibility for dealing with the illness.

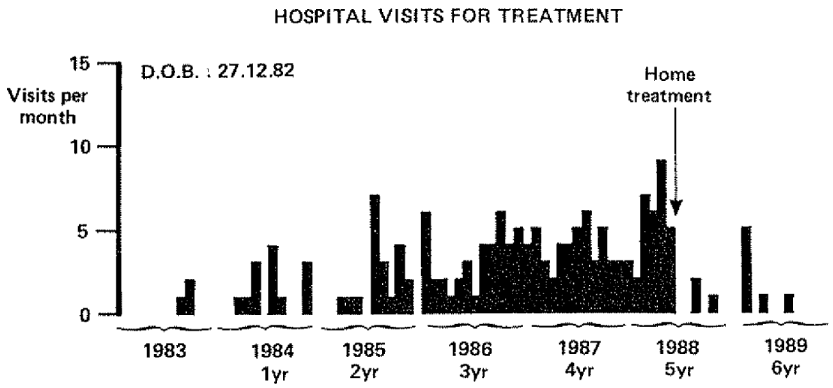

Fig. 1. There is a marked fall in hospital attendances when this 5-year-old boy starts home treatment.

Intravenous technique

Two developments have made the management of intravenous injections much simpler. The first has been the use small plastic venous cannulae, which can be plugged off with an obturator and left in place for several days (Fig. 2).

GRO-C

Fig. 2. Small needles as shown can stay in place for several days.

These minimize the need for repeated injections, such as to cover an operation, and can be left in the arm and used for injection at home.

The second is the development of closed or open venous access systems designed to be left in place for months (Fig. 3). There is a real risk of infection with the open systems, such as the Hickman line (Bard Surgical, UK), but they require no special needles. The closed system, such as the Port-a-Cath (Kabi Pharmacia, UK), reduces the risk of infection, but a special curved needle is needed. Both can be used for continuous or intermittent infusion.

Life expectancy

By 1980, before AIDS began to take its toll, the life expectancy for males in the UK of all ages with less than 2% FVIII was not far short of normal at 69.1 years,

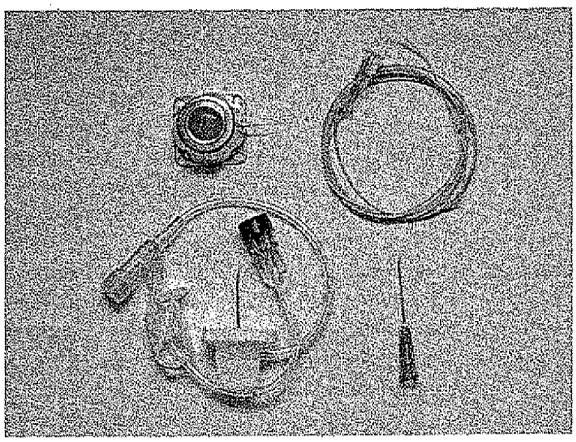

Fig. 3. Hickman apparatus: (a)the subcutaneous access port, (b) the fine cannula that is led from the port subcutaneously into one of the great veins, (c) a needle with bent end for intermittent injection, and (d) a needle with line attached for long-term infusion. The needle is bent to avoid taking a core from the port's diaphragm.

compared with 72.8 years for normal males. Those with 2–10% FVIII fared even better, with a median survival of 79.2 years [5]. The main cause of death was cerebral haemorrhage. Improved survivals were also reported from Sweden [6] and The Netherlands [7].

Prophylactic care

Prophylactic care entails the regular administration of concentrate to prevent bleeds. The original trials were running in Sweden as early as 1968 [8], and were followed in other European countries, but have been slow to develop in the UK. The concept behind prophylaxis is that joint bleeds cause crippling and most damage is done in childhood, before a boy is able to look after himself properly. If bleeds can be prevented during these early years, the boy will grow to manhood with normal joints and will be able to maintain them in good health thereafter. Unfortunately, there is no doubt that regular prophylaxis is more expensive in the amounts of concentrate used than standard on-demand treatment, and it is by no means clear that this increased expense is balanced by a reduction in the amount of material used as an adult, in savings from fewer hospital admissions and joint surgery, or by increased payment of taxes as a result of more regular employment. However, it is a more logical approach to treatment, and popular with patients. Venous access may be difficult, but can be managed with long-term cannulation using subcutaneous access ports. The injections are given three times a week for haemophilia A and twice a week for haemophilia B. The dose needs adjustment to suit the individual. Treatment is usually started when it is clear that a boy has had a minimum number of joint bleeds, usually three, so that those who have low levels of FVIII or FIX, but who bleed infrequently, will not receive unnecessary treatment. It has proved difficult sometimes to know when to stop treatment, as patients like the reassurance and freedom from bleeds that prophylaxis provides.

Continuous infusion

For surgery, it is necessary to maintain a normal clotting factor level during and after the operation, and the short half-life of both FVIII and FIX required that injections be given at 8–12-h intervals in order to maintain haemostasis and wound healing. For a long time, it was customary to give injections intermittently, but seems logical to give factor cover continuously. The concept of giving concentrate by continuous infusion is not new: it was first reported in 1970 [9]; but it is only recently that the practice has become popular, partly because portable syringe drivers capable of maintaining a steady flow of low volume are now readily available and staff have the experience to use them. The technique is now frequently used for operative cover [10]. Continuous infusion may use less clotting factor than intermittent infusion to achieve the same result.

The development of concentrates

FVIII. The past 20 years have seen great improvement in treatment materials. Plasma derivatives have gradually become purer and more soluble. As a result, they cause fewer anaphylactic side-effects. Development has proceeded through several phases.

In the 1970s, concentrates were of intermediate purity. They contained unwanted plasma proteins such as immunoglobulins, fibrinogen, immune complexes and others, additional to the necessary clotting factor. Their specific activity, expressed in international units (IU) of FVIII/mg total protein, varied from 0.5 to 5. Their use revolutionized the management of haemophilia: adequate haemostatic levels could be achieved; treatment could be given promptly, home treatment was introduced, and survival rates increased substantially. There was a 73% decrease in days lost from work or school, a 74% reduction in unemployment, and a 74% decrease in yearly costs per patient [11]. When the risk of HIV infection with concentrates was identified, the need to sterilize the concentrates was recognized, and treatment with heat was introduced between 1983 and 1984. These products remained available after the second generation of products came on the market, both because they remained cheaper, and because they contained von Willebrand factor (vWF) in addition to FVIII:C, and could be used for the treatment of von Willebrand's disease (vWD).

These subsequent materials were made with better methods for both purification and sterilization, which unfortunately led to an increased loss of FVIII. As a result the price was increased, both to cover the fact that, for the same amount of plasma, there was now less FVIII, and also to recover some of the development costs.

It took five or six donations to produce 250 units of FVIII. Soon after heat treatment was introduced, the policy of testing all donors for HIV infection followed in 1985.

Third-generation materials, while still produced from human plasma, were designed to contain as pure a concentrate as possible: they are known as high purity (HP) products. Their specific activity varies from 30 to 50 to as high as 2000 to 4000 IU/mg. FVIII breaks down easily, and as a result it proved necessary to add a stabilizer, which is usually human albumin, and occasionally vWF. So although the production techniques made FVIII of very high specific activity, the concentrates themselves were less concentrated. The techniques used for purification included immunoaffinity chromatography with monoclonal antibodies, usually of mouse origin, and various types of ion exchange chromatography. These products provide an adequate dose in a small volume, which helps when venous access is difficult. They also dissolve quickly.

A major development came with the introduction of recombinant materials (the fourth generation) which soon appeared after 1984 when an active FVIII was produced from recombinant DNA clones [12]. Once the genes for the clotting factors had been isolated, they were inserted into cell cultures, such as baby hamster kidney or Chinese hamster ovary, to direct the system to produce the relevant factor. These products have now passed the various stages of development and are available on the market, but they remain costly. They may not offer any specific advantage over high purity products for management of bleeds. As they contain traces of animal proteins the risk of infection is not completely eliminated, but they should minimize the risk of infection, which is always a remote risk for products derived from human plasma as no method of sterilization is presently in routine use that can eliminate all known viruses. As production is not limited by the availability of plasma, in theory at least they could be produced in unlimited amounts and in the long run entirely replace plasma-derived products.

Soon after their introduction, concern was expressed that they led to the production of inhibitors in a high proportion of cases. Subsequently it became clear that this was noted because the patients given these products had been monitored very closely. It now seems clear that patients may develop inhibitors shortly after starting replacement treatment, but these inhibitors can disappear early on. One new recombinant product uses a modified gene in which the major part of the region encoding the B-domain has been removed. The product retains normal coagulant activity and might be less likely to lead to the development of inhibitors.

Factor IX. The prothrombin complex concentrates used as standard treatment for haemophilia B were originally introduced over 30 years ago. They contain prothrombin and FX as well as FIX. They have enabled many patients to enjoy the benefits of regular twice-weekly prophylactic treatment, as the half-life of FIX is longer than that of FVIII. Unfortunately, large doses of these concentrates have caused thrombosis. The reason why may be connected with high levels of prothrombin and FX, but is not completely understood.

The newest FIX concentrates are purified by affinity chromatography and monoclonal methods which remove the unwanted prothrombin and FIX. They appear to be safer and should be used whenever large doses are needed. Pure FIX is stable, so there is no need to add a stabiliszer to the concentrate. As a result, they have a higher specific activity than the new FVIII concentrates.

