Three problems of hemophilia B : a study of abnormal factor IX molecules with an inhibitor neutralization assay

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THREE PROBLEMS OF HEMOPHILIA B

A STUDY OF ABNORMAL FACTOR IX MOLECULES WITH AN INHIBITOR NEUTRALIZATION ASSAY

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INTRODUCTION

Hemophilia is a sex linked, recessive, hereditary disorder characterized by excessive bleeding. This bleeding tendency manifests itself in spontaneous hemorrhages in the joint cavities and muscles, and in excessive bleeding after trauma or surgical procedures.

The first written references to the disease can be found in the Babylonian Talmud, in which it can be read that Rabbi Judah the Patriarch exempted the third son from circumcision if his mother had already lost two sons because they had bled to death after this operation (1). Rabbi Simon ben Gamaliel even forbade a boy to be circumcised when sons of his mother's three elder sisters had died from bleeding after circumcision (2).

In the 19th century Wardrop discovered the prolonged clotting time of hemophilic blood. For a long time lack of prothrombin was held responsible for the clotting defect until in 1935 Quick found that the prothrombin time of hemophilic plasma was normal (2).

Patek and Taylor reported in 1937 that the prolonged clotting time of hemophilic plasma could be normalized by the addition of a globulin fraction of normal blood. For this reason the lacking clotting component was called antihemophilic globulin; later, by international agreement, it was named clotting factor VIII (2).

In 1944 Pavlovsky observed that a mixture of the blood of two hemophiliacs known to him had a normal clotting time (3, 4). The right interpretation of this finding was given only in 1952 and not by Pavlovsky himself. In that year reports from New York, San Francisco, and Oxford described a disease which was clinically and genetically undistinguishable from hemophilia, but the lacking clotting component was not factor VIII (5-7). The missing factor in this new disorder, PTC-deficiency, Christmas disease or hemophilia B, was later called factor IX.

Hemophilia is a relatively rare disorder with an incidence of approximately 1: 10,000 men if mild cases are also taken into

account. Some 15% of hemophiliacs suffer from hemophilia B (8). The disease frequently causes severe destruction of joints with much suffering and disability to the patients. The economic burden for society that pays for the lifelong and costly treatment of the patients is very heavy. As a consequence, one endeavours all over the world in this field of the medical sciences to prevent irreversible damage to joints or even to prevent the disease altogether by means of genetic counseling of carriers.

In this thesis three aspects of hemophilia B are discussed. The first concerns the heterogeneity of hemophilia B. Some patients with hemophilia B have biologically inactive factor IX molecules in their plasma. These molecules show a cross-reaction with antibodies against normal factor IX. Because of this property these patients are classified as B^+ or CRM-positive. The question as to whether patients lacking factor IX molecules completely, B^- or CRM-negative patients, really exist, or whether absence of factor IX molecules is due to the imperfection of laboratory techniques, is a matter of debate to which we shall add our view. The second problem concerns the detection of carriers of hemophilia B. Carrier detection is an important issue for the female relatives of a hemophilic patient because they have a chance of bearing sons with this potentially disabling disease. In a large proportion of possible carriers it is difficult to ascertain whether such a woman is a carrier or not. We shall describe our attempts to improve carrier detection. Furthermore we studied the in vivo yield of factor IX concentrates. When factor IX concentrates are transfused into patients with hemophilia B for the treatment or prophylaxis of bleeding, a considerable proportion of the transfused factor IX molecules is not recovered in the plasma compartment of the patient. We report the progress of our search for these lost factor IX molecules.

The inhibitor neutralization assay (INA), which is applied for the assay of factor IX-CRM, has been extensively used by several authors who described molecular variants of factor IX. Its application in carrier detection has been reported twice (9, 10), whereas, to our knowledge, it has never been used in the study of factor IX concentrates. Apart from the factor IX activity assay, the INA forms the methodological mainstay of this study. A description of this test is given in Chapter I.

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CHAPTER I

METHODOLOGY

Preparation and properties of an antiserum against factor IX, its use in an inhibitor neutralization assay.

Coagulation methods

Intrinsic clotting factor activities were assayed, as described by Veltkamp et al. (1), using congenitally deficient plasma. The activities of factor VII and X were determined by means of artificially deficient plasmas according to Hemker et al. (2). The assay of factor V was carried out according to Kahn and Hemker (3), and of factor II according to Koller et al. (4).

Preparation of the antiserum

Normal human ACD plasma (200 ml) was treated with 10 g of the absorbent $Al(OH)_3$ after addition of heparin (Organon, Oss, The Netherlands) 10 mg per liter which equals 10^{-7} M (under the assumption of an average molecular weight of 10,000 daltons (5)) to avoid activation of the coagulation system. Benzamidin-HCl (Aldrich-Europe, Beerse, Belgium) 0.006 M and soybean trypsin inhibitor (Sigma, St. Louis, Missouri, USA) 10 mg per liter were added for the same purpose (6). The potency of soybean trypsin inhibitor is given by the fact that 1 mg inhibits the activity of 0.9 mg trypsin. The $Al(OH)_3$ was washed three times at room temperature with 0.2 M sodium citrate after which the absorbed proteins were eluted with 200 ml 0.3 M potassium phosphate buffer pH 8.0, containing 0.01 M benzamidin-HCl and 40 mg soybean trypsin inhibitor per liter. The eluate was dialyzed overnight (at 4°C) against 0.05 M Tris-HCl buffer at pH 7.5. The resulting solution contained 1 unit of factors IX, II, and X, and 3 units of

factor VII per ml; 1 unit clotting factor activity is by definition the amount present in 1 ml of pooled normal plasma. The protein content of the solution estimated by adsorption at 280 nm was approximately 1 mg per ml. The material was stored at —20°C until used.

To enhance purity and immunogenicity, the eluate was adsorbed onto heparin-sepharose (6, 7). Heparin was coupled to cyanogen bromide-activated sepharose 4B (Pharmacia, Upsala, Sweden) according to the instructions of the manufacturer. One ml of the heparin-sepharose suspension was mixed with 10 ml of the partially purified prothrombin complex by stirring carefully. Subsequently the mixture was centrifuged at room temperature for 5 minutes at 1000 x *g*, and the pellet was washed three times with 0.05 M Tris-HCl buffer at pH 7.5 to remove unbound proteins.

Two rabbits (1.5 kg F_1 hybrids of an Alaska x White Vienna mating; TNO, Zeist, Holland) were immunized by subcutaneous injection of the heparin-sepharose-prothrombin complex and 1 ml complete Freund adjuvant (Difco, Detroit, Michigan, USA), at three sites. Before the injections, blood was withdrawn for control experiments with normal rabbit serum. Booster injections were given with the same material mixed with incomplete Freund adjuvant every two weeks. After ten weeks venous blood was drawn for the preparation of the antiserum. To assess the effect of the coupling of the prothrombin complex concentrate to heparin-sepharose, two rabbits were immunized with free prothrombin complex concentrate.

Table 1: Symbols for different rabbit sera.

PRS	:	preimmunization rabbit serum
Anti PC serum	:	antiserum to prothrombin complex
Anti PC-H-S serum	:	antiserum to prothrombin complex-heparin-sepharose-conjugate

For the preparation of all three sera (Table I) blood was allowed to clot in glass tubes overnight at 4°C. After centrifugation, the supernatant sera were adsorbed with $BaSO_4$ 100 mg per ml in order

to remove rabbit prothrombin complex clotting factors and subsequently heated at 56°C for half an hour to inactivate complement factors. Finally the sera were spun for 30 minutes at 20,000 x *g*, 4°C and stored at —20°C.

From later batches of antiserum a globulin fraction was prepared. To this end the antiserum was brought to 37% saturation of $(NH_4)_2SO_4$ and the precipitate was dissolved in half of the original volume of 0.01 M potassium phosphate buffer pH 6.9. After dialysis against the same buffer overnight at 4°C, the antiserum was stored at −20°C.

Properties of the antiserum

The potency of the antiserum was determined by assaying the residual clotting factor activities in mixtures consisting of one part normal pooled plasma and one part of varying antiserum dilutions in Michaelis buffer (0.029 M sodium acetate $3H_2O$, 0.029 M sodium barbiturate, O.117 M NaCl, 0.020 M HCl, pH: 7.4). The residual clotting factor activities proved to be identical after incubating the mixture either for half an hour or 12 hours. Nor was there any difference between incubating at room temperature or at 37°C. Spinning the mixture for 30 minutes at 20,000 x *g* immediately after incubation did not influence the results. We decided, for convenience, to incubate the mixtures at room temperature for half an hour in all experiments to follow, and to omit the centrifugation step.

The antibody activity was expressed in units, the number of units being equal to the inverse value of the dilution factor of the antiserum that consumes 75% of the clotting factor activity present in a mixture of one part normal pooled plasma and one part antiserum dilution. The titration curves of the antibody activities against factors II, VII, IX, and X present in pooled antiserum from two rabbits both immunized with PC-H-S are shown in Figure 1. The antibody activity against factor IX was 9 units, against factor VII 5 units, and against factors X and II 1 unit. The antiserum did not inhibit factor V activity.

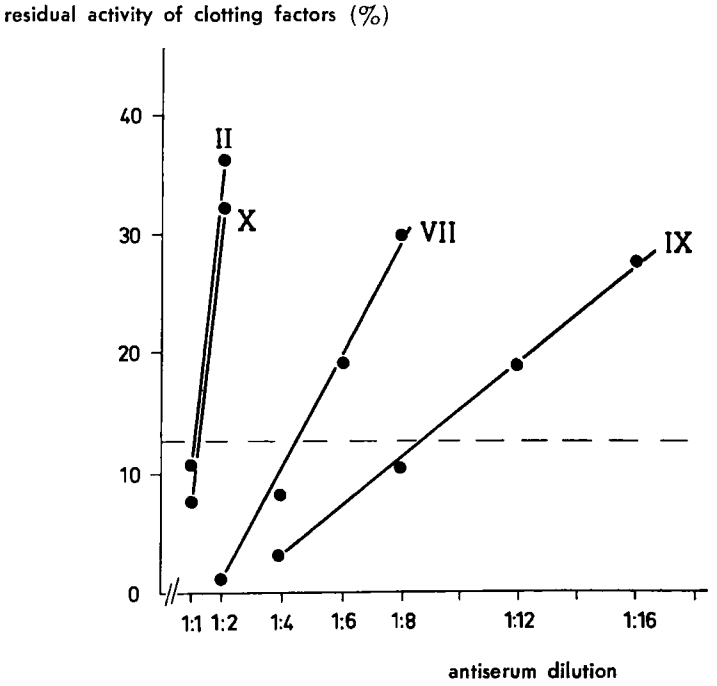

Figure 1: Titration curves demonstrating the activity of anti PC-H-S serum against the clotting factors II, VII, IX, and X. Equal parts of antiserum dilution and normal pooled plasma were incubated at room temperature for 30 min. The expected residual activity was 50% if no inhibitory activity was present. The dashed line indicates the 12.5% residual clotting factor activity level i.e., the point at which 75% of the clotting factor present in the mixture has been neutralized. The potency of antibody activity in units against each clotting factor is read from the intersection with the dashed line.

Similar experiments with PRS provided proof that this type of inhibition is not a property of rabbit serum in general. Table II shows that this serum produces an insignificant reduction in the activity of factors VII and X only.

A non-specific inhibitor of factor IX was detected in earlier batches of antiserum when testing different dilutions of incubation mixtures for factor IX activity. It was removed from later batches by $(NH_4)_2SO_4$ precipitation and dialysis. Experimental results were not influenced by this modification.

Table II: The influence of PRS on human clotting factor activities.

residual clotting factor activity of	observed	expected
f II	50%	50%
f VII	43%	50%
f X	42%	50%
f IX	45%	50%
f V	50%	50%

One part of normal pooled plasma was incubated with one part of undiluted PRS and residual clotting factor activities were assayed.

