Dissertation

British Journal of Haematology, 1974, **26**, 129.

Acquired Factor-IX Inhibitor in a Nonhaemophilic Patient with Autoimmune Disease*

R. LARGO, P. SIGG, A. VON FELTEN AND P. W. STRAUB

Department of Medicine, Kantonsspital, University of Zürich, Switzerland

(*Received* 23 *March* 1973; *accepted for publication* 24 *May* 1973)

SUMMARY. An acquired inhibitor directed against factor IX was demonstrated in the blood of a patient with autoimmune disease. The inhibitor could be removed from the patient's plasma using PPSB or specific antisera insolubilized by polymerization. It could be recovered from these immunoadsorbents and characterized as an IgG-immunoglobulin using specific antisera.

The development of acquired inhibitors directed against clotting factors VIII or IX is a well-known complication of substitution therapy in both haemophilia A and haemophilia B. The spontaneous occurrence of inhibitors against factor VIII in non-haemophilic patients is more rare, and is found primarily in systemic lupus erythematosus, penicillin reactions, after delivery and in old age (Margolius *et al*, 1961; Bidwell, 1969). A factor-IX inhibitor in a non-haemophilic patient was first postulated by Formanék (1957). He described a 4-yr-old girl who developed purpura after recovery from hepatitis. On the basis of the thromboplastin generation test and of the observation that the addition of the patient's blood to normal blood resulted in an incomplete prothrombin consumption, the author concluded that a factor-IX inhibitor was the cause of the abnormality. More recently the presence of a factor-IX inhibitor has been suggested in four patients with systemic lupus erythematosus (Castro *et al*, 1972).

In the present work we describe a non-haemophilic patient who developed a factor-IX inhibitor in the course of autoimmune disease. Using the technique of immunoadsorption originally developed by Avrameas & Ternynck (1969) it was possible to highly purify the inhibitor for further characterization.

CASE REPORT

The patient (C.I.) was a boy without a family history of haemorrhagic disorders who at the age of $6\frac{1}{2}$ yr developed symptoms considered to be of autoimmune origin, namely a pleuritis, hepatosplenomegaly, and purpuric lesions. Six months later he had a pericarditis and massive ascites. Liver biopsy showed thrombosing endophlebitis with focal necrosis, inflammatory reaction and cirrhotic transformation. At the age of $7\frac{1}{2}$ yr he was treated with prednisone,

* Presented at the Annual Meeting of the Swiss Society for Haematology, 8–9 June 1972, in Lausanne, and published in abstract form in *Schweizerische Medizinische Wochenschrift*, **102**, 1612 (1972).

Correspondence: Professor P. W. Straub, Department of Medicine, Kantonsspital University of Zürich, Switzerland.

resulting in a transient improvement. Subsequently hepatosplenomegaly and ascites as well as haemorrhagic skin lesions led to a trial with azathioprine and cyclophosphamide. The child died at the age of 9 yr 10 mth from recurrence of the disease and an associated herpetic infection.

In the early phase of disease the patient had an increased ESR, an elevation of all immunoglobulins, a positive Wassermann reaction, a negative LE-cell preparation, a Coombs-positive haemolytic anaemia with cold agglutinins in the form of complete antibodies of the IgM-type. Subsequently thrombocytopenia was repeatedly demonstrated. Antibodies against pooled normal platelets were demonstrated using a complement binding reaction. Complement consumption (β_1C) was repeatedly demonstrated. Recurrence of the disease was usually associated with a moderate prolongation of prothrombin time and an excessive prolongation of recalcification and partial thromboplastin times.

MATERIALS AND METHODS

Blood was collected directly into siliconized glass tubes containing one-tenth vol of 0.1 M sodium oxalate. For the preparation of Dowex plasma, the ion exchange blood-bag-unit Fenwal JB-2 was used.

The partial thromboplastin time (PTT) was performed by incubation of 0.2 ml plasma with 0.2 ml PTT-reagent (Behringwerke AG, Marburg, Germany) for 2 min at 37°C and subsequent recalcification with 0.2 ml $CaCl_2$ 0.025 M (normal values 40–55 s).

