Clin. exp. Immunol. (1988) **72**, 37–42

Interaction between fibronectin and C1q in rheumatoid synovial fluid and normal plasma

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(Accepted for publication 4 November 1987)

SUMMARY

The interaction between fibronectin and C1q was studied in the presence of normal human plasma and rheumatoid synovial fluid by solid phase binding assay. Fibronectin–C1q binding occurred in the presence of rheumatoid synovial fluid but not in the presence of normal plasma. Binding was strongest at 4°C and in the presence of EDTA. Fibronectin-C1q binding could be induced in the presence of normal plasma by hypotonicity, augmentation of the concentration of solution-phase fibronectin or by the addition of heat-aggregated IgG. The C1q present in rheumatoid synovial fluid bound to both aminoterminal collagen-binding and carboxyterminal noncollagen binding fibronectin fragments although binding to the aminoterminal fragment was stronger. The interaction between fibronectin and C1q in rheumatoid synovial fluid may modulate immune-complex deposition and complement activation in the inflamed joint.

Keywords fibronectin C1q synovial fluid rheumatoid arthritis

INTRODUCTION

Plasma fibronectin appears to play a significant role in host defense (Saba *et al.*, 1978). Fibronectin promotes opsonization of particulate matter and specifically interacts with components of the immune system, including monocytes (Bevilacqua *et al.*, 1981), lymphocytes (Cardarelli & Pierschbacher, 1986), immunoglobulins (Hautanen & Keski-Oja, 1983a) and complement proteins. In particular, the binding of purified fibronectin to purified C1q has been well documented and extensively studied (Anderson *et al.*, 1981; Menzel *et al.*, 1981; Bing *et al.*, 1982; Sorvillo, Gigli & Pearlstein, 1983).

Fibronectin and C1q have been demonstrated in cryoprecipitates formed from systemic lupus erythematosus (SLE) plasma (Beaulieu, Valet & Strevey, 1981) and rheumatoid (RA) synovial fluid suggesting that Fn-C1q binding may occur in body fluid under certain conditions. The characterization of the Fn–C1q interaction in the presence of normal plasma and inflammatory effusions, is important in the assessment of the functional consequences of this interaction *in vivo*. We developed a solid-phase binding assay and have studied Fn–C1q interactions in the presence of normal plasma and RA synovial fluid. The conditions resulting in optimal binding have been identified as well as the regions on the fibronectin molecule responsible for C1q binding.

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MATERIALS AND METHODS

Normal human plasma
Blood obtained from healthy laboratory personnel was collected into tubes containing 6 mM EDTA (final concentration) and centrifuged at 1000 *g*, to remove cellular elements. Supernatant plasma was stored frozen at −70°C.

Synovial fluid
Synovial fluid (SF) was obtained from patients undergoing diagnostic or therapeutic arthrocentesis after informed consent was obtained. Fluid was obtained from nine patients with definite or classical RA and two patients with seronegative RA. Fluids were collected in the absence of anticoagulant, centrifuged at 1000 *g* and supernatants frozen at −70°C. Non-anticoagulated fluids were treated with EDTA (6 mM final concentration) before binding assay.

Fibronectin
This was purified from human plasma by chromatography on Sepharose 4B (Pharmacia) and Gelatin-Sepharose 4B (Carsons *et al.*, 1983). The final preparation yielded a closely spaced doublet at 220 kD on SDS-PAGE under reducing conditions.

C1q

This was purchased from Calbiochem. This preparation was found to be free of fibronectin and other contaminants by SDS-PAGE.

IgG

This was purified from human serum by chromatography on DEAE-Sephacel and Protein-A Sepharose, both purchased from Pharmacia. Soluble aggregates were made by heating a 10 mg/ml solution of IgG at 63°C for 30 min followed by centrifuging at 9600 ***g*** in a Beckman Microfuge. The concentration of soluble aggregates (HAIgG) in the supernatant was determined spectrophotometrically $A1\%_{1cm} = 13{\cdot}6$.

Rabbit anti human C1q

This was purchased from Cappel Labs. Monospecificity was confirmed by SDS-PAGE of whole human plasma under reducing conditions followed by immunoblotting with anti C1q. Only bands corresponding to C1q chains were seen and no reactivity to Fn was noted.

Proteolytic cleavage of Fn

Purified plasma Fn was dialysed into 0·05 M Tris, 0·15 M NaCl, 0·01 M $CaCl_2$, pH 7·0 and then was incubated with chymotrypsin (1:50 wt/wt) for 45 min at 23°C. Digestion was terminated by adding PMSF (2 mM final concentration) and freezing to −20°C. The collagen-binding aminoterminal fragment was separated from the carboxyterminal terminal fragment by chromatography on gelatin-Sepharose.

