Renal Transplantation in the Inbred Rat

II. An Immunohistochemical Study of Acute Allograft Rejection

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THE USE OF inbred strains of rats for kidney allotransplantation now affords the opportunity to study an experimental model which is relatively free from the variabilities inherent in other models of renal transplantation and one in which proper immunogenetic controls can be used. It is the purpose of this paper to present the immunofluorescent observations that have been made with five immunohistochemical reagents in a serial study of acute renal allograft rejection. The system employed is that of renal allotransplantation in inbred rats, utilizing a strain combination which gives a host vs. graft reaction by grafting the (Lewis $\times$ BN) F_1 hybrid kidney into the Lewis rat. This represents strong histoincompatibility which has genetic disparity at 14–16 histocompatibility loci including an incompatibility at the major Ag-B locus.[1,2] The results of staining tissues serially obtained during acute rejection and from matched paired isograft controls with fluorescein-conjugated antibody to immunoglobulin G, immunoglobulin M, β_{1C}-globulin, fibrinogen, and α_2-macroglobulin (as a nonimmunoreactant) are presented.

Materials and Methods

Animals

Young 250- to 350-gm. adult inbred male rats of Lewis/MAI and (Lewis $\times$ BN) F_1 hybrid strains were used in this study. Lewis rats were always the recipients of a renal transplant, and Lewis and (Lewis $\times$ BN) F_1 hybrid rats served as donors of renal isografts and allografts, respectively. The microsurgical technique of renal transplantation in the rat and our modifications have been previously described.[3,4] More than 40 renal transplants were available for serial study from Day 0 to 3 weeks after transplantation. For each transplanted animal, tissue from the recipient's kidney removed at the time of transplantation, the transplanted kidney, the recipient's second kidney, and the donor's opposite kidney was available for immunofluorescent study.

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Immunologic Reagents

Immunoglobulin G (IgG) was isolated by DEAE chromatography.[5,6] Rat serum was dialyzed overnight at 4° C. against 0.007 M phosphate buffer pH 6.3 and applied to a 30 × 750 mm. chromatography column containing DEAE cellulose equilibrated against this same buffer. IgG was eluted with 0.007 M phosphate buffer pH 6.3, concentrated against 30% carbowax, redialyzed, and rechromatographed. Immunologic purity of the collected IgG was verified by immunoelectrophoresis.[7] IgG (10 mg./ml. neutral buffered saline) was emulsified with an equal volume of Freund's incomplete adjuvant and used to immunize rabbits. The rabbits were given an injection of 1 ml. of adjuvant and antigen in their hind foot pads and front shoulders and were bled 21 days later. Antiserum to this antigen gave a strong single precipitin line in the gamma region.

β_{1C}-globulin (C'3) was prepared according to the method of Mardiney and Müller-Eberhard.[8] Zymosan (100 mg. immunologic grade) was boiled for 30 min. in 10 ml. of 0.15 M NaCl; 10 ml. of rat serum was added, and the mixture stirred for 30 min. at 37° C. Following incubation with rat serum, the zymosan was washed 6 times with 50 volumes of cold 0.15 M barbital-buffered saline, pH 7.3. The washed zymosan with absorbed β_{1C}-globulin was suspended in 1 ml. of saline and emulsified with 1 ml. of incomplete Freund's adjuvant for immunization. Rabbits were immunized with a single injection and antiserums were collected at 21 days. Only antiserums that produced a single line on immunoelectrophoresis were used.

Antifibrinogen was prepared by immunizing rabbits with rat plasma emulsified with an equal volume of complete Freund's adjuvant and a booster immunization was given 2 weeks later. The resulting antiserum collected 10 days after the booster immunization was absorbed with an equal volume of rat serum and the precipitate removed after centrifugation. Such antiserums gave a single precipitin line in the β-globulin region on immunoelectrophoresis. In addition, fluorescein isothiocyanate (FITC) rabbit anti-rat fibrin was used.

