Evidence That Fibronectin-Immunoglobulin Complexes Occur Normally in Plasma (43823)

BONNIE ANDERSON BRAY,[1] MOHAMED OSMAN, AND GERARD M. TURINO
Columbia University College of Physicians and Surgeons, New York, New York 10032

Abstract. **This study was done to ascertain whether complexes of plasma fibronectin (PFn) and immunoglobulins (Igs) are normally present in plasma. PFn preparations from plasmas of six random human donors were analyzed for IgG, IgM, and IgA by competitive ELISA procedures. To look for direct evidence of circulating complexes, two of the plasma samples were chromatographed on Sephacryl S-300. PFn prepared from a pool of three plasmas by cryoprecipitation was studied by crossed immunoelectrophoresis to detect PFn-Ig complexes.**

The Ig content of PFn ranged from 0.78% to 9.0% IgG; 0.25% to 4.9% IgM, and 0.15% to 0.71% IgA. The relative amounts were IgG > IgM > IgA in every sample, but the total Ig content of PFn varied with the plasma donor. The Sephacryl S-300 chromatogram of the plasma from which Ig-rich PFn was made showed distortion of the PFn peak by Ig peaks. PFn prepared by cryoprecipitation contained PFn-immunoglobulin complexes.

Plasma from adult beagle dogs was used to prepare canine PFn. IgM was detected in canine PFn by SDS-Page and by double diffusion experiments against antiserum to canine IgM. By crossed immunoelectrophoresis, a PFn-IgM complex was detected in canine plasma. The cryoprecipitate recovered from a Sephacryl S-300 chromatogram of canine plasma contained equivalent amounts of IgM and PFn.

These data suggest that complexes of Igs with PFn are normally present in plasma. [P.S.E.B.M. 1994, Vol 207]

Plasma fibronectin (PFn), a high molecular weight glycoprotein, forms noncovalent associations *in vitro* with molecules of diverse chemical composition, among them fibrinogen and fibrin, collagen, certain glycosaminoglycans, and cell surface proteins (for review see Ref. 1). Recently, PFn has been shown to bind to components of the immune system (e.g., CIq [2–4], immune complexes [5], myeloma immunoglobulin G [IgG] [6], modified polyclonal IgG [7, 8], and to IgM and IgA [9]).

In the course of our studies on tissue fibronectin (10–14), many preparations of fibronectin were made from plasma (human and canine) for use as antigens for the preparation of antisera. We consistently observed in immunoelectrophoresis experiments that the antisera contained antibodies to immunoglobulins as well as to fibronectin (but not to albumin) and had to be absorbed free of anti-Ig antibodies before use. Thus, the PFn preparations all contained Igs, and the suggestion was made that an association between PFn and Igs exists *in vivo* in normal plasma (15). Evidence for such an association has been demonstrated in plasma from patients with a variety of connective tissue, lymphoproliferative, and myeloproliferative disorders (16–21) and in primary IgA nephropathy (22). One could envision that the presence of complexes between PFn and Igs in these diseases is an exaggeration of the normal occurrence of the complexes ordinarily present in small amounts or that form only in response to a special need, for example, the need to remove modified Ig molecules from the circulation via the reticuloendothelial system in which PFn is known to participate (23).

In the present study we have asked whether or not

[1] To whom requests for reprints should be addressed at St. Luke's-Roosevelt Hospital Center, 1000 Tenth Avenue, AJA Room 101, New York, NY 10019.

Received March 11, 1994. [P.S.E.B.M. 1994, Vol 207]
Accepted July 13, 1994.

0037-9727/94/2073–0324$10.50/0

PFn-Ig complexes are normally present in plasma. We have quantified IgM, IgA, and IgG in affinity-purified human PFn specimens stored in the laboratory. PFn that had been prepared under conditions that would preserve associations (i.e., ammonium sulfate precipitation and cryoprecipitation), was examined by crossed immunoelectrophoresis. Intact plasmas were examined by gel permeation chromatography to look for evidence of overlap of the PFn peak with Ig peaks.

Canine PFn was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-Page) and in double diffusion experiments against an antiserum to canine IgM. Plasma from adult beagles was studied in crossed immunoelectrophoresis experiments and by gel permeation chromatography. A cryoprecipitate recovered from the chromatography fractions was analyzed for IgM and PFn content.

The data suggest that PFn-Ig complexes are normally present in plasma.

Materials and Methods

Human Fibronectin Preparations. Human plasma, outdated 5 days or less, was obtained from the blood bank and was kept frozen at −70°C.

Preparations 1–6 (Human). Preparations 1–6 were prepared by affinity chromatography of individual human donor plasmas (20–40 ml) on a 19- to 30-ml column of gelatin-Sepharose (24) obtained from Bio-Rad Laboratories (Richmond, CA), or prepared in this laboratory. After washing out nonbound plasma proteins, the PFn was eluted with 0.2 *M* glycine, pH 2.6–2.8, or 4 *M* urea in 0.05 *M* Tris buffer, pH 7.5. Samples were dialyzed against 0.05 *M* Tris, pH 7.5, containing 0.1 *M* NaCl, and were stored at −70°C. Except where noted, all solutions contained phenylmethylsulfonyl fluoride (PMSF) to prevent proteolysis of PFn during preparation. The concentration of PFn in mg/ml was calculated by subtracting the optical density at 320 nm from that at 280 nm and dividing by 1.28 (25).

