Localization of an Ia-bearing Glomerular Cell in the Mesangium

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ABSTRACT Using trypsin to render intact, isolated rat glomeruli permeable to antibody, and using an electron microscopic immunoperoxidase technique, we have localized a phagocytic immunologically-relevant cell bearing Ia determinants to the renal mesangium. Thus there are at least two functionally distinct cell types in the renal mesangium: one is a contractile smooth musclelike cell, and the other a phagocytic cell that bears immunologically-relevant surface determinants.

We have previously described (1) a subpopulation of rat glomerular cells that bear I region associated (Ia) antigens: membrane antigens, encoded in the major histocompatibility locus, that regulate the immune interactions between phagocytes and lymphocytes. This glomerular cell resembles a mononuclear phagocyte and constitutes 1–2% of the glomerular cell population. Tissue culture experiments have demonstrated that the Ia-positive glomerular cells are capable of processing antigen and activating specifically sensitized lymphocytes in a genetically restricted manner.

Although the Ia-positive cell was present in the normal and uninflamed glomerulus, its precise localization was unknown. In the present study, we have used a combination of ultrastructural immunoperoxidase technique and a staining method that permits the labeling of Ia-positive glomerular cells *in situ*. We report that the Ia-positive cell is situated within the mesangium and is also phagocytic in vivo. The coexistence of Ia-negative cells in the mesangium suggests that there are functionally distinct mesangial cell populations.

MATERIALS AND METHODS

Animals

Female rats, Lewis strain, weighing 100–175 g, and at least 5 wk old, were obtained from Microbiological Associates (Walkersville, MD).

Antisera

Two mouse monoclonal anti-rat Ia antibody preparations, MAS 028b and MAS 029b (Accurate Chemical & Scientific Corp., Westbury, NY), were employed to label Ia antigens of glomerular cells. Their specificities have been extensively characterized (1–3). Each recognizes separate Ia determinants of the Lewis haplotype (RT 1). Labeling with the "sandwich" technique was carried out with F(ab) rabbit-anti-mouse IgG conjugated to horseradish peroxidase (HRPO) (4), at a concentration of 100 μg per ml. This antiserum was a gift from Dr. Donna Mendrick (Harvard Medical School). Control labeling consisted of deleting the anti-Ia antibody and substituting electrophoretically purified mouse IgG from the plasmacytoma line MOPC 195 (Bionetics Laboratory Products, Litton Bionetics Inc., Kensington, MD). The second step of incubation with F(ab)-anti-Ig-HRPO was maintained as before.

Glomerular Isolation

Before harvesting glomeruli, both kidneys were thoroughly perfused to remove circulating cells with a technique modified from Griffith, et al. (5), with the additional step of opening the left renal vein to permit the perfusate to drain. Fifty to 100 ml of a 0.9% NaCl solution was injected via the aorta into both kidneys, resulting in their complete blanching and eventually resulting in a clear perfusate. Mottled kidneys, indicative of incomplete perfusion, were discarded. Glomeruli were obtained by pressing slices of renal cortex through graded sieves (Tyler Inc., Mentor, OH) of 250, 150, and 75 μm with a rubber stopper (1).

Immunoperoxidase Labeling of Intact Glomeruli

Whole glomeruli were placed in a solution containing trypsin, 0.5 mg per ml, and DNAase, 0.01 mg per ml (Sigma Chemical Co., St. Louis, MO), for 30 min at 37°C in HBSS, pH 7.2, and, in some instances, 10^{-5} M chlorpromazine HCl. The glomeruli were then washed twice and incubated first in a 1:4 dilution of pooled anti-Ia antisera for 30 min at 4°C, washed twice, and then incubated in 100 μg per ml of F(ab)-anti-Ig-HRPO for an additional 30 min at 4°C. After washing two further times, the whole but partially digested glomeruli were then fixed and processed for electron microscopic examination after staining for peroxidase activity (see below). Two technical considerations must be mentioned. First, we found that the trypsinized glomeruli are much more permeable to F(ab)-HRPO than to whole IgG-HRPO. Second, trypsinization of glomeruli appeared to increase the frequency of Ia-positive mesangial cell pseudopodia over control nontrypsinized glomeruli; this phenomenon is under further study but was markedly inhibited by adding 10^{-5} M chlorpromazine HCl (Smith, Kline & French Laboratories, Philadelphia, PA) to the trypsin-containing solution. Controls included: (*a*) deleting preincubation with trypsin; (*b*) deleting incubation with anti-Ia but labeling with F(ab)-anti-Ig-HRPO; and (*c*) substituting for the anti-Ia antibody an equal concentration of mouse IgG from plasmacytoma line MOPC 195, detailed above. In none of the controls did we observe surface labeling of glomerular cells.

