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Fc-mediated binding of IgG to vimentin-type intermediate filaments in vascular endothelial cells

(cell injury/cytoskeleton/immunology/mesenchymal cells)

GORAN K. HANSSON*†, GORDON A. STARKEBAUM‡, EARL P. BENDITT*, AND STEPHEN M. SCHWARTZ*

*Department of Pathology, University of Washington, Seattle, WA 98195; and ‡Department of Medicine-Rheumatology, Veterans Administration Hospital, Seattle, WA

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ABSTRACT **Prior studies have shown that vascular endothelial cells bind circulating IgG intracellularly during cell death. We now demonstrate that all endothelial cells have intracellular binding sites for IgG and that these binding sites are exposed to circulating IgG only if the plasma membrane is damaged. The binding sites are located on the cytoskeletal intermediate filaments and can be detected also in other cells containing vimentin-type intermediate filaments. Monoclonal human IgG1 exhibited saturable, high-affinity binding to vimentin-enriched cytoskeletons. Binding was inhibited by Fc fragments but not by Fab, F(ab')$_2$, or pFc' fragments, suggesting that the binding site on IgG is located in the $C_\gamma 2$ domain of the Fc fragment. Binding of IgG to intermediate filaments may be important for the destruction and removal of damaged cells.**

The endothelial lining of the blood vessels is directly exposed to the effectors of immune and inflammatory reactions (1–3). We have previously observed that a small number of endothelial cells in the normal aorta have cytoplasmic IgG deposits (4–6). The presence of cytoplasmic IgG within endothelial cells is associated with intracellular calcium deposits and uptake of trypan blue, which indicates that accumulation of IgG is associated with a damage to the plasma membrane. Such IgG-containing, injured cells occur with a frequency of 0.2% in the normal rat aorta (6).

The correlation between binding of IgG and irreversible cell injury suggested that a damage to the plasma membrane gives IgG access to intracellular binding sites. Our earlier immunoelectron microscopic studies of endothelium had shown that IgG binds to components of the cytoplasmic matrix (5). We have now explored this binding phenomenon in cultivated bovine aortic endothelial (BAE) cells. We report that IgG binds to intermediate filaments of endothelial cells and also of other mesenchymal cells. This binding occurs to a limited number of high-affinity binding sites, and Fc but not Fab fragments compete with labeled IgG for binding. Our data suggest that the binding site is in the $C_\gamma 2$ domain of IgG.

MATERIALS AND METHODS

Cells. BAE cells were isolated and cloned as described (7, 8). They were fed Waymouth's medium with 10% fetal calf serum. Swiss 3T3 cells were fed Dulbecco's modified Eagle's minimum essential medium with 5% fetal calf serum.

Sera. Adult bovine serum, which has an IgG concentration of 10–20 mg/ml (9), was prepared as described by Gajdusek *et al.* (10). Some portions were complement-inactivated by heating to 56°C for 30 min. Fetal calf serum, which has an IgG concentration of ≈0.12 mg/ml (9), was obtained from KC Biological, Lenexa, KS.

IgG and Fragments. Human IgG was prepared from outdated blood plasma by ammonium sulfate precipitation and ion-exchange chromatography on DEAE-cellulose (Whatman) (11). F(ab')$_2$ and pFc' fragments were isolated after cleavage of IgG with pepsin and gel filtration on Sephadex G-150 (Pharmacia, Uppsala, Sweden) (12). Fab and Fc fragments were prepared from IgG subclasses 1, 2, and 4, which were isolated by affinity chromatography on protein A-Sepharose (11). This IgG was cleaved with papain (13) and the fragments were isolated by gel filtration on Sephadex G-150, followed by protein A-Sepharose. Monoclonal human IgG1 was purified from the serum of a patient with multiple myeloma by ion-exchange chromatography on DEAE-cellulose followed by gel filtration on Sephadex G-200. The subclass of paraprotein was kindly determined by John P. Leddy (University of Rochester, Rochester, NY). The IgG was radiolabeled with ^{125}I by the chloramine-T method (14). All preparations of IgG and fragments were analyzed by Ouchterlony double-diffusion and NaDodSO$_4$/polyacrylamide gel electrophoresis.