The α_2-macroglobulin was isolated by gel filtration.[9] After dialyzing rat serum overnight against 0.1 M Tris pH 8.0 buffer, rat serum was placed on a 25 × 900 mm. column containing Sephadex G-200. Elution with 0.1 M Tris-HCl buffer pH 8.0 resulted in 3 protein peaks. The first macroglobulin peak was concentrated to 10 mg. protein per milliliter, stored at −10° C. for 1 month, and emulsified with an equal volume of incomplete Freund's adjuvant for immunization. Rabbits were bled 21 days later and the antiserums analyzed by immunoelectrophoresis. The antiserums gave a single precipitin line in the α-globulin region and no antibody to IgM was seen.

Rabbit antiserum to rat IgM conjugated with FITC was prepared after isolation of IgM by Sephadex G-200 chromatography and Pevikon block electrophoresis.

Antiserums were conjugated to FITC by the method of Clark and Shepherd.[10] The IgG fraction of the antiserums was isolated by DEAE chromatography, adjusted to 10 mg. protein per milliliter, and dialyzed against 0.025 M bicarbonate buffer pH 9.5 and containing 1:20, F:P. After 18 hrs. of conjugation, the antiserums were exhaustively dialyzed against phosphate-buffered saline pH 7.1 at 4° C. Prior to immunohistochemical use, antiserums were absorbed 2 and sometimes 3 times with 50–100 mg. mouse liver or rabbit liver powder per milliliter of antiserum.

Tissue Preparation

Cold ethanol-fixed paraffin-embedded tissue[11] and fresh-frozen tissue were used for immunohistochemical study.[12] Kidneys were quickly removed, sliced into thin slices, and fixed in cold 95% ethanol. Following overnight fixation, the tissue was dehydrated in 3 hourly changes of cold absolute ethanol, cleared in 3 hourly changes of cold xylene, and infiltrated with paraffin in a vacuum oven maintained at

58 ± 1° C. for 1 hr. Sections 5μ thick mounted on clean glass slides without adhesives were deparaffinized in three 1-min. cold xylene baths. After 3 washings of 30 sec. each in cold 95% ethanol and in cold neutral buffered saline, sections were covered with several drops of fluorescent antibody and incubated for 45 min. in a humid chamber. Labeled antibody was washed off with cold neutral buffered saline 3 times for 1 min. each time, fixed for 15 min. in cold 95% ethanol, and finally washed for 5 min. in cold neutral buffered saline. Excessive saline surrounding the tissue section was removed with absorbant tissue and coverslips were mounted with 1:2, glycerol:saline.

Tissue slices from representative kidneys were also frozen on dry ice; 10-μ sections were prepared from the frozen tissue and mounted on room-temperature glass slides. The sections were kept in a humid chamber until a sufficient number was prepared and then they were washed in neutral buffered saline. Soft tissue paper was used to absorb excess saline and the tissue sections were covered with several drops of fluorescein-conjugated antibody. After incubation in a room-temperature humid chamber for 40 min., the antibody was removed with 2 washes of neutral buffered saline each lasting 6 min. The sections were covered with clean coverslips using 1:2, glycerol:saline mounting media.

Fluorescence Microscopy and Photography

Sections were examined by dark-field fluorescence microscopy. Ultraviolet light was supplied to a Reichert microscope equipped with fluorite dark-field objectives and condensors by an Orsam HBO 200-w mercury lamp and filtered by a full-thickness KG-2 5970 exciter filter. Sections were viewed with a W2B barrier filter in place. Photographic recording of the sections was made on high-speed daylight Ecktachrome film by using a Nikon Microflex photomicrographic adaptor. Exposure times varied from 10 to 20 sec., 20 to 40 sec., and 30 to 60 sec. for magnifications of 125, 250, and 500, respectively.

Control Procedures

Tissue controls consisted of (1) renal isografts, (2) each donor's opposite kidney, (3) each recipient's kidney removed at the time of transplantation, and (4) each recipient's opposite kidney if it had remained in place during the course of transplantation.

Two types of blocking experiments were used to determine the specificity of fluorescent-antibody localization. Sections were exposed first to unlabeled antibody, washed, and then exposed to labeled antibody. Blocking was also demonstrated for IgG by adding purified antigen to labeled antibody at equivalence an then removing the precipitate before staining sections.