For the measurement of the prothrombin time according to Quick, recalcification time, residual prothrombin in serum, thromboplastin generation test and of the individual clotting factors I (fibrinogen), II, V, VII-complex, IX and X the methods of Duckert (1958) were used. Factor VIII was measured according to Geiger *et al* (1956), factor XI according to Horowitz *et al* (1963) and factor XII according to Baumann & Straub (1968). The factor-XIII activity was qualitatively estimated using urea solubility according to Loewry & Edsall (1954).

Thrombin and Reptilase clotting times were measured according to Funk *et al* (1971), using bovine thrombin (Hoffman-La Roche, Basel, 5000 NIH u = 100 mg) and Reptilase-reagent of Pentapharm AG, Basel, Switzerland (3–4 thrombin u/ml).

PPSB* (Soulier *et al*, 1968) was obtained from Centraal Laboratorium van de Bloedtransfusiedienst van het Nederlandsche Roode Kruis, Amsterdam. Immunoelectrophoresis was done according to Scheidegger (1955); immunodiffusion according to Ouchterlony (1958).

A rabbit antiserum against human serum was obtained from the Pasteur Institute, Paris, a goat antiserum against human immunoglobulin and rabbit antisera against human IgG, IgM and IgA were obtained from Behringwerke AG, Marburg, Germany.

Preparation of Immunoadsorbents

Polymerization of PPSB. 250 mg of lyophilysed PPSB contained in one 10 ml vial were dissolved in 5 ml distilled water. With constant stirring at room temperature 2 ml of 2.5% (v/v) aqueous glutaraldehyde (Fluka AG, Buchs, Switzerland) were added dropwise, leading

* PPSB = prothrombin, proconvertine, Stuart factor, antihaemophilic factor B, or factors II, VII, X and IX respectively.

to gel formation. After continued stirring for 30 min, the gel was left at room temperature for 3 hr without agitation. After subsequent addition of 100 ml of 0.15 M phosphate buffer, pH 7.2, the suspension was homogenized in a loose-fitting Potter homogenizer and centrifuged at 3000 rpm at 4°C for 15 min. Homogenization and centrifugation were repeated four times. Subsequently the insoluble PPSB was suspended in 200 ml of 0.1 N glycine–HCl buffer, pH 2.8, and centrifuged for 15 min at 3000 rpm at 4°C. It was washed twice in the same medium. In order to check the insolubility the polymerizate was incubated for 15 min at room temperature in the same buffer, centrifuged at 3000 rpm at 4°C for 20 min. If the protein content of the supernatant was not below 0.5 mg per 100 μl (assay according to Lowry *et al*, 1951), washing of the immunoadsorbent was repeated.

Polymerization of anti-IgG-, anti-IgM- and anti IgA-antisera. Five ml antiserum were dialysed overnight at 4°C against 500-fold vol of isotonic saline. 0.5 ml of 0.2 M phosphate buffer, pH 7.0, were added and subsequently 0.5 ml of 2.5% aqueous glutaraldehyde in water were added dropwise at room temperature using constant magnetic stirring. After 30 min of stirring and 3 hr standing at room temperature the sediment was homogenized, washed and tested for insolubility as indicated for the polymerization of PPSB.

Immunoadsorption

One vol of plasma was added to 1 vol of PPSB-immunoadsorbent, stirred for 30 min at room temperature and subsequently centrifuged at 4°C at 3000 rpm for 30 min. The supernatant was passed through a Sartorius membrane filter (Sartorius GmbH, Göttingen, Germany; pore size 2 μm) and kept at 4°C, if the adsorbed plasma was immediately used for further experiments, or at −20°C for subsequent studies.

For elution the immunoadsorbent was washed four times with 40 ml of isotonic saline at 4°C and centrifuged at 3000 rpm at 4°C for 15 min. Subsequently 1 vol of immunoadsorbent was added to 2 vol of 0.1 N glycine–HCl buffer, pH 2.8, and incubated at room temperature for 15 min using constant magnetic stirring; subsequently the suspension was centrifuged at 3000 rpm at 4°C for 20 min. The incubation of immunoadsorbent with the glycine–HCl buffer and the subsequent centrifugation were repeated twice. The supernatants were passed through a Sartorius membrane filter of pore size 0.45 μm at 4°C; they were concentrated using dialysis against methyl cellulose (Aquacide I, mol wt 70 000) to 1/5 or 1/10 of the original volume and dialysed against 400 vol of buffered NaCl (1 vol 0.15 M phosphate buffer, pH 7.2, 4 vol isotonic NaCl) overnight. The eluate was either kept at 4°C for further studies or stored at −20°C. After elution the immunoadsorbent was washed once with 0.1 N glycine-HCl buffer, pH 2.8, and four times with 0.14 M phosphate buffer, pH 7.2, and finally stored at 4°C in buffered NaCl containing one drop of 1% merthiolate. Immunoadsorption with polymerized anti-immunoglobulin antisera was performed as for PPSB. However, the washed and stored immunoadsorbents were used several times (Avrameas & Ternynck, 1969).

