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Adsorption of human antibodies to factor VIII:C to insoluble modified polystyrene from plasma

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Human procoagulant factor VIII (FVIII:C), is a protein that participates in the cascade of blood coagulation. It is absent or defective in haemophiliac A patients. Furthermore, about 5%–10% of severely affected patients who have received FVIII concentrate as treatment, are developing antibodies which neutralize FVIII:C. Some functional polymers with suitable chemical substituents fixed on to their macromolecular chain might be used in extracorporeal circulation to reduce the concentration of these antibodies. For this purpose, insoluble polystyrenes bearing sulphonate and various amino acid sulphamide groups have been synthesized. The affinity constants for the anti VIII:C and the IgG were determined in purified solution, $K_{\text{anti VIII:C}} = 10^8$–$10^9\ \text{l mol}^{-1}$ and $K_{\text{IgG}} = 10^5\ \text{l mol}^{-1}$. The *in vitro* removal of the anti VIII:C from haemophiliac patient plasma with a high level of antibodies, was tested on various polystyrene-derivative resins. This has led to the selection of active polymers, such as polystyrene substituted by glutamic dimethyl ester acid and/or by hydroxyproline.

1. Introduction

Human anti VIII:C neutralizes the factor VIII:C procoagulant activity and it appears in about 10% of severely affected haemophiliac A patients [1, 2]. These patients have to receive treatment with factor VIII concentrate. They present a difficult clinical problem because of the very large quantities of FVIII:C concentrate needed to reach sufficient VIII:C level. Plasma protein fraction exchange is frequently used to reduce the concentration of anti VIII:C immediately before factor VIII injection [3]. But this does not eliminate the risk of contamination by viruses such as those responsible for hepatitis or AIDS, which is an important problem [4, 5].

Therefore our scope was to modify insoluble polystyrene in order to obtain specific interaction with the Fab fragment of the anti VIII:C as shown in Fig. 1 [6, 7]. An antibody is a protein which possesses two main regions: one which is the constant region also named crystallizable fragment; the other, the variable region or the Fab fragment. The Fab fragment is of more importance because it contains the antigen binding site which gives the specificity of an antibody towards a given antigen [8].

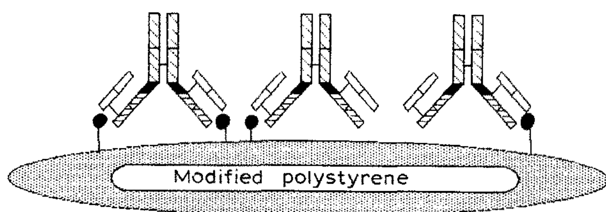

Figure 1 Specific interactions which might be developed between IgG and modified polystyrene.

Based on this idea, cross-linked polystyrenes were modified [6, 7]. To determine the most suitable chemical groups to be fixed on to polystyrene, different types of resin bearing various sulphonate and amino acid sulphamide groups were synthesized. For a given resin it was possible to evaluate the apparent selectivity, *S*, by direct comparison between the adsorption isotherms of the anti VIII:C and the isotherms of the IgG [7]. The more interesting resins were the polystyrene substituted by the glutamic acid dimethyl ester ($PGlOM_2$), and the α hydroxyproline (POHPr). With such resins (150 g) it should be possible to remove the total anti VIII:C antibodies of an haemophiliac patient (200 Bethesda Units/ml) while removing only 1 g IgG (25 g in the whole plasma) [7]. Much of this work was done in buffered solution of single proteins. But studies of protein adsorption from plasma generally show results considerably different from solutions of single proteins [9]. Quantities of individual adsorbed proteins are generally less from plasma and binding affinities may also be modified. Also dynamic effects, involving displacement of one protein by another have been observed [10, 11]. Therefore, the adsorption of the anti VIII:C in the presence of the other plasma proteins is reported. Eight insoluble resins were used. $PGlOM_2$ with different substitution percentages (18%, 24% and 65%) of glutamic dimethyl ester groups, POHPr with different percentages (8%, 16% and

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TABLE I Chemical composition polystyrene resins[a]

	Unsubstituted benzene rings	Sulphonated benzene rings	Amino acid-substituted benzene rings	
			OH–proline	Glu(OMe)$_2$
PGlOM$_2$18	22	60	–	18
PGlOM$_2$24	22	54	–	24
PGlOM$_2$65	22	13	–	65
POHPr8	22	70	8	–
POHPr16	22	62	16	–
POHPr34	22	44	34	–
RM1	22	65	5	8
RM2	22	45	15	18

[a] Data are given as the percentage of benzene rings in the form specified.