Antisera. Rabbit anti-bovine IgG, rabbit anti-bovine serum albumin, and fluorescein isothiocyanate (FITC)-goat anti-guinea pig immunoglobulins were purchased from Cappel Laboratories, Cochranville, PA. FITC-swine anti-rabbit IgG, rhodamine-swine anti-rabbit IgG, rabbit anti-human IgG (heavy and light chains), rabbit anti-human IgG-Fc, rabbit anti-human IgG γ chains, rabbit anti-human complement factor C1q (the first component of complement), and rabbit anti-human serum albumin were bought from Dako (Copenhagen, Denmark). Guinea pig anti-vimentin (15) was a gift from Giulio Gabbiani (University of Geneva, Switzerland). FITC-labeled protein A was obtained from Pharmacia. All antisera were used at optimal dilutions as defined by chessboard titrations on positive tissues, usually 1:50, in calcium-free phosphate-buffered saline (P$_i$/NaCl) with 1% ovalbumin.

Immunofluorescence. Cells were grown on sterile coverslips in 35-mm Petri dishes. For studies of IgG uptake in relation to viability, the cells were incubated for 1 hr with medium containing 10% heat-inactivated adult bovine serum, rinsed repeatedly with Dulbecco's P$_i$/NaCl, incubated for 5 min with 0.1% trypan blue, rinsed again, and fixed with 1% paraformaldehyde in phosphate buffer (pH 7.2). They were incubated with rabbit anti-bovine IgG followed by FITC-swine anti-rabbit IgG, both for 30 min at room temperature, mounted in Tris-buffered polyvinyl alcohol (16), and exam-

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Abbreviations: BAE cells, cultivated bovine aortic endothelial cells; FITC, fluorescein isothiocyanate; C1q, first component of complement.

†Present address: Department of Histology, University of Goteborg, Box 33031, S-400 33, Goteborg, Sweden.

ined in a Zeiss microscope III equipped with an HBO 50 mercury lamp, epi-illumination, and interference filters for FITC. For studies of IgG binding to cytoskeleton, cells were rinsed with Dulbecco's P_i/NaCl and made permeable by treatment with ice-cold methanol for 3 min, followed by ice-cold acetone for 2 min. Alternatively, the cells were permeabilized for 4 min at room temperature with 0.1% Triton X-100 in calcium-free P_i/NaCl (140 mM NaCl/8 mM Na_2HPO_4/1.47 mM KH_2PO_4/2.68 mM KCl, pH 7.2). After rinsing with calcium-free P_i/NaCl, the permeabilized cells were incubated with 10% adult bovine serum or purified rabbit or human IgG or IgG fragments in calcium-free P_i/NaCl for 1 hr at 37°C. After rinsing in calcium-free P_i/NaCl, the cells were stained with rabbit anti-bovine IgG, rabbit anti-human IgG, rabbit anti-human C1q, or rabbit anti-bovine serum albumin, all followed by FITC-swine anti-rabbit IgG. Cells incubated with rabbit IgG or its fragments were stained directly with FITC-swine anti-rabbit IgG.

Double-staining experiments were made by permeabilizing BAE cells with methanol and acetone, incubating with rabbit IgG followed by rhodamine-swine anti-rabbit IgG, and then with guinea pig anti-vimentin, followed by FITC-goat anti-guinea pig immunoglobulins. In some experiments, vimentin-enriched cytoskeletons, prepared as described below, were incubated with adult bovine serum and stained as described above.

