Plasma fibronectin is a component of cryoglobulins from patients with connective tissue and other diseases

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SUMMARY Twenty-four washed cryoglobulin precipitates were examined for the presence of plasma fibronectin, immunoglobulins, complement components C1q and C3, and fibrinogen. Plasma fibronectin was detected in all preparations by immunodiffusion with antifibronectin serum, whereas the other components were found in some but not all of the cryoglobulins.

Cryoglobulins occur with an incidence of approximately 6% in individuals with haematopoietic malignancies, connective tissue diseases, and chronic or acute infections. The cryoglobulinaemias are classified into 3 groups: those containing monoclonal immunoglobulins (Igs), mixed cryoglobulins containing monoclonal Ig with antibody activity to polyclonal immunoglobulin G (IgG), and the polyclonal cryoglobulins containing 1 or more classes of Ig. The latter 2 types of cryoglobulins may contain rheumatoid factor activities, other autoantibody reactivities, complement components, and antigens reactive with the corresponding autoantibody.

Possible factors responsible for the insolubility in the cold of monoclonal cryoimmunoglobulins (cryo-Igs) have recently been discerned by Middaugh et al. They showed that the tertiary structure of cryo-Igs differs from soluble Igs and that the low temperature insolubility of cryo-Igs may be an intrinsic property of the structure which favours associations between cryo-Ig molecules. Although many factors have been studied which affect the solubility of the polyclonal cryoglobulins, there is no evidence to suggest that Igs of the cold-insoluble polyclonal type differ in structure from cold-soluble Igs.

We report here that plasma fibronectin (PFN) is a component of all the cryoglobulins tested. Fibronectin (FN) is a normal plasma constituent whose physical-chemical properties have been extensively studied. The molecule has a large molecular weight (440,000 daltons), probably consists of 2 identical subunits, and contains a small percentage of carbohydrate. FN is also a major constituent of the cell surface components of fibroblastic and endothelial cells, and the cell surface FN differs from PFN by being larger by approximately 10,000 daltons. Viral transformation of tissue cultured cell lines usually results in the loss of FN expression on the cell surface. Several biological activities have been suggested for the FN molecule, including mediating cell-cell and cell-substratum adhesiveness and the function of removal of colloids by liver cells and macrophages. PFN has also been shown to have binding properties to several other molecules, including fibrin-fibrinogen collagen, and heparin. The possibility that the PFN found in the cryoglobulins may be binding to some component of the cryo-Igs and involved in the cryoprecipitation phenomenon is discussed.

Materials and methods

Patients The cryoglobulins from 24 patients were studied. Six of the patients were diagnosed as having systemic lupus erythematosus (SLE), 2 rheumatoid
arthritides (RA), 1 polyarthritis, 4 essential mixed cryoglobulinaemia, and 1 Waldenström's macro-
globulinaemia. Two patients were being seen for chronic infections, 1 identified as lichen myxo-
edematosis and 1 cytomegalovirus. Eight of the patients had different types of clinical features, the
primary disorder not being known. They included: 3 with haemolytic anaemia and thrombocytopenia
and 1 each with thrombophlebitis, chronic urticaria, cardiovascular arrest, renal disease, and platelet
satellite phenomenon.

SERA COLLECTION AND CRYOGLOBULIN
PREPARATION AND ANALYSES

Blood was collected into prewarmed tubes and
allowed to clot at 37°C. The clot was removed by
centrifugation at 1600 g for 30 minutes and the
serum placed at 4°C for 24–48 hours. Precipitated
material was collected by centrifugation at 1600 g
for 20 minutes and 4°C, and the cryoglobulin
precipitates were washed twice with phosphate
buffered saline (PBS), pH 7.2. The cryoglobulins
were dissolved by warming the cryoglobulin pre-
cipitates to 37°C or 56°C in PBS. When the collected
cryoglobulins were kept at 4°C for more than a few
days, they became relatively more difficult to
dissolve by warming. Several of these were dis-
solved by warming to 37°C in the PBS containing
1 M urea. If 37°C or 56°C was sufficient to dissolve
most of the cryoglobulin, the urea solution was not
used. The protein concentrations of the solubilised
portions as determined by the optical density ratios
at 280 and 260 nm varied between 0.045 and 1.7
mg/ml. IgG, IgM, IgA, C1q, C3, and fibrogen
were analysed by double immunodiffusion in
agarose plates (Behring) with monospecific antisera
to the human substances (Cappel Laboratories).

