DEPARTMENT OF CLINICAL HAEMATOLOGY

University
of
Manchester

# CURRENT TOPICS IN HAEMOPHILIA

## PROCEEDINGS OF THE SYMPOSIUM HELD IN MANCHESTER, U.K. ON SEPTEMBER 14th, 1982.

Edited by R. T. Wensley

We wish to thank Armour Pharmaceuticals for making a generous financial contribution towards the cost of the symposium and this publication.

Printed and published by NDT Print, Brunswick Mill, Manchester.



We are grateful to the publishers of Nature for permission to reprint the article of Choo et al, p.27.

# FORWARD

These are exciting times in the haemophilia world. Periods of depression when old complications prove refractory to solution or new complications emerge alternate with periods of elation at the promise of genetic probes and biogenetic concentrates. Our psychiatric colleagues could be forgiven if they conclude that those who care for haemophiliacs are predisposed to manic-depressive psychosis. The present volume gives vent to these frustrations and achievements.

The Haemophilia Centre Directors of the U.K. meet annually to discuss relevant problems. In 1980 our hosts at Glasgow took the opportunity to organise an international symposium in connection with that meeting. Its success was such that our hosts at Manchester this year have followed the Glasgow example, thus establishing a welcome tradition which hopefully will be continued in the future. In addition to reviewing current knowledge on coagulation the Manchester Symposium concentrated on the hepatitis problem and the pathogenesis and treatment of inhibitors both of which constitute major problem areas for the haemophiliac. These are timely presentations because hope has been engendered by the development of hepatitis-reduced concentrates and for the induction of immune tolerance to factor VIII but these therapeutic modalities still need very careful assessment. On another tack the cloning of the human factor IX gene by Professor Brownlee and his colleagues represents a fascinating development which has been one of the highlights of the year. No doubt this success forshadows further developments which will be of vital importance for haemophilia eugenics and treatment.

The organisers of the Symposium are to be congratulated on bringing together such distinguished international experts and the experts are to be congratulated on the lucidity and promise of their presentations. Although much remains to be learned the knowledge imparted in this Symposium sets us on a course that will at least alleviate, if not yet cure the depressive component of our psychiatric syndrome. Unfortunately, true to form, new hazards are appearing on the horizon including the acquired immune deficiency syndrome. The impact of these will no doubt feature in our next Symposium.

A.L.BLOOM

CONTENTS

FACTORS VIII AND IX

The role of factors VIII and IX in blood coagulation.

G. van Dieijen, J. Rosing, J. van Rijn, E. Bevers, H.C. Hemker and R.F.A. Zwaal.

Department of Biochemistry, Rijksuniversiteit Limburg, Maastricht, the Netherlands.

The severity of the bleeding disorders Haemophilia A and B is a reflection of the essential role of factors VIII and IX in blood coagulation. The two proteins participate in the intrinsic activation of factor X , a reaction which further requires the presence of a membrane surface containing negatively charged phospholipids (see ref. 1 for a review). In vivo, blood platelets provide such a surface.

Methods to prepare highly purified factors IX and X are available, and the molecular changes in the zymogens IX and X when they are converted to the active serine proteases factors IXa and Xa are known (1,2). The structure of human factor VIII:C (coagulant), the molecule that corrects haemophilia A is not yet known (see ref 3 for a review). Studies on bovine factor VIII indicate a molecular weight of about 220.000 and the presence of three peptide chains in the activated factor VIII (4). Factor VIII:VWF is a molecule different from factor VIII:C. It is deficient in van Willebrands disease, and functions in the adhesion of blood platelets to damaged endothelium. Factor VIII:VWF is also designated as factor VIII:RAG (factor VIII related antigen) and as the ristocetin cofactor activity. In plasma and cryoprecipitate factor VIII:VWF and factor VIII:C are found associated in the so called factor VIII:complex (3). In our study we have used purified bovine factor VIII:C and factor VIII complex. Until now no differences in factor X activating activity between the preparations have been found. The phospholipids used in this work are unilamellar (single bilayer) vesicles obtained by sonication of lipid suspensions consisting of 25% phosphatidylserine and 75% phosphatidylcholine.

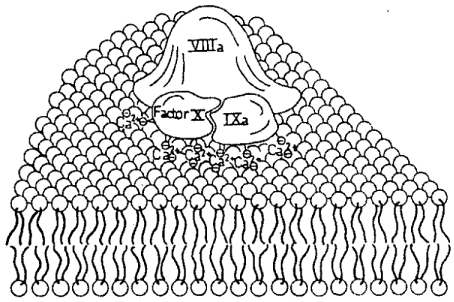



Fig. 1 Assembly of the components of the intrinsic factor X activating complex.

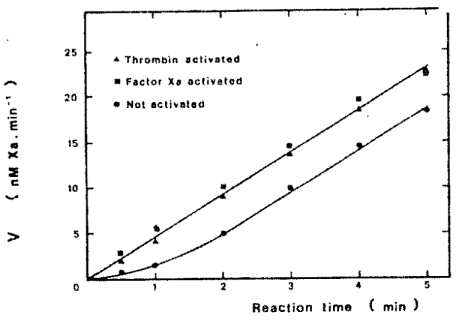



Fig. 2 Effect of preincubation of factor VIII:C with either thrombin or factor Xa on the time course of factor X activation.

It is thought that during activation the factors IXa VIIIa and X are bound at the phospholipid surface. Factors IXa and X are equipped with 10-12 modified glutamic acid residues (gammacarboxy glutamic acid residues), that enables these proteins to bind to the negatively charged phospholipids of the membrane surface via a $Ca^{2+}$-bridge (Fig. 1.) The interaction of factor VIII with lipids has not been investigated in detail.

Factor X activation can be followed on a spectrophotometer using the chromogenic substrate S2337. Factor Xa hydrolyses this substrate liberating paranitroaniline, that absorbs at 405 nm. The rate of S2337 conversion is proportional with the amount of factor Xa present.

Factor VIII must be activated before it can participate in factor X activation. In fig. 2 is shown the time course of factor X activation by factor IXa in the presence of PL, $Ca^{2+}$ and factor VIII. With unactivated factor VIII (closed circles) a lag period is seen which most likely reflects the time required for factor VIII activation in the reaction mixture, since preincubation of factor VIII with factor Xa (1 nM, 5 min. $37^{o}$) in presence of phospholipids and $Ca^{2+}$ prior to addition of factors IXa and X abolished the lag period. The lag is also abolished after a 1 min incubation of factor VIII with 1.5 nM thrombin. In all further experiments we use thrombin activated factor VIII.

Kinetics of factor X activation

To further understand of the roles of factor VIII and of phospholipids and $Ca^{2+}$ in the conversion of factor X to Xa by factor IXa, we have determined the kinetic parameters for several different factor X activating mixtures. The results are summarized in table 1.

| Composition of factor X activating mixture | $Km^{app}$ | Vmax |
|---|---|---|
| | μM | mol Xa.$min^{-1}$.mol $IXa^{-1}$ |
| IXa | 299 | 0.0022 |
| IXa, $CaCl_2$ | 181 | 0.0105 |
| IXa, $CaCl_2$,PL* (10 μM) | 0.058 | 0.00247 |
| IXa, $CaCl_2$, PL (10 μM), VIIIa(11 units/ml)+ | 0.063 | 500 |

* PL, Phospholipid, + VIIIa, factor VIII complex activated with thrombin.

Table 1: Effect of the accessory components (phospholipids, $CaCl_2$ and factor VIIIa) on the kinetic parameters of factor X activation. (data from: van Dieijen et al 1981; ref. 5)

Both phospholipid and factor VIIIa cause important changes of the kinetic parameters of factor X activation. In the presence of phospholipids the Km drops from 181 μM to 0.058 μM, with little change of Vmax. The

effect of factor VIIIa is mainly on Vmax, which is increased about 200.000 fold. The physiological importance of the accessory components is clear, considering the plasma factor X concentration of 0.2 μM. Phospholipids are required to bring the Km for factor X well below the plasma concentration and factor VIII, which increases the Vmax is required to obtain substantial levels of factor Xa.

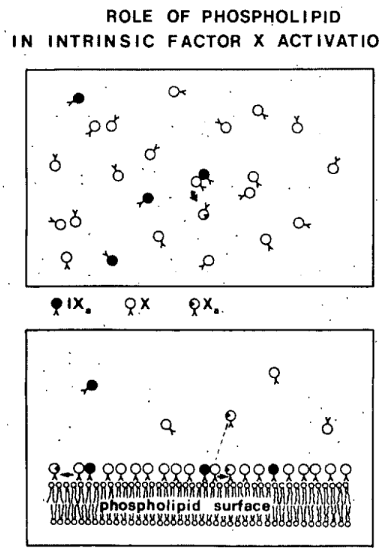



Fig. 3 Model to explain the lowering of Km in presence of a negatively charged phospholipid surface.

Fig. 3. provides an explanation why the Km for factor X is lowered in presence of a phospholipid surface. In solution (upper panel) a high concentration of factor X is required to saturate the enzyme factor Xa. When lipids and $Ca^{2+}$ are present (lower panel) both factor IXa and factor X bind to the lipid surface. Hence, the local factor X concentration is highly increased. Since Km is expressed in terms of added factor X, it is clear that in presence of lipids much lower amounts of factor X are required to obtain half maximal rates of factor X activation.

Assays based on the intrinsic factor X activating system

The knowledge of the kinetic properties of the factor X activating system has enabled us to design assays for phospholipids, factor IXa, and factor VIIIa. In the assays experimental conditions were selected such that the rate of factor X activation is proportional with the component to be assayed.

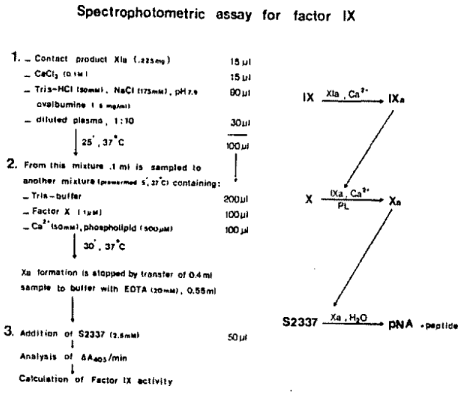



Fig. 4 Principle and experimental conditions for the spectrophotometric determination of factor IX in plasma. Data from ref. 6 and 7.

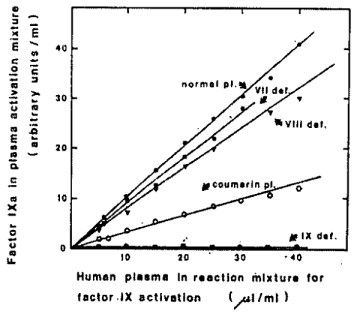



Fig. 5 Calibration curves obtained with various amounts of reference plasma, coumarin plasma and factor IX, VII, and VIII deficient plasmas in the spectrophotometric assay for factor IX.

-Assay of factor IX in plasma. The method consists of three steps. (Fig. 4). Diluted plasma is incubated with contact product (factor XIa), during this step factor IX is completely converted to factor IXa. A sample of the activated plasma is then incubated with factor X in presence of phospholipids and $Ca^{2+}$. After 30 min factor X activation is stopped with EDTA and the amount of factor Xa present is determined with the chromogenic subsrate S2337. From a calibration curve made with known amount of active site titrated factor IXa, the amount of factor IXa in the plasma can be calculated.

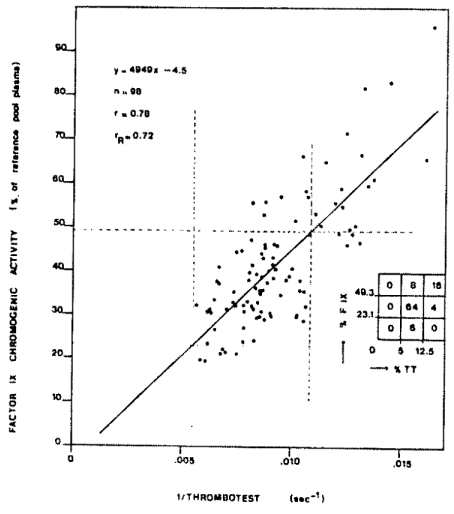



Fig. 6 Relationship between the factor IX activity determined with the chromogenic method and the activity in thrombotest, in plasma of 98 long-term anticoagulated human subjects.

Fig. 5 shows that the factor IX activity in the assay is proportional with the amount of plasma: that factor IX deficient plasma has no activity; that the assay is not influenced by presence or absence of factors VII and VIII and that lower activities are found in plasma from patients receiving coumarin. In Fig. 6 is plotted the factor IX content determined in plasma of 98 patients receiving long term oral anticoagulants, versus the activity as determined with thrombotest. The desirable therapeutical range based on thrombotest is between 5 and 12.5%, which corresponds to 23-49% of the factor IX activity in reference plasma as determined with the chromogenic assay. There is good agreement between the two methods, 80 out of 98 patients, would receive the same instructions with regards to dosage. These results and a comparison with spectrophotometric methods for factor II, VII and X is given in van Dieijen-Visser et al (7). The principle of the method is given in Tans et al. Thrombos. and Haemostas. In press (6).

- Assay of factor VIII in plasma

The principle of the assay is outlined in Fig. 7. Diluted plasma is incubated with thrombin to activate all factor VIII to VIIIa. a sample is added to a mixture of factors IXa, X and phospholipids plus $CaCl_2$.Factor X activation is stopped with EDTA, and the amount of factor Xa formed is determined with S2337.

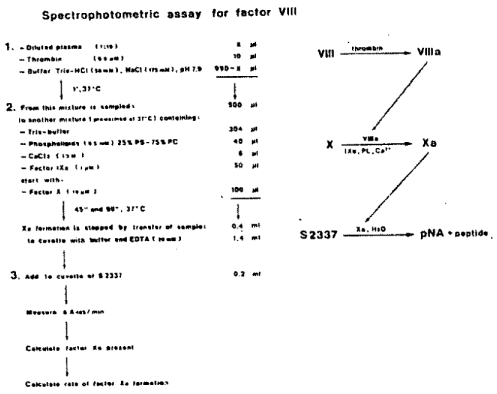



Fig. 7 Principle and experimental conditions for the spectrophotometric determination of factor VIII in plasma.

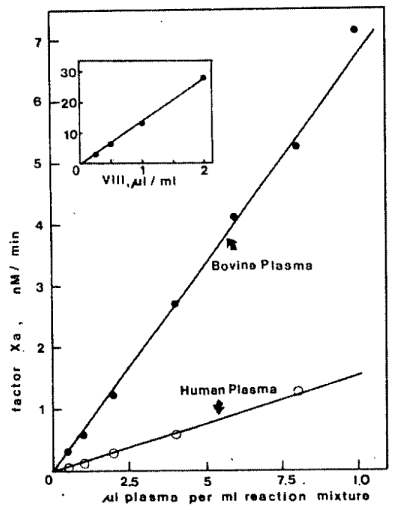



Fig. 8 Calibration curves obtained with various amounts of bovine and human plasma, and with highly purified bovine factor VIII:C, in the spectrophotometric assay for factor VIII.

As shown in fig. 8 a linear relationship is found between the amount of plasma and the rate of factor Xa formation in the test. Bovine plasma gives 5 times higher activities than human plasma. Bovine plasma may contain 5 times more factor VIII, or alternatively the bovine factor VIII molecule is more active than its human counterpart. The insert is the activity of a highly purified bovine factor VIII:C preparation containing 29 nM of factor VIII:C (Method to be published).
This allowed us to conclude that the factor VIII concentration in bovine plasma is 1.1 nM or 0.24 mg/l. When the molecular weight of factor VIII is 220.000 this would result in a specific clotting activity of 4132 U/mg. This is identical to the specific activity reported by Vehar and Davie (4).

Role of blood platelets in intrinsic factor X activation

The activity of platelets in factor X activation was studied using washed human platelets, that were stimulated for various time periods with various agonists. A mixture of factors IXa, VIIIa, X and $Ca^{2+}$ was then added and the rate of factor Xa formation was determined with a chromogenic substrate.

| Platelet or phospholipid preparation | Rate of fXa formation (nM/min) |
|---|---|
| Unstimulated platelets ($2.5 \times 10^6$/ml) | 0.3 |
| Sonicated platelets | 31.5 |
| Platelet lipid extract (1 µM) | 33.6 |
| Vesicles of platelet phospholipid composition (1 µM) | 34.2 |

Table 2: Effect of platelets and platelet phospholipids on the rate of factor Xa formation.

As shown in table 2 unstimulated washed platelets have little activity in factor X activation. Purified phospholipid vesicles, vesicles of platelet lipid composition and platelet phospholipid extract are highly active.

Platelets become active after disruption by sonication. This result is explained by the fact that the platelet phospholipids are asymmetrically distributed over the inner and outer leaflets of the platelet membrane phospholipid bilayer (Fig. 9). The negatively charged phospholipids, phosphatidylserine and phosphatidylinosithol are almost all located at the cytoplasmic side of the platelet membrane and therefore inaccessible for interaction with the clotting factors present in plasma (see ref. 8 from review). However, when platelets are stimulated by the combined action of collagen plus thrombin, activity appears in factor X activation. Thrombin alone is not an efficient stimulant (Fig. 10).

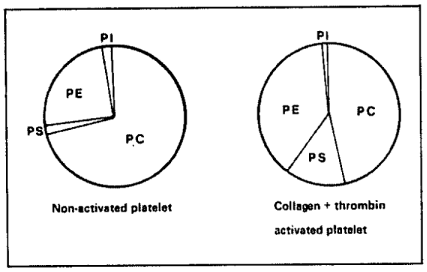



Fig. 9 Glycero-phospholipid composition of the outer monolayer of the platelet plasma membrane. PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, Phosphatidylinosithol. Data from Zwaal and Hemker ref. 8.

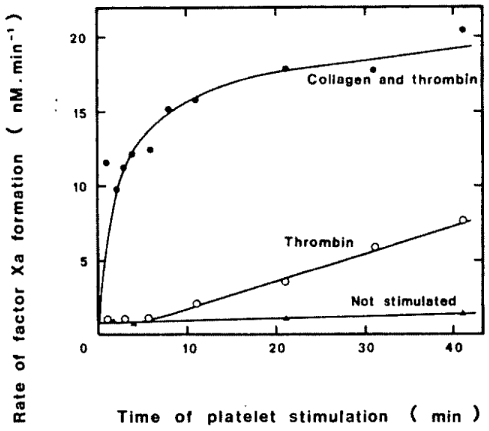



Fig. 10 Time course of appearance of platelet factor X activating activity. Platelets were activated, while stirring, without agonist with IIa or with Collagen/IIa. After the time period as indicated in the figure factors IXa VIIIa and X were added and the rate of factor X activation determined as described in table 3.

Table 3 summarizes the results of all physiological stimulants tested. We find the strongest stimulation by collagen plus thrombin. The non-physiological trigger, the calcium ionophore A23187 is even more active. The results can not be attributed to lysis since the platelets remain intact during activation, and less than 3% of platelet LDH is released during stimulation.

| Platelet stimulator | Rate of fXa formation (nM/min) |
|---|---|
| Unstimulated | 0.4 |
| Thrombin (1.3 nM) | 0.8 |
| Collagen (10 µg/ml) | 2.5 |
| Thrombin plus collagen | 6.0 |
| A23187 (1 µM) | 19.4 |
| Thrombin plus collagen followed by incubation with phospholipase A2 | 0.1 |

Table 3: Effect of platelets on intrinsic factor X activation. Platelet activation was for 10 minutes at 37 $^{o}$C, factor Xa formation was determined from the amount of factor Xa present after 45 and 90 sec. The final concentrations of clotting factors in reactionmixture were: factor IXa, 50 nM; factor VIIIa, 0.1 nM; factor X, 0.5 µM.

Our explanation for the appearance of platelet activity in factor X activation is that after triggering by collagen plus thrombin procoagulant negatively charged phospholipids have become available at the platelet surface. This is supported by several lines of evidence:

a. Collagen and thrombin activated platelets substitute for procoagulant phospholipid vesicles in factor X activation.
b. The activity of activated platelets is abolished after phospholipase A2 digestion (without platelet lysis)(table 3).
c. Direct analysis of the platelet outer surface by non-lytic degradation with phospholipase A2 shows that 25% of the platelet phosphatidylserine has become available at the platelet outer surface. (Bevers et al ref. 9).