Binding Assay. Vimentin-enriched cytoskeletons were prepared (17) by extracting confluent BAE cells grown in 10-mm Falcon multiwells with 1% Triton X-100 in high-salt buffer (600 mM KCl/137 mM NaCl/5 mM EDTA/4.1 mM $NaHCO_3$/1 mM phosphonylmethylsulfonyl fluoride/0.4 mM phosphate buffer, pH 7.4) for 10 min, followed by fixation in ice-cold methanol for 5 min and air drying. The cytoskeletons were incubated for 1 hr at 37°C with ^{125}I-labeled human myeloma IgG1 (^{125}I-IgG1) at various concentrations in calcium-free P_i/NaCl with 1 mg of ovalbumin per ml in a total incubation volume of 250 μl and rinsed three times with calcium-free P_i/NaCl. Bound radioactivity was solubilized by a 20-min incubation with 1 M NaOH and counted for 1 min in a gamma counter. For competition experiments, various amounts of nonradioactive human IgG, F(ab')$_2$, Fab, Fc, pFc', or bovine serum albumin were added to the cytoskeletons together with 1 μg of ^{125}I-IgG1 in calcium-free P_i/NaCl in an incubation volume of 250 μl.

Electrophoresis and Immunoblotting. $NaDodSO_4$/polyacrylamide gel electrophoresis was performed according to Laemmli (18) using a 5% stacking gel and a 10% separating gel with a length of 10 cm. Samples were reduced with 6.25% 2-mercaptoethanol and boiled for 5 min. For electrophoretic studies of serum protein binding, BAE cells were permeabilized with ice-cold methanol for 5 min and incubated with fresh human serum for 1 hr at 37°C. After repeated rinsing in calcium-free P_i/NaCl, material on the dish was scraped off, solubilized in $NaDodSO_4$ sample buffer, and applied to a $NaDodSO_4$/polyacrylamide gel. The gel was analyzed for the presence of IgG by the immunoblotting technique (19), using electrophoretic transfer of proteins to nitrocellulose paper, incubation with rabbit anti-human IgG γ chain, followed by ^{125}I-labeled protein A (New England Nuclear) and detection by autoradiography.

RESULTS

Confluent cultures of BAE cells contained 5% trypan blue-positive cells. No IgG could be detected in cells grown in fetal calf serum. In contrast, ≈5% of cells grown in adult bovine serum contained IgG, as demonstrated by immunofluorescence (Fig. 1). Only cells that were trypan blue-positive contained IgG. Since mitochondrial calcification is correlated with cell death, we examined similar cultures for the ability of cells to bind the calcium probe chlorotetracycline (20). Trypan blue-positive cells also contained intracellular calcium deposits, as indicated by chlorotetracycline uptake (data not shown). These data indicate that IgG is taken up during cell death in unmanipulated BAE cells in culture.

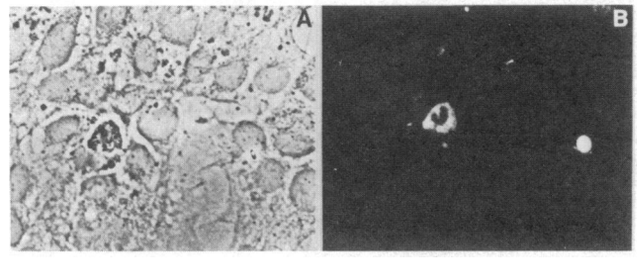

FIG. 1. Confluent culture of BAE cells incubated in 10% adult bovine serum, stained with trypan blue, fixed in paraformaldehyde, and stained with fluorescent anti-IgG. (×285.) (*A*) Phase-contrast micrograph. A trypan blue-positive cell is in center. (*B*) Fluorescence micrograph. The same cell contains IgG. Viable cells, in contrast, have not taken up IgG.

