

Association between coagulation factors VII and X with triglyceride rich lipoproteins

J CARVALHO DE SOUSA,* CLAUDINE SORIA,* MARISE AYRAULT-JARRIER,† DANIELLE PASTIER,† E BRUCKERT,‡ J AMIRAL,§ G BEREZIAT,† J P CAEN*

From *l'Hôpital Lariboisière, Service d'hématologie, Paris, †l'Hôpital Saint-Antoine, Service de biochimie, Paris, ‡l'Hôpital Pitié-Salpêtrière, Service d'endocrinologie, Paris, and §Diagnostic Stago, Franconville.

SUMMARY The association between the concentration of different plasma lipoproteins and plasma factor VII (F VII) was analysed by isolating plasma very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) lipoproteins and assessing their in vitro interaction with F VII by immunoenzyme assay using peroxidase labelled anti-factor VII immunoglobulins to determine whether F VII coagulant activity is prognostic for cardiovascular mortality.

F VII bound to triglyceride rich lipoproteins, the fixation being stronger on chylomicrons and VLDL fractions than on LDL fractions. In our experiments HDL did not bind to F VII. The fixation of coagulation factor X (F X) tested by the same method is comparable with that of F VII. The nature of this fixation seemed to arise from hydrophobic interaction as calcium was not necessary and the use of Tween 20 inhibited the interaction. The binding of factors VII and X was increased when lipids were previously treated by phospholipase C and the interaction seemed to be completely dependent on the lipid part of the lipoproteins.

Hydrophobic fixation is a possible mechanism of interaction of plasma lipoproteins and F VII and X, and it may be of importance in the covariance of triglyceride concentrations and the activity of vitamin K dependent coagulation factors.

Meade *et al* showed that plasma coagulation factor VII (F VII) activity is as reliable a predictor for cardiovascular thrombosis as cholesterol and triglyceride plasma concentrations.¹ It is also well known that F VII activity is positively related to plasma lipid concentrations in healthy adults² and in patients with cardiovascular disease.³

It has previously been shown⁴ that F VII activity is increased in types IIb and IV hyperlipidaemic subjects, but a direct association between this activity and plasma lipoproteins has not yet been established. To our knowledge, correlation of lipoprotein concentrations with F VII have been studied only incidentally. Factor (F X) concentrations have been reported to be higher in type IIb hyperlipidaemia and to have a positive correlation with plasma cholesterol and triglyceride concentrations.^{4,5}

The aim of this study was to investigate the possible direct interaction between plasma lipoproteins and coagulation factor VII. We developed two immunoenzyme assays to probe the adsorption of factors VII and X on lipoproteins.

Material and methods

Blood samples were obtained from five adults. Blood (nine volumes) was collected into polystyrene tubes with 0.13 M sodium citrate (one volume) and immediately centrifuged at 10 000 g for 10 minutes at room temperature. Plasma was kept at 4°C until isolation of lipoproteins was carried out.

Chylomicrons were isolated from plasma by flotation. Lipoproteins were isolated at a maximum of five hours after plasma collection by gradient ultracentrifugation according to the method of Chapman,⁶ and each fraction was collected separately. The lipoproteins were then dialysed against 0.1 M phosphate buffer (pH 7.4) over 12 hours at 4°C. To delipidate very low density lipoproteins (VLDL) and low density lipoproteins (LDL) we used the technique of ether delipidation, as described previously.⁷

IMMUNOENZYME PROCEDURES

We used 96-well polystyrene microtitre plates for enzyme linked immunosorbent assay (ELISA) (Dynatech Laboratory, Paris). Prekonativ (Kabi Flow Laboratories, Paris) was used as factor VII and X

Association between factors VII and X with triglycerides

concentrate. Factors VII and X activities were 0.45 U/mg and 1.15 U/mg, respectively (one U represents the amount contained in one millilitre of normal plasma).

