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Circulating complexes containing IgA and fibronectin in patients with primary IgA nephropathy

(collagen/Berger disease)

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ABSTRACT **IgA antibodies from patients with primary IgA nephropathy bind to collagens I, II, and IV. Here we show that this binding is mediated by the collagen-binding site of fibronectin, which occurs in the circulation in complex with IgA. No antibodies binding directly to collagen were identified. The complexes were isolated by affinity chromatography on gelatin-Sepharose and heparin-Sepharose, both with affinity for fibronectin, followed by adsorption to anti-human IgA immobilized on agarose gel. The presence of fibronectin and IgA antibodies in the isolated complexes is shown by enzyme-linked immunosorbent assay, gel electrophoresis, and electrophoretic transfer followed by immunostaining. The presence of an IgA–fibronectin complex in serum and the binding of this complex to collagen demonstrate the necessity of removing fibronectin from serum prior to identifying anti-collagen antibodies.**

Patients with primary IgA nephropathy, also known as Berger disease, have circulating IgA antibodies that bind to collagen IV prepared from glomerular basement membrane (1). The IgA antibodies bind equally well to collagens I, II, and IV, and denatured collagens bind antibodies most efficiently (1).

Fibronectin, also known as cold-insoluble globulin, is present both as a plasma protein and as a connective tissue protein. The two forms differ slightly in structure but share important functions such as binding to gelatin (collagen), heparin, fibrin, and cell surface receptors (2). Fibronectin is the major plasma component binding to gelatin (3), and this property as well as its heparin-binding properties have been utilized in its isolation (4). Fibronectin is also present in basement membranes. It can be visualized by immunofluorescence, both in the glomerular basement membrane and in the mesangium of healthy individuals (5).

IgA is present in circulating complexes in patients with primary IgA nephropathy and Henoch–Schönlein nephritis (6). However, only the IgA component of the complex has been identified. The present paper establishes that one, apparently major, component in the complex with IgA is fibronectin.

MATERIALS AND METHODS

Antisera. Sera were taken from patients with primary IgA nephropathy—i.e., glomerulonephritis with predominating mesangial IgA deposits detected by immunofluorescence microscopy of renal biopsy specimens. None of the patients had signs of systemic lupus erythematosus (SLE), Henoch–Schönlein purpura, or cirrhotic liver disease. Sera from healthy blood donors were used as controls.

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Affinity-purified swine antibodies to human IgA (Orion Diagnostica, Helsinki, Finland), both native and conjugated to alkaline phosphatase, were further purified by passing the antibodies through a fibronectin column. Rabbit antiserum to human fibronectin (Dakopatts, Copenhagen) was affinity purified on human fibronectin immobilized on divinyl sulfone-agarose (Biocarb, Lund, Sweden). Antibodies used for ELISA were eluted with 3 M KSCN/10 mM Tris·HCl, pH 7.4/0.05% sodium azide.

Isolation of Complexes. Sera (1 ml) were passed through 1-ml columns of Sepharose 4B beads (Pharmacia, Uppsala) prior to chromatography on a 1-ml column of gelatin-Sepharose 4B (Pharmacia). Material bound to the gelatin column was eluted with 4 M urea/0.15 M NaCl/10 mM Tris·HCl, pH 7.4/0.05% sodium azide and immediately chromatographed on a 1-ml column of heparin-Sepharose CL-6B (Pharmacia). Fibronectin and its complexes were eluted from the heparin-Sepharose with 0.5 M NaCl/10 mM Tris·HCl, pH 7.4/0.05% sodium azide. In the final step, the fibronectin complexes with IgA were bound by their IgA component to purified swine anti-human IgA (see above). This immunosorbent was prepared by immobilizing anti-IgA antibodies on divinyl sulfone-agarose and packing it into a 0.5-ml column. The complexes were eluted with 6 M urea/0.15 M NaCl/0.1 M glycine, pH 2.5, and the effluent was immediately neutralized and dialyzed against 10 mM sodium phosphate, pH 7.5/0.15 M NaCl/4 mM KCl/0.05% Tween 20 (ELISA buffer). Leakage of rabbit IgG from the column was negligible.

Electrophoresis and Immunoblotting. These techniques were performed essentially as described elsewhere (1).