Once the gene for FIX was cloned in 1982 [13], the path was opened to produce a recombinant FIX, but difficulties with production have delayed its introduction until 1996. One interesting development however was the breeding of transgenic sheep and mice who produce FIX in their milk (Fig. 4). The yield in sheep, at ~1 μg/ml, was too low for commercial development; but the latest studies have produced mice with at least 27 μg/ml of biologically active FIX. If this could be achieved in sheep, a relatively small flock could provide the estimated worldwide demand for FIX of ~3 kg per year [14].

Sterilization of blood products. Soon after clotting factor concentrates were first introduced, it was noted that occasional patients developed frank jaundice, and the majority showed transient changes in liver function tests. This initiated the study of methods for sterilizing clotting factor concentrates; but the development of AIDS and HIV infection greatly stimulated their development and introduction. Heat treatments were introduced first, but heating the concentrate dry at low temperatures for relatively short periods (e.g. 60°C for 24–30 h) failed to prevent HIV seroconversion, leading to longer treatment at higher temperatures, such as 68–80°C for 72 h, or completely

Fig. 4. Transgenic sheep at the Institute of Animal Physiology and Genetic Research, Edinburgh. The sheep produce small amounts of FIX in their milk. © *The Scotsman* Publications, Edinburgh.

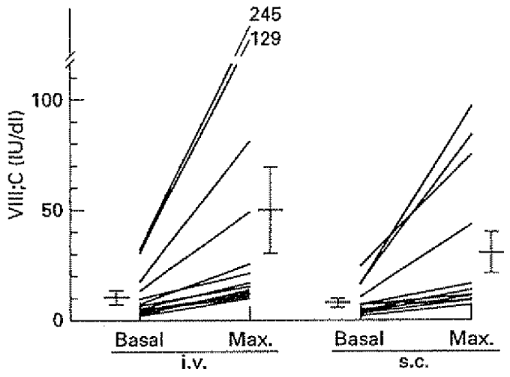

Fig. 5. The effect of intravenous (i.v.) and subcutaneous (s.c.) administration of 0.3 μg/kg DDAVP on FVIIIC levels in 14 patients with mild haemophilia A. Max = peak levels after DDAVP. (Reprinted from Mannucci *et al.* [168] with permission.)

different methods. Heating the freeze-dried concentrate in the dry state ('dry heat') needs a higher temperature and longer time than heating the concentrate before drying ('wet heat' or 'pasteurization'). Other methods include heating in an organic slurry of *n*-heptane, solvent–detergent treatment such as tri-[*n*-butyl] phosphate and sodium cholate, vapour heating, and the use of immunoaffinity chromatography together with one of the previous procedures. Although these methods proved satisfactory in the prevention of HIV transmission, hepatitis A resists the solvent–detergent process. These methods have also been used to sterilize FIX concentrates.

DDAVP. Wherever possible, FVIII concentrates, even though sterilized, should be avoided in the treatment of mild or moderate haemophilia A or vWD, because of the risk of infection. The treatment of choice is desmopressin (DDAVP) a synthetic vasopressin which boosts blood levels of FVIII in patients who already produce modest but sub-normal amounts of FVIII. Its use was introduced in 1977 [15]. It may be given intravenously or subcutaneously: similar results are obtained (Fig. 5). In vWD, it usually works well with mild type I but is less effective in type III and type IIA, and is contra-indicated in type IIB as it often provokes thrombocytopenia. Most FVIII concentrates do not contain vWF, and when DDAVP is ineffective, concentrates containing vWF multimers as well as FVIII should give better results. Simple FVIII concentrates may provide initial haemostasis in most cases, but their control of the bleeding time is very variable and transient, and they may not be suitable for long-term wound healing and prevention of late haemorrhage [16]. Cryoprecipitate is effective; but its use is not recommended as it is not treated with either heat or detergent.

Products used to treat patients with inhibitors

Porcine FVIII concentrate. FVIII concentrates made from the plasma of cattle and pigs were first developed in the 1950s. Porcine FVIII was used most. It was relatively impure, and its use led to immune reactions with pyrexia, thrombocytopenia, and failure to respond due to development of antibodies. A new porcine FVIII concentrate (Hyate:C, Speywood Pharmaceuticals, Maidenhead, UK), purified with polyelectrolytes, which separate FVIII:C from vWF, was introduced in the early 1980s [17]. Although it lacks the platelet aggregating factors that proved so troublesome with the earlier concentrates, it may still cause thrombocytopenia in a few cases, but other adverse reactions are rare [18]. It has been used mainly for the treatment of haemophilia A patients with inhibitors, as the inhibitor usually reacts less strongly with porcine than with human FVIII. It was also used for ordinary haemophilia in the early 1980s after AIDS was reported for some patients who wanted the reassurance of a product that was not derived from human sources.

Activated prothrombin complex concentrates. Some patients with inhibitors to FVIII respond to treatment with simple prothrombin complex concentrates used routinely for haemophilia B. Many however do not, and this led to the development of activated concentrates which contain FII, IX and X, and sometimes VII, in an activated form. Two are widely used: FEIBA (Immuno, Sevenoaks, UK) and Autoplex (Baxter Healthcare, Newbury, Berks, UK). They shorten the partial thromboplastin time without increasing FVIII levels. They may be effective because they contain activated FVII and FIX [19, 20].

Recombinant FVIIa. FVII reacts with tissue factor to activate the intrinsic pathway. FVIIa from human plasma was first used successfully as long ago as 1983 [21], as it by-passes the FVIII and FIX stages in the extrinsic pathway, and the effects of FVIII and IX antibodies. A recombinant FVIIa has been developed. Its successful use to cover surgery was first reported in 1988 [22], and case reports continue to appear [23]. It could be used for the treatment of other clotting factor deficiencies that involve only this pathway. Its half-life is even shorter than that of FVIII, which necessitates injections every 3 h, but it offers the prospect of treatment for cases who do not respond to any other therapy.

Other drugs

There are two groups of drugs used in haemophilia: those used to help stop bleeding and those used for pain and chronic joint disease. The anti-fibrinolytic drug tranexamic acid (Cyklokapron; Kabi Pharmacia, UK) has shown no benefit for long-term prophylaxis, but has proved useful for oral and gastro-intestinal bleeding, and for menorrhagia.

By prolonging the stability of the clot, the requirement for clotting factor may be reduced.

A variety of analgesics are used for acute pain, with the well-known exception of aspirin. The the long-term use of the non-steroidal anti-inflammatory drugs (NSAIDs) commonly prescribed for non-haemophilic arthritis was considered dangerous because of their potential for causing bleeding by depressing platelet function. However, this anxiety has not been confirmed in clinical trials, and drugs such as ibuprofen, available under many proprietary names, have been found to be both safe and beneficial [24].

Management of joint disease

Acute haemarthrosis. Although modern treatment should reduce or even eliminate acute joint bleeds, they remain a major problem. No clear policy has evolved whether or not to aspirate every acute joint bleed, although it is more generally agreed that there is a role for aspiration in joints that are very swollen or painful. Early factor replacement is essential, and may allow an individual to nip the bleed in the bud. A lower dose of concentrate, around 7 units/kg [25, 26] may arrest the bleed when treatment started later will not. If the early signs are ignored, the joint will swell, and time will be needed to allow absorption of blood. This may call for bandages or splints: but this too is still not a matter of general agreement. Nor has any clear opinion evolved about the role of intra-articular steroids [27]; but the risk of infection limits enthusiasm.

Frequent joint bleeds, particularly in one joint, call for prophylactic treatment. The dose must be adjusted to prevent break-through bleeds. This approach is effective for some, but not all, children with chronic synovitis [28].

Chronic arthropathy. Synovectomy was the operation originally used to treat chronic haemophilic arthropathy. It reduces but does not abolish further bleeds at the expense of reduced joint mobility [29, 30], and should be avoided when other means of treatment are available. Intra-articular injection of osmic acid or gold, or joint irradiation, have not become popular, and the best approach appears to be regular injections of concentrate in doses high enough to prevent bleeds completely for long enough to allow natural recovery. It is becoming more and more evident that regular short- or long-term prophylactic treatment should prevent the development of chronic joint disease. For the many older men whose chronic joint disease developed before prophylactic treatment became available, joint replacement appears to be the preferred treatment.

Inhibitors in patients with haemophilia

Incidence. About 10% of patients with haemophilia A fail to respond to normal doses of concentrate because they have developed an inhibitor. The transfused protein is recognized as foreign and an anti-FVIII antibody develops. It rapidly neutralizes infused FVIII and makes bleeding harder to stop. FVIII inhibitors are circulating immunoglobulins of restricted heterogeneity. Many contain predominantly or exclusively gamma-G3 or gamma-G4 heavy chains. They are specific for the procoagulant component of the FVIII complex [31]. The antibodies vary in their dependency on time and temperature, and a variety of times and temperatures have been used in different assay methods. In the UK, the Old Oxford method [32] was followed by the New Oxford method [33], but in most centres the Bethesda assay has become the standard. For this test, the ability of 1 ml inhibitor plasma when mixed with 1 ml normal plasma to reduce the FVIII concentration by half after 2 h at 37°C comprises one unit [34].

Two types of patient are recognized: low and high responders. The former have low antibody levels that rarely rise above 10 Bethesda units (BU); the latter have high levels of antibody that rise further with an anamnestic response after renewed exposure to FVIII, reaching titres as high as 10,000 BU or more. Many patients develop low levels of short-lived antibody after first exposure to FVIII, but only a minority develop long-term problems. The response may be genetic, and depend on the innate ability to produce antibodies or the particular mutation leading to haemophilia.