Since the PC-H-S antiserum is not specifically directed against factor IX activity, one might think that the antibodies against factors VII, X, and II could influence the results of factor IX assays. This is not likely because after incubation the mixtures are diluted 1: 10 for the final clotting factor assay and the final antiserum dilution is then more than 1: 60. As it appears from Figure 1, this antibody concentration is far too low to consume substantial amounts of factor II or X from the factor IX deficient substrate plasma used in the final clotting factor assay and, consequently, too low to cause prolongation of the clotting time. It is obvious that activity against factor VII as the only factor exclusively acting in the extrinsic system does not influence results of intrinsic clotting tests.

To assess the effect of adsorbing the prothrombin complex concentrate onto heparin-sepharose on the titer and on the specificity of the antiserum, the anti PC sera were tested in the same way. The titers of antibody against factors II, VII, IX, and X are given in Table III together with those of the anti PC-H-S serum. The difference in antibody activity against factor IX is quite marked.

Having established that the antiserum displayed a definite inhibition of factor IX clotting activity, we investigated its immunoprecipitation properties. We tested the antiserum by means of the

immunoelectrophoresis technique as described by Laurell (8). In this test the plasma samples of all our hemophilia B patients showed "rockets" which could not be distinguished from those obtained with normal plasma. $Al(OH)_3$ adsorbed plasma, which we expected to be negative, was definitely positive, just as our artificially prepared (2) factor X deficient reagent which contained only 5% factor IX activity of human origin. The only material giving a negative result was the artificially prepared (2) factor VII deficient reagent which contained 20% human factor IX activity. These results suggested that the antiserum might form a precipitate with factor VII, but not with factor IX. However, two congenitally factor VII deficient plasma samples ($< 1\%$) from two unrelated patients gave both rockets of the same size as those obtained from normal plasma. One of the two patients was CRM-positive and the other CRM-negative as we had established by means of an inhibitor neutralization assay (9). The antiserum was adsorbed with plasma of all our severe hemophilia B patients whom we con-

Table III: Activities of two different antisera to clotting factors II, VII, IX, and X.

	anti PC serum	anti PC-H-S serum
f II	1 U	1 U
f VII	4 U	5 U
f IX	2 U	9 U
f X	1 U	1 U

sider to belong to the B− variety. Factor IX inhibitory activity remained but precipitation lines were no longer obtained whether tests were carried out with the Ouchterlony technique, the Laurell electrophoresis, or the two-dimensional crossed electrophoresis. Negative results were obtained also with factor IX concentrates. On this basis we concluded that the antibody to factor IX did not have precipitating properties.

Inhibitor neutralization assay

The procedure of the inhibitor neutralization assay (INA) as described by Roberts et al. (10) is outlined in Figure 2. The principle underlying the test is that plasma containing factor IX molecules binds the antibody against factor IX. The quantity of antibody neutralized is proportional to the quantity of factor IX

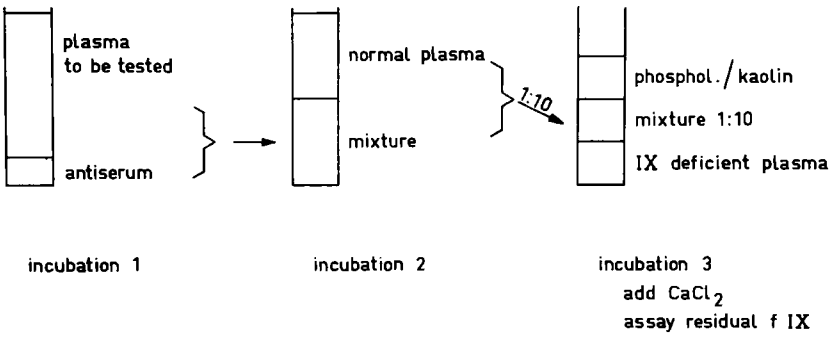

Figure 2: Schematic representation of the inhibitor neutralization assay.

molecules in the sample. The quantity of antibody neutralized can be determined from the quantity of antibody that is left. This residual amount of antibody is assessed by testing the ability of the incubation-mixture to diminish the factor IX activity in normal plasma. It follows that the residual factor IX activity is proportional to the concentration of factor IX molecules in the experimental plasma.

The amount of antibody in the first incubation mixture is chosen in such a way, that after the first incubation with normal pooled plasma a factor IX activity is found between 1 and 5%. This small, yet significant amount proves that all anti-factor IX activity has been exhausted. We used this amount of antibody in all further tests. In the second step, an equal amount of normal pooled plasma is added in all experiments. Consequently the differences in the final test result depend on the only variable in the system i.e., the amount of factor IX molecules in the experimental plasma. Serial

dilutions of normal pooled plasma in Michaelis buffer are assayed simultaneously with each series of experimental samples in order

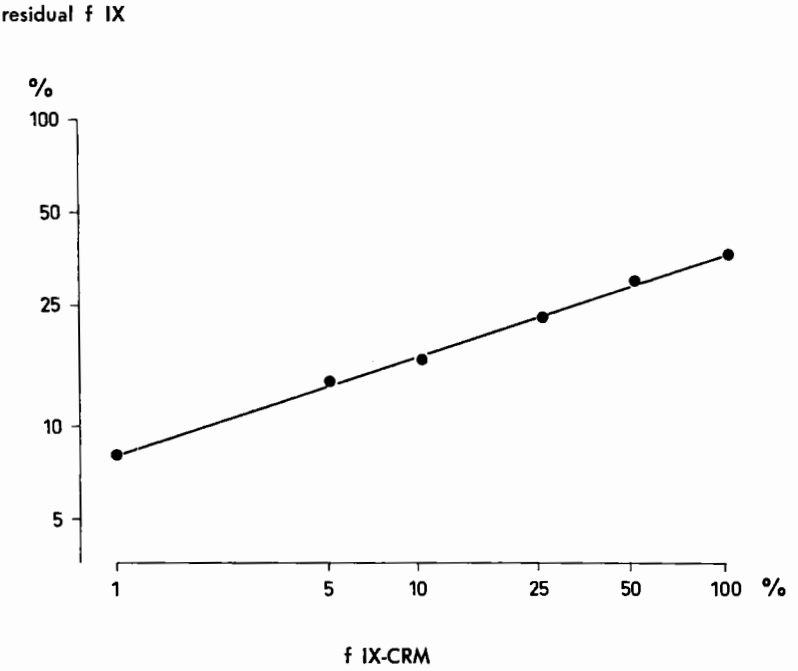

Figure 3: An example of a reference curve of the inhibitor neutralization assay.

to obtain points of reference. Figure 3 shows an example of a reference curve for the conversion of residual factor IX activity into factor IX-CRM concentration. Similar reference curves are obtained if the dilutions are made in the plasma of a patient with severe hemophilia B$^-$. This proves that the assay is specific for factor IX molecules.

A question to be solved concerned the possible influence of the factor IX deficient reagent used for the factor IX assay. One might suppose that the inactive factor IX molecules in a CRM-positive reagent could compete in the equilibrium between antibody and antigen thereby causing release of active molecules during the incubation period of the factor IX assay. This would result in a falsely high yield of residual factor IX. However, such a competition

could not be demonstrated and when plasmas of CRM-negative patients were tested with a CRM-positive reagent, the results were not different from the values found when a CRM-negative reagent was used. A CRM-negative reagent was used in all experiments to be described.

To assess the precision of the INA, we calculated the coefficient of variation from multiplicate (varying from 2 to 8) determinations of thirty-eight samples. This was found to be no less than 18.5%, while the analytic error also expressed as coefficient of variation in the assays of factor VIII and IX activity amounts to 5-10% (11).

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CHAPTER II

GENETIC HETEROGENEITY OF HEMOPHILIA B

INTRODUCTION

Hemophilia B cannot be considered a homogeneous disease because of the variability in:

1. the severity of the disease;
2. the concentration of inactive factor IX molecules;
3. the sensitivity of the plasma to ox-brain thromboplastin;
4. the increase of the factor IX activity level during life.

It is generally accepted that this heterogeneity has a genetic basis because affected members of one family all suffer from the same variant of hemophilia B.

In this chapter we shall give a survey of the literature concerning the biochemical peculiarities underlying these variants and the combinations in which these characteristics occur. We will also discuss the findings in our patients.

Clinical severity

The clinical severity of hemophilia B is correlated with the level of factor IX activity in the patient's plasma: patients who have less than 1% of the normal factor IX activity level suffer from recurrent spontaneous hemorrhages, especially in the joint cavities, and this type is called severe hemophilia B. When the factor IX activity level is between 1 and 5%, the patient has rarely spontaneous bleeding although minor trauma may provoke severe hemorrhages, a condition which is called hemophilia of the moderate type. Mild hemophilia is found in patients with factor IX activity levels of 5 to 25%; the bleeding tendency is only revealed by a challenge of the

hemostatic mechanism, such as surgical intervention or accidental trauma. Hemophiliacs of one kindred all have a similar severity of their disease. The variability between kindreds may be explained by multiple allelomorphic genes for the hemophilia B locus.

Inactive factor IX molecules

The second parameter is the inhibitor neutralizing capacity of plasma. In some patients with severe hemophilia B the plasma contains a substance that can react with antibodies to factor IX circulating in the blood of some (rare) patients with severe hemophilia B, or with antibodies against factor IX raised in rabbits. This property of cross-reacting is ascribed to the presence of a structurally altered factor IX molecule devoid of procoagulant activity (1) and is called inhibitor neutralizing capacity (INC) (2). Depending on whether or not there is an excess of INC over clotting factor activity, a case may be classified as being B^+ or B^- (3) or alternatively CRM^+ and CRM^- respectively (2).

The physico-chemical properties of CRM present in hemophilia B^+ plasma as studied by Twomey et al. (4) and by Somer and Castaldi (5) do not differ from those of normal factor IX.

The concentration of CRM in the plasma of B^+ patients, however, is not always as high as that found in normal plasma (6, 7).

Ox-brain thromboplastin-time

The third feature was first described by Kidd et al. (8), and studied more extensively by Hougie and Twomey (9). It is characterized by the fact, that the patient's plasma has an abnormally slow reactivity with ox-brain thromboplastin; the slow reacting group is given the suffix M (hemophilia B_M), after the surname of the first two patients whose plasma showed this feature.

Hemophilia B Leyden

The last parameter seems to set apart only a very small number of rather privileged patients who are born with a severe or moderate form of hemophilia B, but whose factor IX activity level begins to

rise during puberty and continues to do so with advancing years. This form was described by Veltkamp et al. (10) in three families originating from Drente, a province in the Netherlands, and has been called hemophilia B Leyden.

Does hemophilia B⁻ exist?

Tests for CRM or "factor IX-like material" have been carried out largely with the inhibitor neutralization assay (INA) (11). This is a laborious test and its accuracy is limited. The incidence of the B^+ variant varies considerably in different reports (Table I) and this

Table I: Variants of hemophilia B in the literature.

investigator (first author) with reference number	number of kindreds investigated	number of kindreds		
		Bm	B+ detected by	
			heterologous antiserum	homologous inhibitor
Kidd 1963 (8)	6	1	nt	nt
Hougie 1967 (9)	5	1	nt	nt
Roberts 1968 (2)	25	nt	nt	4
Denson 1968 (3)	27	3	3	3
Minami 1969*(22)	50	3	nt	nt
Pfueller 1969 (12)	11	0	4	nt
Bithell 1970 (23)	6	1	nt	nt
Brown 1970 (13)	23	nt	nt	6
Veltkamp 1970 (10)	3	0	nt	0
George 1971 (24)	10	0	nt	2
Meyer 1972*(5)	19	5	18	8
Elödi 1972*(18)	18	3	2	nt
Ørstavik 1975 (16)	11	1	5	nt

* Some details not given in the publication were obtained by personal communication with the author.

nt: not tested.

might, apart from reflecting true differences in the incidence in patient groups from various regions in the world, be dependent on the type and source of antisera used in the INA. Some heterologous antibodies produced in response to the injection of partially purified factor IX in rabbits (7, 12) appear to detect a higher percentage of B^+ kindreds than homologous inhibitors (2, 6, 13). Meyer et al. (7) demonstrated the presence of cross-reacting material in 18 out of the 19 families they had examined. When they used a homologous inhibitor to factor IX instead of a heterologous antiserum, only 8 out of the 19 families were found to have inhibitor neutralizing material in their plasma. Zimmerman and Edgington (14) have demonstrated that after insolubilization of a heterologous antibody to agarose beads (so-called "antibody beads"), 100% of hemophilia A patients exhibited factor VIII-CRM in the INA, while a much lower incidence of CRM-positive patients was found when the same antiserum was used in a fluid phase system. The explanation they offered for this discrepancy is, that antibodies directed against antigenic determinants remote from the active site of the molecule do not necessarily interfere with clotting activity. In the fluid phase system these antigenic determinants are not detected because residual clotting factor activity will not be influenced as long as the antibody-clotting protein complex is soluble. From this we might draw the inference that the factor IX molecules whose antigenic determinants at or near the active site have been subjected to alteration by the mutation, might not be detectable by antisera in a fluid phase INA. The large proportion of CRM-positive families found by Meyer et al. (7) in the fluid phase assay is not due to selection of the patient material (15). It might be caused by the fact that their heterologous antiserum is directed against more antigenic determinants of the factor IX molecule than the sera used by other investigators. An immunoelectrophoretic assay with a precipitating antibody is expected to give the same results as the solid phase INA because the biological activity of the molecules does not come into play. Ørstavik et al. (16) used a heterologous antiserum and found only 3 out of 11 families to be CRM-positive, both with a fluid phase INA and with an immunoelectrophoretic assay. This observation might be used as an argument for the existence of hemophilia B^-.

