Studies on the Pathogenesis of Experimental Anti-Tubular Basement Membrane Nephritis in the Guinea Pig

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Using the model of renal disease induced in guinea pigs by immunization with bovine TBM preparations in adjuvant, the following observations were made. Animals with actively induced disease show bright staining for IgG along the TBM and only faint, inconstant staining along the GBM. Following transfer of serum from animals with anti-TBM disease to normal recipients, accumulation of IgG was found predominantly in glomeruli at 4 hours, but at Days 3 and 5, IgG was seen predominantly along the TBM. There was no appreciable accumulation of neutrophils in the kidneys of recipients of anti-TBM serum, even at early intervals (4 and 24 hours) after transfer. However, within 2 days, small numbers of mononuclear cells were found. By Day 3, mononuclear cells were numerous, and multinucleate giant cells and tubular cell damage were present. After that, the lesions increased in severity and by 10 days were indistinguishable from those found in actively immunized animals at 14 to 21 days. Study of frozen sections of kidneys obtained from animals with active disease at 14 days, employing sheep cells coated with rabbit antibody (IgG EA) revealed rosettes around many of the mononuclear cells in the infiltrate, indicating that they are mononuclear phagocytes (monocytes or macrophages). IgM complexed with sheep cells and complement (EAC) did not react and thus failed to provide evidence for the presence of B lymphocytes. Transfer of $7 \times 10^4$ lymph node cells from the TBM-immunized Strain 13 donors to normal Strain 13 recipients failed to result in renal lesions. The findings are interpreted as indicating that anti-TBM antibodies mediate the renal disease without the participation of cell-mediated immunity and further that these antibodies bring about an influx of circulating mononuclear cells, predominantly monocytes, without attracting appreciable numbers of neutrophils. (Am J Pathol 83:531-546. 1976)

Recently, models of experimentally induced interstitial nephritis associated with anti-tubular basement membrane (TBM) antibodies have been reported in guinea pigs and rats. The immunofluorescence findings are distinctive: linear accumulation of IgG, and sometimes of C3, is seen along tubular basement membranes. Histologically, tubular damage and interstitial inflammation are present, with accumulation of mononuclear cells and peritubular multinucleate giant cells. Following description of these models, similar forms of renal disease were recognized in man.

It has been shown that the experimental disease can be transferred to

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normal animals with serum from affected donors. The renal findings in the recipients are very similar to those in actively immunized animals. Thus, although characterized by a predominantly mononuclear cell infiltrate, the disease appears to be initiated by antibodies and can appropriately be called anti-TBM disease. Several antibody-mediated lesions have been described with a predominantly mononuclear infiltrate, but in these cases either there has been recognized an earlier phase during which neutrophils predominate, or the models have not been examined at an early enough stage to exclude such a possibility. The nature of the mononuclear cells (i.e., proportion of T and B lymphocytes and mononuclear phagocytes) in such lesions has not been studied.

Although the interstitial nephritis is initiated by antibodies, it is still possible that delayed sensitivity could account for the mononuclear cell accumulation. One way in which this could occur is if cell-mediated immunity develops against a conjugate formed by TBM constituents and anti-TBM antibodies.

The present studies on guinea pig anti-TBM disease were undertaken to investigate: a) if there is an early influx of neutrophils (heterophils) preceding the mononuclear cell infiltrate in recipients of anti-TBM serum, b) the time of appearance of mononuclear cells following anti-TBM serum transfer, c) the nature of the infiltrating mononuclear cells and of the giant cells, and d) whether transfer of lymph node cells from donors with anti-TBM disease to normal recipients would result in interstitial nephritis.

**Material and Methods**

**Animals**

Female Hartley strain guinea pigs, weighing 300 to 500 g, were obtained from Camm Research, N.J. and from local commercial suppliers. In some experiments, Strain 13 guinea pigs were used that were obtained from the same sources. Animals were fed standard guinea pig laboratory chow, supplemented with lettuce and alfalfa hay, and had free access to water at all times.

