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Monoclonal Antibodies to Coagulation Factor IX Define a High-Frequency Polymorphism by Immunoassays

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SUMMARY

Monoclonal antibodies have been used to demonstrate a polymorphism of human plasma coagulation factor IX antigen in double antibody solid-phase immunoradiometric assays. This polymorphism is detected in an assay where a monoclonal antibody (A-1) adsorbed to microtiter wells is used to bind factor IX from diluted plasma samples. Plasma samples with the factor IX polymorphism have less than 0.2 U/ml of apparent antigen when tested with the A-1 antibody, while assays with other monoclonal antibodies and assays with goat antisera to factor IX show normal amounts of factor IX antigen. Factor IX coagulant activity was normal in samples from donors with the polymorphism. The thin-layer polyacrylamide gel isoelectric focusing pattern of factor IX purified from a donor with the factor IX polymorphism (IXp) was identical to that obtained with factor IX prepared from a donor who did not have the polymorphism (IXn). Purified radiolabeled factor IX prepared from a donor with the polymorphism showed a Ka for the A-1 antibody that was threefold less than that measured for IXn. The gene frequency of IXp in male blood donors is 0.25. This polymorphism may be useful as a marker for the X chromosome in genetic studies on plasma samples. Further studies are necessary to determine the explanation for decreased reaction of IXp with the A-1 monoclonal antibody.

INTRODUCTION

Deficiency of coagulation factor IX, hemophilia B, causes a hemorrhagic disease that is clinically indistinguishable from hemophilia A (factor VIII:C

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deficiency). Factor IX is coded by a single copy of a 34-kilobase (kb) gene on the q26-qter region of the X chromosome [1, 2]. A restriction site polymorphism, *Taq*I, can be demonstrated on genomic DNA [1, 3, 4]. The less frequent allele (1.3-kb fragment) can be found in 0.29–0.40 of samples [1, 3, 4]. This restriction site polymorphism is on the 5′ region of genomic DNA, in a region near an exon specific for residues 47–84 of factor IX [3, 5]. The *Taq*I polymorphism, however, is probably intronic.

Previous studies have described plasma factor IX polymorphisms. Isoelectric focusing variants have been found in factor IX purified from normals [6] and genetic variation in factor IX activity levels has been described in population studies [7]. A recent abstract has described a 4:1 threonine:alanine polymorphism in normal factor IX at residue 148 [8].

Monoclonal antibodies have been raised to human coagulation factor IX for immunoassays and for use in purification of factor IX [9]. In the course of these studies, samples from some normal donors were found to have decreased reaction with the A-1 monoclonal antibody to factor IX when different combinations of monoclonal antibodies and goat antisera to factor IX were used in factor IX immunoassays. Coagulant activity from donors with the polymorphism (IXp) was normal. The studies presented here describe the detection of this polymorphism with immunoassays. The frequency of the polymorphism in male and female blood donors has been determined.

MATERIALS AND METHODS

Monoclonal Antibodies and Antisera

Monoclonal antibodies to human coagulation factor IX were prepared and partially purified as described [9]. Antibodies A-1, A-4, and A-5 react with the heavy chain (residues 181–415) of activated factor IX (IXa) in immunoblotting experiments while the A-7 antibody reacts with the light chain (residues 1–145) of factor IXa [9]. Residues 146–185 comprise the activation peptide region of the molecule.

Goat antibody to factor IX was produced by injecting a female goat with 200 μg of factor IX in complete Freund's adjuvant (Difco, Detroit, Mich.) followed by injections at 30 and 60 days with 100 μg factor IX in incomplete adjuvant (Difco). Affinity purified goat antihuman factor IX was prepared by passing antisera over factor IX coupled to agarose (Sepharose 4B, Pharmacia, Piscataway, N.J.) by cyanogen bromide technique [10] at a concentration of 1 mg factor IX per ml of packed gel. Bound antibody was eluted with 6 M guanidine in 0.15 M NaCl-0.01 M Tris HC1 (TBS), pH 7.2, and dialyzed against TBS overnight.

Samples for Immunoradiometric Assay

Plasma samples for the pooled plasma standard and family studies were drawn in 3.8% trisodium citrate at a 1:9 ratio of anticoagulant to blood. Plasma samples and normal pooled plasma were processed as described [11] and stored at −70°C. Samples obtained from New Mexico blood donors were collected in a 1:7.4 ratio of CPDA-1 to blood. Plasma was obtained from blood centrifuged at 5,000 *g* for 8 min at 4°C. Samples were stored at −70°C. Factor IX clotting activity was measured as described [9].