Treatment of inhibitor patients. The approach to the treatment of patients with inhibitors is two-fold. The first is to achieve haemostasis by giving higher doses of human FVIII concentrate or normal doses of porcine concentrate, by using non-activated or activated prothrombin complex concentrates, or activated FVII. Immediate reduction of antibody levels may be achieved by plasma separation for exchange or antibody absorption with protein A Sepharose [35]; these methods have sometimes been combined. For some patients the antibody titre falls with time, even with continued treatment, and this has led to long-term attempts to eliminate inhibitors by inducing tolerance with courses of normal concentrate given in high [36] or low [37] dosage over a variable period of time, usually of many months' duration. Although treatment with steroids, immunosuppressive drugs and immunoglobulin given singly appears to have little effect, combined treatment with high doses of FVIII, immunoglobulin and cyclophosphamide has proved successful [35].

Inhibitors may occasionally develop in patients with haemophilia B and vWD. The principles of management are similar.

Acquired haemophilia

Haemophilia can develop as an acquired disease due to the spontaneous development of antibodies to FVIII. With the exception of women who become affected during or after pregnancy, patients tend to be elderly. Although rheumatoid arthritis and auto-immune disorders may be predisposing

factors, in most cases there is no associated condition. Soft-tissue bleeding is common: joint bleeding is rare. The antibodies are always IgG and react more strongly with human than with animal FVIII.

Bleeding may be life-threatening: 10–22% of cases die [38]. High doses of human or porcine FVIII may be effective for immediate treatment. Plasma exchange may be helpful; but there is no consensus for the role of immunosuppressive treatment in long-term management.

Infection

HIV and AIDS

History of AIDS. In the early 1980s reports started to appear in the USA of a new disease called acquired immune deficiency syndrome (AIDS). Over 800 cases had been reported by the end of 1982, with a very high mortality rate. Eight haemophiliacs, treated with clotting factor concentrates, were included, which raised the possibility that an agent was transmitted by blood products. Most haemophiliacs became infected between 1982 and 1984 [39]. The aetiology was not known until 1984, when a new retrovirus called human T-lymphotrophic virus type III (HTLV-III) or lymphadenopathy-associated virus (LAV) was found to be the cause [40, 41]. The virus came to be known as human immunodeficiency virus (HIV). The first ELISA test for anti-HIV was licensed in the USA in 1985.

Although a second human immunodeficiency virus (HIV-2) has now been described [42], it predominates in West Africa and has not been responsible for infection in haemophilia.

Haemophilia A. It was apparent by 1983 that the risk of infection was greater with concentrates derived from large donor pools, and advice was given to change from large-pool products to cryoprecipitate [43]; but because AIDS was still rare, the cause was unknown, the benefits of home treatment with concentrate were substantial, and a change back to cryoprecipitate would have disrupted the arrangements for making freeze-dried concentrate in the UK, this advice was not followed. For mildly affected patients, the Haemophilia Centre Directors recommended DDAVP.

In the same year, the Committee of Ministers of the Council of Europe noted that imported blood products from countries with paid donors considerably increased the risk of contamination and recommended that they should be avoided wherever possible, and the first two British cases of AIDS in haemophilia were reported [44].

By 1984, 64% of American haemophiliacs were anti-HIV-positive [45]. Heat-treated imported concentrates were coming on the market, but supplies and budgets were limited. The NHS concentrate was not heat-treated, and it would not be for a couple of years that adequate heat-treated NHS FVIII was available. In 1985 it was confirmed that dry heat prevented HIV transmission [46], and anti-HIV screening was introduced at all UK transfusion centres. The new NHS intermediate purity product, 8Y, dry-heated to 80°C for 72 h, became available in 1985. An activated prothrombin complex concentrate for treating inhibitor patients proved not to transmit HIV. It was treated with 20% ethanol for 10 h [47]. Methods then developed using wet treatment in heptane slurry [48] and lipid solvents and detergents [49]. These methods remain in use.

Haemophilia B. Initially, it was thought that patients with haemophilia B might not develop HIV infection, but in 1984 AIDS was reported in European patients [50]. It became clear that the risk was lower. There was anxiety that heating FIX concentrate would increase the risk of thrombosis, and this delayed the introduction of heat-treated FIX in the UK until 1985.

The third survey of UK patients showed that of 1196 severely affected patients tested, 58% with haemophilia A were HIV-antibody positive compared with only 9% with haemophilia B [51]. In the USA, the incidence in haemophilia B was lower than for haemophilia A, but rose from 31% in 1983 to 69% in 1985 [52]. The same donor pools are used to produce both FVIII and FIX, but the technique used to produce FIX may be less likely to transmit HIV. However, the differences are more likely to be due to the fact that haemophilia B cases tend to use less concentrate than those with haemophilia A, and more importantly, the British patients with haemophilia B were largely treated with concentrates made from UK donors, whose carrier rate was lower than that of US donors.

Clinical signs. In haemophilia, AIDS presented with clinical disease from immune deficiency, such as immune thrombocytopenia, leucopenia and anaemia, *Pneumocystis carinii* pneumonia, candidiasis, herpes zoster, lymphadenopathy, and growth failure in boys. There were very few cases of Kaposi's sarcoma, and neurological disease such as cerebral toxoplasmosis, progressive multifocal leucoencephalopathy and cerebral cytomegalovirus infection were much less common than in homosexual men [53].

Auto-immune thrombocytopenia affects about 13% of HIV-positive haemophiliacs, 82% of whom bleed as a result, with a distinct risk of cerebral haemorrhage [54]. It causes particular difficulties with treatment, as splenectomy is difficult and the response to steroids and intravenous gammaglobulin has been short-lived. Zidovudine and interferon offer most promise [55].

Septic arthritis is another problem of particular relevance to haemophilia. In Texas, of 139 children with haemophilia, four developed septic arthritis of whom three were HIV-positive [56]. It is a complication that develops more frequently in haemophiliacs with previous joint surgery.

Laboratory studies and prognosis. Infection is characterized by a depression of helper (CD4) lymphocytes that progresses with time. This measurement was used in the early years to identify affected cases until reliable tests for HIV became available, and changes in the ratio of CD4 to CD8 cells were reported in haemophilia first in 1983 [57]. The fall of the CD4 count and progression to AIDS tend to go together, and the CD4 count is widely used to measure the progress and prognosis of HIV infection. In 1987, the Centres for Disease Control in the USA specified a list of symptoms that confirmed a diagnosis of AIDS [58]. In 1992 this was modified to include all patients with a CD4 count of $<200 \times 10^6$/l [59].

The presence of the p24 antigen of HIV and antibodies to cytomegalovirus are further indicators of disease progression [60]. Other factors which correlate with disease progression include a raised level of serum beta-2 microglobulin, high plasma IgM pre-exposure and the pattern of specific IgM and IgA anti-HIV response around the time of IgG seroconversion, the number and percentage of circulating activated (DR+ve) T cells, and the levels of plasma interleukin-2 receptor [61]. These are all measures of immune reactivity, which appears to relate closely to disease progression.

Initial experience with homosexual men suggested that those positive for HIV would soon progress to AIDS and death. This has not proved to be the case with haemophilia. 12 years after seroconversion, the estimated progression to AIDS at the Royal Free Hospital, London, was 45% [60]. It was faster (63%) in patients aged 25 and over and in those with previous cytomegalovirus infection. 59% of those with p24 antigenaemia developed AIDS compared to 20% of those without. The risk of AIDS rises substantially once the CD4 count falls to 200×10^6/l . The risk of AIDS within 30 months was 50% for haemophiliacs with a CD4 count of $<200 \times 10^6$/l, 22% for those with counts of $200–290 \times 10^6$/l, and 3-6% for those with counts over 300×10^6/l [62]. This effect is in part due to prophylactic treatment against the infections which characterize AIDS. The latest studies suggest that, with current management, up to a quarter of patients with HIV infection will survive for 20 years without developing AIDS and 15% will be free after 25 years [63].

Family studies. Haemophiliacs and others infected by blood products comprised 5.7% of the males with AIDS in the UK in 1991 [64], whereas 82% of male AIDS cases are homosexual. Haemophiliacs attend their doctors regularly and have been followed up for many years, often with regular blood tests. They are a reliable source of information about HIV transmission. Studies of men with haemophilia may give a better all-round view of the progress of the disease than studies of homosexual men.

The risk of heterosexual transmission was only 3.3% in London in 1986 [65], even though only a quarter of the contacts of seropositive cases regularly used barrier methods of contraception. There is a very small risk of needlestick injury of family members with home treatment.

Treatment of HIV and AIDS. Treatment has been two-fold. The first approach has attempted to eliminate the virus with anti-viral and other drugs, for symptomatic treatment and prophylaxis, and to modify progression of the disease by using purer concentrates in the hope that they will reduce the immunosuppressive effects of the virus. The second approach has been to manage the social and psychological effects of infection.

Anti-viral therapy has proved neither more nor less successful in haemophiliacs than in others. There have been extensive trials of zidovudine (formerly AZT). It produces a short-lived improvement of the CD4 count if given late in the disease when AIDS has developed or earlier when the CD4 count is low [66, 67]; but it remains to be seen if such treatment actually improves survival. A range of other antiviral drugs have been tried and are under investigation, but none has so far emerged as a treatment of choice. At present, combined drug therapy appears to offer most promise.

Prophylactic treatment for HIV-related infection is common. Many patients with a CD4 count below 400 or 500×10^6/l are given drugs such as pentamidine or co-trimoxazole to prevent *Pneumocystis carinii* pneumonia and fluconazole for candidiasis. If tolerated, such treatment has helped improve long-term survival [59].

