Virchows Archiv [Pathol Anat] (1984) 404:139–158

Virchows Archiv A

IgG Fc membrane receptor on normal human glomerular visceral epithelial cells

Raoul Mancilla-Jimenez, Marie-Dominique Appay, Blanche Bellon, Joelle Kuhn, Jean Bariety, and Philippe Druet

Hôpital Broussais INSERM U 28 CNRS ERA 48, Laboratoire de morphologie et d'immunopathologie rénale 96rue Didot, F-75674 Paris, France

Summary. This study demonstrates that human glomerular epithelial cells are able to bind heat aggregated immunoglobulins and antigen-antibody complexes. This has been observed on kidney cryostat sections, on whole glomeruli and on cultured visceral epithelial cells. Binding depends on the presence of the Fc portion of IgG and occurs in the absence of complement, showing that the IgG Fc receptor is different from the C3b receptor. The use of heat aggregated anti-peroxidase IgG and of peroxidase anti-peroxidase complexes allowed us to demonstrate, at the ultrastructural level, that the binding of the reagents at the plasma membrane was followed by their internalization within coated pits of vesicles. These observations strongly suggest that glomerular visceral epithelial cells are capable of receptor mediated endocytosis. The role of this process in glomerular diseases remains to be established.

Key words: Human glomerulus – Fc receptor – Immunoelectron microscopy – Anti-peroxydase antibodies

In human glomerulonephritis it is common to observe granular subepithelial deposits (SED) of immune material between the glomerular basement membrane and the visceral epithelial cells (VEC). Based on experimental acute and chronic serum sickness (Dixon et al. 1961; Germuth et al. 1972), it has been widely believed that SED result from the trapping of circulating immune complexes. However, this explanation is not entirely satisfactory, since injection of preformed immune complexes does not usually result in subepithelial deposition (Fleuren et al. 1980; Haakenstad et al. 1976; Okumura et al. 1971). This has stimulated the search for other possible mechanisms. There is now evidence based on in vivo and in vitro experiments that SED may result from the in situ interaction between antibodies and antigens at the capillary wall (Couser et al. 1980; Couser et al. 1978; Fleuren

Offprint requests to: P. Druet at the above address

et al. 1980; Fleuren et al. 1978; Van Damme et al. 1978) and the role of the negative charge on the capillary wall has recently been demonstrated (Batsford et al. 1980; Couser et al. 1980; Oite et al. 1982). Another intriguing question concerns the fate of immune deposits located in the subepithelial position. The demonstration of a specific receptor for C3 on human VEC by Gelfand et al. (1975 and 1976) and later by others (Burkholder et al. 1977; Girard et al. 1977; Kazatchkine et al. 1982; Moran et al. 1977; Petterson et al. 1978; Sobel et al. 1976) introduced the possibility that, in the trapping and sequestration of immune complexes, specific molecular interactions could be at play. The role of the C3 receptor in glomerulonephritis remains controversial. Finally, there have been various attempts to demonstrate a receptor for the Fc portion of IgG on VEC of the glomerulus, which could participate in immune complex trapping. Most of these were negative (Gelfand et al. 1979; Matre et al. 1980; Moran et al. 1977; Petterson et al. 1978); however, convincing evidence for a Fc receptor on VEC of the human glomerulus was recently published (Mizoguchi and Horiuchi 1982).

In this work, we undertook the search for IgG-Fc receptors in cultured human glomerular epithelial cells. For this, we used as probes human and sheep gamma globulins and sheep anti-horseradish peroxidase (HRP) IgG aggregated by heating, and antigen-antibody complexes prepared with sheep anti-HRP antibodies and HRP. By immunohistochemical methods it was observed that these macromolecular aggregates bind to the membrane of epithelial cells in the absence of complement, through the FC fragment. The use of anti-HRP aggregates or of HRP anti-HRP immune complexes allowed us to document, at the ultrastructural level, membrane binding followed by interiorization within membrane bound bodies. This process showed features of receptor-mediated endocytosis. An IgG-Fc receptor was also documented on crysostat sections of frozen human kidney and in whole fresh decapsulated glomeruli in suspension.

Materials and methods

Isolation and culture of glomeruli. The uninvolved portion of six kidneys removed because of renal cell carcinoma was used throughout these experiments. Under sterile conditions the cortex was excised, finely minced and washed in Hank's balanced salt solution (HBSS) for 10 min at 37° C. The tissue was digested as described by Foidart et al. (1979). The minced tissue was incubated in 0.18% trypsin (Flow Laboratories, United Kingdom) in calcium and magnesium free phosphate buffered saline, pH 7.3 (PBS). Digestion was carried out under constant stirring for 30 min at 37° C. After digestion, the mixture was decanted and the supernatant containing the digested tissue was centrifuged at 400 G for 10 min at 10° C. The resulting pellet was washed twice in HBSS. The undigested tissue was treated with trypsin for 5–7 times. Then, the pellets were pooled and successively passed through three cheese clothes, a 80 and then a 200 mesh stainless steel sieves. Glomeruli retained in the 200 mesh sieve were virtually free from other tissue contaminants and over 95% free from Bowman's capsules. Glomeruli were explanted in Falcon 25 cm^2 tissue culture flasks (Falcon Plastics Company, Oxnard, California) containing 5 ml of culture medium RPMI 1640 (Flow Laboratories, United Kingdom). Medium was supplemented with penicillin (60 U per ml), streptomycin (0.060 mg per ml), L-glutamine, and with 15% fetal calf serum heat inactivated at 56° C for 45 min (Grand Island Biological Company, Grand Island, NY, USA). The medium was buffered

Table 1. Immunoglobulin preparations used in this study

Reagent tested	Abbreviation
Aggregated human gamma globulin	Agg HGG
Aggregated human gamma globulin plus normal human serum	Agg HGG + C
Fluoresceinated aggregated human gamma globulin	FITC Agg HGG
Monomeric human gamma globulin	HGG
Aggregated normal sheep gamma globulin	Agg SGG
Aggregated anti-HRP affinity purified sheep IgG	Agg a-HRP SIgG
Monomeric anti-HRP affinity purified sheep IgG	a-HRP SIgG
Aggregated anti-HRP affinity purified sheep IgG plus fresh human serum	Agg a-HRP SIgG + C
HRP anti-HRP immune complexes prepared with affinity purified sheep IgG	HRP a-HRP SIgG
HRP anti-HRP immune complexes prepared with affinity purified sheep IgG plus fresh human serum	HRP a-HRP SIgG + C
HRP anti-HRP immune complexes prepared with F(ab′)2 fragments from sheep anti-HRP IgG	HRP a-HRP F(ab′)2
HRP anti-HRP immune complexes prepared with F(ab′)2 fragments from sheep IgG plus fresh human serum	HRP a-HRP F(ab′)2 + C
Aggregated Fc fragments from sheep IgG	Agg SIgG-Fc

with 20 mM of N-2-hydroxi-ethylpiperazine-N-2-ethanesulfonic acid (HEPES). It was changed every two days. Cell were grown to confluence and then used for incubation experiments or released with 0.05% trypsin in EDTA and subcultured.

Anti-horseradish peroxidase (HRP) antibodies and fragments. An anti-HRP antiserum was raised in a sheep as previously described (Druet et al. 1978). From this antiserum the gammaglobulin fraction was precipitated at 40% ammonium sulfate saturation. Pure anti-HRP IgG was isolated by immuno-adsorption using HRP insolubilized with BSA (Druet et al. 1978) using glutaraldehyde (Avrameas and Ternynck 1969). Fc and F (ab′)2 fragments were obtained by digestion of pure anti-HRP IgG with papain (Porter 1981) or pepsin (Nisonoff et al. 1960). F (ab′)2 fragments were separated from undigested IgG by gel filtration using a G-200 Sephadex column (Pharmacia, Upsala, Sweden) and their purity was ascertained by immunoelectrophoresis. Fc fragments were isolated by the method described by Franklin (1960).