The nature of hemophilia B_M

By testing mixtures of normal plasma with either untreated hemophilia B_M plasma or hemophilia B_M plasma treated with $BaSO_4$, evidence was produced that the prolonged ox-brain thromboplastin-time of hemophilia B_M was caused by an inhibitor adsorbable onto $BaSO_4$. It might, therefore, be identical to a structurally altered prothrombin complex factor, presumably an abnormal factor IX molecule (3, 9, 17). An almost conclusive experiment in this context was carried out by Denson et al. (3), who showed that adsorption of hemophilia B_M plasma with a circulating inhibitor to factor IX followed by centrifugation normalized the ox-brain thromboplastin-time. In contrast with these authors, Elödi and Puskas (18) found that the ox-brain thromboplastin-time of their hemophilia B_M patients was completely normalized when the plasma was mixed with normal plasma, suggesting a deficiency rather than an inhibitor. Testing for factor VII activity in an assay using ox-brain thromboplastin resulted in a significantly lower level than with the use of human thromboplastin which gave a normal result. Therefore it was concluded that slow reactivity of factor VII rather than an inhibiting factor IX molecule was responsible for the prolonged ox-brain thromboplastin-time in hemophilia B_M (19). Of all kindreds with hemophilia B published, about 10% seems to have the B_M variant (Table I). It should be stressed that the frequency of the variants has to be expressed in a percentage of kindreds, because all patients in a single kindred suffer from the same variant of hemophilia B. This is one of the major indications that we are dealing with different mutations resulting in sex-linked inherited hemophilia B (2, 13).

Clinical severity and molecular variants

The relationship of the clinical severity of the hemophilia to its variants appears to be as follows. Both the B^+ and the B^- variants have been found to occur in combination with all levels of factor IX deficiency (2, 6, 12, 18). Hemophilia B_M has been described most frequently in combination with the severe form (6, 8, 9, 17), but also with moderate (7) and with mild hemophilia B (19).

Is hemophilia B^+ identical to hemophilia B_M?

As long as tests for factor IX-like material are far from perfect, theories concerning the relation of the B^+ and the B^- variants on the one hand, and the B_M and the "not B_M" variants on the other remain somewhat speculative. In 1968 Denson et al. (3) found that his patients with hemophilia B_M belonged to the B^+ category and vice versa (Table I). This finding nicely fitted the hypothesis, that the prolonged thromboplastin-time of hemophilia B_M is caused by inhibition due to a structurally abnormal factor IX, and that all patients with hemophilia B^+ have prolonged ox-brain thromboplastin-times. The finding of a group of patients with hemophilia B^+ without prolonged ox-brain thromboplastin-time by Pfueller et al. (12) and Brown et al. (13) falsified this hypothesis. So did the report of Meyer et al. (4), who surprisingly found patients with hemophilia B_M without material cross-reacting with the human antibody; however, when they used a heterologous antibody instead of a homologous inhibitor, they demonstrated that the group belonged to the B^+_M variety. In conclusion: all B_M patients are B^+, but this proposition cannot be inverted.

Hemophilia B Leyden and molecular variants

With respect to hemophilia B Leyden it can be observed that no patient fell into the B_M category and no excess of factor IX-like material over factor IX activity could be detected in any of them by means of a homologous factor IX inhibitor (10).

In the following paragraphs the results are described of analogous investigations in our patient material.

MATERIALS AND METHODS

Coagulation methods have been described in Chapter I.

We studied the plasma samples of 35 patients from 20 unrelated families. The three hemophilia B Leyden families were considered to belong to the same pedigree and therefore to represent a single

mutation (10). Patients were venipunctured in our hospital or at home, from where the blood samples were transported to Leiden by car; the time between venipuncture and centrifuging of the blood sample never exceeded four hours. Platelet free plasma samples were stored at —70°C before performing the assays.

Dr. Ørstavik from Oslo kindly tested 21 samples using a specific heterologous antiserum to factor IX containing a precipitating antibody in a fused rocked immunoelectrophoretic assay (16). For this purpose the plasma samples were shipped to Oslo after lyophilization.

RESULTS

Factor IX activity (Table II)

Patients from 13 out of the 20 families showed factor IX activities below one percent of normal. The hemophilia B Leyden patients were divided into two groups, one under and one over the age of 14; the younger group exhibited approximately one percent of factor IX activity, while above 14 the average factor IX activity amounted to 41%. Family 3 and family 19 have moderately severe hemophilia B (1.6% and 1.0% factor IX activity respectively) and the families 6, 10, 12, and 18 mild hemophilia B (factor IX activities ranging from 6 to 28%).

Factor IX-CRM (Table II)

Patients from 8 out of the 13 families carrying the severe mutant gene had no detectable factor IX-CRM, while patients of the other 5 families had levels of 50 to 100% of normal. In none of the severely affected youngsters with hemophilia B Leyden factor IX-CRM could be detected, while the elder patients had factor IX-CRM levels roughly proportionate to their factor IX activities. The 6 families with moderate and mild hemophilia B all had factor IX-CRM in excess of factor IX activities.

Comparison of three assays for factor IX-CRM

The results obtained with the two different antisera, homologous and heterologous, in the fluid phase INA are more or less the same

Table II: The assay of factor IX activity and factor IX-CRM in 35 patients with hemophilia B.

pedigree	initials	age (yrs)	f IX act. (%)	f IX CRM (%)		
				fluid phase		immunoelectrophoresis*
				heterol.	homol.	
1 (B Leyden)	A. K.	56	68	31	nt	<
	G. U.	41	34	20	28	29
	G. J.	31	20	16	nt	<?
	J. H. J. v. G.	37	49	31	35	nt
	A. H. v. G.	33	43	30	58	nt
	H. B.	24	41	16.5	35	28
	H. H.	20	20	30	18	<
	L. d. J.	14	<1	<	<	<
	J. K.	12	1.4	<	<	nt
	H. A.	11	<1	<	<	<
	J. d. J.	9	1.4	<	<	<
	H. K.	7	1.3	<	<	nt
2	S. v. D.	9	<1	<	<	<
3	C. v. E.	29	1.5	52	98	142
	H. v. d. B.	14	1.8	51	70	nt
4	P. H. H.	22	<1	<	<	<
5	A. G. M. v. W.	35	<1	<	<	<
6	U. S.	33	24	71	72	80
7	A. d. W.	23	<1	65	56	66
8	E. K.	60	<1	<	<	<
9	A. M.	4	<1	50	56	67
10	C. S. K.	25	28	73	70	131
	Th. A. K.	13	24	100	nt	127
11	B. H.	49	<1	<	<	<
12	L. M. D.	30	10	33	74	nt
13	H. v. L.	28	<1	<	<	<
14	A. J. S.	38	<1	101	65	180
15	P. T.	17	<1	95	90	nt
	E. A.	16	<1	91	85	nt
16	W. v. E.	25	<1	65	78	nt
17	H. S.	9	<1	<	<	nt
	E. J. S.	8	<1	<	<	nt
18	A. J. R.	62	6	36	44	nt
19	B. L.	7	1	63	35	nt
20	M. B.	10	<1	<	<	nt

<: below level of detectability.
nt: not tested.
* Carried out by Dr. Ørstavik, Oslo.

(Table II). No patient was classified as B$^-$ on the basis of the test with one antiserum and B$^+$ with the other. Moreover, the results for 21 samples in the immunoelectrophoretic assay were consistent with both the fluid phase assays.

Ox-brain thromboplastin-time studies using the Thrombotest^R reagent

Only one patient (A. J. S.) showed a significantly prolonged ox-brain thromboplastin-time. The prolongation was not normalized by admixing normal plasma. After absorption by $Al(OH)_3$ the patient's plasma behaved exactly like normal absorbed plasma (Table III). The factor IX activity in this patient's plasma was $< 1\%$. In the thrombotest dilution curve test (20), which is a suitable method to detect inhibitory activity in prothrombinase formation, this patient's plasma contained 6.5 units of inhibitor activity (Fig. 1). Levels of extrinsic clotting factors were normal, whether they were assayed with human-, ox-, or rat-brain thromboplastin.

DISCUSSION

The existence of hemophilia B$^-$

Although we applied two different antisera in the fluid phase test and a third in an immunoelectrophoretic assay, we found nine out of twenty families to be CRM-negative. All methods yielded the same result. Therefore we tend to believe that hemophilia B$^-$ in fact exists.

On the basis of our results it appears unlikely that antibodies can react with the factor IX molecule without destroying its biological activity. Consequently, tests for factor IX antigen independent of factor IX activity, like the INA with antibody beads or the immunoelectrophoretic assays, cannot be expected to demonstrate CRM if it has not been found in a fluid phase INA. So the finding of hemophilia B$^-$ cannot be ascribed to the use of the wrong test.
Should we seek the answer in the antisera perhaps?

The finding of Meyer et al. (7) of 18 B$^+$ families from a total of 19 suggests that an appropriate antiserum might dispose of the idea

Table III: Thrombotest® studies of patient A. J. S. (pedigree 14).

testmaterial	thrombotest seconds
patient	102
normal plasma (NP)	44
NP + pat (1 : 1)	62
NP + $Al(OH)_3$ ads. NP (1 : 1)	52
NP + $Al(OH)_3$ ads. pat. (1 : 1)	53

of hemophilia B– altogether. It is possible that the antibodies in their antiserum combine with more antigenic determinants of the factor IX molecule than those in the antisera used by other investigators. In that case misshapen molecules with only a few normal antigenic determinants react only with this particular antiserum. Alternatively it might be that their assay is not specific for

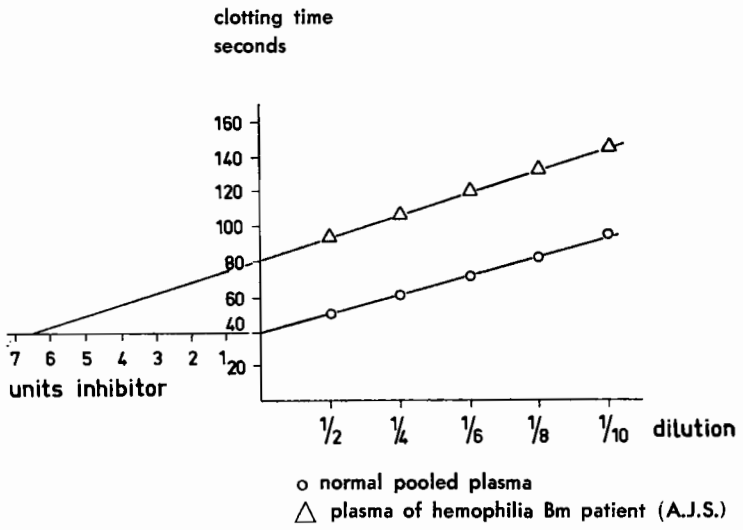

Figure 1: Thrombotest dilution curves of normal pooled plasma and the plasma of patient A. J. S. (pedigree 14) which contains 6.5 units of inhibitor in this system.

factor IX. Another, rather obvious explanation could be the difference in the population of patients. Only the exchange of antisera between different investigators can give the final answer to this problem.