Fibronectin quantification

This was performed in plasma and synovial fluid by electro-immunoassay as described by Carsons *et al.* (1981).

C1q quantification

This was performed by radial immunodiffusion (Ouchterlony & Nilsson, 1973) in 1% agarose containing 1 mM EDTA and 0·01% anti-C1q (v/v).

Solid phase binding assay

Ninety-six-well microtitre plates (Sarstedt) were coated with Fn or Fn fragments (500 ng/well). Wells were then incubated with serial dilutions of either purified C1q, normal plasma or RASF in isotonic PBS for 16 h at 4°C. To assess the temperature dependence of binding, the assay was also performed at 37°C. For some experiments samples were diluted in hypotonic Tris buffer or hypotonic phosphate buffer instead of PBS. Following this incubation, the wells were decanted and washed three times with PBS-0·05% Triton-X-100. The amount of C1q bound was determined by incubation with a 1:2000 dilution of anti-C1q for 1 h. After washing in PBS-Triton, wells were incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (1:1000 in PBS-BSA) for 45 min at room temperature. Phosphatase substrate (*p*-nitrophenyl phosphate) was then added for 15 min at 37°C and absorbance at 405 nm was determined using a Titertek ELISA plate reader. No reaction was seen in the absence of specific antigen despite the presence of non-immune rabbit serum or specific anti-C1q. Similarly no reaction was seen in the presence of antigen if non-immune rabbit serum was substituted for anti-C1q.

To compare binding among different samples, dilutions were made in parallel on the same plate and dilution-binding curves were constructed and normalized to the C1q concentration of the sample.

Electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970). Western blotting was performed according to the method of Towbin, Staehelin & Gordon (1979). Electrophoresis equipment and reagents were from Bio-Rad.

RESULTS

Binding of C1q in normal plasma and RA synovial fluid to solid phase Fn

Purified C1q diluted into isotonic PBS bound to Fn-coated microtitre-plate wells in a dose-dependent manner but showed no binding to BSA coated wells (Fig. 1a). C1q present in EDTA-treated pooled normal human plasma (NHP) did not demonstrate binding to Fn coated wells whereas C1q present in all (11/11) RASF bound Fn in a dose-dependent manner. C1q present in RASF did not bind to BSA coated control wells.

A comparison of dilution-binding curves demonstrated that C1q in RASF did not bind Fn as strongly as equivalent amounts of purified C1q (Fig. 1b). In order to estimate binding affinity, the amount of C1q required to produce 50% maximal Fn binding was determined. Approximately 800 ng of purified C1q was required to produce 50% binding whereas approximately 14 μg of C1q in SF was required, an 18-fold difference. Among the 11 RASF studied there was no correlation between the degree of Fn-C1q binding, the Fn concentration or the C1q concentration of the fluid.

In three of 12 RASF, a biphasic curve was observed demonstrating reduced binding at higher C1q concentrations (Fig. 1b, open circles).

Effect of EDTA and temperature on Fn-C1q binding

EDTA can enhance C1q interactions by dissociating the Ca^{2+} dependent C1qrs complex (Yonemasu & Stroud, 1971). Although C1q present in freshly collected, non-anticoagulated RASF bound Fn, the addition of 6 mM EDTA enhanced binding approximately 5-fold (Fig. 2a).

Fn–C1q binding was examined at body temperature (37°C) and at cryoprecipitation temperature (4°C). Fn and C1q did interact in the presence of RASF at 37°C, but binding was enhanced at 4°C (Fig. 2b).

Conditions resulting in enhancement of Fn–C1q binding in NHP

Differences in the method of sample preparation were noted to affect Fn–C1q binding. Dilution of NHP into either hypotonic phosphate or Tris buffers (50 mM) instead of PBS (150 mM NaCl) resulted in Fn–C1q binding (Fig. 3a). Binding was maximal at a 5-fold plasma dilution corresponding to a C1q concentration of 36 μg/ml and was still detected at a 50-fold dilution (3·6 μg/ml). Hypotonicity did not result in non-specific C1q binding or precipitation since C1q in NHP did not bind BSA in the presence of 50 mM phosphate or Tris buffer (Fig. 3a).