Buffer and reagent used for immunoenzyme assay were as follows: phosphate buffered saline-albumin buffer, pH 7.6 (0.15 M NaCl, 0.07 M PO₄ HNa₂, 0.0015 M PO₄ H₂ K, 1 g bovine albumin in one litre of distilled water). In the experiments with Tween 20, 0.5 ml of this was added to one litre of buffer. Tris-imidazole buffer was made up with imidazole (1.36 g/l), Tris (0.1 M), pH 7.3, 1 g bovine albumin in one litre of distilled water. This buffer was also made with and without calcium (M/80). Carbon/bicarbonate buffer (pH 9.6) was made up with sodium bicarbonate 8 g and sodium carbonate 14.6 g in one litre of distilled water.

Reagent for the determination of peroxidase activity comprised 40 mg of orthophenylene diamine (OPD) dissolved in 100 ml pH 5 buffer consisting of 0.05 M citric acid and 0.1 M Na₂HPO₄, to which were added 40 µl of 30% hydrogen peroxide.⁸

Anti-F VII and anti-F X peroxidase-labelled immunoglobulins were obtained from Diagnostic Stago Laboratories, Asnières, France. Anti-Apo AI, anti-Apo AII, and anti-Apo B immunoglobulins were obtained from antisera as described previously.⁹ Anti-Apo AI, anti-Apo AII, anti-Apo B, anti-Apo CIII, and anti-Apo E monoclonal antibodies were kindly given by Professor Fruchart, Service de Biochimie, Institut Pasteur de Lille, France. Phospholipase C from *Bacillus Cereus* was purchased from Sigma, St Louis, Missouri, USA.

DIRECT ASSAY (fig 1)

Ninety six-well polystyrene microplates (Dynatech-Microelisa) were coated by adding 150 µl of lipoprotein fractions to each well and incubating overnight at 37°C. The plates were washed four times with 0.15 M NaCl containing (or not) 0.05% Tween 20, using a Dynatech washer. Another incubation with bovine albumin (30 mg/ml in distilled water) was done in all wells for two hours at 37°C to avoid subsequent non-specific adsorption. Prekonativ was dissolved in Tris-imidazole buffer (2 mg/ml) with and without Tween 20, and 200 µl was added to each well.

After two hours' incubation at 37°C the plates were washed four times with 0.15 M NaCl containing (or not) Tween 20. Two hundred µl of peroxidase-labelled anti-F VII or anti-F X Ig G in the PBS-albumin buffer was added. After two hours' incubation at 37°C the plates were washed four times and peroxidase-labelled IgG bound to the wells was determined by adding into each well 200 µl orthophenylene diamine (OPD) solution: the colour development was stopped after exactly 240 seconds by adding 50 µl of 2.5 N sulphuric acid. The absorbance was measured at 492 nm using an autoreader (Dynatech).

In another series of experiments a solution of phospholipase C (100 µg/ml final concentration in PBS buffer) was added to each well before F VII or F X concentrates and then the same method was applied.

Each test in each of the five different plasmas was performed in triplicate and results were taken as the mean value (differences between the three determinations were always less than 5%).