Labeling of Isolated Glomerular Cells

Isolated glomerular cells were obtained by subjecting sieve-purified glomeruli to sequential enzymatic digestion (1). Glomeruli were placed in a solution of trypsin, DNAase, and collagenase followed by incubation in EDTA followed by an incubation in collagenase and DNAase. After repeated washing, the suspension of single cells was incubated with anti-Ia antisera followed by F(ab)-anti-Ig-HRPO, as detailed above, for glomeruli.

Electron Microscopy

Cell suspensions and glomerular preparations were fixed in 1.25% glutaraldehyde in 0.1 M cacodylate buffer for 0.5 and 1.5 h, respectively. They were then

rinsed three times in 0.05 M Tris buffer at pH 7.6, incubated first in 0.05 gm% diaminobenzidine (DAB) in Tris buffer for 20–30 min, and subsequently in 0.5% DAB containing 0.01% H_2O_2 for 20–30 min. Following further rinsing in Tris buffer, the preparations were fixed in 2% OsO_4, dehydrated, and embedded in Epon. Some preparations were embedded in pellets of agar gel prior to dehydration. Thin sections were examined with a Philips 201 electron microscope either unstained or stained lightly with lead citrate.

In Vivo Phagocytosis

Soluble, heat-aggregated human γ-globulin (HGG) (Miles Laboratories Inc., Elkhart, IN) was prepared according to the method of Michael et al. (6) and conjugated to rhodamine isothiocyanate (RITC) by standard methods. Rats were injected intravenously with 8 mg of RITC-HGG and sacrificed 90 min later. The kidneys were perfused and glomeruli harvested and stained for Ia antigens with fluoresceinisothiocyanate-conjugated $F(ab)_2$-anti-Ig, as detailed previously (1). Intact glomeruli were examined for the presence of green membrane-staining, indicating a cell-bearing Ia antigen, and red cytoplasmic staining, indicating recent phagocytic uptake of the circulating HGG; a Leitz microscope with appropriate filters was used and the percentage of Ia-positive cells containing RITC-HGG determined in 50 glomeruli.

RESULTS

In a previous report, we demonstrated that isolated, intact glomeruli can be rendered permeable to anti-Ia antibody by mild trypsinization, without distortion of overall cytoarchitecture (1). Because the Ia determinants under study are trypsin insensitive, trypsin-treated glomeruli can be stained for the presence of Ia-positive cells using labeled antibody in the sandwich method outlined in Materials and Methods. The membrane staining is sufficiently clear that the presence of a very small number of cells in an entire glomerulus can be detected. By light microscopy, whole glomeruli stained with the immunoperoxidase technique showed an average of 6–9 Ia-positive cells in different experiments. Fig. 1 demonstrates six Ia-positive cells in the same plane of focus; the membrane labeling with peroxidase produces a rim staining pattern, clearly distinguishable from the staining of endogenous peroxidase in occasional red cells that persisted after renal perfusion. Staining glomeruli after exposure to nonspecific mouse Ia followed by anti-Ig-HRPO results in the labeling of occasional erythrocytes but no other focal staining.