Similarly, collection of normal plasma into heparin (0·5 mg/ml) also resulted in the induction of Fn–C1q binding. Maximal binding was seen at a C1q concentration of 10 μg/ml corres-

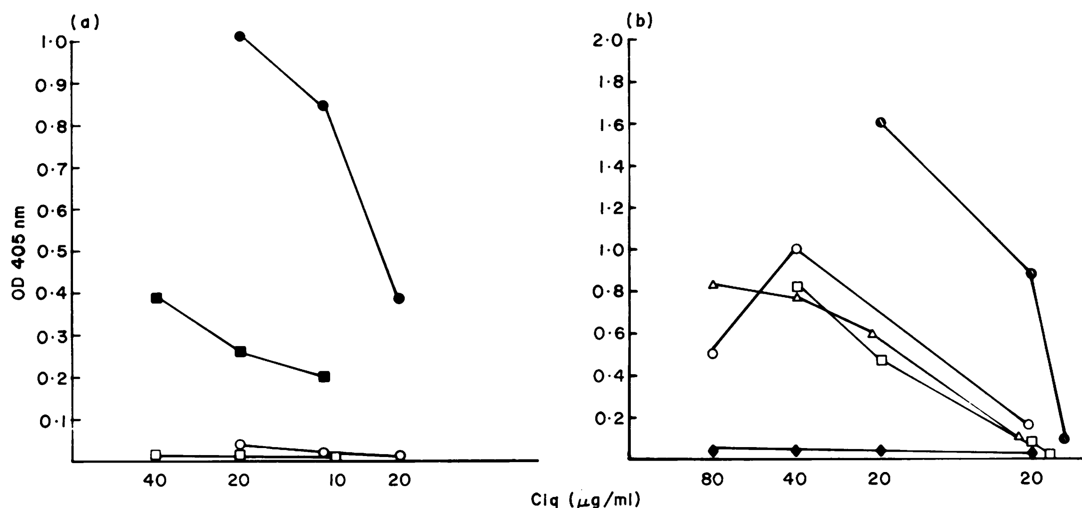

Fig. 1. (a) Fn–C1q interaction in the solid-phase binding assay. Samples were incubated in Fn or albumin coated wells for 16 h at 4°C. Bound C1q was detected with anti-C1q antibody followed by alkaline phosphatase labelled goat-anti rabbit IgG. Ordinate represents OD measured at 405 nm. Abscissa represents C1q concentration of sample. (●) Purified C1q, Fn-coated wells; (○) purified C1q, BSA-coated control wells. (■) RA synovial fluid, Fn-coated wells. (□) RA synovial fluid, BSA-coated control wells. (b) Fn-C1q interaction in normal plasma and RA synovial fluid. Solid-phase binding assay as described in Fig. 1a and methods. (♦) pooled normal EDTA human plasma. (□, ○, △) three RA synovial fluids. (⊘) binding of purified C1q.

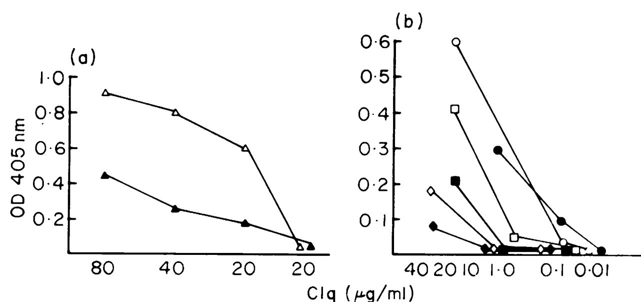

Fig. 2. (a) Fn-C1q interaction in the presence and absence of EDTA. Assay as described in Fig. 1a and Materials and Methods. (▲) synovial fluid collected in the absence of any anticoagulant. (△) synovial fluid collected as above except with the addition of EDTA (6 mm final concentration). (b) Fn–C1q solid phase binding assay performed at 4°C and 37°C. Assay performed as described in Fig. 1(a) except a replicate plate was kept in a 37°C incubator for 16 hr in addition to the plate normally kept at 4°C for 16 h. Open symbols, 4°C incubation; closed symbols, 37°C incubation; diamond, normal human plasma; square and circle, two RA synovial fluid samples.

ponding to a 1/10 dilution (Fig. 3b). Heparin (0·5 μg/ml) did not increase the binding of purified C1q to solid phase Fn.

