Preparation of Factor IX Deficient Human Plasma by Immunoaffinity Chromatography Using a Monoclonal Antibody


A murine hybridoma clone is described that grows continuously in culture and produces a monoclonal antibody we have called Royal Free Monoclonal Antibody to factor IX No. 1 (RFF-IX/1). This has high affinity for a coagulation site on factor IX. RFF-IX/1 immobilised on sepharose can be used to deplete factor IX from normal human plasma. This immunoaffinity depleted plasma is indistinguishable from severe Christmas disease plasma and can be used as the substrate in a one stage coagulation assay for factor IX. The affinity column has high capacity and can be regenerated so that large scale production from normal plasma of factor IX deficient plasma as a diagnostic reagent is now feasible.

Materials and Methods

Coagulation Factor Assays

Human plasma deficient in factors VIII, IX or XI was obtained from patients attending our centre and preactivated for use in one stage coagulation assays by incubation of 100 μl of plasma with 100 μl of kaolin phospholipid suspension (1% (w/v) centrollex P (Dey-Duncan & Co. Ltd., Haslemere, Surrey, UK) homogenized in isotonic saline with 5 mg/ml kaolin) at 37°C for 7.5 min. Then 100 μl of test plasma, diluted from 1:10 to 1:500 in pH 7.5 barbital buffered saline was added and 30 sec later 100 μl of 0.03 M CaCl₂ was added with instantaneous mixing. The time from recalcification to formation of a visible clot was recorded. Clotting times for normal pooled plasma dilutions provided the standard curve and defined the units. Factors II, V, VII, and X were measured by similar assays in which activation was achieved by Taipan venom (Diagnostic Reagents Ltd., Thame, England), tissue factor and Russell viper venom (Wellcome Reagents Ltd., Beckenham, England) respectively. Normal pooled plasma was prepared from at least 20 normal donor blood samples taken into 3.8% sodium citrate, separated rapidly at 4°C, aliquoted and stored in liquid nitrogen and was assumed to contain one unit of each procoagulant activity per ml.

Factor IX

Human factor IX concentrate was kindly provided by the Plasma Fractionation Laboratory, Oxford, in the form of material that had not passed pharmaceutical tests for clinical use. Ten bottles of factor IX concentrate were reconstituted in distilled water to a total volume of 200 ml. An equal volume of 3.3% citrate was added and the final mixture was made 1 μM in DFP (diisopropyl fluorophosphonate). Barium chloride (135 mls of a 1 M solution) was added dropwise with stirring. After stirring for 1 hour at 4°C, the precipitate was collected by centrifugation at 3000 × g for 20 min. The supernatant was decanted and the precipitate was resuspended in 75 mls of 0.1 M sodium chloride containing 10 μM barium chloride. The precipitate was again collected by centrifugation and resuspended in 200 ml of 20 μM Tris-Cl pH 7.4.

The suspension was made 0.1% in DFP and 50 ml 2 M ammonium sulphate (pH 7.5 with ammonium hydroxide) was added dropwise. After stirring for 1 hr at 4°C, the suspension was centrifuged for 10 min at 10,000 × g to remove the barium sulphate.

The supernatant was decanted and dialysed against 20 m M Tris/Cl containing 0.15 M sodium chloride and 1 mM benzamidine hydrochloride pH 7.4. After dialysis the solution was divided into three portions, each of approximately 100 ml, two of which were frozen and stored at −40°C for later use, and one of which was processed as follows:

All procedures were performed at 4°C.
The protein solution was applied to a Whatman D.E.52 cellulose column (3.2 x 20 cm) which had previously been equilibrated in 20 mM Tris-HCl containing 0.15 M sodium chloride and 1 mM benzamidine hydrochloride, pH 7.4, and the column was washed with 250 ml of the equilibrating buffer. Two litres of the benzamidine containing Tris buffer with a linear sodium chloride gradient (0.15–0.4 M) were used to develop the column using a flow rate of 0.9 ml/min. The fractions containing factors IX, X, and II were pooled and concentrated to approximately 60 ml using polyethylene glycol 6000.