We propose the following model for appearance of platelet procoagulant activity (Fig.11).

MODEL FOR PLATELET PROCOAGULANT ACTIVITY

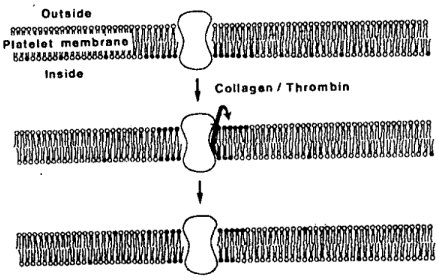



Fig. 11 Model for the appearance of platelet procoagulant activity. Schematically shown is the platelet plasma membrane lipid bilayer. The filled circles in the bilayer denote negatively charged polar head groups of procoagulant phospholipid, e.g. phosphatidylserine (PS), that interact with clotting factors. Platelet stimulation with Collagen and IIa results in PS exposure, resulting from PS transport from the inside to the platelet outside (flip-flop hypothesis; for details see ref. 8.

In the unstimulated platelet the procoagulant phospholipid, phosphatidylserine PS is located at the platelet inside. Triggering by collagen plus thrombin leads to appearance of PS at the platelet outside. the translayer transport of PS (flip-flop) may be facilitated by proteins. Membrane proteins may be present that have affinity for PS, such that domains with a high PS content are formed. As could not be shown here, activated platelets also stimulate the activation of prothrombin into thrombin by factor Xa and Va. Bevers et al (9). A detailed study on the activity of platelets in factor X and prothrombin activation is forthcoming. (Van Rijn et al. Manuscript in preparation).

Importance of intrinsic factor X activation in clotting pathways.

Intrinsic factor X activation must be a physiological important mechanism considering the seriousness of factors VIII and IX deficiencies. However the intrinsic and extrinsic clotting pathways do not readily explain this prominence (Fig. 12).

The extrinsic pathway of factor X activation bypasses the intrinsic route and does not explain why haemophilia is a disorder. The intrinsic pathway explains the importance of factors IX and VIII, but leaves no role for thromboplastin release as initiator of clotting. The importance of the contactsystem for the initiation of clotting is further complicated by the fact that deficiencies in factor XII, high molecular weight kininogen, and prekallikrein are not major bleeding disorders. The alternative pathway was discovered by Østerud and Rapaport in 1977, who showed that factor IX can

be activated to factor IXa by thromboplastin and factor VIIa (10). This pathway leaves a role for factors IX and VIII, however it must be explained why the indirect alternative route should be advantageous over direct factor X activation.

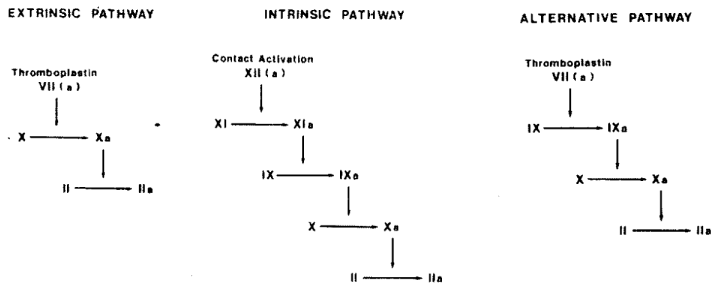



Fig. 12 Major clotting pathways in plasma.

The answer may be amplification. Jesty and Silverberg (11) have determined that with a given amount of thromboplastin, factor Xa is formed at 6-fold higher rates as factor IXa. However, our results (table 1) indicate that under optimal conditions, each molecule of factor IXa produces 500 molecules of factor Xa per minute. Therefore, the alternative pathway may produce factor Xa at 100 fold higher rates as the direct extrinsic pathway.

Acknowledgement: We thank G. Tans and M.P. van Dieijen-Visser for permission to reproduce data; KABI AB for donating part of the chromogenic substrates that were used in our work; T. Janssen-Claessen, J. Govers-Riemslag, J. Franssen and P. Comfurius for help in protein purification and expert technical assistance.

References

1. Jackson, C.M. and Nemerson, Y (1980) Ann. Rev. Biochem. 49, 765-811.
2. Davie, E.W. (1981): Methods in Enzymology vol 80 (L. Lorand, Ed) pp. 153-157. Academic Press, New York.
3. Hoyer, L.W. (1981) Blood, 58, 1-13.
4. Vehar, G.A. and Davie, E.W. (1980). Biochemistry 19, 401-410.

5. van Dieijen, G., Tans, G., Rosing, J. and Hemker, H.C. (1981) J. Biol. Chem. 256, 3433-3442.
6. Tans, G., Janssen-Claessen, T., van Dieijen, G., Hemker, H.C. and Rosing, J. (1982). Thromb. Haemostas. (Stuttgart) 48 (in press).
7. van Dieijen-Visser, M.P., van Wersch, J., Brombacher, P.J., Rosing, J., Hemker, H.C. and van Dieijen, G. (1982) Haemostasis, 12, 241-255.
8. Zwaal, R.F.A. and Hemker, H.C. (1982) Haemostasis 11, 12-39.
9. Bevers, E.M., Comfurius, P., van Rijn, J.L.M.L., Hemker, H.C. and Zwaal, R.F.A. (1982) Eur. J. Biochem. 122, 429-436.
10. Østerud, B. and Rapaport, S.J. (1977) Proc. Natl. Acad. Sci. USA 74, 5260-5264.
11. Jesty, J. and Silverberg S.A. (1979). J. Biol. Chem. 254, 12337-12345.

# Immunological Approaches to the Study of the Haemophilias.

Howard M. Reisner, Ph.D. Associate Professor of Pathology, University of North Carolina at Chapel Hill, Chapel Hill, N.C. U.S.A. 27514.

Richard Lewis, Ph.D. Post-doctoral Fellow, Department of Haematology, New England Medical Center, Boston, Mass.

Ernest Briet, M.D., Ph.D. Chief of Hemostasis and Thrombosis Laboratory, University Hospital, Leiden, The Netherlands.

Suzan de Serres, Research Associate, Department of Pathology, University of North Carolina at Chapel Hill, Chapel Hill, N.C. U.S.A. 27514

Harold R. Roberts, M.D. Professor of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, N.C. U.S.A. 27514.

Key words : Factor IX, alloantibody, inhibitor, Haemophilia B

## Summary

Inhibitors to clotting factors VIII and IX which arise in multiply transfused haemophiliacs are now recognized as being alloantibodies directed toward these proteins infused in therapeutic concentrates. Such alloantibodies may be used in sensitive radioimmunoassays to detect and measure levels of clotting factors. Two antibodies to FIX, one previously reported to be exclusively IgG4 lambda, appear to be highly polyclonal using sensitive isoelectrophoretic techniques. An alloantibody to FIX has been separated into two populations, one of which reacts with a conformeric determinant expressed only in the presence of calcium ions. These antibodies have proven to be valuable probes of structural variants of FIX in the plasma milieu.

Immunological studies of clotting factors VIII and IX presuppose the existence of immune probes, antibodies, which can recognize these respective molecules with high specificity. Our laboratory in Chapel Hill, North Carolina, has had a long standing interest in inhibitors of factors VIII and IX. Such inhibitors are now clearly recognized as being human alloantibodies most often produced in hemophiliacs in response to coagulation factors given as part of replacement therapy (1). Such antibodies may tell us much about the human immune response in general, but for the purpose of this review, we will be interested in what they tell us about the structure and function of mutant coagulation proteins.

Let me start by reviewing some of the studies undertaken by ourselves, Dr. Harold Roberts, and many others, on inhibitors of coagulation FIX. To date two such alloantibodies, both of the IgG class, have been characterized in some detail. One antibody, PWB, appeared to be restricted to the IgG4 subclass of immunoglobulins and to have only lambda type light chains. This was determined by adding monospecific anti-gamma chain subclass or light chain type antisera to PWB and measuring the amount of residual anti FIX activity in clotting assays. This "inhibitor neutralization" assay showed that only anti-gamma 4 removed the inhibitor activity allowing for the recovery of residual FIX. A second antibody, RJ, showed a very different pattern in inhibitor neutralization assays. Neither anti kappa nor anti lambda sera were able to totally abrogate the ability of this inhibitor to neutralize FIX. Likewise, no one anti subclass antisera totally neutralized the inhibitor activity in RJ. Hence, we hypothesized that inhibitor RJ was a polyclonal antibody represented in all immunoglobulin subclasses, while PWB was restricted in subclass distribution being oligo- or possibly monoclonal; a hypothesis which we will demonstrate to be quite incorrect (2,3).

A powerful measure of clonal diversity is the technique of isoelectric focusing. Using this technique IgG can be found to have a pI range from about 9.5 to less than 6.0. When inhibitor RJ plasma was isoelectric focused, inhibitor activity as measured in a coagulation inhibition assay was found over this wide range of values. As an internal control, anti HLA-A1 activity also present in plasma RJ, focused as two sharp, clonally restricted peaks. A sample of RJ plasma taken prior to anamnesis producing therapy had inhibitor activity restricted into two peaks. This would be consistent with RJ's having had a clonally restricted antibody which became broadened through repeated anamnesis induced by therapy (3).

The previously mentioned techniques for measuring clonal diversity have relied on the ability of alloantibodies to inhibit the coagulation activity of FIX for detection. These techniques suffer from at least two drawbacks; they are relatively low in resolving power, and they will not detect antibody clones which bind FIX but do not inhibit its coagulant activity. To obviate these problems we have combined high resolution thin layer agarose gel isoelectric focusing of antibody with the "Western" Blotting technique [Fig. 1]. In this technique the completed isoelectric focusing pattern is transferred to a protein binding nitrocellulose filter by blotting under pressure. The ampholytes used in isoelectric focusing are not retained by the membrane. The membrane, now containing the replicate of the isoelectric focusing pattern is incubated with radiolabelled FIX, washed and autoradiographed. Black bands on the x-ray film represent zones of antigen binding. Such an autoradiograph is shown in figure 2. Normal pooled plasma shows no significant binding of FIX. A mouse monoclonal antibody prepared by us (9/30) shows a highly restricted band pattern of rather low pI. Both RJ and PWB show complex patterns characteristic of a diverse, polyclonal

secondary immune response. Ironically, the goat heteroantibody seems more charge restricted than either human antibody. Of what significance are these observations? Early reports by ourselves and others regarding the clonal restriction of inhibitors to FIX as measured in coagulation based assays appear exaggerated. Even PWB, reported to be restricted to the IgG4 subclass, appears highly polyclonal.

Do these findings imply that human inhibitors are not useful probes into the structure and function of FIX? Certainly hybridoma antibodies such as 9/30 will be extremely valuable as "by definition" they recognize single antigenic determinants; nevertheless, antibody RJ has proven useful in defining the molecular defect in some forms of Hemophilia B. About four years ago Dr. Richard Lewis, then a graduate student in our laboratory devised a simple radioimmunoassay procedure for detecting antibodies to FIX using a direct binding technique. In this method radiolabeled FIX and human antibody are mixed and incubated. Immune complexes are separated from free iodinated FIX by precipitation with ammonium sulfate, or by using killed and fixed Staph a to bind antibody-antigen complexes. We were aware of work by the Furies and others (4) suggesting that certain antigenic determinants on the prothrombin molecule required divalent calcium ($Ca^{++}$) for expression and decided to see if either alloantibody to FIX contained a population which recognized such a $Ca^{++}$ dependent conformeric determinant. When PWB was titered in the presence of $Ca^{++}$ a slight but reproducible increase in activity was seen compared to titering in the absence of $Ca^{++}$. When RJ was similarly titered a two to threefold enhancement of antibody activity was seen in the presence vs. the absence of $Ca^{++}$. These findings suggested to Dr. Lewis and myself that an antibody subpopulation of RJ inhibitor might detect a $Ca^{++}$ dependent conformeric antigen of FIX (5). Once more we turn to Western

Blots, in this case incubating the nitrocellulose filter with radiolabeled FIX in the presence or absence of $Ca^{++}$. There is no detectable binding of FIX by normal pool. In the presence of $Ca^{++}$ there is much enhanced binding of FIX to antibody in all observable bands. Several bands of antibody in the lower pI range appear to be either very reduced, or completely absent in the presence of EDTA versus $Ca^{++}$ (Fig. 3). This can be seen much more clearly when the autoradiographs are scanned and the height of the major triplet of bands are equalized. Clearly there is much reduced binding of the relatively acidic clones when $Ca^{++}$ is not present. One clone may totally lack the ability to bind FIX unless $Ca^{++}$ is present in the incubation mixture (Fig. 4).

If there are clones of antibody in RJ which bind to antigen only in the presence of $Ca^{++}$, we should be able to separate RJ antibody into two or more subpopulations. In a simple procedure devised by Dr. Lewis, FIX is coupled to Sepharose and equilibrated in EDTA. Antibody recognizing $Ca^{++}$ dependent conformers of FIX does not bind to the solid phase resin under these conditions and is eluted with the bulk of non-antibody protein. Antibody binding to FIX in the absence of $Ca^{++}$ remains bound to the affinity matrix to be subsequently eluted with low pH buffer. This simple procedure functions remarkably well (Fig. 5). In the top panel we see the starting immunoglobulin pool showing enhancement of binding in the presence of $Ca^{++}$. Antibody eluted in pool 1 (middle panel) binds to FIX only in the presence of $Ca^{++}$. There is no detectable binding in buffer. Lastly, the acid eluate shown in the bottom panel binds identically in $Ca^{++}$ or buffer. Hence, we have succeeded in isolating an immune probe for FIX which binds to an antigenic determinant expressed only in the presence of $Ca^{++}$ (6).

Let me remind you that FIX is a single chain serine protease which upon activation forms IXa beta through the release of an internal activation peptide. The light chain contains the post-synthetically modified gamma-carboxyglutamic acid residues important in $Ca^{++}$ binding while the disulfide bonded heavy chain contains the active enzyme site. Presumably the light chain, possibly its amino terminal gamma-carboxyglutamic acid containing region, is important in forming the ion-dependent conformeric antigen. Is there any evidence to support the role of gamma-carboxylation in formation of the conformeric determinant? The anticoagulant Warfarin is known to block gamma-carboxylation of all Vitamin K dependent clotting factors by interfering with the post-synthetic modification of glutamic acids. Using competitive radioimmunoassays we determined that thirty-three normal individuals showed identical levels of FIX using either 1. whole IgG, 2. the acid eluted antibodies detecting the non-conformeric, non-ion dependent antigenic determinants (referred to as Non ConAg), or 3. the antibodies, binding to the $Ca^{++}$ requiring site (ConAg) (Table 1) (7). Dramatically different results were observed with Warfarinized patients, as can be seen in the second column. The level of non-conformeric antigens was reduced to about 2/3 of normal while the $Ca^{++}$ dependent conformeric antigen was reduced to about 1/2 this value or 1/3 of normal. These data suggest that the inhibition of gamma-carboxylation reduces the level of $Ca^{++}$ dependent conformeric antigens significantly more than non-ion dependent antigens.

Can the antibody probes tell us anything about the nature of the molecular defect in Hemophilia B? As is true of other clotting factors, molecular variants of FIX may be classified as CRM negative, having no antigenically detectable abnormal protein, or as CRM reduced or CRM positive, having reduced or normal levels of an immunologically detectable, defective

protein. For FIX, about 50% of all kindred synthesize some level of antigenically detectable protein. CRM negative kindred will, of course, be of great interest to the molecular biologist for study at the DNA level. Such mutations may be considered the "Thallassemias" of hemophilia B. The use of immune probes however requires an abnormal protein. In collaboration with Dr. Briët of Leiden, visiting at UNC, we studied 45 hemophilia B patients all having less than 20% of FIX coagulant activity. These samples had between 10 and 160% of normal FIX antigen measured using whole RJ in the absence of $Ca^{++}$. In each abnormal (closed circles) and the 33 normal control samples (open squares), levels of ConAg and Non ConAg were measured and the results plotted on a scattergraph (Fig. 5). Most hemophilic samples and the normals have paired antigen values which lie near a line with the slope of 1 (although there is considerable scatter at high antigen levels). Thus, most samples have similar levels of conformeric and non-conformeric antigen on their FIX molecules. Three numbered samples are clear outliers, having much reduced levels of the $Ca^{++}$ dependent antigen in relation to non-$Ca^{++}$ dependent antigen. In a statistical evaluation of the scattergram data, a frequency distribution of the log transformed values of antigen ratios for the 33 normal samples was constructed and a 99% tolerance interval calculated. Application of the tolerance interval to the patient data showed hemophilia B patients 1, 2 and 3 to be clearly outlying samples.

What do we know of these three patients? None of them had hemophilia Bm, a hemophilia B variant showing an abnormal ox brain PT. The first patient has been previously described by Dr. Bertina as having FIX of an abnormally high molecular weight and not binding $Ca^{++}$. Patients 2 and 3 were non-related individuals seen by Dr. Manucci. Both showed severe to moderate clinical disease. Interestingly, FIX from patient 2 does show some degree of $Ca^{++}$

binding in a crude electrophoretic system, suggesting that the molecular lesion is not related to a total loss of gamma-carboxylation. FIX from patients 2 and 3 is currently being purified for biochemical analysis by Drs. Roberts, Griffith and coworkers. We feel certain that based on the observed antigenic abnormality, the $Ca^{++}$ binding region will be involved in the molecular lesion in all the patients.

I hope in this brief review that the utility of alloantibodies as structural probes for clotting factors has been demonstrated. With the application of monoclonal antibodies to these proteins we can be certain that the use of antibodies as structural probes for abnormal clotting factors will continue to increase.

## References


1. Lewis, R.M., Zeitler, K., Blatt, P., Reisner, H.M. and Roberts, H.: Manual of Clinical Immunology 2nd edition, American Society for Microbiology, Washington, D.C. 1980 pp. 750-763.

2. Pike, I.M., Yount, W.J. and Puritz, E.M.: Blood 40:1-10; 1972.

3. Reisner, H.M., Roberts, H.R., Krumholz, S. and Young, W.J.: Blood 50: 11-19, 1977.

4. Furie, B. and Furie, B.C.: J. Biol. Chem. 254:9766, 1979.

5. Lewis, R.M., Reisner, H.M., Chung, B.S. and Roberts, H.R.: Blood 56: 608-614, 1980.

6. Lewis, R.M., Reisner, H.M. and Roberts, H.R.: In preparation.

7. Briet, E., Reisner, H.M. and Roberts, H.R.: In preparation.


**TABLE**

FACTOR IX ANTIGEN LEVELS IN NORMALS AND PATIENTS ON WARFARIN (U/ml)

| | NORMALS (n = 33) | PATIENTS ON WARFARIN (n = 10) |
|---|---|---|
| Complete IgG | 1.02 (0.14) | n.d. |
| anti NonConAg | 1.08 (0.21) | 0.62 (0.33) |
| anti ConAg | 1.05 (0.19) | 0.32 (0.21) |

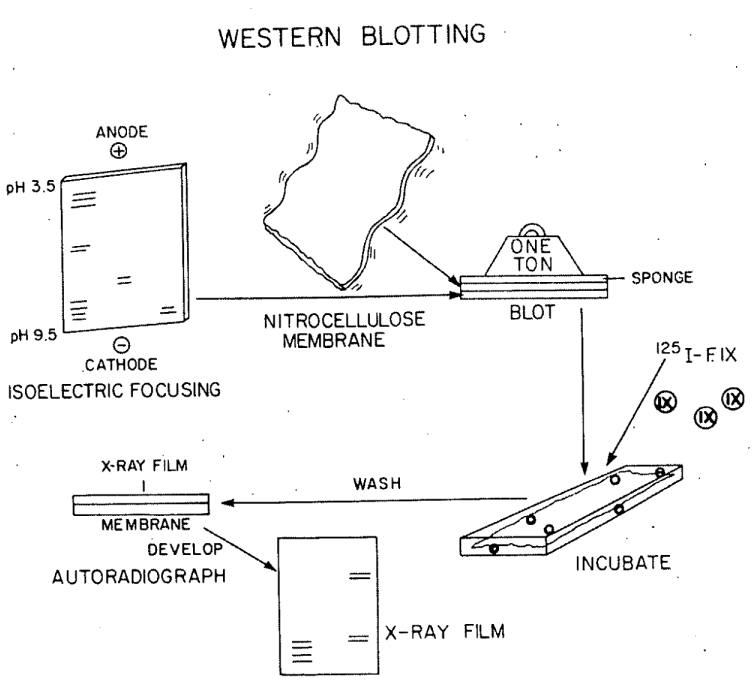



Figure 1. The Western blotting technique for detecting antibodies to F.IX.