In the meantime we accept the possibility that CRM-negative patients do not produce factor IX molecules at all, which means that hemophilia B− exists. However, it will be impossible to disprove that CRM-negative patients produce factor IX molecules so different from normal ones that they cannot be identified by the existing tests. Secondly, it could be argued that CRM-negative patients produce abnormal factor IX molecules with a very rapid turnover, which was demonstrated for PIVKA II (21), causing plasma levels too low for detection. The fact, that the amount of factor IX-CRM in hemophilia B^+ is often not as high as in normals, may also be explained by one of these hypotheses.

The nature of hemophilia B_M

Regarding the one patient with hemophilia B_M our findings suggest, as do other reports, that the abnormal factor IX behaves as an inhibitor in the assay of the ox-brain thromboplastin-time. Unlike Elödi (19) we could not detect an abnormality of factor VII. The combination of a peculiarity of factor VII with a disorder of factor IX, which Elödi puts forward as an explanation for the prolonged ox-brain thromboplastin-time in hemophilia B^+, is difficult to understand on genetical grounds. Although it has been taken for granted that the prothrombin complex factors have a common ancestor in the evolution, it is impossible that the product of an autosomal gene (factor VII) could have been altered in linkage with an X-chromosomal trait unless this mutation occurred early in the phylogeny of man and was inherited for thousands of generations.

Clinical severity and the occurrence of molecular variants

Our findings do not add new combinations to those already reported in the literature. A graphical representation of the existing combinations is shown in Table IV.

Table IV: Variants of hemophilia B.

severity	molecular variants		
	B+		B–
	B_M	"not B_M"	
severe	+	+	+
moderate and mild	+	+	+
B Leyden (variable depending on age)	—	—	+

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CHAPTER III

CARRIER DETECTION IN HEMOPHILIA B

INTRODUCTION

Discrimination of carriers of hemophilia B is based on two data: the chance of the woman being a carrier based on genetical grounds and her factor IX activity level. Unfortunately, however, the ranges of the clotting factor activity levels of the two reference groups i.e., a group of obligatory carriers and one of women without a family history of hemophilia, are not clearly separated and the overlapping area is relatively large. So large in fact, that carrier detection in the hemophilias is a difficult task (1-4). Factors adding to this problem are the random inactivation of one of the X-chromosomes in each female cell in early embryonic life (Lyon hypothesis), age, and the use of oral contraceptives.

1. Random inactivation of either one or the other X-chromosome in female cells early in embryonic development is undoubtedly an important cause of the large range of factor IX activity levels in hemophilia B carriers; the same holds true for factor VIII activity levels in carriers of hemophilia A (5-7). This mechanism has been named lyonization after Mary Lyon, who forwarded the hypothesis. Lyon's hypothesis offers an explanation for dosage compensation i.e., the fact that normal men and women have the same levels of antihemophilic clotting factor activities despite their having a different number of X-chromosomes, because in female cells only one X-chromosome is active just like in male cells. Dosage compensation is present even in individuals with multiple X-chromosomes, which means that all X-chromosomes but one are inactivated. Moreover, it explains for the occurrence of hemophilic symptomatology in heterozygous females i.e., hemophilia carriers who do not simultane-

ously suffer from Turner's syndrome. In such carriers the majority of normal X-chromosomes has by chance been inactivated, leaving them in a state comparable to the hemophilic male. On the other hand, the inactivation of abnormal X-chromosomes explains the existence of obligatory carriers who display normal or almost normal factor IX activity levels in their plasma.

2. Age influences the factor IX activity level in both normals and carriers (8), the level rising slightly with advancing years. Although symptomatology in hemophilia becomes milder in adulthood, a rise in factor VIII or IX activity is not demonstrable. Only in the case of hemophilia B Leyden (9) age has a profound influence on the factor IX activity level of the affected males. Whether this age related rise also occurs in carriers of this disorder, has not yet been established.

3. Estrogen containing oral contraceptives are known to alter the level of many plasma proteins. A rise of the factor IX activity level has been reported by many authors (10-15). Although their influence on the factor IX activity level in carriers of hemophilia B has not been reported there is no reason to deny such an influence. This is important because many potential carriers use these medicaments at the moment they are being examined for carriership.

Carrier detection in hemophilia A has been improved by the simultaneous assay and comparison of factor VIII activity and factor VIII-like antigen (16-25), although the relation between these entities is not yet completely understood (16, 26, 27). It has been reported that the plasma of carriers of hemophilia B^+ who theoretically produce two populations of factor IX molecules, one of which is biologically active and the other inactive, shows in fact an excess of factor IX antigen over factor IX activity (28-30). If factor IX antigen is determined and used as a third parameter to distinguish between carriers and normals, in addition to the factor IX activity level and the genetic chance of carriership, the efficiency of detection might improve. In hemophilia B, however, Elödi expects the improvement to be less than in hemophilia A because of the low incidence of the CRM-positive variant (28, 29).

In this chapter we try to provide an answer to four questions pertinent to carrier detection in hemophilia B.

1. Does the factor IX activity level in carriers of hemophilia B Leyden rise with advancing years?

2. Does oral contraceptive medication influence the level of factor IX activity and of factor IX antigen both in normal women and in carriers of hemophilia B?

3. To what extent does the assay of factor IX antigen contribute to the detection rate of carriers of hemophilia B^+?

4. Is it possible to obtain a good discrimination between obligatory carriers of hemophilia B and normal women using the assays now available and, as a consequence, what may we expect of the detection rate if we apply these methods to a group of possible carriers?

MATERIALS AND METHODS

Two groups of twenty normal women, one group using oral contraceptives, were examined for their factor IX activity and factor IX antigen levels. The average age of the women in the group using the pill was 22 years, the average age in the other group 21 years. The same tests were done on the plasma samples of 37 obligatory carriers of hemophilia B coming from 14 families. Details concerning age, use of the pill, and type and severity of hemophilia are given in Table I and II. Individuals were classified as B^+ on the basis of a significant excess of factor IX antigen over factor IX activity found in male patients belonging to the same kindred (31-33). Carriers from the B Leyden variant were considered to belong to one family, although kinship between the three originally described pedigrees has not yet been proved (9).

Factor IX activity was assayed as described by Veltkamp (3) using a congenitally deficient substrate plasma. For the reference curve, dilutions were made of normal pooled plasma from 30 healthy donors. This group consisted of 15 men and 15 women with an average age of 30 years. Three of these women used contraceptive pills. Factor IX antigen was assayed as described in chapter I.

Table I: Obligatory hemophilia B carriers.

variant	initials	pedigree	age	factor IX activity %	factor IX antigen %	pill
B Leyden	K.-J.	1	30	40	38	—
	K.-Z.	1	41	109	99	+
	K.-H.	1	42	54	59	—
	U.-B.	1	70	43	49	—
	A.-K.	1	40	24	31	—
	O.-T.	1	69	65	77	—
	d. J.-O.	1	36	74	113	+
	K.-T.	1	71	48	57	—
	S.-E.	1	76	124	113	—
	J. S.	1	14	66	71	—
	B.-K.	1	48	77	62	+
	J.-K.	1	57	89	65	—
	H.-K.	1	45	76	80	—
	K. v. G.	1	10	47	42	—
	M. v. G.	1	2	37	25	—
	B.-R.	1	21	66	75	+
	F.R.	1	22	75	87	—
	v. R.-R.	1	27	106	55	+
	H.-R.	1	28	72	98	+
B—	v. B.-H.	2	67	57	74	—
	d. V.-v. B.	2	42	38	40	—
	v. D.-v. B.	2	34	79	74	+
	H.-S.	4	60	53	52	—
	E.-K.	8	29	67	56	+
	S.-G.	17	35	39	61	—
	B.-R.	20	36	56	46	—
B+	v. E.-Z.	3	66	51	86	—
	v. d. B.-v. E.	3	40	42	88	+
	L.S.	6	9	53	76	—
	F.S.	6	5	58	62	—
	d. W.-B.	7	46	20	68	—
	M.-d. V.	9	27	30	62	+
	K.-G.	10	59	102	95	—
	T.-S.	15	40	41	110	—
	A.-S.	15	36	85	102	—
	v. E.-I.	16	51	52	94	—
	M.-R.	18	28	88	81	+

Table II: Normal women.

number	age	pill	factor IX activity %	factor IX antigen %
1	26	+	111	124
2	22	+	155	87
3	19	+	159	140
4	21	+	109	101
5	21	+	141	170
6	21	+	130	118
7	23	+	103	116
8	24	+	135	170
9	25	+	130	136
10	23	+	116	90
11	19	+	95	110
12	19	+	130	116
13	21	+	91	77
14	22	+	121	90
15	22	+	130	136
16	19	+	120	80
17	21	+	124	120
18	19	+	104	122
19	23	+	110	82
20	21	+	100	100
1	22	—	115	102
2	18	—	115	102
3	19	—	115	82
4	16	—	90	65
5	23	—	78	86
6	21	—	88	122
7	20	—	82	96
8	22	—	109	140
9	27	—	120	85
10	18	—	65	54
11	23	—	89	88
12	23	—	49	59
13	24	—	82	76
14	23	—	72	90
15	21	—	100	140
16	19	—	84	79
17	19	—	98	83
18	18	—	81	82
19	18	—	81	122
20	21	—	114	140

RESULTS

Factor IX activity

Figure 1 shows a plot of the factor IX activity levels of 37 obligatory carriers of hemophilia B against their age. The slope of the regression line was not significantly different from zero in any of the four groups indicated. Nine adult carriers of hemophilia B Leyden had been examined at an earlier occasion varying from

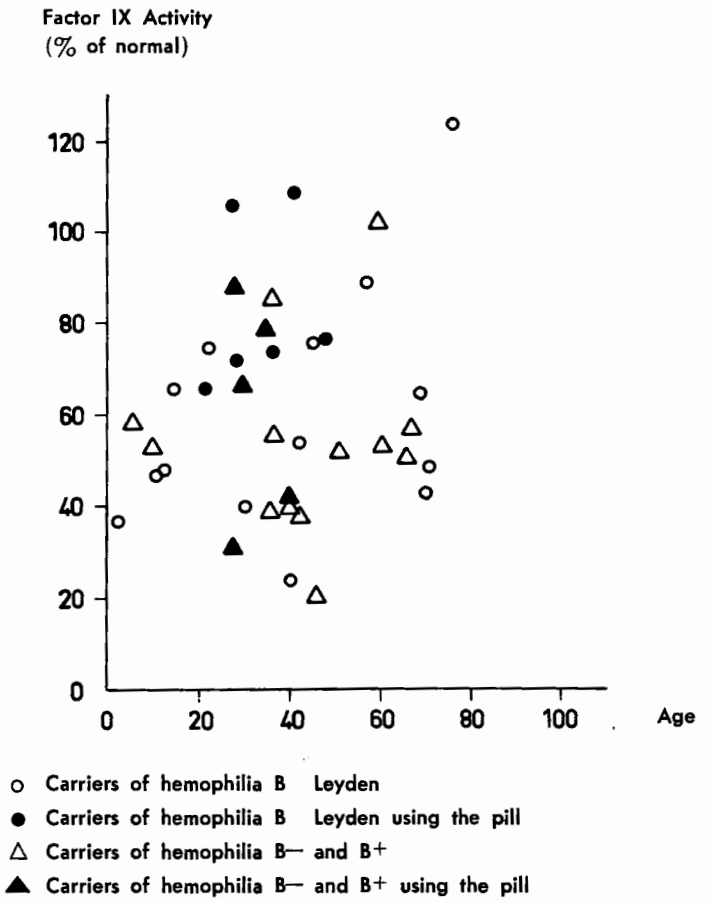

Figure 1: A plot of the factor IX activity level and age of 37 obligatory carriers of hemophilia B.