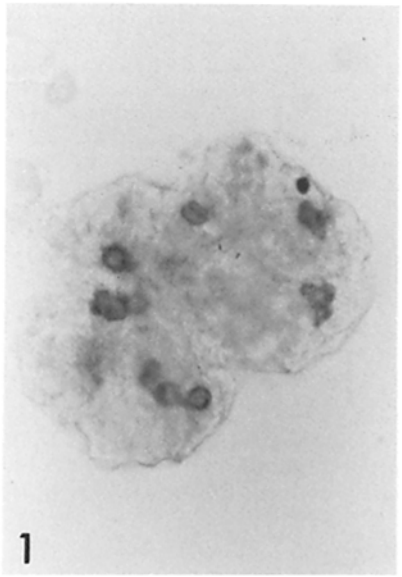

FIGURE 1 Light micrograph of a glomerulus stained with the immunoperoxidase method for Ia antigen. There are six Ia-positive cells in the plane of focus. The dark cell at the right upper corner is a red cell showing endogenous peroxidase activity. × 580.

Trypsinization resulted in several morphological alterations on the ultrastructural level. The visceral epithelium was usually totally detached, and the mesangial matrix appeared expanded and partially digested. Nonetheless, the capillary lumens, endothelial cells, and basement membrane remained well-preserved, permitting clear localization of Ia-positive cells to the mesangial region (Fig. 2).

Fig. 2 shows a cell with heavy membrane staining for Ia. It is located within the mesangial region, identified by its relationships to the basement membrane and the urinary space stripped of its epithelial cells, with three adjacent capillary profiles. The cell has extended irregular, cytoplasmic processes through the mesangial matrix; one of the cytoplasmic projections has insinuated itself beneath the Ia-negative endothelial cell (arrows). The capillary lumens are empty and the typical fenestrated endothelial cells are not labeled.

Ia-positive cells are frequently seen in close apposition to Ia-negative cells in the mesangium. In Fig. 3, the Ia-positive cell has extended tortuous cytoplasmic processes that protrude marginally into a neighboring capillary lumen. The surface of Ia-negative cells in contact with Ia-positive cells shows slight staining due to the leeching effect of the reaction product (Fig. 3). Compared to the Ia-negative mesangial cell, the Ia-positive mesangial cells manifest a somewhat more open nuclear chromatic pattern and have many more peripheral vesicles and vacuoles, most of them stained for Ia.

Note that, whereas trypsinization is required to render the glomeruli permeable to antibody, it is not necessary for the detection of Ia on cell membrane. Ia determinants, recognized by the monoclonal antibodies, of membrane of peritoneal and splenic cells isolated without enzymatic digestion, did not change with pretreatment with trypsin (1).

Ia-positive mesangial cells were also examined in suspension after enzymatically digesting a preparation of isolated glomeruli. A representative Ia-positive glomerular cell is shown in Fig. 4. Its characteristics are similar to those observed in whole glomeruli. The densely labeled cell displays tortuous, elongated cytoplasmic processes and deep invaginations. There is considerable peripheral vacuolization, with some vacuoles stained for Ia and others negative for Ia. The nucleus is relatively large and has a fine chromatin pattern.

When intact glomeruli were stained for Ia antigens 90 min after in vivo administration of rhodamine-labeled aggregated HGG, 24% of glomerular Ia-bearing cells demonstrated uptake of the labeled protein when examined by fluorescent microscopy (Fig. 5). There was evidence of uptake by Ia-negative cells, albeit to a lesser extent, but such uptake could not be quantitated accurately in the absence of a membrane label.

DISCUSSION

We have demonstrated by electron microscopy that a recently-defined mononuclear phagocytic cell bearing Ia antigens is located within the normal glomerular mesangium. Previous experiments have demonstrated that these glomerular cells are phagocytic in vitro and here we show them to be phagocytic *in situ*. They are able to initiate immune reactions in vitro by presenting antigen to sensitized lymphocytes. Consistent with their display of Ia antigens, these glomerular cells strongly stimulate allogeneic lymphocytes in mixed cultures (1).