RESULTS

Routine Coagulation Studies

As shown in Table I, the prothrombin time, recalcification time and partial thromboplastin

time (PTT) were repeatedly prolonged during 2½ yr. These findings as well as thrombocytopenia with impaired clot retraction were usually closely associated with clinical exacerbation of the autoimmune disease. A moderate depression of factors II, VII and X could easily be interpreted as a consequence of liver disease. However, impairment of liver function was not severe enough to account for the more massive depression of factors IX, XI and XII. The other clotting factors, as well as thrombin and Reptilase times were normal. The clotting time according to Lee and White, the Ivy-bleeding time and the Rumpel-Leede test were performed three times and were normal.

TABLE I. Clotting function tests

	Test					
	23.3.69	12.3.70	26.5.71	2.9.71	19.10.71	*Normal range*
Prothrombin time (s)	13	11.9	12.9	13.3	14.2	11.5
Recalcification time (s)	180	201	220	200	300	70–120
Partial thromboplastin time (s)			112	102	182	40–55
Prothrombin consumption (%)			13	<4	<4	<7
Fibrinogen (mg%)			430	310	360	150–500
Factor II			88	60	60	70–100
Factor V			54	60	66	50–100
Factor VII			60	55	48	70–100
Factor VIII			58	52	49	50–100
Factor IX			13	17	4	50–100
Factor X			86	80	86	70–100
Factor XI			30		15	50–100
Factor XII			<5		12	50–100
Factor XIII (urea solubility)			Insoluble		Insoluble	Insoluble
Thrombin clotting time (s)			18	16	14	13–18
Reptilase clotting time (s)			17	18	18	18–18
Platelet count (per μl)			215000		138000	150–500000
Clot reaction (%)			Reduced		Reduced	Normal

In the thromboplastin generation test the incubation mixtures of patient serum with adsorbed normal plasma and of normal serum with adsorbed patient plasma gave results compatible with a factor-IX disturbance (Fig 1). If the patient's plasma was used as a substrate, it behaved like normal plasma. This excludes the presence of an antithromboplastin in the patient's plasma (Jung *et al*, 1961). Furthermore, in Quick's prothrombin-complex assay dilution of the patient's plasma did not lead to a normalization as one would expect in the case of an antithromboplastin (Jung *et al*, 1961). Finally the complement β_1C level was normal in plasma and in serum, whereas Jung *et al* (1961) described complement consumption during the coagulation of patient's blood in their case of antithromboplastin.

Mixing Experiments in the PTT-Assay System

As shown in Fig 2, the patient's plasma, when mixed with normal plasma, did not behave like haemophilia B plasma: while the PTT of haemophilia B plasma was virtually normalized

by addition of 25% normal plasma, addition of more than 87.5% normal plasma did not normalize the PTT of the patient's plasma.

After adsorption with the insoluble PPSB-immunoadsorbent the curve of the patient's plasma became similar to that of haemophilia B plasma, although some inhibitor action was

