EVIDENCE FOR A PATHOGENIC ROLE OF A CELL-MEDIATED IMMUNE MECHANISM IN EXPERIMENTAL GLOMERULONEPHRITIS*

By ATUL K. BHAN, EVELINE E. SCHNEEBERGER, ALFRED B. COLLINS, AND ROBERT T. McCLUSKEY

(From the Departments of Pathology, Massachusetts General Hospital, and Harvard Medical School, Boston, Massachusetts 02114, and The Children's Hospital Medical Center, Boston, Massachusetts 02115)

Studies performed in the past two decades in animal models and in man have established the occurrence of two major forms of immunologically mediated glomerular disease; one results from the accumulation of antigen-antibody complexes in glomeruli (immune complex glomerulonephritis) and the other from the reaction of antibodies with constituents of the glomerular basement membrane (anti-GBM disease). Although it has been suggested that cell-mediated immunity (delayed hypersensitivity) may also produce glomerular injury (1, 2) clear-cut evidence for this mechanism has not been presented, except in renal allografts, and even in this setting only under special circumstances (3). Furthermore, in most morphologic studies of glomerulonephritis no mention is made of accumulation of mononuclear cells in glomeruli, and this has been used as an argument against a role of cell-mediated mechanisms (2, 4). Infiltrating mononuclear cells have, however, been described in glomeruli in some forms of experimental glomerular disease (5-10), although this provides, at best, indirect evidence for the operation of cell-mediated mechanisms.

We have carried out studies based on a model of glomerular disease in rats that is characterized by conspicuous glomerular accumulation of mononuclear cells (10, 11). The disease is produced by intravenous injection of subnephritogenic doses of rabbit anti-rat GBM antiserum into rats that had been previously immunized with rabbit gamma globulin in adjuvant. Employing a modification of this model in which nonimmunized rats that had received subnephritogenic doses of rabbit anti-GBM antiserum were given lymph node cells from donors immunized with rabbit gamma globulin, we have obtained evidence that cell-mediated mechanisms participate in the production of glomerular lesions.

Materials and Methods

Animals. Female Lewis strain rats, weighing between 140 and 200 g (Microbiological Associates, Walkersville, Md.), were used in all experiments.

* Supported by U. S. Public Health Service grants CA 19393, AM 18729, and AM 16392.

Abbreviations used in this paper: C3, third component of complement; GBM, glomerular basement membrane; HBSS, Hanks' balanced salt solution; OVA, ovalbumin; PBS, phosphate-buffered saline; RER, rough endoplasmic reticulum; RGG, rabbit gamma globulin.
Preparation of Rabbit Anti-Rat GBM Antiserum. Rat GBM antigen preparation was obtained by a modification of the method of Krakower and Greenspon (12), using Sprague-Dawley rats of both sexes (Charles River Breeding Laboratories, Inc., Wilmington, Mass.). The kidneys were perfused with ice-cold phosphate-buffered saline (PBS), pH 7.3. Pooled cortical tissue was forced through stacked mold metal sieves, 150, 100, and 200 mesh respectively, yielding a glomerular-rich fraction. The fraction was then washed four times with PBS, centrifuged at 800 rpm for 5 min, suspended in a small volume of distilled water, and then sonicated to disrupt glomerular cells. The resultant preparation was washed several times with distilled water and lyophilized. New Zealand white rabbits were injected in the foot pads or subcutaneously with the GBM preparation in complete Freund's adjuvant, each rabbit receiving the equivalent of the GBM obtained from 10 rat kidneys; they were boosted on days 14 and 21 with the same dose, and bled on day 28. All lots of sera were combined into two pools (A and B), which were absorbed with washed rat erythrocytes and with a lyophilized rat tubular basement membrane preparation. The capacity of the antisera to react with rat GBM in vivo and to produce histologic lesions was investigated by intravenous injections of 0.25-1 ml of the antisera into Sprague-Dawley rats; the animals were sacrificed 4 h to 4 days later. Frozen sections of kidney were stained with fluorescein-labeled goat anti-rabbit gamma globulin. Typical linear staining of the GBM was seen after injection of all doses of antisera. No histologic abnormalities were present in the kidneys in the 4-day period of observation.