PREPARATION OF PLASMA FIBRONECTIN
AND ANTIPLASMA FIBRONECTIN SERA

Plasma fibronectin (PFN) was purified by a modi-
fication of the procedure of Engvall and Ruuslahti.12
Gelatin (8 g, Sigma Chemical Co.) was dissolved in
a mixture of 60 ml of PBS and 10 ml of 1 M sodium
acetate, pH 5–0, and 4 ml of 25% glutaraldehyde
was immediately added dropwise. The reaction
mixture was stirred at room temperature overnight.
The insolubilised gelatin was placed in a 2 × 60 cm
column, and the gelatin was washed with 1% Triton
X-100 in PBS, 3 M KCl, 1 M urea in PBS, and 8 M
urea in PBS. Plasma (500 ml) was passed through
the column and the gelatin washed with 2 column
volumes each of PBS, 1% Triton X-100 in PBS,
3 M KCl, and 1 M urea in PBS. PFN was eluted
with 8 M urea in PBS. The eluate was concentrated
with a filter cone (Amicon Corporation) and dialysed
against 0.5 M urea in PBS at 4°C for 24 hours. The
precipitate was collected by centrifugation and
dissolved by warming in 0.5 M urea in PBS. PFN
was also obtained as a gift from Dr Isaac
Cohen, Northwestern University Medical School.
The PFN preparations showed no reactivity in double
immunodiffusion with antisera to human Igs,
complement, or clotting components.

Antisera were prepared to PFN by multiple site
injections (subcutaneous, intramuscular, inter-
peritoneal) of rabbits with 100–200 μg of PFN
emulsified in complete Freund's adjuvant (Miles
Laboratories). Subsequent immunisations were
done biweekly with the same amounts of PFN in
incomplete Freund's adjuvant.

Serum was obtained 7–10 days after each immuni-
sation, and by 8 weeks strong precipitin lines were
seen in double immunodiffusion of the antisera
against purified PFN and human plasma. A single
precipitin band was obtained with the latter 2
antigen sources with no additional precipitin
formation or spurring. The anti-PFN sera showed no
reactivity to purified human Igs, complement, or
clotting components. The commercial antiserum to
the latter components showed no indications of
cross-reactions with the line formed by anti-PFN
sera, and plasma or purified PFN.

Quantitations of PFN were done by a method
similar to that published by Ruoslahti et al.14 PFN
(10 μg) was radiolabelled with 2mCi of Na125I
(Amersham Corporation) plus 0.04 mg of chloro-
mine-T for 1 minute at 23°C. The reaction was
stopped by the addition of 0.03 mg sodium meta-
bisulphite. KI (0.1 of a 0.1 N Solution) and PBS
containing 0.1% bovine serum albumin (BSA) were
also added to the reaction mixture, and the 125I–PFN
and free 125I separated on a Sephadex G–25 column.
The 125I–PFN was further purified by adsorption
to glutaraldehyde-insolubilised gelatin by incubation
with the gelatin for 1 hour at 23°C. The gelatin was
washed extensively with PBS and 125I–PFN removed
with 4 M urea in PBS containing 1% BSA. 80% of
the purified 125I–PFN bound to antibody in the
radioimmunoassay (RIA). The second antibody
(goat anti-rabbit IgG serum. Miles Laboratories)
was absorbed by incubation of 2 ml of the antisera
with 1 ml of glutaraldehyde-insolubilised gelatin.
The RIA reaction mixtures consisted of 15 μl of
125I–PFN (specific activity approximately 80 000
cpm/ng), and anti-PFN serum diluted to titre
in 1:100 diluted normal rabbit serum (300 μl) in
PBS containing 1% BSA. After incubation at 37°C
for 2 hours 40 μl of the absorbed second antibody
was added, and incubation was continued for 1 hour
at 37°C. The precipitates were removed by centri-
fugation at 1500 xg for 45 minutes and counted in a
gamma counter. Known amounts of purified PFN were added to construct an inhibition curve, and varying amounts of dissolved cryoglobulins were quantitated for PFN concentration.