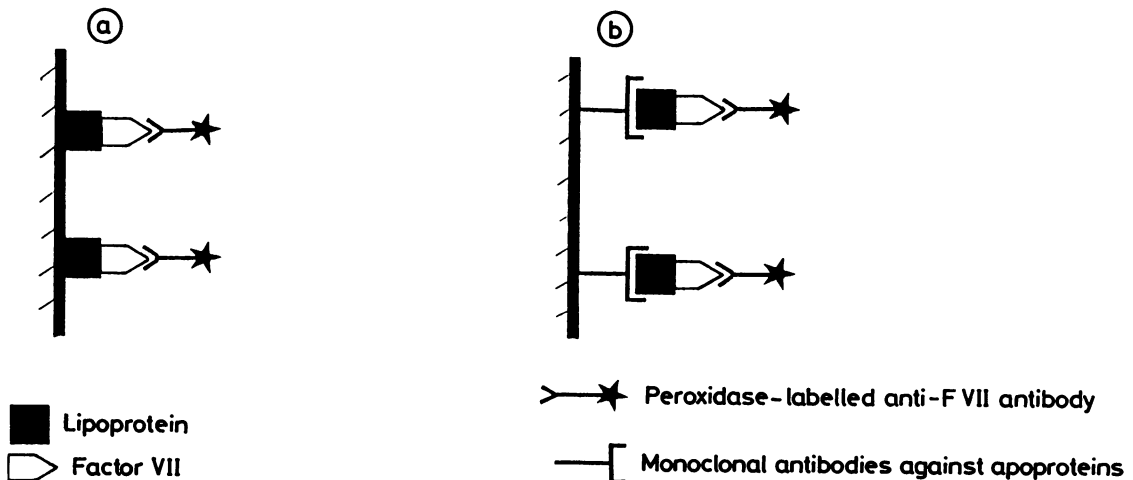


Fig 1 Direct (a) and indirect (b) immunoassays of FVII interaction with plasma lipoproteins. ■ Lipoprotein fractions. ◇ Factor VII. ➔★ Peroxidase-labelled anti-F VII antibody. —[Monoclonal antibodies against apoproteins.

INDIRECT ASSAY

A procedure using polystyrene bound polyclonal and monoclonal antibodies against apolipoproteins as capture antibodies was used. Ninety six-well polystyrene microplates (Dynatech-Microelisa) were coated by adding to each well 150 μ l of each antibody dissolved in carbonate/bicarbonate buffer, pH 9.6 (50 μ g/ml) and incubated overnight at 37°C. The plates were then washed four times with 0.15 M NaCl, and another incubation with bovine albumin (3% in PBS buffer) was done in all wells (200 μ l/well) for two hours at 37°C to avoid future non-specific adsorption. Two hundred μ l/well of several dilutions of a normal citrated plasma in PBS-albumin buffer were then added to all wells and another two hours' incubation at 37°C was carried out. After this the plates were washed four times with 0.15 M NaCl. Prekonativ dissolved in Tris-imidazole buffer (2 mg/ml) was added to each well. The experiment was then continued as described for the direct assay.

For all the experiments negative controls were carried out with bovine albumin (3% in PBS buffer) coated wells.

Results

We first analysed the interaction of F VII with isolated lipoprotein fractions in a purified system. The results of these tests carried out with anti-F VII peroxidase-labelled immunoglobulin showed binding of F VII to chylomicrons, VLDL, and LDL. This binding was greater for VLDL than for LDL (fig 2). HDL fractions bound minimal amounts of F VII.

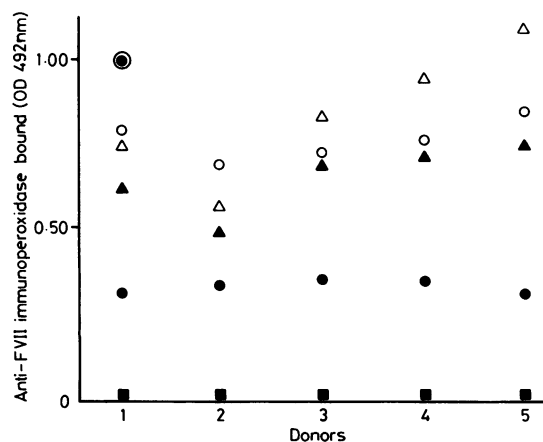


Fig 2 Interaction of F VII with isolated lipoproteins from five donors tested before and after phospholipase C treatment. ■ HDL coated plates before treatment; ● LDL coated plates before and after (○) treatment; ▲ VLDL coated plates before and after (△) treatment; ⊙ chylomicrons coated plates from donor 1.

The uncoated wells showed no binding (optical density less than 0.01).