Donor Rats. Groups of female Lewis rats were immunized with 100 µg of rabbit gamma globulin (RGG) or ovalbumin (OVA) (Miles Laboratories, Inc., Elkhart, Ind.) in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) in a total volume of 0.4 ml, divided equally among the four foot pads. In addition, 0.05 ml of Bordetella pertussis vaccine (Massachusetts State Health Labs., Jamaica Plain, Mass.), at a concentration of $2.0 \times 10^{10}$ organisms/ml, was injected subcutaneously in the dorsum of each foot. 7 days later, the rats were killed to provide lymph node cells or serum for use in transfer experiments. Cell suspensions were prepared from popliteal and axillary lymph nodes by mincing; the cell suspensions were passed through 60-gauge mesh and washed twice with Hanks' balanced salt solution (HBSS). In one experiment, nylon wool columns were used according to the method of Julius et al. (13) to obtain preparations enriched in T cells. 5% of the recovered cells showed surface immunofluorescence for Ig using fluorescein-labeled goat anti-rat gamma globulin, as compared with 22% of the whole lymph node cell suspension. Cell viability was assessed by the trypan blue exclusion test and was always >80%. The level of antibodies against RGG in the pooled sera of the RGG donors was determined by the quantitative precipitin technique (14) and was found to be 0.14 mg/ml.

Autoradiography. Estimation of the degree of increase in cells or the presence of recently proliferated cells within glomeruli was facilitated by the study of autoradiographs, prepared from renal tissue of the recipient rats that received three injections of [3H]thymidine (1 µCi/g body weight, sp act 6.7 Ci/mmol; New England Nuclear, Boston, Mass.) after the time of cell transfer. Autoradiographs were prepared both from paraffin- and plastic-embedded tissue. With paraffin-embedded material, 4-µm unstained sections were dipped in NBT-2 Kodak Nuclear track emulsion and exposed for 2 wk. After development, the sections were stained with hematoxylin and eosin. With plastic-embedded tissue, 1-µm thick sections were mounted on glass slides and stained with periodic acid-Schiff-iron hematoxylin (15). The slides were dipped in total darkness in undiluted NTB-2 emulsion, air-dried for 24 h, packed in light tight Bakelite boxes containing Drierite, and exposed for 3-5 wk. All autoradiographs were developed with Dektol developer (Eastman Kodak Co., Rochester, N. Y.).

Processing of Tissue. For immunofluorescence studies, specimens of renal cortex were obtained immediately after sacrifice and frozen in OCT Compound (Ames Co., Div. of Miles Lab., Inc., Elkhart, Ind.) on a cryostat holder; 4-µm sections were cut and stained with fluorescein-conjugated antisera to RGG, rat IgG, rat third component of complement (C3), and rat fibrinogen (N. L. Cappel Laboratories Inc., Cochranville, Pa.).

For electronmicroscopic and autoradiographic studies, tissue was obtained under ether anesthesia just before sacrifice. The left renal pedicle was clamped and the kidney was removed. 1-mm thick slices were immediately fixed in formaldehyde-glutaraldehyde fixative (16), diluted 1:1 with 0.1 M cacodylate buffer, pH 7.3, for 3 h at 4°C. They were then washed overnight in 0.15 M cacodylate buffer, pH 7.3. Small fragments of tissue (1-mm³) were post-fixed in 1.3% OsO₄ in s-collidine buffer for 1 h at 4°C. They were dehydrated in graded alcohol, and embedded in Epon.
Thin sections were cut, picked up on carbon-coated grids, and stained with uranyl acetate and lead citrate. They were examined in a Philips EM-300 electron microscope (Philips Electronic Instruments, Inc., Mount Vernon, N. Y.).

For light microscopy, thin slices of renal cortex obtained immediately after sacrifice were fixed in 10% buffered formalin, embedded in paraffin, cut at 4 μm, and stained with hematoxylin and eosin.