34%) of hydroxyproline groups, and two resins substituted with sulphonate, glutamic dimethyl ester and hydroxyproline sulphamide groups (RM) with different percentages of amino acid groups.

2. Materials and methods

2.1. Preparation of functional resins

L-glutamic dimethyl ester sulphamide polystyrene (PGlOM$_2$), *L*-hydroxyproline sulphamide polystyrene (POHPr) and *L*-glutamic dimethyl ester, hydroxyproline sulphamide polystyrene (RM) were prepared as previously described [6, 7]. They were successively washed with 1.5 M NaCl solution and Michaelis buffer, pH 7.4. The mean diameter of the remaining polymer particles was about 100 μm. Their structures are shown in Fig. 2, and their composition are reported in Table I.

2.2. Proteins

Plasma samples were prepared from the blood of normal subjects and from two deficient patients (R and G), R has developed antibodies to FVIII:C (200 Bethesda U/ml). Plasma was collected into 3.8% trisodium citrate, and stored at $-70\,^{\circ}C$ before use; immunoglobulins G were isolated from one haemophiliac's plasma with an anti VIII:C titre of 640 Bethesda U/ml. The final preparation obtained contained 50 Bethesda U anti VIII:C/mg of IgG [7]. The horseradish peroxidase conjugate of the haemophiliac antibody to factor VIII, prepared as F(ab')$_2$ fragment, was kindly provided by Dr J. Ingerslev [12]. This conjugate is stable at $-70\,^{\circ}C$ over more than 2 years.

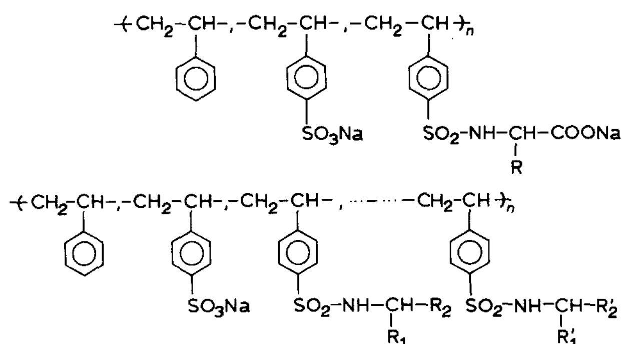

Figure 2 Structure of the modified polystyrene resins.

2.3. Preparation of F(ab')$_2$ fragments

Digestion of anti FVIII:C IgG with pepsin was performed using an enzyme/protein ratio of 2/100 (vol/wt). IgG (2.5 mg/ml plasma R) and pepsin (Sigma 1:60000) were incubated under sterile conditions at 37 °C for 20 h in 0.07 M acetate buffer at pH 4.2 containing 0.05 M NaCl. The reaction was stopped by the addition of solid Tris to obtain a pH value of 7.0 [12]. After 24 h dialysis against acetate buffer, the pepsin digest was subjected to Sephadex G-150 gel chromatography (2.5 × 90 cm) at a flow rate of 30 ml h^{-1}. The F(ab')$_2$-containing fractions were pooled and concentrated using sodium carboxymethyl cellulose (500 kd). Anti VIII:C activity was measured and expressed in Bethesda units [7]. The F(ab')$_2$ fragments solution contained 54 Bethesda U/ml. The solution was kept at $-70\,^{\circ}C$ before use.

2.4. Adsorption procedures

In a purified system. This procedure has been previously described [6].

In plasma. Plasma from the two haemophiliac A patients, R and G, were mixed together in order to obtain plasma samples with different concentrations of anti VIII:C (Table II).

50 μl plasma samples (at various concentrations of anti VIII:C) were incubated with 50 μl polymer suspension (50 mg ml^{-1}) or Michaelis buffer, for 1 h at 37 °C. After centrifugation (900 g for 10 min), the supernatant was diluted 1:100 or 1:200 in Michaelis buffer in order to reach a residual concentration of anti VIII:C varying from 0–2 Bethesda U/ml, compatible with an ELISA experiment. This solution was used to determine simultaneously the residual concentration of anti VIII:C (by ELISA analysis [6, 7]) and of IgG (by Laurell analysis [6, 7]). The assays performed in the presence of buffer allowed the determination of initial antibody concentrations before adsorption.