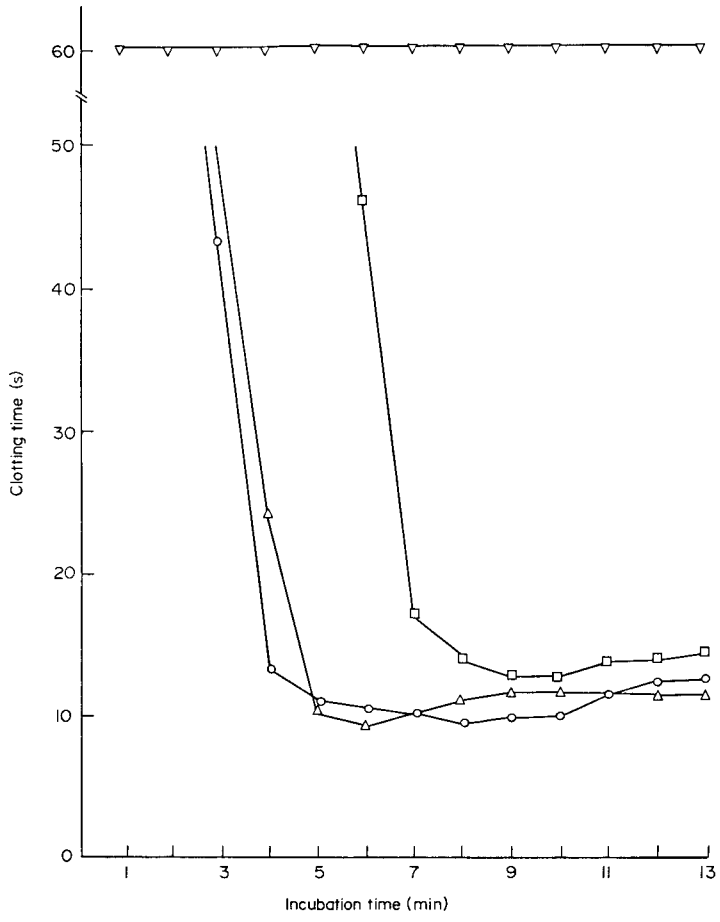

FIG 1. Thromboplastin generation test.

Incubation mixture	*Dowex plasma substrate*
▽ Ads. normal plasma/patient serum	Normal plasma
□ Ads. patient's plasma/normal serum	Normal plasma
○ Ads. normal plasma/normal serum	Normal plasma
△ Ads. normal plasma/normal serum	Patient's plasma

still present. The behaviour of the patient's plasma incubated with insoluble albumin obtained in the same manner as the insoluble PPSB, was not modified. Normal plasma incubated with insoluble PPSB behaved as before incubation.

Results of mixing experiment using PPSB-eluate and normal plasma are shown in Fig 3. The eluate of PPSB previously incubated with the patient's plasma led to a prolongation of the PTT of normal plasma. The eluate of PPSB previously incubated with normal plasma

did not prolong the PTT of normal plasma. As shown in Table II, the eluate of insoluble anti-IgG-antisera previously used for immunoadsorption of the patient's plasma, did prolong the PTT of normal plasma, while eluates of similarly incubated insoluble anti-IgM and anti-gA antisera gave control values.

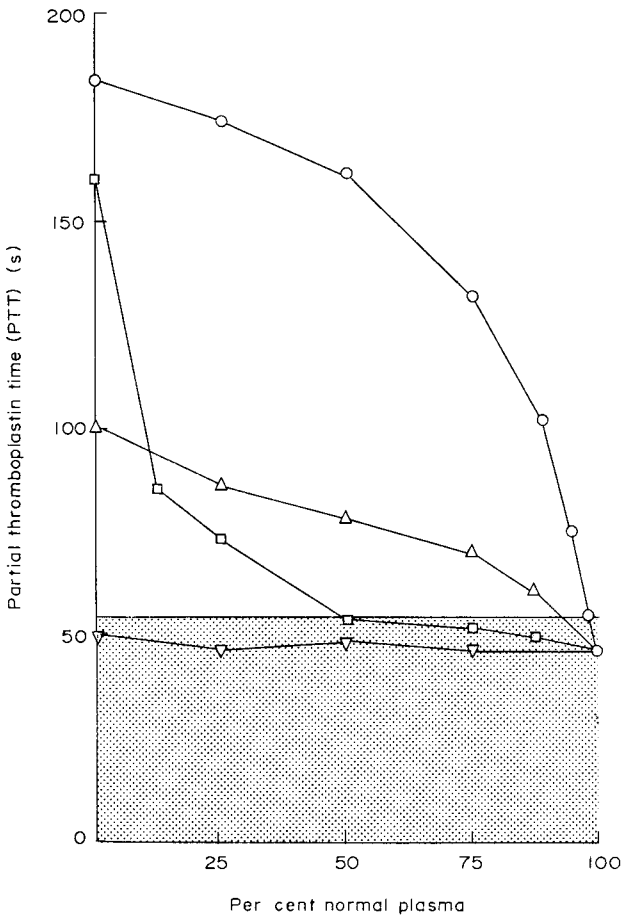

FIG 2. Mixing experiments in the PTT system. ○, Normal plasma in non-adsorbed patient's plasma; △, normal plasma in adsorbed patient's plasma; □, normal plasma in non-adsorbed factor-IX deficient plasma; ▽, normal plasma in adsorbed normal plasma.