To assess the type of interaction we analysed the effect of calcium and detergents. When F VII or X were added to lipoprotein coated wells in Tris-imidazole buffers with or without calcium (M/80) no difference was found between experiments (optical density difference less than 5%). When Tween 20 was used in NaCl or in reagent buffers after coating no immunoperoxidase-labelled Ig bound to any of the lipoproteins.

To test the specificity of this association between triglyceride rich plasma lipoproteins and F VII, we performed the same experiments with F X. The binding of F X follows the same pattern as that of F VII (fig 3). F X also binds preferentially to triglyceride rich lipoproteins. In an attempt to identify the molecules implicated in this interaction we investigated the effect of phospholipase C. It seemed to amplify the binding of F VII and X (figs 2 and 3).

Ether delipidation of VLDL and LDL abolished the binding of F VII and greatly decreased that of F X (fig 4).

Finally, in indirect assays we tested the adsorption of F VII to plasma lipoproteins captured by polyclonal and monoclonal antibodies. Lipoproteins bound to anti-Apo B, anti-Apo CIII, and anti-Apo E monoclonal antibodies (table). Results obtained using polyclonal anti-Apo B antibodies are similar to those obtained with monoclonal antibodies. Lipoproteins recognised by anti-Apo AI and anti-Apo AII polyclonal and monoclonal antibodies did not bind F VII in measurable amounts for plasma dilutions from 1/10

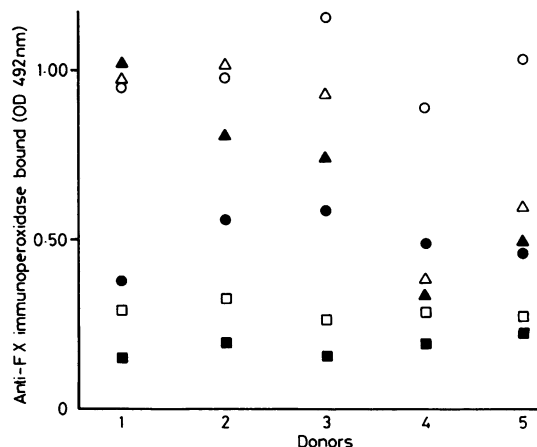


Fig 3 Interaction of F X with isolated plasma lipoproteins from five donors tested before and after phospholipase C treatment. ■ HDL coated plates before and after (□) treatment; ● LDL coated plates before and after (○) treatment; ▲ VLDL coated plates before and after (△) Plc treatment.

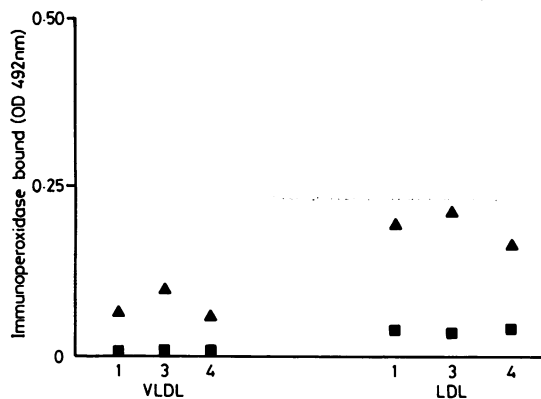


Fig 4 Interaction of F VII and X with plasma lipoproteins treated with ether from donors 1, 3, and 4. Anti-F VII fixation on delipidated VLDL is almost undetectable; note scale amplification on optical density. ■ Anti-F VII immunoperoxidase; ▲ anti-F X immunoperoxidase.

to 1/10 000, but these HDL lipoproteins were recognised by coated antibodies as we were able to identify them by peroxidase-labelled immunoglobulins to Apo AI (results not shown).

Repeating the experiments with Tween 20 added after lipoprotein capture (as for direct assays) showed that no immunoperoxidase-labelled Ig was bound to any of the lipoproteins (optical density always less than 0.001).