After concentration the solution was applied to a Sephacyrl S-200 column (2.6 x 86 cm). The column was developed using 50 mM Iris (0.9 mI/mm). The fractions containing factors IX, X, and II were pooled and dialyzed against 0.1 M sodium chloride and 10 mM citrate, pH 7.4. The flow rate was 0.7 ml/min. The fractions containing factors IX, X, and II were pooled and concentrated to 25 ml using polyethylene glycol 6000 and applied to a heparin sepharose column (1.6 x 20 cm) which had previously been equilibrated in the sodium chloride/calcium chloride/benzamidine/Mes-Tris buffer. The column was washed with 40 ml of the equilibrating buffer and the column was washed with 0.15 M sodium chloride and 1 mM benzamidine hydrochloride pH 7.4, and the column was washed with 2.5 mM calcium chloride and 2 mM benzamidine, pH 5.9. The solution was concentrated to 25 ml using polyethylene glycol 6000 and applied to a heparin sepharose column (1.6 x 20 cm) which had previously been equilibrated in the sodium chloride/calcium chloride/benzamidine/Mes-Tris buffer. The column was washed with 40 ml of the equilibrating buffer and the column was washed with a linear gradient of sodium chloride (50 mM–1.5 M) in the Mes–Tris buffer containing 2.5 mM CaCl2, 2 mMMes-Tris buffer, pH 5.9, then concentrated using PEG. The fractions containing factor IX were pooled and concentrated to approximately 60 ml using polyethylene glycol 6000.

Detection of Antibody Activity of Factor IX

Antibody activity against functional sites of factor IX was detected by neutralization of zymogen in a specific inhibitor assay. Equal volumes of normal plasma and of culture supernatant, mouse sera, or ascitic fluid were incubated at 37°C for 15 min then subsampled into kaolin activated factor IX deficient plasma in the presence of phospholipid and the assay performed as described above. The clotting time after recalification is then inversely related to residual factor IX. Antibody binding to the totality of sites on the factor IX molecule was determined in a solid phase radioassay.

Radiometric Screening Assay

For this assay, purified factor IX was coupled to polystyrene beads (Northumbria Biologicals Ltd., Cramlington, Northumberland, UK) by gently agitating the beads for 1 hr at 20°C with 10 μg/ml factor IX in bicarbonate buffer pH 9.6 (0.035M NaHCO3; 0.01 M Na2CO3; 0.02% w/v NaN3). Coated beads were stored at 4°C in the same buffer and could be kept under these conditions for many weeks without loss of antigenicity of the factor IX. To assay for monoclonal antibodies to factor IX the beads were first washed 5x in PBS containing 0.05% (v/v) Tween 20 and unoccupied protein binding sites blocked by a 1 hr incubation at 20°C, with 10% (w/v) ovalbumin in PBS. Following a further five washes with PBS + Tween the beads were incubated, in multiwell trays with 200 μl aliquots of antibody containing culture supernatant, ascitic fluid or mouse serum, for 2 hr at 20°C. The beads were washed, again 5x with PBS + Tween, and incubated for a further 1 hr, at 4°C, with 200 μl of 125I-labeled goat antimouse IgG diluted in PBS + 1% (w/v) ovalbumin, containing a total activity of 100,000 cpm per assay, washed and counted. The goat antimouse IgG reagent was prepared by affinity purification on a mouse μg sepharose column and was radiolabeled with 125I using Chloramine T.

Immobilized Antibody Column

Mouse ascitic fluid containing RFF–IX/1 antibody was heat inactivated by a 30 min incubation at 56°C and adsorbed with

Monoclonal Antibody Production

Balb/c mice were immunized by intraperitoneal (i.p.) injections of 15 μg of factor IX preparation in complete Freund's adjuvant. Additional injections were given after 3 and 14 wk with complete Freund's adjuvant. At 6 mo after the initial immunization a mouse whose serum contained 200 Bethesda units/ml of antifactor IX activity12 was injected with 15 μg of factor IX, i.p. without adjuvant. Three days after this last injection, the spleen was removed from the mouse and 3.9 x 106 spleen cells were fused with 3.9 x 106 mouse myeloma cells of the line P3-NS/I-Ag 4-113 by a 7 min incubation with 40% (w/w) PEG 1500 and aliquoted into 211, 2 ml cultures. HAT medium14 was added 24 hr after fusion and hybrid colonies appeared on day 11 in culture. Antibodies to factor IX were detected in the supernatants using two separate assays. Antibody secreting cells were cloned three times by limiting dilution. Monoclonal antibodies were obtained either from the culture fluid in which the cloned cells had been growing or from ascitic fluid of Balb/c mice in which the antibody producing cells had been grown as an ascitic tumour.