If cells were permeabilized with 0.1% Triton X-100 or with acetone before incubation with adult bovine serum, all cells bound IgG (Fig. 2*A*). The fibrillar staining pattern suggested binding of IgG to components of the cytoskeleton. In contrast, staining for albumin using antibodies at optimal dilutions with low concentration of antibody immunoglobulin showed only trace amounts of binding and a diffuse binding pattern (Fig. 2*B*). Staining with anti-albumin antibodies at high concentrations of antibody immunoglobulin, on the other hand, resulted in a cytoskeletal binding pattern superimposed on the diffuse one. This was probably due to nonimmune binding of immunoglobulin (see below). Staining for complement factor C1q showed a similar pattern as IgG, even when low antibody concentrations were used and therefore probably reflected a true cytoskeletal binding of C1q (Fig. 2*C*). The binding of C1q, but not that of IgG, was abolished by heating serum to 56°C for 30 min before incubation with permeabilized cells. This suggests that binding of IgG was independent of C1q binding, and this conclusion was supported by the fact that IgG binding was detected also when purified IgG was used instead of serum and when either FITC-labeled protein A or FITC-labeled antibodies were used as a second reagent.

Binding of IgG to permeabilized cells was confirmed by immunoblotting. Permeabilized BAE cells were incubated with fresh serum and the material remaining on the dish after rinsing was solubilized in $NaDodSO_4$ sample buffer and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. Samples incubated with serum showed several new bands that were absent in similar preparations of cells not incubated with serum (data not shown). The proteins were electrophoretically transferred to nitrocellulose paper and incubated with specific antibodies against IgG γ chains. One of the

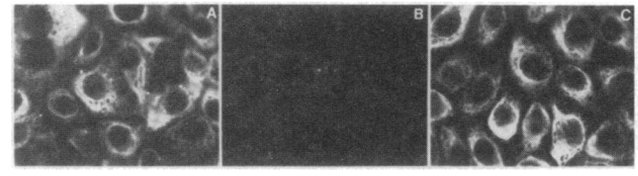

FIG. 2. BAE cells that were permeabilized with acetone, incubated with adult bovine serum, and stained for plasma proteins with indirect immunofluorescence. (×200.) (*A*) Staining for IgG shows binding in a cytoskeletal pattern. (*B*) Staining for albumin shows only a faint, diffuse binding. (*C*) Staining for C1q demonstrates binding in a cytoskeletal pattern.

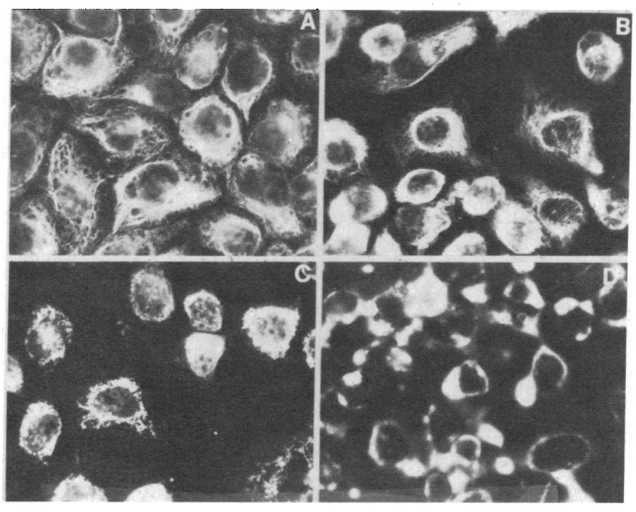

FIG. 3. BAE cells treated with demecolcine before permeabilization, incubation with adult bovine serum, and staining for IgG. (×285.) (*A*) No demecolcine. Control. (*B*) Demecolcine treated for 30 min. IgG binding sites have formed a perinuclear ring with fine radiating filaments. (*C*) Demecolcine for 4 hr. IgG binding sites are entirely perinuclear. (*D*) Demecolcine for 24 hr, resulting in perinuclear caps of IgG binding sites.

new bands was identified as the heavy chain of IgG (data not shown).

The cytoskeletal binding sites for IgG were explored in three sets of experiments. In the first, BAE cells were treated with drugs that affect the organization of the cytoskeleton, permeabilized, incubated with serum, and stained as described above to visualize the IgG binding sites (Fig. 3*A*). Pretreatment with cytochalasin D, which depolymerizes microfilaments (21), did not alter the binding pattern. Pretreatment with 1 μM demecolcine (Colcemid) for 30 min resulted in an aggregation of the binding sites into a perinuclear ring with fine radiating filaments (Fig. 3*B*). When demecolcine treatment was extended to 4 hr, the IgG binding sites formed a perinuclear ring with no radiating filaments (Fig. 3*C*), and after 24 hr of treatment IgG was bound only to a perinuclear cap structure (Fig. 3*D*). This change of the IgG binding pattern is identical to the changes in the distribution of intermediate filaments following treatment with demecolcine (22) and suggest that IgG binds to intermediate filaments.