Preparation 7 (Human). Three units of plasma were combined and centrifuged at 27,000*g*. To 550 ml of the clear mixture (cooled to −4°C [ice-salt bath]), was added 70.9 g ammonium sulfate in small portions with stirring (approximately 18% saturation). The precipitate was collected by centrifugation at 27,000*g* and was washed twice with 25% saturated ammonium sulfate. The washed precipitate was dissolved in 100 ml of 0.05 *M* phosphate containing 0.01 *M* ethylenediaminetetraacetic acid (EDTA), pH 6.7 at 37°C. The solution was cooled to −2°C (ice-salt bath), whereupon a large amount of cryoprecipitate formed. This precipitate was collected by centrifugation at 27,000*g* and was dissolved in 100 ml of 0.01 *M* Tris, pH 7.4 containing 0.1 *M* NaCl. Fibrinogen was removed as fibrin formed upon the addition of 100 units of bovine thrombin in 0.05 *M* Tris, pH 8, containing 0.002 *M* $CaCl_2$. The clot (fibrin) was removed after 15 min, and the supernatant was centrifuged at 27,000*g* to remove any small pieces of fibrin. The clear supernatant in a plastic tube was cooled to −10°C (ice-salt bath) and was kept at that temperature for several hours. The white precipitate which formed on the walls of the centrifuge tube was collected by centrifugation at 2000*g* at 0°C and was dissolved in 25 ml of 0.01 *M* Tris, pH 7.4 containing 0.1 *M* NaCl. This was a true cryoprecipitate in that it came out of solution upon standing at 4°C and readily redissolved at 37°C. Fibrinogen was shown to be absent by immunoelectrophoresis and immunodiffusion experiments with antifibrinogen.

Canine Fibronectin Preparations. Canine blood was obtained from healthy, adult purpose-bred beagles from Marshall Farm, Inc. (North Rose, NY). They had been vaccinated against canine distemper, hepatitis, parvo viruses, and leptospirosis, and were also heart worm negative. These dogs were used in an acute experiment and were deeply anesthetized with pentobarbital sodium (35 mg/kg) intravenously. A butterfly catheter was inserted into the cephalic vein for both anesthesia administration and blood collection. For blood collection the butterfly was connected into a 20-ml syringe containing 2 ml of a mixture of 3.8% sodium citrate and 5 m*M* benzamidine hydrochloride. The amount of blood collected varied from 60 to 100 ml, depending on the protocol for each experiment. After centrifugation at 2000*g* to settle rbc, white cells, and platelets, clear plasma was removed and stored at −70°C until used.

Preparation 1 (C1). Canine plasma (25 ml) was chromatographed on a column of gelatin-Sepharose with a resin bed volume of 30 ml. After washing out nonbound plasma proteins the PFn was eluted with 0.2 *M* glycine, pH 2.6, and was dialyzed against 0.05 *M* Tris, pH 7.4 containing 0.1 *M* NaCl. All buffers contained PMSF (1 m*M*).

Preparation 2 (C2). The procedure of Vuento and Vaheri (26) was adapted. All buffers were made in 0.05 *M* Tris, pH 7.5, containing 5 m*M* benzamidine HCl (Tris). The plasma (50 ml) was first passed through a column of Sepharose 4B (100 ml resin bed equilibrated with Tris −0.02% sodium azide) to remove plasma proteins which might bind nonspecifically to Sepharose 4B and was then chromatographed on gelatin-Sepharose 4B (preeluted with 1 *M* arginine) to which PFn adhered. The column was washed sequentially with Tris, Tris containing 1 *M* NaCl and then Tris containing 0.2 *M* arginine, and these washes were discarded. The first fraction of PFn was eluted with Tris containing 1 *M* arginine. Twenty-five percent of the PFn remained adherent to the gelatin-Sepharose after the 1 *M* arginine elution step. This PFn (C2) was eluted

with Tris containing 8 *M* urea and was dialyzed against 0.05 *M* Tris, pH 7.5, containing 0.1 *M* NaCl.

Preparations C1 and C2 were examined by SDS-Page and in double immunodiffusion experiments and were used to immunize rabbits.

Antisera. Antisera to selected human PFn preparations and to canine PFn preparations C1 and C2 (urea eluate) were raised in rabbits and were absorbed with fibronectin-free plasma, which had been insolubilized (27), until the only antibodies detectable in immunoelectrophoresis experiments were to fibronectin. Rabbit anti-human IgG (heavy and light chains) was obtained from Cappel (Organon Teknika-Cappel Corp., Durham, NC) as was goat anti-canine IgM (μ chain specific). Rabbit anti-human fibrinogen was obtained from Dr. Konrad Hsu of Columbia University and from Hyland Laboratories.