Results

The cryoglobulins in this study were defined as the portion of sera drawn and clotted at 37°C which precipitated at 4°C for 24 to 48 hours. The precipitated fractions showed differences in solubility, some readily dissolving on warming to 37°C and others leaving small amounts of precipitate on warming to 56°C or 37°C in 1 M urea-PBS. Only the soluble portions were examined for their components. As shown in Table 1, all of the PBS washed and dissolved fractions of the cryoglobulins contained a component which was reactive with antisera prepared to PFN. In addition, 17 of the 20 cryoglobulins tested for IgG contained that component. The cryoglobulin derived from the patient with Waldenström's macroglobulinaemia was positive for IgM but not IgG or IgA. Several other cryoglobulins contained either IgM or IgA or both. C1q and C3 were detected in 2 and 8, respectively, of the cryoglobulins. Fibrinogen was detected in only 1 of all the dissolved cryoglobulin fractions. The components negative by double immunodiffusion may be present but below the limits of detection of that method. As shown in Fig. 1, anti-PFN serum formed single-precipitin bands with solubilised cryoglobulins which fused with the precipitin band formed with purified PFN and plasma and serum.

Several experiments were done to ascertain whether PFN was loosely adherent to other components of the cryoglobulin. Several cryoglobulin precipitates were solubilised by warming and reprecipitated at 4°C repeatedly and an aliquot removed at each solubilisation stage. All of the solubilised portions were positive by precipitin band formation in double immunodiffusion against anti-PFN serum. Moreover, extensive washing of cryoglobulin precipitates at 4°C with PBS did not remove the PFN.

Table 1 Components detected in washed cryoglobulin precipitates*

<table>
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<tr>
<th>Cryoglobulin</th>
<th>PFN</th>
<th>IgG</th>
<th>IgM</th>
<th>IgA</th>
<th>C1q</th>
<th>C3</th>
<th>Fibrinogen</th>
<th>Diagnosis†</th>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>LM</td>
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* Blood specimens were collected and clotted at 37°C and the sera placed at 4°C for 24 or 48 hours. The cryoglobulins were collected by centrifugation and washed 3 times at 4°C with PBS. The precipitates were then redissolved and tested for the various components listed by double immunodiffusion analysis. + = positive as defined by precipitin band formation, - = negative; ND = not determined.

† Abbreviations for diagnoses: RA, rheumatoid arthritis; PA, polyarthritis; SLE, systemic lupus erythematosus; WM, Waldenström's macroglobulinaemia; EMC, essential mixed cryoglobulinaemia; CU, chronic urticaria; HA, TCP, haemolytic anaemia and thrombocytopenia; CMV, cytomegalovirus; TP, thrombophlebitis; CVA, cardiovascular arrest; PSP, platelet satellite phenomenon; RD, renal disease; LM, lichen myxoedematosus.

Fig. 1 Double immunodiffusion analysis of solubilised cryoglobulin fractions against antiplasma fibronectin serum. Wells 1 and 2 contained the solubilised cryoglobulins of 2 different individuals with essential mixed cryoglobulinaemia; wells 3 and 6 contained purified PFN; well 4, 1:2 diluted normal human serum; well 5, 1:3 diluted normal human plasma; the centre well contained anti-PFN serum. A single precipitin band formed with all antigen sources which fused without apparent spurring. The appearance of a spur-like precipitin with the human serum and plasma antigens is due to a prozone effect near those wells.
In one experiment (Table 2) $^{125}$I-PFN was added to the freshly drawn serum from a patient with essential mixed cryoglobulinaemia and the $^{125}$I-PFN followed through stages of precipitation, solubilisation, and reprecipitation. As seen in the Table, 7.7% of the total $^{125}$I-PFN remained in the cryoprecipitated fraction of serum.

If it is assumed that the original concentration of PFN in the serum was 150 μg/ml the precipitate would contain approximately 12 μg of PFN. The second and third PBS washes of the precipitate contained negligible counts, again showing that the PFN probably was not loosely adherent to other cryoglobulin components. When the redissolved first precipitate was allowed to precipitate in the cold, approximately 50% of the $^{125}$I-PFN reprecipitated, and approximately 70% of the latter reprecipitated a third time. Thus, the PFN becomes insoluble in the cold with other cryoglobulin components after removal of other serum factors and solubilisation.