Discussion

Several studies¹⁰ have shown increased F VII coagulant activity in hyperlipidemic states, but a direct interaction between this glycoprotein and circulating lipids has not yet been shown. For these reasons we decided to study the interaction of F VII with different circulating lipoproteins.

Our results clearly show that lipoproteins interact with F VII to a degree that depends on the type of lipoprotein. The interaction is stronger with triglyceride rich lipoproteins. The association of F VII

with VLDL and LDL shows a positive relation between the amount of bound F VII and the triglyceride content of those lipoproteins.¹¹ The chylomicrons tested in one of the plasma samples also bound the highest amount of F VII.

This interaction was hydrophobic because it was inhibited by Tween 20 and is calcium independent. The inhibiting effect of Tween 20 is not related to a release of lipoproteins from polystyrene wells as the same results were obtained in the two systems. In indirect assays where lipoproteins are not directly immobilised on plastic but captured by antiapolipoprotein antibodies Tween 20 also inhibited the adsorption of coagulation factors, and it is known that antigen-antibody reactions are not abolished by Tween 20 in such conditions. This is an important difference for the formation of enzymatically active clotting factor-phospholipid complexes, as has already been noted for coagulation F V and VIII.^{12,13}

This difference is also apparent in the experiments with phospholipase C. Phospholipase C action increases the fixation of F VII, in contrast to its action on procoagulant complexes.¹⁴ The increased fixation might be due to the greater availability of neutral lipids after phospholipase action. The role of neutral lipids in this form of adsorption is implicated not only by their distribution among the lipoproteins but also by the effect of ether treatment, which abolishes the adsorption—probably because it removes neutral lipids. The results of indirect assays with polyclonal and monoclonal anti-apoprotein antibodies show that conformational lipoprotein changes could not have been responsible for the interaction we described.

F VII and X bind to lipoproteins in a very similar way. For F X we found a slight fixation on HDL and a slight interaction with ether delipidated lipoproteins which was not seen with F VII. The molar concentrations of F X, however, are 10 times higher than those of F VII, which could partially explain the differences.

It is not inconceivable that this interaction can also occur in vivo, but no direct evidence was obtained using separation of lipoproteins by ultracentrifugation as we were not able to detect any F VII or X in the

Table Immunoperoxidase anti-F VII bound to monoclonal antibodies coated plates.*

Monoclonal antibodies	Anti-Apo A ₁	Anti-Apo A ₂	Anti-Apo B	Anti-Apo C _{III}	Anti-Apo E
Plasma dilutions added to coated plates					
1/10	0.085	0.137	0.435	0.995	1.920
1/50	0.089	0.121	0.375	0.820	1.145
1/250	0.090	0.116	0.070	0.160	0.575
1/500	0.050	0.093	0.065	0.086	0.230
1/1000	0.060	0.075	0.068	0.087	0.200
1/5000	0.070	0.060	0.059	0.063	0.085
1/10 000	0.065	0.080	0.073	0.050	0.078

*The results are expressed in absorbance at 492 nm and all are the mean value of two determinations.

isolated lipoproteins (results not shown). These negative results do not exclude an interaction *in vivo* as the labile adsorption of coagulation factors can be successfully abolished by the ultracentrifugal procedure.

Additionally, there is some indirect evidence of *in vivo* interaction between F VII and triglycerides from clinical studies which show a positive correlation between F VII and plasma triglyceride concentration. The functional activity of F VII bound to immobilised triglyceride rich lipoproteins should be investigated to see if fixation affects activation of the molecule.

The capacity of VLDL and LDL atherogenic lipoproteins to bind F VII in a different way from procoagulant phospholipids may well be of potential importance. This could be reflected in epidemiological correlations between lipids, F VII, and cardiovascular thrombosis.

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Requests for reprints to: Dr J Carvalho de Sousa, Hôpital Lariboisière, Service d'hématologie, 75475 Paris, Cedex 10, France.