Determination of Clotting Factors Before and After Incubation of the Patient's Plasma with Insoluble PPSB

Factors I, II, VII, IX and X were not modified by incubation with PPSB. Particularly, no increase of factor IX was found. Factor V and factor VIII were reduced by 15% and 20% respectively, a finding which is due to the lability of these two factors since control plasma showed a similar drop of these two clotting factors. Factors XI and XII, however, were definitely increased after adsorption with insoluble PPSB (Table III). Control studies using normal plasma and incubation with PPSB gave no such increase but rather a moderate drop of activity of factors XI and XII.

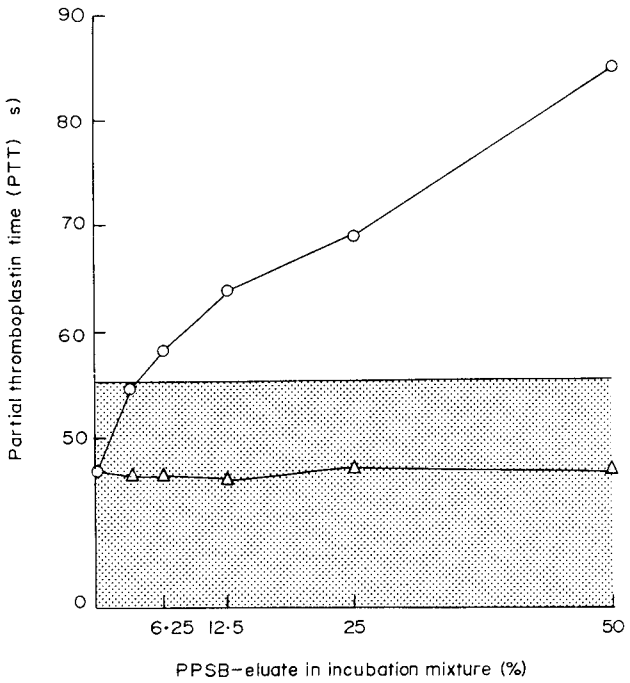

FIG 3. PTT of mixtures of eluates with normal plasma. ○, PPSB-eluate of patient's plasma; △, PPSB-eluate of normal plasma.

TABLE II. Mixing experiments with eluates of insoluble antisera

Mixture	*PTT (s)*
0.1 ml normal plasma + 0.1 ml eluate (IgG)	68
0.1 ml normal plasma + 0.1 ml eluate (IgM)	51
0.1 ml normal plasma + 0.1 ml eluate (IgA)	50
0.1 ml normal plasma + 0.1 ml NaCl	49
0.1 ml normal plasma + 0.1 ml normal plasma	45

TABLE III. Factors XI and XII before and after PPSB-absorption of the patient's plasma

Patient's plasma	*Factor XI* (50–100%)	*Factor XII* (50–100%)
Non PPSB-absorbed	15%	12%
PPSB-absorbed	54%	38%

Physicochemical Properties of the Inhibitor

The inhibitor was present both in plasma and serum, it was not dialysable, it was entirely precipitable with 30% (w/v) ammonium sulphate and it was not adsorbed by $BaSO_4$. It was almost completely destroyed by heating for 5 min at 70°C; however, its activity was not affected by heating for 30 min at 56°C and by storage for several months at −20°C.