Preparation of macromolecular aggregates. The various preparations used and their abbreviations are listed in Table 1.

Heat aggregated and non-aggregated immunoglobulins. Two per cent solutions of the following immunoglobulins were aggregated by heating: human gammaglobulin (Agg HGG) (Fraction II Cohn; United States Biochemical Corporation, Cleveland Ohio), human gammaglobulin labeled with fluorescein isothiocynate (FITC-Agg HGG), normal sheep gammaglobulin (Agg SGG), pure anti-HRP sheep IgG (Agg a-HRP SIgG), and sheep IgG Fc fragments (Agg SIgG-Fc). Aggregation was carried out at 63° C for 30 min as previously described (Mancilla-Jimenez et al. 1982). Agg HGG and Agg a-HRP SIgG were incubated at 37° C for one hour with fresh human serum as a source of complement (Agg HGG + C, Agg a-HRP SIgG + C). Four mg per ml solutions of human gammaglobulin (HGG) and of pure anti-HRP IgG (a-HRP SIgG) were ultracentrifuged at 27,000 r.p.m. for one hour to eliminate spontaneous aggregates. The upper two thirds of the supernatant were used for incubation experiments shortly after centrifugation.

Antigen-antibody and antigen-complement-complexes. HRP anti-HRP immune complexes were prepared the day before use with intact pure anti-HRP sheep IgG (HRP a-HRP SIgG), and

with its F (ab')2 fragments (HRP a-HRP F (ab')2). Precipitation curves were constructed with fixed amounts of antibodies and with increasing amounts of HRP in PBS (from 0.001 mg per ml to 2 mg per ml). Incubation was carried out at 37° C for one hour and then at 4° C for 18 h. The equivalence point was obtained with 0.300 mg of anti-HRP IgG and 0.010 mg of HRP and with 0.300 mg of F (ab')2 anti-HRP fragments of 0.015 mg of HRP. Complexes in five or ten times antibody excess were used for incubations. HRP a-HRP SIgG + C and HRP a-HRP F(ab')2 + C complexes were prepared as described for aggregated IgG. Activation of the complement system by the above immune complexes including those prepared with F(ab')2 fragments was demonstrated by incubating fresh normal rat serum with the supernatants of the precipitation curve tubes and measuring the CH50 levels (Mayer 1972).

Search for IgG receptors on cultured glomerular cells. Confluent primary cultures or subcultures with homogeneous epithelial cell populations as determined by phase contrast microscopy and electronmicroscopy were selected for incubation with the various immunoglobulin preparations. The amount of immunoglobulin in the test preparation varied from 0.150–0.250 g per ml of incubation medium. Incubation procedure was as follows: monolayers were washed in PBS (5 min × 3) and then incubated in the immunoglobulin preparation for one hour at 22° C or 37° C; this was done with Agg HGG, Agg a-HRP SIgG and with HRP anti-HRP SIgG immune complexes both with and without complement. The affinity of glomerular cells for each immunoglobulin preparation was tested several times with cells from at least 2 different cultures. For comparative studies, neighbouring portions of the same monolayer were used. Monolayers from one culture were incubated at 4° C for one hour with Agg HGG, Agg a-HRP SIgG, Agg SIgG-Fc or with HRP a-HRP SIgG. At the end of the incubation period the medium was eliminated and the monolayers were rinsed in PBS (5 min × 3).

Immunofluorescence studies. For immunofluorescence studies, incubations of monolayers were made, these were growing on glass slides placed within the culture flasks at the time of glomerular explantation or subculture. After rinsing, cell monolayers were fixed in −20° C methanol for 15 min, washed in PBS and incubated for 30 min at room temperature with fluoresceinated (FITC) antisera to either human IgG, sheep IgG, or human C3. FITC labeled antisera to human IgG and human C3 were purchased from Behringwerke (Marburg, West Germany). FITC labeled antiserum to sheep IgG was prepared as described elsewhere (Druet et al. 1978). After incubation, cells were rinsed in PBS. For semi-quantitative estimation of labeled cells, nuclear counterstaining was achieved with paraphenylene-diamine-glycerol mounting media (Oriol and Mancilla-Jimenez 1983).

Immunoperoxidase studies. Light microscopic immunoperoxidase studies were made with monolayers grown on glass slides or directly on the plastic. Monolayers challenged with anti-HRP antibodies, either heat aggregated or antigen complexed, were processed as follows: incubation medium was eliminated and cells were rinsed with 0.1 M phosphate buffer pH 7.2. Cells were fixed within the tissue culture flasks with 1.25% glutaraldehyde in the above phosphate buffer for 45 min. After fixation and overnight rinsing at 4° C, monolayers were incubated in a 0.05% HRP solution in PBS for one hour at room temperature. After rinsing in phosphate buffer for 30 min, HRP was revealed in 0.1% 3,3'-diaminobenzidine (DAB) and H_2O_2 in 0.1 M tris-(hydroxymethyl aminomethane)-HCL buffer pH 7.4. Incubation in this solution was carried out for 15 min at room temperature. Thereafter, monolayers were examined by light microscopy or further processed for electron microscopy. For this, monolayers were postfixed in osmium tetroxide, dehydrated in graded ethanol solutions and embedded in Epon. After polymerization, selected areas of the plastic bottom were punched out and mounted on blocks of hardened Epon with a thin layer of fresh Epon. After further polymerization, sections were taken parallel or perpendicular to the monolayer surface. Semithin sections were examined and selected areas were thin sectioned. Grids with and without counterstaining with uranyl acetate and lead citrate were examined comparatively with an EM 10 Zeiss electron microscope operating at 50 KV.

Blocking specificity studies were conducted incubating cells with Agg SIgG-Fc or Agg HGG and, after rinsing, with macromolecular aggregates prepared with anti-HRP antibodies (Agg a-HRP SIgG, HRP anti-HRP SIgG). Thereafter, monolayers were incubated with 0.05%

HRP and DAB-H_2O_2. Binding under these conditions was compared with that seen without blocking.

Search for IgG Fc receptors on cryostat sections. Portions of 4 of the kidneys used for glomerular cultures and 3 percutaneous renal biopsies were used. Tissues were rapidly frozen in liquid nitrogen and 4 μm thick cryostat sections were taken and processed. Direct immunofluorescence with antisera to human IgG, IgM, IgA and C3 (Behringwerke – Marburg – RFA) showed no glomerular deposits. Light microscopy examination revealed no significant pathologic changes in the glomeruli. After a 10 min washing in PBS, sections were incubated with Agg HGG, FITC-Agg HGG, Agg a-HRP SIgG, Agg SIgG-Fc, HRP a-HRP SIgG, HRP a-HRP SIgG + C, HRP a-HRP F (ab′)2, HRP a-HRP F(ab′)2 + C, HGG and with a-HRP SIgG. After several trials, it was found that overnight incubation at 4° C gave the best results. Incubation at room temperature or at 37° C even for short periods resulted in heavy non-specific binding which made impossible to evaluate glomerular binding. Short incubations at 4° C gave varying results. After incubation and rinsing, the slides were fixed in −20° C methanol for 15 min and washed in PBS. Then, the FITC-labeled antisera to human or sheep IgG or to human C3 were applied to the slides for a 30 min incubation at 4° C. After rinsing, the slides were mounted with either glycerol or paraphenylenediamine-glycerol which helped to identify the labeled cells (Oriol and Mancilla-Jimenez 1983).