The spleen of a rat immunized with intravenous bovine serum albumin and a kidney containing intravascular thrombosis served as positive controls for IgG and for fibrin localization, respectively.

Results

The light-microscopic features of allograft rejection have been previously presented for this model.[4] The salient features will be mentioned for orientation of the immunohistochemical findings.

Vessels

Frequent mononuclear cells with large vesicular nuclei and one or more prominent nucleoli are seen within peritubular capillaries 2–3 days following renal transplantation (Fig. 7). Some of these cells are in con-

tact with and adherent to the endothelial lining of the peritubular capillaries. Although occasional large mononuclear cells are seen passing through the walls of capillaries, destruction of peritubular capillaries is not a prominent feature of unsensitized renal allograft rejection.

Many mononuclear cells within peritubular capillaries contain abundant cytoplasmic IgG as demonstrated by positive fluorescence when the sections are exposed to FITC rabbit anti-rat IgG (Fig. 1). IgG is also demonstrated on the walls of many peritubular capillaries (Fig. 2). It is seen in capillaries with and without intraluminal mononuclear cells. The IgG is localized in some peritubular capillaries as thin homogeneous layer following the inner aspect of the capillary's wall. In the other capillaries and veins, a slightly granular pattern of fluorescence is apparent. β_{1C}-globulin—but not IgM, fibrinogen, or α_2-macroglobulin—is also localized on peritubular capillaries. Mononuclear cell cytoplasm contains only IgG. The intensity of fluorescence in peritubular capillaries is diminished at Day 5, and by Day 7 capillary fluorescence for the presence of IgG and β_{1C}-globulin becomes obscured by other marked morphologic alterations seen at this time in the allograft rejection. Isogeneic tissue shows no localization with these reagents.

Five days after transplantation, IgG and β_{1C}-globulin are localized on medium- and large-sized veins (Fig. 3). They are located on the endothelial lining, not in the walls, of these vessels. Although some α_2-macroglobulin is occasionally seen on the walls of veins, it is always intimately associated with intravascular serum precipitates. Except for an occasional intravascular thrombus, no fluorescence is observed when sections are treated with FITC-labeled rabbit anti-rat fibrinogen. IgM localization is not noted.

Arteries and arterioles do not demonstrate localization of IgG, IgM, β_{1C}-globulin, α_2-macroglobulin, or fibrinogen. Even vessels with frank fibrinoid necrosis, which is regularly seen 2–3 weeks after allografting, do not contain immunohistochemically demonstrable IgG, IgM, β_{1C}-globulin, α_2-macroglobulin, or fibrinogen. Medial vacuolization of arteries is seen both in isografts and allografts. Not infrequently these vacuoles contain inspissated material, but localization of IgG, IgM, β_{1C}-globulin, fibrinogen, or α_2-macroglobulin is not observed.

Glomeruli

Glomerular alterations characterized by mesangial- and endothelial-cell swelling and proliferation characteristically develop in allografted kidneys 5 days after transplantation (Fig. 8). Some increase in mesangial matrix and focal capillary-wall thickening also occurs at this time. Sim-

ilar, though less severe, glomerular alterations are occasionally seen in the recipient's own kidney if it remains in place. Glomerular localization of IgG occurs concomitantly with the glomerular hypercellularity seen at Day 5. Small, irregular, granular fluorescent deposits on capillary walls and in mesangial areas appear after sections are exposed to labeled anti-rat IgG. Glomerular tufts also contain granular deposits of β_{1C}-globulin in a mesangial- and capillary-wall pattern (Fig. 4). Fibrinogen is also seen in glomerular tufts at this time; however, its pattern of localization contrasts with IgG and β_{1C}-globulin. Fibrinogen is located solely in mesangial areas and is diffuse rather than granular. Glomerular tufts do not contain α_2-macroglobulin or IgM. Frequent polymorphonuclear leukocytes appear within glomerular tufts on post-transplant Day 7, and IgG and β_{1C}-globulin are demonstrable within the cytoplasm of the polymorphonuclear leukocytes. Additional glomerular alterations at this time consist of focal necrosis and occlusion of capillary loops by fibrillar PAS-positive material (Fig. 9). Glomerular localization of IgG and β_{1C}-globulin is no longer demonstrable by immunohistochemical methods in many 7-day allografts. In the few animals with persisting granular deposits of IgG and β_{1C}-globulin at this time, polymorphonuclear leukocytes staining positively for IgG and β_{1C}-globulin are not seen. Fluorescence for localization of fibrinogen remains in a mesangial pattern and is most intense at 7 days (Fig. 5).