ELISA. The techniques used were essentially those previously described (1). Antigen was coated on polystyrene 96-well microtiter plates (NUNC immunoplate I, NUNC, Roskilde, Denmark). Samples of column eluates (Fig. 1), affinity-purified swine antibodies against IgA (1 μg/ml), or affinity-purified antibodies against fibronectin (1 μg/ml) (Fig. 2) were coated overnight under nondenaturing conditions in 50 mM sodium carbonate buffer, pH 9.6, containing 0.05% sodium azide. To prevent nonspecific binding and reduce background, coating was followed by incubation for 1 hr with the same buffer supplemented with 2% bovine serum albumin (blocking buffer). Collagen I (1 μg/ml) was coated overnight under denaturing conditions in 6 M guanidine hydrochloride/50 mM Tris·HCl, pH 7.4 (Fig. 1). In this case incubation with blocking buffer was not needed. Column effluents were diluted in ELISA buffer and incubated for 3 hr in the collagen I- or antibody-coated microtiter plates. To minimize nonspecific interactions the buffer also contained 0.25 M guanidine hydrochloride.

Bound IgA antibodies were detected by incubation for 1 hr with affinity-purified anti-human IgA conjugated to alkaline phosphatase (see above). Fibronectin was detected by incubation with affinity-purified anti-human fibronectin antibodies (see above) followed by incubation with affinity-purified

anti-rabbit IgG conjugated to alkaline phosphatase (Fig. 1) or anti-human fibronectin conjugated to peroxidase (Fig. 2). Enzyme activity was determined by using *p*-nitrophenyl phosphate or 1,2-phenylenediamine dihydrochloride as the substrate for alkaline phosphatase or peroxidase. Absorbance at 405 or 492 nm, respectively, was measured by using a Titertek Multiskan photometer. Microtiter plates were rinsed between each step with 0.15 M NaCl containing 0.05% Tween 20. All samples were analyzed in triplicate.

RESULTS AND DISCUSSION

Interaction of IgA Antibodies with Collagen. We have previously found that IgA antibodies from patients with primary IgA nephropathy bind to collagens I, II, and IV (1). Further studies revealed that those CNBr fragments of collagen I, which bind the IgA antibodies, are identical to the fragments containing the fibronectin-binding domains (7). We also showed that the collagen-binding IgA antibodies from the patients were retained on a heparin column. The data indicate that the IgA antibodies form a complex with fibronectin. We therefore decided to purify the complexes, using columns with affinity for fibronectin and IgA, respectively, in sequence.

Adsorption of Fibronectin to Gelatin-Sepharose. Fibronectin binds to collagen by its collagen-binding domain, located in the N-terminal part of the molecule (2). This property is useful in the purification of fibronectin (4), the major collagen-binding protein in plasma (3). In the present study it is shown to be useful in the purification of IgA–fibronectin complexes also.

Gelatin-Sepharose was used in the initial step for the purification of IgA–fibronectin complexes from human serum (Fig. 1). As demonstrated in ELISA, using the fractions as coat, less than 1% of the IgA was retained on the gelatin column (data not shown). In contrast the fibronectin bound to the gelatin column and was eluted with a 4 M urea buffer (Fig. 1). Note that the sensitivity of the assay was adjusted to make it possible to compare the unbound and bound fractions.

The collagen-binding property of fibronectin was also utilized in an ELISA (denoted as IgA complex in Fig. 1) to demonstrate the presence of IgA–fibronectin complexes in the eluates from the affinity columns. Fractions from the columns were incubated in microtiter wells coated with denatured collagen I (3). Bound IgA antibodies were detected by using the purified anti-IgA antibody conjugate. Using this test, we found that only the material that bound to the gelatin affinity column contained IgA antibody binding to collagen. Most likely this represents IgA–fibronectin complexes in which the IgA antibodies are bound to fibronectin and fibronectin mediates binding to collagen by its collagen-binding domain.

Expectedly, no complexes were detected in a serum sample from a healthy blood donor (Fig. 1).

Adsorption of Fibronectin to Heparin-Sepharose. Heparin-Sepharose (4) was used as the second step for the purification of IgA–fibronectin complexes from human serum. The material eluted from the gelatin-Sepharose by 4 M urea was directly applied to the heparin-Sepharose column. The major portion of the fibronectin bound to the heparin column and was eluted by 0.5 M NaCl (Fig. 1). IgA antibodies were found only in the patient. These antibodies all bound to heparin-Sepharose, apparently representing IgA in complex with fibronectin. In support, complexes detected by using ELISA with collagen I coat (Fig. 1) were found only in the bound material from the patient.