Search for IgG Fc receptors in suspensions of whole glomeruli. Glomeruli were isolated from two non used kidneys provided by the French Transplant Program. Kidneys had been kept in Collin's solution for 24 h before isolation of glomeruli. The isolated cortex was finely minced and buttered successively through a 80 then a 115 mesh sieve. Glomeruli retained on the second sieve were washed twice in HBSS and aliquots of 5000 glomeruli per ml were incubated in Agg HGG and Agg a-HRP-IgG. The protein concentration of the incubation media was 0.50 mg per ml. Incubation was carried out at room temperature for 3 h under gentle stirring. After rinsing in HBSS, glomeruli were incubated with the corresponding fluoresceinated antibodies. Aggregated anti-HRP IgG was revealed by incubation in a HRP solution and then DAP-H_2O_2 as done with glomerular cells in vitro. Thereafter glomeruli were layered on glass slides, mounted and examined under ultraviolet light or by conventional light microscopy. Aliquots were processed for electronmicroscopy following basically the same steps as described before.

Control studies. Various studies were performed to test the specificity of binding. To rule out non specific binding of the various FITC-labeled antisera, these were applied to cell monolayers, cryostat sections or glomeruli in suspension without prior incubation with the immunoglobulin test preparation. Endogenous HRP activity was investigated by treating cells incubated in macromolecular aggregates with and without anti-HRP activity in DAB-H_2O_2. In these controls, incubation with HRP before DAB-H_2O_2 was omitted. Since HRP could bind non-specifically to fixed cells and/or to aggregated IgG bound to cells, monolayers incubated in macromolecular aggregates without anti-HRP activity (Agg SGG and Agg HGG) were incubated in a HRP solution before treatment with DAB-H_2O_2. To test whether HRP alone could bind to living cells, monolayers were incubated with a HRP solution in PBS containing 0.250 mg of enzyme per ml.

Results

Patterns of cell outgrowth from explanted glomeruli

Serial examination by phase contrast microscopy was done. Twenty four hours after explantation, cells started to grow around the attached glomeruli. Most cells were elongated spindle-shaped and measured approximately 100 μm in diameter. Other cells had a balloon-like appearance and were larger; these cells were attached to the outer part of the glomeruli by finger-

Table 2. Demonstration of Fc-dependent binding on cultured glomerular epithelial cells

Incubation medium[a]	Method to reveal bound reagent	Membrane binding
Agg HGG	FITC anti-human IgG	+
	FITC anti-human C3	−
	HRP/DAB/H_2O_2	−
Agg HGG+C	FITC anti-human IgG	+
	FITC anti-human C3	+
FITC Agg HGG	none	+
HGG	FITC anti-human IgG	−
Agg SGG	FITC anti-sheep IgG	+
	HRP/DAB/H_2O_2	−
Agg a-HRP SIgG	FITC anti-sheep IgG	+
	FITC anti-human C3	−
	HRP/DAB/H_2O_2	+
	DAB/H_2O_2	−
Agg a-HRP SIgG+C	FITC anti-sheep IgG	+
	FITC anti-human C3	+
	HRP/DAB/H_2O_2	+
Agg SIgG-Fc	FITC anti-sheep IgG	+
a-HRP SIgG	FITC anti-sheep IgG	−
HRP a-HRP SIgG	FITC anti-sheep IgG	+
	FITC anti-human C3	−
	HRP/DAB/H_2O_2	+
HRP a-HRP SIgG+C	FITC anti-sheep IgG	+
	FITC anti-human C3	+
	HRP/DAB/H_2O_2	+
HRP a-HRP F(ab′)$_2$	FITC anti-sheep IgG	−
	HRP/DAB/H_2O_2	−
HRP a-HRP F(ab′)$_2$+C	FITC anti-sheep IgG	−
	FITC anti-human C3	−
	HRP/DAB/H_2O_2	−
HRP	DAB/H_2O	−
Medium alone	DAB/H_2O	−
	FITC anti-human IgG	−
	FITC anti-human C3	−
	FITC anti-sheep IgG	−

[a] Incubations were made at 22 or 37° C for 1 h

FITC: Fluorescein isothyocyanate

like cytoplasmic prolongations. In the following days, cells gradually increased in number, reaching confluence within 7–10 days after explantation. At this time most cells were smaller and round or cuboidal in shape and were focally arranged in a whirled pattern. They showed extensive cell to cell contacts and by standard electronmicroscopy the great majority of them

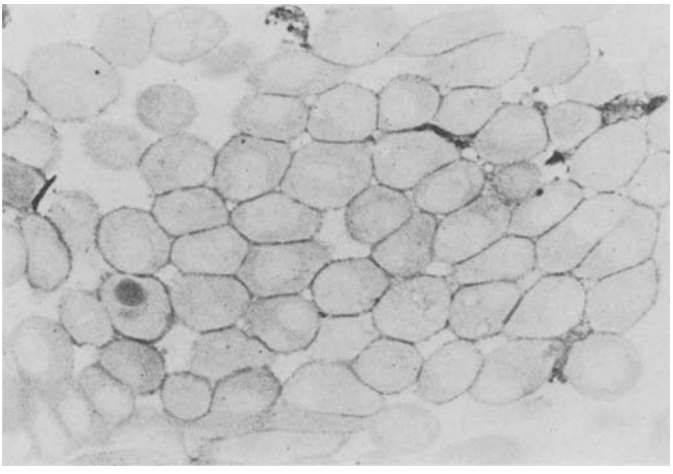

Fig. 1. Semithin section of cultured glomerular epithelial cells that had been incubated for one hour at 37° C with Agg a-HRP SIgG. Monolayers were fixed with glutaraldehyde prior to incubation with HRP and DAB/H_2O_2. *Uninterrupted labeling* of the plasma membrane of most cells is evident. × 800

displayed features of epithelial differentiation such as junctional complexes including desmosomes, hemidesmosomes and particularly tight junctions. Complex cytoplasmic interdigitations between neighbouring cells, and microvilli at the free surface were common. Subcultured cells had the same features and reached confluence from 7–10 days after trypsinization. At this time, no cells with features suggestive of mesangial origin or macrophages were seen.

Detection of IgG binding on cultured glomerular cells (Table 2)

Immunofluorescence and light microscopy immunoperoxidase studies gave similar results. In monolayers incubated with Agg HGG, FITC Agg HGG or SGG and Agg a-HRP SIgG for one hour at 22° C or at 37° C, IgG bound to the plasma membrane of epithelial cells was found consistently. Similar results were obtained whether the bound IgG was revealed using fluoresceinated antisera or, as in the case of monolayers incubated with complexed anti-HRP IgG, by incubation in HRP and then in DAB and H_2O. Aggregated Fc fragments were also found to bind to epithelial cells as revealed by immunofluorescence. Generally, binding displayed a fine granular uninterrupted pattern (Fig. 1). Coarse granules were seen in some monolayers incubated with Agg HGG. Cells incubated at 4° C displayed also membrane binding but this was less intense and finer. Incubation with HRP anti-HRP complexes gave results similar to those obtained with heat aggregated immunoglobulins provided that they were prepared with intact IgG molecules (Fig. 2). When cells were incubated with aggregates or immune complexes which had been exposed to complement, positive results