5 to 10 years before. Figure 2 shows that during this period a rise in factor IX activity level could not be demonstrated. However, a rise of the factor IX activity level during puberty in carriers of hemophilia B Leyden is probable because we found rather low levels in the youngest girls. In order to exclude age as a con-

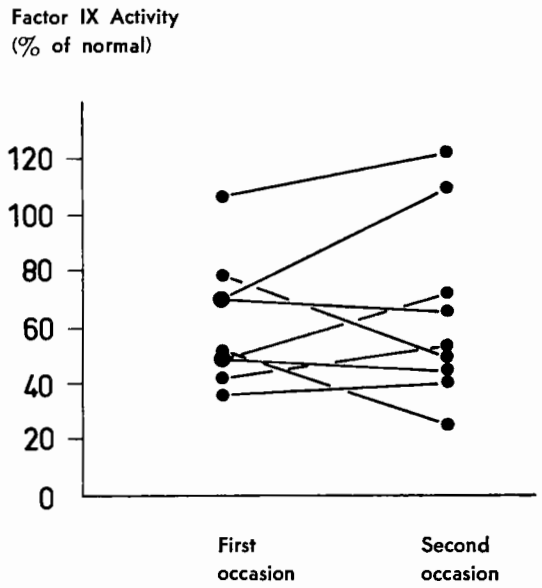

Figure 2: Factor IX activity levels of 9 obligatory carriers of hemophilia B Leyden at two occasions; the time interval varying from 5 to 10 years.

tributing factor to the large range of factor IX activity levels in carriers of hemophilia B, we must restrict further considerations to persons over 15 years of age.

Figure 3 shows the factor IX activity levels in groups of normal women and carriers with and without oral contraceptive medication. An analysis of variance on these data was performed to study the influence of carriership as well as the use of oral contraceptives on the level of factor IX activity. The results of this analysis are demonstrated in Table III. Interaction between carriership and use of the pill as to their influence on the factor IX activity level may be considered absent ($p > 0.10$). It may be concluded therefore that the positive influence of estrogen containing oral contraceptives on

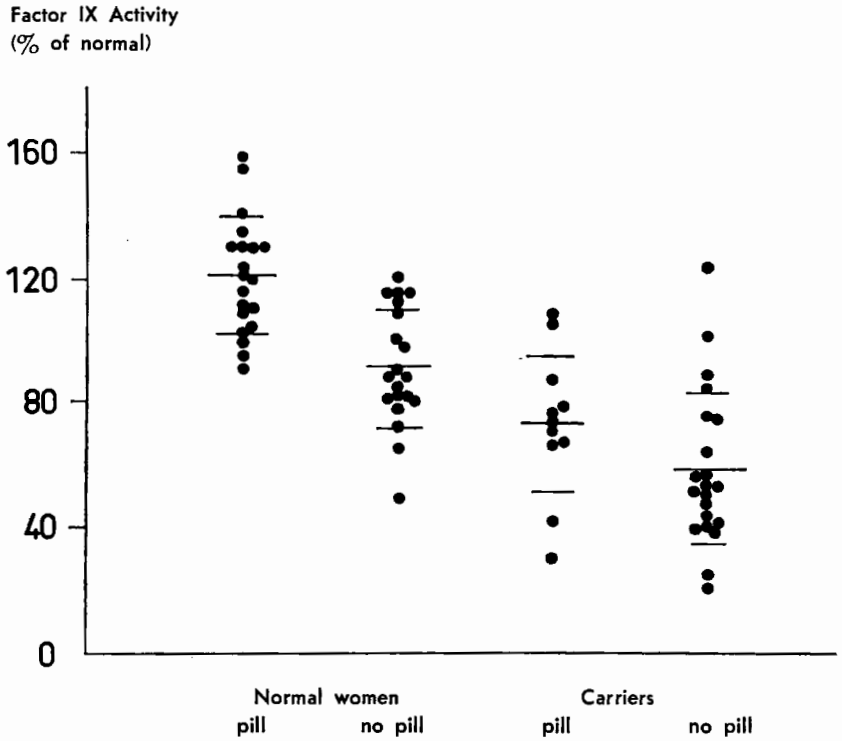

Figure 3: The factor IX activity levels of normal women and obligatory carriers with and without oral contraceptive medication. Mean values and one standard deviation have been indicated. An analysis of variance showed a significant difference between women who use oral contraceptives and those who do not, both for normal women and carriers ($p < 0.0001$).

Table III: Variance analysis of the influence of carriership and the use of oral contraceptives on the factor IX activity level.

source	degrees of freedom	mean square error	F	P
normals vs carriers	1	26661	56.46	$<<10^{-4}$
pills vs no pill	1	8235	17.44	$<10^{-4}$
interaction	1	867	1.84	>0.10
residual	70	472		

the factor IX activity level is significant in the group of normal women as well as in the group of carriers ($p < 0.0001$).

Factor IX antigen

The levels of factor IX antigen in the samples tested are given in Table I and II. We used the ratio of the factor IX antigen and the factor IX activity levels to express the relation between these entities. For reasons of symmetry logarithms of the ratios are shown in Figure 4 and 5 instead of the ratios themselves. Figure 4 shows

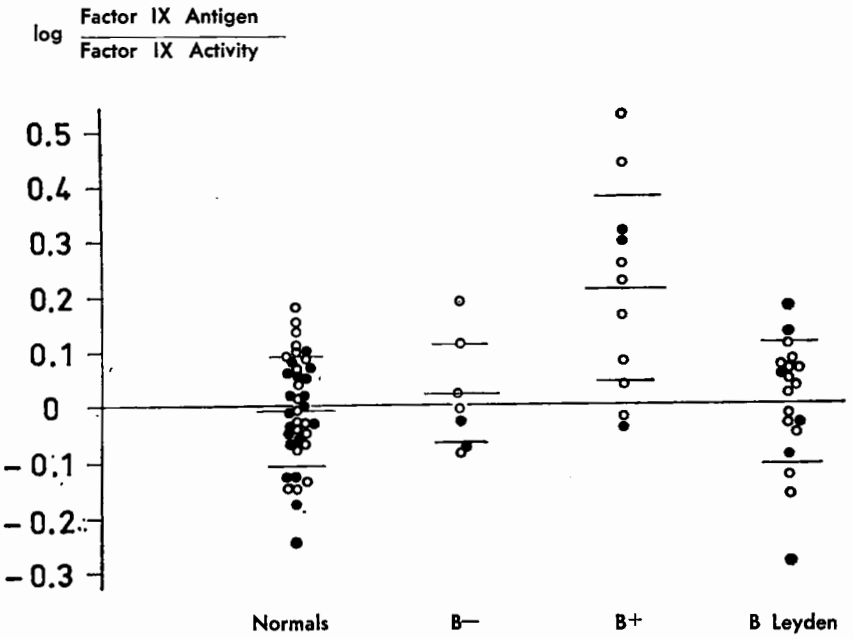

Figure 4: The log ratio of the factor IX antigen level to the factor IX activity level in normal women and obligatory carriers of hemophilia B with (●) and without (○) oral contraceptive medication. The difference between the carriers of hemophilia B+ and the three other groups is significant ($p = 0.001$).

the log ratios of all the persons examined. The average log ratio deviated significantly from zero only in the carrier group of hemophilia B+. In this group the average log ratio is 0.21 ± 0.18 (s.d.)

(antilog: 1.8) as compared to an average log ratio in normal women of -0.01 ± 0.10 (s.d.) (antilog: 1.0). The difference is definitely significant ($p = 0.001$; Student's t test). The use of oral contraceptives does not appear to influence the ratio of the factor IX antigen level to the factor IX activity level.

DISCUSSION

Hemophilia B Leyden

We were unable to prove that the factor IX activity level of hemophilia B Leyden carriers rises with the age. Neither did we establish an age-related rise in the other investigated groups. Nevertheless, the fact that 4 prepubertal carriers of hemophilia B Leyden had a mean factor IX activity level of 49% while the mean factor IX activity level of the whole group amounted to $68 \pm 26\%$ (s.d.) as compared to $55 \pm 10\%$ (s.d.) of the other carriers, points in this direction. Possibly, the factor IX level rises sharply during and shortly after puberty, just as it does in the male patients of hemophilia B Leyden. In order to give conclusive evidence more women should be examined before and after puberty.

The pill

The influence of oral contraceptives on the factor IX activity level of normal women is evident as Figure 3 shows. The analysis of variance suggests that this influence is of the same magnitude in carriers. For the purpose of carrier detection it appears rational, therefore, to compare the factor IX activity level of a potential carrier using the pill with the levels found in normal women and obligatory carriers who also use oral contraceptives. However, it is not impossible that the hormonal influence on the factor IX activity level shows a large individual and unpredictable variation, depending on variables like estrogen content of the pill, age of the woman, etcetera.

The difference between the factor IX antigen levels of users and non-users of oral contraceptives is equal to the difference between the factor IX activity levels of both groups. This applies both to normal women and carriers. Figure 4 illustrates this phenomenon in

that the ratio of the factor IX antigen level to the factor IX activity level is not influenced by the use of the pill.

The value of the assay of factor IX antigen

The value of the assay of factor IX antigen levels for the purpose of carrier detection is limited to women coming from families with hemophilia B^+ (29, 30). An excess of factor IX antigen over factor IX activity would point to carriership even if the level of factor IX activity were normal. However, in our group of 11 carriers of hemophilia B^+ an excess of factor IX antigen over factor IX activity was

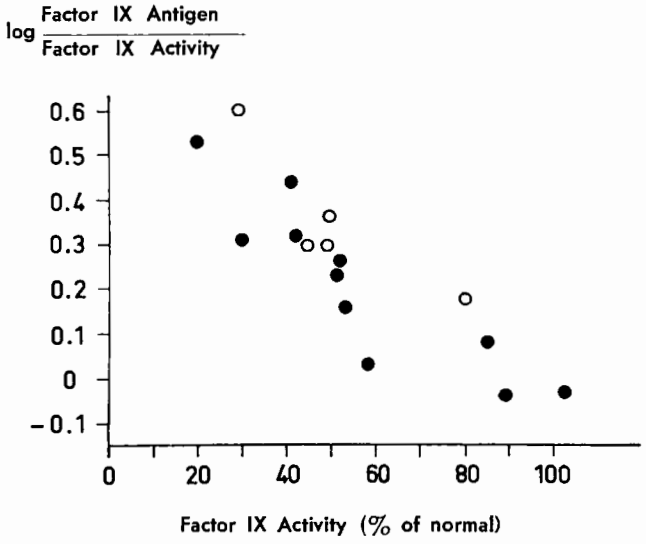

Figure 5: A plot of $\log \frac{\text{factor IX antigen level}}{\text{factor IX activity level}}$ against the factor IX activity level of 11 carriers of hemophilia B^+ (●). Open circles (o) indicate the 5 carriers described in the literature (29, 31, 33).

found only in carriers with low levels of factor IX activity and not in carriers with a high level. The 5 obligatory carriers of hemophilia B^+ described in the literature (29, 31, 33) showed the same phenomenon (Fig. 5). Apparently, a normal amount of factor IX molecules is produced in carriers of hemophilia B^+ of which a variable proportion is biologically inactive depending on the fraction of

normal X-chromosomes that has been inactivated. We may conclude that the determination of the level of factor IX antigen by means of the INA does not improve the detection of carriers of hemophilia B, even if they come from families with the B^+ variants. This situation is by no means comparable to the one in hemophilia A. In carriers of hemophilia A the level of factor VIII antigen often exceeds that of normal women (19). In hemophilia B the level of factor IX antigen represents the sum of normal and abnormal factor IX molecules while the relation of factor VIII activity to factor VIII antigen in hemophilia A is not quite as simple.

The differentiation between carriers and normal women

For practical purposes we have investigated to what extent the two laboratory parameters enabled us to differentiate between the 40 normal women and the 32 obligatory carriers over 15 years of age included in this study. Figure 6 and 7 show a graphic representation of the factor IX activity and antigen levels of all the persons who were examined. Figure 6 contains the data of the women without oral anticonception, Figure 7 those of the women with oral anticonception. In both figures tolerance ellipses have been drawn containing 90% of the values for the normal women and for the carriers (34).