ELISA Reagents. Human IgG, IgM, and IgA were obtained from Cappel. Alkaline phosphatase conjugates of antibodies to human IgG, IgA, and IgM were obtained from Sigma Chemical Co. (St. Louis, MO), as was the substrate disodium p-nitrophenylphosphate.

Quantification of Immunoglobulins. The immunoglobulin content of the human PFn preparations was determined by a competitive ELISA assay. For IgG each flat-bottom well of a polystyrene microtiter plate (Costar tissue culture clusters; Costar, Cambridge, MA) was coated with the antigen, human IgG (Cappel) by incubation with 0.2 ml of IgG at 2000 ng/ml in sodium carbonate-bicarbonate coating buffer, pH 9.6, overnight in a moist chamber at 4°C. Excess antigen was removed, and the wells were rinsed three times (3 min/rinse) with 1% bovine serum albumin (BSA) containing 0.9% NaCl, and 0.05% Tween-20. Unreacted sites on the plastic were blocked by filling the wells with 5% BSA containing 0.9% NaCl, and 0.05% Tween-20, and incubating the plate for 30 min at room temperature. After removal of the blocking solution, the wells were rinsed three times as above.

Competition of known amounts of antigen (standard curve) or unknown samples for anti-human IgG alkaline phosphatase conjugate was carried out in triplicate wells of another plate which had been blocked with BSA and rinsed as above. To 110 μl anti-human IgG-alkaline phosphatase conjugate (1 → 1000 dilution) was added 110 μl of human IgG of known concentration or 110 μl of an appropriate dilution of the sample being analyzed or of PBS-Tween (control). The mixtures were incubated in a moist chamber at 4°C overnight, and 0.2 ml of each mixture was transferred to a well of the antigen-coated plate, and the plate was incubated for 2 hr at 25°C. The competition mixture was removed, and the plate was rinsed as before. Bound alkaline phosphatase conjugated anti-human IgG was detected by incubation with the substrate disodium p-nitrophenylphosphate at 1 mg/ml in 0.05 *M* sodium carbonate and 1 m*M* magnesium chloride, pH 9.8, for 15 min, and the reaction was stopped by the addition of 50 μl 3 *M* NaOH to each well. Optical densities were read on a microelisa minireader (Dynatech). IgM was determined by the same procedures using pooled myeloma IgM as antigen and alkaline phosphatase-conjugated anti-human IgM as antibody. Likewise, for IgA determination the antigen was IgA and the antibody was anti-human IgA-alkaline phosphatase conjugate. The coefficients of variation of the procedures were always less than 10%.

Crossed Immunoelectrophoresis (28) of Preparation 7. In the first dimension, the samples (15 μl of isolated PFn [Preparation 7]) were electrophoresed into agarose at pH 8.6. For the second dimension, the strips of agarose were positioned so that the components would migrate into agarose (12 ml) containing antifibronectin (50 μl) (Fig. 1, left panel) or into agarose (12 ml) containing the same antifibronectin (50 μl) admixed with 50 μl of antiserum to both the heavy and light chains of IgG (Fig. 1, right panel). After soaking to remove reagents other than those in immune precipitates, the plates were dried and stained with Coomassie Brilliant Blue R250. Tracings were made of the peaks, and these tracings were cut out and weighed.

Crossed Immunoelectrophoresis of Canine Plasma. Canine plasma (5 μl) was electrophoresed in the horizontal dimension. In the vertical dimension the separated components were electrophoresed through an intermediate gel containing either goat anti-canine IgM (50 μl/4 ml agarose) or 50 μl buffer/4 ml agarose, and then into agarose containing a monospecific rabbit antibody to canine fibronectin (75 μl/8 ml agarose). The plates were soaked to remove components not bound in immune precipitates and were dried and stained as above.

SDS-Page. All PFn preparations were examined in SDS-PAGE experiments by the procedure of Furthmayer and Timpl (29).

Chromatography of Plasma on Sephacryl S-300. Plasma 1 (2.5 ml) and 6 (3 ml) were separately chromatographed on a column of Sephacryl S-300 of 1-cm radius and 87-cm height (273-ml volume), and fractions (2.4 and 2.7 ml, respectively) were collected. Aliquots of the fractions were analyzed for PFn, IgM, IgA, and IgG by quantitative immunodiffusion (30).

Canine plasma (25 ml containing citrate and benzamidine) was chromatographed on the same column in 0.05 *M* Tris containing 0.1 *M* NaCl, pH 7.6, and 6.4-ml fractions were collected. The cryoprecipitate that formed in fractions of the IgM and PFn regions (109–154 ml) was collected by centrifugation at 2000*g* at 2°C and was washed three times with the buffer and was dissolved in 5-ml buffer by warming to 37°C. PFn in

the cryoprecipitate was determined by an ELISA method (14) and the IgM present by quantitative immunodiffusion (30).

Results

Human PFn. *Quantitation of immunoglobulins in human PFn preparations.* IgM, IgA, and IgG were all present in PFn obtained from plasma of six random, individual donors by affinity chromatography on gelatin-Sepharose. These data are presented in Table I as the percentage of total protein in the isolated PFn preparation and are arranged in order of increasing Ig content. Overall, the content varied as follows: IgG, 0.78%–9.0%; IgM, 0.25%–4.9%; and IgA, 0.15%–0.71%. In every sample, there was more IgG than IgM, and more IgM than IgA.