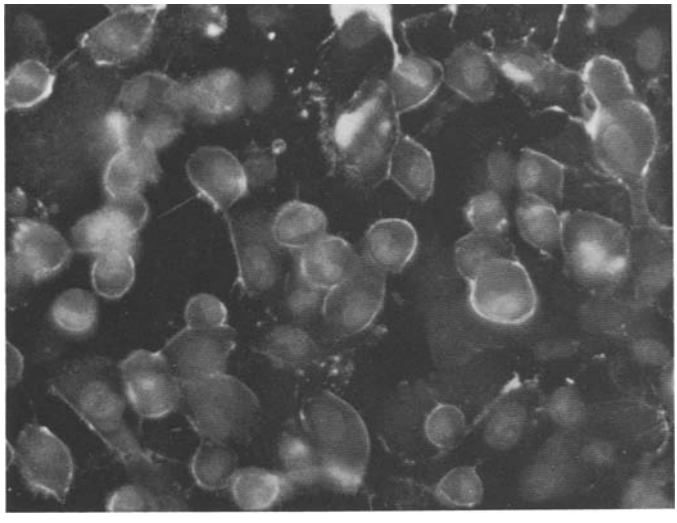

Fig. 2. Immunofluorescence micrograph of epithelial cultured cells incubated with HRP a-HRP SIgG complexes for 1 h at 37° C and treated with FITC labeled anti-sheep IgG antiserum. In most cells fine fluorescent granules outline the plasma membrane. Cell nuclei show paraphenylenediamine induced fluorescence. × 800

were obtained by immunofluorescence with both the anti-IgG and the anti-C3 conjugates. There was no fixation of the anti-C3 antiserum to cells when aggregates were not exposed to complement before incubation while fixation of the anti-IgG conjugate was unchanged. Complexes prepared with F(ab′)2 fragments with or without complement did not bind to cells (Fig. 3). This was comfirmed several times with cells from 3 different cultures, although cells from neighbouring portions of the same monolayer fixed HRP a-HRP SIgG complexes. Binding was not observed on cells incubated with non heated, ultracentrifuged HGG and SIgG.

The number of reactive cells varied from approximately one third to virtually 100%. The extent of IgG binding varied according with the morphology of the VEC tested. Binding was always present in small round or cuboidal cells at confluence, the intensity of binding being greater in cells arranged in whirls. Large spindle-shaped, romboidal or balloon-like cells were usually negative or presented non-specific paranuclear or diffuse fixation. Examined by immunoelectronmicroscopy, these cells had reaction product freely distributed throughout their cytoplasm. Since these cells exhibited shrunken nuclei and other degenerative features, this phenomenon could be ascribed to free penetration of reagents in to dead or damaged cells. Non-specific fixation of labeled antibodies on cultured glomerular cells has been noticed before by others (Scheinmann and Fish 1978).

In monolayers incubated at 37° C for twelve hours, the number of cells with IgG membrane binding was less than at one hour and clustering of granules at the cell surface was common. Most cells exhibited patchy binding often restricted to the areas of cell to cell contact. In many of these

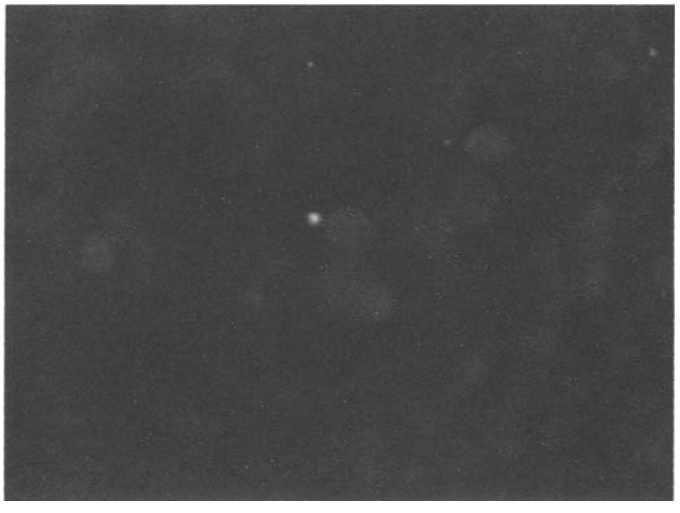

Fig. 3. Micrograph of a monolayer of epithelial cultured cells incubated with HRP a-HRP F(ab′)2 complexes at 37° C for one hour and then incubated with fluoresceinated anti-sheep IgG antisera. No specific staining is observed. × 800

Table 3. Blocking specificity studies on cultured glomerular cells

1st incubation	2nd incubation[a]	Membrane binding
Agg a-HRP SIgG	none	+
HRP a-HRP SIgG	none	+
Agg HGG	Agg a-HRP SIgG	−
Agg HGG	HRP a-HRP SIgG	−
Agg SIgG-Fc	Agg a-HRP SIgG	±
Agg SIgG-Fc	HRP a-HRP SIgG	±

[a] After the second incubation monolayers were incubated in HRP and then in DAB-H_2O_2

cells, distinct positive granules or vacuoles within the cytoplasma were common. This was better seen by immunoperoxidase. Blocking specificity studies (Table 3) showed that prior incubation of cultured cells in either Agg HGG or Agg SIgG-Fc prevented the binding of subsequently administered complexes. The extent of blocking was variable: in some observations it was virtually complete and in other some cells still displayed membrane binding.

Immunoelectronmicroscopic observations were made in duplicate and triplicate in cells incubated for one and 12 h at 37° C with Agg a-HRP SIgG, HRP a-HRP SIgG, Agg a-HRP SIgG+C and with HRP a-HRP SIgG+C. The surface and intracellular distribution of IgG was the same whether heat aggregated immunoglobulins or antigen-antibody complexes, with or without complement, were used. At 1 h, 30–90% of epithelial cells showed reaction product uniformly distributed as fine granules on the entire plasma membrane facing the culture medium (Fig. 4). Labeling of the inter-

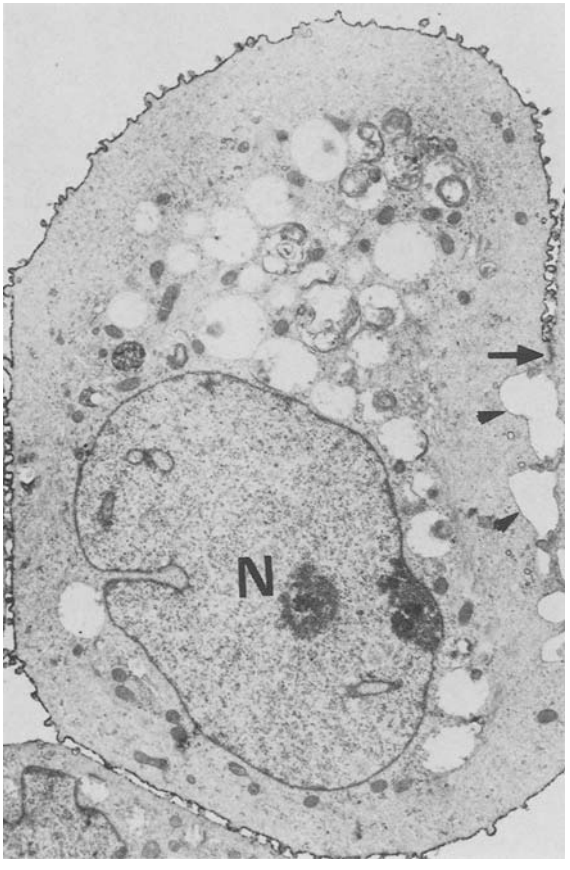

Fig. 4. Electronmicrograph of a cultured glomerular epithelial cell incubated with HRP a-HRP SIgG complexes, for 1 h at 37° C (and then processed as described in "Materials and methods"). Most of the plasma membrane is labeled by reaction product displaying a fine granular, continuous pattern. The intercellular membrane (*arrowheads*) and a tight junction (*arrow*) are not labeled. Numerous electronlucent non labeled vacuoles are seen. *N*: nucleus. × 9,000

cellular membranes was less common and never seen at the junctional complexes (Fig. 4). In many cells, unlabelled coated vesicles measuring about 100 nm in diameter were common underneath the labeled cell surface (Fig. 5a). In some cells, there was intense labelling of small endocytic invagination covered with bristles (coated pits) (Fig. 5b). In other cells reaction product was heavily concentrated within coated vesicles whereas the overlying plasma membrane was almost free of the label (Fig. 5c). In some cells empty coated vesicles alternated with others partially or completely filled with reaction product (Fig. 5d). After incubation for one hour, reaction product was rarely seen in non coated vesicles or lysosomes. In monolayers incubated for twelve hours, a quite different labeling pattern was observed. In most cells the plasma membrane was entirely negative or the binding was patchy and labeled coated pits and vesicles were rare. In most cells the reaction product was confined within uncoated vesicles which were surrounded by a continuous single, uniform membrane. These vesicles measured from 200–500 nm, had no electron dense matrix and the aggregates were characteristically accumulated in a peripheral segment leaving the rest