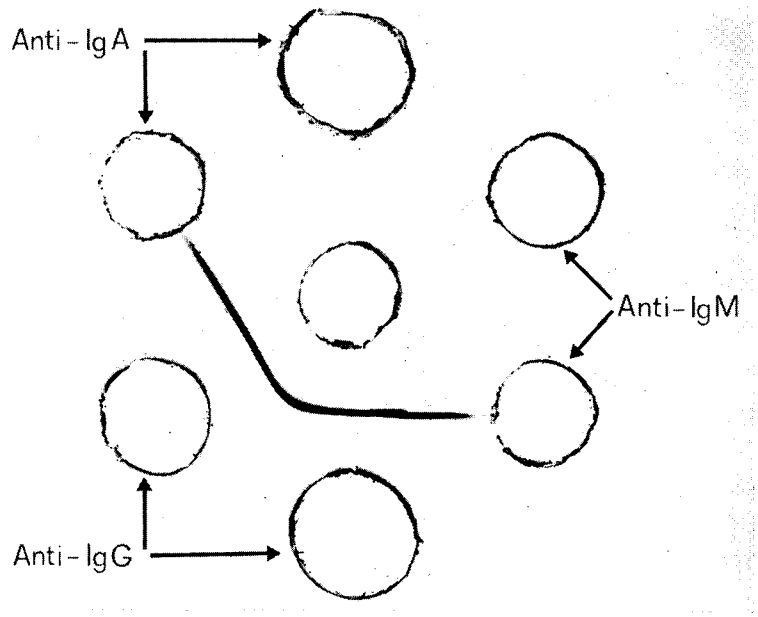

FIG 4. Immunodiffusion according to Ouchterlony. Centre well: PPSB-eluate after incubation of polymerized PPSB with the patient's plasma.

Immunological Characterization of the Inhibitor

PPSB-eluate. In order to obtain a high inhibitor concentration, insoluble PPSB was repeatedly incubated with a 20-fold vol of the patient's plasma. The inhibitor was then eluted (Fig 3) and reduced to 1/10 or 1/20 of the original volume by dialysis against methyl cellulose. Using this concentrate and specific antisera against IgG, IgM and IgA in the immunodiffusion test of Ouchterlony (1958) the inhibitor could be identified as an IgG-immunoglobulin (Fig 4).

The results of the assay for IgG subclasses in the above eluate are given in Table IV. Although the PPSB eluate contained all four IgG subclasses, IgG 1 was present in a slightly higher relative concentration than in the original serum.

TABLE IV. Immunoglobin subclasses in the patient's serum and in PPSB-eluate

	Concentration (mg/ml) of:			
	IgG 1	*IgG 2*	*IgG 3*	*IgG 4*
Serum	6.2	1.8	0.3	0.3
PPSB-eluate	0.096	0.018	0.005	0.004