We have outlined a method for labeling glomerular cells by trypsinizing isolated glomeruli, a process that renders them

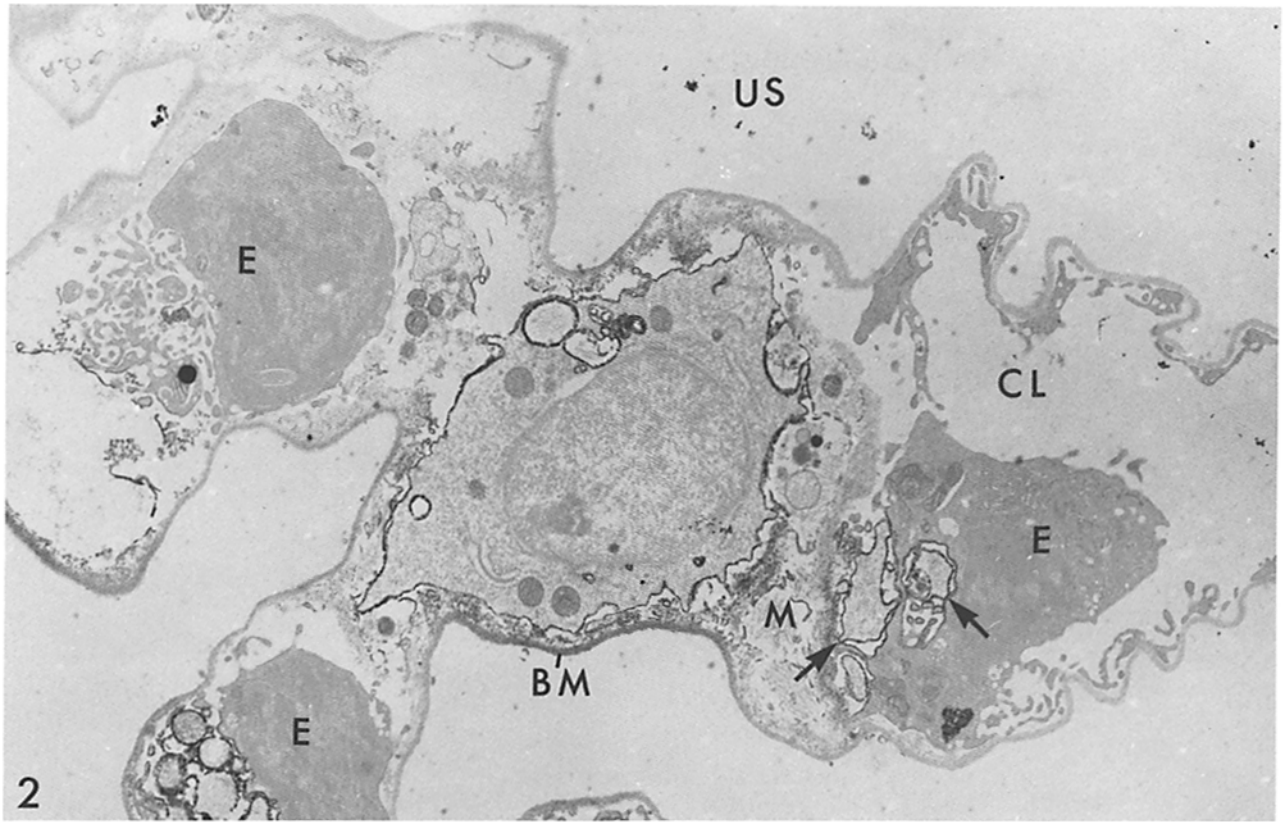

FIGURE 2 Electron micrograph of a portion of glomerulus stained for Ia antigen. The visceral epithelial cells have been digested off the urinary space (*US*), but the basement membrane (*BM*), endothelium (*E*), and mesangium (*M*) are relatively well-preserved. Note heavy surface labeling of cell in the mesangium. Mesangial cell processes (arrows) beneath the endothelium are also stained. Focal staining of the *BM* is due to leeching of reaction product, and staining of free organelles in capillary lumen (lower left) represents nonspecific adsorption of peroxidase to injured membranes. *CL*, capillary lumen. × 9,500.