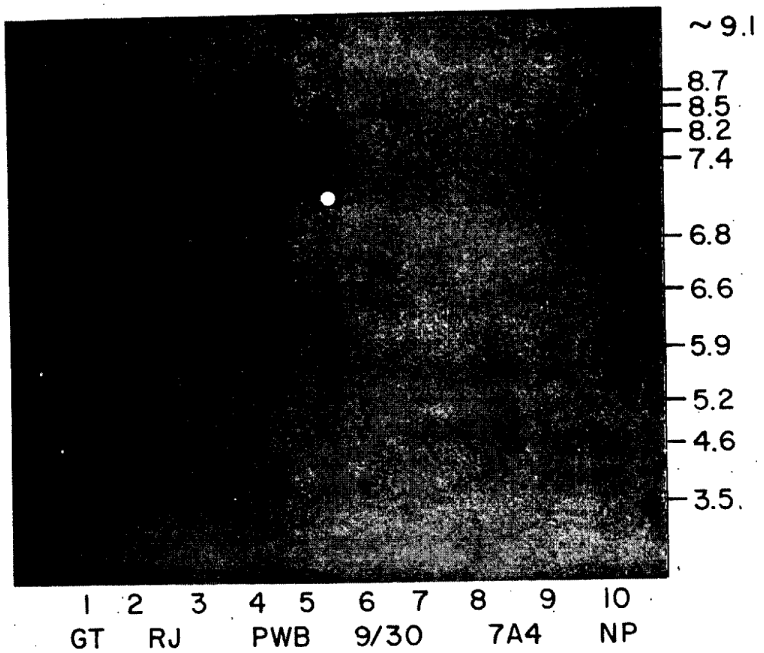



Figure 2. Western blotting technique as in Figure 3. Lanes contain the following:
1. Goat anti-F.IX; 2. 3. RJ (two dilutions); 4, 5, PWB; 6,7 monoclonal antibody to F.IX; 10 normal pooled plasma.

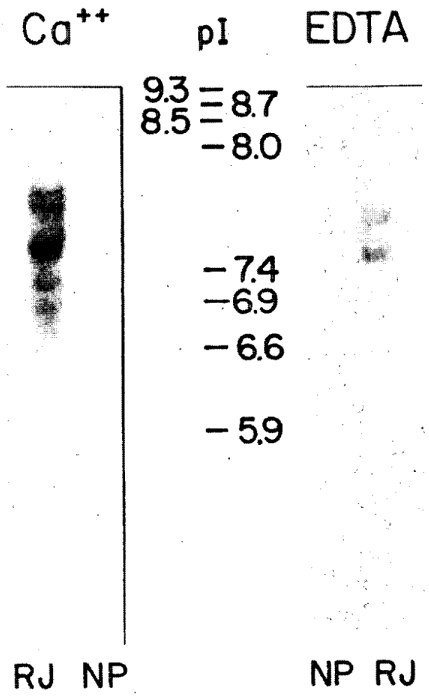



Figure 4. Densitometric scan of Figure 7. Peak height of major doublet bands equalized electronically.

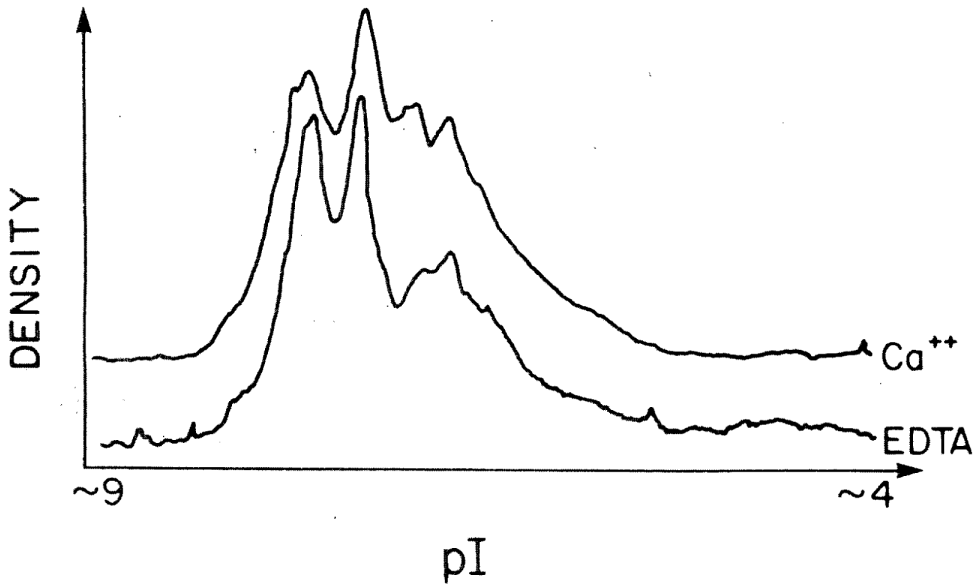



Figure 3. Western blotting of RJ and normal pool (NP) in the presence and absence of calcium

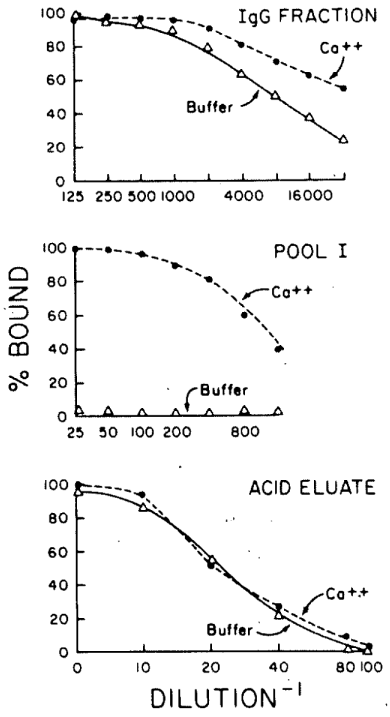



Figure 5. Titration of RJ fractions in the presence and absence of calcium

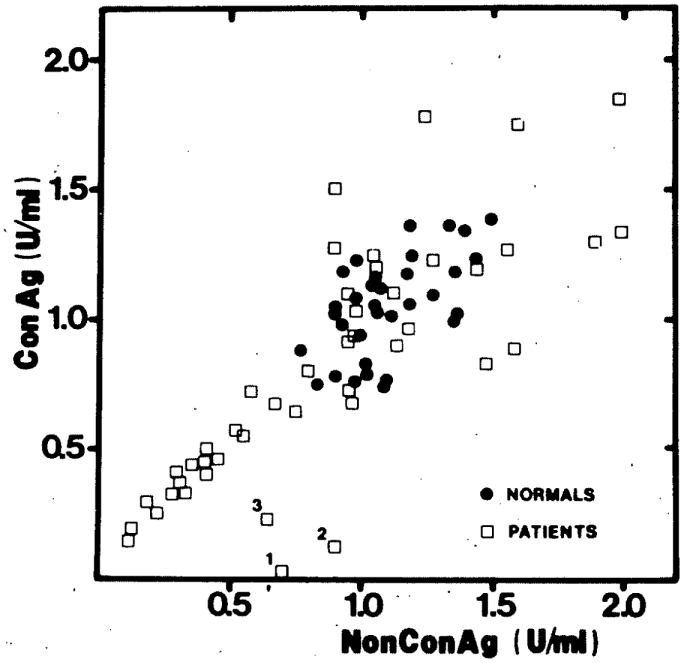



Figure 6. Levels of calcium dependent (Con Ag) and calcium independent. (Non Con Ag) F. IX antigens in 33 normal and 45 hemophilia B patients plasmas.

# MOLECULAR CLONING OF THE GENE FOR HUMAN ANTI-HAEMOPHILIC FACTOR IX

(This article originally appeared in Nature, September 9th, 1982 299 178-180).

K.H.Choo, K.G.Gould, D.J.G.Rees and G.G.Brownlee

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK

As a first step in the isolation of a cDNA clone for bovine factor IX, bovine liver was used to prepare total mRNA by the guanidine hydrochloride method[3]. A preparation enriched in factor IX-specific mRNA was obtained by oligo (dT)-cellulose chromatography, followed by two successive separations by sucrose gradient centrifugation. The enrichment was monitored by a specific immunological assay based on cell-free translation using rabbit reticulocyte lysates (Fig.1). A sucrose gradient fraction sedimenting at 20-22S was found to be enriched (about 10-fold) for the bovine factor IX mRNA, and was therefore used in the following cloning experiments.

Synthetic oligonucleotides were used[4] to produce a cloned cDNA library. From the available amino acid sequence data for bovine factor IX[5], two different sets of oligonucleotides were synthesized. The first set (designated oligo (N-1) was deduced from the amino acid sequence residues 348-352 (His-Met-Phe-Cys-Ala) and was synthesized using the solid phase phosphotriester method[6], as a mixture of eight different sequences (5'-GC$^{A}_{G}$CA$^{A}_{G}$AACAT$^{G}_{A}$TG-3'). The second set of oligonucleotides (oligo (N-2) ) was deduced from amino acids 70-75 (Glu-Cys-Trp-Cys-Gln-Ala); this was synthesized as two mixtures of eight different sequences each (5'-GCTTG$^{A}_{G}$CACCA$^{A}_{G}$CA$^{T}_{C}$TC-3' and 5'-GCCTG$^{A}_{G}$CACCA$^{A}_{G}$CA$^{T}_{C}$TC-3'). From a knowledge of the concentration of factor IX in blood plasma ($\sim$ 2-4$\mu$g ml$^{-1}$) and of the amount of $^{35}$S-cysteine counts precipitable by anti-factor IX antibodies, the abundance of factor IX mRNA was estimated to be as low as 0.01%. Therefore, to achieve further enrichment for factor IX mRNA, oligo (N-1) was used as a primer to synthesize cDNA from the sucrose gradient-enriched poly(A)-mRNA preparation. A library of 7,000 colonies was prepared and screened using $^{32}$P-labelled oligo (N-2) (see Fig.2 legend). One colony (designated BIX-1) gave a positive hybridization signal above background. Characterization of this colony by restriction endonuclease cleavage (data not shown) indicated that it contained a DNA insert of $\sim$ 430 base pairs (bp). Figure 2 shows part of the nucleotide sequence, that is, 304 residues of the BIX-1 insert, and confirms that it is a bovine factor IX sequence. It is interesting that only part of the insert sequence (amino acid residues 52-139) corresponds

directly to the nucleotide sequence predicted from the known bovine amino acid sequence data[5]. Over this region, there are no discrepancies between BIX-1 and the published data for factor IX, except at nucleotides 38-40 which code for Asp instead of Thr. This amino acid change was also observed in a second, independent cDNA clone. (This latter clone, which has a DNA insert spanning nucleotides 7-108 as shown in Fig.2, was isolated using the procedure described for the BIX-1 clone, except that oligo $(dT)_{12-18}$ was used to initiate cDNA synthesis, and the cloned cDNA library was prepared following the homopolymer tailing method of Roychoudhury and Wu[7].) The origin of the adjacent sequence starting at residue 140 (see Fig.2) which codes for an amino acid sequence unrelated to factor IX is not clear.

A DNA fragment (see Fig.3 legend) was isolated from the bovine BIX-1 clone and used as a hybridization probe to screen a cloned human gene library; a positive clone λ HIX1b, was isolated and characterized by restriction endonuclease cleavage and Southern blotting (see Fig.3). From these results, a partial restriction map for λ HIX1b was constructed (Fig.4A). A segment of the DNA insert containing the region that hybridizes to the bovine cDNA probe, was identified and isolated for direct sequencing[8]. The results (Fig.4B) reveal that, for the 96 nucleotides determined, there is 85% nucleotide sequence homology between λHIX1b and bovine factor IX. Comparison of the amino acid sequences deduced from the nucleotide sequences shows 78% homology between the two species; note that three of the nucleotide changes are not accompanied by an amino acid change and are therefore silent changes. The degree of homology is compatible with the cross-hybridization observed between the bovine factor IX cDNA probe and the λHIX1b clone in the rather stringent conditions used, and provides direct evidence that the latter carries an authentic DNA sequence coding for the human factor IX gene.

The present data are equivocal as to whether the λHIX1b clone carries a complete factor IX gene. The restriction map (Fig.4A) shows however, that the cloned segment includes more than six kilobases (kb) of DNA on one side and $>$ 10kb on the other side of the sequenced region (or the 'connecting peptide region')[5], and suggests that this segment potentially contains a complete human factor IX gene. Further sequencing should clarify this and also elucidate the structure of this gene.

Recent reports[9,10] have described the use of specific gene probes for prenatal and antenatal diagnosis of genetic disorders. These methods are based on the observation of the existence of polymorphisms for restriction

endonuclease sites which are closely linked to a particular gene locus. It is hoped that the genomic clone reported here will be similarly useful for the diagnosis and understanding of the mutations in patients with haemophilia B, as well as of heterozygote mothers who are carriers of the factor IX mutation.

We thank Dr.M.P.Esnouf for gifts of the crude anti-bovine factor IX antiserum and pure bovine factor IX; Drs.F.E. Baralle, N.J. Proudfoot and F.Giannelli for their help and useful discussion; and Dr.M.C.Carrol for providing the Bam-HI-cut and phosphatase-treated pBR322 vector. This work was supported in part by MRC project grant G8103835 to G.G.B. K.H.C. is a recipient of Uncle Bobs Travelling Scholarship and a C.J. Martin Research Fellowship from the National Health and MRC of Australia.

Note added in proof: Further sequence of clone λHIX1b suggests that there is a splice point after residue 101 of the sequence (Fig.4B, line d). The TTAG (nucleotides 98-101 may therefore derive from an intron and may not code for the amino acids L and D.

The origin of the sequence unrelated to factor IX mRNA shown within square brackets in Fig.2 could reflect either a cloning artefact or may derive from an intron region assuming we have cloned a region of a pre-mRNA molecule. Further sequencing of the genuine clone should resolve this.

Summary

A functional deficiency of factor IX, one of the coagulation factors involved in blood clotting[1], leads to the bleeding disorder known as Christmas disease[2], or haemophilia B. Both this disease and haemophilia A (factor VIII (C) deficiency) are X chromosome-linked and together occur at a frequency of ~1 in 10,000 males. The molecular basis for the functional alteration of factor IX in Christmas disease is not clearly understood. As a first step towards the elucidation of the molecular events involved, we have attempted molecular cloning of the factor IX gene. We used a bovine factor IX cDNA clone, isolated using synthetic oligonucleotides as probes, to screen a cloned human gene library. Here we report the isolation and partial characterization of a λ recombinant phage containing the human factor IX gene.

## REFERENCES


1. Jackson, C.M. and Nemerson, Y.A. Rev. Biochem, 49, 765-811 (1980).
2. Ratnoff, O.D. in The Metabolic Basis of Inherited Disease (eds. Stanbury,J.B. Wyngarden,J.B. and Frederickson, D.S.) 1755-1791 (McGraw-Hill, New York, 1978).
3. Chirgwin,J.M. Przybyla,A.E., MacDonald,R.J. and Rutter,W.J. Biochem 18, 5294-5299 (1979).
4. Wallace, R.B. et al. Nucleic Acids Res. 9, 879-894 (1981).
5. Katayama,K. et al. Proc. natn. Acad. Sci. U.S.A. 76, 4990-4994(1979).
6. Duckworth,M.L. et al. Nucleic Acids.Res. 9, 1691-1706 (1981).
7. Roychoudhury, R and Wu. R. Meth.Enzym. 65, 43-62 (1980).
8. Maxam, A. and Gilbert, W. Proc. natn. Acad.Sci. U.S.A. 74, 560-564 (1980).
9. Kan, Y.W., Lee, K.Y., Furbetta,M., Angius, A and Cas, A. New Engl.J.Med. 302, 185-188 (1980).
10. Little, P.F.R. et al. Nature 285, 144-147 (1980).
11. Caton, A.J. and Robertson, J.S. Nucleic Acids Res. 7, 1445-1456 (1979).
12. Pelham, H.R.B. and Jackson, R.J. Eur.J.Biochem, 67, 247-256 (1976).
13. Choo, K.H. Cotton, R.G.H., Danks, D.M. and Jennings, I.G. Biochem J. 181, 285-294 (1979).
14. Choo,K.H., Jennings,I.G. and Cotton, R.G.H. Biochem.J. 199, 527-535 (1981).
15. Huddleston,J.A. and Brownlee, G.G. Nucleic Acids.Res.10, 1029-1038 (1981).
16. Cassadaban, M.J. and Cohen, S.N. J.molec.Biol. 138, 179-207 (1980).
17. Gergen,J.P., Stern, R.H. and Wensink, P.C. Nucleic Acids Res. 7, 2115-2136 (1979).
18. Lawn, R.M., Fritsch,E.F., Parker, R.C., Blake, G and Maniatis, T. Cell, 15, 1157-1174 (1978).
19. Southern, E.M. J. molec Biol. 98, 503-517 (1975).

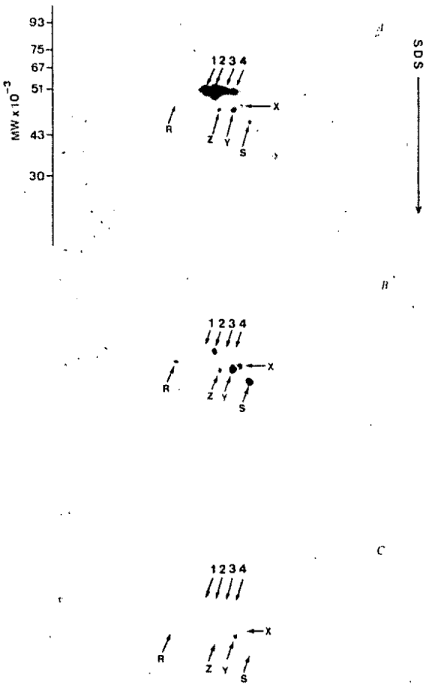



Fig.1 Two-dimensional polyacrylamide gel electrophoretic analysis of immunoprecipitated cell-free translation products of bovine liver mRNA. mRNA purified through an oligo (dT)- cellulose column[11] was translated in a rabbit reticulocyte cell free system[12] in the presence of $^{35}$S-cysteine. After incubation at 29°C for 90 min, samples were treated for immunoprecipitation as described previously[13]. Crude antiserum against pure bovine factor IX was raised in rabbit. Specific anti-factor IX immunoglobulins used for immunoprecipitation were purified as described previously[14] by passage of the crude antiserum through a Sepharose-4B column to which pure bovine factor IX had been coupled. After incubation, the antibody-antigen complex was precipitated by the addition of protein A-Sepharose CL-4B resin (Pharmacia), washed and resolved on a two-dimensional polyacrylamide gel[13]. A, immunoprecipitation in the presence of purified antibodies; B, same as for A, except that immunoprecipitation was performed in the presence of both antibodies and a competing amount of unlabelled pure bovine factor IX; C, same as for A, except that immunoprecipitation was performed in the presence of a control antiserum from a rabbit which had not been immunized with factor IX. 1-4, Factor IX polypeptide spots; evidence for this comes from the fact that (1) these are the predominant spots immunoprecipitated by the specific antibody; (2) their molecular weight ($\sim$ 50,000, a single polypeptide chain plus a possible pre-peptide signal sequence) is compatible with published data[5], (3) the spots are specifically competed for by pure factor IX; and (4) they are absent from the gel when control serum is used. (Partial chymotryptic

digestion of polypeptide spots 1 and 2 (data not shown) suggests that they are related and may have arisen as a result of post-translational modification; the amount of material recovered from spots 3 and 4 was insufficient for these to be compared). R, S, X, Y and Z designate nonspecific background polypeptide spots which form useful reference points on the two-dimensional gel. The molecular weights (MWs) of the polypeptide markers on the second dimension gel are shown on the left. IEF designates isoelectric focusing in the first dimension[13], SDS, polyacrylamide gel electrophoresis in the second dimension[13].