Immunoradiometric Assays for Factor IX

Monoclonal antibodies and polyclonal antibodies to factor IX were used for immunoradiometric assays in a modification of methods described [9]. Partially purified

monoclonals were diluted in 0.05 M $NaHCO_3/Na_2CO_3$, pH 8.6, to a concentration of 0.025 mg/ml. Polystryene microtiter wells (Immulon I, Dynatech, Alexandria, Va.) were filled with 0.10 ml of the antibody solution, and plates were stored overnight at 4°C. The plates were then washed twice with 200 μl of TBS per well. Nonspecific binding was blocked by coating the plates with 3% ovalbumin (Sigma Crude Ovalbumin, Grade II, Sigma, St. Louis, Mo.) and 0.01% sodium azide in TBS for 4–6 hrs at room temperature. Plates were again washed twice with TBS. A normal curve was constructed on each plate with dilutions of pooled normal plasma (from 1:20 to 1:400) from 23 donors in TBS-0.1% ovalbumin (Sigma Grade III). One-tenth ml amounts of normal plasma and tested plasma were added in triplicate to microtiter wells, and plates were incubated for 2 hrs at 37°C and overnight at 4°C. Since the A-7 antibody reacts with a Ca^{2+} dependent epitope, plates were washed five times with TBS containing 5 mM $CaCl_2$, prior to addition of 0.1 ml of radiolabeled monoclonal antibody in TBS-0.1% ovalbumin with 5 mM $CaCl_2$ containing a minimum of 1×10^5 cpm. The antibody specific activity was 4 μCi/μg after the chloramine-T labeling procedure. After 6-hrs incubation at room temperature, plates were washed six times with TBS containing 5 mM $CaCl_2$ prior to counting bound radioactivity in wells separated from the plates. In some experiments, affinity purified goat antihuman factor IX was used in the solid phase at a concentration of 0.5 μg/ml.

Data were analyzed by fitting the standard curve by a polynomial least-squares method using an Apple IIe computer and software from Interactive Microware, State College, Pa.

Purification of Factor IX from Samples of Plasma from Individual Donors

Factor IX was purified from plasma obtained from individuals for study of reactions with monoclonal antibodies. Plasma was collected in a 500-ml 2-unit plasmapheresis from one donor using 0.14 M sodium citrate in a ratio of 1:10 anticoagulant to blood, while 3,000 ml of plasma was obtained from another individual donor after therapeutic plasmapheresis for polyneuritis. During therapeutic plasmapheresis, the anticoagulant was ACD-A at a 1:14 ratio to blood. Procedures for plasma collection were approved by the Human Subjects Committee of the University of New Mexico School of Medicine.

Benzamidine-HCl (Aldrich, Milwaukee, Wis.) and PMSF (phenylmethylsulfonylfluoride, Sigma) were added to plasma at final concentrations of 4 mM and 0.1 mM, respectively. A barium citrate precipitate was obtained by adding 17.48 g of $BaCl_2 \cdot 2H_2O$ per liter of plasma. After stirring for 1 hr at 4°C, the precipitate was recovered by centrifugation at 5,000 *g* for 15 min and washed with 0.15 M $BaCl_2$ containing the same inhibitors as the plasma. Protein was eluted from the barium citrate precipitate twice with 0.15 M trisodium citrate-0.05 M Tris, pH 7.5, with proteolytic inhibitors. The final volume of the eluate after two elutions was one-third of the starting plasma volume. Sodium EDTA, pH 7.5, was added to the eluate at a final concentration of 0.01 M, and the eluate was dialyzed overnight against 0.15 M NaCl-0.025 M trisodium citrate-0.01 M Tris, pH 7.2, containing inhibitors. The dialyzed eluate was passed over a 20-ml immunoaffinitiy column of monoclonal A-4 coupled to Sepharose 4B by cyanogen bromide technique at a concentration of 3 mg/ml antibody packed gel. The column was washed with 2 column volumes of 0.5 M NaCl-0.01 Tris, pH 7.2, with 0.1% Tween 20 and then with 2 column volumes of the same buffer without Tween. Bound material was eluted with 6 M guanidine in TBS and 1 mM benzamidine. Samples were recovered from the eluate by immediate gel filtration over Sephadex G-25 in TBS with 1 mM benzamidine. Samples were greater than 90% pure by SDS-PAGE. There was no detectable prothrombin in these preparations, but apparent contamination with factor X and protein C could be detected by quantitative immunodiffusion at a level of less than 0.5% of the total protein.