**Preparations of TBM Antigen and Immunization**

Bovine renal cortical tubules were isolated from minced, washed cortex by the sieving method of Krakower and Greenspon, as modified by Spiro. Cortical TBM was prepared by ultrasonic disruption of isolated tubules. After repeated washings in 1.0 M NaCl, followed by distilled water, the TBM antigen was lyophilized and stored at −20°C.

For immunization, each animal received 1 mg TBM antigen in complete Freund’s adjuvant (CFA) (4 mg ml Mycobacterium tuberculosis H37Ra Strain. (Difco Laboratories, Detroit, Mich.) in 1 ml, divided between the two hind footpads and three to four subcutaneous sites on the back. In most experiments in which the active lesions were studied, the animals were sacrificed 14 days after immunization.
Preparation of Anti-TBM Sera for Passive Transfer Experiments

Guinea pigs were immunized as before on Day 0, and then on Day 14 they received an additional 1 mg of TBM antigen in adjuvant, administered at multiple subcutaneous sites. They were exsanguinated via cardiac puncture on Day 21. Sera from groups of 20 to 30 animals were pooled, sterilized either by filtration or by the addition of 1 ml PenStrep Fungizone (Grand Island Biological Company, Grand Island, N.Y.) per 100 ml of serum, divided into 20-ml aliquots, and stored at −70°C until used. The sera had titers of 1:640 or greater of anti-TBM activity, as measured by indirect immunofluorescence.

For passive transfer of disease, animals were given 20 ml of anti-TBM serum, divided equally between intravenous and intraperitoneal routes. Earlier experiments had shown that this amount of anti-TBM serum resulted in a reproducible lesion which affected 25 to 50% of the cortex within 5 days of transfer.

Cell Transfer Experiments

For cell transfer studies, donor Strain 13 guinea pigs were immunized as before, except that the immunizing dose was divided equally among the 4 foot pads. Control donor animals received 1 mg of bovine liver powder in CFA. On Day 9 after immunization, donor animals were anesthetized with ether and bled out via cardiac puncture. All peripheral lymph nodes were harvested and pooled. Cell suspensions were prepared and washed twice with Hanks' balanced salt solution.

Histology

Tissues for light microscopy were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin, the periodic acid–Schiff (PAS) method, and PAS silver methenamine (PASM) method.13 In some experiments, kidneys were also fixed in 0.15 M cacodylate-buffered 2.5% glutaraldehyde (pH 7.3), postfixed in 1.6% osmium tetroxide, dehydrated, embedded in Epon, cut at 1 μ, and stained with toluidine blue.13

Immunofluorescence

Kidney blocks, approximately 3 to 4 mm cubed, were embedded in OCT compound (Tissue Tek, Ames Co., Elkhart, Ind.) and rapidly frozen on the chuck of a cryostat. Three-micron sections were cut, air dried, washed in phosphate-buffered saline (PBS) (pH 7.4), and stained with rabbit anti–guinea pig IgG, rabbit anti–guinea pig C3, and rabbit anti–guinea pig albumin (Cappel Laboratories, Inc., Downington, Pa.). Kidney sections from normal Hartley strain guinea pigs of similar age were used to detect anti-TBM antibodies in serum or eluates by indirect immunofluorescence.

Elution Studies

Antibodies were eluted from kidneys of actively immunized animals with 0.02 M citrate buffer at pH 3.2, according to the method of McPhaul and Dixon.14

Identification of Mononuclear Cells in Tissue Sections

A sheep red cell rosetting technique, performed as previously described,16 was used to attempt to identify receptors on mononuclear phagocytes and B lymphocytes in renal cortical infiltrates and lymphoid tissue. Briefly, sheep red cells were coated with either purified IgM or IgG rabbit antibodies to boiled sheep red cell stromata (donated by Dr. Harvey Colten). IgM–EAC complexes were formed by treating IgM EAC with 1:10 dilution of fresh mouse serum. Four-micron-thick frozen sections of kidneys or spleen were
overlayered with 1% suspensions of IgM EAC, IgM EA, or IgG EA in veronal-buffered saline, pH 7.4 for 30 minutes at 37 C. Sections were washed with PBS, fixed with 3% glutaraldehyde in PBS for 1 hour and stained with hematoxylin and eosin.