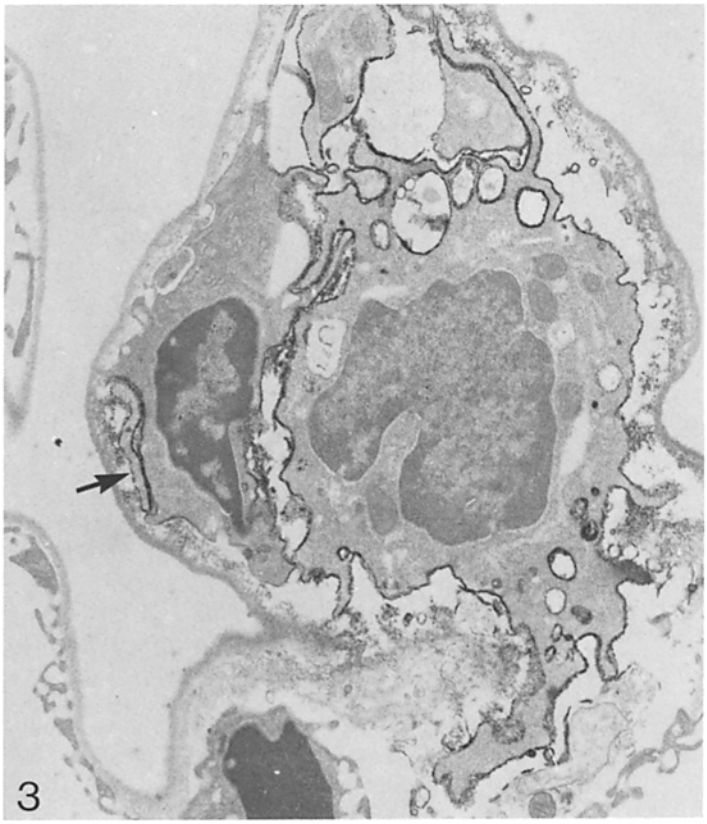

FIGURE 3 The mesangial area contains an Ia-positive and Ia-negative cell, in close apposition. Note process (arrow), probably from Ia-positive cell around the Ia-negative cell. × 11,500.

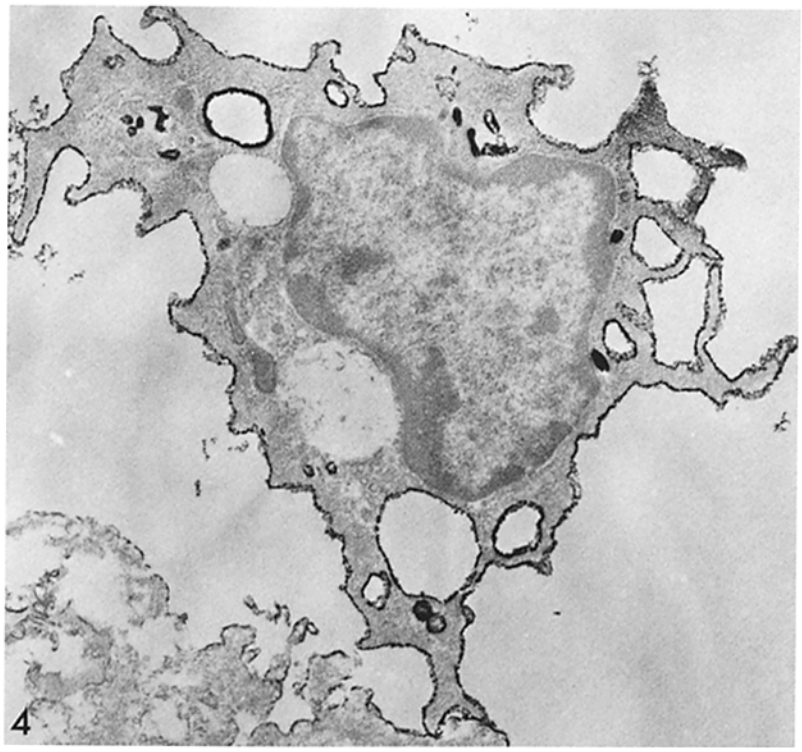

FIGURE 4 An Ia-positive cell from a glomerular cell suspension stained for Ia. The cell displays cytoplasmic processes and deep positively stained surface invaginations and peripheral vacuoles. × 14,000.