Competitive Binding Experiments

Polystyrene microtiter wells were coated as described above but overnight incubation with mixtures of antigen and radiolabled antigen was at 4°C. Purified factor IX from

plasma donors with and without the factor IX polymorphism was radiolabeled with the chloramine-T technique. One × 10^5 CPM of labeled factor IX was added to wells containing increasing amounts of purified factor IX diluted in TBS-0.1% ovalbumin. Mixtures were incubated overnight at 4°C and then plates were washed and bound antibody counted as described above for the immunoradiometric assay.

Isoelectric Focusing

Thin (0.4 mm) 5% polyacryamide gels were prepared using ampholytes and a gel casting device from Bio-Rad (Richmond, Calif.) following the manufacturer's directions. Two- to 5-μg samples were applied after being treated with 10 mM sodium EDTA followed by dialysis against 1% glycine. Focusing was done at 6 W for 1 hr with limits of 2,500 V and 15 mA after plates had been prefocused at 3 W with limits of 2,200 V and 15 mA. Markers were used for calibration of the gel (Isoelectric Focusing Calibration Kit, Pharmacia). Gels were fixed with 5% sulfosalicilic acid in 10% trichloro acetic acid and stained with methanol:acetic acid:H_2O 3:1:6 with 0.2% R-250 Coomassie Brilliant Blue, then destained with methanol:acetic acid:H_2O 3:1:6.

Antibody Titration Curves

Polypropylene tubes of 0.3 ml (Sarstadt, Princeton, N.J.) were used to study binding to monoclonal antibodies in the fluid phase. Triplicate samples of 0.025 ml of monoclonal antibody dilution in TBS-3% ovalbumin, 0.025 ml of diluted antigen in TBS-3% ovalbumin, and 0.05 ml of radiolabeled factor IX in TBS-1% heat-inactivated mouse serum were incubated at 37°C for 1 hr and then overnight at 4°C. Fifty microliters of 1:8 dilution of goat antimouse IgG (Pel-Freeze Biologicals, Rogers, Ark.) in TBS was added, and after further incubation at room temperature for 4 hrs. tubes were centrifuged in a Beckman Microfuge B (Beckman, Palo Alto, Calif.) at 11,000 *g* for 5 min. Precipitated radioactivity was determined, and binding in the absence of monoclonal antibody was subtracted.

Population Studies of the Factor IX Polymorphism

Plasma samples were analyzed from 118 blood donors in New Mexico. A presumptive classification of the factor IX antigenic phenotype was made by comparing two different combinations of monoclonal antibodies in the immunometric assay described above. The antibodies used in the solid phase, A-1 and A-4, both react with the heavy chain of factor IXa. In both combinations, the A-7 antibody that reacts with the light chain of factor IXa was used as the radiolabled antibody. Samples were classified as having the polymorphism (IXp) when they had the generally undetectable (< 0.17 U/ml) antigen in the assay system when the diluted samples were added to A-1–coated microtiter wells at 1:33 and 1:66 dilutions. These samples had normal levels of antigen when added to plates coated with the A-5 antibody, as discussed below.

Since classification of male donors as normal (IXn) or (IXp) was straightforward, the relationship between the A-1 solid phase [(A-1)–(A-7)] assay and the A-5 solid phase [(A-5)–(A-7)] assays on the same samples was analyzed by linear regression for 43 male donors without the IXp polymorphism [12]. Ninety-five percent confidence limits from this analysis were used to classify antigen results on 61 female blood donors where classification between heterozygotes (IXp, IXn) and homozygotes (IXn, IXn) was less clear. Identical classification results were obtained with 95% confidence limits for ratios of A-1/A-5 assay results or 95% confidence limits from linear regression analysis.

The A-4 antibody that reacts with the heavy chain of factor IXa ws substituted for the A-5 antibody in some initial experiments as the solid-phase antibody. Confidence limits for the IXp and IXn gene frequencies were calculated using the binomial distribution [13].