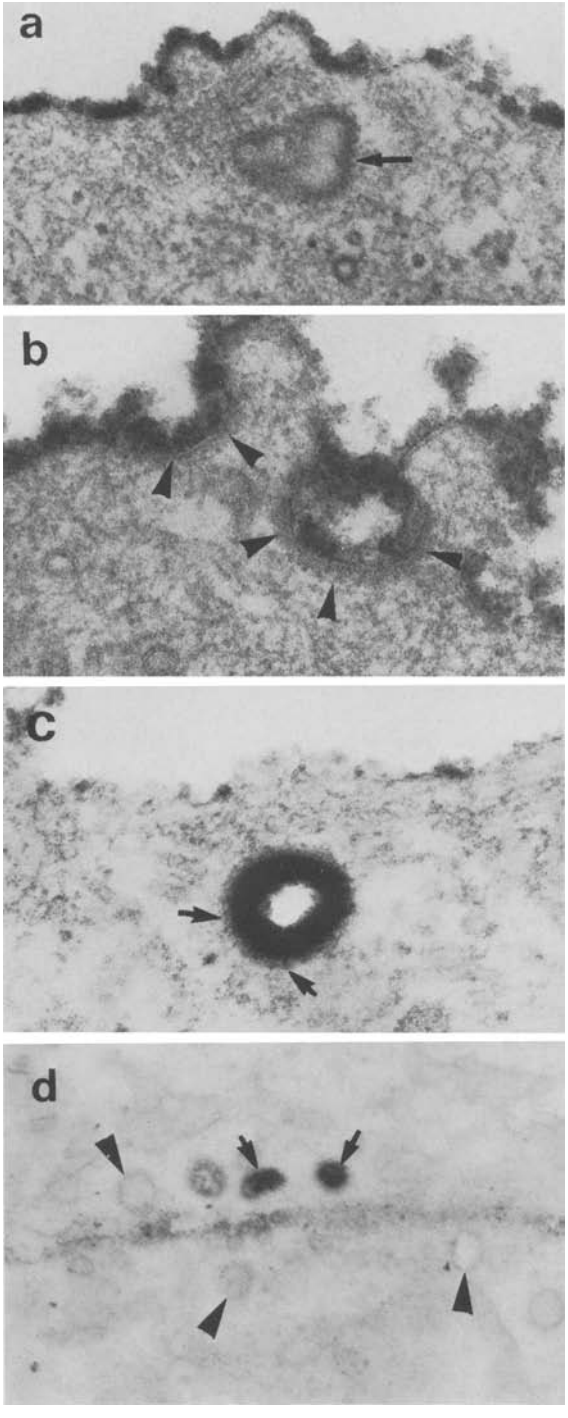

Fig. 5a–d. Composite figure to illustrate the internalization of macromolecular aggregates within coated pits. These electron micrographs were taken from different cultured glomerular epithelial cells that had been incubated at 37° C for 1 h with Agg a-HRP SIgG.
a The cell surface is heavily labeled; a coated vesicle (*arrow*) is free of reaction product.
b Reaction product is abundant within coated pits; the cytoplasmic bristle coat is evident (*arrowheads*).
c Heavily labeled apparently intracytoplasmic vesicle surrounded by a continuous bristle coat (*arrow*). The overlying cell membrane is virtually unlabelled.
d Several small coated vesicles underneath the plasma membranes of two neighbouring cells. Two vesicles are heavily labeled (*arrows*) and the others are negative (*arrowheads*). **a**, **b**, **c** $\times 150{,}000$, **d** $\times 60{,}000$

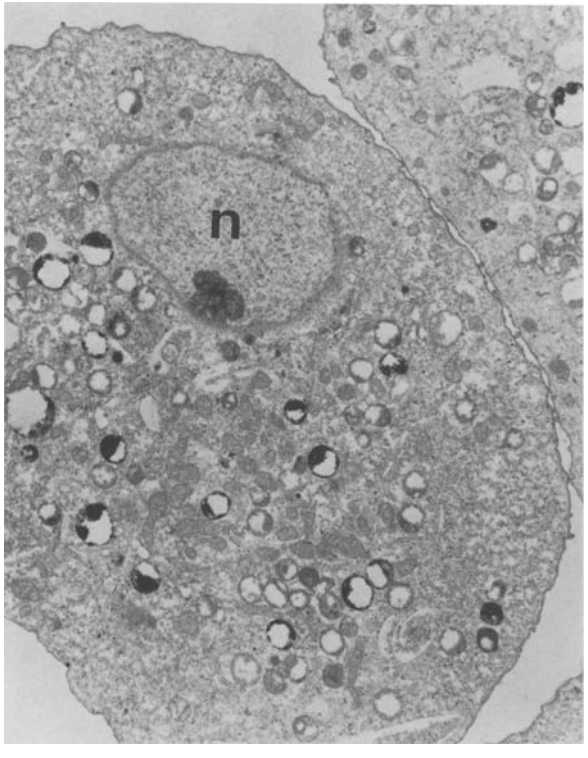

Fig. 6. Glomerular epithelial cell incubated for 12 h at 37° C with Agg a-HRP SIgG, and then incubated successively with HRP and DAB-H_2O. Numerous uncoated vesicles containing reaction product are seen throughout the cytoplasm. Reaction product fills the vesicle partially. The rest is electron lucent. The plasma membrane is free of label and no labeled coated pits or vesicles are seen. *N*: nucleus. ×6,200

of the cavity empty (Fig. 6). In some cells, large vesicles filled with reaction product were observed (Fig. 7). Labeled lysosomes were occasionally observed.

IgG binding to glomerular cells on tissue sections and on whole glomeruli (Table 4)

All heat aggregated immunoglobulin preparations including the Fc portion of sheep IgG adhered to glomeruli. Antigen-antibody complexes prepared with intact antibodies were also bound. That IgG binding to glomeruli is Fc dependent is supported by the lack of fixation of HRP a-HRP F(ab′)2 complexes in the absence of complement. Different results were obtained when HRP a-HRP F(ab′)2 complexes were exposed to fresh serum. Under these conditions, both IgG and C3 were demonstrated by immunofluorescence displaying identical binding patterns. Nuclear counter-staining with paraphenylenediamine aided in identifying the binding structure as the VEC. In some cases labelling of cells lining the Bowman's capsule was also seen. Binding to tubular cells and to interstitium was seen in some sections but it was weak and did not interfere with the identification of glomerular binding. Glomerular cells of tissues stored for 3 weeks or longer at −20° C lost their IgG binding capacity.