High-purity concentrates undoubtedly help to delay the fall of the CD4 count. The early concentrates contained contaminating proteins and other substances that tended to depress immunity. An outbreak of tuberculosis in 1981/82 in Birmingham, UK, affected an undue proportion of haemophilic boys, and was related to the amount of treatment they received [68]. Severe haemophiliacs who are HIV-negative show depressed cell-mediated immunity and impaired ability to produce interleukin-2. Intermediate purity concentrate impairs the ability of lymphocytes to respond to phytohaemagglutinin *in vitro*; and impairs activation and proliferation of peripheral blood mononuclear cells from HIV-negative haemophiliacs [69]. 32% of HIV-negative haemophilia A patients treated with intermediate-purity concentrate had CD4 counts below 500×10^6/l. This was strongly related to annual concentrate usage [70]. Thus it is not surprising that haemophiliacs treated with high purity products do not show the same progressive fall of CD4 counts as those treated with intermediate-purity products [71, 72]. There has been pressure to introduce high-purity products from patients and manufacturers. It is sensible to use as pure a drug as possible, but the newer products are more expensive and there is no evidence yet that they retard the development of AIDS [73].

Other aspects of management of HIV-positive cases. Infection with HIV has produced enormous anxiety for patients and their families and increased work for their carers. Counsellors have been appointed and self-help groups have developed. In the UK, affected patients have sued the various authorities responsible for care. Without acknowledging responsibility, the Government in 1990 made a grant of £10 million. The Macfarlane Trust was set up to administer the funds and over £90 million has now been paid out. A similar fund has been set up for patients infected as a result of blood transfusion. In France, four doctors involved with haemophilia care were found guilty of doing too little to prevent the spread of HIV: two went to prison. There seems little doubt that the French authorities reacted to public demand for something to be done and that the doctors were found guilty unjustly. In the UK the Royal College of Pathologists took the unusual step of issuing a statement defending the position of one of them [74]. Doctors in the USA, Spain and other countries have also had problems defending the treatment they recommended in the early days after HIV infection was first recognized.

Hepatitis

Freeze-dried concentrates were coming into use by the mid-1970s. It was soon found that such treatment was followed by both acute hepatitis [75] and long-term abnormalities of liver function tests [76], with chronic liver disease. Because of the risks associated with liver biopsy in haemophilia, and the fact that patients with abnormal liver function tests remained clinically well, these abnormalities were at first not regarded seriously. Chronic persistent hepatitis proved to be the most common lesion, but a significant proportion of patients, both adults and children, showed chronic aggressive hepatitis and even cirrhosis [77].

Before 1974, most British patients were treated with cryoprecipitate. The amount of freeze-dried concentrate prepared from pools of modest size from volunteer UK donors was small. The incidence of hepatitis was low, about 2–3%, and did not differ significantly whether cryoprecipitate or freeze-dried concentrate was given [78]. Therefore, when treatment with commercial concentrates was first introduced into the UK in 1972, most patients were given products produced in the USA, where donors were often paid and donor pools were much larger, and had a higher incidence of infection with hepatitis viruses. The Haemophilia Centre Directors would have preferred to use concentrates produced in the UK, but supplies were inadequate. However, subsequent events have shown that hepatitis C is the major problem, and is as likely to be spread by domestic as by imported products, so their anxiety was misplaced.

Hepatitis B (HBV), known earlier as homologous serum jaundice, had been known for a long time to be transmitted by blood and blood products [79]. Up to the end of the 1960s it was a major hazard in the management of haemophilia [80] as substantial amounts of blood products were contaminated [81]. Over 50% of haemophiliacs showed evidence of infection [82, 83], but as plasma collectors eliminated HBV-positive donors from their donor pools between 1971 and 1973, the risk fell, and was further improved by the introduction of more sensitive tests. Immunization of haemophiliacs with anti-HBV vaccine was a further help. Although the change to products tested for HBV reduced the incidence of infection from 30 to 10%, 55–65% of patients continued to have abnormal liver function tests [84, 85]. It was clear that there was something else in concentrates that caused hepatitis, which could be a reaction to proteins or contaminants, but was more likely to be another virus.

Non-A Non-B (NANB) hepatitis was the term used to describe the changes identified by abnormal liver function tests. A 7-year study from Sheffield recorded a significant progression from chronic persistent hepatitis through chronic active hepatitis to cirrhosis [86] and the long-term risks became apparent. Seven of 28 haemophiliacs in London showed oesophageal varices [87]. The death rate from liver disease in the UK rose from three in 1988 and three in 1989 to 10 in 1992 and 12 in 1993 (Fig. 6) (UK Haemophilia Centre Directors' annual returns), and from three in 1976–81 to 11 in 1981–86 in Pennsylvania [88]. Once hepatitis C virus (HCV) was identified [89], it soon became apparent that it was the cause of most cases of NANB hepatitis [90]. Furthermore, it became clear that the minor changes found on liver biopsy of patients with NANB hepatitis, which were at one time considered to be benign, were in fact the early signs of potentially serious infection with hepatitis C (Fig. 7).

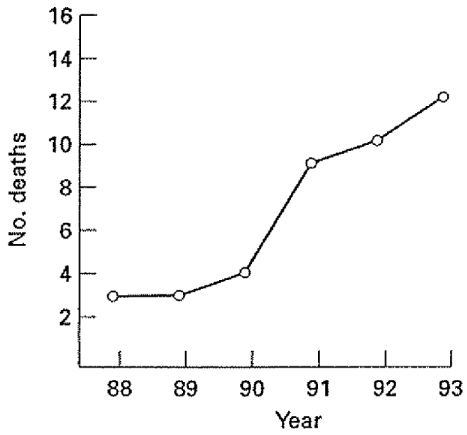

Fig. 6. Deaths from liver disease 1988 to 1993. Data from the UKHCDO. The 1994 figures report 14 deaths from liver disease and two from liver cancer (both following cirrhosis).

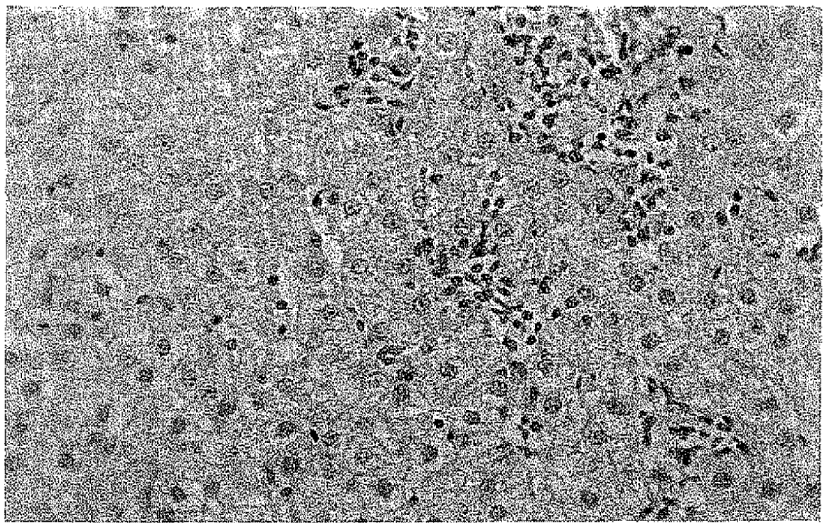

Fig. 7. Liver biopsy taken in 1984 from an anti-HIV-positive 25-year-old man with severe haemophilia in good general health. The serum alanine transferase was 136 IU/ml (n=5–40). He had been immunized against hepatitis B in 1981. The liver biopsy shows minor inflammatory changes, diagnosed as chronic persistent hepatitis. He was later found to be positive for hepatitis C. By 1995 he was in liver failure with a rising prothrombin time and ascites, and received a liver transplant. (Reproduced by permission of Dr R. F. Stevens, Royal Manchester Children's Hospital.)

Hepatitis C proved to be a major problem. Unlike HIV, there is an appreciable incidence of infection in the British donor population: about 1 in 2000 are positive for antibodies [91]. Infection became more widespread with the development of intravenous drug addiction from the 1960s. Because all clotting factor concentrates were probably contaminated with the virus until sterilization methods were generally adopted, nearly 100% of haemophiliacs became infected [92]. Hepatitis C antibodies are more common in haemophiliacs who are already positive for HIV and hepatitis B [90]. Heat treatment and chemical inactivation with solvent–detergent methods destroy the virus, so hepatitis C should not trouble younger patients, but those treated with concentrates earlier face the risk that about 50% will have persistent infection and progressive disease. About 60% of those with persistent infection are shown by liver biopsy to have chronic active hepatitis and 20–30% will develop cirrhosis [93, 94]. A small number are at risk of developing hepatocellular carcinoma: 10 cases were found in a study of 11,801 haemophiliacs [95]. Fortunately, the risk of sexual transmission is low [96].

Treatment. Alpha-interferon has been used for treatment, but has really been no more successful in haemophiliacs than was earlier treatment with corticosteroids. In studies of patients infected by exposure to blood and blood products, abnormal liver function tests and liver histology showed initial improvement in about half the cases treated for 6 months with 1, 2 or 3 million IU three times a week, but 50% of these relapsed when treatment was stopped [97, 98]. Repeat courses of treatment, even at the recommended full dose of 3 million IU three times a week, do not appear to improve the long-term response rate [99]. There are side-effects, with pyrexia, headache, irritability, rash, and alopecia, as well as leucopenia and thrombocytopenia, and lowered haemoglobin. The need for regular injections has been a further difficulty in treating patients who already need injections for their underlying disease, but haemophiliacs have shown improvement of liver enzymes and histology [100]. There is also the problem of paying for extra treatment in a group of patients whose basic treatment is already expensive. HIV infection may [101] or may not [90] accelerate the progression of hepatitis, but patients with dual infection appear to respond to and tolerate treatment with interferon as well as those without HIV [102]. The treatment of last resort for liver disease is liver transplantation, which has been successfully undertaken [103]. Although it is complicated and expensive, it has the advantage that no other treatment of haemophilia has, of curing the disease, as the normal liver sinusoidal cells in the graft produce normal FVIII.