RESULTS

Solid-Phase Immunoradiometric Assays

During preliminary studies on factor IX antigen level in normal blood donors and patients with hemophilia B, samples from three normal male donors had low levels of factor IX antigen in comparison to factor IX coagulant activity when plasma samples were tested with the combination of the A-4 monoclonal antibody in the solid-phase and radiolabeled A-1 monoclonal antibody. Antigen and activity levels on these three samples and a fourth male donor later classified by IXp are shown in table 1. These results suggested that reaction with the A-1 antibody was attenuated in some plasma samples. Repeat samples were obtained from two of the male donors, and further testing confirmed the original results. When the antigen content in these plasma samples was tested with the A-1 antibody radiolabeled, there was only a modest reduction in the antigen content compared to assays using other monoclonals or goat antisera. The mean antigen content of nine plasmas without the factor IX polymorphism was 1.13 U/ml with the A-7 antibody radiolabeled and 1.06 with the A-1 antibody as the labeled antibody (table 1). There was no difference in mean antigen level of these samples by paired t-test ($t = 1.68$, 8 df) for immunoassays including or not including A-1. When samples from four male donors with the IX polymorphism were assayed using the A-4 antibody in the solid phase and using A-7 or A-1 as the labeled antibodies, there was a reduced mean antigen content with means of 0.64 for the [(A-4)–(A-1)] combination and 0.82 for the [(A-4)–(A-7)] combination. This difference in means was significant at $P < .05$ ($t = 5$, df = 3). Clotting activity was normal in samples with the A-1 polymorphism.

In initial studies, the A-4 antibody was absorbed to polystyrene plates and the A-1 antibody was radiolabeled. When combinations of available monoclonal antibodies were used to test samples from IXp donors and the solid-

TABLE 1

FACTOR IX COAGULANT ACTIVITY U/ml AND ANTIGEN CONTENT WITH SOLID-PHASE A-4 AND RADIOLABELED A-1 OR A-7 (U/ml) IN SAMPLES FROM MALE BLOOD DONORS

	Normal				IX Polymorphism		
	^{125}I-A-1	^{125}I-A-7	Clotting activity		^{125}I-A-1	^{125}I-A-7	Clotting activity
1	1.18	1.23	0.88	10	0.58	0.87	1.54
2	1.28	1.13	1.08	11	0.80	0.91	1.06
3	1.26	1.44	1.37	12	0.57	0.75	1.29
4	1.16	0.85	1.36	13	0.60	0.75	1.26
5	1.16	1.26	1.42				
6	1.10	0.86	0.94	Mean	0.64	0.82	1.28
7	1.09	0.85	0.91				
8	1.29	0.71	0.97				
9	1.23	1.22	1.26				
Mean	1.13	1.06	1.13				

phase and radiolabeled antibodies in the two antibody assay were varied, a strikingly decreased level of measurable factor IX antigen was detected when the A-1 monoclonal antibody was the antibody adsorbed to plastic microtiter wells when IXp samples were tested (table 2). Samples from most donors, however, showed only minor variations in antigen levels when different combinations of monoclonal antibodies were used to compare antigen levels in individual samples to a pooled plasma standard. In table 2, three samples from male donors with the factor IX polymorphism (404, 409, 421), one sample from a presumed female heterozygote (406), and one sample from a male donor without the factor IX polymorphism (415) were tested in multiple immunoassays. As shown in table 2, when the A-1 monoclonal was in the solid phase, more striking differences for assay values on IXp samples were detected and the antigen content was below the limits of detection of the assay system, usually less than 0.10 U/ml. There were no aberrant reactions in this antibody system when other monoclonal antibodies, including one obtained from Dr. A. Thompson (*Blood* 62:1027–1034, 1983), were used.

Isoelectric Focusing

Factor IX was purified from 500 ml of plasma from a donor with the A-1 polymorphism and 3 liters of plasma obtained from a patient known to have normal levels of factor IX antigen when measured with the A-1 monoclonal. Factor IX purified using monoclonal antibody affinity chromatography was approximately 90% pure as judged by SDS-PAGE. The isoelectric focusing pattern of factor IX from these two normal individuals was identical to purified

TABLE 2

ANTIGEN CONTENT COMPARED TO POOLED PLASMA (U/ml)