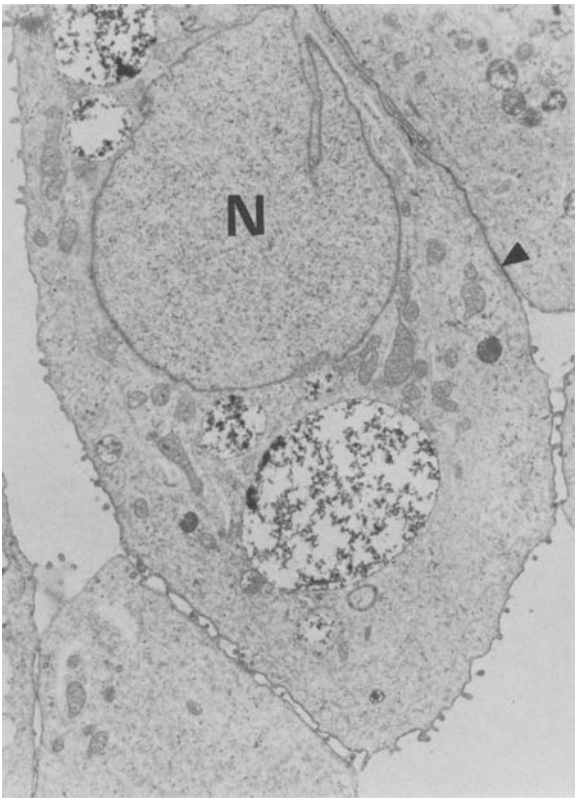

Fig. 7. Electronmicrograph of monolayer incubated with HRP a-HRP SIgG complexes. A few large vesicles contain abundant granular labeled material. The plasma membrane is negative. A junctional complex between two cells is seen (*arrow*). *N*: nucleus. ×6,600

Incubations of fresh, decapsulated, whole glomeruli, allowed to characterize further the VEC as the IgG binding cell. In glomeruli incubated with Agg HGG and with Agg a-HRP SIgG, FITC-labeled anti-IgG antisera revealed intense global fluorescence of glomeruli scattered throughout the tuft and particularly at the periphery there were intensely fluorescent cells. Immuno-electronmicroscopic studies of glomeruli incubated with Agg a-HRP SIgG were carried out. In semithin sections, membrane labelling of VEC was conspicuous particularly on those cells located peripherally; most deeply located VEC were negative, indicating poor reagent penetration. Electronmicroscopic examination confirmed localization of reaction product at the VEC membrane. Mesangial and endothelial cells were negative.

Control Studies (Tables 2 and 4)

Incubation of monolayers, kidney sections and whole glomeruli with fluoresceinated antisera without prior incubation with macromolecular aggregates gave consistently negative results. On cultured cells incubated with HRP alone, with Agg a-HRP SIgG or with medium alone and then with DAB-H_2O, endogenous HRP activity was seen in a few cells as scanty intracyto-

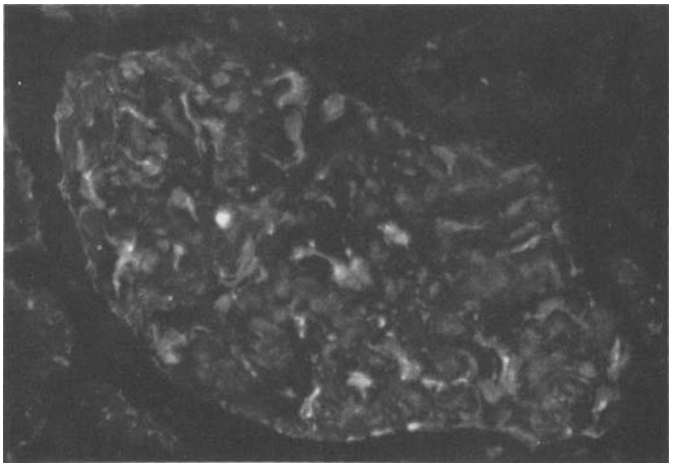

Fig. 8. Cryostat section of human kidney incubated with heat aggregated sheep IgG Fc fragments and then treated with FITC labelled anti-sheep IgG antiserum. Labelling of visceral epithelial cells is observed. × 250

Table 4. Demonstration of IgG and C3-dependent binding to glomerular cells on cryostat sections

Incubation	Glomerular cell binding by I.F.	
	Anti-IgG	Anti-C3
Medium alone	–	–
HGG	–	ND
a-HRP SIgG	–	ND
Agg HGG	+	–
FITC Agg HGG	+	ND
Agg a-HRP SIgG	+	–
Agg SIgG-Fc	+	ND
HRP a-HRP SIgG	+	–
HRP a-HRP SIgG + C	+	+
HRP a-HRP $F(ab')_2$	–	–
HRP a-HRP $F(ab')_2$ + C	+	+

ND: not done; I.F.: immunofluorescence

plasmic granules. There was no membrane labelling seen. Only endogenous HRP activity was seen on cells incubated with aggregated IgG without anti-HRP activity (Agg HGG and Agg SGG) and then treated successively with HRP and DAB-H_2O. No membrane labelling was seen by immunofluorescence on cells or cryostat sections incubated with monomeric HGG or anti-HRP SIgG.

Discussion

In this study we have documented membrane affinity of cultured glomerular cells for complexed IgG. The binding of heat aggregated immunoglobulins

and antigen-antibody complexes appears to be dependent upon the presence of the Fc portion of the IgG molecule and occurs in the absence of complement. Isolated heated Fc fragments were capable of membrane binding and of blocking subsequently administered aggregates. Furthermore, antigen-antibody complexes made with F(ab')2 fragments, did not bind to cells. For binding to occur, it was also necessary for IgG to be in an aggregated form. There was no binding of non complexed immunoglobulins. Thus clustering of Fc sites is required for membrane binding to take place. The binding of IgG containing macromolecular aggregates to cultured cells was not related to the C3b surface receptor, since binding occurred in the absence of complement. Moreover, the fact that both the C3b and the IgG-Fc receptor can be demonstrated in vivo while on cultured glomerular cells only the IgG-Fc receptor is present, indicates that the ability of VEC to bind macromolecular aggregates through either C3b or Fc fragments resides in different molecules. That the C3b receptor is lost during the culturing process is supported by several data: 1) immunofluorescence studies with anti-C3b antibodies made simultaneously with our studies (J. Bariety, unpublished observations), were negative. 2) Other groups using different techniques have obtained similar negative results (Scheinmann et al. 1978; Striker et al. 1976). 3) In the present study, HRP a-HRP F(ab')2+C complexes which were shown to activate complement did not bind to cultured cells whereas they were bound to VEC on cryostat sections.

Therefore the above immunohistochemical observations document an IgG-Fc receptor on the membrane of human cultured glomerular epithelial cells. That this is not a property acquired during in vitro growing is supported by the demonstration of a similar affinity on cryostat sections of frozen renal tissue and on whole fresh glomeruli in suspension.

As far as the identity of the IgG binding cell, several lines of evidence suggest it is the VEC. At the time of the challenge, monolayers were composed almost exclusively of cuboidal or round cells displaying extensive and intimate intercellular contacts (see Fig. 1). This is the pattern in which epithelia are organized both in vivo and in vitro. This impression was confirmed by immunoelectronmicroscopy as the cells with IgG bound to their membranes displayed an array of junctional complexes including desmosomes. It is widely accepted that junctional complexes of desmosomal type are markers of epithelial cells (Dubois et al. 1981; Foidart et al. 1979; Staehelin 1974; Striker et al. 1980). Moreover, counterstaining of nuclei with paraphenylenediamine was very useful in locating the reactive cell in the outer aspect of the glomerular basement membrane (Oriol and Mancilla-Jimenez 1983) in a pattern comparable to that revealed by immunofluorescence with the anti-GP205 antibody that recognizes the C3b receptor on human VEC (Kazatchkine et al. 1982). Finally, in whole glomeruli IgG binding was seen on the membrane of VEC and not on other cells.