Hepatitis A (HAV) has also been transmitted by concentrates [104]. 85 cases were reported in 1992 and 1993 from Italy, Germany, Belgium and Ireland [105]. Some solvent–detergent methods may not eliminate this virus, which does not have a lipid envelope. Normally it is transmitted by the oro-faecal route, and is not considered a complication of transfusion. Fortunately, although jaundice is common, and the disease has an acute onset, it does not lead to chronic liver disease. The illness is milder in children than in adults. It has been recommended that all haemophiliacs be immunized with an anti-HAV vaccine.

Other infections

Parvovirus B19 has also been transmitted by concentrates [106]. This is usually a minor infection, which commonly presents in children as erythema infectiosum (Fifth disease)

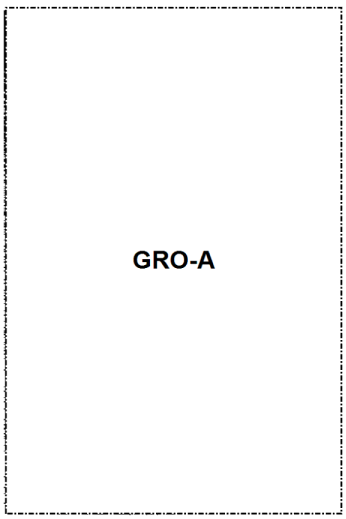

Fig. 8. Child with erythema infectiosum, to show the 'slapped cheek' rash. (Reprinted from Levene [169] with permission.)

with an initial facial rash sometimes resembling a slapped cheek (Fig. 8) followed by an itchy maculopapular rash on the rest of the body. Adults are more prone to develop some of the other signs of the disease, such as myalgia, arthritis, headache, upper respiratory and gastro-intestinal symptoms. This is a minor illness with few complications. However, reticulocytopenia can occur. It does not cause anaemia in patients with normal red cell survival, but individuals with haemolytic anemia may become anaemic, and those with immune suppression from HIV may develop chronic anaemia. There is also a risk of transmission of infection to the fetus with consequent anaemia. The virus resists sterilization by both solvent–detergent and heat treatments. By the age of 10 years, 35% of children show evidence of past infection, and 30–80% of blood donors may be expected to have had an infection [107]. Parvovirus infection reminds us that other, at present unrecognized, infections may be transmitted by concentrates derived from human donors.

Creutzfeld-Jacob disease (CJD) is a progressive, fatal neurodegenerative disease thought to be transmitted by a prion, which is an agent that can modify normal proteins into a form identical to itself. It has been transmitted by injection of growth hormone extracted from human pituitary glands, transplantation of corneas and dura mater from infected individuals, and by contaminated EEG electrodes implanted in the brain. So far it has only been transmitted to humans by nervous tissue. There is no evidence that it has been transmitted by transfusion of blood products: indeed, several individuals have had blood transfusions from donors subsequently found to have CJD, yet none has become affected [108]. The injection of whole blood or leucocytes from patients with CJD into chimpanzees failed to transmit the disease, but when spinal cord extracts were given intravenously, four of six animals were affected [109]. Buffy coats from the blood of two patients with CJD were injected into guinea-pig and hamster brains and produced changes characteristic of CJD [110].

The incubation period is 10 to 30 years, and there is anxiety that CJD might be transmitted by clotting factor concentrates. The chance appears to be remote, but concentrates derived from individuals who are subsequently found to have CJD should be withdrawn [111].

Structure and function of the factors involved in haemophilia A and B, and in von Willebrand's disease

Haemophilia A and FVIII

A deficiency of FVIII leads to haemophilia A. It affects 1–2 per 10,000 male births in all ethnic groups [112]. FVIII circulates in a complex with vWF, which stabilizes it. Proteolytic activation frees it from vWF, allowing it to act as a protein co-factor in the intrinsic FXase complex. This complex, which consists of the serine protease FIXa and FVIIIa, when assembled on a phospholipid membrane, catalyses the conversion of FX to factor Xa. In this, it resembles factor V in prothrombinase; but FVIIIa substantially increases the catalytic activity of FIX towards FX [113].

FVIII is a large protein of ~300 kDa and 2332 residues [113]. The concentration in plasma is around 100–200 ng/ml, equivalent to about 1 nM. There are three A domains homologous to regions in factor V and caeruloplasmin, two C domains that share 20% amino-acid homology with the lipid-binding region of a cellular agglutinin from the slime mould *Dictyostelium discoideum* (Fig. 9) and a large B domain that is not homologous to any other known structures, and which has no known function. The structure of the domains runs (NH_2)A1-A2-B-A3-C1-C2(COOH). The

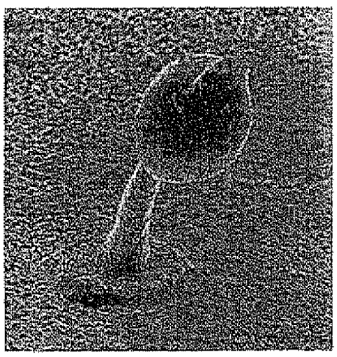

Fig. 9. Scanning electron micrograph of sporangium of *Dictyostelium discoideum*: the protoplasm has aggregated into spores. (Reprinted from Mauseth [170] with permission.)

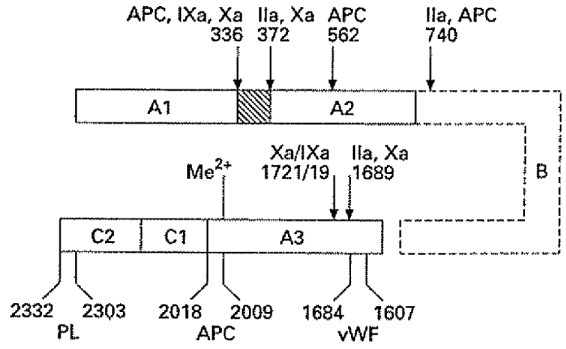

Fig. 10. Scheme of the FVIII heterodimer. The heavy chain (A1-A2-B domains) is linked by divalent metal ion (Me^{2+}) to light chain (A3-C1-C2 domains) via residues present in the A1 and A3 domains. The A1 and A2 domains are separated by a connecting region (hatched area) that contains a high concentration of acidic residues. The B domain is represented by the broken line to indicate its size, variability and dispensibility to cofactor function. Cleavage sites for thrombin (IIa), FIXa (IXa) FXa (Xa) and activated protein C (APC) are indicated by arrows. The brackets on the light chain show regions in macromolecular binding interaction. (Reproduced from Fay [113] modified from Walker & Fay [171].)

homology with caeruloplasmin is very close and suggests a common origin for the two proteins, as with factor V; but it is difficult to see how the similarity with the lectin from *D. discoideum* (which is a cell surface recognition protein and agglutinates sheep red cells [115]) arises other than by chance. Purified FVIII preparations consist of a mixture of polypeptides ranging from 80 to 210 kDa, representing sub-units for a series of heterodimers, which are formed by proteolysis at the B-A3 junction plus additional cleavage sites within the B domain [113,116] (Fig.10).

FVIII consists of a light chain of constant size comprising the A3-C1-C2 domains, and a heavy chain comprising the A1-A2 domains plus a variable amout of the B domain. The two chains are bound in a non-covalent manner, by divalent metal ion linkages with the responsible residues in the A1 and A3 domains. The heterodimers are dissociated by chelating agents, and thus lose coagulant activity. They can be recombined with buffers containing Mn^{++} or Ca^{++} to yield active FVIII. This is enhanced by vWF. FVIII binds on its light chain to vWF near its NH_2 terminus, and to phospholipid at the C-terminal region.

Thrombin and FXa both activate FVIII, but FXa has only about 20% efficiency of the former, and thrombin may be the sole activator of FVIII (and factor V) in tissue factor-activated plasma, as FXa does not activate FVIII bound to vWF. FVIII is very labile and decays spontaneously in the absence of further proteolysis, which probably reflects the dissociation of the A2 sub-unit. Protein C inhibits coagulation by inactivating factors Va and VIIIa in the presence of phospholipid and Ca^{++}.

Haemophilia B and factor IX

Haemophilia B, also known as Christmas disease, is clinically indistinguishable from haemophilia A, but is less frequent, affecting 1 in 25,000 males. It is due to a deficiency or defect of FIX, which when activated is one of the enzymes converting FX to FXa. FIXa is a serine protease with close homology with the vitamin K-dependent factors: its three-dimensional structure appears to be similar to that of other serine proteases such as trypsin and thrombin. FIX is smaller than FVIII, and although haemophilia B affects only up to a quarter of men with haemophilia, it proved easier to study than FVIII and information about its molecular structure and function became available earlier (for reviews see Thompson [117] and Roberts [118]).

FIX is a single chain glycoprotein, and represents about 0.0001 of the total protein circulating in plasma. It is activated by FXIa: this is the first Ca^{++}-dependent reaction in the intrinsic clotting pathway. No protein co-factor or phospholipid is required for this step. It can also be activated by FVIIa with its co-factor, tissue thromboplastin. The molecular weight of FIX is about 54,000, about 17% being carbohydrate, with 415 amino-acid residues.