By 2 and 3 weeks all glomerular tufts are bloodless and occluded by fibrillar eosinophilic material. Only a few viable mesangial- and endothelial-cells remain. In contrast, epithelial cells are numerous and contain large vesicular nuclei and abundant cytoplasm. Polymorphonuclear leukocytes remain, although decreased in number. The few remaining polymorphonuclear leukocytes contain cytoplasmic IgG and β_{1C}-globulin at 2 and 3 weeks after transplantation.

Tubules

Significant tubular alterations are not seen in routine hematoxylin-and-eosin-stained sections until 5 days after allotransplantation. Tubular epithelial cells contain coarse vacuoles and protein droplets. Qualitatively similar and quantitatively fewer alterations are seen in isografts. Frank tubular necrosis appears in allografts by Day 7 and many tubules are dilated and contain eosinophilic casts. By 2 and 3 weeks after transplantation, tubular necrosis is complete. Tubular epithelial cells and casts contain IgG, β_{1C}-globulin, fibrinogen, and α_2-macroglobulin. Several patterns of localization within the renal tubular epithelial cells are observed. In some tubules, just the apical portions of the tubular

epithelial cells contain these proteins. In others, the proteins are localized as discrete 0.3- to 3.0-μ spherical granules scattered throughout the epithelial cells. In still other cells, these proteins are distributed diffusely throughout the cytoplasm. Although less intense, qualitatively similar tubular patterns of protein localization are found in isogeneic transplants. The amount of proteins demonstrable in both isogeneic and allogeneic grafts by immunohistochemical techniques frequently correlates with the number of protein-reabsorption droplets seen in routine slides.

Interstitium

Mononuclear cells with large vesicular nuclei, prominent nucleoli, and pyroninophylic cytoplasm are in obvious accumulations in perivascular areas 2 days after allografting (Fig. 10). By Day 5 these mononuclear cells have diffusely infiltrated the renal cortex. At 2 and 3 weeks after transplantation, the mononuclear cells frequently contain PAS-positive material. Interstitial edema—first confined to perivascular areas—diffusely involves the cortex by post-transplant Day 7.

IgG is not seen in the mononuclear cells of the interstitial infiltrate during the first week in spite of the large number of cells with pyroninophilic cytoplasm. IgG-containing mononuclear cells are seen 2–3 weeks after transplantation when the kidneys are functionally and morphologically completely rejected. However, the mononuclear cells also contain β_{1C}-globulin, fibrinogen, and α_2-macroglobulin (Fig. 6). Areas of interstitial edema contain abundant IgG, β_{1C}-globulin, fibrinogen, and α_2-macroglobulin.

Discussion

The importance of peritubular capillary damage in the rejection mechanism of renal allografts has been suggested from electron-microscopic study of the phenomenon.[13] The presented data-demonstrating IgG localization on the walls of peritubular capillaries soon after allografts are transplanted, and preceding the functional and morphologic alterations of allograft rejection—suggest that an immunologic reaction has taken place at this site. Supporting evidence for an immunologic reaction in this region is found in the concomitant localization of β_{1C}-globulin, presumably bound after antibody-antigen interaction, and the absence of fibrinogen and α_2-macroglobulin localization along the wall of these vessels. However, the possibility of localization of IgG and β_{1C}-globulin due to the nonspecific aggregation of protein in areas of damaged endothelial cells cannot be definitely eliminated. The participation of cell-bound antibody in renal allograft rejection is suggested by the frequent intracapillary mononuclear cells whose cytoplasm is positively stained for

IgG. Some of these cells appear adherent to capillary walls and possibly transfer their antibody at this site. This finding certainly does not completely rule out the possibility that antibody can be manufactured at distant sites and reach the target organ through the circulation without either carriage or local manufacture by these intravascular mononuclear cells. The relatively small number of cells containing cytoplasmic IgG in comparison to the more extensive localization of IgG along peritubular capillary walls suggests that an additional mechanism of antibody deposition may be involved.