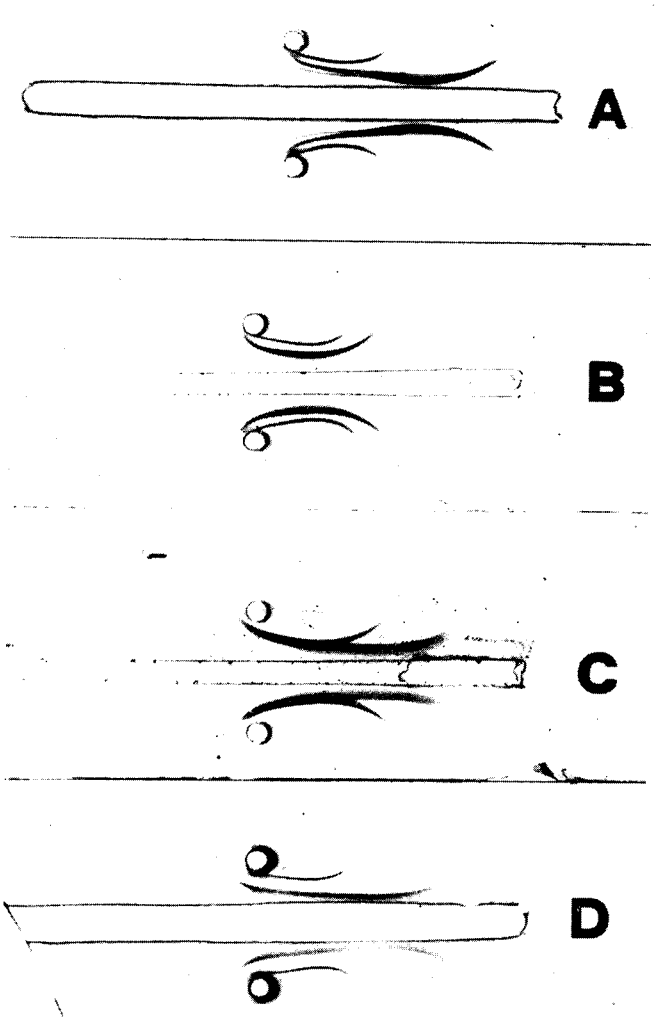

FIG 5. Immunoelectrophoresis of patient's plasma after absorption with: (A) insoluble rabbit serum; (B) insoluble anti-IgG-antiserum; (C) insoluble anti-IgM-antiserum; (D) insoluble anti-IgA-antiserum. Trough: anti-Ig antiserum; anode to the right.

Removal of inhibitor with anti-immunoglobulin-immunoadsorbents. In order to remove the individual immunoglobulins from the patient's serum, immunoadsorption had to be repeated up to 12 times (Fig 5). The collected eluates were concentrated 10-fold using methyl cellulose, the immunoglobulins were identified in the eluates using immunoelectrophoresis (Fig 6) and tested for inhibitor activity (Table II).

DISCUSSION

The present patient showed various clinical manifestations and laboratory findings compatible with an autoimmune disease. The coagulation abnormalities were accentuated during

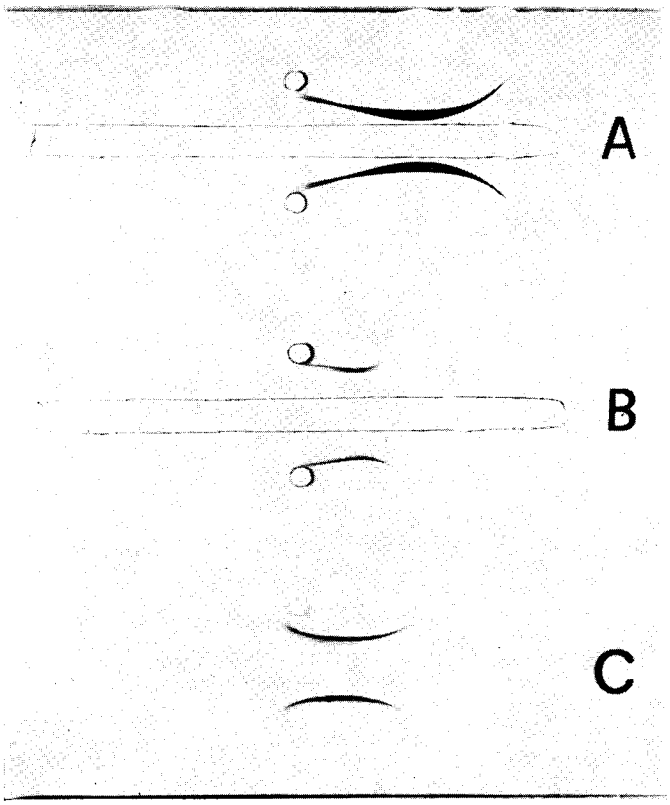

FIG 6. Immunoelectrophoresis of eluates from insoluble anti-immunoglobulins against an anti Ig-antiserum. Wells: A, anti-IgG eluate; B, anti-IgM eluate; C, anti-IgA eluate. Anode to the right.

recurrences and were thus probably also related to the basic disorder. The abnormality of prothrombin time and of the level of vitamin K dependent factors is explained by definite impairment of liver function. However, liver disease did not explain the massive prolongation of recalcification time, of PTT and the depression of factors IX, XI and XII. PTT-mixing experiments allowed to exclude a clotting factor-deficiency as a cause of the latter abnormalities and to establish the presence of an inhibitor.