Binding of IgG to intermediate filaments was further supported by a double-tracer experiment. Acetone-permeabilized BAE cells were incubated with normal rabbit serum, stained for IgG (with diluted rhodamine-labeled swine anti-rabbit IgG), and then stained for vimentin (with a specific, diluted guinea pig anti-vimentin followed by FITC-labeled goat anti-guinea pig IgG). IgG from the nonimmunized rabbit stained the same structures as the anti-vimentin antibodies (Fig. 4 *A* and *B*). A similar cytoskeletal binding of IgG was observed in 3T3 cells, which also contain vimentin-type intermediate filaments (data not shown).

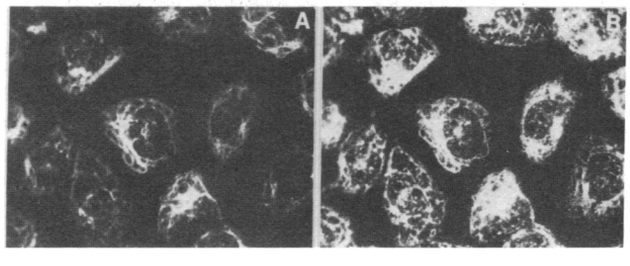

FIG. 4. Double staining of permeabilized BAE cells after incubation with rabbit IgG. (×285.) (*A*) Staining with FITC-anti-rabbit IgG shows IgG decoration of intermediate filaments. (*B*) Staining with guinea pig anti-vimentin and rhodamine-anti-guinea pig immunoglobulins shows that anti-vimentin antibodies decorate the same structures as rabbit IgG.

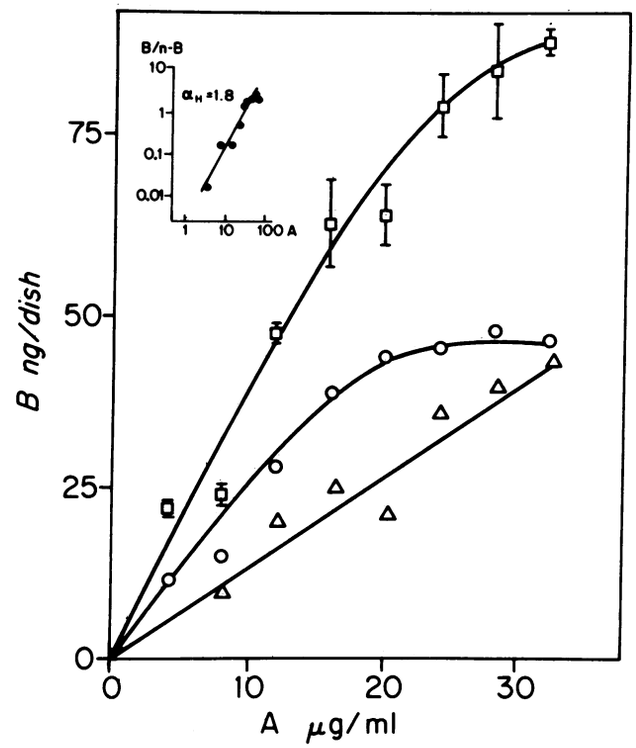

FIG. 5. Binding of IgG to vimentin-enriched cytoskeletons. Binding (B) of ^{125}I-IgG1 to vimentin-enriched cytoskeletons. Total binding (□, ng per dish) and specific binding (○, ng per dish) after subtraction of binding to serum-coated dishes (△, ng per dish) are plotted vs. concentration (A) of IgG1 added (ng/μl). In a typical experiment, addition of 20 ng of ^{125}I-IgG1 per ml gave a total binding of 2000 cpm. (*Inset*) Hill plot of the binding data shown. A value of $n = 2 \times 10^5$ per cell was used for this analysis.