**Results**

**Active Lesions**

**Pathologic Findings**

The descriptions are based on histologic examination of 84 guinea pigs, consisting of 14 Strain 13 and 70 Hartley strain guinea pigs. The lesions in kidneys of animals immunized once with 1 mg TBM in CFA 14 days prior to sacrifice, or of animals immunized twice, 14 days apart, and then sacrificed 7 days later, are similar to those previously reported.1-2

Histologically, the kidneys showed a multifocal to diffuse interstitial infiltrate, which was limited to the cortex. The extent of involvement varied from approximately 50 to 90%. On morphologic grounds the majority of the mononuclear cells were considered to be mononuclear phagocytes, since they possessed abundant, pale pink, sometimes finely vacuolated cytoplasm and oval or slightly indented vesicular nuclei (Figure 1). Interspersed between these cells were smaller cells with the morphologic appearance of small lymphocytes. Occasional plasma cells were also present. Only rare neutrophils were seen (Table 1). Peritubular giant cells were always present. Although the percentage of giant cells among infiltrating cells was quite small (Table 1), the giant cells appeared quite prominent since most of them contained three to fifteen nuclei (in cross sections). In 1-μ Epon-embedded sections, occasional mast cells were seen, but no basophils were found. In the areas where there were interstitial infiltrates the tubules showed degenerative changes characterized by vacuolation, swelling, increased acidophilia of cytoplasm, and nuclear pyknosis (Figure 1A). In PAS- and PASM-stained sections, the TBM of such tubules was often fragmented or missing entirely around a portion of the tubule (Figure 1C and 1D).

**Immunofluorescence Findings**

In all actively immunized animals studied, immunofluorescence showed continuous linear staining along the TBM with anti-IgG, and more focal, segmental linear staining with anti-C3, as has been previously reported.1,6 Although some staining for C3 was seen along the TBM in control animals, this was not as bright or as extensive as in the experimental animals. In some animals, faint linear staining for IgG was seen along
Table 1—Differential Leukocyte Count in Renal Interstitial Infiltrates

<table>
<thead>
<tr>
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<th>14-Day actively immunized animals</th>
<th>5-Day TBM serum transfer recipients</th>
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<tbody>
<tr>
<td>Mononuclear cells*</td>
<td>96.7 (95.1–98.3)†</td>
<td>97.3 (96.6–98.2)‡</td>
</tr>
<tr>
<td>Plasma cells</td>
<td>1.1 (0.3–1.9)</td>
<td>0.6 (0.1–0.9)</td>
</tr>
<tr>
<td>Giant cells</td>
<td>1.5 (1.3–1.9)</td>
<td>1.1 (0.4–1.8)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.8 (0.3–1.7)</td>
<td>1.2 (0.8–1.7)</td>
</tr>
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* Mononuclear cells includes monocyte-macrophages and large and small lymphocytes.
† Percent of cells in 1000 total infiltrating cells counted in each of 5 animals; figures represented are means and ranges.
‡ Percent of cells in 1000 total infiltrating cells counted in each of 3 animals; figures represented are means and ranges.

the glomerular basement membrane (GBM). Staining with anti–guinea pig albumin was limited to tubular reabsorption droplets.

Eluted antibody, when tested by indirect immunofluorescence on normal kidney, gave a continuous linear staining pattern along the TBM, as previously reported.¹

Passive Transfer Lesions

Pathologic Findings

The following descriptions are based on histologic examination of 25 guinea pigs that received anti-TBM serum, including 4 Strain 13 and 21 Hartley strain guinea pigs. The renal lesions were qualitatively the same as those in actively immunized animals and differed only in severity.

The earliest lesions were detectable by Day 2 following transfer and consisted of sparse infiltrates of medium to large mononuclear cells around groups of two to three tubules, especially adjacent to inner or mid cortical glomeruli. No giant cells were seen. By Day 3, the lesions were well established (Figure 2), and had increased to involve five to ten tubules per focus. Peritubular giant cells were present. The number of foci had increased so as to involve 5 to 10% of the cortex. There was evidence of tubular cell damage in the areas of cellular infiltration, in the form of increased eosinophilia of tubular cells and vacuolation of cytoplasm.