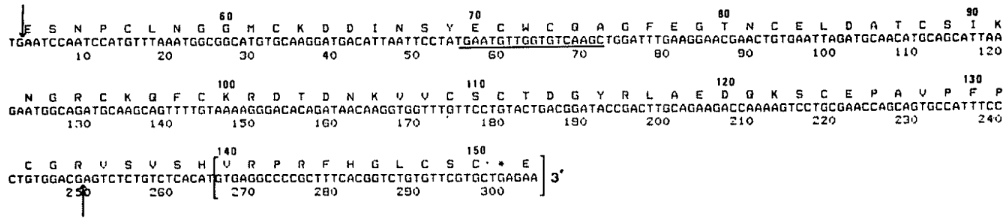



Fig.2. Isolation and characterization of bovine BIX-1 clone. A library of clones containing bovine cDNA inserts was prepared as follows. First, cDNA strands were synthesized as described elsewhere[15] except that 2 µg of oligo (N-1), 20-30µg of sucrose gradient-enriched poly $(A)^+$ mRNA, 10 µCi ($\alpha^{-32}$)dATP (3,000 Ci $mmol^{-1}$; Amersham) and 50 U of reverse transcriptase were used in a 50 µl reaction mixture. The cDNA was phenol-extracted, desalted on a Sephadex-G100 column, then treated with alkali (0.1M aOH, 1mM EDTA) at 60°C for 30 min to remove the mRNA strand. Second strand DNA was synthesized as described elsewhere[15]. The double-stranded DNA was then cleaved with the restriction enzyme MboI and ligated to pBR322 which had been cut with BamHI and treated with calf intestinal alkaline phosphatase to minimize vector self-religation. The ligated DNA was used to transform Escherichia coli strain MC1061 (ref.16). A library of 7,000 ampicillin-resistant colonies was obtained. Of these, ~ 85% were tetracycline-sensitive. The library of colonies were transferred in an unordered fashion to 13 Whatman 541 filter papers, amplified with choloramphenicol, and prepared for hybridization as described by Gergen et al[17]. The filters were prehybridized at 65°C for 4 h in 6 x NET, 5 x Denhardt's, 0.5% NP-40 and 1 µg $ml^{-1}$ yeast RNA described by Wallace et al.[4] Hybridization was at 47°C for 20 h in the same solution, containing 3 x $10^5$ c.p.m. (0.7 ng)$ml^{-1}$ of labelled oligo (N-2) probe. Labelling was achieved by phosphorylating the oligonucleotides at the 5' hydroxyl end using ($\gamma^{-32}$P)ATP and $T_4$ phosphokinase (Boehringer)[15]. At the end of the hybridization, filters were washed successively at 0-4°C (2h), 25°C (10 min) 37°C (10 min) and 47°C (10 min). On fluorography of the filters

from this screening, one colony showed a positive signal above background; this clone was designated BIX-1 and part of the DNA insert was characterized by sequencing performed according to Maxam and Gilbert[8]. Only the coding strand is shown and this is numbered in the opposite orientation to the tetracycline resistance gene of the plasmid. The deduced amino acid sequence (top row) has been numbered according to Katayama et al[5]. The 5' Sau3AI cloning site in the BIX-1 clone is eight nucleotides to the left of the start of the above sequence. The arrows indicate two HinfI sites which give rise to a 247-nucleotide fragment (see text). Brackets indicate the start of a sequence which does not correspond to published amino acid data[5] (see text). The region corresponding to the oligo (N-2) probe is underlined. *Designates a nonsense codon. IXNET is 0.15 M NaCl, 1 mMEDTA, 15 mM Tris-chloride, pH 7.5.

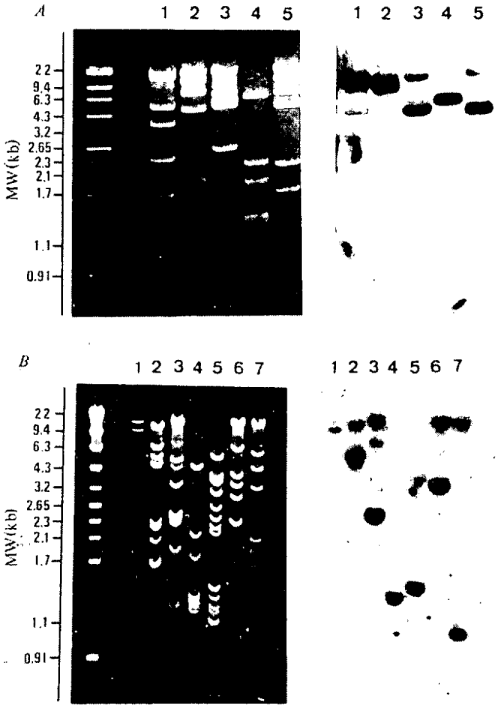



Fig.3 Isolation and characterization of the human λ HIX1b clone. The cloned human DNA library used as a HaeIII/AluI λ Charon 4a library prepared by Lawn et al[18]. Phage recombinants ($10^6$) from this library were screened using the in situ plaque hybridization procedure described previously[18]. Prehybridization and hybridization were carried out at 42°C in 50% formamide. After hybridization, filters were washed at room temperature with 2 x SSC, 0.1% SDS, then at 65°C with 1 x SSC, 0.1% SDS. A 325-nucleotide fragment from the bovine BIX-1 cDNA clone was initially used as a probe in the hybridization. This fragment

corresponds to nucleotides -8 to 317 as shown in Fig.2, and was isolated by Sau3AI digestion of BIX-1 plasmid DNA. The isolated DNA was labelled to high specific activity by incorporation of ($\alpha^{-32}$P)ATP using Amersham's nick-translation kit; 10 clones were isolated. These were plaque-purified and re-screened with a 247-nucleotide fragment from BIX-1 clone. This fragment, derived from nucleotides 3-249 (see Fig.2), contains only sequences corresponding to the published bovine factor IX amino acid sequence[5], and was isolated by HinfI digestion of BIX-1 plasmid DNA. Only a single clone gave a positive hybridization signal with this probe. This clone was further plaque-purified and the resulting clone (designated λHIX1b) characterized by digestion with various restriction endonucleases. Ethidium bromide-stained gels are shown on the left and their corresponding fluorograms after Southern transfer[19] and hybridization with factor IX-specific 247-nucleotide probe are shown on the right. A, 1-5, cleavage pattern with BamHI, BglII, HindIII, HpaII and EcoRI, respectively. The sizes of the fragments showing predominant hybridization signals for these endonucleases are ~16, 11, 5.5, 6.5 and 5.5 kb respectively. B, 1, cleavage with BglII alone; 2-7, cleavage with BglII in combination with BamHI, HindIII, HpaII, PvuII, SstI and EcoRI, respectively. The sizes of the fragments showing predominant hybridization signals for these endonucleases (1-7) are ~11, 5.5, 2.5, 1.4, 1.5, 3.4 and 1.0kb, respectively. The MW markers (kb) shown on the left are fragments from pBR322 plasmid DNA cleaved with AluI, BamHI + PvuII and BamHI + PstI, plus λ DNA cleaved with HindIII.

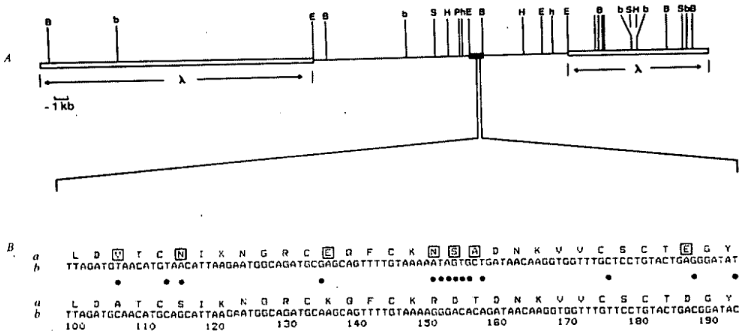


Fig.4 A partial restriction map of λ HIX 1b phage DNA. B = BglII; b, BamHI; E, EcoRI; S, RstI; H,HindIII, h, HpaII, P, PvuII. The Charon 4A λ veetor long arm is on the left and the short arm is on the right. Restriction sites for BglII, BamHI, EcoII, SstI and HindIII on the vector arms are shown. The smallest fragment (~ 1 kb) within the human DNA insert which hybridizes to the bovine cDNA probe (Fig.3) is a BglII-EcoRI fragment (indicated by a solid box). The construction of this map was based on the data shown in Fig.3 and that derived from restriction analysis of a pBR322 subclone of the 10-12-kb BglII fragment containing the 1-kb BglII-EcoRI probe region. This map shows the approximate

positions of the nearest restriction sites to the left and right of the probe region (with the exception of SstI and Pvu where their sites to the right have not been determined). B, Sequence of 96 nucleotides located within a region of the human DNA insert which hybridizes to the bovine cDNA probe. a, Amino acid sequence from the nucleotide sequence b, for λ HIX1b. These two sequences are compared with the amino acid c and nucleotide d sequence of bovine factor IX. The bovine nucleotide sequence has been numbered similarly to that shown in Fig.2. ● Indicates a nucleotide difference bteween the human and bovine sequences. □ Indicates a difference in amino acid between the two species. Note that the orientation of this sequence within the genomic clone is 5' to 3' from right to left.

HEPATITIS AND HAEMOPHILIA

# THE SPECTRUM OF LIVER DISEASE IN HAEMOPHILIA

R. F. STEVENS. B.Sc., M.B., Ch.B., M.R.C.P. M.R.C.Path

As a result of relatively recent developments in plasma collection and fractionation, many haemophiliacs now receive potent, easily re-constituted, convenient blood products which have resulted in an improved life style for the severe haemophiliac. But at what price?

Over the past decade clinicians have become increasingly aware of clinical, sub-clinical and laboratory abnormalities in multi-transfused haemophiliacs suggestive of hepatocellular damage, and there is increasing evidence that such damage is the result of viral infection, particularly Non-A, Non-B hepatitis.

The problems associated with liver dysfunction in the haemophiliac can be considered under the following headings.

1. Clinical stigmata of liver disease in haemophiliacs
2. Abnormal liver function tests in haemophiliacs
3. Liver histology in haemophiliacs
4. Therapeutic approach to liver dysfunction in haemophiliacs

## Clinical stigmata of liver disease in haemophiliacs

It must be emphasised that haemophiliacs represent a highly selected group of patients. They attend hospital over prolonged periods of time, are heavily investigated, and it is not surprising that any biochemical abnormality in liver function is detected early. However, it is difficult to correlate these biochemical changes with clinical disease and long term hepatic function.

One of the first clinical signs of liver disease in any patient with or without haemophilia is that of jaundice. Several studies have reported the incidence of jaundice as follows :-

| | |
|---|---|
| Biggs (1974)[1] | 3.5% over 3 years |
| Lewis (1973)[2] | 11.7% over 6 years |
| Kasper (1972)[3] | 31% with previous history |
| Mannucci (1975)[4] | 17.6% with previous history |

The causes of jaundice in haemophiliacs are several and include haemolysis (transfusion of haemolytic iso-antibodies in blood products), many viral agents not including Non-A, Non-B hepatitis (e.g. Hepatitis B, Hepatitis A, CMV, Toxoplasma, E.B. virus) and occasionally cholestasis. However, Non-A, Non-B hepatitis is becoming increasingly implicated, although the majority of cases are non-icteric.

In general terms, it would appear that the annual incidence of overt jaundice in multi-transfused haemophiliacs in the U.K. prior to the introduction of large pool commercial concentrates was approximately 2.5% per annum, rising to approximately 5% per annum after 1974 when these products first became readily available. Since then the number of cases of overt jaundice has tended to fall as fewer patients per year are exposed to large pool concentrates for the first time.

Hepatosplenomegaly is another clinical feature not uncommon in haemophiliacs.

Hepatosplenomegaly in Haemophilia

| | Liver | Spleen |
|---|---|---|
| Mannucci (1975)[4] | 5% | 2% |
| Levine (1977)[5] | NIL | 27% |
| Stevens (1982)[6] | 23% | 60% |

Levine et al in 1977 reported that approximately one quarter of multi-transfused haemophiliacs have splenomegaly. A recent survey in the Manchester region showed that in haemophiliacs with persistently abnormal transaminase levels, 60% had splenomegaly and 23% hepatomegaly on the basis of radio-isotope liver and spleen scan although clinical examination proved much less informative.

Direct questioning will not infrequently reveal symptoms compatible with a viral hepatitis like illness but such symptoms are not usually volunteered by haemophiliacs and the suggestion that they may possibly have some degree of liver dysfunction usually comes as a surprise to the patient.

Although most clinicians have seen or have heard about isolated haemophiliacs with severe liver disease associated with other clinical features, such cases are very infrequent, and in general terms the symptoms and signs of significant liver disease are found relatively infrequently in haemophiliacs. I am glad to say that the number of deaths due to liver disease so far reported in the U.K. remains very small.

## Abnormal liver function tests in haemophiliacs

Several reports have now been produced indicating the presence of raised transaminase levels in multi-transfused haemophiliacs.

| Report | Abnormal LFTS % | Abnormal LFTS > 6/12 % |
|---|---|---|
| Mannucci (1975)[4] | 45 | |
| Hasiba (1977)[7] | | 51 |
| Hilgartner (1977)[8] | | 52 |
| Levine (1977)[5] | 68 | 51 |
| McVerry (1977)[9] | 55 | |
| Preston (1978)[10] | | 70 |
| Rickard (1982)[11] | 34 | 8 |

It can be seen that up to 50% of multi-transfused haemophiliacs show persistently abnormal transaminase levels.

It is worth discussing a little further the recent report by Rickard et al on Hepatitis and Haemophilia Therapy in Australia. In this survey, haemophiliacs were receiving replacement therapy almost entirely in the form of cryoprecipitate. The more severely affected patients receiving the higher dosage regimens had a significantly higher frequency of liver transaminase abnormality than the milder haemophiliacs receiving less therapy. Of the severe haemophiliacs 49% had raised transaminase during the study and 13% had raised levels for more than 6 months. If the conclusions of Rickard et al prove to be correct then the exclusive use of cryoprecipitate is no guarantee of a reduced risk of liver dysfunction and that the hepatitis risk is not a valid reason for prohibiting the use of factor VIII concentrates.

Recent figures from the Oxford Haemophilia Centre (courtesy of Dr. J. Craske and Dr. C. Rizza) indicate that in mild haemophiliacs first time exposure to large pool concentrate is associated with a very high incidence of presumed Non-A, Non-B hepatitis, and that in cases where the haemophiliac had been previously exposed to concentrate, it was usually of a different brand.

| | Previous Concentrate | No Previous Concentrate |
|---|---|---|
| Total | 17 | 9 |
| NA NB Hepatitis | 6 | 9 |
| No Hepatitis | 11 | 0 |

All nine haemophiliacs not previously exposed to large pool factor concentrates developed clinical and laboratory features suggestive of Non-A, Non-B hepatitis.

To summarise the studies on persistently abnormal transaminase levels in haemophiliacs, it can be stated that they are :

i) Present in a high percentage of haemophiliacs

ii) Not usually associated with symptomatic liver disease

iii) Tend to persist far in excess of six months, although fluctuations in transaminase levels are typical

iv) Not related to the age of the patient, nature of the coagulation defect, or quantity and duration of replacement therapy once the patient has been exposed to the presumed infective agent

In Manchester we have recently carried out a trial of dynamic liver function tests in multi-transfused haemophiliacs. These have consisted of the Galactose Elimination Capacity (GEP-a measure of the functional liver cell mass) and the prolonged Bromosulphthalein test (BSP).

| Diagnosis | Number | GEP | BSP |
|---|---|---|---|
| CAH/Cirrhosis | 1 | Impaired | Reduced |
| Very mild CAH | 4 | 3 Impaired | 1 Reduced |
| Chronic Persistent | 1 | N | N |
| Chronic Lobular | 1 | Impaired | Slightly reduced |
| Non-Specific | 5 | 5N | 1 Reduced |

There was a tendency for patients with more serious liver histology to show more deranged dynamic liver function tests but the numbers are too small to draw any firm conclusions. Nevertheless, these relatively non-invasive tests may be useful in detecting deteriorating liver function in haemophiliacs.

Liver histology in haemophiliacs

The realisation that many multi-transfused haemophiliacs have persistently abnormal liver function tests resulted approximately 5 years ago in various haemophilia centres undertaking programmes of routine liver biopsy. The rationale for such an invasive procedure in patients who were in the main asymptomatic and have a haemostatic defect was primarily the differentiation of chronic persistent hepatitis from chronic active hepatitis and cirrhosis which may benefit from specific treatment, and which may also place a new prognosis on the life expectancy of the haemophiliac.

The following table reviews some of the initial results.

| Centre | Number of cases | Number of cases with CAH/Cirrhosis | % |
|---|---|---|---|
| Lesesne (Chapel Hill)[12] | 6 | 3 | 50 |
| Spero (Pittsburgh)[13] | 13 | 5 | 38 |
| Mannucci (Milan)[14] | 11 | 6 | 55 |
| Preston (Sheffield)[10] | 8 | 4 | 50 |
| Schimpf (Heidleburg)[15] | 32 | 10 | 31 |

It can be seen that early reports suggested that up to 50% of multi-transfused haemophiliacs have significant histological liver disease, either with chronic active hepatitis or cirrhosis, although histological interpretation may account for some variation in these exact figures.

Only the Heidelburg survey has reported the results of repeated liver biopsies in 6 haemophiliacs, repeated a minimum of 3 and a maximum of 5 years after the first biopsy, and in all cases there proved to be no significant deterioration.

We in Manchester have recently successfully carried out liver biopsies on twelve haemophiliacs, all of whom were multi-transfused and had persistently abnormal transaminase levels.

Manchester Haemophilia Liver Biopsy Trial

| | | |
|---|---|---|
| Number of haemophiliacs biopsied | 12 | |
| Chronic active hepatitis and cirrhosis | 1 | (8%) |
| Very mild chronic active hepatitis | 4 | (33%) |
| Chronic lobular hepatitis | 1 | (8%) |
| Chronic persistent hepatitis | 1 | (8%) |
| Non-specific changes | 5 | (42%) |

In our survey, however, only one patient had chronic active hepatitis and cirrhosis, although four other cases were considered to have very mild chronic active hepatitis. We also found that there was no relationship between the nature or dosage of the previously received plasma product.

A recent report from the Ad Hoc Haemophilia Study Group[16] in the U.S.A. reviewed the results of liver histology from 155 haemophiliacs.

U.S.A. Ad Hoc Haemophilia Study Group[16]

| | | |
|---|---|---|
| Number of liver biopsies | 115 | |
| Number of Autopsy liver biopsies | 40 | |
| Total number of cases | 155 | |
| Trivial disease | 6% | |
| Mild disease | 59% | |
| Acute hepatitis | 6% | |

| | | |
|---|---|---|
| Chronic active hepatitis | 9% ) | 16% |
| Cirrhosis | 7% ) | |
| Suspicious/inadequate | 15% | |
| Others | 3% | |

No relationship was found between the histological diagnosis and the previous treatment history.

In the 40 autopsy cases, of whom 21 had never been exposed to commercial concentrates, the distribution of histological diagnosis was similar to the biopsied cases. This may also be an indicator that small donor pool products may also carry a significant risk of hepatitis.