These data were used to estimate the fraction of PFn molecules that could have been present as PFn-Ig complexes. It was assumed that every Ig molecule in the PFn preparation was complexed with PFn at the time the plasma was applied to gelatin-Sepharose and that PFn-Ig complexes have the same affinity for gelatin-Sepharose as does free PFn. The molecular weights used were 440 kDa for PFn, 150 kDa for IgG, 1000 kDa for IgM, and 160 kDa for IgA. For Preparation 1a from Plasma 1, there were 18 molecules of IgG, 1 of IgM, and 3.2 of IgA for 776 molecules of PFn. Thus, around 3% of the PFn molecules could have been complexed with Ig. For Plasma 6, however, for every 193 molecules of PFn there were 60 of IgG, 5 of IgM, and 4 of IgA, which amounted to 36% of the PFn molecules. Since these donors were randomly chosen and no other information was available on them, we can not explain this variation in total Ig content between the donors.

Evidence for PFn-Ig complexes in cryoprecipitable PFn. In PFn Preparation 7, prepared by ammonium sulfate precipitation and cryoprecipitation of PFn, the yield of PFn was only 25% theoretical, but the content of Ig was increased as indicated by the analysis for IgG (11% IgG). IgM was shown to be present in the preparation by immunoelectrophoresis (not shown), but quantitative data for IgM and IgA are not available. In this preparation, complexes between PFn and Igs were detected by crossed immunoelectrophoresis (Fig. 1). In both panels of Figure 1, PFn 7 was first electrophoresed at pH 8.6 in the horizontal direction. The separated components were then electrophoresed in the vertical direction into agarose containing (Fig. 1, left panel) monospecific anti-human PFn or (Fig. 1, right panel) a mixture of the anti-PFn and anti-human IgG. In the left panel, a large peak at the expected location for PFn was present. Additionally, the peak had a shoulder of partial identity on the cathodal edge. By planimetry, 30% of the PFn was in the shoulder. The fact that the shoulder of partial identity was cathodal to the PFn peak suggests that the PFn was complexed with IgG which, when free, migrates cathodal to the origin. In the complex, the net negative charge of PFn was reduced by the positive charges on IgG.

The pattern of peaks detected with anti-PFn (Fig. 1, left panel) was markedly changed by the presence of the antibodies to Igs (Fig. 1, right panel). The width of the main peak was broadened and by planimetry had an area of 10.58 units compared with an area of 9.44 units with anti-PFn only (Fig. 1, left panel). Thus, all the fibronectin reactive material was accounted for in the peak, and additional material was detected by the anti-IgG. More importantly, there was no crossing over of the precipitin lines, suggesting reactive species to both antisera were present in the same component (a complex).

Knowing that the preparation contained 11% IgG

Table I. Immunoglobulin Content of Fibronectin Preparations from Human Plasma

Plasma donor	Preparation	Method	Eluent	Immunoglobulins (%)		
				IgG	IgM	IgA
1	1a[a]	A	G	0.78	0.29	0.15
	1b	A	G	ND	0.34	0.17
2	2[a]	A	G	1.8	0.25	0.20
3	3a	A	G	3.7	1.3	0.58
	3b	A	G	2.7	0.57	0.52
4	4	A	U	3.8	0.69	ND
5	5a	A	G	ND	0.39	0.24
	5b	A	G	5.4	0.59	ND
6	6[a]	A	U	9.0	4.9	0.71
7–9[b]	7	C	—	11	ND	ND

Note. A, affinity for gelatin-Sepharose (24); G, glycine (0.2 *M*, pH 2.6–2.8); U, urea (4 *M* in 0.05 *M* Tris, pH 7.5); C, cold insolubility; ND, not determined.
[a] No PMSF.
[b] These three plasmas were pooled.

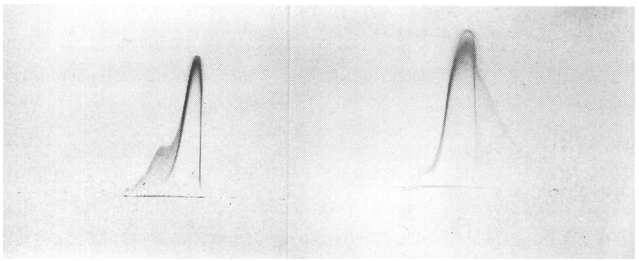

Figure 1. Evidence for an IgG-PFn complex in fibronectin Preparation 7. The second-dimension electrophoresis (anode at top) was run into agarose gel that contained anti-PFn (Left Panel) or a mixture of anti-PFn and anti-human IgG (Right Panel). The shoulder on the fibronectin peak in the left panel indicates the presence of a substance giving a reaction of partial identity with anti-PFn. The position of the shoulder and its distortion by anti-IgG (Right Panel) suggests the shoulder was a complex of fibronectin with IgG.

and that the molecular weight of PFn is 440 kDa and that of IgG is 150 kDa, it can be estimated that in the peak of partial identity, in which 30% of the fibronectin was present, one molecule of PFn was complexed with one molecule of IgG.