We see that the overlapping area of the two ellipses in Figure 6 is considerable. Of all the examined women without oral contraconception about two-third is located within the overlap which applies equally to the normal women and to the carriers. It turns out that the values of 7 of the 21 obligatory carriers (33%) are outside the normal region. One obligatory carrier is outside the carrier region but inside the normal region and should be considered as misclassified. On the other hand, 5 out of the 20 normal women (25%) are located outside the carrier region. It follows that for 69 out of 100 possible carriers with a 50% genetic chance of carriership not using oral contraception no conclusive answer can be obtained. For women using the pill the differentiation seems to be somewhat better, as Figure 7 shows. Of the 11 obligatory carriers 7 cases (64%) are outside the normal region, while 10 of the 20 normals (50%) are outside the carrier region. This means that in a group of possible carriers with a 50% genetic chance on carriership 32%

will be identified as carrier, 25% as normal, and that 43% cannot be given a conclusive answer as to carriership or normality.

In order to find out whether the determination of the factor IX

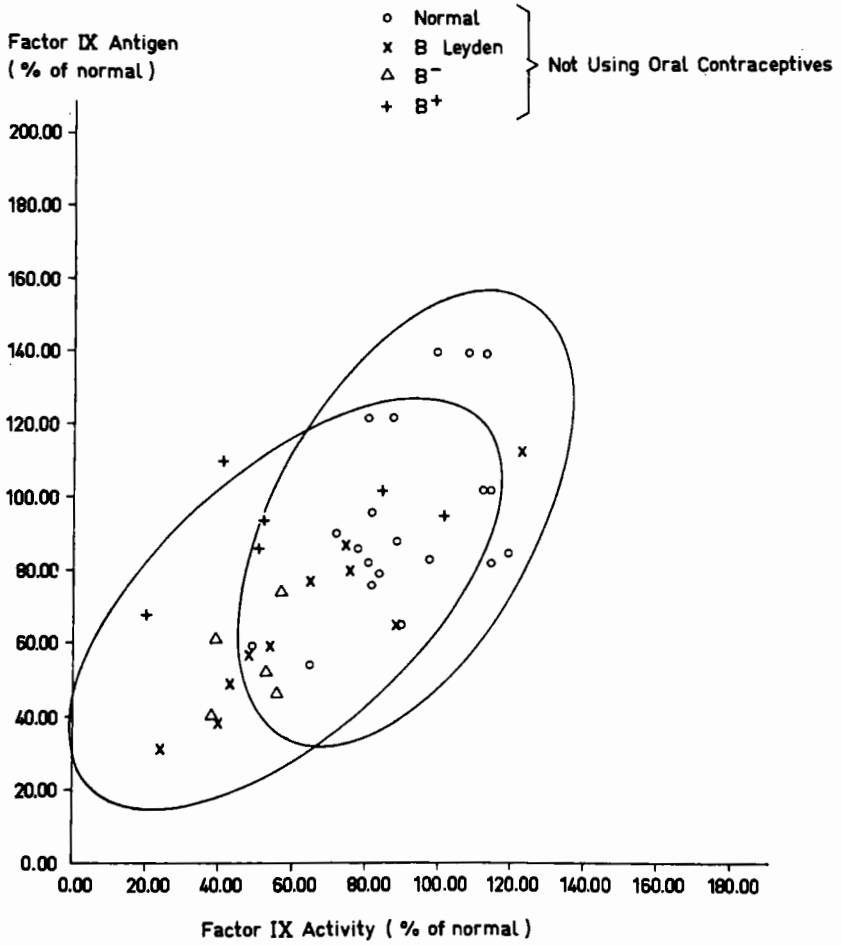

Figure 6: A plot of the levels of factor IX antigen against the levels of factor IX activity in women without oral contraceptive medication.

antigen levels yields additional information, we made the same analysis based on the factor IX activity levels only. Here 90% tolerance intervals were calculated to characterize the activity range

of each category. The results are summarized in Table IV. It can be concluded that the differentiation of true carriers and normals in a potential carrier population is not facilitated by assaying both factor IX activity and factor IX antigen by means of the INA.

The detection of carriers appears to be equally difficult for the three variants of hemophilia B. Notably we identified only 4 out of

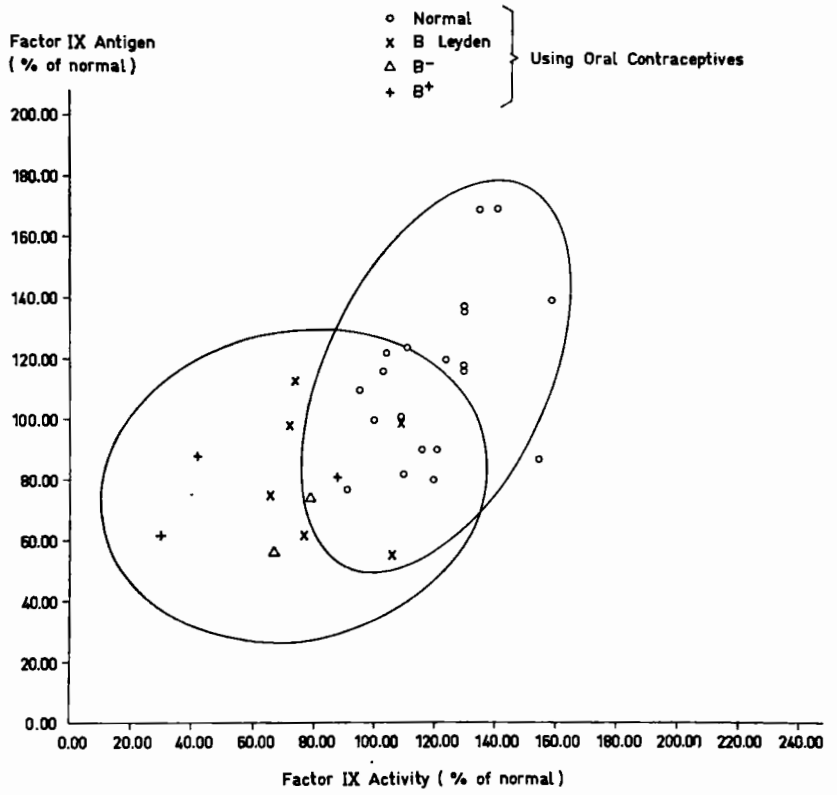

Figure 7: A plot of the levels of factor IX antigen against the levels of factor IX activity in women using oral contraceptives.

9 carriers of hemophilia B^+, the category in which we hoped to improve the detection rate by the application of the factor IX antigen assay. This outcome was expected when it appeared that with higher factor IX activities the ratio between factor IX antigen and factor IX activity decreases (Figure 5) and thereby the discriminatory power of the combined assay.

Table IV: Carrier detection by means of the factor IX activity assay with or without the factor IX antigen assay.

	no oral contraceptives			using oral contraceptives		
	both assays	versus	activity assay only	both assays	versus	activity assay only
fraction of obligatory carriers outside 90% region of normals (identified as carriers)	7/21	vs	14/21	7/11	vs	8/11
fraction of normals outside 90% region of obligatory carriers (identified as normals)	5/20	vs	6/20	10/20	vs	11/20
fraction of individuals (normals and carriers) in area of overlap (no identification)	28/41	vs	19/41	14/31	vs	12/31
misclassified as normal	1/41	vs	1/41	0	vs	0
misclassified as carrier	0	vs	1/41	0	vs	0

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CHAPTER IV

THE IN VIVO YIELD OF FACTOR IX CONCENTRATES

INTRODUCTION

The in vivo yield of factor IX concentrates is on the average less than one would expect on the basis of the factor IX activity assay in the preparation and the plasma volume of the patient studied. The molecular weight of human factor IX is 66.000, if determined by means of sedimentation equilibrium (1), which is too high to allow rapid diffusion into the extravascular compartment. Table I shows that in the literature the yield of the various factor IX concentrates in vivo is reported to be between 20 and 100% (median 36%) of the value expected on the basis of in vitro assays. The method used to prepare the concentrate does not seem to be the cause of this phenomenon, although the lower in vivo yield is most striking with DEAE cellulose preparations. Breen and Tullis (2) found a 2.5% rise of the factor IX activity level after the administration of one unit factor IX activity per kg body weight of their DEAE sephadex preparation. This would mean a complete in vivo recovery, a unique finding, which was not confirmed in 5 other studies using smilar material (3-7).

It is not quite clear whether this discrepancy between in vitro measurements and in vivo recovery also occurs when plasma is transfused. Biggs and Denson (8) state that only 30% of the transfused factor IX activity in citrated plasma is recovered in the patient's circulation, whereas Aggeler et al. (9) reported a 95% recovery of the factor IX activity that was present in the transfused plasma. Loeliger et al. (10) transfused equivalent amounts of factor IX as plasma and as factor IX concentrate (the French product PPSB) into the same patients and found that both materials had the same low yield of factor IX activity in vivo.

Several hypotheses have been put forward to account for the low

Table I: In vivo yield of factor IX preparations

investigator (first author) with reference number		starting material	adsorbent used	heparin added	in vivo yield in % of expected rise
v. Creveld	1970 (19)	cryoprecipitate supernatant	DEAE cellulose	—	20
Gilchrist	1969 (12)	Cohn fraction III	$Ca_3(PO_4)_2$	+	23
Shanbrom	1970 (21)	Cohn fraction III	$Ca_3(PO_4)_2$	+	25
Dike	1972 (20)	cryoprecipitate supernatant	DEAE cellulose	—	25
Bidwell	1967 (22)	G2 precipitate from ether fractionation of plasma	$Ca_3(PO_4)_2$	+	26
Middleton	1973 (23)	cryoprecipitate supernatant	DEAE cellulose	—	28
Bidwell	1967 (22)	precipitate P from alcohol fractionation of plasma	$Ca_3(PO_4)_2$	+	32
Bruning	1971 (11)	cryoprecipitate supernatant	$Al(OH)_3$	—	32
Biggs	1961 (24)	G2 precipitate from ether fractionation of plasma	$Ca_3(PO_4)_2$	+	32
Gunay	1973 (6)	fraction IV-I	$CA_3(PO_4)_2$	+	33
Nilsson	1971 (3)	fraction I-O supernatant	DEAE sephadex	—	36
Hoag	1969 (4)	Cohn fraction I	DEAE sephadex	—	38
Aggeler	1970 (5)	Cohn fraction I	DEAE sephadex	—	40
Dike	1972 (20)	EDTA plasma	$Ca_3(PO_4)_2$	+	40
Barrowcliffe	1973 (13)	cryoprecipitate supernatant	$Al(OH)_3$	—	44
Gunay	1973 (6)	Cohn fraction I	DEAE sephadex	—	44
Loeliger	1967 (10)	EDTA plasma	$Ca_3(PO_4)_2$	+	48
Allain	1972 (25)	EDTA plasma	$Ca_3(PO_4)_2$	+	48
Suomela	1977 (7)	cryoprecipitate supernatant	DEAE sephadex	—	48
Menaché	1963 (26)	serum	$Ca_3(PO_4)_2$	+	76
Breen	1969 (2)	resin plasma	DEAE sephadex	—	100

Authors are placed in order of the in vivo yield they found. Values are expressed in % of expected. The expected yield was calculated from volume and the activity of the transfused material in combination with the plasma volume of the patients. In the literature recoveries are mostly expressed as % rise per unit transfused per kg body weight. If no details were given we calculated the expected yields (= % rise per unit transfused per ml plasma volume) by assuming a plasma volume of 41 ml per kg body weight.

in vivo recovery of transfused factor IX activity. Loeliger et al. (10) suggested the existence of a distribution space, about twice as large as the plasma volume, in which distribution of the transfused factor IX takes place instantaneously. Others (4, 11, 12) hypothesized that part of the transfused factor IX molecules is in an activated state and therefore labile, which permits rapid elimination in vivo. Barrowcliffe (13), however, demonstrated that only 2% of the factor IX in his $Al(OH)_3$-adsorbed concentrate proved to be in an activated state. Furthermore, the addition of heparin to the concentrate to prevent activation does not appear to influence the in vivo yield of factor IX activity (6). Bruning and Loeliger (11) suggest that activated molecules might return to the inactive form, thus accounting for the relatively low activity found after transfusion. If we assume that factor IX in citrated plasma is still in its native i.e., unactivated state, both studies (8, 10) reporting identical in vivo recoveries with equivalent amounts of plasma and factor IX concentrate could plead against the activated state hypothesis.