On the basis of this Manchester trial and the results of this recent world-wide collaborative study, I would like to suggest the following conclusions :-

i) that hepatic histology among multi-transfused haemophiliacs may not be related to specific blood product replacement therapy and although not yet proven, long term use of small donor pool products (e.g. cryoprecipitate) may carry as much risk of transmitting Non-A Non-B hepatitis as that of large donor pool concentrates.

ii) that the frequency with which therapeutically useful information is obtained from liver histology in haemophiliacs is limited and may not justify the risk of this procedure. Out of 115 liver biopsy cases in the recent collaborative study there were two deaths as compared with a mortality of 1 in 5,000 in non-haemophiliacs.

Therapeutic approach to liver disease in haemophiliacs

At this time we do not know how much of a problem liver disease represents itself in haemophilia. We do know that despite the exclusion of Hepatitis B to a large degree, the dangers of viral disease transmission, presumably Non-A Non-B, associated with intensive transfusion are far from over, and that even small donor pool cryoprecipitate may not be exempt from this problem. Although as yet, mortality and the advances clinical sequaelae of liver disease in haemophiliacs are thankfully rare, the potential risks remain, and all multi-transfused haemophiliacs must be followed closely for any clinical or laboratory evidence of hepatic deterioration. Dynamic liver function tests may prove useful in predicting deteriorating liver function.

Whether or not this follow up should include liver biopsy is debatable. Although this procedure can be carried out safely in the majority of cases, deaths have been reported, and in the absence of any proven specific therapy for chronic active hepatitis and cirrhosis, many

people feel that percutaneous biopsy is not indicated, although it might be reasonable to carry out biopsy under direct 'vision' during abdominal surgery for another reason.

The prospect of a new "clean" replacement products either of human origin or from genetic engineering leave hope for the future but at the present time it appears that the clinical risks associated with replacement therapy may be overstated and are certainly outweighed by the need to treat the haemophiliac by the rapid replacement of his deficient clotting factor.

## REFERENCES


1. BIGGS, R. Jaundice and antibodies against Factors VIII and IX in patients treated for haemophilia or christmas disease in the United Kingdom. Brit.J.Haemat. 1974; 26: 313-329.

2. LEWIS, J.H., MAXWELL, N.G., BRANDON, J.M.: Jaundice and Hepatitis B Antigen/Antibody in Haemophilia. Transfusion 1974; 14: 203-211.

3. KASPER, C.K., KIPNIS, S.A.: Hepatitis and clotting factor concentrates. 1972; JAMA 221: 510.

4. MANNUCCI, P.M., CAPITANIO, A., DEL DINNO, E., et al. Asymptomatic Liver Disease in Haemophiliacs. J.Clin.Path. 1975; 28: 620-624.

5. LEVINE, P.H., McVERRY, B.A., ALLOCK, B., DORMANDY, K.M.: Health of the intensively treated haemophiliac, with special reference to abnormal liver chemistries and splenomegaly. Blood 1977; 50: 1-9.

6. STEVENS, R.F., CUTHBERT, A., PERERA, P.R., et al. Hepatic function and histology in haemophiliacs. An overstated problem? 1982; In Press.

7. HASIBA, V.W., SPERO, J.A., LEWIS, J.H.: Chronic liver dysfunction in multi-transfused haemophiliacs. Transfusion 1977; 17: 490-494

8. HILGARTNER, M.W., and GIARDINA, P.: Liver dysfunction in patients with haemophilia A, B and von Willebrands disease. Transfusion 1977; 17: 495-499.

9. McVERRY, B.A., VOKE, J., MOHAMMED, I., DORMANDY, K.M., and HOLBOROW, E.J: Immune complexes and abnormal liver function in haemophilia. J.Clin.Path. 1977; 30: 1142-1146.

10. PRESTON, F.E., UNDERWOOD, J.C.E., MITCHELL, V.E., TRIGER, D.R., BARDHAM, G., STEWART, R.M., BLACKBURN, E.K.: Percutaneous liver biopsy and chronic liver disease in haemophiliacs. Lancet 1978; ii: 592-594.

11. RICKARD, K.A., DORITY, P., CAMPBELL, J. et al. Hepatitis and haemophilia therapy in Australia. Lancet 1982: ii; 146-148.

12. LESESNE, H.R., MORGAN, J.E., BLATT, P.M., WEBSTER, W.P., ROBERTS, H.R.: Liver biopsy in Haemophilia A. Ann. Intern. Med. 1977; 86: 703-707.

13. SPERO, J.A., LEWIS, J.H., VAN THIEL, D.H., HASIBA, U., RABIN, B.S.: Asymptomatic structural liver disease in Haemophilia. 1978; N.Engl.J.Med. 1978; 298: 1373-1378.

14. MANNUCCI, P.M., RONCHI, G., ROTA, L., COLOMBO, M.: A clinicopathological study of liver disease in Haemophiliacs. J.Clin. Path. 1978; 31: 779-783.

15. SCHIMPF, K., ZIMMERMANN, K., BLEYL, V., DÖHNERT, G.: Liver biopsy findings in Haemophilia. In: SELIGSOHN, V., RIMON, A., HOROSZOWSKI, H, Eds. Haemophilia: Castle House Publications, 1981: 149-153.

16. ALEDORT, L.M., LEVINE, P., HILGARTNER, M. et al. A stud[illegible] of liver disease among haemophiliacs. Blood 1981; 58: Supp.1; 210a.

# VIRAL HEPATITIS

Professor A.J. Zuckerman

Department of Medical Microbiology and the WHO Collaborating Centre for Reference and Research on Viral Hepatitis, London School of Hygiene and Tropical Medicine, London WCIE 7HT

## Hepatitis A virus

Hepatitis A virus is spread by the faecal-oral route, most commonly by person to person contact, and infection occurs readily in conditions of poor sanitation and overcrowding.

The infection has been transmitted to certain species of marmosets and to susceptible chimpanzees. The virus has been identified as a small cubic particle 25-28 nm in diameter. Hepatitis A virus has a linear single-stranded RNA and polypeptides which are closely similar to those of the enteroviruses. The virus is relatively resistant to inactivation by heat, to ether and to acid; but it is inactivated by formalin at a concentration of 1:4000 at 37°C for 72 hr. and by chlorine at 1 ppm for 30 min.

Only one serotype has been identified in volunteers infected experimentally with hepatitis A, in patients from several different outbreaks of hepatitis in different geographical regions, in sporadic cases of hepatitis A and in naturally and experimentally infected chimpanzees.

Specific laboratory tests for hepatitis A antigen and antibodies have been developed including immune electron microscopy, immune adherence haemagglutination, radioimmunoassay and enzyme-linked immunosorbent assay (ELISA or enzyme immunoassay).

Recent surveys have shown that the age-standardized prevalence of hepatitis A antibody was 24% in Switzerland, 40% in the U.S.A., 75% in Senegal, 88% in Belgium, 88% in Taiwan, 94% in Israel and 97% in Yugoslavia, confirming that infection with hepatitis A virus is widespread throughout the world.

The spread of infection is reduced by simple hygienic measures and the sanitary disposal of excreta. Normal human immunoglobulin may prevent or attenuate a clinical illness, while not always preventing the infection.

An adapted strain of hepatitis A virus (CR 326) has been cultivated in tissue culture. Other strains have now been cultured.

A vaccine against hepatitis A is not available, but vaccines are under development.

Hepatitis B virus

Infection with hepatitis B virus is associated with the appearance in the serum of a specific antigen, hepatitis B surface antigen. A second antigen is present in the core (nucleocapsid) of the virus. A third antigen, hepatitis B e antigen appears to correlate with the number of virus particles and the degree of infectivity of surface antigen-positive sera.

The surface antigen appears in the sera of most patients during the incubation period of the acute infection, some 2-8 weeks before biochemical evidence of liver damage or the onset of jaundice. The surface antigen is usually cleared from the circulation in adolescents and adults during convalescence. Free core antigen has not been detected in serum. Next to appear in the circulation is the associated DNA polymerase activity immediately before or at the time of raised serum transaminases. The polymerase activity persists for days or weeks in acute cases and for months or years in a proportion of persistent surface antigen carriers. Antibody to the core is found in the serum 2-10 weeks after the appearance of the surface antigen and it is frequently detectable during the acute infection and for some time after recovery has taken place, although with declining titres. Core antibody appears to be correlated with the amount and duration of replication of the virus. Finally, surface antibody appears.

Surface antigen-antibody complexes may be found in the sera of some patients during the incubation period and during the acute phase of illness.

Cell-mediated immunity appears to be important in terminating hepatitis B infection and, under certain circumstances, in promoting liver damage and in the genesis of autoimmunity.

There are some 176 million persistent carriers of hepatitis B surface antigen in the world and there is an urgent need to define the mechanisms which lead to the high carrier rate in endemic areas.

The surface antigen represents excess virus-coat material. The 42 nm particle is the complete virion. The core of the virus particle contains DNA-dependent DNA polymerase, closely associated with a DNA template. The DNA has a molecular weight of 1.8 to 2.3 x $10^6$. The DNA structure has been characterised by gel electrophoresis and restriction enzyme cleavage and shown to be double-stranded and circular structure approximately 3600 nucleotides in length containing a single-stranded gap of 600-2100 nucleotides. The endogenous DNA polymerase reaction appears to repair the gap.

Hepatitis B immunoglobulin is of value in conferring temporary passive immunity under certain circumstances and guidelines for passive immunisation against hepatitis B have been proposed by the WHO Expert Committee on Viral Hepatitis in 1977.

Information obtained from the sequencing of the 226 amino acids of hepatitis B surface antigen, using cloned DNA of hepatitis B virus, has opened the way to the development of chemically synthesized peptides corresponding to amino acid sequences predicted from the nucleotide map. Several such synthetic peptides elicit antibodies in experimental animals which react with the surface antigen. The potential of chemically synthesized vaccines is under intensive investigation.

Investigations are in progress on the effect of exogenous and endogenous interferon for the treatment of chronic hepatitis B infection. Several antiviral preparations, including arabinosyl nucleosides, are being studied.

The new human hepatitis viruses

There is clear evidence that there are at least two new forms of viral hepatitis that are clinically indistinguishable from hepatitis A or B (non-A, non-B hepatitis) but antigenically unrelated to either type. The infection is transmissible by blood transfusion and by blood products such as Factor VIII and Factor IX. There are at least two different types of parenterally-transmitted non-A, non-B hepatitis viruses. Epidemic water-borne and endemic forms of non-A, non-B hepatitis have been described in the sub-continent of India. 15-25% of adult patients with sporadic viral hepatitis in industrialised countries are diagnosed as non-A, non-B hepatitis, not necessarily nor commonly associated with apparent parental transmission.

Suggestions for further reading

Zuckerman, A.J. (1979) Specific serological diagnosis of viral hepatitis. British Medical Journal, 2, 84-86

Hepatitis Viruses of Man. A.J. Zuckerman and C.R. Howard. Academic Press, London and New York, December 1979

S. Sherlock (Ed.) Viral Hepatitis. Clinics in Gastroenterology, volume 9. Saunders 1980

Viral Hepatitis: Report on a WHO Informal Consultation (1981) Journal of Medical Virology, 8, 1-29

Zuckerman, A.J. (1982) Priorities for immunisation against hepatitis B. British Medical Journal, 284, 686-688

Szmuness, W., Alter, H.J. and Maynard, J.E. (Eds.) Viral Hepatitis. (Proceedings of the 1981 New York Symposium). Franklin Institute Press, Philadelphia, 1982.

Non-A, non-B hepatitis remains a diagnosis by exclusion. There is considerable evidence for at least two types of non-A, non-B agents transmitted by blood and blood products, and the infection has been induced experimentally in non-human primates.

Distinct ultrastructural changes have been found in the liver of experimentally infected chimpanzees, and less predictably nuclear changes in the hepatocytes of certain species of marmosets. The cytoplasmic changes in the smooth endoplasmic reticulum of hepatocytes consist of fusion of opposing membranes, deposition of electron-dense material along the membrane system and the formation of dense tubular structures. Recently it was shown that the electron-dense material within the tubular structures was arranged in the form of well-defined fibrillar-like meshwork with a periodicity of approximately 15 nm, which was enhanced by staining with potassium permanganate. Potassium permanganate has an intense staining affinity for membranous structures containing glycoproteins and lipoproteins (McCaul et al, 1982a).

Electron-beam X-ray microanalysis of the tubular structures did not reveal the presence of inorganic or metal components and no deviation of the energy spectrum when compared to the background or control analysis (McCaul et al, 1982b). The formation of tubular structures may therefore reflect either a disordered synthesis or an abnormal incorporation of organic compounds such as sugars into glycoproteins or glycolipids or their abnormal metabolism.

Clusters of intranuclear particles, varying in diameter from 15-20 nm, may be found and nuclear alterations are usually detected after the cytoplasmic changes. The nuclear and cytoplasmic changes in the hepatocytes of infected chimpanzees are not mutually exclusive (Tsiquaye et al, 1981). The variation in size of the nuclear particles may perhaps be an indication of incomplete virus formation with manifest involvement secondary to attempted cytoplasmic elaboration of viral protein capsular elements.

Crystalline arrays, 25-30 nm in diameter, have been found in the endothelial cells and Kupffer cells in association with experimental non-A, non-B hepatitis. Examination of individual crystalline particles, which possess units in a four-point symmetrical pattern, by the Markham rotation technique, showed

that the particles consisted of an electron-dense core approximately 12 nm in diameter, surrounded by an envelope measuring 5.5 nm in thickness. The outer structure possessed 16-18 divisions. However, the individual crystals are probably non-viral and the presence of the crystalline arrays is a reflection of the pathological response of the host cell to infection (McCaul et al, 1982c).

REFERENCES

McCaul, T.F. Tsiquaye, K.N. Tovey, C, Hames, C and Zuckerman, A.J. Application of electron microscopy to the study of structural changes in the liver in non-A, non-B hepatitis. Journal of Virological Methods. 1982a, 4, 87-106

McCaul, T.F. Tsiquaye, K.N. Tovey, G, Hames, C. Lu X-Z, Hobgen, D.N. and Zuckerman, A.J. A study of ultrastructural alterations in experimental non-A, non-B hepatitis by electron-beam analysis. British Journal of Experimental Pathology. 1982b, 63, 325-329.

McCaul, T.F. Tsiquaye,K.N. Tovey, G. Duermeyer, W and Zuckerman, A.J. Examination of crystalline arrays in non-A, non-B hepatitis. Journal of Medical Virology. 1982c. 9, 185-188.

Tsiquaye, K.N. Amini, S, Kessler, H, Bird, R.G, Tovey, G and Zuckerman, A.J. Ultrastructural changes in the liver in experimental non-A, non-B hepatitis. British Journal of Experimental Pathology. 1981, 62, 41-51.

# THE REMOVAL OF VIRAL CONTAMINANTS

# FROM COAGULATION FACTOR CONCENTRATES

T.J.SNAPE, BA., Ph.D., C.Chem., M.R.S.C.
(Head of Quality Control, BPL, Elstree).

J.K.SMITH, B.Sc., Ph.D., C.Chem., F.R.S.C.
(Head of Coagulation Factor Production,
BPL, Elstree).

Abstract :

Viral Hepatitis is the most frequent serious complication of transfusion therapy, and of coagulation factor replacement therapy in particular. This paper reviews the approaches currently available for the elimination of viral infectivity, and comments critically on the claimed effectiveness.

Screening for HBsAg by third generation test methods has made an enormous impact on the rate of transmission of hepatitis B, despite the fact that the detection limit of such tests is many times higher than the infective concentration. More intensive screening of a small population of accredited donors can provide very low risk plasma for special purposes, but is neither practically nor economically feasible for routine application.

Elimination of infectivity of an established infective product may possibly be achieved by :

i. removal of virus by selective fractionation methods,
ii. neutralization of virus by immunological methods,
iii. inactivation of virus by physical or physico-chemical methods.

Success has been claimed in all of these areas, but validation of such claims is far from complete. Given the detection limit of available tests (or lack of a suitable test in the case of non-A non-B hepatitis), and given the fact that the chosen marker may not coincide with infectivity, the only proof of non-infectivity of treated products lies in infusion studies in animals or man.

## Introduction : Transmission of Viral Hepatitis by Coagulation Factor Concentrates

Viral hepatitis is the only disease commonly transmitted by coagulation factor concentrates. If someone who has recently received coagulation factor therapy shows clinical or laboratory signs of hepatitis, a reasonable first hypothesis must be that the concentrate was responsible. Unfortunately, the incubation period is so variable that many batches of concentrate may have been given during the relevant period. Even in situations where only one batch of concentrate is implicated, it is most usual to find that the whole batch has been used before recall procedure can be initiated.

We are not, of course, talking about a single infectious agent. Current or recent hepatitis B infection is recognised by well characterised marker antigens or antibodies in the patients blood. Coincidental infection with hepatitis A, which is not usually transmitted by blood products, may be diagnosed serologically by detection of IgM antibody to hepatitis A. Physical, and drug induced jaundice, as well as jaundice resulting from infection with cyto-megalovirus or EpsteinBar virus must also be excluded. After all such exclusions have been made, it can be assumed that non-A, non-B (NANB) hepatitis has been transmitted by the concentrate. Furthermore, since there are several well documented cases of repeated NANB hepatitis infections, one must assume that there are several NANB viruses which do not confer cross-immunity.

How big a problem are we dealing with? One must acknowledge that the problem is large enough to make a clinician hesitate, and balance the potential risks and benefits, before using a large pool coagulation factor concentrate. This is particularly true if the patient in question has had little or no exposure to coagulation factor concentrates in the past. Thus fibrinogen concentrates are now no longer licensed in the United States. In this country, the use of factor IX concentrates for, say, anti-coagulant reversal would be discouraged, and the use of large pool factor VIII concentrates, for treatment of von Willebrand's disease or carriers of mild haemophilia, would be similarly discouraged. Nevertheless, 70% of all NANB hepatitis has been associated with first time treatment with a large pool concentrate, often where a safer alternative product, cryo-precipitate for example, might have been used.

More than 70% of the U.K. population of haemophiliacs have shown evidence at some time of challenge with either hepatitis B, or NANB hepatitis, and one must assume that this is attributable to treatment with large pool concentrates. The balance of the problem is, of course, shifting from hepatitis B to NANB hepatitis. Thus there is some evidence that all factor VIII concentrates, commercial and NHS alike, have a probable 100% infection rate for NANB hepatitis on first time exposure. Confirmed transmission of hepatitis B, by current NHS concentrates at least, is by comparison a singularly rare event.

Although not common, particularly in multi-transfused haemophiliacs, it must be recognized that infection with either hepatitis B or NANB virus from a large pool concentrate may lead to fulminant hepatitis. In addition, 5-10% of those affected with hepatitis B become long term carriers and show some signs of progressing to cirrhosis later in life. Even when the infection with hepatitis B or NANB hepatitis has been symptomless, and detected only by disturbance of serum enzyme levels, biopsy evidence points to a high incidence of liver damage.

## Towards Coagulation Factor Concentrates with Reduced Risk of Hepatitis Transmission

These then are the stimuli to making coagulation factor concentrates safer in respect of transmission of viral hepatitis. What can the fractionator do about it? Four possible approaches may be distinguished :

i. Exclude (hepatitis) virus from the pool.
ii. Remove (hepatitis) virus by selective fractionation.
iii Neutralize (hepatitis) virus by immunological methods.
iv. Inactivate (hepatitis) virus by physical/chemical methods.

These four approaches will be considered in turn.

### Exclude (hepatitis) virus from the pool :

Screening for HBsAg by third generation test methods has had an enormous effect on the rate of transmission of hepatitis B. It is a fact however, that the detection limit of these tests is up to 1000 times higher than the infective concentration. This means that if just one donation, containing hepatitis B virus at a concentration just below the detection limit, is included in a 1000 litre pool of plasma for fractionation, the coagulation factor concentrates produced from that pool may still be infective.

Despite the promise of recent advances there is still no selective screen for NANB hepatitis, although determination of serum enzyme levels may be indicative.

For certain programmes it has been worthwhile to screen a small population of plasma donors more intensively than usual, including careful selection from well known donors, serial estimation of transaminases, clinical surveillance and quarantining of plasma for more than three months as a final precaution. This is currently done by N.W. Thames R.T.C. to provide BPL with about 100 litres of plasma each year, for the preparation of highly purified fibrinogen for isotopic labelling.