Although in autoimmune diseases inhibitors of blood or tissue thromboplastin are the most likely finding, we could exclude this possibility. On the basis of the depression of factors IX, XI and XII and the results of the thromboplastin generation test an inhibitor against either factor IX, factor XI or factor XII was suspected. Assuming a factor-IX inhibitor, we attempted to remove the inhibitor with polymerized PPSB previously shown to contain more than 200-fold the activity of factor IX than of factors XI or XII. Whereas immunoadsorption with PPSB did not affect the low factor-IX activity, it led to a marked increase of the activity of factors XI and XII. This is comprehensible since the reaction sequence in the one-stage assay of factors XI and XII included an adequate activation of factor IX. It thus appears likely that the low factor XI and XII activities in our case were due to the effect of the factor-IX inhibitor in the assay system. A factor XI or XII inhibitor could hardly

explain the low factor-IX activity, even after PPSB adsorption, in the presence of a normal activity of other clotting factors of the intrinsic coagulation sequence, especially of factor VIII. For the further characterization of the inhibitor various immunologic techniques were considered. According to previous studies, the inhibitor concentration in plasma is too small to use antigen–antibody precipitation reactions (Denson, 1967; McLester *et al*, 1965). Furthermore, the results of such attempts have been questioned (Penalver *et al*, 1957; Ehrenworth, 1963; Denson, 1967; Bidwell, 1966; Berglund, 1963). Passive haemagglutination as used by several authors (Colombani & Terrier, 1962; Roberts *et al*, 1965) was found unsuccessful in our case as well as in previous studies (Biggs & Bidwell, 1959; Ratnoff, 1957). Various authors have added specific anti-immunoglobulin-antisera to the inhibitor plasma and subsequently demonstrated neutralization of the inhibitor by one antiserum. This method is not satisfactory since even the best antisera are not able to block more than a part of the inhibitor. Furthermore, the antiserum contains coagulation-active substances which will give rise to difficulties in the interpretation of results. Therefore we attempted the characterization of the inhibitor using immunoadsorption (Avrameas & Ternynck, 1969), a technique which allows insolubilization of proteins without loss of antigenicity. Thus immunoadsorbents prepared from PPSB and from antisera against IgG, IgM and IgA could be added to plasma repeatedly and in high amounts and removed by centrifugation without modifying the plasma quantitatively or qualitatively. Indeed, incubation of the patient's plasma with polymerized PPSB, which contains factor IX but no factor XI or XII, removed a considerable part of the inhibitor. Furthermore the adsorbed plasma showed an improved activity of the previously depressed factors XI and XII, while the low factor-IX level remained unchanged. Finally, the eluate of the insoluble PPSB contained the inhibitor which could then be characterized as IgG-immunoglobulin using specific antisera in the immuno-diffusion assay. This indicates that the clotting abnormality of the intrinsic system was due to an antibody directed against factor IX.

Some clotting inhibitors have been shown to be immunoglobulins of the IgG 4 subclass (Bidwell, 1969). Although in the present patient immunoadsorption allowed a fair purification of the inhibitor, it reacted with all four subclass antisera.

While immunoadsorption with polymerized antigen in the form of insoluble PPSB gave good results, the use of polymerized antisera against IgG, IgM and IgA proved much less satisfactory. This may be explained in the following way. In the case of insoluble PPSB the antibody (factor-IX inhibitor) could be selectively adsorbed by a very high amount of insoluble antigen (factor IX). In the case of insoluble anti-immunoglobin antisera the entire IgG-, IgM- or IgA-immunoglobulins had to be adsorbed. Therefore, in contrast to the adsorption with insoluble PPSB, a very large amount of antigen other than the inhibitor had also to be removed by a small quantity of insoluble antibodies, the adsorption thus being non-selective. Moreover, immunoadsorption is generally more efficient when the antigen is insolubilized, as in the case of PPSB, than with insoluble antibody. In fact, in order to remove an immunoglobulin from the patient's plasma to a degree that it could no more be demonstrated by immunoelectrophoresis, the plasma had to be adsorbed up to 12 times with the insoluble antiserum. This precluded a subsequent precise analysis of the clotting activity of the adsorbed plasma. Still, the inhibitor could be eluted from anti-IgG-antiserum and not from the other antisera. This is an additional argument for the IgG-nature of the in-

hibitor. Immunoadsorption proved to be an efficient method to highly purify a factor-IX inhibitor. The procedure may be promising for the study of other clotting inhibitors.

ACKNOWLEDGMENTS

We wish to thank Dr Andreas Morell, Tiefenauspital, University of Berne, for the assay of immunoglobulin subclasses. The skilful technical assistance of Mrs V. Heller is gratefully acknowledged.

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