Differences in protein composition between NHP and RASF might also affect C1q–Fn interactions and could result in the enhanced binding seen in RASF. The role of two such alterations—the elevated levels of Fn and the presence of immune complex-like material in RASF—were examined. Dilution of NHP into a concentrated solution of purified plasma Fn in isotonic PBS resulted in Fn–C1q binding as did dilution of NHP into a solution of heat-aggregated IgG in PBS. Binding was noted at Fn and HAIgG concentrations exceeding 680 and 550 μg/ml respectively (Fig. 3c). Dilution of NHP in isotonic PBS from 1/10 to 1/10,000 corresponding to C1q concentrations of 30 μg/ml–30 ng/ml did not itself result in Fn–C1q binding.

Binding of C1q to fibronectin fragments

In order to determine the Fn domains contributing to C1q binding in the presence of RASF, C1q binding to collagen binding and non-binding Fn chymotryptic fragments was studied (Fig. 4a). C1q in RASF bound to both Fn fragments, but binding to the collagen-binding fragments was usually greater (Fig. 4b). The binding of C1q in RASF to Fn fragments was weaker than binding to intact Fn (Compare Fig. 3b with Fig. 1b).

DISCUSSION

Purified Fn has been shown to bind purified C1q *in vitro* (Anderson *et al.*, 1981; Menzel *et al.*, 1981; Bing *et al.*, 1982; Sorvillo *et al.*, 1983). The studies reported here were designed to determine whether this potentially important interaction would occur in normal plasma or pathological (inflammatory) body fluid. Using a solid-phase binding assay, we demonstrated Fn–C1q binding in all RASF studied. We chose to study binding to solid-phase Fn because Fn has been shown to be an early constituent of the extracellular matrix in the inflammatory response (Clark, Dvorak & Colvin, 1981). Preliminary data using an Fab′ antifibronectin antibody, however, has shown that binding of Fn in solution to solid phase C1q occurs in the presence of RASF (S. Schwartzman & S.E. Carsons, unpublished results).

Fn-C1q binding was not detected in pooled NHP. This is consistent with results obtained by Hautanen & Keski-Oja (1983a,b) who, using a similar assay, demonstrated binding of Fn and C3 but not Fn and C1q in the presence of normal human serum.

Fn–C1q binding in RASF did occur at body temperature (37°C), but maximal binding occurred at 4°C. Fn and C1q are present in cryoprotein formed from RASF, and prior depletion of synovial fluid fibronectin has resulted in loss of C1q from

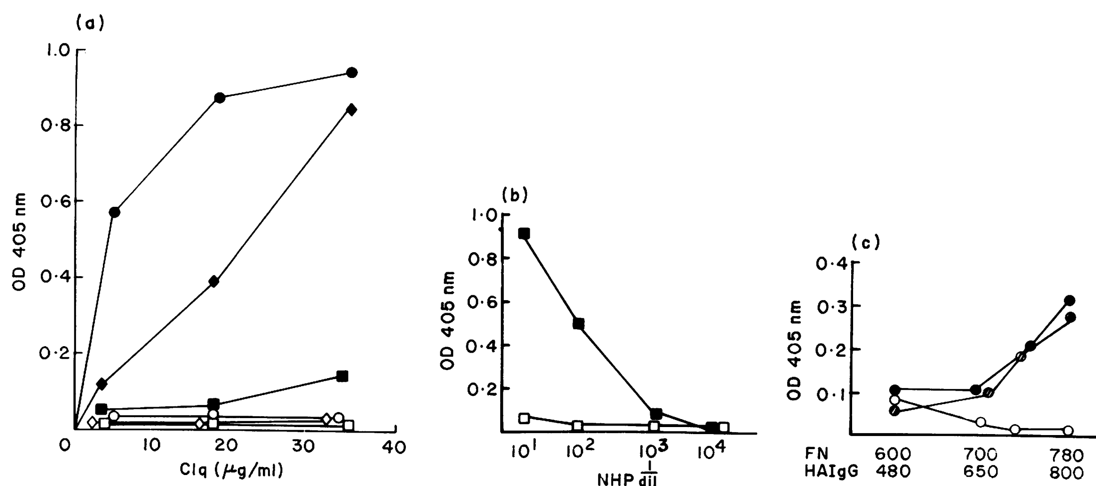

Fig. 3. Fn-C1q solid phase binding assay in the presence of normal human plasma. Assay performed as described in Materials and Methods. (a) Induction of Fn–C1q binding by hypotonic solution: open symbols, binding to BSA coated wells; C1q present in plasma did not bind BSA in the presence of PBS (150 mM NaCl) (□); phosphate, 50 mM NaCl (○); or 50 mM Tris (◇). Closed symbols, binding to Fn coated wells; C1q in plasma did not bind Fn in presence of PBS (150 mM NaCl) (■) but did bind in the presence of hypotonic buffers: phosphate, 50 mM NaCl (●) or 50 mM Tris (♦) (b) Induction of Fn-C1q binding by heparin. (□) EDTA plasma; (■) heparin treated EDTA plasma. (c) Induction of Fn-C1q binding in NHP by increasing the concentration of Fn or by the addition of heat aggregated IgG. (○) NHP diluted in isotonic PBS. (⊘) NHP diluted into PBS containing purified Fn (1 mg/ml). (●) NHP diluted into PBS containing heat aggregated IgG (800 μg/ml).