The new peak, which was more anodal than PFn, is another Ig-related component detected by antibodies to the light chains of IgG which would also react with the light chains of IgA and IgM. These data demonstrate that the IgG in Preparation 7 was present as a complex with PFn and that at least one other PFn-Ig complex was present.

Gel permeation chromatography of human plasmas. Two of the plasma specimens—1 (least Ig content in PFn) and 6 (highest Ig content in PFn)—were chromatographed on Sephacryl S-300. The elution patterns of PFn, IgM, IgA, and IgG are presented in Figure 2. In Panel A (Plasma 1), the shape of the PFn peak was symmetrical, and this indicates it was not affected by the Igs in the plasma. However, small amounts of Igs especially aggregated IgA, were under the peak. In Panel B (Plasma 6), the PFn peak was distorted, and the shape of the PFn peak mimics both the trailing edge of IgM and a peak of higher molecular weight IgA. There is also more high molecular weight IgG under the PFn peak. The distortion of the peak indicates the PFn was interacting with something else in the plasma. The locations of the shoulder over IgM, of the spike over aggregated IgA, and of the entire PFn region over aggregated IgG suggest the interaction is with Igs, and this is reasonable when one considers that the PFn prepared from Plasma 6 had the highest content of all Igs.

Canine PFn. As was true for all the human PFns, the canine PFns, including C2, also elicited antibodies to canine Igs as well as to canine PFn, but not to albumin, when injected into rabbits.

Detection of immunoglobulins in canine PFn.

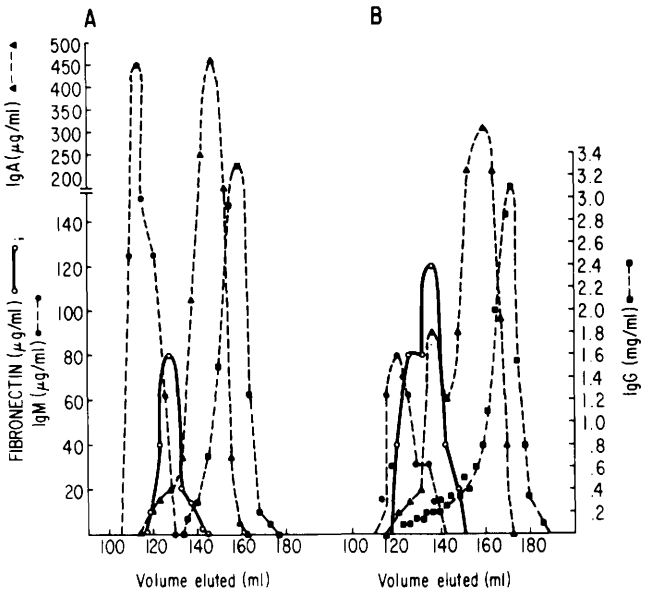

Figure 2. Elution profile of human plasma chromatographed on Sephacryl S-300. By immunodiffusion against specific antisera fractions were screened for PFn (solid line), IgM (●–●–●), IgA (▲–▲–▲), and IgG (■–■–■). (Panel A): Plasma from Donor 1; (Panel B): plasma from Donor 6. In Panel A, the PFn peak is symmetrical and appears not to be affected by the Igs. In Panel B, the PFn peak is distorted. It has a shoulder overlying an IgM shoulder. The main PFn peak is directly over a peak of high molecular weight IgA. High molecular weight IgG is under the entire PFn area. Thus, PFn appears to be interacting with all three classes of Ig in Plasma 6.

By SDS-PAGE under reducing conditions (Fig. 3) it could be seen that C1 (gel on the left) contained peptides with the mobility of immunoglobulin light chains and the μ chain of IgM. That IgM was present was confirmed in double diffusion experiments with anti-canine IgM specific for the μ chain (not shown). The quantity of Igs in C2 (urea eluate) was too small to detect by these methods, but, as noted above, this PFn also elicited antibodies to Igs. The plasma from which PFn C2 was made contained a complex of PFn with IgM as detected by crossed immunoelectrophoresis (see below).

Evidence for PFn-IgM complexes in canine plasma: Crossed immunoelectrophoresis. The canine plasma from which C2 was prepared was examined by crossed immunoelectrophoresis with an intermediate gel containing antibodies to canine IgM (Fig. 4). The canine plasma was first electrophoresed at pH 8.6 into agarose in the horizontal direction. The separated components were electrophoresed through an intermediate gel containing anti-canine IgM (Fig. 4, left panel) or buffer only (Fig. 4, right panel) and then into agarose containing monospecific anti-canine PFn. The right panel shows that the PFn peak trails cathodically to the point of origin, where a definite peak was formed with anti-PFn. Contact with anti-canine IgM in the intermediate gel (Fig. 4, left panel) removed the peak at the origin and much other material from the