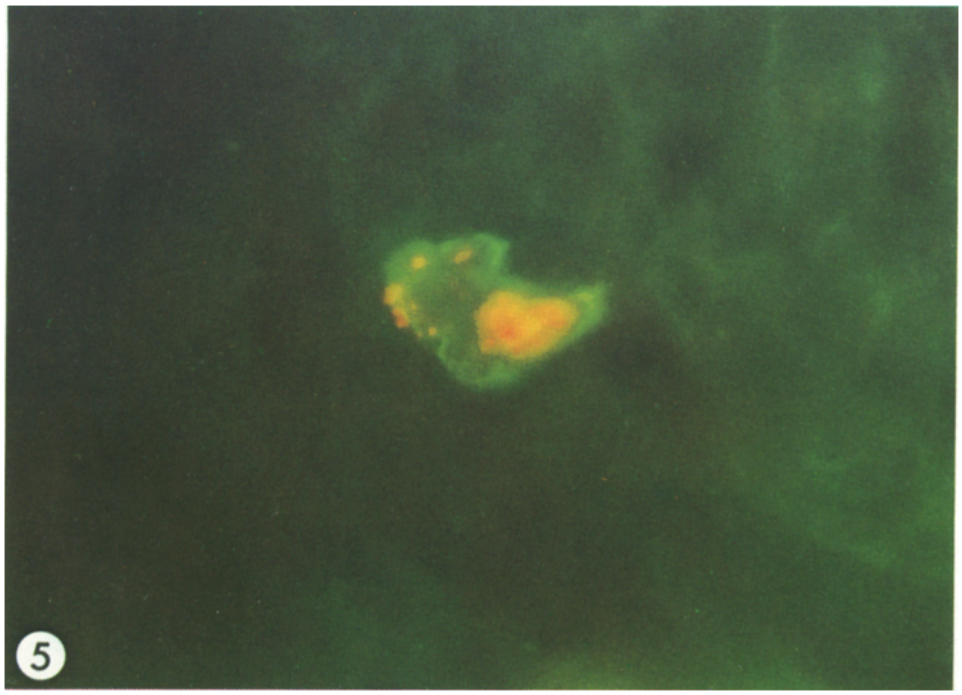

FIGURE 5 Fluorescent micrograph of an Ia-positive mesangial cell, within an intact glomerulus, 90 min after in vivo administration of RITC-labeled aggregated γ-globulin. The cytoplasmic fluorescence (orange-red under fluorescence microscope) indicates phagocytic uptake; the Ia-labeling is represented by the rim staining of the membrane (green under fluorescence microscope). × 6000.

permeable to antibody, which we employ in a sandwich-labeling technique. This method offers three advantages. First, it permits the detection of very small numbers of cells, *in situ*, in tissue fragments. With a mean of 6–9 Ia-positive cells per glomerulus, a range of 3–19 cells per glomerulus, and with an estimated total glomerular cell count of 600, one can accurately assess a population of cells comprising ~1–2% of total glomerular cells. Secondly, one can direct sectioning for electron microscopy for prior scanning under low power of stained, mounted, intact glomeruli, thus increasing the efficiency of electron microscopy for ultrastructural studies. Finally, although there is some distortion caused by loss or alteration of trypsin-sensitive structures, the morphology of the trypsinized, stained glomerulus is adequate for detailed study.

The mesangial Ia-bearing cells are phagocytic in vivo, with respect to circulating aggregates of γ-globulin. The fact that, in

this assay, only 24% took up the aggregate may reflect three considerations. The first is that there is heterogeneity among Ia-bearing glomerular cells with regard to phagocytic capacity. We have shown that in culture there are functional supopulations of Ia-bearing cells, with ~35% demonstrating a phagocytic capacity after 2 h in culture, that percentge doubling after the activation of overnight in vitro culture (1). In addition to the nonphagocytic spindle-shaped Ia-negative mesangial cells, cultures of kidney cells contain phagocytic cells that are morphologically indistinguishable from Ia-positive phagocytes but do not display Ia antigens. Because such cells can be induced to express membrane Ia antigens in culture, they may represent a separate functional stage rather than a separate cell population. Finally, we have negatively biased the assay by choosing an extremely short time-course for glomerular isolation after a single bolus phagocytic challenge. In a similar model, Striker (7) found no evidence for infiltrating cells in the first 24 h after the injection of immune complexes; we chose a time-course of 90 min to minimize the possibility of monocytic infiltration. Additionally, circulating rat monocytes are Ia-negative.