		SEX, GENOTYPE CLASSIFICATION AND INDIVIDUAL SAMPLE NOS.				
SOLID-PHASE ANTIBODY	LABELED ANTIBODIES	Male 404 (IXp)	Female 406 (IXp, IXn)	Male 409 (IXp)	Male 415 (IXn)	Male 421 (IXp)
A-1	A-4	Low	0.49	Low	0.99	Low
	A-5	Low	0.38	Low	1.47	Low
	A-7	Low	0.35	Low	1.03	Low
	Seattle	Low	0.47	Low	1.48	Low
A-4	A-1	0.58	0.91	0.80	1.10	0.60
	A-4	*	*	*	*	*
	A-5	*	*	*	*	*
	A-7	0.87	1.04	0.91	0.86	0.75
Goat anti-IX	A-1	0.23	0.57	0.23	1.92	0.24
	A-4	*	*	*	*	*
	A-5	*	*	*	*	*
	A-7	0.87	0.88	1.67	1.04	1.09
	Seattle	N.T.	N.T.	1.12	N.T.	1.12

NOTE: N.T. = not tested. Low = less than 0.10 U/ml. Seattle = antibody obtained from Dr. A. Thompson, Seattle, Washington (*Blood* 62:1027–1034, 1983).
* Competition between antibodies.

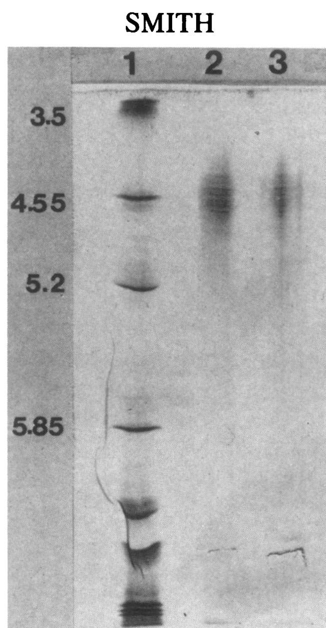

FIG. 1.—Isoelectric focusing of normal and polymorphic factor IX. Thin-layer isoelectric focusing analysis of calibration standards (*lane 1*), affinity purified normal (*lane 2*), and polymorphic factor IX (*lane 3*) are shown. Multiple bands separated by 0.04 pH units may be due to variable sialic acid content.

factor IX prepared from pooled normal plasma (fig. 1). The seven or eight bands observed were separated by 0.04 pH units. This microheterogeneity may be due to triantennary sugar chains leading to incomplete sialylation of factor IX [14].

Competititive Binding Experiments

When mixtures of labeled and nonlabeled affinity purified factor IX were compared in binding to monoclonal antibodies adsorbed to the solid-phase and Langmuir plots [15] were constructed, it was found that factor IX from a donor with the A-1 polymorphism had reduced binding to the A-1 antibody, while binding to other monoclonal antibodies was comparable to that seen with factor IX purified from a patient without the A-1 polymorphism.

The A-1 association constant for purified factor IX from a donor with the polymorphism (IXp) was 0.15×10^9 M^{-1} vs. 0.41×10^9 M^{-1} with factor IX from a patient without the polymorphism (IXn). When other monoclonal antibodies were used, the association constants were generally higher for the factor IX from the donor with the polymorphism. Factor IXp showed an association constant of 1.3×10^9 M^{-1} (with both the A-4 and A-5 monoclonals), while the constants were 0.80×10^9 M^{-1} and 0.69×10^9 M^{-1} for A-4 and A-5, respectively, with IXn. Scatchard analysis of these data gave similar results.

Binding to Monoclonal Antibodies in the Fluid Phase

When radiolabeled factor IX from a donor with the A-1 polymorphism was compared to radiolabeled factor IX from a normal donor, there was again a

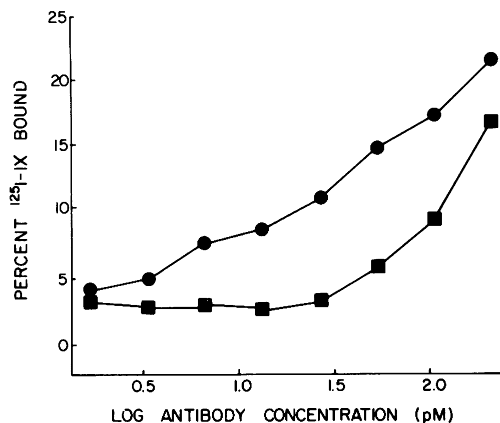

FIG. 2.—Differential binding of normal and polymorphic factor IX to the A-1 monoclonal antibody in solution. Factor IX was purified by monoclonal antibody affinity chromatography from a normal donor (*circles*) and a donor with the factor IX polymorphism (*squares*). Binding of both types of radiolabeled factor IX to the monoclonal antibody was tested in a double antibody RIA system. At low concentrations of antibody, there was decreased binding of the radiolabeled IXp sample (*squares*) to the A-1 antibody when compared to binding of IXn (*circles*).

decreased binding to the A-1 monoclonal antibody, while binding to the other monoclonals tested was comparable for the two samples of factor IX (fig. 2).