Haemophilia B is not due to a simple deficiency of FIX. Early immunological studies with antibodies to FIX showed that about 60% of patients have an excess of FIX antigen over FIX coagulant, and 40% have reduced levels of both [119]. The former have been classed as haemophilia B+ and the latter haemophilia B–. The disorder in severely affected patients is further characterized by the clotting time with bovine thromboplastin: about two-thirds have a prolonged bovine thromboplastin time, and in a third it is only slightly prolonged [120]. Mildly affected patients do not have a prolonged bovine thromboplastin time, but about one-third have an excess of FIX antigen over coagulant activity. Kasper *et al.* [121] were able to identify four groups of patients with haemophilia B on the basis of these tests (Table 1).

FIX is low in the neonate: in term babies on Day 1 the plasma level is about 0.3 IU/ml. The FIX coagulant level is similar to the antigen level. The level rises to around 0.5 IU/ml on Day 5 and remains constant to Day 30, slowly rising with age.

Table 1. Classication of haemophilia B by FIX antigen and bovine thromboplastin (based on Kasper *et al.* [120]).

Group	Level of FIX antigen	Clotting time with bovine thromboplastin	Percentage
1	Normal	Greatly prolonged	9
2	25% - normal	Slightly prolonged	21
3	25% - normal	Normal	10
4	No excess	Normal	60

A rare variant, known as haemophilia B Leyden, is unusual in that it improves spontaneously with puberty. Although affected boys have <1% FIX activity, their clinical symptoms improve after puberty and FIX levels gradually rise to around 50% [122].

Haemophilia B has occasionally been reported in females. These women may be homozygous for haemophilia B; they may show extreme Lyonization [123]; they may be sporadic; or they may have a chromosome defect such as XO [124].

Von Willebrand's disease and von Willebrand factor

von Willebrand factor. In addition to being the carrier protein for FVIII, and hence having a role in coagulation, von Willebrand factor (vWF) is also involved with platelet adhesion and thrombus formation. Abnormalities of vWF lead to von Willebrand's disease (vWD); and as vWF is a large and complicated factor, the disorders now classified as von Willebrand's disease have proved to be extensive and complicated too. Several authors have recently reviewed the structure and function of vWF [125–127] (Fig. 11). Whereas FVIII is produced in the liver and assembled in endothelial cells, vWF is produced only in megakaryocytes and endothelial cells where it is found in the Weibel–Palade bodies; but is also found in platelet alpha-granules, plasma and subendothelium. The largest multimers are restricted to endothelial cells, subendothelium and platelets. They are only transiently found in normal blood. They are released from injured blood vessels and play an important role in haemostasis via platelet adhesion and aggregation. Proteolysis of vWF produces several fragments, and study of these fragments has made it possible to identify the active domains.

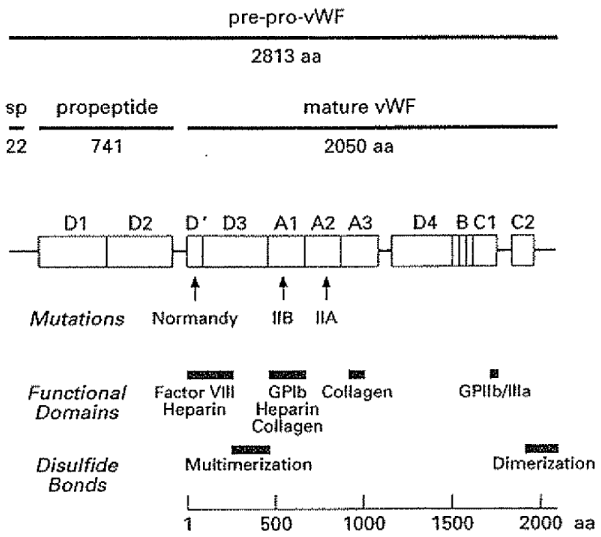

Fig. 11. Schematic representation of the structural and functional features of vWF. At the top the single chain pre-pro-vWF precursor protein of 2813 amino-acids (aa) and the loctation of the signal peptide (sp), the vWF propeptide and the mature vWF subunit are shown. In the middle the repeated domains (a–D) are indicated as well as the location of regions with clustered vWD mutations (arrows). The diagram at the bottom represents the functional domains of vWF and the regions containing intersubunit disulphide bonds involved in dimerization and multimerization. (Reproduced from Eikenboom *et al.* [127] with permission.)

vWF has two major roles. Its first is to bind to and stabilize FVIII, protecting it from inactivation by activated protein C or FXa. Individuals who cannot produce enough vWF have low levels of FVIII because there is too little vWF to carry a normal amount. The normal FVIII binding domain is a 272 amino acid tryptic fragment at the N-terminus of vWF. FVIII-binding defects are found at the D' domain, while defects causing vWD type IIB are found at the A1 domain, and IIA at the A2 domain (Fig. 11).

The second role is to bridge specific binding sites on platelets and the vessel wall, causing platelet adhesion, and on platelets themselves, causing platelet aggregation and thrombus formation. This occurs at a 48/52-Kd tryptic fragment between Val449 and Lys 728. It interacts in the high shear stress conditions found in small blood vessels with collagen, heparin and sulphatides, but it is not certain exactly which type of collagen is the most important in binding vWF. A further potential collagen binding site is found at residues 911–1114. Binding to subendothelium induces a change of conformation which is essential for its interaction with the platelet glycoprotein GPIb, which is lacking in the Bernard–Soulier syndrome: this accounts for the defects of platelet function found in this disease. Ristocetin is an antibiotic that never found an anti-bacterial role because it caused thrombocytopenia in normal people but not in some types of vWD. This occurs because it binds to platelet GPIb causing aggregation. A similar property is found in Botrocetin, an extract of venom from a snake, *Bothrops jararaca.* These agents are used in the diagnosis of the different types of vWD. Binding of vWF with GPIb then generates the transduction of an intraplatelet signal which activates platelet factor GPIIb/IIIa, which then binds to vWF causing platelet spreading and irreversible platelet adhesion. This may occur on a site at amino-acids 1744-1747. GPIIb/IIIa is deficient in patients with Glanzmann's thrombasthenia.

von Willebrand's disease. At one time, a diagnosis of vWD was based on the presence of a long bleeding time and low FVIII coagulant, but as more sophisticated tests have come into use, including assessment of vWF multimers, the vWF antigen (vWF:Ag) and the ristocetin co-factor vWF:RCo), a wide variety of sub-types have become recognized. In 1987, Ruggeri & Zimmerman [128] made a classification based on levels of FVIIIC, vWF:Ag, and vWF:RCo, which stood

Description*	Future types?	Revises type*	Previous type†
Quantitative deficiency of vWF	1	1	1 platelet normal/low 1-1, 1-2, 1-3
Simple heterozygosity for a null allele	1S	1	—
Compound hterozygosity for null alleles	1C	3	III
Dominant-negative defect	1D	1	1A
Qualitative deficiency of vWF	2	2	—
Decreased platelet-dependent function that is associated with the absence of high molecular weight vWF multimers	2A	2A	1 platelet discordant IB IIA-1, IIA-2, IIA-3
Increased affinity for platelet GPIb	2B	2B	IIB 1 New York, Malmo
Decreased platelet-dependent function that is not caused b the absence of high molecular weight vWF multimers			IC, ID
Markedly decreased affinity for FVIII	2N	2N	Normandy

*Table and descriptions are adapted from the 1994 revised classification of vWD for the Subcommittee on vWF of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis [129].
†The previous types correspond mainly to the classification as reviewed by Ruggeri & Zimmermann [128].

Table 2. Classification of von Willebrand's disease. (Reproduced from Eikenboom *et al.* [127] with permission.)

the test of time until the recognition of many other subgroups required the Subcommittee on von Willebrand factor of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis to revise the classification in 1994 [129] (Table 2).

Type I is the commonest. It affects over 70% of cases of vWD and involves 1–30 per 1000 of the population. It is inherited in an autosomal dominant manner with variable penetrance. Family studies of clinically affected cases may show minor changes in parents and others who remain symptom-free. It seems to be a matter of opinion whether these individuals should be classed as mildly affected cases or as asymptomatic carriers. Furthermore, the laboratory changes may not be consistent in every affected family member. Type III involves 1–5 per million; it is inherited in an autosomal recessive manner. Type IIA affects 10–15% of cases of vWD, while type IIB affects less than 5%. Both are autosomal dominants with more complete penetrance than type I. The other types are all rare.

Haemophilia and genetics

Ante-natal diagnosis

Only a minority of women likely to have a baby with haemophilia wish to have the fetus tested with a view to having a termination if the test is positive [130], but for those who do, the means have gradually become available. At first, it was only possible to distinguish the male from the female fetus by sexing cells shed into the amniotic fluid. Carrier mothers at risk of having a baby with haemophilia A or B had to decide whether or not to terminate a pregnancy in the knowledge that there was a 50 : 50 chance that a male baby would be normal. The introduction of fetal blood sampling *in utero* in the second trimester of pregnancy in 1978 [131] soon allowed direct examination of the baby, not only to confirm its sex, but also to check its level of FVIII or IX, at a stage when it was not too late for termination of a pregnancy with an affected child [132]. This was relatively straightforward for haemophilia A, as the level of FVIII in the fetus is similar to that of a term baby or older child. It was more difficult for haemophilia B, as FIX levels are lower in the pre-term and newborn baby than in the older child, with the result that mild or moderate haemophilia B could not be diagnosed with confidence. The reliability of sampling for haemophilia A was increased by assaying both FVIII:CAg and FVIII:C [133].

Unfortunately, such tests should only be applied to women who are known carriers; and some women at risk cannot be sure if they are carriers or not. More reliable methods had to await the identification of the FVIII gene and gene markers.