Following antigen-antibody interaction—well described in other systems—increased vascular permeability and vascular spasm ensued.[14] Similar spasm and increased permeability may occur in renal allografts following the deposition of antibody. The early perivascular and later diffuse interstitial localization of IgG, β_{1C}-globulin, α_2-macroglobulin, and fibrinogen is evidence in rejecting allografts of the increased vascular permeability to serum proteins with nonspecific accumulation of these proteins in the renal interstitium.

The absence of IgG localization in arteries in the present studies contrasts with the results obtained in dog and man. In both unmodified canine renal allografts[15] and some renal allografts in the immunosuppressed human,[16] deposits of IgG are occasionally found in the muscular wall of arteries. Horowitz *et al.*[15] observed localization of IgG within vacuoles in the muscular layer of arteries. Although vacuolization of arterial walls is seen in rat renal allografts, similar vacuoles are seen in isografts and control kidneys.[4] A localization of IgG, β_{1C}-globulin, fibrinogen, or α_2-macroglobulin was not found in these vacuoles. Even vessels with obvious fibrinoid necrosis do not show the localization of these proteins. A false-negative result in this study tends to be minimized by the finding of IgG localization and localization of other immunoreactants in other parts of the kidney at various times during rejection. This negative finding was observed in both 95% ethanol-fixed and in fresh-frozen tissue.

The results with staining the tissues for IgM were uniformly negative. Since one expects that the primary response to antigen would reveal an early 19S antibody response, the failure to find IgM localization very early in acute allograft rejection in disturbing, particularly if destruction of the kidney by humoral factors is postulated. However, several possibilities may explain the absence of immunohistochemically demonstrable IgM. Fewer 19S antibody molecules may be involved early in the stage of rejection, and the immunofluorescent technique may be too insensitive to detect these.[17] Secondly, it has been observed that some bacterial antigens may sensitize to subsequent allografts implying antigen cross-

reaction.[18,19] Therefore, the renal allograft may not be an instance of typical primary immunization and one might not expect an early 19S-antibody response to the antigenic stimuli involved.

This and a previous study have drawn attention to the glomerular alterations which develop in allogeneic renal transplants.[4] Functionally, morphologically, and immunohistochemically these glomerular alterations are similar to the lesions seen in acute "one-shot" serum sickness.[20] Circulating immune complexes consisting of soluble organ-specific and genetically specific transplantation antigens and antibodies with complement probably are deposited in glomerular tufts and initiate mesangial- and endothelial-cell swelling and proliferation. The localization of IgG and β_{1C}-globulin—but not α_2-macroglobulin or fibrinogen—in a granular pattern throughout the glomerulus supports this view. The pathogenetic role of circulating antigen-antibody complexes in initiating glomerular alterations has been established.[20–23] Since similar granular deposits of IgG and β_{1C}-globulin are found in the glomerular alterations in the host's own kidney if it remains in place, the deposition of immune complexes in glomerular tufts as occurs in serum sickness is emphasized and is not immunogenetically directed. In the final stages of allograft rejection, the appearance of PAS-positive fibrillar material occluding glomerular capillary loops correlates directly with the deterioration of renal function.[4] This study would seem to indicate that fibrinogen (or fibrin) plays a prominent role in occluding the glomerular capillary loops. The significance of fibrin in the pathogenesis of the glomerular alterations that develop in some long-term human renal allografts has been suggested.[16]