subsequently formed cryoprotein (Carsons, Lavietes & Diamond, 1983). These data suggest that the Fn–C1q interaction may be in part responsible for the formation of cold-insoluble precipitates seen in serum and synovial fluid of patients with rheumatic disease.

Since C1q in NHP did not bind Fn whereas C1q in RASF did, it is likely that conditions present in RASF but not in plasma enhanced binding. The addition of heparin to RASF has been shown to enhance the concentration of Fn in the cryoprecipitate subsequently formed (Carsons *et al.*, 1983). In the data presented here, heparin was able to induce Fn–C1q binding in normal human plasma perhaps by complexing to Fn and/or C1q. It is possible that glycosaminoglycans present in synovial fluid and other body fluids may similarly enhance Fn–C1q binding.

Hypotonicity alone was able to induce Fn–C1q binding in normal plasma. This is in agreement with previous studies on the interaction between purified Fn and C1q where binding was shown to be enhanced in the presence of low ionic strength buffers (Sorvillo *et al.*, 1983). Perhaps the isotonicity of plasma is one factor that inhibits Fn–C1q interactions in the normal circulation. It is also likely that normal plasma proteins inhibit the Fn–C1q interaction since strong binding of purified Fn and C1q occurred in the presence of isotonic buffer but not in isotonic plasma.

Although albumin is the most abundant plasma protein it is unlikely that it alone inhibited Fn-C1q binding in NHP since albumin levels in RASF are also significant (1 to 5 g/dl) and we observed Fn-C1q binding in an RASF with an albumin content of 5·1 g/dl.

Levels of Fn in RA fluids are generally 2–3-fold higher than corresponding plasma Fn levels (Carsons *et al.*, 1981). To determine whether alterations in the concentration of soluble Fn would influence C1q–Fn binding, we augmented the Fn level of NHP and found induction of Fn–C1q binding at levels exceeding 680 μg/ml—approximately twice normal plasma concentration. Fn contains a self-association site near the aminoterminal (Vartio, 1983) so that interactions between soluble and solid phase Fn may promote interaction with C1q or other macromolecules.

A major difference in the protein composition of NHP and RASF is the presence of immune-complex-like material in the RA fluid. When immune-complex-like material was added to NHP, C1q–Fn complex formation occured. Consistent with this observation, Sorvillo, Gigli & Pearlstein (1986) have shown that Fn–C1q binding occurred in EDTA-plasma supplemented with BSA anti-BSA immune complexes. Possibly, the immune-complex-bound C1q has enhanced affinity for Fn enabling binding to occur in the presence of NHP.

C1q is capable of binding to multiple different fragments of the Fn molecule and *in vitro*, the Fn collagen-binding domain serves as the major binding site for C1q (Sorvillo, Gigli & Pearlstein, 1985). The presence of a biphasic binding curve in several of our SF samples suggested that interactions at more than one site were possible. When C1q binding to collagen-binding and non-binding Fn fragments was examined, C1q in RASF bound to both domains of the Fn molecule although binding was generally greater to the collagen binding domain (Fig. 4).

We have shown that C1q present in RASF binds solid phase Fn with maximal binding occurring under conditions conducive to cryoprotein formation. Surface bound deposits of Fn, such as those present on the lining of RA synovium (Scott *et al.*, 1981), therefore, could bind C1q present in synovial fluid. Interaction of the globular portion of C1q with immune complex-like material present in RASF may enhance C1q binding to Fn. Since Kratz, Borsos & Isliker (1985) have shown that interaction of Fn with C1q can inhibit subsequent complement haemolytic activity, Fn–C1q interactions may modulate immune-complex deposition and complement activation in areas of inflammation.