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aluminium hydroxide. The gamma globulins were separated by rivanol and ammonium sulphate precipitation and covalently linked to Cyanogen bromide activated sepharose 2B in the ratio of 392 Bethesda units inhibitory activity per ml of beads (8 mg/ml; RFF-IX/1-sepharose). Coupling to activated sepharose has been carried out on four occasions with uniform success.

**Characterization of RFF-IX/1 Antibody**

The immunoglobulin class of RFF-IX/1 was determined by Ouchterlony double immunodiffusion and by immunoelectrophoresis against rabbit antisera to mouse µg classes (Miles Laboratories, Slough, England). Affinity was measured in free solution by incubating a fixed concentration of RFF-IX/1 with varying amounts of purified factor IX. The residual factor IX at equilibrium (after 30 min see Fig. 6) then represents free antigen and the bound antigen is the amount inactivated assuming that the complex [Ab - Ag] is nonfunctional. The latter assumption is supported by the observation that in antibody excess factor IX coagulant activity was totally destroyed. Molar concentrations of factor IX were calculated from the measured coagulant activity and the specific activity of the particular purified factor IX batch used in the experiment.

\[
\frac{1}{[\text{bound}]} \text{ was plotted against } \frac{1}{[\text{free}]} \text{ and the affinity } K_A \text{ derived from the Langumuir equation.}^{18}
\]

\[
\frac{1}{[\text{bound}]} = K_A \frac{[\text{free}]}{[\text{total antibody}]} + \frac{1}{[\text{total antibody}]}
\]

See Fig. 5.

**Purification of RFF-IX/1 Antibody**

RFF-IX/1 antibody was purified to homogeneity by passage over a Protein A Sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden) and by elution with pH 5.5, 0.1 M phosphate-citrate buffer.¹⁹

**RESULTS**

**Factor IX Preparations**

The purity of the factor IX preparations used for immunization is demonstrated by the single peaks obtained for reduced and unreduced ¹²⁵I-labeled factor IX run in polyacrylamide gels (Fig. 1). The factor IX had an apparent molecular weight of 59,000 daltons and specific coagulant activity ranging from 2.05 to 10.9 µg/unit. No high molecular weight aggregated material was detected in the gels. Table 1 shows one such purification.

![Fig. 1. SDS-polyacrylamide disc gel electrophoresis of ¹²⁵I-factor IX: Profiles of radioactivity measured in 1mm slices of SDS-polyacrylamide gels into which were electrophoresed reduced or unreduced ¹²⁵I-factor IX preparations as described in methods. Migration towards anode at right (slice 70). Molecular weight of protein standards (Daltons)](image)

**Mouse Immunizations**

All seven mice immunised with pure factor IX showed significant levels of serum antifactor IX activity as shown in Fig. 2. These results show the antibody levels in the mice at 20 wk. Subsequent immunisations

**Table 1. Purification of Factor IX**

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Volume (ml)</th>
<th>Activity</th>
<th>Recovery %</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IX concentrate</td>
<td>90</td>
<td>30</td>
<td>2.36</td>
<td>100</td>
</tr>
<tr>
<td>Barium sulphate eluate</td>
<td>120</td>
<td>24</td>
<td>6.25</td>
<td>97</td>
</tr>
<tr>
<td>DE-52 pool</td>
<td>100</td>
<td>23</td>
<td>11.9</td>
<td>77</td>
</tr>
<tr>
<td>Sephacryl S-200 pool</td>
<td>30</td>
<td>60</td>
<td>14.1</td>
<td>61</td>
</tr>
<tr>
<td>Heparin sepharose pool</td>
<td>58</td>
<td>24</td>
<td>400</td>
<td>47</td>
</tr>
<tr>
<td>Final product</td>
<td>8</td>
<td>170</td>
<td>486</td>
<td>46</td>
</tr>
</tbody>
</table>
with similar quantities of the antigen have consistently produced antibody activity to factor IX, detectable in both the coagulation and radiometric assays.

Production of Monoclonal Antibodies

A fusion was performed on the spleen cells that were taken from mouse 4 (Fig. 2) three days following the final boost with factor IX. Of the 211 wells initially plated, 169 wells showed growth of hybrid cells. Screening of these 169 hybrids was carried out in the specific inhibitor assay, to detect the production of monoclonal antibodies specific for the coagulation site of factor IX. As shown in Fig. 3, four of these cultures produced antifactor IX activity, one of which has remained stable and has continued to produce an antibody to factor IX (RFF–IX/1) following repeated cloning and passage through Balb/c mice. A comparison of the antibody activity in the culture supernatants from these four original hybridomas as detected by the functional assay and by the radiometric assay for anti factor IX antibodies (Fig. 4) demonstrated a differential activity of the antibodies in the two assays. For example, clone 3 consistently gave significant inhibition of factor IX activity whilst relatively small amounts of mouse antibody bound to factor IX were detected in the radiometric assay. RFF–IX/1 antibody however, showed high levels of activity in both assays. All monoclonal antibodies and all mouse sera that showed inhibitory activity against factor IX were consistently found to be positive in the radiometric assay.