Although the mononuclear cells infiltrating the renal interstitium have been called immunocytes, these results indicate that they are not engaged in antibody synthesis, as there is little or no immunohistochemically detectable IgG or IgM in these cells at a time when rejection is occurring. Low-level synthesis of antibody below the sensitivity of immunohistochemical methods seems unlikely. Since active proliferation occurs among the interstitial mononuclear cells, the abundant cytoplasmic ribosomes—the appearance of which are frequently cited as evidence for antibody synthesis in these cells—may be synthesizing cellular proteins necessary for growth and replication rather than antibody. The contrast of interstitial cells lacking cytoplasmic IgG with the IgG-containing cells within peritubular capillaries may be explained on a basis of a proliferative role of the interstitial cells. Only at a later date when kidneys are functionally and morphologically completely rejected do the infiltrating mononuclear cells contain IgG. However, even then

only a small percentage of these cells contain this material. A similar paucity of IgG-containing cells has been noted in the human renal allografts.[16] The additional demonstration of β_{1C}-globulin, α_2-macroglobulin, and fibrinogen localized to foci of interstitial cells at the time when IgG is seen suggests a phagocytic role for these cells rather than their being engaged in the manufacture of antibody.

Summary

Renal allograft rejection was examined in inbred rats by immunohistochemical techniques for the localization of immunoglobulin G (IgG), immunoglobulin M (IgM), β_{1C}-globulin, fibrinogen, and α_2-macroglobulin. Kidneys were transplanted from Lewis and (Lewis $\times$ BN) F_1 donors to Lewis recipients for renal isografts and allografts. Two days after allotransplantation, mononuclear cells were seen within peritubular capillaries and some were adherent to vascular endothelium. Some of these cells contained cytoplasmic IgG. IgG was also located on the walls of many peritubular capillaries and vein walls. β_{1C}-globulin (C'3), but not fibrinogen or α_2-macroglobulin, was also on vessel walls. Arteries and arterioles did not contain immunohistochemically demonstrable IgG, IgM, β_{1C}-globulin, fibrinogen, or α_2-macroglobulin. Glomerular alterations characterized by mesangial- and endothelial-cell swelling and proliferation, mesangial matrix increase, and some capillary-wall thickening developed in allograft kidneys 5 days after transplantation. Concomitantly, IgG and β_{1C}-globulin appeared on glomerular capillary walls and mesangial areas as irregular granular deposits. Fibrinogen was also located in glomerular tufts at this time; however, it was located in a mesangial pattern and was diffuse rather than granular. Perivascular infiltrates of mononuclear cells with large vesicular nuclei, prominent nucleoli, and pyroninophilic cytoplasm appeared 2–3 days after allografting and diffusely infiltrated the renal cortex by Day 5. None of the immunohistochemical reagents localized in these interstitial cells while acute rejection was occurring. Only after kidneys were morphologically and functionally completely rejected 2–3 weeks after allotransplantation did IgG appear within cells; however, β_{1C}-globulin, α_2-macroglobulin, and fibrinogen also appeared within mononuclear cells at this time.

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[*Illustrations follow*]

Legends for Figures

Fig. 1. IgG localization in rat renal allograft 2 days after transplantation. Cytoplasm of mononuclear cells located within peritubular capillary contains abundant IgG. These cells are in contact with vascular endothelium. IgG also seen as a thin layer coating the endothelium. × 250.