By Day 5, the interstitial foci had increased in size and number, involving 25 to 50% of the cortex in most instances (Figure 3), with occasional kidneys showing more severe involvement. The infiltrate included cells with the appearance of lymphocytes, as well as occasional plasma cells. More extensive tubular damage was present, and in some areas epithelial cells with pyknotic nuclei were seen. By Day 10 following serum transfer, the lesions were indistinguishable from lesions in 14 or 21 day actively immunized animals.
The question of whether an early neutrophil phase preceded the mononuclear infiltrate was examined. Neutrophils were counted in 100 glomeruli and also in the interstitium in 100 fields of cortical tissue (excluding glomeruli) as examined with a 40× objective lens. Sections were examined from 12 animals at 4 hours, 24 hours, and 5 days following transfer of anti-TBM serum or normal guinea pig serum (2 anti-TBM serum recipients and 2 normal guinea pig serum recipients at each time interval). In addition, neutrophils were counted in kidney sections from 6 actively immunized guinea pigs (2 animals each at 7, 9, and 14 days following immunization) and in 2 normal uninjected control guinea pigs. The results are presented in Table 2. The numbers of neutrophils in recipients of anti-TBM serum did not differ significantly (Z test) from those in recipients of normal serum. The numbers of neutrophils in glomeruli and interstitium of actively immunized animals examined 7, 9, and 14 days following immunization were similar to those in recipients of serum.

The percentage of neutrophils in actively immunized animals was less than 2% in the areas of cellular infiltrate (Table 1). The proportion of various cell types, namely mononuclear cells, plasma cells, giant cells and neutrophils, was similar in actively immunized animals and serum transfer recipients, as examined at 5 days.

Immunofluorescence Findings

In one experiment, animals were examined by immunofluorescence 4 hours and 1, 3, and 5 days following transfer with guinea pig anti-TBM

<table>
<thead>
<tr>
<th>Table 2—Neutrophil Counts in Anti-TBM Nephritis</th>
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<tr>
<td><strong>PMNs/100 Glomeruli</strong></td>
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<tr>
<td>------------------------</td>
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<tr>
<td><strong>Serum transfer recipients</strong></td>
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<tr>
<td>4 hours after</td>
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<tr>
<td>Anti-TBM serum</td>
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<tr>
<td>Normal GP serum</td>
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<tr>
<td>24 hours after</td>
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<tr>
<td>Anti-TBM serum</td>
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<tr>
<td>Normal GP serum</td>
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<tr>
<td>5 days after</td>
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<tr>
<td>Anti-TBM serum</td>
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<tr>
<td>Normal GP serum</td>
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<tr>
<td><strong>Actively immunized animals</strong></td>
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<tr>
<td>7 days</td>
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<tr>
<td>9 days</td>
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<td>14 days</td>
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<td>Normal uninjected controls</td>
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* Counts were performed on 4 to 6 μ, H&E-stained sections at 400× magnification.
† The figures represent the average of two determinations from 2 animals.
serum. The greatest intensity of staining with anti-guinea pig IgG at 4 hours was along the GBM; at this time the TBM showed either no staining or very faint staining, especially in tubules immediately adjacent to glomeruli (Figure 4). At Days 3 and 5, a change in staining pattern was noted. By Day 3, the TBM stained as intensely as the GBM (Figure 5), and by Day 5, the TBM stained more brightly, although the GBM continued to exhibit some staining.

Staining for C3 was patchy and irregularly linear in glomeruli and TBM, but tended to follow the same pattern as IgG. It is not certain that staining for C3 exceeded that in control animals.

Cell Transfer Studies

Lymph node cells from 17 donor Strain 13 guinea pigs immunized with TBM antigen were transferred to 8 normal Strain 13 recipients. In addition, cells from 8 donors immunized with bovine liver powder were transferred to 4 normal recipients. Each recipient received approximately $7.7 \times 10^6$ lymph node cells intravenously. Kidneys were obtained by nephrectomy on Day 2 (4 experimental animals), Day 5 (2 experimental, 2 control), Day 7 (2 experimental, 2 controls), or at sacrifice on Day 7 (4 experimental) or Day 10 (4 experimental, 4 control).