In an attempt to test some of the above-mentioned hypotheses further, we studied the in vivo recovery of the factor IX concentrate currently in use at our institution. We also examined the factor IX concentrate for a surplus of factor IX activity as compared to the factor IX antigen concentration, which, if present, would support the activation hypothesis. Heystek et al. (12) found a substantial excess of the prothrombin activity over the prothrombin antigen levels in the concentrate, but a modification of the preparation procedure resulted in the disappearance of this discrepancy. The batches used in our study were all prepared according to this modified procedure. Furthermore, a comparison was made of the in vivo rise of factor IX activity and factor IX antigen levels after transfusion. Finally we carried out in vitro mixing experiments to see whether an important fraction of added factor IX molecules was adsorbed onto the blood cells in hemophilic blood, explaining the low recovery in the plasma compartment.

MATERIALS AND METHODS

Coagulation methods and the determination of the factor IX antigen level have been described in Chapter I. Factor IX concen-

trates were obtained from the Central Laboratory of the Netherlands Red Cross Blood Tranfusion Service (Amsterdam), where it was prepared from cryoprecipitate supernatant by DEAE Sephadex adsorption and elution as described by Heystek et al. (14, 15). Various batches of this material were used during the years 1974, 1975, and 1976. The transfusion studies were carried out in patients with hemophilia B^-, B^+, and B Leyden, and in patients with coumarin-induced hypocoagulability. The concentrate was administered by syringe or intravenous drip within 15 minutes. A venous blood sample was drawn from the other arm 5 to 10 minutes after termination of the transfusion.

In the in vitro mixing experiments a certain volume of the concentrate was incubated with the citrated blood of a patient with hemophilia B^- or B^+. The respective volumes were chosen in such a way, that in the case of a 100% recovery the rise of the factor IX level in the plasma had to be about 50% of a normal plasma level. The mixture was incubated for 10 minutes at 37°C. After incubation the factor IX activity level of the plasma was assayed. The "in vitro yield" was calculated from the plasma volume of the blood sample and from the amount of factor IX added. The plasma volume of the blood sample was calculated from the hematocrit and the volume of the blood sample.

Patient samples and samples of the transfused material were stored at —70°C and tested later. The plasma volume of the patients was estimated from their blood volume and body hematocrit. The blood volume was read from the nomogram of Dagher et al. (16) given the age, sex and body weight of the patient; the body hematocrit was calculated from: venous Ht x 0.88 = body Ht (17).

RESULTS

The average recovery of factor IX activity in 27 transfusion studies with factor IX concentrate was 70 ± 21% (s.d.) of the expected value.

If patients are divided in CRM-positive (hemophilia B^+ and coumarin treatment) and CRM-negative (hemophilia B^- and hemophilia B Leyden), we find a significant difference between the average recoveries in both groups. The average recovery of 21

transfusions of factor IX in CRM-positive patients is 76 ± 19% (s.d.) as compared to a recovery of 51 ± 17% (s.d.) after 6 transfusions in CRM-negative patients ($p < 0.01$; Student's t test).

We did not find a discrepancy between the factor IX antigen level and the factor IX activity level in the concentrates. The average factor IX antigen level in 14 samples of several batches of the concentrate was 21 ± 4 U/ml (s.d.), while the average factor IX activity level was 19 ± 4 U/ml (s.d). The difference is not significant ($p > 0.10$; paired t test).

The rise of the factor IX activity levels measured after transfusion of the concentrate was not different from the rise of the factor IX antigen levels. The average rise of the factor IX activity level after 14 transfusions was 29 ± 4% (s.e.m.) as compared to an average rise of the factor IX antigen level of 28 ± 4.5% (s.e.m.). In the paired t test the difference was not significant ($p > 0.10$).

The average yield of factor IX activity in 14 in vitro mixing experiments was 107 ± 27% (s.d.). The difference between the in vivo yield and the "in vitro yield" is significant as shown in Figure 1 ($p < 0.001$; Student's t test).

DISCUSSION

The in vivo yield of factor IX transfusions presented here is higher than most authors have reported in the literature. The following factors might account for this outcome.

In the first place, the assay of factor IX activity in concentrates is critical in the sense that proper dilutions have to be used in order to avoid extrapolation on the graph for the conversion of clotting times into activity, which otherwise might lead to overestimation of the activity contained in the concentrate. We found on the average 19 U/ml in the concentrate we used, whereas its producers mention 20-25 U/ml in the instructions for use. This could be explained by previous findings included in a study for the testing of a putative WHO-standard of factor IX activity (18). It appeared that the determinations of the factor IX activity in a concentrate by 15 different laboratories showed a range from 4.57 to 6.80 arbitrary units. Therefore, the difference between the 19 U/ml we found in the concentrate and the 20-25 U/ml as given by its producers is

probably not significant. In the second place, the material was frozen and stored at —70°C before determining the factor IX activity and the factor IX antigen level in the concentrate. Although

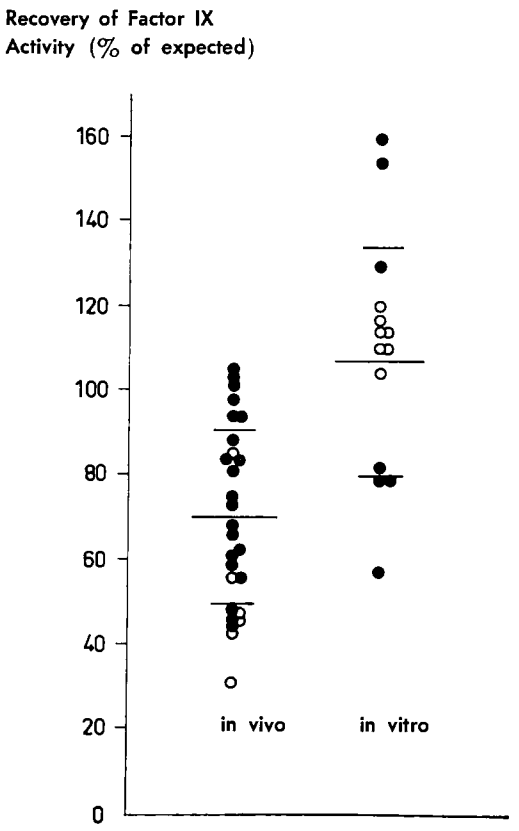

Figure 1: The recovery of factor IX activity in 27 transfusion studies with factor IX concentrate and in 14 mixing experiments. CRM-negative patients have been indicated by open circles (●) and CRM-positive patients by closed circles (○).

factor IX activity is rather stable, it is possible that as a result of the freezing and subsequent thawing the factor IX activity level in the concentrate diminishes. If this is an important factor, we would expect excess factor IX antigen over factor IX activity, as

Heystek et al. (14) described for prothrombin. Moreover, it appears from Heystek's article that omitting freezing during the production phase causes only a slight rise of the factor IX activity level — from 29.1 U/ml to 31 U/ml, indicating the relative unimportance of freezing for loss of activity of factor IX. The third and most interesting factor which is related to the higher yield we found, is the fact that most of the transfusions were given to patients with hemophilia B^+ and patients on coumarin treatment. In patients with hemophilia B^- we found a significantly lower yield of the transfusions than in patients with hemophilia B^+ or in patients with oral anticoagulant therapy, both categories of patients with significant amounts of factor IX antigen in their circulation. In Figure 2 the in vivo yield of factor IX activity is plotted against the pre-transfusion plasma level of factor IX antigen. There is a significant ($p < 0.025$) albeit rather low correlation ($r = 0.56$) between the two parameters. These findings correspond with an observation in our laboratory by Bruning and Loeliger (11), who found a 100% in vivo recovery of factor IX activity after transfusion of Prothrombal, their home-made factor IX concentrate, in a patient with hemophilia B_M. This result is also in agreement with observations of van Creveld et al. (19) and Dike et al. (20). These investigators found a lower in vivo yield of factor IX activity after first transfusions than after subsequent transfusions of factor IX concentrates, although the difference was not significant. Their observations concerned patients who received several transfusions within a short time because of tooth extractions and spontaneous or traumatic bleeding.

Although the loss of transfused factor IX molecules is somewhat smaller than most authors reported in the literature, the phenomenon is fairly constant. Our results provide a tentative solution for this problem. The fact that we do not find a discrepancy, as it can be observed in serum, between the levels of factor IX activity and antigen in the concentrates used in this study, pleads strongly against the presence of activated coagulation factors. It may well be that the presence of activated coagulation factors, which are, presumably, rapidly eliminated from the bloodstream, is partly responsible for the low yield obtained with other preparations. In view of our findings it is unlikely that this is the only or the most

important explanation. If we assume, as Loeliger et al. (10) do, that the distribution space for transfused factor IX molecules is larger than the plasma volume, the question arises what the anatomi-

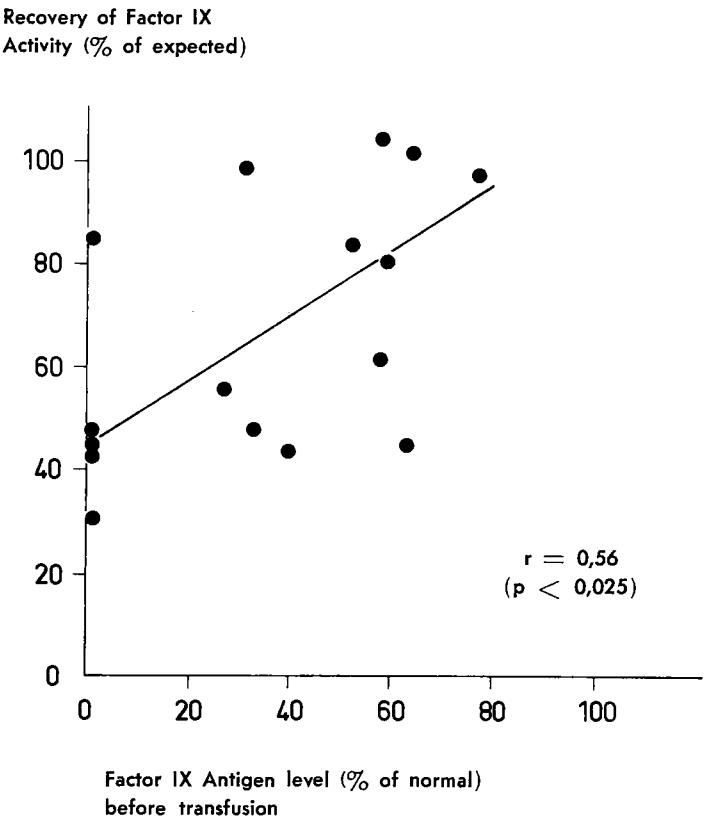

Figure 2: A plot of the recovery of factor IX activity against the level of factor IX antigen before transfusion in 16 transfusions of factor IX concentrate.

cal substrate of this space is. It appears from our mixing experiments that the factor IX molecules are not absorbed by the cellular elements of the hemophilic blood. The endothelial lining of the vascular system is the surface onto which factor IX molecules could very well be adsorbed. Immunofluorescent examination of the

endothelium, which might demonstrate the presence of factor IX molecules, is not yet possible. In view of the positive correlation between the pre-transfusion factor IX antigen level and the in vivo recovery and our finding of a significantly lower in vivo yield of factor IX activity in CRM-negative patients it is tempting to suppose that the "endothelial reservoir" or any other hypothetical space is empty in the case of hemophilia B^- and more or less saturated with factor IX antigen in the case of hemophilia B^+.