In the past, several studies have failed to demonstrate glomerular IgG-Fc receptors on both cryostat sections of renal tissue (Gelfand et al. 1979; Matre et al. 1980; Moran et al. 1977; Petterson et al. 1978) and cultured cells (Burkholder et al. 1977) using Ig coated SRBC. The different methodo-

logy employed could explain the difference between these and our studies. With SRBC as indicator particles, only a limited number of antibody molecules can be used to coat cells without producing agglutination and it may be insufficient for the detection of Fc receptors on glomerular cells. On tissue sections, only a rim of cell membrane is exposed with few receptors available for binding. It could be also that IgG Fc receptors on glomerular cells are labile as suggested by the fact that tissues loose their IgG binding properties upon storage at −20° C for a few weeks. The fact that IgG binding on cryostat sections is better accomplished by prolonged incubation at 4° C could indicate that VEC carry a low affinity Fc receptor. The failure to demonstrate Fc receptors in vitro, in other studies could also derive from the different methodology employed to isolate and to grow cells. It is known that the capacity of cells to express surface receptors can be influenced by the method of culture (Killen and Striker 1979; Scheinmann et al. 1978).

In any event, it seems that macromolecular aggregates are a good alternative to demonstrate IgG-Fc receptors on human glomerular cells. The apparent superiority of this method lies perhaps in the fact that clusters of Fc sites are readily available for binding. These sites would be stabilized by a multimeric interaction, thus making possible its immunohistochemical demonstration. Our current observations are supported by data published during the course of this study by Mizogouchi and Horiuchi (1982). These authors found convincing evidence for the presence of an IgG-Fc receptor in VEC of human glomerulus by incubating cryostat sections of renal tissue with IgG coated polystyrene latex particles. From scanning electronmicroscopy they concluded that the latex particles were attached to VEC.

The use of complexed antiperoxidase antibodies allows both the localization of the binding structure and the characterization of the associated subcellular events. Indeed, our ultrastructural observations indicate that IgG-Fc receptors on cultured glomerular epithelial cells promote both membrane binding and interiorization of the complexes. Complement does not participate in any of these phenomena. Our sequential observations revealed a drastic shift in the location of IgG. At one hour, most of it was seen to be bound to the plasma membrane, whereas after twelve hours, virtually all of the IgG had been transported to the interior of the cell. Current information on the mechanisms cells use to interiorize materials suggests the existence of two major pathways. Materials for which there are no specific membrane receptors enter cells by non specific endocytosis in organelles named pinosomes; these are formed from plasma ruffle movements (Silverstein et al. 1977). These organelles are characteristically surrounded by actin microfilaments and have a regular smooth uninterrupted membrane and their lumina are clear. Shortly after their formation, pinosomes fuse with lysosomes, and their contents begin to be degraded. Recently it has been realized that the interiorization of substances bound to specific receptors follows a different pathway, called adsorptive or receptor mediated endocytosis (Goldstein et al. 1979; Kaplan 1981; Pastan et al. 1981; Silver-

stein et al. 1977). It has been observed that after binding of ligand to some but not all (Schlessinger et al. 1978) surface receptors there is a random lateral movement of ligant-receptor complexes within the plasma membrane. This results in their concentration into specialized regions of the membrane called coated pits presumed to contain clathrin (Pearse 1975). After clustering, ligand receptor complexes are interiorized within vesicles known as receptosomes which may transport the ligand to lysosomes or the Golgi region.

Our structural observations on cultured glomerular epithelial cells incubated with anti-HRP IgG complexed by heating or by antigen, strongly suggest that these cells are capable of receptor-mediated endocytosis. Indeed after a one hour incubation many cells, beside having IgG bound to their plasma membrane, had concentrated the ligand within coated pits or coated vesicles. After a twelve hour incubation, cell plasma membranes were virtually free of IgG which was contained within intracytoplasmic uncoated vesicles, probably receptosomes, and within lysosomes. Thus IgG-Fc receptors on human VEC should be added to the growing list of surface receptors capable of promoting the interiorization of substances through highly specialized specific pathways (Goldstein et al. 1979; Kaplan 1981; Pastan and Willingham 1981). Other examples are receptors binding low density lipoprotein (Anderson et al. 1977) α_2 macroglobulin (Willingham et al. 1979) and epidermal growth factor (Gordon et al. 1978) which are capable of mediating the endocytosis of these ligands within coated pits. More recently receptor-mediated endocytosis has been shown to occur in cultured human lymphoblastoid cells after the linking of surface IgM antibodies (Salisbury et al. 1980) and on polymorphonuclear leukocytes labeled with F(ab')2 anti C3b receptors (Abrahamson and Fearon 1983). Although in these studies physiologic ligands were not used, they indicate that immunoglobulins can also be interiorized via coated pits.

Although it has been suggested from electronmicroscopic observations that VEC are capable of eliminating immune complexes in human post-streptococcal glomerulonephritis (Tornroth 1976), there has not been any direct demonstration of this phenomenon. There are however, studies in animal models, showing that VEC can interiorize dextran (James and Ashworth 1961), protamin-heparin aggregates (Schwartz et al. 1981) and perhaps immune complexes (Rantala 1981). These observations added to the current study demonstrating endocytosis of immune complexes via an IgG-Fc receptor on cultured human glomerular cells, suggest very strongly that one of the functions of VEC could be the elimination of IgG containing macromolecular aggregates from the outer aspect of the glomerular capillary wall. But it is clear that the role of the IgG-Fc receptors in health and disease awaits further investigation.

References

Abrahamson DR, Fearon DT (1983) Endocytosis of the C3b receptor of complement coated pits in human polymorphonuclear leukocytes and monocytes. Lab Invest 48:162–168

Anderson RGW, Brown MS, Goldstein JL (1977) Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts. Cell 10:351–364

Avrameas S, Ternynck T (1969) The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. Immunochemistry 6:53–66

Batsford SR, Takamiya H, Vogt A (1980) A model of in situ immune complex glomerulonephritis in the rat employing cationized ferritin. Clin Nephrol 14:211–216

Burkholder PM, Oberley TD, Barber TA, Beacom A, Koehler C (1977) Immune adherence in renal glomeruli. Complement receptor sites in glomerular capillary wall. Am J Pathol 86:635–654

Couser WG, Salant DJ (1980) In situ immune complex formation and glomerular injury. Kidney Int 17:1–13

Couser WG, Steinmuller DR, Stilmant MM, Salant DJ, Lowenstein LM (1978) Experimental glomerulonephritis in the isolated perfused rat kidney. J Clin Invest 62:1275–1287

Dixon FJ, Feldman JD, Vazquez JJ (1961) Experimental glomerulonephritis: the pathogenesis of a laboratory model ressembling the spectrum of human glomerulonephritis. J Exp Med 113:899–920

Druet P, Bariety J, Laliberte F, Bellon B, Belair MF, Paing M (1978) Distribution of heterologous antiperoxidase antibodies and their fragments in the superficial renal cortex of normal Wistar-Munich rat: an ultra-structural study. Lab Invest 39:623–631

Dubois CH, Foidart JB, Hautier MB, Dechenne CA, Lemaire MJ, Mahieu P (1981) Proliferative glomerulonephritis in rats: evidence that mononuclear phagocytes infiltrating glomeruli stimulate the proliferation of endothelial and mesangial cells. Eur J Clin Invest 11:91–104

Fleuren GJ, Grond J, Hoedemaeker PJ (1980) In situ formation of subepithelial glomerular immune complexes in passive serum sickness. Kidney Int 17:631–637