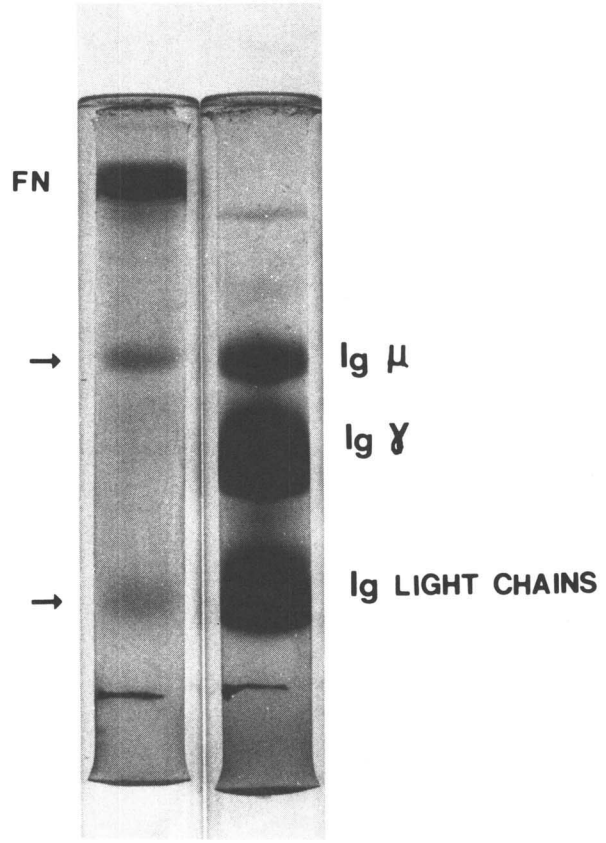

Figure 3. Demonstration by SDS-PAGE (5% disc gels) that Preparation C1 (canine PFn) contained subunits of the same molecular size as immunoglobulin light chains and μ chains. Dithiothreitol was present during denaturation to reduce disulfide bonds. The samples electrophoresed were (Left Gel) canine PFn C1 and (Right Gel) immunoglobulin fraction from canine serum.

main PFn peak. The material removed in the intermediate gel contained IgM and PFn, suggesting the IgM and PFn were present together in complexes.

Further evidence: Gel permeation chromatography. When a large sample (25 ml) canine plasma was chromatographed on the same column used for human plasma a cryoprecipitate formed in the IgM and PFn region (109–154 ml). The washed precipitate was dissolved in 5 ml of buffer and by analysis the solution contained 124 μg IgM/ml and 62 μg/ml of PFn (i.e., a ratio of 2.0). The ratio of the molecular weights of IgM (1000 kDa) and PFn (440 kDa) is 2.27. Thus, the cryoprecipitate contained nearly equivalent amounts of PFn and IgM. Only 2.5% of the plasma IgM was present in the cryoprecipitate.

Discussion

The data in this study clearly show that all the PFn preparations contained Igs. Although the presence of Igs in PFn preparations has been noted before, the extent of their presence (up to 9% IgG and 4.9% IgM) may not have been appreciated.

Because of the high concentrations of Igs in normal human serum (IgG: 8–16 mg/ml; IgA: 1.4–4.2 mg/ml; IgM: 0.5–1.9 mg/ml) (31), it is a reasonable first assumption that any Ig present in PFn preparations is merely a contaminant. The data presented here do not rule out that interpretation since all three classes of Ig were present in each preparation and IgG was present in the highest concentrations. There were, however, indications that the presence of Igs reflected the presence of complexes between PFn and Igs in the plasmas from which the preparations were made. For instance, it can be seen in Table I that on a weight basis more IgM was present than IgA, a reversal of their concentrations in plasma. The *in vitro* data of Rostagno, Frangione, and Gold (9) show a preferential quantitative binding order of PFn to the major Ig classes to be IgG > IgM > IgA. The correlation of the relative amounts of IgM and IgA in PFn preparations with this binding order supports the idea that the amounts of IgG, IgM, and IgA we have found in PFn reflects associations in the plasma from which the PFn was prepared.

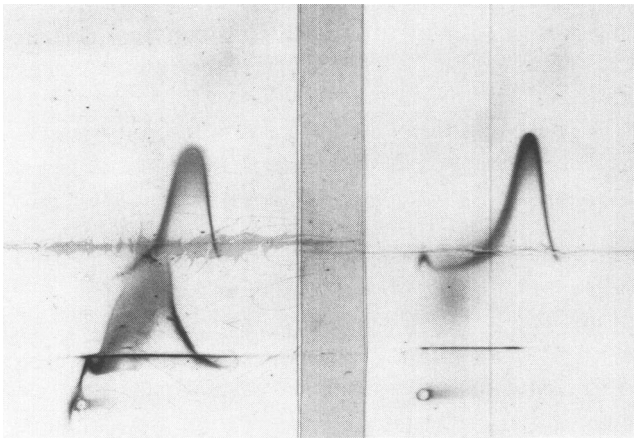

Figure 4. Evidence for an IgM-PFn complex in canine plasma. Canine plasma (5 μl) was electrophoresed into agarose at pH 8.6 in the horizontal dimension. In the vertical dimension, the separated components were electrophoresed through an intermediate gel containing (Left Panel) goat anti-canine IgM (50 μl/4 ml agarose) or (Right Panel) buffer (50 μl/4 ml agarose) and then for both panels into agarose containing a monospecific rabbit antibody to canine PFn (75 μl/8 ml agarose).