It should be pointed out that the sensitivity of the ELISA of effluents from the heparin and the anti-IgA columns is enhanced as compared with the assay of the gelatin column effluents. This was achieved by incubating with less diluted samples of the fractions. Incubation time with the substrate was also extended.

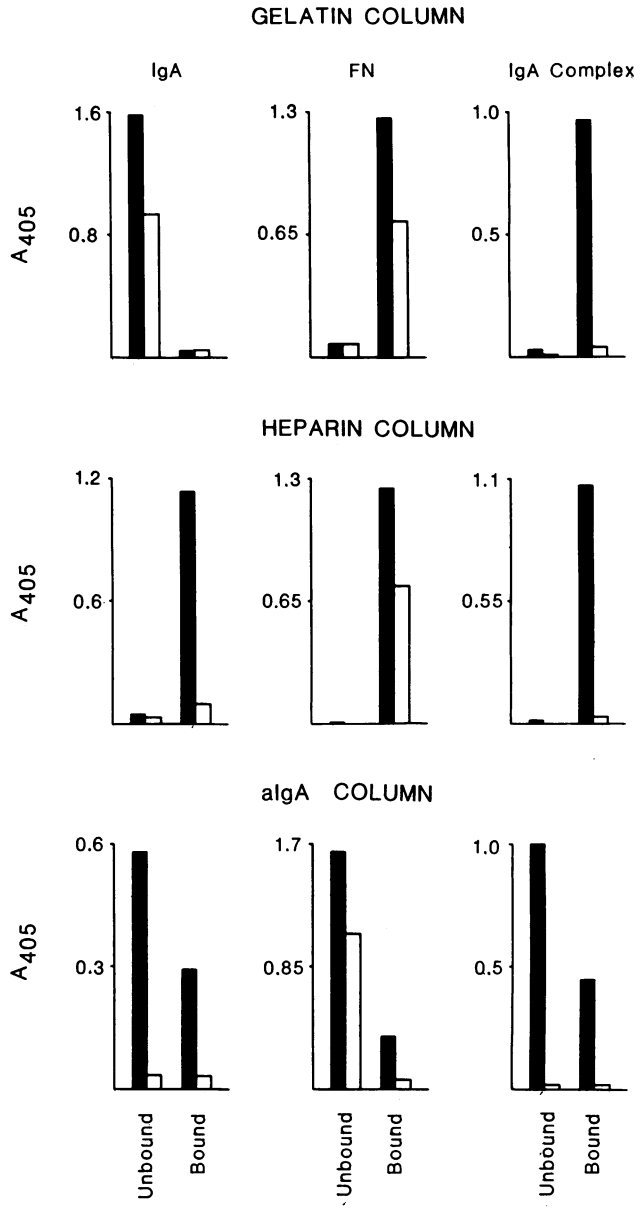

FIG. 1. Affinity chromatography of patient and control sera on Sepharose conjugated with gelatin (*Top*), heparin (*Middle*), and anti-IgA (*Bottom*). Aliquots of column effluents were coated on a microtiter plate (*Left* and *Center*) or incubated on plates coated with denatured collagen I (*Right*). FN, fibronectin; IgA complex, IgA component of the fibronectin–IgA complexes; solid bars, patient; open bars, control.

Adsorption of the IgA Component to Anti-IgA Agarose. The two purification steps described selected for both IgA–fibronectin complexes and fibronectin that did not participate in complex formation. A third step for specific adsorption of IgA was therefore utilized. Anti-human IgA antibodies were immobilized on agarose gel, and a column was prepared for affinity purification.

The major portion of fibronectin, both in patient and control samples, was not retained on the column (Fig. 1). Expectedly, IgA antibodies and complexes were found only in samples from the patients (Fig. 1). In support, binding of fibronectin to anti-fibronectin antibodies coated on microtiter plates showed IgA antibodies bound in complex only in

patient samples (Fig. 2 *Left*). When the plates were coated with anti-IgA antibodies, only patient samples showed binding of fibronectin (Fig. 2 *Right*).

The amount of complexes recovered from the anti-IgA column, compared to the level in the original serum, was measured in ELISA with collagen I coat and found to be 0.4% in the unbound and 0.2% in the bound material. It is possible that some of the complexes were dissociated during the purification, since rather harsh conditions were used.

A substantial portion of the complexes did not bind to the anti-IgA column (Fig. 1). This was not due to overloading of the column, since chromatographing unbound material a second time failed to bind more than a small fraction (data not shown). Interestingly, the antibody used for affinity chromatography was the same antibody as that used in the ELISA (Figs. 1 and 2). It is somewhat surprising that the results in the two systems are different, but similar results were obtained when antibodies from two other suppliers were tested.