Genetic studies in Haemophilia A

Carrier detection: early studies. By the early 1970s it was recognized that the ratio of FVIII:C to vWF:Ag could identify carriers; but the test is not consistent as some families cannot be identified. FVIII levels fluctuate in normal women, and it is sometimes difficult to distinguish haemophilia A from vWD. In order to increase precision, the World Health

Organization in 1977 classified the types of carrier [134] and emphasized the need to establish international, national and laboratory standards of FVIII and its calibration in international units. Careful details were given of the statistical analysis of pedigree and laboratory data, and the problems and responsibilities in counselling were discussed.

Studies were then extended to use the FVIII clotting antigen (FVIII:CAg), but there was no great difference between the ratio FVIII:CAg/vWF:Ag and FVIII:C/vWF:Ag. Both ratios misclassed carriers as normal. There was no difference in the antigen ratio of women whose relatives were severely affected from those whose relatives were mildly affected, nor in the FVIII:C levels of women who inherited the gene from their fathers and those who inherited the gene from their mothers. Discrimination was more reliable if the median of three values for each variable was analysed [135]. Another approach to improve accuracy involved stimulating the production of FVIII with DDAVP [136]. There is no difference in the levels of vWF:Ag of normal women and either type of carrier. Age has an important effect on both FVIII:C and vWF:Ag: they are higher at very young and very old ages. Women of blood groups A, B and AB, both normals and carriers, have significantly higher levels of both factors than women of blood group O. Lastly, there is a significant difference in the results of assays of both FVIII:C and vWF:Ag between laboratories [137].

The initial methods used for measurement of vWF:Ag included Laurell immunoelectrophoresis, haemagglutination inhibition and radioimmunoassay, but each had its drawbacks. When ELISA was introduced, it was considered to be easier and more reproducible [138] and could be used for measurement of FVIII:CAg with advantage too [139]. Each laboratory was advised to construct its own discriminant. A discriminant function is a mathematical expression used to discriminate between groups on the basis of more than one type of measurement or variate. However, an international study reported that a universal linear discriminant was nearly as effective as laboratory-specific discriminants, including the age and ABO group; and linear discriminants were more effective than those based on FVIII:C/vWF:Ag ratios [140].

Carrier detection: gene studies. In 1984 *Nature* reported cloning [116, 141] and characterization [141] of the FVIII gene. It proved to be extremely large. FVIII genomic DNA is 186 kb long and contains 26 exons. The nucleotide sequence of the exons, intron–exon boundaries and 5' and 3' untranslated regions have been determined. The exon length varies from 69 to 262 nucleotides except for exon 14 which is 3106 nucleotides long and exon 26 which has 1958 nucleotides. There are six large introns of more than 14 kb, such as IVS22 which is 32 kb long [112] (Fig. 12).

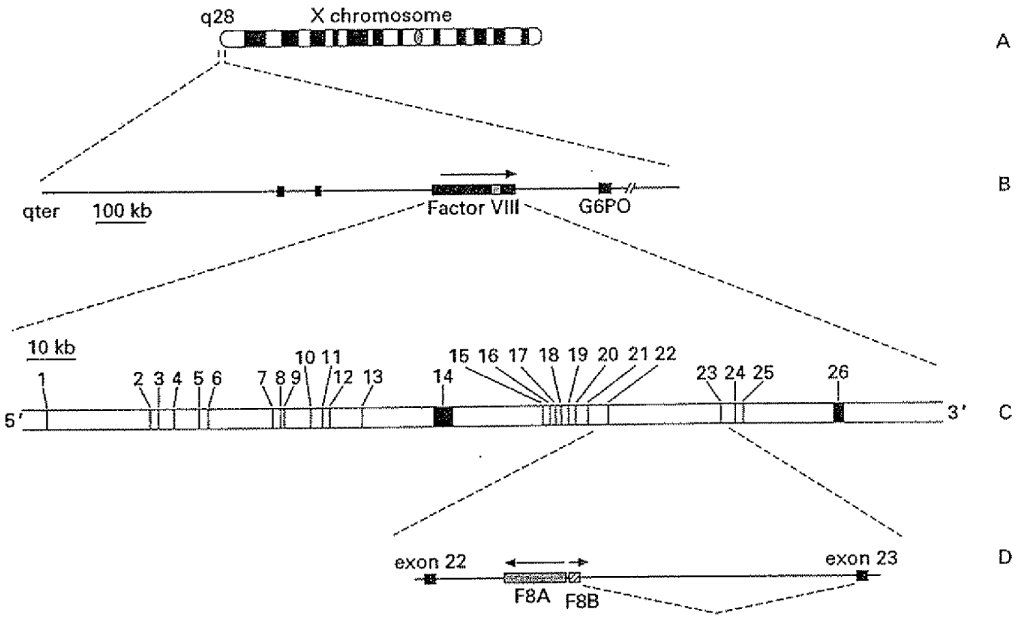

Fig. 12. Schematic representation of the chromosomal localization and structure of the FVIII gene. The gene is located about 1000 kb from the Xqter (B). It is 186 kb long and contains 26 exons (C). The large IVS22 contains two nested genes, the intronless F8A and F8B, which uses the exon of 23 of FVIII gene as its second exon (D). There are two further copies of sequences homologous to F8A on Xq28, as shown by the grey boxes in B. (Reproduced from Antonarakis *et al.* [112] with permission.)

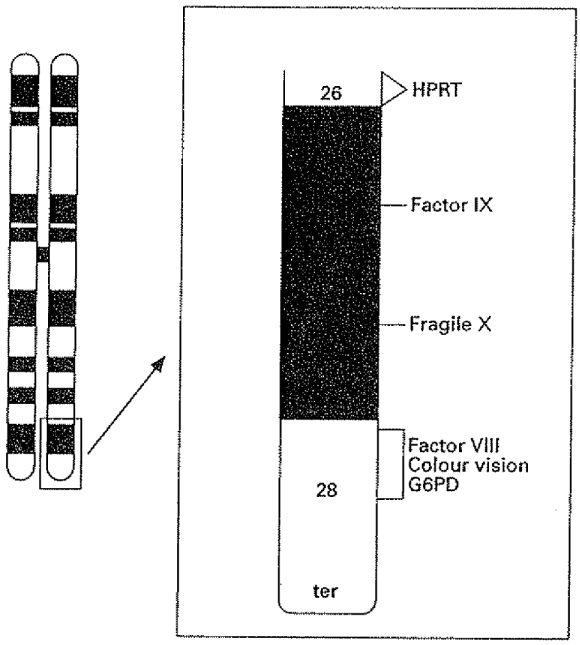

Fig. 13. Location of the FVIII and FIX genes on the X chromosomes. The inset is an enlargement of the distal part of the long arm and shows the location of the FVIII gene in relation to the colour vision and G6PD genes, and the FIX gene in relation to the fragile X gene. (Reproduced from Roberts [118] with permission.)

It has long been known that the gene lies on the X chromosome, but it has now been localized to a site on the long arm in or near band q28 [143], distal to glucose-6-phosphate-dehydrogenase (Fig. 13). The gene for FIX is separated from it by the gene for fragile X. Normal FVIII mRNA is approximately 9 kb long with a coding sequence of 7053 nucleotides. The function of much of the gene is unknown. FVIII is expressed in the liver (mainly in the hepatocyte), spleen, lymph nodes and a variety of human tissues, but not the bone marrow, peripheral blood lymphocytes or endothelial cells [144]. So liver transplantation offers a prospect of cure; but bone marrow transplantation does not.

Application of molecular genetics to haemophilia. The early hope that gene studies would provide an easy and precise tool for identifying haemophilia were soon dashed: apart from the cost, an enormous variety of gene defects became apparent [145], which is not surprising in view of the size of the FVIII gene. Because of the difficulties in identifying them in individual patients with haemophilia A, workers took to other ways of identifying an abnormal gene. Restriction length polymorphisms (RFLPs) proved to be very useful and the following probes have been commonly used: the intragenic polymorphic *Bcl* I at intron 18, *Xba* I at intron 22, and *Bgl* I at sites within the FVIII gene, the extragenic multiallelic *Taq* I system at the St14 locus, and the extragenic *Bgl* II site at the DX13 locus. The random X chromosome-specific DNA probes, DX13 and St14, are closely linked to the FVIII gene. Another locus, DXS52, sited approximately 5cM from FVIII, has also been used. In one study the FVIII probe *Bcl* I was informative in 30%, St14 in 82% and DX13 in 60% of obligate carriers. The combination of FVIII-*Bcl* I and St14-*Taq* I showed that 91% of obligate carriers were heterozygous for one or both [146].

Abnormalities of the FVIII gene. The subject has been recently reviewed by Antonarakis *et al.* [112]. There are several domains with internal homology. They are arranged from the amino to the carboxy-terminus in the order A1-A2-B-A3-C1-C2. A1, A2 and A3 have an amino-acid sequence homology of about 30% and are homologous with caeruloplasmin and factor V. The B domain, which separates the A2 and A3 domains, has no known function. At the carboxyl-terminus of ther mature protein there are two homologous domains, C1 and C2, which are homologous to the A,C and D chains of discoidin I, the C domains of factor V and mouse milk fat globular membrane protein. Mouse FVIII cDNA encodes a protein with 2319 amino-acids with 74% overall identity to the human sequence [147]. Its B domain contains 19 potential *N*-glycosylation sites which is the same number as in the human, which suggests that glycosylation of the B domain is important for the biosynthesis of FVIII.

DNA from more than 1000 patients has now been examined for molecular defects [148]. Owing to the complexity of the gene, progress has been slow. Southern blotting, cloning and sequencing were used first, but the introduction of the polymerase chain reaction (PCR) has been revolutionary.