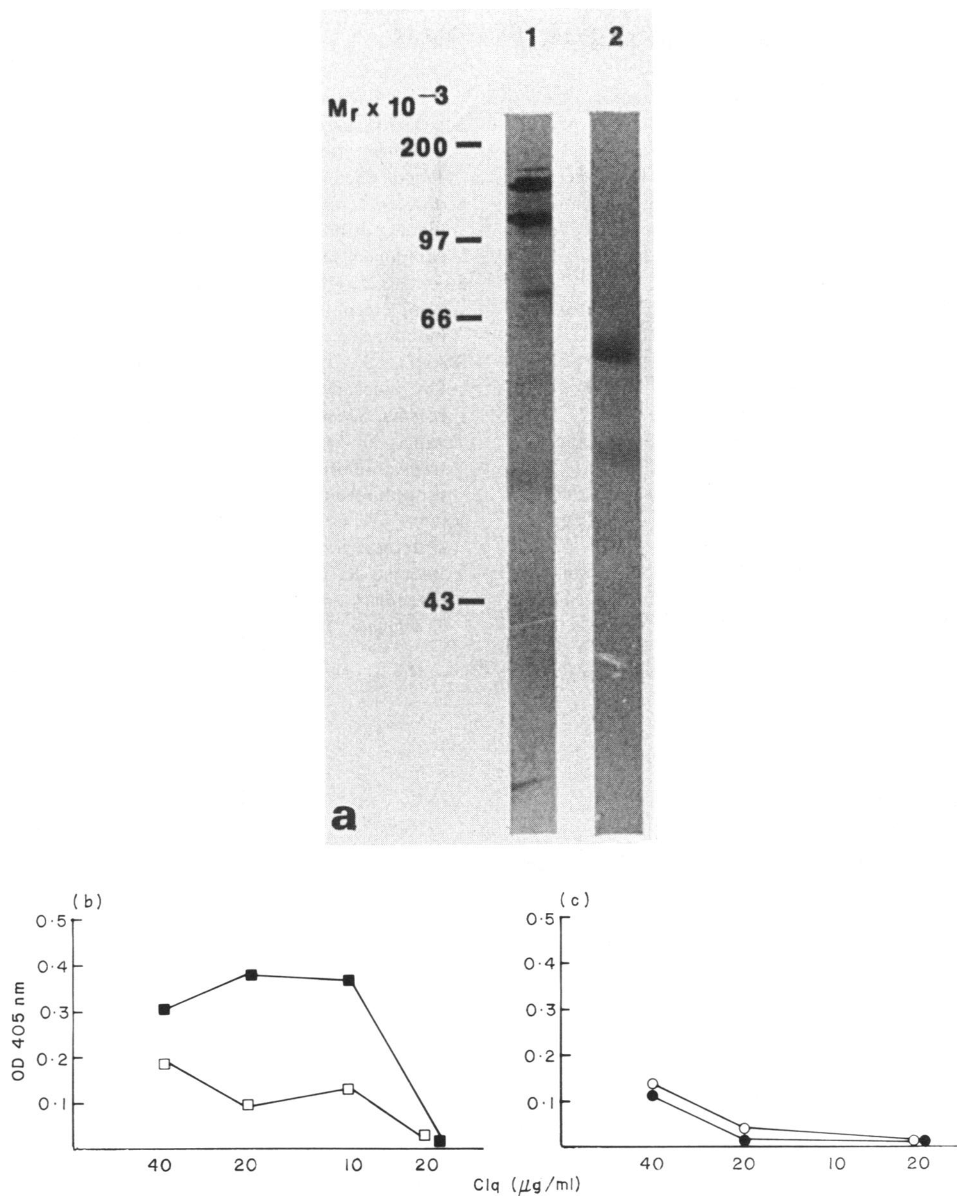

Fig. 4. (a) SDS-PAGE (10% gel) of Fn chymotryptic digest separated into collagen-binding and non-binding fragments by gelatin-Sepharose affinity chromatography. Lane 1, nonbinding 140–160 kD fragments. Lane 2, collagen binding 60 kD fragment. (b) Binding of C1q to solid phase Fn fragments in the presence of RA synovial fluid. (□) wells coated with 140 kD fragment. (■) wells coated with 60 kD collagen binding fragment. (c) Binding of C1q to solid phase Fn fragments (500 ng/well) in the presence of normal human plasma. (○) wells coated with 40 kD fragment; (●) wells coated with 60 kD collagen binding fragment.

ACKNOWLEDGMENTS

We thank Barbara Diamond for photographic assistance, and Ann Marchesiello for preparation of the manuscript. This work was supported in part by a grant from the New York Chapter of the Arthritis Foundation.

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