Histologic examination of kidneys revealed no lesions in any of the experimental or control animals. Immunofluorescence studies were not performed.

Sheep RBC Rosetting

In 5 of 6 cases the IgG EA adhered to some of the mononuclear cells in the interstitial infiltrate (Figure 6) but did not adhere to any giant cells. IgM EAC and IgM EA did not adhere to any of the cells in the infiltrate. Sections of spleen treated similarly showed adherence of IgM EAC to the lymphoid follicles but not to sinus macrophages. IgG EA adhered only to macrophages in the red pulp.

Discussion

The results obtained in the present study confirm earlier reports on the transferability by serum of the form of tubulointerstitial disease that can be induced in guinea pigs by immunization with heterologous TBM preparations in adjuvant, showing that the disease is initiated by anti-TBM antibodies. Nevertheless, it is apparent that deposition of anti-TBM antibodies is not sufficient in itself to bring about renal damage, but that secondary pathogenic mechanisms are required. Thus, Hyman et al. reported that Strain 2 guinea pigs show renal accumulation of anti-TBM
antibodies following serum transfer and yet fail to develop lesions.\textsuperscript{16} under similar circumstances Strain 13 recipients develop severe lesions. These authors suggested that sensitized cells are essential for the full expression of the disease. Similarly, immunization of several strains of mice with bovine TBM preparations in adjuvant results in striking accumulation of mouse immunoglobulins along TBM, but not in renal lesions.\textsuperscript{17} The participation of delayed sensitivity also seemed worth exploring because the infiltrate is composed principally of mononuclear cells. However, in the present study no evidence could be obtained for a role of cell-mediated immunity; transfer of $7 \times 10^8$ lymph node cells from TBM-immunized donors to normal Strain 13 guinea pigs failed to result in lesions. Nevertheless, because of the inconstancy of positive results in cell transfer experiments in other autoimmune diseases, the evidence obtained here cannot be considered incontrovertible. Further, as mentioned earlier, it is possible that the delayed reactivity is directed not against normal TBM, but rather against a complex composed of TBM constituents and anti-TBM antibodies. However, the appearance of mononuclear cells in the kidneys of recipients of anti-TBM serum as early as 2 days after transfer argues against this hypothesis. Rudofsky and Pollara have presented evidence that the renal damage depends on infiltration by circulating leukocytes; thus, irradiation of prospective recipients results in failure of development of tubular or interstitial lesions following transfer of anti-TBM serum, despite the accumulation of antibodies in the kidney.\textsuperscript{18} Further, in both actively and passively produced disease, tubular cell damage is apparent only in areas of cellular infiltration.

The nature of the infiltrating leukocytes was explored in the present study. Histologic studies failed to disclose any appreciable accumulation of neutrophils, even when animals were examined shortly after transfer of anti-TBM serum. In experimentally induced anti-TBM induced disease in rats,\textsuperscript{3,4} neutrophils are often conspicuous early, as in most other forms of antibody-mediated tissue damage, even in those cases where mononuclear cells eventually predominate.\textsuperscript{7,9} On histologic grounds, most of the mononuclear cells in guinea pig anti-TBM disease appear to be mononuclear phagocytes (monocytes or macrophages), although some cells with the appearance of small lymphocytes are also present. However, the distinction between monocytes and large lymphocytes is not always possible in histologic sections.

Recently, techniques have been developed that permit the identification of surface receptors on mononuclear phagocytes and on B lymphocytes in frozen tissue sections through the use of sheep erythrocytes
(E) coated with rabbit antibody (EA) or with antibody and complement (EAC), which form rosettes around the appropriate cells. The reagent IgG EA is used to detect receptors for Fc on mononuclear phagocytes, whereas the reagent IgM EAC is used to reveal receptors for C3 present on both mononuclear phagocytes and B lymphocytes. For some unexplained reason, however, IgM EAC does not react with all mononuclear phagocytes in tissue sections, as in the spleen, or in some cases in inflammatory infiltrates. In the present study, it was shown that many of the infiltrating cells react with IgG EA, indicating that they are monocytes or macrophages. The importance of mononuclear phagocytes is also supported by studies of Rudofsky and Pollara, who showed that reconstitution of irradiated recipients by bone marrow (but not by spleen) restored their capacity to develop infiltrates after transfer of anti-TBM serum.