These observations could be explained by the presence of autoantibodies to vimentin, which have been reported to occur frequently in several species (23, 24). To explore this possibility, purified human IgG was cleaved by papain. The isolated Fab and Fc fragments were incubated with permeabilized BAE cells and then stained with FITC-labeled anti-IgG (heavy and light chains). Binding of Fab fragments could only be detected at high concentrations (2 mg/ml), but Fc

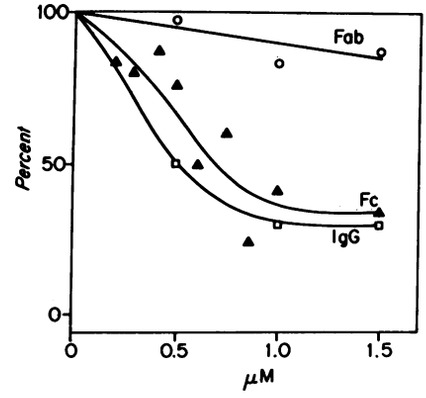

FIG. 6. Competition for binding to vimentin-enriched cytoskeletons of 26 mM (4 ng/μl) ^{125}I-IgG1 by unlabeled human IgG (□), Fab (○), and Fc (△) fragments.

fragment binding to the cytoskeleton was detected with concentrations as low as 100 μg/ml.

The binding was further characterized by using intermediate filaments prepared from BAE cells by extraction with Triton X-100 and a high-salt buffer. $NaDodSO_4$/polyacrylamide gel electrophoresis of the residual material scraped off the dish showed that the M_r 58,000 intermediate filament protein, vimentin, was the dominating protein remaining in these preparations (data not shown). A binding assay was constructed with ^{125}I-labeled monoclonal human IgG1 as the ligand and the vimentin-enriched cytoskeletons as receptors. The specific binding curve, obtained after subtracting binding to serum-coated dishes from total binding to the cytoskeletons, showed saturation (Fig. 5). Analysis of a Scatchard plot (25) indicated a K_d of 1×10^{-10} M, and the number of binding sites was estimated to be 2×10^5 per cell, assuming monovalent binding. Since there was a tendency towards a sigmoidal shape of the binding curve, the data were examined for inter-binding site cooperativity with a Hill plot (26), shown in Fig. 5 *Inset*. The Hill constant was 1.8, which suggests a positive inter-binding site cooperativity during binding of ligand.

The binding of radiolabeled IgG1 was inhibited by unlabeled IgG or unlabeled Fc fragments in a competition assay but not by unlabeled Fab fragments (Fig. 6). The binding site within the Fc fragment was probably in the $C_\gamma 2$ domain, since only Fc but not $F(ab')_2$ or pFc' fragments inhibited binding (Table 1).

DISCUSSION

We reported previously that intracellular binding of IgG in endothelial cells could be correlated with evidence for cell death (4–6). This observation suggested the presence of a cytoplasmic binding site normally hidden by the intact plasma membrane.

This hypothesis was tested by identifying a cytoplasmic binding site for IgG in permeabilized cells. The pattern shown by immunofluorescence, redistribution in response to Colcemid, and ability of vimentin-enriched cytoskeletons to bind IgG supports the conclusion that at least one site for cytoplasmic binding of IgG is on the intermediate filaments of the cytoskeleton.

At first glance, these observations are not surprising. The literature contains a large number of reports on spontaneous antibodies to this protein (23, 24, 27). We demonstrated, however, binding both of monoclonal human IgG and of Fc fragments, implying that binding is *not* a function of the antigen-binding sites of the variable regions of IgG.