This small pool approach is simply not feasible in the production of factor VIII for treating the majority of haemophiliacs who are bound to receive the equivalent of many thousands of donations per year in any case. To put the costs of the two products into perspective, small pool fibrinogen from special plasma costs about £900 per gram, perhaps twenty times the cost of large pool factor VIII. It is possible however, to fractionate comparatively large batches of plasma obtained by plasmapheresis of a limited donor panel, though one is still talking about several hundred donors.

Fresh frozen plasma despatched to BPL for the production of coagulation factor concentrates may, under present circumstances, be fractionated within a few weeks of its receipt at BPL. As a result, a disturbing number of batches may be held up, or even need to be abandoned, as a result of reports of unwell donors, or infection thought to have arisen from transfusion of red cells associated with a plasma donation in a fractionated plasma pool. Clearly a longer holding period for fresh frozen plasma prior to fractionation is desirable, and indeed plans for expansion at BPL include the provision of extended $-40^{o}C$ quarantine storage, to allow holding of incoming fresh frozen plasma for at least three months, with possible extension to six months.

Clearly then, for the foreseeable future, the starting plasma for routine fractionation to produce coagulation factor concentrate has to be accepted as possibly containing hepatitis B virus, below the detection limit of the screening test, and NANB virus at any level.

Remove (hepatitis) virus by selective fractionation :

Quite a lot of work has been done following markers of hepatitis B through the fractionation of known infective plasma. Most of this work has been done with HBsAg as a marker, but other markers of potential infectivity tell much the same story; if the plasma is infective, coagulation factor concentrates made from it will probably also be infective. One therefore cannot rely on the fractionation process per se to remove hepatitis B virus.

One of the earliest, and more convincing descriptions of a method for removing HBsAg from plasma fractions, in this case factor IX, was the published work of Johnson et al (1976). The procedure involved selective precipitation with polyethylene glycol 4000. Glycine and sodium chloride were added to a factor IX concentrate produced by elution from DEAE-cellulose. These additions were designed to increase the solubility of factor IX for the subsequent stages. PEG4000 was then added to a final concentration of 20% w/v, and the precipitate brought down by the PEG was removed by centrifugation. The authors report quantitative recovery of HBsAg in the precipitate. The yield of factor IX obtained by subsequent precipitation from the PEG supernatant was claimed to be of the order of 60%. Residual HBsAg was said to be undetectable in the concentrate.

The technique involves a delicate balance of conditions. PEG 4000 precipitates proteins and virus, but whereas virus tends to be precipitated at about 20% PEG irrespective of the conditions, the precipitation of proteins may be affected by simultaneous manipulations of pH ionic strength and temperature. In this example factor IX was held in solution at 20% PEG by the addition of glycine and sodium chloride - additions which did not affect precipitation of virus. The concentration of the virus marker was apparently reduced by a factor of approximately $10^4$ at each precipitation, so that one or two reprecipitations of factor IX eluate reduced HBsAg below detectable levels.

There are several published claims for the removal of HBsAg from concentrates, usually at an intermediate stage of production, by the use of an adsorbant semi-specific for the protein of interest. Thus it has been claimed that the adsorption of factor VIII to polyelectrolytes or to amino-acyl sepharose (Austin & Smith, 1982) may leave HBsAg and hepatitis B virus unbound, allowing the factor VIII to be eluted later with, of course, greatly reduced content of antigen and presumably virus. Unfortunately these claims have not yet been convincingly tested and the fractionation methods themselves are still unattractive in terms of the yield of factor VIII available.

The work of Einarsson and her colleagues at KABI (Einarsson et al, 1982) on the removal of hepatitis B virus from factor IX concentrates by the technique of hydrophobic interaction chromatography is interesting. Optimum conditions appear to involve column adsorption of HBsAg and hepatitis B virus using octanoic acid hydrazide sepharose 4B. The non cross-linked variant of the gel appeared to be more effective. Reduction of HBsAg and hepatitis B virus concentration by a factor of $10^4$ to $10^5$ was claimed with approximately 85% of the applied factor IX being recovered. The authors claimed that the results of tests for activated factors were unaffected by the procedure.

All of the foregoing procedures for removal of HBsAg and hepatitis virus from concentrates depend on properties which are probably attributable to all viruses. They are therefore attractive in that they might be generally applicable to viruses other than hepatitis B, and in particular to NANB virus. This, of course, remains to be established. It is also important to acknowledge a weakness in the way these methods have been tested. The fact that a technique will decrease a marker concentration by a factor of $10^4$ or $10^5$ from a high concentration added to the product deliberately, does not mean that the same process will work equally well at the much lower, but still infective starting level of virus in many concentrates. Nor does removal of marker guarantee elimination of infectivity.

Neutralize (hepatitis) virus by immunological methods :

There are a number of possible immunological approaches to neutralizing hepatitis virus in plasma or plasma products. The most obvious problems in this area are the number of serotypes associated with viral hepatitis, the difficulty of accumulating enough antibody conventionally from human plasma and balancing that, the risks of using antibodies from animal or monoclonal sources.

It is possible to envisage four possible immunological methods for neutralization of hepatitis virus:

i. ensure excess antibody in the starting pool.
ii. add excess antibody to the product.
iii. solid phase immunosorption.
iv. passive/active immunization.

The proportion of hepatitis antibody to undetected virus in plasma is obviously affected by blood screening and by donor selection. It is difficult to imagine that we would ever be in the happy situation of being able to select for fractionation only those pools containing demonstrable excess of antibody. If this were possible, questions would arise about the stability of the antibody-antigen complex during the fractionation procedure.

Tabor et al (1980) demonstrated that hepatitis B virus infectivity could be removed from prothrombin complex concentrate by the addition of hepatitis B immunoglobulin. Studies in chimpanzees were used to prove non-infectivity of the treated concentrate. The Dutch Red Cross add hepatitis B antibody to therapeutic preparations of C1 esterase inhibitor, and it is planned to extend this approach to other concentrates, in particular concentrates of factor IX. This approach would be quite attractive for first time, or once only, users of such concentrates, though in multiply transfused individuals one might consider the risk of reactions from conventional IgG antibodies administered intravenously.

Charm and Wong (1977) reported the use of immunoadsorption for the removal of hepatitis B surface antigen from plasma. However they observed rather low affinity of HBsAg for the anti-HBs Sepharose, and, as a result, contact times might be inconveniently high at the low temperature one would consider using, and given the minute but infective concentration of virus to be removed.

Probably the most important immunological approach will be the imunization of high risk individuals such as multi-transfused haemophiliacs. Currently, of course, the only immunoglobulins available for passive immunization are those for prophylaxis against the development of hepatitis A and hepatitis B. Also it remains to be seen whether such immunization would be effective when the patient is repeatedly challenged with large pool concentrate. The question of active immunization using a vaccine of the Merck, Sharp & Dohm type is a subject of current debate.

Inactivate (hepatitis) virus by physical/chemical methods :

Varying degrees of success have been claimed by proponents of two such methods. The ß-propiolactone/UV treatment of plasma was first introduced by LoGrippo (1967) and has since been adopted by Biotest. Plasma is treated with the virucidal agent ß-PL, and the virucidal effect enhanced by UV radiation. Excessive reagent is said to be rapidly hydrolyzed by plasma enzymes. Biotest claim good recovery of prothrombin complex factors on fractionation of plasma so treated, but do concede that factor VIII in plasma so treated is inactivated (Stephan et al, 1981). The two principle reasons why this method has not been adopted by other fractionators in Europe and in the United States are :

1. Treatment with ß-PL results in chemical modification of proteins. This reagent acylates and esterifies susceptible amino acids in proteins, and may result in the appearance of new antigenic determinants, or reduced effectiveness in vivo.

2. The reagent has been found to be carcinogenic and mutagenic when applied to experimental animals by a variety of routes.

The effectiveness of the procedure for comprehensive removal of viral infectivity remains to be satisfactorily demonstrated.

Pasteurization for 10 hours at 60°C, the conditions found experimentally to destroy infectivity of hepatitis B virus, results in the denaturation of most isolated plasma proteins. The most important exception to this is, of course, albumin in albumin products such as PPF, which is heated in its final container under the protection of fatty acids as part of the normal production process. Immunoglobulin and plasminogen may also be subjected to heat treatment under certain conditions, and antithrombin III can be recovered in good yield following heat treatment in the presence of a high concentration of citrate ions (Holleman et al, 1977). Workers from Behringwerke (Heimburger et al, 1981)have recently claimed that they can pasteurize factor VIII in the presence of sucrose and glycine, and that the product so treated does not transmit hepatitis. Unfortunately the yield of factor VIII activity claimed is only about 8%, hardly generally acceptable.

Conclusions :

In conclusion, it is possible to suggest guidelines as to the probable most fruitful approaches to eliminating viral infectivity for the different types of coagulation factor concentrates. In the case of factor VIII and fibrinogen, infrequent users would be protected by encouraging the use of small pool cryoprecipitate and possibly by the addition of antibody to the concentrate. As discussed earlier such an approach would be impossible for regular users of factor VIII concentrate and here one would have to rely on improvements in donor screening and securing the quality of the starting plasma by an extended quarantine period. Vaccination of suitable groups might also play an important part. BPL are pursuing methods for inactivation with the factor VIII protected by suitable non-toxic reagents.

For concentrates of factors of the prothrombin complex, and thrombin, the approach would be similar but the technique of PEG precipitation of virus becomes more important.

The Plasma Fractionation Laboratory at Oxford is currently developing factor XIII and antithrombin III concentrates and for these preparations heat treatment would be the obvious approach to reducing viral infectivity.

These are the possibilities then, but lets recap on the overlying problems. It is one thing to develop ideas for the possible treatment of plasma or concentrates that will inactivate or remove virus, in particular hepatitis virus. It is quite another to demonstrate the non-infectivity of a product so treated. Donations of plasma containing infected levels of hepatitis B virus still find their way into plasma pools, and we have not even begun to eliminate donations contributing NANB virus. With that sort of record at single donor level what chance have we of finding virus in a pool of thousands of plasma donations, or in the product?

Many studies performed in the past rely on the disappearance of a marker, upon application of a given treatment, to demonstrate removal of infectivity. Unfortunately only injection of the product into susceptible animals can prove non-infectivity, and this must surely be the most severe limitation on progress at the moment. If, as seems likely, the only convincingly susceptible animals prove to be those of an endangered species, then we may have no alternative but to fall back upon very difficult clinical trials for most of the proof we need.

INHIBITORS TO FACTORS VIII AND IX IN HAEMOHPILIACS

# IMMUNOLOGICAL BASIS FOR THE INDUCTION OF SPECIFIC TOLERANCE TO FACTOR VIII.

M.D.KAZATCHKINE. INSERM U 28, Hôpital Broussais, 75014 Paris France.

Induction of specific tolerance to Factor VIII would represent the most satisfactory but as yet theoretical treatment of hemophilic patients with Factor VIII inhibitors in that it would mimick natural tolerance to a self-antigen and prevent possible adverse effects of non-specific manipulation of the immune system (Table I). In an immunocompetent host, induced tolerance (specific unresponsiveness) can be defined as the acquired inability of the individual to express immunity towards a specific antigen to which it would otherwise respond (e.g. procoagulant Factor VIII in patients with antibodies to Factor VIII) although the capacity to respond to other antigens is preserved.

Table I. Therapeutic suppression of immune response to Factor VIII.

| | |
|---|---|
| I982 | Steroids<br>Cyclophosphamide<br>Plasmapheresis<br>Monoclonal antibodies to T cells and to T cell subsets |
| I9?? | Soluble suppressor factors<br>Anti-idiotypic immunization<br>Specific tolerance |

Antibodies to Factor VIII do not occur in patients who are not transfused. Thus Factor VIII inhibitors may be considered as analogous to alloantibodies towards antigenic determinants of (or close to) the procoagulant site of Factor VIII that are missing in severe hemophiliacs but are

present in the immunogenic Factor VIII concentrates and cryoprecipitates. It is assumed that these determinants to which induction of tolerance should be directed, are present on the Factor VIII procoagulant molecule which circulates in plasma and results in natural tolerance in normal individuals.

Natural tolerance to autologous soluble proteins is considered as resulting from an active T cell-dependent process leading to suppression and establishment of an equilibrium between effector cells, suppressors and self-antigens in the immune network, rather than being dependent on deletion of anti-self reactive clones. Thus, the inability of normal (C5-sufficient) mice to respond to the Complement protein C5 was shown to be dependent on a specific T cell defect of these animals from which T cells, in contrast to T cells from C5-deficient mice, were unable to cooperate with B lymphocytes to produce anti-C5 (1). B cells from tolerant (C5-sufficient) and non tolerant (C5-deficient) animals retained the capacity to produce autoantibody. In this and in other models, induction and maintenance of tolerance were clearly dependent on the presence of the antigen. Therefore any attempt to induce tolerance to Factor VIII would require prolonged contact with the antigenic determinants of the procoagulant Factor VIII molecule against which the inhibitor is directed, but under conditions which lead to specific tolerance rather than antibody formation.

Several mechanisms by which specific unresponsiveness can be experimentally achieved in adult animals are listed in Table II.

Table II. Acquired unresponsiveness

| |
|---|
| "Peripheral" inhibition of the immune response |
| Generation of T suppressor cells |
| Antibody or immune-complex mediated suppression |
| Anti-idiotypic suppression |
| Antigen blockade of B lymphocytes |
| "Central" unresponsiveness |
| Immunosuppressive drugs |
| Low doses or high doses of soluble antigen |
| Non-immunogenic (e.g. non-polymerized) forms of the antigen |

"Peripheral" inhibition results in blocking of the capacity of immunocompetent cells to produce antibody by interfering with the physiological regulatory mechanisms of the immune response (2). In contrast, "central unresponsiveness" (2) is characterized by a tolerant state probably identical to tolerance to self where immunocompetent cells of the host are incapable of reacting with the tolerogen and where lymphocytes transferred to a neutral host remain tolerant. These two types of acquired unresponsiveness will be briefly discussed below.

One of the regulatory mechanisms of the immune response is generation of T suppressor cells specific for the inducing antigen and for immunoglobulin idiotypes. Suppressor cells have been associated with tolerance to thymus-dependent and thymus-independent antigens, both with peripheral inhibition of the immune response and with central unresponsiveness to antigen (3). However tolerance to protein antigens has also been established in the absence of detectable suppressor cells. Two examples are : increased activity of "contra-suppressive" circuits (4) and unresponsiveness induced in the absence of T cells such as unresponsiveness to human gammaglobulin produced in nude mice (5).

Another regulatory mechanism of the immune response is antibody itself which may facilitate or depress the immune response in vivo and in vitro by interfering with T-B cell interactions through Fc-dependent and independent mechanisms. Thus, addition of anti-POL (i.e. polymerized flagellin) antibody to spleen cultures that have been immunized in vitro with POL or with an irrelevant antigen, was shown to selectively inhibit the anti-POL response (6). Complexed antibody also provides both positive and negative signals that modulate the immune response (7); suppressive effects may result from interactions between immune complexes and receptors for antigen or Fc fragments on B cells, from activation of T suppressor circuits or from blockade of antigen receptors on T cells and of T-B cell interactions. Immune complexes may form between procoagulant Factor VIII and human antibodies to Factor VIII from multitransfused hemophiliacs (8) and circulating immune complexes, some of which have VIII-anti-VIII or IgG-Rheumatoid Factoe specificity, are found in the serum of hemophilic patients with inhibitors (9).

Generation of auto-antiidiotypic antibodies following exposure to antigen (10) provides another regulatory circuit of the immune response with potential therapeutic applications. Tolerance to foreign protein antigens in adult animals and to transplantation antigens has been induced with anti-idiotypic

immunization (11). However, although some plasmas from patients with Factor VIII inhibitors completely inhibit the binding of the indicator antibody in an immunoradiometric assay of VIII CAg (12), it is not yet known how heterogeneous are anti-Factor VIII antibodies from hemophilic patients with regard to idiotypic specificity.

B cells see the antigen by means of immunoglobulin and can be made tolerant by reversible blockade of antigen receptor. For example, B cell tolerance to hapten can be experimentally produced with haptens that had been conjugated to non-immunogenic or poorly immunogenic carriers for T cells such as some synthetic amino acid polymers (D-GL) or the pneumococcal polysaccharide S III. These models may be relevant to the Factor VIII situation in that pneumococcal polysaccharidic antigens or small amino acid polymers elicit in animals an immune response of restricted heterogeneity that is analogous to the restricted human response to procoagulant Factor VIII. Among the poorly immunogenic carriers, autologous IgG (13) has proven to be a powerful tolerogenic carrier in several experimental models of acquired tolerance to haptens or even to complete proteins such as horseradish peroxidase ; critical are : the mode of linkage and the molar ratio of hapten to IgG and the chemical integrity of the IgG molecule. Hapten bound to IgG may induce both B and T cell tolerance and thus may represent a model of peripheral inhibition as well as of central unresponsiveness.

Experimental induction of central unresponsiveness to soluble proteins in adults is dependent on a number of parameters such as the physico-chemical state and the dose of the antigen, the inherent immunogenicity of the antigen (i.e. a weak immunogen more readily induces tolerance) and the route of administration (preferentially i.v. for tolerance induction).

Immunosuppressive agents have been disappointing in the treatment of patients with Factor VIII inhibitors. One of the reasons may be that in animal models, specific tolerance could only be established with immunosuppressive drugs such as cyclophosphamide in defined experimental conditions where, for example, cyclophosphamide should be administered in a narrow time span of 48 hours before or after or at the time that the antigen is injected (14).

The use of low doses of antigen below the treshold of immunogenicity or of high doses far above the immunogenic dose has been known for many years to favor induction of tolerance to soluble protein antigens (15). Little evidence is available

as yet on decreased inhibitor response to Factor VIII in patients receiving biweekly infusions of "low doses" of Factor VIII; however decreased inhibitor titers, suppression of inhibitor and of the anamnestic response to Factor VIII have been shown in patients that had received daily infusions of large amounts of Factor VIII concentrates for several months (16). These results are suggestive of an acquired unresponsiveness induced with high doses of antigen although a restricted specificity to Factor VIII of the "tolerant state" has not been demonstrated in these patients. Other suppressive mechanisms may also be involved such as feed-back inhibition of the immune response by high amounts of IgG contaminating the Factor VIII preparations and non-specific suppression from other contaminating competing antigens (17).

Another model of induced tolerance to proteins in adult animals that may be relevant to Factor VIII, is tolerance induced by aggregate-free gammaglobulin. Thus, ultracentrifuged bovine gammaglobulin (BGG) or human gammaglobulin (18) (HGG) induce specific unresponsiveness to BGG and HGG when injected into adult mice. Tolerance to deaggregated proteins is associated with stimulation of T suppressor circuits and may involve lack of stimulation of T helper cells because of by-pass of the appropriate macrophage presentation of antigen to a helper/inducer cell. Obviou: the tight link of procoagulant Factor VIII with the high molecular weight von Willebrand Factor contributes to the immunogenicity of infused Factor VIII, whereas it may be speculated that a von Willebrand Factor and aggregate-free small procoagulant moiety could be a good tolerogen since it is a relatively poor immunogen that elicits immune responses of restricted heterogeneity.

A major difficulty that one would encounter in attempting to induce specific unresponsiveness to Factor VIII is that acquired tolerant states only exist for a finite time and that maintenance of tolerance is necessarily associated with the presence or the repeated exposure of the antigen in it's tolerogenic form. Recovery from T-dependent ("central") tolerance may also occur following contact of the tolerant host with the tolerogenic hapten coupled to an unrelated carrier, with an altered tolerogenic protein, or with cross reactive proteins. Heterogeneity between Factor VIII preparations and the presence in the infused material of contaminating proteins would thus limit efforts to induce specific tolerance with most of the protocols that have just been reviewed.