Of gross DNA rearrangements partial inversions prove to be by far the most common. There is a small number of large deletions, of which 39% of cases have developed inhibitors to FVIII. The insertion of retrotransposons, duplications and chromosomal re-arrangement breakpoints involving the FVIII gene are all rare. The inversions originate almost entirely in male meiosis. They are present in 46% of severe haemophiliacs [149] and account for about 25% of all patients with haemophilia. In about 5% of haemophiliacs there are large deletions of more than 100 nucleotides, mostly responsible for severe disease. 66 have been described to date, and in most the deletion breakpoint has not been precisely characterized.

Regarding *point mutations and small deletions/insertions*, of 411 point mutations recorded, 39 small deletions in 34 different sites have been noted. Small deletions or insertions resulting in frameshifts causing severe haemophilia A have been reported in 49 patients. About one-third of the small

deletions and half of the insertions were found on the large exon 14. The majority of the small deletions/insertions occur in DNA regions of short direct repeats.

Nonsense mutations comprise about 20% of the total number of point mutations; 85 have been described, in 25 different codons.

CpG dinucleotides are a frequent site of point mutations, occurring in 46% of the point mutation database. CG is often substituted to TG. 189 independent mutations in 42 sites have been identified. The mutation usually occurs in tissues in which the gene is not expressed. This mutation hotspot has been noted in a wide variety of other human genes related to disease phenotypes.

Exon skipping due to nonsense mutations occurs rarely.

Missense mutations are spread throughout the gene except for exon 14, which encodes the B domain, and where none has been reported. 139 mutations are recorded in 268 unrelated patients. They occur at sites for thrombin cleavage and *N*-glycosylation, at the vWF binding site. They occur in both CRM-positive and -negative patients, predominantly the latter, affecting the folding and stability of the protein. Many of the CRM-positive mutations occur in the A2 domain comprising about 10% of the coding region of FVIII. This region must be important in procoagulant activity. Although there are 50 splice junctions in the FVIII gene, splicing errors are few. No mutations have been reported in the 5' untranslated region of the gene.

Mutations in patients with FVIII inhibitors are characterized in 93% of cases studies by nonsense mutations or deletions in the gene, but no clear picture has emerged for any correlation between the size of the breakpoints of the deletions and the presence of inhibitors. Nor have these studies yet shown if and why abnormalities of the gene lead to the development of inhibitors in some cases and not in others.

DNA polymorphisms have been identified in the FVIII gene and can be used for the indirect detection of defective genes.

Genetic studies in haemophilia B

Although haemophilia B is clinically identical to haemophilia A, the FIX locus on the X chromosome is fairly distant from the FVIII locus as it is not linked to the loci for colour blindness or glucose-6-phosphate-dehydrogenase deficiency. It is sited on Xq 27.1, close to the site for the fragile X gene [150] (Fig. 13).

Carriers for haemophilia B have low levels of FIX coagulant (FIX:C), but only a minority appears to have low levels of FIX antigen (FIX:Ag), so the use of the ratio of coagulant to antigen proved to be less successful than it was with haemophilia A [151].

The measurement of FIX activity alone enables some, but not all, carriers to be identified. Most men with haemophilia B have no excess FIX antigen, and immuno-assay of FIX in their relatives will not help increase the accuracy of carrier detection. Fortunately, because the FIX gene is smaller and less complicated than the gene for FVIII, gene studies of FIX progressed faster than those for FVIII.

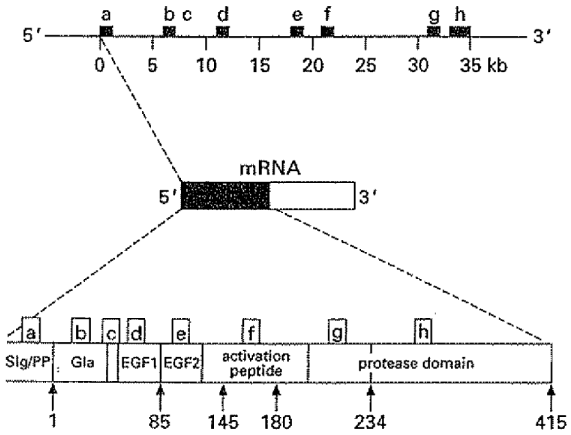

Fig. 14. Schematic drawing of the FIX gene (top) showing exons a–h and intervening sequences (introns). mRNA is shown in the middle. The FIX protein with domains corresponding to exons is shown at the bottom. Numbers correspond to amino-acid positions. (Reproduced from Roberts [118] with permission.)

The gene for FIX is about 34 kilobases, comprising eight exons (a to h) and seven introns (Fig. 14). The exons are highly homologous with the other vitamin K-dependent factors. The gene was cloned in 1982 [13] and several DNA probes have been identified to analyse the gene defect. Gross deletions in the region of the FIX gene were found to be associated with the development of inhibitors [152], but patients without gross deletions could not be identified by simple probing and, as for haemophilia A, recourse was made to the use of RFLPs.

The first recognized polymorphism within the FIX gene was the presence of an additional *Taq* I site near the 5' end on intron D [153]. It identified 40% of affected families. Other sites followed, including a 50-base pair insertion element polymorphic for *Hinf* I and *Dde* I near the 3' end of intron A, a site polymorphic to *Xmn* I near the 5' end of intron C, *Bam* HI in the 5' flanking region, and others identified with *Msp* I , *OP* ! *Hha* I and the Ala-Thr polymorphism within exon VI [154]. Using these in different combinations allows carrier diagnosis in 89% of cases in the Caucasian population. The percentage can be increased to 94% by adding another RFLP *Mse* I in the 5' flanking region of the FIX gene [155]. Only *Hha* I is present in Asian populations with any significant frequency; and *Bam* HI is only detected at a significant level in American Blacks. This approach suffers when tested women are homozygous for all the available markers.

The limitations of the indirect approach using RFLPs have prompted a return to the direct detection of FIX mutations.

Two genetically determined molecular variants of normal human FIX have been identified using monoclonal FIX antibodies with different properties. This has been used to complement carrier diagnosis, but its ethnic variation limits its usefulness [156]. More sophisticated approaches include amplification mismatch detection (AMD) [157], denaturing gradient gel electrophoresis (DGGE) [158], and standard single stranded conformation polymorphism (SSCP) [159].

von Willebrand's disease

vWD is inherited in an autosomal manner. Standard laboratory tests include the bleeding time, FVIII:C, vWF:RiCoF, and vWF:Ag. Type III vWD is homozygous: but in the parents of an affected case, these tests may be normal or only slightly abnormal. When very minor changes are met in individuals with no family history of bleeding, it is difficult to know if they represent a slight variation from normal, or if they are truly carriers. Most patients have type I disease, which is inherited in an autosomal dominant manner and yet the results in an affected family are often inconsistent. We can hope that gene studies will give more reliable results.

The gene for vWF was cloned by several groups in 1985 [160–163] (Fig. 15). It is situated on chromosome 12p12>pter. Localization studies using a cDNA probe from the midportion of vWF identified not only the authentic gene on chromosome 12, but also a second sequence on chromosome 22 localised to 22q.11.22q11.23 [164]. The complete exon/intron structure has been established [165]. There are 52 exons spanning 178 kb, approximately 0.1% of chromosome 12. Exons range from 40 bp to 1.4 kb for exon 28, the largest, which encodes the entire A1 and A2 repeats containing most of the mutations responsible for type IIA and type IIB vWD. The A3 domain extends across five exons.

The pseudogene on chromosome 22 shows 97% homology to the authentic gene on chromosome 12. It is not a functional gene in humans, as there are multiple stop codons; and it is not found in many other mammals. Its high degree of sequence identity with the authentic gene causes difficulty with the identification of mutations [166].

Because of the large size of vWF, and the variety of types of vWD, there is a large number of genetic defects responsible for clinical disease, and a large number of RFLPs within the gene has been recognized. vWD appears to be generally due to defects within the gene itself.

Gene deletions have only been reported in six families [166] associated with type III vWD in five. This is not a common variant.

Mutations resulting in quantitative decreases in vWF are difficult to identify owing to the size of the gene, and only a few families have been studied. The molecular defect causing type I vWD is unknown. The first point mutations were reported for the type IIA variant. By 1992 11 mutations in 17 families had been identified [166]. They are generally clustered within the A2 homologous repeat.

Expression of mutant vWF sequences by transfection in mammalian cells has allowed type IIA vWD to be classed into two subgroups. In Group 1, the associated point mutation leads to a defect in intracellular transport with vWF observed to be retained within the endoplasmic reticulum. In Group 2, expression of recombinant vWF results in vWF multimers that appear normal in tissue culture cells [167]. Type IIB vWD is characterized by increased reactivity with the platelet receptor GPIb, whose binding domain is localized to a peptide fragment within vWF exon 28. In this type of vWD, efforts have therefore been concentrated on this site, and seven specific single amino-acid substitutions and one insertion have been identified, all within a small segment of the vWF A1 repeat. Four mutations account for nearly 90% of the type IIB patients studied to date. There are several other minor variants of vWD which have been classified on the basis of slight abnormalities of the vWF multimeric structure.

The future

The enthusiasm which followed the introduction of treatment with concentrates in the developed world has, in the past 25 years, been tempered by disastrous complications, first of HIV and AIDS, and now of liver disease due to hepatitis C. For the next 25 years we must look forward to treatment which must be 100% safe.

Long-term prophylactic care could enable boys to grow up with normal joints, and for many might replace treatment on demand, particularly if economies of scale enabled recombinant clotting factors to be available for treatment at a low price. Perhaps modified liver transplantation or gene therapy might in time produce a basic cure of haemophilia in all its forms.

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