Characterization of RFF–IX/1 Antibody

RFF–IX/1 is of the murine IgG1 class. The affinity ($K_a$) of RFF–IX/1 measured by fluid phase neutralisation (see “Methods”) was found to be $K_a = 4 \times 10^6$ M$^{-1}$ (Fig. 5). This affinity is within the range established for hapten-antihapten interactions.20

Inhibition of Factor IX by RFF–IX/1

Kinetic studies of the reactivity of RFF–IX/1 with a coagulation site of factor IX demonstrated that maxi-
Fig. 4. Comparison of radiometric and inhibitor screening assays run in parallel from four positive clones (see Materials and Methods).

Fig. 5. Binding of RFF-IX/1 to purified factor IX in free solution. Affinity calculated according to Langmuir equation (see Materials and Methods).

Fig. 6. Prolongation of clotting time (secs) for RFF-IX/1 and clones 2, 3, and 4.

Affinity Depletion of Factor IX from Plasma

Factor IX could be totally removed from 280 ml of human plasma from single donations (five individuals to date) by passage over a 10 ml column of RFF-IX/1-sepharose whilst other coagulation factors were not depleted (Table 2B). Larger volumes of human plasma (up to 500 ml) could be passed over the column with approximately 97% depletion of factor IX. The RFF-IX/1-sepharose column could be regenerated by sequential washes with 2 M NaCl in barbital buffer (pH 7.4) followed by pH 10 borate saline buffer and finally 3 M potassium isothiocyanate. Under these conditions RFF-IX/1 is stable but factor IX is denatured. This column has been regenerated five times without appreciable loss of efficiency.

The factor IX deficient plasma obtained by immunoaffinity depletion has been used as a deficient substrate in a one stage factor IX coagulation assay. Fig. 7, shows that the results obtained with this reagent are highly correlated with those obtained when the same samples of plasma were analysed in an assay using congenital factor IX deficient plasma.

Discussion

The establishment of a clone of hybrid cells that secretes an antibody to factor IX has made possible the production of large amounts of antibody. This in turn can be applied to the large scale production of factor IX deficient plasma for use as a diagnostic reagent. The high affinity of RFF-IX/1 for factor IX ensures that the removal of factor IX from considerable vol-
individual plasmas by affinity depletion. The specific activity of factor IX used in these studies varied somewhat from batch to batch. Possibly this was due to the nature of the starting material (clinical concentrate). However, that the material was apparently homogeneous and free of activated factor IX or any other coagulation factor.
The affinity of RFF-IX/1 for factor IX is comparable to that found for hapten-antihapten interactions\textsuperscript{20} though lower than sometimes found for hyperimmune polyvalent antisera. Hapten antigen interactions involve few sites so that the effective valency of the antigen is small or unity. Obviously with a monoclonal IgG antibody to a monomeric protein without repeating subunits the maximum number of combining sites per antigen molecule is unity and this restricts the affinity compared to that seen with conventional (polyvalent) antisera.\textsuperscript{22} The nature of the antigen antibody interaction could however vary widely with different monoclonals and different epitopes. This particular monoclonal RFF-IX/1 has so far proved most useful for affinity depletion but we anticipate that further fusions will produce monoclonal antibodies that can be used for purification (as has already been reported in relation to human factor V\textsuperscript{23}) or in various combinations for antigen assays.

In principle, hybridoma technology can therefore be expected to yield useful antibodies to each of the thirteen or more zymogens and cofactors involved in plasma coagulation or its inhibition.

REFERENCES

19. Ey PL, Prowse SJ, Jenkin GR: Isolation of pure IgG\textsubscript{1}, IgG\textsubscript{2}, and IgG\textsubscript{3} immunoglobulins from mouse serum using protein A-sepharose. Immunochem 15:429, 1978
Preparation of factor IX deficient human plasma by immunoaffinity chromatography using a monoclonal antibody

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