References

1. HARRIS D.E., L. CAIRNS, F. ROSEN and Y. BOREL. 1982. A natural model of immunologic tolerance. Tolerance to murine C5 is mediated by T cells and antigen is required to maintain unresponsiveness. J.Exp.Med. 156: 567

2. WEIGLE O. 1980. Analysis of autoimmunity through experimental models of thyroiditis and allergic encephalomyelitis. Adv.Immunol. 31: 159

3. KATZ D.H. and B. BENACERRAF. 1974. Immunological tolerance : mechanisms and potential therapeutic applications. Academic Press, New York

4. ETLINGER HM. and J.M. CHILLER. 1977. Induction of tolerance in athymic mice with an antigen which is highly immunogenic in euthymic mice. Cell.Immunol. 33: 297

5. GREEN D.R. and R.K. GERSHON. 1982. Hyperimmunity and the decision to be intolerant. Ann.N.Y.Acad.Sci. 392: 318

6. DIENER E. and M. FELDMANN. 1970. Antibody-mediated suppression of the immune response in vitro. II. A new approach to the phenomenon of immunological tolerance. J.Exp.Med. 131: 31

7. THEOPHILOPOULOS A.N and F.J. DIXON. 1979. The biology and detection of immune complexes. Adv.Immunol. 28: 89

8. LAZARCHICK J. and L.W. HOYER. 1977. The properties of immune complexes formed by human antibodies to Factor VIII. J.Clin.Invest. 60: 1070

9. KAZATCHKINE M.D., Y. SULTAN, E.J. BURTON-KEE and J.F. MOWBRAY. 1980. Circulating immune complexes containing anti-VIII antibodies in multitransfused patients with hemophilia A. Clin.Exp.Immunol. 39: 315

10. SCHRATER A.F., E.A. GOIDL, G.J. THORBECKE and G.W. SISKIND. 1979 Production of auto anti-idiotypic antibody during the normal immune response to TNP-Ficoll. I. Occurence in AKR/J and BALB/c mice of hapten-augmentable, anti-TNP plaque forming cells and their accelerated appearance in recipients of immune spleen cells. J.Exp.Med. 150: 138

11. JANEWAY C.A. 1980. Manipulation of the immune response by anti-idiotype. in "Immunology 80" edited by J.DAUSSET and M.FOUGEREAU, Academic Press, New York: 1148

12. GAWRYL M.S. and L.W. HOYER. 1982. Immunological studies of antihemophilic Factor (AHF, VIII C). IV. Characterization of antigenic determinants using human antibodies. Clin.Immunol.Immunopathol. 23: 521

13. BOREL Y., D.T. GOLAN, L. KILHAM and H. BOREL. 1976. Carrier determined tolerance with various subclasses of murine myeloma IgG. J.Immunol. 116: 854

14. AISENBERG A.C. 1967. Studies on cyclophosphamide-induced tolerance to sheep erythrocytes. J.Exp.Med. 125: 833

15. MITCHISON N.A. 1964. Induction of immunological paralysis in two zones of dosage. Proc.Royal Soc.Biol. 161: 275

16. MARIANI G., M.A. RUSSO and F. MENDELLI. 1982. Activated prothrombin complex concentrates. Managing hemophilia with Factor VIII inhibitor. Praeger pub., New York

17. RADOVITCH J. and D.W. TALMADGE. 1967. Antigenic competition : cellular or humoral. Science. 158: 512

18. PARKS D.E. and W.O. WEIGLE. 1979. Regulation of B cell unresponsiveness by suppressor cells. Immunological Rev. 43: 217

## THE EFFECT OF FACTOR VIII REPLACEMENT ON THE LEVEL OF FACTOR VIII ANTIBODIES IN HAEMOPHILIA

C.R. Rizza, Oxford Haemophilia Centre, Churchill Hospital, Oxford.

Antibodies to factor VIII remain one of the most important complications of haemophilia. They arise in approximately 6% of our patients(12% of severely affected patients) and are thought to appear as a response to factor VIII replacement therapy. During the past 13 years during which the U.K. Haemophilia Directors have been collecting data on haemophilia and its management, there seems to have been no increase in the incidence of antibodies despite the fact that increasing amounts and different kinds of factor VIII have been transfused into haemophiliacs during that period. In almost all cases studied the antibodies have been IgG immunoglobulins of restricted heterogeneity. Most have been found to consist of kappa light chains and G4 heavy chains. Mechanism of the inactivation of factor VIII by these antibodies is still not fully understood. The reaction, however, is known to be time dependent and temperature dependent and does not result in a visible immuno precipitate.

When factor VIII is transfused to patients who have factor VIII antibodies there is usually a rise in the level of antibody which commences 5-7 days after the injection reaching a maximum during the following week. This anamnestic response in the patients varies from patient to patient and often in the same patient depending on his recent transfusion history. If further factor VIII is not given the level of antibody after reaching its peak falls progressively and after many months may, in some patients, fall to zero but in others persist at a low but detectable level.

There are now several different approaches to the management of haemophiliacs with antibody (Table I). The fact that so many forms of treatment have been tried and are still being tried suggests that no single form of treatment has been found to be uniformly successful. Certainly none of the methods is as successful in controlling haemorrhage in patients who have antibody as is factor VIII in patients with antibody.

In Oxford our experience during the past 10 years has been mainly with the use of factor VIII. On a very few occasions Porcine factor VIII has been used and on one occasion a seriously ill patient was treated by plasmapheresis. We have not used prothrombin complex concentrates nor have we used immunosuppressive drugs in haemophiliacs. Before 1972 our policy was to withhold factor VIII except for severe and life endangering haemorrhages but since then we have used factor VIII for the treatment of all but the most minor of bleeds in patients with antibodies. The amount of factor VIII given depends to some extent on the level of antibody in the patient's plasma at the time of treatment and on the severity of the haemorrhage being treated. As a consequence of this change of treatment many patients who have antibodies who had been infrequently transfused now receive frequent injections of factor VIII. This change in treatment, in addition to producing a satisfactory clinical effect in many patients, especially those with lower levels of antibody, has also been accompanied by a change in the pattern of antibody responses.

Patients studied: 28 severely affected haemophiliacs with antibodies to factor VIII attend the Oxford Haemophilia Centre; 24 of those patients are regular attenders about whom we have collected over the years detailed information about antibody levels, patterns of antibody response and types and amounts of factor VIII administered to control haemorrhage. Table 2 shows some details of the patients we have studied.

Dosage regime: All of the patients, apart from 2, were receiving on demand treatment for episodes of bleeding and there was, therefore, no regular pattern of dosage. In the early 1970s most patients were receiving very infrequent factor replacement but in recent years many patients have received frequent factor VIII replacement amounting to at least one dose a month and sometimes several doses each month. The types of material used has included cryoprecipitate and freeze-dried

factor VIII concentrates prepared from human plasma.

Methods

The factor VIII antibody was measured by the method of Rizza & Biggs (1973) and was carried out by incubating dilutions of antibody containing plasma with factor VIII at 37°C for 4 hours. The residual factor VIII is then assayed. One unit of antibody is defined as the amount which destroys 0.5 units of factor VIII in 4 hours at 37°C.

Results.

If one looks at the patients from the point of view of antibody responses over the years they can be grouped broadly and arbitrarily into 3 groups: in group I consisting of 7 patients (patient 1-7, Table 2) there has been a progressive fall in the level of factor VIII antibody until it is now no longer detectable. The maximum antibody level assayed by us in those patients ranged from 4 to 69 units. The pattern of antibody response in 2 of those patients is shown in figs. 1 and 2. Since the disappearance of antibodies from their blood 3 patients (1, 3 and 7) in this group have undergone dental extractions or corrective orthopaedic surgery. All 3 patients showed a normal factor VIII response after transfusion. With regard to ½-life of transfused factor VIII, in one patient the half-life was 10-12 hours and in the other 2 patients it was found to be 6-8 hours. All 7 patients in this group are now on home therapy. The next group (group II) consists of 6 patients (patients 8-13, Table 2) who have all shown a gradual downward trend in the level of antibody since beginning to receive more frequent doses of factor VIII. The maximum observed levels of antibody in this group ranged from 29 to 485 units. It is notable that although some of the patients still show some anamnestic response this has become less in recent years. Two examples of patients in this group are shown in figs. 3 and 4. All of the patients in this group are now on home therapy.

The final group of patients (group III) consists of the remaining 11

(patients 14-24, Table 2) who had maximum antibody levels ranging from 22 to 7000 units and who, apart from two, have shown no decrease in antibody titre over the years. The majority of patients in this group have been relatively infrequently transfused when compared with patients in the previous two groups. This was particularly so with patients 14, 15, 17, 18, 20 and 21 in whom there was an anamnestic response following most transfusion of factor VIII. Patients 19, 22, 23 and 24 all received frequent factor VIII replacement but showed no reduction in the titre of their antibody. The pattern of antibody response in two of those patients is shown in figs. 5 and 6. Two patients included in this group, namely patients 14 and 17, are of particular interest and are now discussed in more detail. Patient 14 had until 1980 received very few transfusions of factor VIII and on the rare occasions when he did receive factor VIII his level of antibody rose to more than 100 units and on one occasion to more than 2000 units. In February 1980 he began to receive regular factor VIII replacement amounting to 1500-3000 units weekly, depending on his needs. After an initial sharp increase in the level of antibody, the level fell progressively until October, 1980 by which time it was approximately 50 units/ml. At this point the patient decided to return to a more conservative form of therapy and did not receive factor VIII replacement for 18 months. During that 18 months period without treatment his antibody remained in the region of 20-50 units. In August, 1982 he sustained an injury to his right knee which resulted in a severe, painful haemarthrosis for which he required intensive factor VIII replacement following which the level of his antibody rose to 2000 units (Fig. 7). Patient 17 had received very few transfusions in the past and when challenged with factor VIII there was usually an increase in the antibody level rising sometimes to more than 1000 units. At the end of 1981 he requested regular factor VIII replacement for joint and muscle

bleeds. Since then he has been receiving on average 1500 units of factor VIII weekly. There was an initial rise in his level of factor VIII antibody to more than 300 units but since then the level of factor VIII antibody in his plasma has fallen progressively and the last estimate in September, 1982 showed it to be 50 units/ml. (Fig. 8).

Amount of factor used: The total amount of factor VIII used during the past 3 years to treat each of the 24 patients discussed here is shown in table 3 along with the average amount of factor VIII used per annum per patient. On average the patients with antibodies received approximately twice as much factor VIII per annum as haemophiliacs without antibodies who attend the Oxford Centre. When broken down by groups, as described above, the usage per patient per annum in group I, II and III was 43,324 units, 57,970 units and 32,270 units respectively.

Summary and conclusion: Regular administration of relatively small doses of factor VIII to haemophiliacs with antibodies to factor VIII has been accompanied by a reduction in antibody titre to undetectable levels in seven patients and to very low levels in a further 6 patients. In nine of the remaining eleven patients, some of whom were receiving frequent doses of factor VIII, there was no fall in the level of antibody during the period of the study. In the remaining two patients a fall in antibody titre was seen during the administration of 1500-3000 units of factor VIII weekly. Brackmann and his colleagues (1977) have obtained similar results but using very much larger doses of factor VIII in conjunction with an activated prothrombin complex concentrate. The dosage used by us is more akin to that used by other workers (Sultan 1981, Stenbjerg & Jorgensen 1981) who have found a reduction in antibody level during prolonged administration of relatively low doses of factor VIII.

The reason for the fall in antibody levels in our patients is

not clear but is possibly due to some form of induced tolerance to factor VIII. Nor do we know the optimum conditions with regard to amount and frequency of dosage required to bring about a fall in antibody titre.

REFERENCES

1. BRACKMANN, H.H. & GORMSEN, J. (1977) Massive factor VIII infusion in a haemophiliac with factor VIII inhibitor, high responder. Lancet, ii, 933.

2. RIZZA, C.R. & BIGGS, R. (1973) The treatment of patients who have factor VIII antibodies. British Journal of Haematology, 24, 65-82.

3. STENBJERG, S. & JORGENSEN, J. (1981) Immunotolerance by long term factor VIII administration. In: Abstracts of International Medicine on Activated Prothrombin Complex Concentrates: The State of the Art of Managing Hemophilia with factor VIII Inhibitor, Rome.

4. SULTAN, Y. (1981) Immunotolerance by long term factor VIII administration. In: Abstracts of International Meeting on Activated Prothrombin Complex Concentrates: The State of the Art of Managing Hemophilia with factor VIII Inhibitor. Rome.

---

**TABLE I**

**Treatment of haemophiliacs with factor VIII antibodies**

1. **Factor VIII - Human or Porcine**
2. **Prothrombin complex concentrate - activated or non-activated**
3. **Plasmapheresis**
4. **Immunosuppressive therapy**
5. **Various combinations of two or more of the above forms of treatment**
6. **Antifibrinolytic drugs**

TABLE 2

| Patient | Year of birth | Age (yrs.) when antibody first detected. | Highest antibody level every recorded. (units/ml). |
|---|---|---|---|
| GRO-A | 1958 | 7 | 5 |
| | 1944 | 27 | 7 |
| | 1958 | 7 | 23 |
| | 1970 | 1 | 40 |
| | 1959 | 15 | 4 |
| | 1965 | 9 | 8 |
| | 1958 | 8 | 69 |
| | 1965 | 8 | 48 |
| | 1959 | 12 | 74 |
| | 1958 | 16 | 33 |
| | 1942 | 26 | 29 |
| | 1969 | 2 | 117 |
| | 1954 | 13 | 485 |
| | 1944 | 24 | 2560 |
| | 1914 | 53 | 350 |
| | 1961 | 9 | 22 |
| | 1935 | 33 | 1850 |
| | 1943 | 21 | 1600 |
| | 1936 | 32 | 35 |
| | 1932 | 35 | 7000 |
| | 1964 | 4 | 385 |
| | 1945 | 16 | 70 |
| | 1977 | 1 | 50 |
| | 1965 | 5 | 740 |

TABLE 3

| Patient | Total units during 3 years | Average units/year | Units/kg body weight/year |
|---|---|---|---|
| 1 | 167593 | 55864 | 830 |
| 2 | 53357 | 17785 | 237 |
| 3 | 227188 | 75729 | 850 |
| 4 | 64585 | 21528 | 861 |
| 5 | 27085 | 9028 | 129 |
| 6 | 247479 | 82493 | 1528 |
| 7 | 122521 | 40840 | 756 |
| 8 | 305546 | 101848 | 3395 |
| 9 | 181190 | 60397 | 863 |
| 10 | 98620 | 32873 | 548 |
| 11 | 97295 | 32432 | 416 |
| 12 | 307421 | 102474 | 2928 |
| 13 | 53386 | 17795 | 323 |
| 14 | 122560 | 40853 | 851 |
| 15 | 5425 | 1808 | 28 |
| 16 | 26765 | 8922 | 127 |
| 17 | 56617 | 18872 | 236 |
| 18 | 33182 | 11061 | 158 |
| 19 | 159150 | 53050 | 680 |
| 20 | 153328 | 51109 | 786 |
| 21 | 72836 | 24279 | 639 |
| 22 | 274249 | 91416 | 1904 |
| 23 | 40531 | 13510 | 900 |
| 24 | 120313 | 40104 | 1146 |

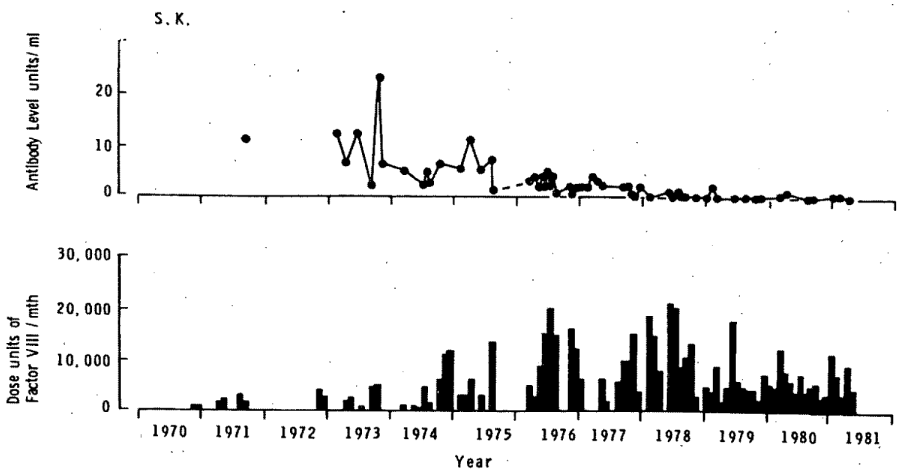
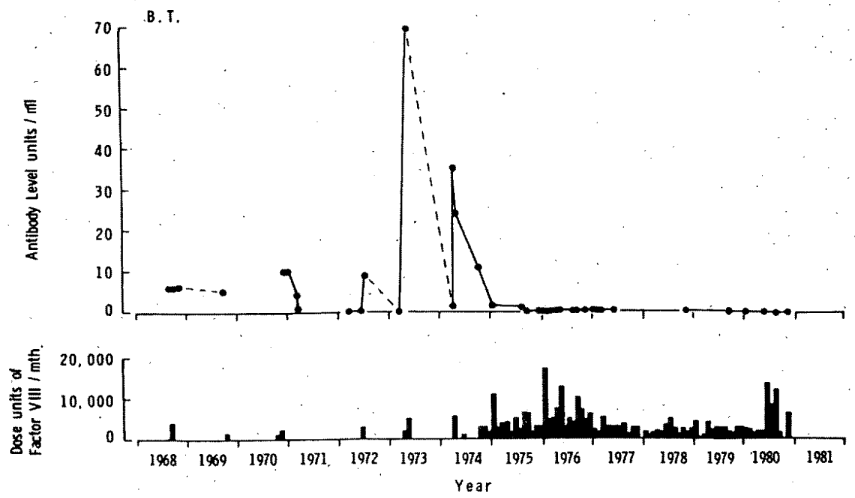



Figure 1

Amount of factor VIII administered (units/month) and level of factor VIII antibody.

Figure 2



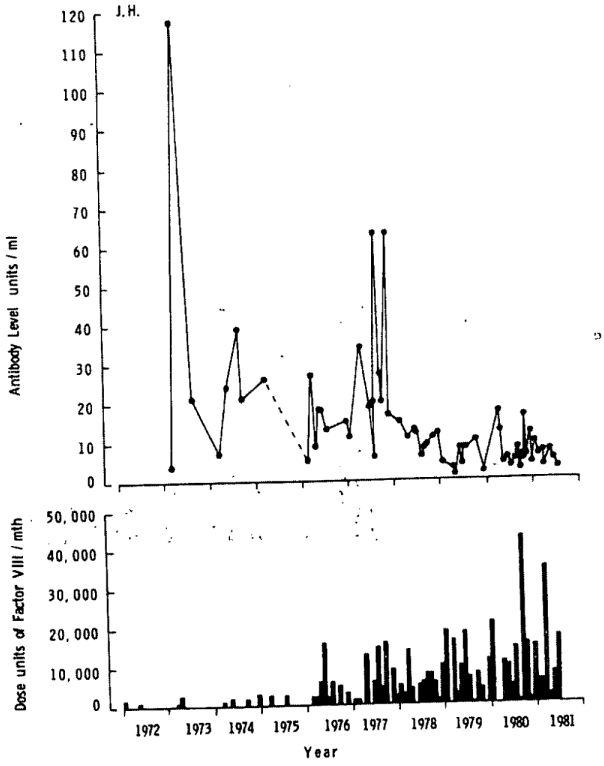
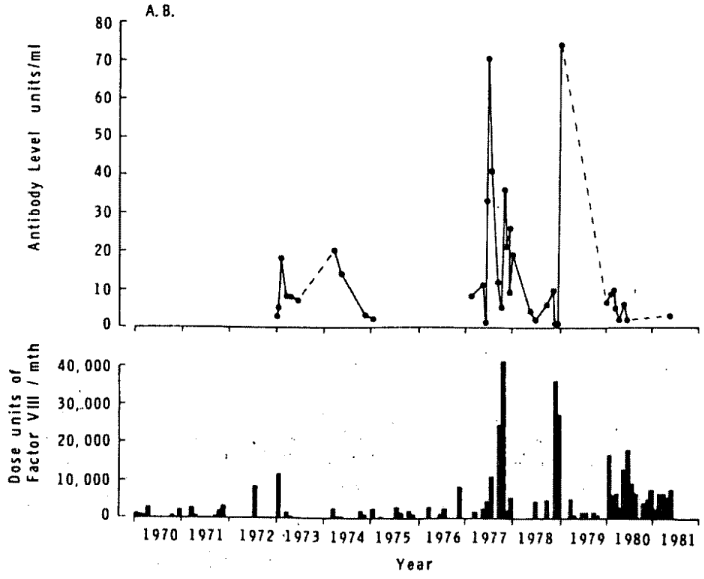



**Figure 3**

**Amount of factor VIII administered (units/month) and level of factor VIII antibody**

**Figure 4**



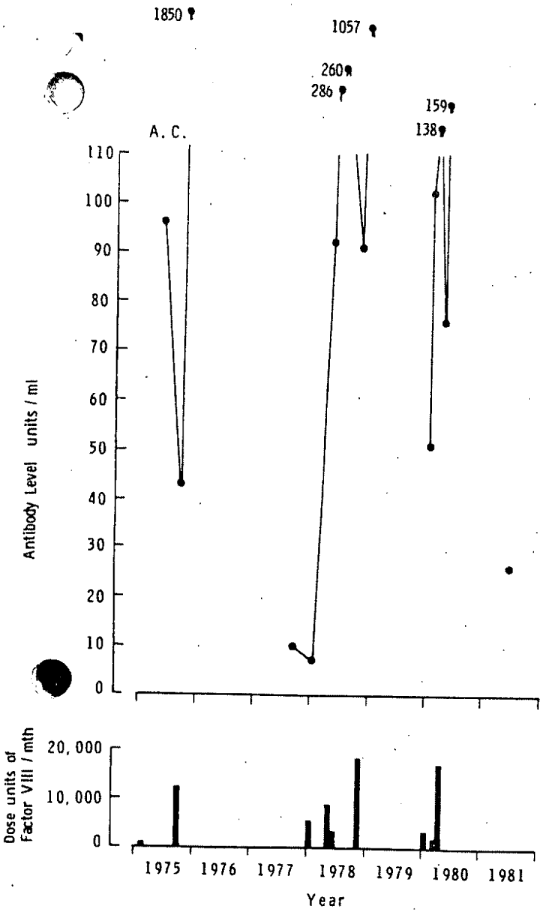



Figure 5.