Fleuren GJ, Van der Lee RJ, Greben HA, Van Damme BJC, Hoedemaeker PJ (1978) Experimental glomerulonephritis in the rat induced by antibodies directed against tubular antigens: IV. Investigations into the pathogenesis of the model. Lab Invest 38:496–501

Foidart JB, Dechenne CA, Mahieu P (1979) Tissue culture of normal rat glomeruli. Isolation and characterization of two different cell populations. Invest Cell Pathol 2:15–26

Franklin EC (1960) Structural units of human 7S gamma globulin. J Clin Invest 39:1933–1941

Gelfand MC, Franck MM, Green I (1975) A receptor for the third component of complement in the human renal glomerulus. J Exp Med 142:1029–1034

Gelfand MC, Franck, MM, Green I, Shin ML (1979) Binding sites for immune complexes containing IgG in the renal interstitium. Clin Immunol Immunopathol 13:19–29

Gelfand MC, Shin ML, Nagle RB, Green I, Franck MM (1976) The glomerular complement receptor in immunologically mediated renal glomerular injury. N Engl J Med 295:10–14

Germuth FG, Senterfit LB, Dreesman GR (1972) Immune complex disease: V. The nature of the circulating immune complexes associated with glomerular alteration in the chronic BSA-rabbit system. Johns Hopkins Med J 130:344–357

Girard JF, Ayed K, Druet P (1977) Complement receptors in human renal glomeruli: Further evidence by immunofluorescence. J Immunol Methods 17:1–6

Goldstein JL, Anderson RGW, Brown MS (1979) Coated pits, coated vesicles and receptor mediated endocytosis. Nature 279:679–685

Gordon PO, Carpentier JL, Cohen S, Orci L (1978) Epidermal growth factor: morphologic demonstration of binding, internalisation and lysosomal association in human fibroblasts. Proc Natl Acad Sci USA 75:5025–5029

Haakenstad AO, Striker GE, Mannick M (1976) The glomerular deposition of soluble immune complexes prepared with reduced and alkylated antibodies and with intact antibodies in mice. Lab Invest 35:293–301

James A, Ashworth CT (1961) Some features of glomerular filtration and permeability revealed by electron microscopy after intraperitoneal injection of dextran in rats. Am J Pathol 38:515–520

Kaplan J (1981) Polypeptide-binding membrane receptors analysis and classification. Science 212:15–20

Kazatchkine M, Fearon DT, Appay MD, Mandet Ch, Bariety J (1982) Immunochemical study of the human glomerular C3b receptor in normal kidney and in seventy-five cases of renal disease. J Clin Invest 69:900–912

Killen PD, Striker GE (1979) Human glomerular visceral epithelial cells synthesize a basal lamina collagen in vitro. Proc Natl Acad Sci USA 76:3518–3522
Mancilla-Jimenez R, Bellon B, Kuhn J, Belair MF, Rouchon M, Druet P, Bariety J (1982) Phagocytosis of heat-aggregated immunoglobulins by mesangial cells. An immunoperoxidase and acid phosphatase study. Lab Invest 46:243–253
Matre R, Tonder O, Wesenberg F (1980) Human renal glomeruli possess no Fc gamma receptors. Clin Immunol Immunopathol 17:157–162
Mayer MM (1972) Complement and complement fixation. In: Experimental Immunochemistry, edited by Kabat EA and Mayer MM, p 133, Thomas Springfield
Mizoguchi Y, Horiuchi Y (1982) Localization of IgG-Fc receptors in human glomeruli. Clin Immunol Immunopathol 24:320–329
Moran J, Colasanti G, Amos N, Peters DK (1977) C3b receptors in glomerular disease. Clin Exp Immunol 28:212–217
Nisonoff A, Wissler FC, Lipman LN, Woernley DL (1960) Separation of univalent fragments from the bivalent rabbit antibody molecule by reduction of disulfide bonds. Arch Biochem Biophys 89:230–244
Oberley TD, Mosher DF, Mills MD (1979) Localization of fibronectin within the renal glomerulus and its production by cultured glomerular cells. Am J Pathol 96:651–662
Oite T, Batsford SR, Mihatsch MJ, Takamiya H, Vogt A (1982) Quantitative studies of in situ immune complex glomerulonephritis in the rat induced by planted cationized antigen. J Exp Med 155:460–474
Okumura K, Kondo Y, Tada T (1971) Studies on passive serum sickness: The glomerular fine structure of serum sickness nephritis induced by preformed antigen-antibody complexes in the mouse. Lab Invest 24:383–391
Oriol R, Mancilla-Jimenez R (1983) Fluorescent staining of nuclei and amyloid substance. Two useful properties of p-Phenylenediamine. J Immunol Meth 62:185–192
Pastan IH, Willingham MC (1981) Journey to the center of the cell. Science 214:504–509
Pearse BMF (1975) Coated vesicles from pig brain: purification and biochemical characterization. J Mol Biol 97:93–98
Petterson EE, Bhan AK, Schneeberger EE, Collins AB, Colvin RB, McCluskey RT (1978) Glomerular C3 receptors in human renal disease. Kidney Int. 13:245–252
Porter RR (1959) The hydrolysis of rabbit gamma globulin antibodies with crystalline papain. Biochem J 73:119–126
Rantala I (1981) Glomerular epithelial cell endocytosis of immune deposits in the nephrotic rat. An ultrastructural immunoperoxidase study. Nephron 29:239–244
Salisbury JL, Condeelis JS, Satir P (1980) Role of coated vesicles, microfilaments and calmodulin in receptor-mediated endocytosis by cultured B lymphoblastoid cells. J Cell Biol 87:132–141
Scheinmann JI, Fish AJ (1978) Human glomerular cells in culture. Three subcultured cell types bearing glomerular antigens. Am J Pathol 92:125–146
Scheinmann JI, Fish AJ, Kim Y, Michael AF (1978) C3b receptors on human glomeruli in vitro. Loss in culture. Am J Pathol 92:147–154
Schlessinger J, Shechter Y, Cuatrecasas P, Willingham MC, Pastan I (1978) Quantitative determination of the lateral diffusion coefficients of the hormone-receptor complexes of insulin and epidermal growth factor in the plasma membrane of cultured fibroblasts. Proc Natl Acad Sci USA 75:5353–5357
Schwartz MM, Sharon Z, Bidani AK, Pauli BU, Lewis EJ (1981) Evidence for glomerular epithelial cell endocytosis in vitro. Lab Invest 44:502–506
Silverstein SC, Steinman RM, Cohn ZA (1977) Endocytosis. Ann Rev Biochem 46:669–722
Sobel AT, Gabay YE, Lagrue G (1976) Analysis of glomerular complement receptors in various types of glomerulonephritis. Clin Immunol Immunopathol 6:94–101
Staehelin LA (1974) Structure and functions of intercellular junctions. Int Rev Cytol 39:191–283
Striker GE, Killen PD, Farin FM (1980) Human glomerular cells in vitro. Isolation and characterization. Transplant Proc 12:88–99
Tornroth T (1976) The fate of subepithelial deposits in acute poststreptococcal glomerulonephritis. Lab Invest 35:461–474

Van Damme BJC, Fleuren GJ, Bakker WW, Vernier RL, Hoedemaeler PJ (1978) Experimental glomerulonephritis in the rat induced by antibodies directed against tubular antigens: V. Fixed glomerular antigens in the pathogenesis of heterologous immune complex glomerulonephritis. Lab Invest 38:502–510

Willingham MC, Maxfield FR, Pastan IH (1979) α_2 macroglobulin binding to the plasma membrane of cultured fibroblasts: diffuse binding followed by clustering in coated regions. J Cell Biol 82:614–625

Accepted May 21, 1984