Frequency of the A-1 Polymorphism

One hundred eighteen samples from blood donors in New Mexico were tested in a combination of assays using the A-1 and A-5 monoclonal antibodies in the solid phase combined with radiolabeled A-7. In order to establish the fraction of female heterozygotes, the results of immunoassays in 43 male blood donors without the polymorphism were used to establish 95% confidence intervals for the assays using A-5 or A-1 in the solid-phase antibody and A-7 as the radiolabeled antibody (see MATERIALS AND METHODS). In 43 males, the 95% confidence limits for the A-1/A-5 assay ratio were 1.75–0.86. When these confidence intervals were applied to results from antigen determinations on female donors, females outside the confidence limits were classified as heterozygotes with partial deficiency of the A-1 epitope or homozygotes if factor IX antigen was undetectable when the A-1 was the solid-phase antibody. Figures 3*A* and *B* show the results of this testing. The frequencies of the IXp and IXn genotype are listed in table 3.

Fourteen of 57 male blood donors demonstrated the IXp variant. Six female homozygotes were found in 61 female blood donors, while 11 females were classified as heterozygotes.

The regression line does not intersect the origin because the antigen content is expressed in U/ml referring to a pooled plasma standard. Samples without the A-1 polymorphism would be expected to have high antigen content in assays using A-1 as the solid-phase antibody when compared to pooled factor IX that contains plasma from donors with the A-1 polymorphism.

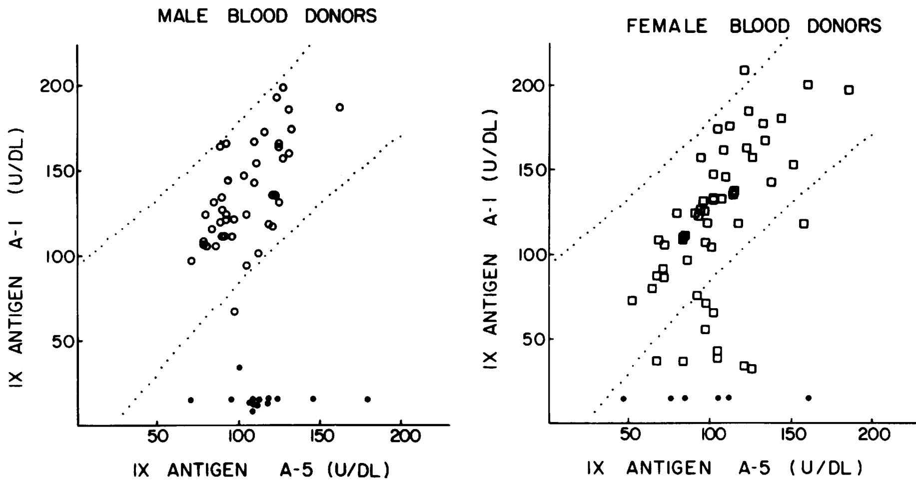

FIG. 3.—Definition of the factor IX polymorphism by monoclonal antibody immunoassays. Plasma samples from male (*open circles*) and female (*open squares*) blood donors were tested in a two-assay system. The A-5 and A-1 antibodies were used in the solid phase while the A-7 antibody (specific for the light chain of factor IXa) was used as the radiolabeled antibody for both assays. Some male blood donors and female homozygotes with the factor IX polymorphism had undetectable antigen levels when the A-1 antibody was in the solid phase. These individuals are shown as *solid circles* in *A* and *B*. Ninety-five percent confidence limits are shown (. . . . .) for prediction of the [(A-1)–(A-7)] antigen level (*y axis*) given results with an assay system not sensitive to the polymorphism [(A-5)–(A-7)] (*x axis*). These confidence intervals in males without the polymorphism were used to classify samples from female blood donors.

Family Studies

Samples obtained from families seen for renal transplant evaluation or from families seen in the clinical laboratory for evaluation of von Willebrand disease. In these samples, the A-1 polymorphism showed the expected pattern; 10 families comprising 34 individuals were tested. In no family did the A-1 polymorphism appear in children without a detectable IXp in a parent. As expected, obligate female carriers were found in daughters of a male with IXp.