In a separate experiment a different serum was processed in the same manner and the PFN in the various fractions was quantitated by RIA. As can be seen in Table 2, results were obtained which were similar to those as in the experiment where $^{125}$I-PFN was added to serum. These results confirmed the observations that a fraction of PFN coprecipitated in the cold with the other components of cryoglobulins and that the PFN was reprecipitable in several cycles of precipitation-solubilisation.

**Table 2** Percentages of $^{125}$I-labelled PFN added to serum precipitating with cryoglobulins and of PFN assayed in the cryoglobulin fractions by RIA

<table>
<thead>
<tr>
<th>Per cent of total PFN</th>
<th>$^{125}$I-PFN*</th>
<th>PFN†</th>
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<tr>
<td>1. Serum</td>
<td>100</td>
<td>100</td>
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<tr>
<td>2. 1st precipitate‡</td>
<td>7.5</td>
<td>9.4</td>
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<tr>
<td>supernatant</td>
<td>90.2</td>
<td>89</td>
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<tr>
<td>1st wash</td>
<td>2.1</td>
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<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>3rd wash</td>
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</tr>
<tr>
<td>3. 2nd precipitate‡</td>
<td>3.9</td>
<td>7.1</td>
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<tr>
<td>supernatant</td>
<td>3.7</td>
<td>2.6</td>
</tr>
<tr>
<td>3rd precipitate‡</td>
<td>3.0</td>
<td>6.9</td>
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<td>1.2</td>
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<td>0.1</td>
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<tr>
<td>supernatant</td>
<td>6.6</td>
<td>0.2</td>
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*A total of $2.7 \times 10^{5}$ cpm of $^{125}$I-PFN ($7.2 \times 10^{4}$ cpm/ng) were added to the 1 ml of serum.

†Serum (14 ml) was treated in the same manner as the serum to which the $^{125}$I-PFN was added and the various fractions assayed by RIA for PFN as described in the text.

‡The 1st precipitate was collected by centrifugation after the serum remained at 4°C for 48 hours and was washed 3 times with PBS (1st, 2nd, and 3rd washes). The 2nd, 3rd, and 4th precipitates were those obtained after redissolving the preceding precipitate in PBS at 37°C and reprecipitation at 4°C for 24 hours.

**Discussion**

Cryoglobulins may consist of many components. In individuals with autoantibodies the cryoglobulins can include IgG or IgM with the autoantibody specificity; IgA and various complement components are also detectable.4-6,15 We report here that PFN was also a component of all the cryoglobulins tested, most of which were apparently of the polyclonal type. We have not tested monoclonal cryo-Igs nor have we directly ascertained whether a rheumatoid factor active cryo-IgG precipitate with IgG contains PFN. It is possible that either of the 2 cryoglobulins from patients with RA reported here may have contained rheumatoid factor activity. Because PFN associates with many other proteins, including fibrin and collagen,18 it is pertinent to ask to which component of the cryoglobulins PFN is binding. A portion may be binding to C1q as C1q contains collagen-like segments. Moreover, we have noted that some IgG copurifies with PFN in certain stages of the glutaraldehyde-polymersed gelatin method (unpublished observations); thus PFN may associate to some extent with Igs or a fraction of Igs. Alternatively, PFN, which is defined as a cold-insoluble globulin, may simply coprecipitate at 4°C with other components of cryoglobulins. However, it has been shown that the cell surface form of purified FN is fractionally soluble at neutral pH and 0°C at a concentration of 0.5 mg/ml.16 Further, the purified PFN used in these studies remained soluble in PBS at concentrations of 0.2-0.8 mg/ml over extended periods of time at 4°C. Therefore it would be expected that in the repeated precipitation-solubilisation experiments PFN would be removed from the cryoglobulin precipitates.

It will be of interest to determine to what component(s) of the cryoglobulins PFN is associated and also to ascertain if the presence of PFN affects the cryoprecipitability of cryoglobulins. These questions are being investigated.

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**References**

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