Components with mobilities corresponding to fibronectin and IgA in the material that bound to the anti-IgA column were demonstrated by using $NaDodSO_4$/PAGE (Fig. 3). Further proof for the identity of the components was obtained by immunoblotting, using anti-IgA and anti-fibronectin antisera (Fig. 3). The anti-fibronectin antiserum stained a component at the expected position of fibronectin, and it also stained components having higher mobilities. It is likely that the latter components represent degradation products of the fibronectin molecule still bound by IgA in a complex. No IgA antibodies were identified in control samples (Fig. 3).

Trace amounts of fibronectin were seen in the control on $NaDodSO_4$/PAGE and immunoblotting. This is most likely due to cross-reactivity between fibronectin and the anti-IgA antibodies used for affinity chromatography. The use of affinity-purified anti-human IgA antibodies adsorbed on fibronectin for affinity chromatography reduced the nonspecific binding of fibronectin to less than 1% compared to

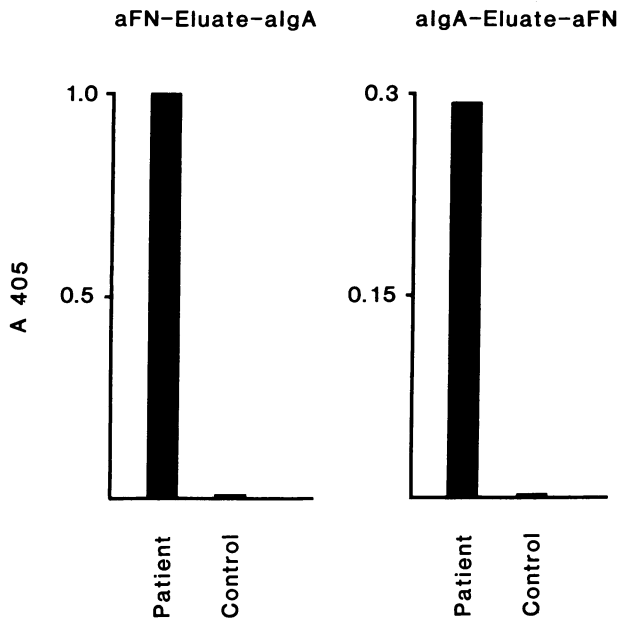

FIG. 2. Presence of complexes in the eluate from the anti-IgA column (Fig. 1). aIgA, anti-immunoglobulin A antibody; aFN, anti-fibronectin antibody; Eluate, bound material (patient/control) of anti-IgA column (Fig. 1). (*Left*) The fibronectin component of the complexes was bound to the coat and the IgA component was detected by the use of anti-IgA as a second antibody. (*Right*) The IgA component was bound to the coat and the fibronectin component was detected by a second antibody.

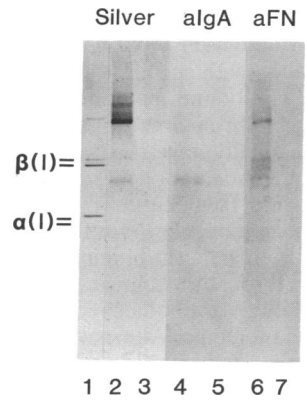

FIG. 3. $NaDodSO_4$/PAGE on 3–16% gels (lanes 1–3) and electrophoretic transfer followed by immunostaining (lanes 4–7) of material eluted from the anti-IgA column (Fig. 1). The positions of collagen I α and β chains are indicated by bars. Samples applied were collagen I, used as a reference (lane 1), immune complexes containing IgA and fibronectin from the patient (lanes 2, 4, and 6), and a sample from a blood donor representing an equal volume of serum (lanes 3, 5, and 7). Proteins were silver stained (lanes 1–3). Proteins separated on identical $NaDodSO_4$/PAGE gels were electrotransferred onto nitrocellulose paper and incubated with either antibodies against human IgA (lanes 4 and 5) or antibodies against human fibronectin (lanes 6 and 7). IgA was identified only in the patient sample (lanes 2 and 4). A faint staining for fibronectin was seen also in the control serum due to cross-reactivity between fibronectin and the anti-IgA antibodies used (see text).

nonadsorbed, not-affinity-purified antiserum. Three anti-IgA antisera (two affinity purified), from different manufacturers, were tested. All three showed cross-reactivity with fibronectin to various extents. The affinity for fibronectin was probably low, since it was detected only by affinity chromatography and not detected in the ELISA when the sera were tested. It was not possible to block the cross-reactivity by adding 0.1% Tween, 0.2 M galactose, or 1 M urea to the buffer. Neither varying the concentration of NaCl (0–0.5 M) nor exchanging phosphate for Tris·HCl buffer (4) altered the result. It is possible that the rabbit IgG immobilized on the column interacts with fibronectin in the sample. However, addition of 0.2 mg of rabbit IgG per ml sample did not significantly reduce binding of fibronectin.