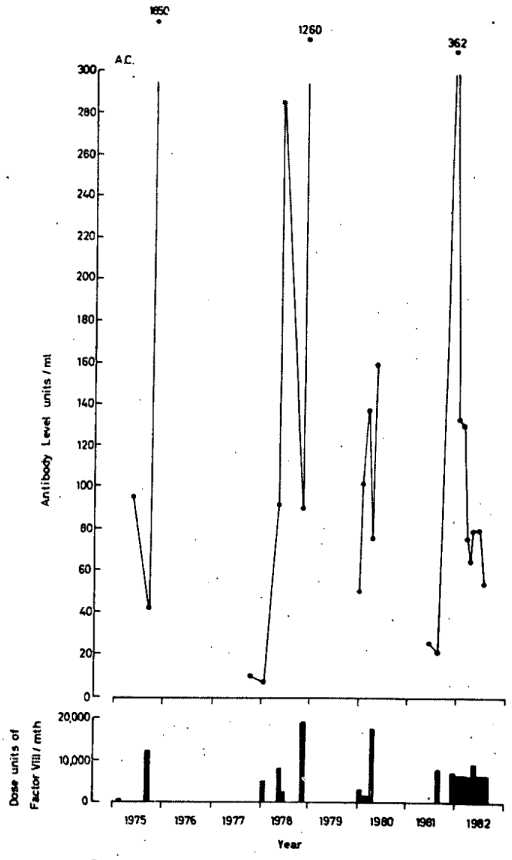



Figure 8.

Amount of factor VIII administered (units/month) and level of factor VIII antibody

Figure 6.

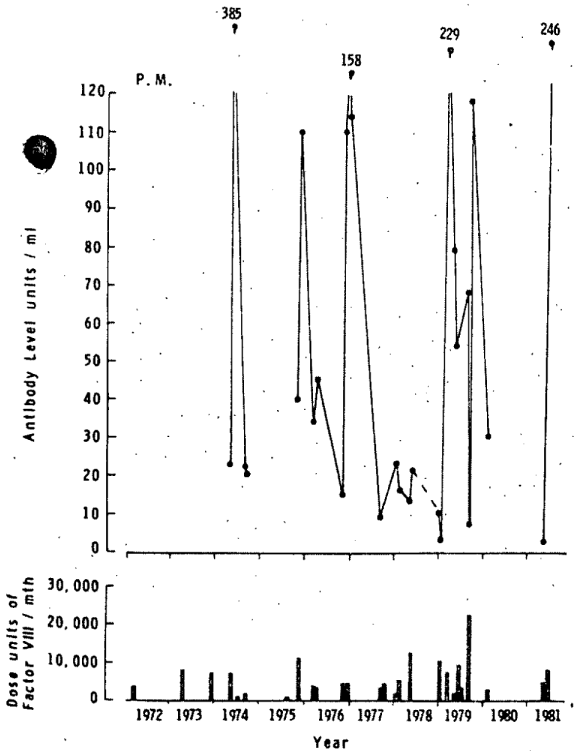
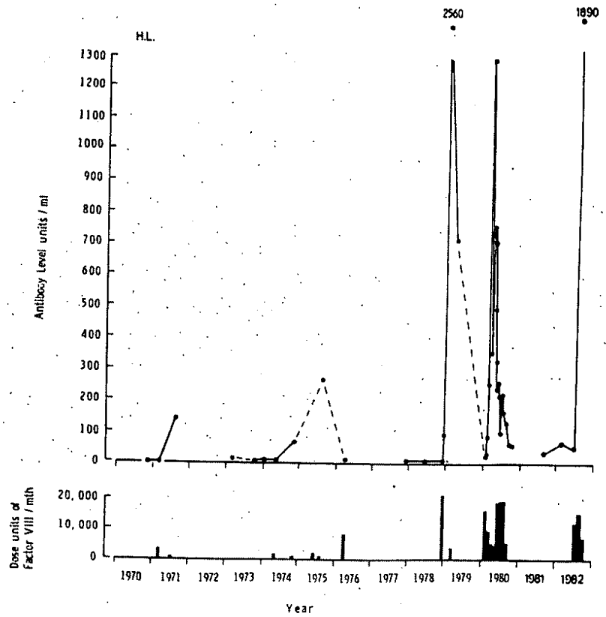



Figure 7.



# MANAGEMENT OF PATIENTS WITH INHIBITORS AT THE ROYAL FREE HOSPITAL

P B A KERNOFF

During the period 1978-82, there have been 20 haemophiliacs with inhibitors under our regular care. One of these patients has died from uncontrollable haemorrhage. In the same period, we have seen seven new patients with acquired haemophilia. Most of these have been elderly, and in none could any specific underlying disease be identified. Four patients have died, and in none was death directly attributable to bleeding. One patient has undergone a spontaneous remission.

Reported bleeding episodes in patients with inhibitors are almost invariably treated with blood products, the choice of material and dosage depending upon previously observed clinical responses and consideration of the patients anti-VIII level and its response to therapy. The three products in routine use are human factor VIII concentrate, polyelectrolyte-fractionated porcine factor VIII concentrate (PE porcine VIII, Hyate:C, Speywood), and Feiba (Immuno). Plasma exchange by discontinuous or continuous flow has occasionally been used in serious bleeding episodes where an inadequate therapeutic response has been obtained to standard regimes, but logistic constraints and difficulties with venous access prevent its routine application. Conservative measures alone are rarely employed although some patients are known not to report mild bleeding episodes, relying on rest and analgesia at home. Immunosuppressive drugs and steroids have been used in attempts to suppress anti-VIII production in patients with acquired haemophilia, but are not currently given to patients with the congenital disease. Autoplex (Travenol) has not yet been tried, both for financial reasons and because it is considered that there is a lack of evidence of advantage over Feiba. Factor IX concentrate is currently being assessed in patients with high level inhibitors, but we have at present little experience of its use. Treatment with platelet concentrates in the same group of patients is under consideration.

For management purposes, three broad groups of patients with the congenital disease are recognised. These have low, intermediate and high level inhibitors.

The six patients with low level inhibitors (usually less than 10 Bethesda units) rarely or never show classical anamnestic rises in anti-VIII, and are routinely treated with human VIII concentrate in conventional or moderately increased dosage. Five of these patients usually treat themselves at home, and have been on long or short term prophylaxis. Clinical responses are similar to those seen in patients without inhibitors and are in accord with measured post-infusion plasma levels of factor VIII. There is evidence of a

general decline in anti-VIII levels over the period that regular treatment with human VIII has been given, and in some patients anti-VIII is no longer detectable by an in vitro assay.

The seven patients with high level inhibitors (usually greater than 50 Bethesda units) generally show marked anamnestic responses, and infrequently respond favourably to either human or porcine VIII. Treatment is usually with Feiba, in doses of about 100 u/kg. Objective evidence of clinical improvement is less common than in patients with lower level inhibitors treated with human or porcine VIII, the overall success rate being about 50%. Where treatment with Feiba fails, and the severity of the bleeding episodes warrants further intervention, human or porcine VIII or human IX concentrate have been tried, sometimes combined with plasma exchange.

The seven patients with intermediate level inhibitors (usually between 10 and 50 Bethesda units) show generally poor clinical responses to human VIII, although high dosage therapy (100-200 u/kg) can be of clear benefit when the anti-VIII level is relatively low. These patients show variable anamnestic responses, which in some cases appear dose-dependent. Since mid-1980, most bleeding episodes have been treated with PE porcine VIII usually in doses of 20-50 u/kg. Over this two year period, 9 patients have received 62 courses of treatment requiring a total of 405 infusions. One of these patients had acquired haemophilia. Reasons for treatment have been very varied, including life-threatening bleeding episodes, surgery, and joint and muscle haemorrhages. Duration of courses of therapy has ranged from 1-27 days, and number of infusions per course from 1-59.

The rationale of treatment with porcine VIII is that anti-VIII activity in patients with inhibitors is almost invariably weaker against porcine than human factor VIII. This is particularly so in patients with acquired haemophilia. At similar dosage, therefore, porcine VIII has the potential to produce a greater rise in the plasma factor VIII level than human VIII. In our experience this potential is realised in practice. Porcine VIII can frequently cause a rise in the plasma factor VIII level when human VIII has either failed to produce a rise or would be unlikely to do so. As would be expected, the magnitude of the post-infusion rise in plasma factor VIII is inversely related to the pre-infusion anti-PE porcine VIII level. The occurrence of any detectable post-infusion level of factor VIII is a good predictor of a favourable clinical outcome, which is often comparable to that which would be expected in a patient without inhibitors receiving treatment with human VIII. Above 13 old Oxford units of anti-PE porcine VIII and 50 Bethesda units of anti-human VIII, no post-infusion rises were detected and clinical responses were generally poor.

As with any therapeutic agent, best results with PE porcine VIII are obtained after careful patient selection and an appreciation of potential hazards. While PE porcine VIII is clearly a much improved version of the conventional product, it still has disadvantages which in our view should limit its use to major haemophilia centres, where adequate facilities and expertise are available for stringent monitoring. The main problem is the relatively high incidence of infusion reactions (10%) which, although rarely severe enough to justify stopping treatment, may occasionally be alarming. A post-infusion fall in the platelet count is most unusual and, unlike conventional porcine VIII, many patients may be treated with multiple course of PE-fractionated material without evidence of a deteriorating response or an increasing tendency to reactions. If given in sufficient dosage to a susceptible patient, PE porcine VIII has the capability to provoke an anamnestic rise in the anti-VIII level, although its overall immunogenicity appears less than human factor VIII. In the longer term, repeated courses of PE porcine VIII may cause narrowing of the difference between levels of anti-human and anti-porcine inhibitor activity.

Use of PE porcine VIII to treat patients with intermediate-level inhibitors has increased the proportion of patients we are able to treat effectively and predictably from one-third to two-thirds, and we therefore regard the introduction of this material as a significant therapeutic advance. We have still, however, no satisfactory solution to the problem of management of patients with very high level inhibitors.

# THE MANAGEMENT OF HAEMOPHILIA A PATIENTS WITH FACTOR VIII INHIBITORS AT MANCHESTER ROYAL INFIRMARY

R.T.Wensley

Universiry Department of Clinical Haematology, Manchester Royal Infirmary

It is our policy to treat bleeding episodes in haemophilia A patients who have past or present evidence of factor VIII inhibitors with human factor VIII in appropriate doses.

Knowledge of the peak level of the inhibitor following previous exposure to factor VIII therapy enables the prediction of the maximum level of inhibitor 'anamnestic' response to be expected. Our experience shows broadly that high inhibitor level responders[1] remain high responders and similarly for low level responders. Following an anamnestic antibody response there is a gradual decay in inhibitor level, but a high level responder will remain with high levels of inhibitor for longer and is, therefore, more difficult to treat successfully if he should bleed during this phase of his anamnestic response.

At our centre, two modes of therapy have been devised to enable us to more successfully manage inhibitor patients who are high level responders. These are plasma exchange[2] and induction of tolerance to factor VIII. Each will now be described.

## Plasma Exchange

Using a cell separator, providing good venous access can be acquired, it is possible to exchange up to 6 litres of circulating blood plasma with a suitable replacement medium such as plasma protein fraction, 5% albumin solution or citrated human plasma. Approximately 40 % of IgG is contained within the intra-vascular space. A one-plasma-volume (calculated from the patients weight) plasma exchange will remove 50% of the intra-vascular inhibitor and 20% of the inhibitor within the body at a given time. For four to five days after commencing factor VIII therapy there is no enhanced synthesis of inhibitor, but after this latent period massive antibody synthesis will outpace any removal by plasma-exchange.

A major bleeding episode in a haemophilia A patient with inhibitors must be controlled as soon as possible, and certainly within five days. We combine high-level factor VIII therapy (to effect antibody neutralisation) with daily plasma-exchanges (to remove antibody and immune complexes, although

we have no evidence that the latter are harmful in this situation) to eliminate all the antibody to give a temporary period of normal haemostasis lasting up to five days. This 'haemostatic interval' should allow the majority of bleeds to be controlled and the healing process to commence.

Induction of immune tolerance to factor VIII

Following unsuccessful experience with repeated sub-cutaneous injections of factor VIII in an attempt to 'desensitise' haemophilia A inhibitor patients to factor VIII, we have been able to devise a simple programme for inducing immune tolerance to factor VIII using daily injections of factor VIII. The details will be published elsewhere, but following the successful treatment of bleeding episodes as described in the previous section, factor VIII was continued daily for prolonged periods. Eight patients have been followed and all developed anamnestic responses. Six of these suddenly ceased the increase in their plasma levels of antibody and the subsequent fall of plasma antibody level showed a steady fall with a $t_{\frac{1}{2}}$ averaging 18 days. This is similar to the $t_{\frac{1}{2}}$ rate of plasma decay of IgG.[3] We believe that antibody synthesis completely ceased in these patients. The inhibitor response to factor VIII of the first patient treated is shown in figure 1.

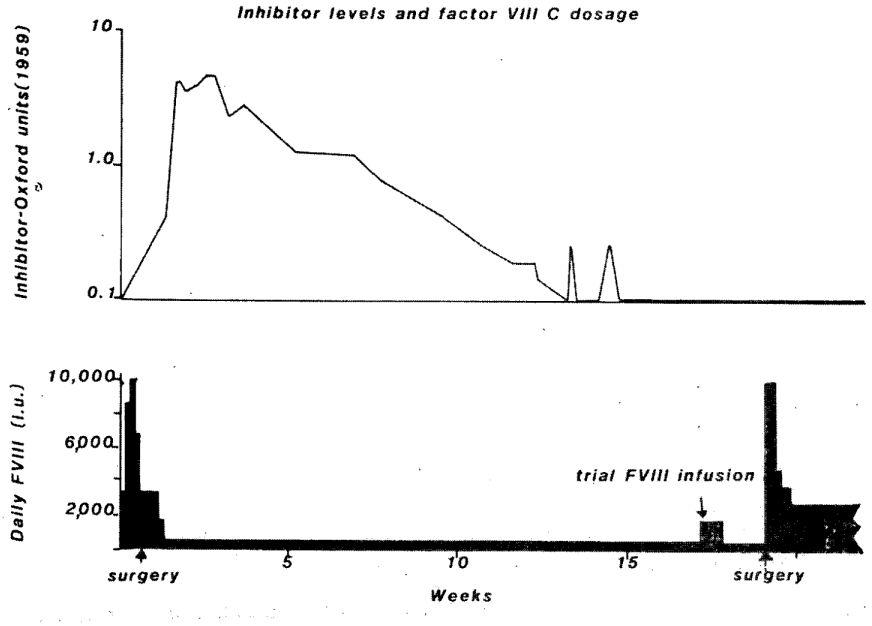
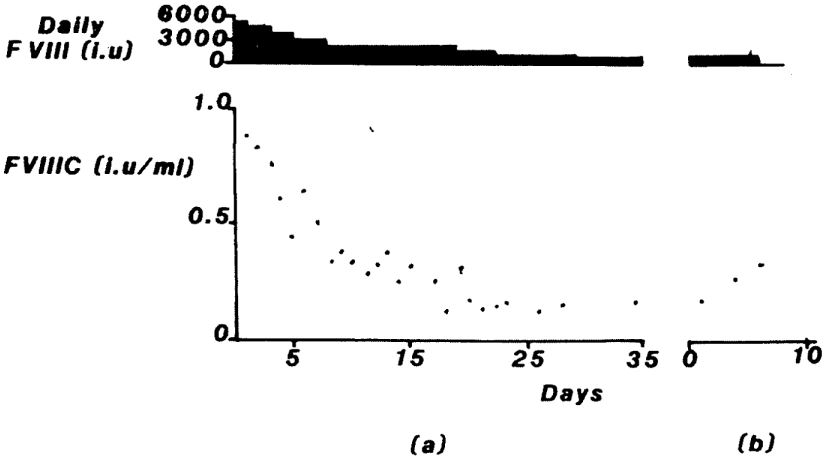



Figure 1

When he had completely lost his inhibitors, he had an operation carried out (pinning of femur with bone grafting). Haemostasis was normal at operation and factor VIII recoveries were as expected in a haemophiliac patient without an inhibitor. (Figure 2[a]).

***Fig-2 Pre-infusion plasma factor VIII C levels on 12 hourly factor VIII***




After thirteen months factor VIII was discontinued. Three months later he re-presented with a vertebral musculature bleed. Factor VIII was again given and recoveries were normal. (Figure 2[b]). There was no anamnestic antibody response. Although he is our only re-treated patient so far, we are now hopeful that if factor VIII is administered for a period after the successful induction of immune tolerance the tolerance may become permanent and further factor VIII injections will not be required, except for the treatment of bleeds.

Our method of inducing immune tolerance to factor VIII employs factor VIII only and concurrent administration of FEIBA is not required. I estimate that the costs of our schedule are about one-twentieth of that of a previously described regimen[4].

Earlier in this symposium Dr. Kazatshkine comments "Induction of specific tolerance to factor VIII would represent the most satisfactory (but as yet theoretical) treatment of haemophilia patients with factor VIII inhibitors." It now seems possible that those words in brackets can be deleted.

## REFERENCES


1. ALLAIN, J.P.; FROMMEL, D.: Antibodies to factor VIII v Pattern of immune response to factor VIII in haemophilia A. Blood 1976, 47: 975-982.

2. WENSLEY, R.T.; STEVENS, R.F.; BURN, A.M.; DELAMORE, I.W.: Plasma exchange and human factor VIII concentrate in managing haemophilia A with factor VIII antibodies. Br. Med. J. 1980; 281: 1388-1389.

3. SPIEGELBURG, H.L.: Biological activities of immunoglobulins of different classes and subclasses. Advances in Immunology 1974; 19: 259.

4. BRACKMAN,H.H.; GORMSEN,J.: Massive factor VIII infusion in a haemophiliac with factor VIII inhibitor, high responder. Lancet, 1977; ii: 933.