TABLE II

Plasma R (μl)	Plasma G (μl)	Anti VIII:C (BU/ml)	IgG (mg ml^{-1})
50	200	20	7.3
75	175	30	7.2
100	150	40	7.4
175	75	70	7.3
200	50	86	7.3
215	35	100	7.5

TABLE III Adsorption of anti VIII:C to modified polystyrene resins

	$PGlOM_224$	$PGlOM_265$	POHPr8	POHPr16
K_{aff} (l mol^{-1}) plasma system	2.7×10^8	4.3×10^8	4.6×10^8	4.7×10^8

3. Results and discussion

3.1. Interaction of anti VIII:C and IgG with $PGlOM_2$ and POHPr resins

3.1.1. *Purified system*

Adsorption isotherms were generated from measurements of anti VIII:C and IgG adsorption after 1 h (data not shown). The affinity constants for anti VIII:C and IgG binding, computed on the basis of the Langmuir equation, were determined. They are of the same order of magnitude for $PGlOM_218$ and POHPr34 resins; $K_{anti\ VIII:C} = 10^8$–$10^9\ l\ mol^{-1}$ and $K_{IgG} = 10^5\ l\ mol^{-1}$. These resins exhibit higher affinity for anti VIII:C than for IgG. Furthermore, comparison of the selectivity previously determined for various insoluble modified polystyrene resins, shows that the best substituents for the resins are *L*-glutamic dimethyl ester and hydroxyproline [7]. In order to improve this selectivity, we studied different resins substituted with different levels of the same amino acid derivative. Moreover, Turkova [13] showed that lower content of amino acid derivatives should minimalize non-specific sorption of undesirable proteins on to synthetic sorbents.

3.1.2. *Plasma system*

Adsorption isotherms of anti VIII:C on $PGlOM_218$, $PGlOM_224$, $PGlOM_265$, POHPr8, POHPr16 and POHPr34 resins are shown in Fig. 3. Isotherms were generated from measurements of anti VIII:C binding after 1 h, because it was determined that this was a suitable time in which to reach the steady state [7]. The adsorption isotherms are langmuirian. The affinity constants are shown in Table III.

In the case of $PGlOM_2$ resins, the best results were obtained with the less substituted resin ($PGlOM_218$). The curves reach a plateau at an initial concentration of anti VIII:C of about 60 Bethesda U/ml for both $PGlOM_224$ and $PGlOM_265$. The plateau value of 10–15 Bethesda U/ml presumably represents the limiting capacity of resins for anti VIII:C adsorption under the experimental conditions. For $PGlOM_218$, this capacity is higher, and we did not succeed in reaching the plateau.

In the case of POHPr resins, different adsorption patterns were observed for the higher substituted resins (POHPr34). This is related to a competition with plasma proteins which decrease with increasing concentration of anti VIII:C (Vroman effect) [10]. For POHPr8 and POHPr16, the curves reach a plateau at an initial concentration of antibodies of 70 Bethesda U/ml. The plateau value of 40 Bethesda U/ml is higher than that obtained for $PGlOM_224$. With such resins, it is possible to remove about 50% of inhibitors from a plasma with an anti VIII:C titre of 100 Bethesda U/ml.

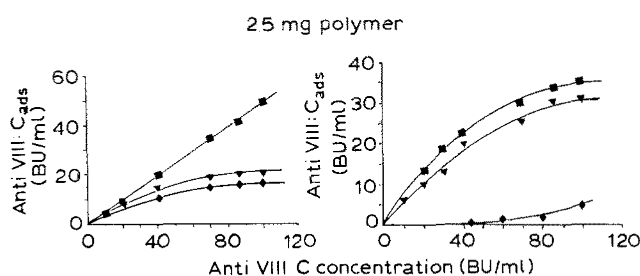

Figure 3 Adsorption isotherms at 37 °C for anti VIII:C in plasma on (■) $PGlOM_218$ (0.7 meq g^{-1}), (▼) $PGlOM_224$ (1.1 meq g^{-1}), (◆) $PGlOM_265$ (2.5 meq g^{-1}), (▼) POHPr8 (0.4 meq g^{-1}), (■) POHPr16 (0.75 meq g^{-1}), and (◆) POHPr34 (1.6 meq g^{-1}).