Secondly, antisera to the PFn preparations, all of which contained antibodies to Igs, did not contain antibodies to albumin. Since albumin is a good antigen, one can infer from this that albumin was absent from the PFn preparations. Thus, the preparations were not contaminated with other plasma components merely as a result of improper washing of the gelatin Sepharose column after loading on the plasma. Albumin of low molecular weight (67 kDa) would be one of the last things to be washed out of the resin, even after monomeric IgG (150 kDa).

The explanations remaining for the presence of the Igs require that they be specifically interacting with

the PFn or with the gelatin and/or Sepharose 4B. The data presented in this paper demonstrate PFn-Ig interactions. (I) Under nondissociative conditions, a preparation of PFn was made in which a complex of PFn with IgG could be demonstrated in crossed immunoelectrophoresis experiments (Fig. 1). (II) The crossed immunoelectrophoresis experiments on intact canine plasma showing that an intermediate gel containing anti-IgM removed one peak and weakened another is quite good evidence for a PFn-IgM complex in canine plasma (Fig. 4). (III) Gel permeation chromatography of both human and canine plasma suggests interactions of PFn and Igs. For human plasma, the argument is based on the comparison of patterns produced by Plasma 6 with those produced by Plasma 1. For the plasma which provided Ig-rich PFn (Plasma 6), the PFn peak was distorted and the location and shapes of the shoulders of the PFn peak followed those of underlying Igs. For canine plasma the argument is even stronger. A cryoprecipitate was obtained from a gel permeation chromatogram, and this cryoprecipitate contained nearly equivalent amounts of IgM and PFn.

The conditions under which the putative complexes will form are not known. From *in vitro* published experiments it appears that either the PFn or the Ig must be modified in some way. If the latter is the case, the alteration probably occurs in one of the shared light chains since all three classes are involved. Vuento *et al.* (32) showed that when the carboxyl group of a number of proteins, among them IgG, was altered, the proteins then readily bound to PFn. Purified human myeloma IgG proteins of all four subclasses were shown to bind to solid phase fibronectin, whereas polyclonal IgG had much less affinity (6). Salvarrey and Rostagno (7) showed that for direct binding of fibronectin and polyclonal IgG to occur it was necessary that the IgG be modified (i.e., immobilized on Sepharose) and that binding was enhanced under nonphysiological conditions such as low ionic strength and slightly acidic pH. Recently (9), that laboratory has demonstrated binding under physiological conditions to IgG, IgM, and IgA—all three to one site on the PFn molecule. In all cases, binding was increased when the ionic strength or the pH was lowered. Aggregated, but not monomeric, IgG competitively inhibited the binding. From their data, the authors conclude that PFn has the potential to interact with immune complexes and aggregates of all Igs in the circulation.

In their study of IgA-Fn complexes Davin *et al.* (33) conclude that these complexes result from a normal binding process which is enhanced in primary IgA nephropathy. Under the conditions they used to study binding of Igs to Fn-coated plates (pH 7.5 at 37°C), the binding of both polyclonal IgG and polyclonal IgM was only about a fifth that of polymeric IgA. It is clear from such differences in binding data that further study is required to define the necessary conditions for complex formation.

The possible participation of other plasma components in the formation of circulating PFn-Ig complexes also needs to be addressed. Some third component may be required to stabilize the complex or, conversely, to inhibit its formation. In this regard, Davin *et al.* (33) noted that the binding capacity of purified human secretory IgA or of human polymeric IgA1 was much higher when these IgA molecules were added to plasma rather than to the corresponding serum. They suggested that a plasma cofactor(s) lacking in serum, may facilitate the interactions between IgA and solid-phase PFn. They also suggested from other evidence that thermolabile cofactors in normal plasma might inhibit the fibronectin binding capacity of circulating IgA.

The overall sense that emerges from the published reports and from the present study is that low levels of PFn-Ig complexes involving all three classes of Igs circulate normally in plasma and are markedly enhanced in certain disorders. We can envision quantification of Igs in PFn—easily isolated on gelatin-Sepharose—as another diagnostic tool in these disorders.

This work was supported by USPHS Grant HL 15832.

Anita Hersh and Lillian Rodriguez provided the skilled technical assistance required in this study.