Radiolabeled IgG1 bound to vimentin-enriched cytoskeletons in a saturable fashion and with high affinity. Further analysis of binding data suggested a positive inter-binding site cooperativity with a concentration-dependent increase in affinity for IgG. This could explain why nonimmune binding of IgG to the cytoskeleton was observed only at high concentrations of IgG in the immunofluorescent assay. It should, however, also be noted that the result of an immunofluorescent assay is dependent not only on the affinity of binding sites for ligand but also on the two-dimensional distribution of binding sites. It is therefore difficult to relate these data to those of the radiochemical binding assay in a quantitative fashion, although they both demonstrate the same phenomenon—i.e., Fc-mediated binding of IgG to intermediate filaments.

Table 1. Competition of binding to vimentin-enriched cytoskeletons by ^{125}I-IgG1 and unlabeled proteins

Competing protein	^{125}I-IgG1 bound, ng
Bovine serum albumin	8.994 ± 0.539
Fab	9.330 ± 1.148
$F(ab')_2$	8.921 ± 0.634
Fc	4.286 ± 0.703*
pFc'	8.085 ± 0.648

^{125}I-IgG1 (26 nM; 4 ng/μl) was incubated with vimentin-enriched cytoskeletons in the presence of 1.60 μM unlabeled protein. Values are shown as mean ± SEM.

*Significantly different from ^{125}I-IgG1 binding in the presence of bovine serum albumin ($P < 0.01$).

Both native IgG and Fc fragments competed with radiolabeled IgG1 for the binding sites, but the competition was not equimolar. This might imply a higher affinity of the binding sites for IgG1 than for the pooled human IgG and Fc that were used as competitors. Further competition experiments suggested that binding to intermediate filaments is most likely mediated by the $C_\gamma 2$ domain of the Fc fragment.

These data make it important to reevaluate the reports of a very high frequency of low-titer autoantibodies to intermediate filaments. Although it has been proven beyond a doubt that autoantibodies to vimentin may occur (e.g., ref. 24), some of the positive reactions observed by immunofluorescence could be due to Fc binding. Our data also raise the possibility that Fc-mediated binding of IgG to virally infected cells (28, 29) reflects intracellular, cytoskeletal IgG-Fc binding in injured cells rather than an expression of cell surface Fc receptors.

Our data do not rule out the possibility that IgG might bind to other components of the cell in addition to intermediate filaments. Immunoelectron microscopy (5) showed IgG on the cytoplasmic side of intracellular vesicles and this might represent a different type of binding. It is also possible that other plasma proteins bind to intracellular binding sites. In addition to IgG, complement factor C1q also binds to intermediate filaments, as shown by Linder (30). These two plasma proteins have complementary binding sites for each other (31, 32) and the fact that both of them bind to intermediate filaments may therefore suggest the presence of complementary binding sites on the filaments resembling IgG and C1q, respectively. We do not, however, know the precise location or structure of these binding sites on intermediate filaments. C1q also binds in a saturable fashion to specific binding sites on mitochondrial membranes (33) as does nerve growth factor, which can mimic the function of C1q in the complement cascade (34) and also binds to actin microfilaments and to microtubules (35). All these data suggest important functions for the interaction between humoral components of the immune system and the cytoskeleton.

Finally, we want to consider some pathophysiologic aspects of these phenomena. Mechanisms for the removal of damaged and dead cells remain enigmatic. This is particularly true for mesenchymal tissues, where such cells cannot be eliminated by being sloughed off from surfaces continuous with the external environment, as is the case in the elimination of many epithelial cells. Recent studies of cell death indicate that autolysis may be a very slow and limited process (36) and largely intact cells can be seen in the center of infarcts days or weeks after ischemia (37). It may therefore be important to elicit some specific responses that facilitate the destruction and removal of damaged mesenchymal cells. They all contain vimentin as a major cellular protein (17, 38), which would be exposed to plasma proteins in cells with a damaged plasma membrane. In this way, cytoskeletal activation of the IgG-complement cascade could generate localized proteolytic and membranolytic activity (32, 39), as well as chemotactic and opsonizing factors that would enhance phagocytosis of the remnants of the dead cells.

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