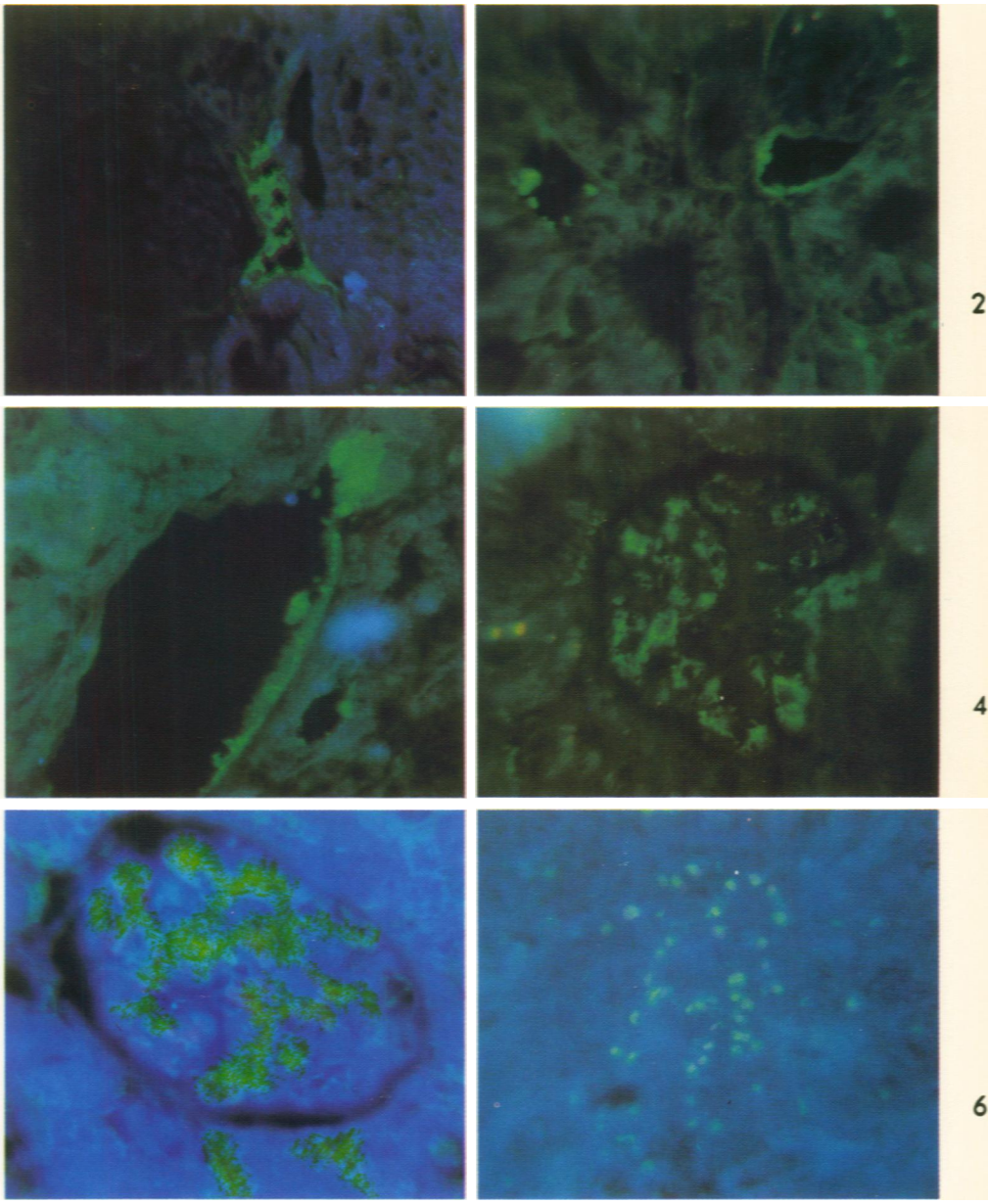
Fig. 2. β_{1C}-globulin (C'3) localization in rat renal allograft 2 days after transplantation. β_{1C}-globulin is localized on peritubular capillaries both as thin homogeneous layer (right) and granular deposits (left). × 500.

Fig. 3. β_{1C}-globulin localization in rat renal allograft 5 days after transplantation. Granular deposits of β_{1C}-globulin line inner aspect of wall of medium-sized vein. × 500.

Fig. 4. IgG localization in rat renal allograft 5 days after transplantation. Granular deposits of IgG are located on glomerular capillary walls and in mesangium. Glomerular localization of β_{1C}-globulin, but not fibrinogen of α_2-macroglobulin, is also seen in a similar pattern. × 500.

Fig. 5. Fibrinogen localization in rat renal allograft 7 days after transplantation. Fibrinogen is located in mesangial area of glomerulus. × 500.