1. Ruoslahti E. Fibronectin and its receptors. Annu Rev Biochem **57**:375–413, 1988.
2. Anderson B, Entwistle R, Puyat L, Davis L, Schmid FR. Fibronectin associated with C1q in a C1q isolation procedure. Immunol Comm **10**:687–696, 1981.
3. Bing DH, Almeda S, Isliker H, Lahav J, Hynes RO. Fibronectin binds to the C1q component of complement. Proc Natl Acad Sci USA **79**:4198–4201, 1982.
4. Pearlstein E, Sorvillo J, Gigli I. The interaction of human plasma fibronectin with a subunit of the first component of complement, C1q. J Immunol **128**:2036–2039, 1982.
5. Cosio FG, Bakaletz AP. Binding of human fibronectin to antigen-antibody complexes. J Lab Clin Med **107**:453–458, 1986.
6. Hautanen A, Keski-Oja J. Affinity of myeloma IgG proteins for fibronectin. Clin Exp Immunol **53**:233–238, 1983.
7. Salvarrey M, Rostagno A. Affinity of fibronectin for polyclonal IgG. Clin Exp Immunol **76**:92–96, 1989.
8. Rostagno AA, Frangione B, Gold LI. Biochemical characterization of the fibronectin binding sites for IgG. J Immunol **143**:3277–3282, 1989.
9. Rostagno A, Frangione B, Gold L. Biochemical studies on the interaction of fibronectin with Ig. J Immunol **146**:2687–2693, 1991.
10. Bray BA. Presence of fibronectin in basement membranes and acidic structural glycoproteins from human placenta and lung. Ann NY Acad Sci **312**:142–150, 1978.
11. Bray BA. Cold-insoluble globulin (fibronectin) in connective tissues of adult human lung and in trophoblast basement membrane. J Clin Invest **62**:745–752, 1978.

12. Bray BA, Mandl I, Turino GM. Heparin facilitates the extraction of tissue fibronectin. Science **214**:793–795, 1981.
13. Bray BA. Quantification of tissue fibronectin from terminal villi of placenta. Biochem J **226**:811–815, 1985.
14. Bray BA, Osman M, Ashtyani H, Mandl I, Turino GM. The fibronectin content of canine lungs is increased in bleomycin-induced fibrosis. Exp Mol Pathol **44**:353–363, 1986.
15. Bray BA. Fibronectin associates with immunoglobulins. Fed Proc **42**:1890, 1983.
16. Wood G, Rucker M, Davis JW, Entwistle R, Anderson B. Interaction of plasma fibronectin with selected cryoglobulins. Clin Exp Immunol **40**:358–364, 1980.
17. Anderson B, Rucker M, Entwistle R, Schmid FR, Wood GW. Plasma fibronectin is a component of cryoglobulins from patients with connective tissue and other diseases. Ann Rheumatic Dis **40**:50–54, 1981.
18. Beaulieu AD, Valet JP, Strevey J. The influence of fibronectin on cryoprecipitate formation in rheumatoid arthritis and systemic lupus erythematosus. Arthritis Rheumatism **24**:1383–1388, 1981.
19. Strevey J, Beaulieu AD, Menard C, Valet JP, Latulippe L, Hebert J. The role of fibronectin in the cryoprecipitation of monoclonal cryoglobulins. Clin Exp Immunol **55**:340–346, 1984.
20. Baglin TP, Price SM, Boughton BJ. Circulating high molecular weight IgG fibronectin complexes in myeloproliferative disorders. J Clin Pathol **43**:102–105, 1990.
21. Villar M, Garcia-Bragado F, Joven J, Biosca M, Antolin M, Rodrigo MJ, Schwartz S. Cryoglobulinemia and fibronectin. Acta Haemat **74**:161–163, 1985.
22. Cederholm B, Wieslander J, Bygren P, Heinegard D. Circulating complexes containing IgA and fibronectin in patients with primary IgA nephropathy. Proc Natl Acad Sci USA **85**:4865–4868, 1988.
23. Saba TM, Jaffe E. Plasma fibronectin (opsonic glycoprotein): Its synthesis by vascular endothelial cells and role in cardiopulmonary integrity after trauma as related to reticuloendothelial function. Am J Med **68**:577–594, 1980.
24. Engvall E, Ruoslahti E. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. Int J Cancer **20**:1–5, 1977.
25. Mosesson MW, Umfleet RA. The cold-insoluble globulin of human plasma. I. Purification, primary characterization, and relationship to fibrinogen and other cold-insoluble fraction components. J Biol Chem **245**:5728–5736, 1970.
26. Vuento M, Vaheri A. Purification of fibronectin from human plasma by affinity chromatography under non-denaturing conditions. Biochem J **183**:331–337, 1979.
27. Avrameas S, Ternynck T. The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. Immunochem **6**:53–66, 1969.
28. Laurell C-B. Antigen-antibody crossed electrophoresis. Anal Biochem **10**:358–361, 1965.
29. Furthmayer H, Timpl R. Characterization of collagen peptides by sodium dodecylsulfate-polyacrylamide electrophoresis. Anal Biochem **41**:510–516, 1971.
30. Mancini G, Carbonara AO, Heremans JF. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochem **2**:235–254, 1965.
31. Fudenberg HH, Pink JRL, Wang A-C, Douglas SD. Basic Immunogenetics. New York: Oxford University Press, p16, 1978.
32. Veunto M, Korkolainen M, Stenman U-H. Association of fibronectin with carboxy-group-modified proteins *in vitro*. Biochem J **205**:303–311, 1982.
33. Davin J-C, Vecchi ML, Nagy J, Foidart JM, Foidart JB, Sangiorgi GB, Malaise M, Mahieu P. Evidence that the interaction between circulating IgA and fibronectin is a normal process enhanced in primary IgA nephropathy. J Clin Immunology **11**:78–94, 1991.