Thus, the glomerular mesangium appears to contain at least two, and perhaps more, subpopulations of cells. The first might be termed the "classical" mesangial cell. It is the predominant mesangial cell type, of renal origin and resembling smooth muscle; it is contractile, and bears angiotensin II receptors (8, 9). This mesangial cell has been studied in culture by Kreisberg et al. (8), who noted it is nonphagocytic, as we have. The second mesangial cell type is much less common, phagocytic, adherent to glass, and capable of immunologically specific interactions with lymphocytes. Under in vitro conditions, 50–60% of phagocytic glomerular cells bear Ia determinants. Assuming that one-third of glomerular cells are mesangial (1) and that 1–2% of glomerular cells bear Ia determinants, one can estimate that up to 12% of mesangial cells are a distinct subpopulation capable of phagocytosis, Ia determinant expression, or both. The phagocytic cell is located in approximation to the contractile cell, raising the possibility of physiological interactions between the two cell types. Preliminary experiments utilizing bone marrow transplants indicate that the Ia-positive cell originates outside the kidney, migrates into the mesangium, and resides there for an unknown period.

These observations of phenotypically discrete mesangial cell populations with different properties may, to some extent, reconcile recent divergent opinions as to the nature of "the" mesangial cell. Early ultrastructural studies had indicated apparent phagocytosis by at least some mesangial cells of tracer substances such as ferritin (10) and thorium (11). More recently, the existence of a phagocytic mesangial cell has been questioned (12), based on failure of the contractile mesangial cell to phagocytose in vitro, the difficulties in distinguishing pinocytosis in in vivo studies, and the possible contribution of infiltrating blood monocytes to glomerular phagocytosis (7). Our approach has shown that there is at least one type of phagocytic mesangial cell type, the Ia-positive cell, but one that occurs sufficiently infrequently so as to render detection by other techniques difficult. Whether the Ia-negative blood monocytes that infiltrate the rat glomerulus under pathologic conditions (7) acquire Ia determinants in the renal mesangium is now under study.

Endogenous renal Ia antigens have been demonstrated via absorption techniques in mouse renal homogenates (13), and by immunohistologic methods in a dendriticlike cell, in the renal interstitium of rats (14). We have observed a similar Ia-positive cell in the renal interstitium after fluorescent antibody labeling of frozen sections. Of particular importance is that in both intact glomeruli and suspensions, endothelial cells are Ia-negative, as defined by the two monoclonal antibodies employed in this study which recognize the Ia determinant. Recent immunofluorescent studies on frozen sections of human kidney or on isolated cells have suggested the presence of DR antigens (thought to be analogous to rodent Ia antigens [15]) on glomerular endothelial cells, employing monoclonal antibodies (16) or polyvalent, heterologous antisera (17). In human renal tissue, differences in labeling between public and private DR specifications (16), between monoclonal antibody and polyvalent antisera (16), and between heterologous antisera and alloantisera (17) have been noted. Whether the apparent difference in the distribution of I-region antigens between man and rat represents species differences, antisera differences, or undefined cross-reactive specificities uncovered in polyvalent antisera requires further clarification. It is possible that there is differential expression of I-subregion determinants among glomerular cells and that antisera recognizing Ia determinants other than those studied in this report may stain rat endothelium positively, as is the case in humans. Nonetheless, the Ia-positive phagocytic glomerular cell described herein is a mesangial cell and not a component of capillary endothelium.

It has been clearly established that marrow-derived mononuclear phagocytes, whether circulating cells or resident macrophages, engage in reciprocal, modulatory interactions with lymphocytes that are critical to a host's immune reactivity to an antigen (18). The observation that such cells can be found within the mesangium may be significant with respect to possible mechanisms of immunologically mediated injury to the glomerulus. Their presence also suggests an accessible source of endogenous donor Ia antigens necessary for the induction of host immunity against transplanted kidneys.

We are indebted to Dr. Emil R. Unanue for invaluable advice and support during the course of this study. We wish to thank Ms. Deborah Sandstrom for excellent technical assistance.

This research was supported by grants #AM-2-7787-01 and HL-2-2602-04 from the National Institutes of Health.

Received for publication 24 February 1982, and in revised form 6 May 1982.

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