Fig. 6. β_{1C}-globulin localization in rat renal allograft 3 weeks after transplantation. Interstitial mononuclear cells contain abundant β_{1C}-globulin within their cytoplasm. IgG, fibrinogen, and α_2-macroglobulin is also seen within these cells. × 500.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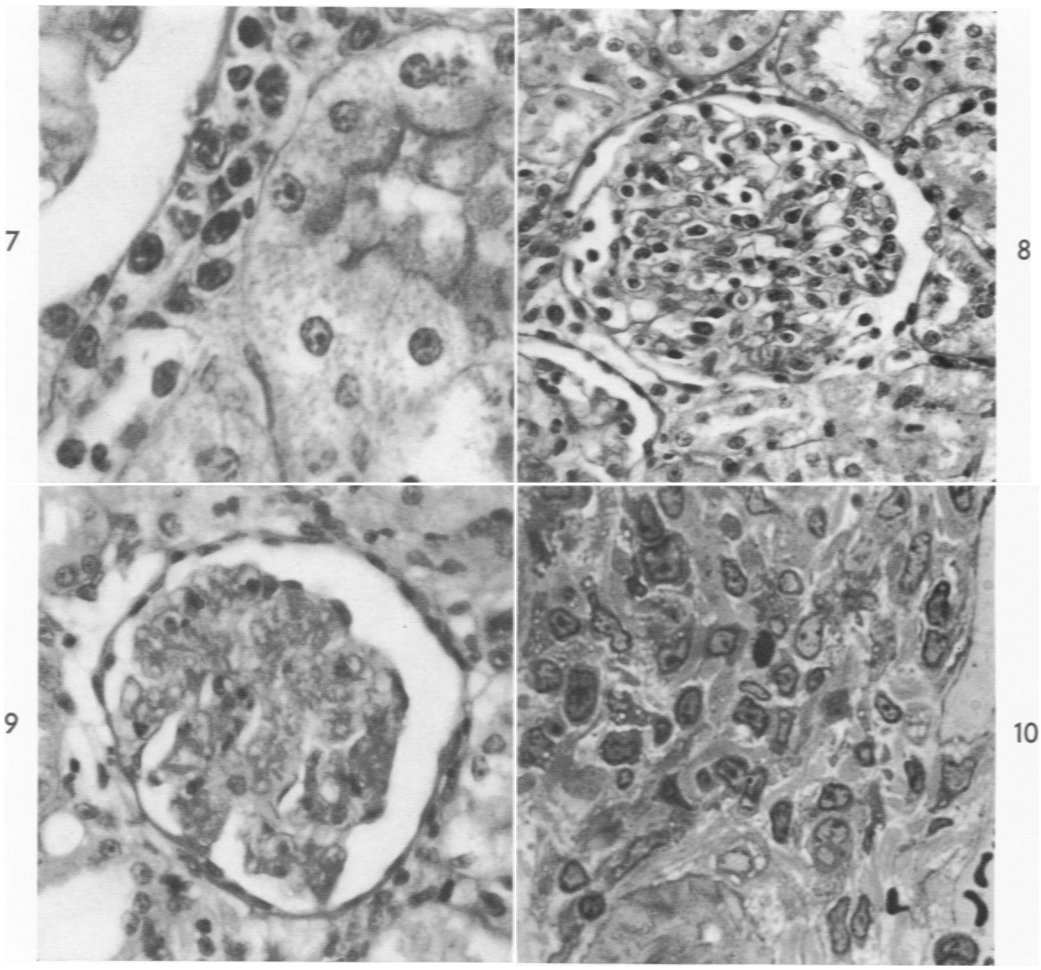

Fig. 7. Three-day renal allograft in rat. Thin-walled vessel contains several mononuclear cells with vesicular nuclei and 1 or 2 prominent nucleoli. Some of these cells are in contact with vascular endothelium. Hematoxylin and eosin. × 560. **Fig. 8.** Five-day renal allograft in rat. Glomerulus is hypercellular and glomerular capillaries are occluded by mesangial and endothelial cells. Hematoxylin and eosin. × 310. **Fig. 9.** Seven-day renal allograft in rat. PAS-positive fibrillar material occludes glomerular capillaries and is deposited in mesangial region. Glomerular cells are decreased in number at this time. Periodic acid Schiff. × 310. **Fig. 10.** Three-day renal allograft in rat. Mononuclear cells with vesicular nuclei and one or more prominent nucleoli accumulate in perivascular areas. Epon-embedded, 1-μ section. Toluidine blue O-borax. × 560.