3.2. Interaction of anti VIII:C and IgG with RM resins

Adsorption isotherms of anti VIII:C on RM resins substituted with both glutamic dimethyl ester and hydroxyproline groups were established in the purified and plasma systems, and compared with those of $PGlOM_2$ and POHPr resins. They were generated from measurement of anti VIII:C adsorption after 1 h, because again it was determined that this was a suitable time in which to reach the steady state. The antibody concentration varied from 10–100 Bethesda U/ml. Fig. 4 shows the isotherms of anti VIII:C on RM1 and RM2, in both IgG with 10% plasma G solution and in plasma samples. For both resins, data are not sufficiently extensive that plateaux are not well defined. The affinity constants cannot be determined.

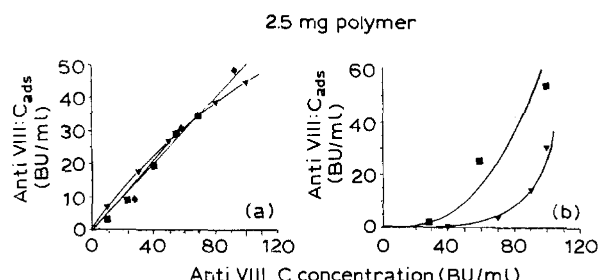

Figure 4 Adsorption isotherms at 37 °C for anti VIII:C in (▼) plasma or (■, ◆) purified systems on (a) RM1 (RM18) and (b) RM2 (RM27). RM18:OHPro 0.28 meq g^{-1}, GlOMe$_2$ 0.45 meq g^{-1}; RM27:OHPro 0.83 meq g^{-1}, GlOMe$_2$ 1.2 meq g^{-1}.

The best results are obtained with RM1 with a low degree of amino acid substitution. We should note that the exact chemical nature of the glutamic dimethyl ester and of the hydroxyproline binding sites is unknown and that a number of possibilities exist, including various sequences of sulphonate, glutamic dimethyl ester and hydroxyproline of different lengths. The results obtained in the purified system are similar to those obtained in plasma. RM1 resin retained its high capacity and strong affinity for anti VIII:C even in the presence of many other plasma proteins and enzymes. This result is in agreement with the value of the affinity constant previously found, $K_{\text{anti VIII:C}} = 10^9 \text{ l mol}^{-1}$. The presence in this resin of a range of adsorption sites of different types is almost certain, and it may be that sites specific to anti VIII:C are unavailable to other proteins when anti VIII:C is present. Furthermore, we notice that the results obtained for RM1 are almost the same as those obtained with PGlOM$_2$18.

RM2 shows a greater difference in adsorption kinetics between the plasma and the purified system than do RM1. The adsorbed anti VIII:C concentrations are lower than the corresponding values obtained in the purified system and are attained less rapidly. This effect looks like a Vroman effect [10] and is an indication of a competition between the anti VIII:C and the other plasmatic proteins. This suggests its distinctive behaviour is due presumably to the higher amino acid group substitution.

3.3. Adsorption of F(ab')$_2$ fragments

Adsorption of F(ab')$_2$ fragments to POHPr8 was studied. The results were obtained from measurements of F(ab')$_2$ adsorption after 1 h, for an initial concentration of antibodies high enough to reach the saturation plateau. These values were chosen after study of the anti VIII:C adsorption isotherms (Figs 3 and 4). The results are reported in Table IV.

For an initial anti VIII:C concentration of 57 Bethesda U/ml, the adsorbed F(ab')$_2$ fragment concentration obtained at saturation is of the same order of magnitude as that determined for anti VIII:C, although consistently smaller. For the second concentration of antibodies studied, the adsorbed F(ab')$_2$ fragment concentration is two times less than the corresponding value obtained for the anti VIII:C. This may indicate that a small part of anti VIII:C is adsorbed through non-specific binding.

TABLE IV Adsorption of F(ab')$_2$ fragment to POHPr8

	Initial concentration anti VIII:C (BU/ml)	
	57	40
Adsorbed anti VIII:C (BU/ml)	24 ± 3	19 ± 3
Adsorbed F (ab')$_2$ (BU/ml)	18 ± 3	7 ± 3

The major conclusion from the present data is that PGlOM and RM1 resins retain their high capacity and strong affinity for anti VIII:C even in the presence of plasmatic proteins. Furthermore, these results suggest that anti VIII:C interacts with the polymer surface via its variable region.

Acknowledgements

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