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SUMMARY

In this thesis a method is described for the immunological assay of clotting factor IX. The test is based on the principle that antibodies against factor IX molecules are bound by these molecules. This binding is not related to the biological coagulation activity of these molecules. As a consequence molecules lacking procoagulant activity (cross-reacting material, CRM) can also be demonstrated. The antiserum used in this assay was produced by immunizing rabbits with a preparation containing the clotting factors II, VII, IX, and X. The serum was not specifically directed against factor IX, but it also displayed activity towards factor VII. The poor specificity and the absence of a precipitation reaction with factor IX rendered the serum unfit for use in an electrophoretic assay, but it could be applied in an inhibitor neutralization assay.

By means of this assay the factor IX-CRM levels where determined in 35 patients with hemophilia B from 20 unrelated families. In 9 out of the 20 families we did not find a discrepancy between the levels of factor IX activity and factor IX-CRM (hemophilia B^-). Identical results were obtained when a human inhibitor of factor IX was used. Moreover, 21 samples were tested elsewhere in an electrophoretic assay by means of a specific antiserum against factor IX, which yielded the same results as the inhibitor neutralization assay. On this basis it seems probable that hemophilia B^- cannot be considered to be an artifact of laboratory techniques. We could not confirm the results of some investigators who found only one hemophilia B^- family among 19 families (see Chapter II). A small proportion of patients with hemophilia B^+ show a prolonged thromboplastin-time with ox-brain thromboplastin (hemophilia B_M). It is probable that defective factor IX molecules act as inhibitors. However, it has been claimed that abnormalities in the reactivity of factor VII in some patients with hemophilia B_M are responsible

for the prolonged thromboplastin-time. The reactivity of factor VII in our patient with hemophilia B_M was found to be normal.

In a study of 40 normal women and 37 obligatory carriers from 14 hemophilia B families, we investigated whether carrier detection could be improved if, in addition to the factor IX activity level and the genetic chance of the woman to be a true carrier, the factor IX antigen level was taken into consideration as well. Furthermore, we tried to establish whether age or the use of oral contraceptives exert any influence on carrier detection. Age had no influence on the levels of factor IX activity and antigen in the group of carriers, although it is likely that during puberty the factor IX activity level in carriers of hemophilia B Leyden rises sharply. The use of oral contraceptives has a definitive influence on factor IX activity and antigen. The average factor IX activity level in the normal women was 92% against 121% in the group who used the pill. A similar difference was found in the levels of factor IX antigen. As to the 37 carriers, we also found significantly higher levels in the group using oral contraceptives than in the other group. This finding is important for future carrier detection programs. As a reference one should consider the values found in normal women and obligatory carriers, subdividing each group in those using the pill and those who do not. The laboratory values of a potential carrier should then be compared to those of the normal women and the obligatory carriers. The reference groups are chosen in accordance with the potential carrier taking oral contraceptives or not. On the basis of factor IX activity assays we may expect to identify with 95% confidence 27 normal women and 36 carriers out of a population of 100 potential carriers all using oral contraceptives and with a genetical chance on carriership of 50%. As to women who do not use the pill, the number that may be identified is smaller. When the level of factor IX antigen is considered in combination with the factor IX activity level, the number of women that can be identified is not higher than after determining the factor IX activity level only.

Finally, we tried to find an explanation for the recurrent phenomenon that after transfusion of factor IX concentrates less factor IX activity is observed in the patient's plasma than one would expect on the basis of the transfused amount. We did not find any discrepancy between the levels of factor IX activity and antigen in

the concentrates. This could be, apart from other considerations, an argument against activation and instability of the transfused factor IX as an explanation for the low yield. Yet, in patients with hemophilia B^+ a higher yield was found than in patients with hemophilia B^- (76%, respectively 51% of the yield expected). Moreover, a significant positive correlation could be demonstrated between the level of factor IX antigen before the transfusion and the yield of a transfusion. As the molecular weight of factor IX (66,000) is too high to cause a rapid diffusion from the capillaries, the explanation for the low yields might be sought in adsorption of the transfused molecules onto membranes which are in contact with the plasma. From the relation between yield and factor IX antigen levels it might be deduced that these membranes in patients with hemophilia B^+ and in normals are saturated, partly or completely, by factor IX molecules. Mixing experiments in vitro showed that factor IX molecules are not absorbed by the cellular elements in the blood of hemophilia B patients. It is possible, that the factor IX molecules are absorbed by the endothelium.

SAMENVATTING

In dit proefschrift wordt een methode beschreven voor de immunologische bepaling van stollingsfactor IX. De test berust op het feit dat antilichamen tegen factor IX moleculen door deze moleculen worden gebonden. Deze binding is niet afhankelijk van de biologische stollingsactiviteit van de factor IX moleculen zodat ook inactieve moleculen (cross-reacting material, CRM) kunnen worden aangetoond. Het voor deze proef benodigde antiserum werd opgewekt door immunisatie van konijnen met een preparaat waarin de stollingsfactoren II, VII, IX en X aanwezig waren. Het antiserum was dan ook niet specifiek tegen factor IX gericht, doch toonde ook activiteit tegen factor VII. Het gebrek aan specificiteit en het ontbreken van een herkenbare precipitatie reactie met factor IX maakte dat dit serum niet in een electroforetische bepaling kon worden gebruikt, maar wel in een inhibitor neutralisatie proef.

Met behulp van deze bepalingsmethode werd de factor IX-CRM spiegel bepaald bij 35 hemofilie B patiënten uit 20 families. In 9 van de 20 families vonden wij geen wezenlijk verschil tussen de spiegels van factor IX-CRM en factor IX activiteit (hemofilie B^-). Dezelfde uitkomst werd verkregen wanneer een humaan antilichaam tegen factor IX werd gebruikt en ook wanneer de bepaling werd verricht met een precipiterend specifiek antilichaam in een electroforetische bepaling. Op grond hiervan lijkt het waarschijnlijk dat hemofilie B^- niet kan worden beschouwd als een artefact van de gebruikte techniek. Wij konden de lage frequentie van de hemofilie B^-, slechts één op 19 families zoals gevonden door Franse onderzoekers, niet bevestigen. Sommige patiënten met hemofilie B^+ vertonen een verlengde thromboplastinetijd indien de test met runderhersen thromboplastine wordt uitgevoerd (hemofilie B_M). Waarschijnlijk is het defecte factor IX molecuul verantwoordelijk voor remming van de activiteit van dit thromboplastine. In de literatuur wordt melding gemaakt van enige hemofilie B_M patiënten bij

wie een afwijking van factor VII verantwoordelijk zou zijn voor de verlengde thromboplastinetijd. Bij onze patiënt met hemofilie B_M werd zo'n afwijking van factor VII niet gevonden.

In een onderzoek van 40 normale vrouwen en 37 obligate draagsters uit 14 hemofilie B families werd nagegaan of het aantonen van draagsterschap gemakkelijker wordt indien naast de genetische kans en het factor IX activiteitsniveau ook de plasmaspiegel van factor IX antigeen in de kansberekening wordt betrokken. Verder werd onderzocht in hoeverre de leeftijd en het gebruik van orale anticonceptiva van invloed zijn op het draagsteronderzoek. De niveaus van factor IX activiteit en antigeen werden in het onderzochte patiëntenmateriaal niet door de leeftijd beïnvloed. Wel echter geldt waarschijnlijk voor draagsters van hemofilie B Leyden dat in de puberteit een vrij plotselinge stijging optreedt van de factor IX activiteit en antigeen spiegels. Het gebruik van orale anticonceptiva heeft een significante invloed op de spiegels van factor IX activiteit en antigeen. De gemiddelde factor IX activiteitsspiegel van normale vrouwen bedroeg 92% bij hen die geen oraal anticonceptivum gebruikten en 121% bij de vrouwen die dat wel deden. Een dergelijk verschil werd eveneens voor de factor IX antigeen spiegels gevonden. Ook bij de 37 draagsters vonden wij significant hogere waarden in de groep die orale anticonceptiva gebruikte. Deze bevinding is van betekenis voor het draagsteronderzoek. Als referentie dient men gebruik te maken van de waarden gevonden bij normale vrouwen en obligate draagsters en een scheiding aan te brengen tussen hen die de pil gebruiken en degenen die dat niet doen. Een te onderzoeken mogelijke draagster dient dan te worden vergeleken met één van beide referentiegroepen al naar gelang zij wèl of niet de pil gebruikt. Op grond van factor IX activiteitsbepalingen mag worden verwacht dat van 100 mogelijke draagsters met een a priori kans op draagsterschap van 50% die gebruik maken van orale anticonceptiva, 27 vrouwen kunnen worden geïdentificeerd als normaal, 36 als draagster en dat aan de rest van hen geen uitsluitsel kan worden gegeven met meer dan 95% betrouwbaarheid. Voor vrouwen die geen orale anticonceptiva gebruiken is het aantal dat kan worden geïdentificeerd kleiner. Wanneer zowel de spiegels van factor IX activiteit als van antigeen in de beschouwing worden betrokken is het aantal te identificeren

vrouwen niet groter dan wanneer alleen de activiteit als maatstaf wordt gebruikt.

Tenslotte werd gezocht naar een verklaring voor het bekende verschijnsel dat na transfusie van factor IX concentraten minder factor IX activiteit in het plasma van de patiënt wordt teruggevonden dan verwacht mag worden op grond van de getransfundeerde hoeveelheid. Wij vonden geen discrepantie tussen de spiegels van factor IX activiteit en antigeen in de concentraten. Naast een aantal andere overwegingen is dit een argument tegen activatie en labiliteit van het getransfundeerde factor IX als verklaring voor de lage opbrengsten. Wel werd bij patiënten met hemofilie B^+ een hogere opbrengst gevonden dan bij patiënten met hemofilie B^- (76% resp. 51% van de verwachte opbrengst). Bovendien bleek er een significante positieve correlatie te bestaan tussen de spiegel van factor IX antigeen vóór de transfusie en de opbrengst van een transfusie. Daar het molecuul gewicht van factor IX (66.000) te groot is om een zeer snelle diffusie uit de capillairen mogelijk te maken, moet de verklaring voor de geringe opbrengsten wellicht worden gezocht in adsorptie van de getransfundeerde moleculen aan membranen die met het plasma in contact zijn. Het verband tussen opbrengst en factor IX antigeen spiegels suggereert dat deze membranen bij patiënten met hemofilie B^+ en bij normalen geheel of gedeeltelijk met factor IX moleculen zijn bezet. Uit mengproeven in vitro kan niet worden geconcludeerd dat factor IX moleculen geabsorbeerd worden door de cellulaire elementen in het bloed van hemofilie B patiënten. Mogelijk worden de factor IX moleculen door endotheel geabsorbeerd.

GLOSSARY OF TERMS AND ABBREVIATIONS

CRM-negative	—	plasma in which the immunologically estimated level of a protein and the level of its biological activity are equal.
CRM-positive	—	plasma in which a significant excess is found of the immunologically estimated level of a protein over the level of its biological activity.
factor IX activity	—	clotting factor IX measured by its biological activity in the blood clotting mechanism.
factor IX antigen	—	factor IX protein measured by an immunological assay, is therefore a synonym for factor IX-CRM i.e., material that produces a cross-reaction with antibodies against factor IX.
factor IX inhibitor	—	antibody against factor IX present in the plasma of approximately 5% of patients with severe hemophilia B after treatment with factor IX transfusions.
hemophilia B—	—	variant of hemophilia B in which the plasma is CRM-negative.
hemophilia B+	—	variant of hemophilia B in which the plasma is CRM-positive.
hemophilia B Leyden	—	variant of hemophilia B in which the bleeding symptoms disappear after puberty along with a gradual increase of both factor IX activity and antigen level.
hemophilia B_M	—	variant of hemophilia B+ in which a prolonged ox-brain thromboplastin-time is found.

INA	—	inhibitor neutralization assay.
obligatory hemophilia carrier	—	a women who is definitely a carrier of hemophilia on the basis of her family history i.e.: a daughter of a hemophiliac or a woman with hemophilic relatives who has at least one hemophilic son, or a woman who has two or more hemophilic sons.
PIVKA	—	*P*rotein *I*nduced by *V*itamen *K* *A*bsence (or in the presence of *V*itamin *K* *A*ntagonist); biologically inactive precursor of the vitamin K dependent clotting factors (II, VII, IX, X) that can be demonstrated by immunological assays.

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