DISCUSSION

Monoclonal antibodies have proved to be useful in demonstrating polymorphisms in human serum proteins even when these polymoprhisms cannot be distinguished by electrophoretic techniques [16]. Although electrophoretic variants [6], polymorphism at residue 148 [8], and genetic variation in factor IX clotting activity levels [7] have been described, most studies on coagulation factor IX variants have characterized samples from patients with deficient enzymatic activity.

Hybridization studies have shown that there is a restriction enzyme site polymorphism in the factor IX genome [1, 3], but this site is probably not intronic. Although the DNA sequence of factor IX has been published by three

TABLE 3

POPULATION STUDIES ON THE FACTOR IX POLYMORPHISM

Sample	Assigned genotype		No.
61 females	IXn/IXn		42
	IXp/IXn		13
	IXp/IXp		6
57 males	IXp		14
	IXn		43

	Gene frequency		
	IXp	IXn	95% confidence intervals
Males	$p = .246$	$q = .754$	$P = .112–.358$
Females	$p = .205$	$q = .795$	$P = .133–.277$
Males and females	$p = .218$	$q = .782$	$P = .158–.278$

groups [5, 17, 18], the only remaining inconsistency is seen in a single amino acid substitution in the activation peptide [5] that is now thought to be a true polymorphism.

In the present study, a polymorphism in human factor IX has been found using monoclonal antibody immunoassays. The antibody that detects the polymorphism is specific for the C terminal region of factor IX found on the heavy chain of factor IXa and probably not related to the *Taq*I polymorphism or the residue 148 polymorphism on the activation peptide [5].

Despite the use of factor IX purified from pooled plasmas as the immunogen for monoclonal antibody production, the A-1 antibody clearly has reduced reaction with some samples of factor IX in plasma. Limited studies on families and on samples from a blood-donor population indicate that this is a genetic variant found in 25% of males. Samples from females have been classified population studies, but the accuracy of this method can be established only by family studies.

The nature of the variation in primary structure, glycosylation, or conformation responsible for the attenuated reaction of polymorphic factor IX with the A-1 antibody is now known. If there is a single amino acid substitution that accounts for the IX polymorphism, it has not altered the overall charge of the molecule given the results on isoelectric focusing.

Two further observations on the A-1 epitope have been made in this report. When factor IX preparations from donors with and without the polymorphism were compared in experiments to determine affinity for solid-phase antibody, the decreased affinity of the polymorphic factor IX was not as dramatic as expected from immunoassays. Also, the reduced reaction with polymorphic factor IX was not as pronounced when the A-1 antibody was the radiolabeled

species in the double antibody immunoradiometric assay compared with assay results when A-1 was in the solid phase.

Although it is not possible to explain disparate results with radiolabeled factor IX, it may be that iodination or purification has altered factor IX antigenic properties. There may be subtle differences in antigen-antibody reactions when an antibody is adsorbed to plastic rather than free in solution, resulting in less binding of factor IX to the A-1 antibody in the solid phase than when both antibody and antigen are in solution.

Although the basis for the IX antigen polymorphism is not understood, immunoradiometric assays on plasma or serum samples can be used to classify patients in both population and family studies. This marker for a polymorphism of factor IX may be quite useful in carrier studies in families where the polymorphism is identified. In informative families, this polymorphism could be useful in studying linkage to loci in the q26-qter region of the X chromosome [1, 2]. The HGPRT locus is thought to be in this region of the X chromosome, for example.

In families where a carrier female is heterozygous for both hemophilia B and IXp, it would be possible to use a fetal sample to establish whether a male fetus has hemophilia B. To date, we have not found any samples from patients with hemophilia B with the IX polymorphism expressed on a circulating dysfunctional factor IX.

More family studies are planned in order to accurately define the detection of the carrier state for the polymorphism in females, and studies are currently in progress with antigen determinations on families of patients with hemophilia B. Purification of factor IX from donors with and without the polymorphism is needed to determine if peptide mapping techniques can demonstrate a biochemical basis for the difference between normal and polymorphic factor IX.

If peptide mapping techniques and sequence determinations on mapped peptides demonstrate a difference between normal and polymorphic factor IX, it will be of interest to test DNA from patients with the IXp polymorphism for possible restriciton site polymorphisms.

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