The generality of fibronectin-binding IgA antibodies in patients with primary IgA nephropathy was studied. Sera from five patients, containing IgA antibodies binding to collagen I-coated microtiter plates, were applied to a heparin-Sepharose column. In all five patients the IgA antibodies binding to the collagen I-coated plates were retained on the column, indicating presence of IgA complexed with fibronectin. The material not adsorbed on the column contained most of the IgA antibodies but showed no binding to collagen I in the ELISA. This shows that the patients do not have anti-collagen antibodies.

No IgG or IgM antibodies were detected in complex with fibronectin (data not shown).

To provide information on the size of the complexes, the material eluted from the heparin column was applied to a Superose 6 (Pharmacia) molecular sieve column. The major portion of IgA binding to collagen I was eluted before, but close to, the major fibronectin and IgA peaks (data not shown), indicating the presence of a rather small complex. None of the sera from 100 healthy blood donors showed increased titers of IgA binding to collagen I when tested by ELISA.

General Discussion. IgA–fibronectin complexes were isolated in a three-step procedure. The initial step was binding of fibronectin and complexes containing fibronectin and IgA

to gelatin-Sepharose. Binding of fibronectin was repeated in the second step, which used heparin-Sepharose. It is unlikely that an IgA antibody with specificity for collagen would also bind to heparin. The bound material, therefore, most likely contains IgA and fibronectin linked in a complex. The complexes were finally adsorbed to an anti-IgA affinity column by their IgA component, thereby separating the complexes from free fibronectin. The procedure yields the IgA–fibronectin complexes in pure form.

Interestingly, both heparin-binding and collagen-binding domains of the fibronectin appear free to interact in the complex. Thus the IgA antibodies recognize other structures on the fibronectin molecule. Alternatively, the dimeric fibronectin has at least one gelatin- and one heparin-binding domain free.

The presence of circulating complexes of fibronectin and immunoglobulins has to be considered when measuring antibodies to collagens. A procedure for removal of fibronectin from the antiserum should be included prior to assaying for anti-collagen antibodies.

At the present stage we cannot claim with certainty that the binding between IgA and fibronectin is a true antigen–antibody binding. It is possible, although in view of its stability unlikely, that the binding is mediated by the Fc region of the IgA molecule. The binding between IgA and fibronectin is not covalent, since it is reversed during incubation in the Na-DodSO_4-containing sample buffer prior to electrophoresis (Fig. 3). Preliminary experiments have shown that it is possible to dissociate the complexes and bind the IgA antibodies to fibronectin purified from blood donor plasma (data not shown).

Circulating antibodies in complex with fibronectin have been studied in other conditions. Thus patients with secondary syphilis have circulating complexes containing fragments of fibronectin, IgG, and IgM antibodies against these fragments and a treponemal fibronectin-binding protein (8). We have, however, not been able to demonstrate foreign proteins in the complexes isolated from patients with IgA nephropathy (Fig. 3). Available data suggest that fibronectin–IgA complexes show a preferential deposition in mesangial structures. Experimental evidence was obtained by injecting artificial complexes binding to fibronectin. These showed a preferential localization to kidney as well as some other tissues (9). In addition, injection of heterologous antibodies to plasma fibronectin gave deposits of immunoglobulins in the mesangium (10). A similar mechanism may be operative in primary IgA nephropathy. Deposition of IgA preferentially in the mesangium could be of pathogenic significance, since it has been found that injection of denatured fibronectin causes production of IgG autoantibodies to fibronectin and glomerular injury (11).

The present study shows that patients with IgA nephropathy have circulating complexes containing IgA antibodies and fibronectin. This is in agreement with a proposed immune complex mechanism for primary IgA nephropathy (6). Further studies are necessary to identify the structure of the fibronectin molecule responsible for binding of the IgA antibodies.

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