The lack of rosetting with IgM EAC fails to provide evidence for the presence of B lymphocytes. However, this does not exclude any participation of B cells: first, because lesions were examined at only one interval (14 days), second, because the C3 receptors may have been blocked, and third, because either very immature or highly differentiated B cells (plasma cells) may lack the surface receptor. Indeed, plasma cells were found in small numbers at various stages of the actively or passively produced disease. The techniques used here would not permit detection of T lymphocytes.

The nature of the giant cells was not clarified in the present study; the lack of reactivity with IgG EA or IgM EAC fails to provide evidence that they were derived from macrophages but does not exclude this. Electron microscopic studies of rat anti-TBM disease led to the conclusion that the giant cells originate by fusion of monocytes.

The mechanisms responsible for the accumulation of monocytes were not elucidated in the present or previous studies. Although factors chemotactic for monocytes are generated by the complement system, it is not clear that these are obligatory in anti-TBM disease, since typical infiltrates can be seen in the absence of demonstrable C3 in renal tissue.

The sequence of accumulation of immunoglobulins in renal tissue following transfer of anti-TBM serum was unexpected. Initially (at 4 hours), there was fairly bright staining of glomerular basement membranes for IgG, and staining along tubular basement membranes was seen only in tubules adjacent to glomeruli. Later, glomerular staining disappeared and there was bright staining along all proximal tubular basement membranes. These findings can be explained by assuming that antibodies are directed against constituents of both GBM and TBM, but predominantly the latter (as evidenced also by their in vivo staining pattern
in actively immunized animals), and that the initial GBM staining results from the greater access of antibodies to the GBM because of the filtration function of the glomerulus.

References

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Figure 1A—Section of renal cortex from a guinea pig immunized 14 days earlier with bovine TBM in CFA. There is a marked interstitial infiltration by macrophages, lymphocytes, peritubular giant cells, and occasional plasma cells. Several tubules are collapsed, with some cells containing pyknotic nuclei. (H&E, × 250)  
B—Epon-embedded section of renal cortex from a 14-day actively immunized guinea pig, with a similar interstitial infiltrate as in A, showing more clearly the nature of these infiltrating cells. Slight interstitial edema is also present. (Toluidine blue, × 400)
Figure 1C—Renal cortex from the same animal shown in A, demonstrating fragmentation and disappearance of TBM (arrows). (PASM, x 400)  
D—Same animal shown in A and C, depicting a giant cell, associated with absence of the TBM (arrows). (PASM, x 400)  
Figure 2—Section of renal cortex from a guinea pig 3 days following passive transfer of 20 ml of guinea pig anti-TBM serum. There is a mild mononuclear interstitial infiltrate with giant cell formation. (H&E, x 250)
Figure 3—Section of renal cortex from a guinea pig 5 days following passive transfer of 20 ml of guinea pig anti-TBM serum. The infiltrate is more extensive and contains more giant cells than in Figure 2, but is similar in nature. (H&E, × 100)

Figure 4—Frozen section of kidney obtained 4 hours following transfer of guinea pig anti-TBM serum. Staining for IgG is seen predominantly in the glomerulus, in a linear pattern. There is only minimal, irregular TBM staining. (× 250)
Figure 5—Guinea pig kidney, stained for IgG, 3 days following transfer of guinea pig anti-TBM serum. The TBM and GBM show bright staining. (× 250)

Figure 6—Section of guinea pig kidney with anti-TBM nephritis, showing adherence of IgG EA to mononuclear cells in the interstitial infiltrate. The adherent red blood cells are seen as bright discs. (Darkfield illumination, × 400)