Statistical analysis. The observed counts were transformed to stabilize the variances using a square-root transformation (17). Groups within an experiment were compared by one-way analysis of variance on the transformed data, and then the statistical significance between all pair-wise comparisons of group means was performed by Tukey’s t test (18). This test was used instead of the standard Student’s t test, because it takes into consideration the fact that more than two groups are to be compared. The means of the transformed data were squared to convert them to the original counts, and are shown in the tables as “mean converted to original scale.” The level of significance used was P < 0.05.

Results

Experiment I. The basic aim of this experiment was to determine if the transfer of lymph node cells from rats sensitized to RGG into nonimmunized recipients that had received subnephritogenic doses of rabbit anti-GBM antiserum (and which had therefore RGG fixed in their glomeruli) would result in glomerular lesions. Normal Lewis rats were injected intravenously with 0.5 or 1.0 ml of anti-GBM antiserum (pool A). 24 h later, these animals were divided into five groups which were treated as follows: rats in groups I and II were injected intravenously with 400-500 x 10⁶ lymph node cells from donors immunized with RGG or OVA, respectively; rats in groups III and IV were injected with 7 ml of sera (4 ml intraperitoneally and 3 ml intravenously) from the RGG or OVA donors, respectively; rats in group V were injected intravenously with 2 ml of HBSS. Rats in group VI were not given anti-GBM antiserum and received only HBSS (2 ml intravenously). Most of the animals were sacrificed 48 h after the time of transfer; a few were killed at 96 h.

Histologic findings (Table I). Unequivocal histologic abnormalities in the kidneys were found only in rats that received anti-GBM antiserum followed by lymph node cells from donors immunized with RGG (group I); among rats killed 48 h after cell transfer, abnormalities were seen in 11 surviving rats (from a group of 12) that had received 1 ml of anti-GBM antiserum, and in two of four animals that had received 0.5 ml. The abnormalities were confined to glomeruli. The lesions were irregular and were characterized by segmental hypercellularity in most glomeruli (Fig. 1) and by segmental necrosis in a small percentage of glomeruli (Fig. 2). Two additional rats (not shown in Table I) received 1 ml of anti-GBM serum and were killed 96 h after cell transfer; in both, more severe glomerular abnormalities were seen, affecting all glomeruli. The areas of hypercellularity contained mononuclear cells, but it could not be determined to what extent they were infiltrating cells or mesangial and endothelial cells that had proliferated.

Several donor and recipient rats (in group I) were skin tested 24 h before sacrifice with 25-100 μg of RGG. Histologic sections of the sites obtained 24 h after challenge showed inflammatory reactions with a predominantly mononuclear cell infiltrate, consistent with delayed hypersensitivity reactions.

Immunofluorescence findings. In all rats that received rabbit anti-rat GBM antiserum (groups I-V), intense linear staining for RGG was found along
### Table I

*Experiment I: Renal Histologic and Immunofluorescence Findings in Rats Given Anti-GBM Antiserum with or without Cell or Serum Transfer 24 h Later*

| Experimental group                     | Amount of anti-GBM antiserum (pool A) | Number of animals | Number with histologic glomerular lesions | Linear GMB staining |
|----------------------------------------|---------------------------------------|-------------------|------------------------------------------|---------------------|
| I. Anti-GBM antiserum + LN cells from RGG donors§ | 1 ml                                  | 12†               | 11                                       | ++++                |
|                                      | 0.5 ml                                | 4                 | 2                                        | ++++               |
| II. Anti-GBM antiserum + LN cells from OVA donors | 1 ml                                  | 4                 | 0                                        | ++++               |
|                                      | 0.5 ml                                | 4                 | 0                                        | ++++               |
| III. Anti-GBM antiserum + serum from RGG donors | 1 ml                                  | 8                 | 0                                        | ++++               |
| IV. Anti-GBM antiserum + serum from OVA donors | 1 ml                                  | 3                 | 0                                        | ++++               |
| V. Anti-GBM antiserum + HBSS           | 1 ml                                  | 12†               | 0                                        | ++++               |
|                                      | 0.5 ml                                | 4                 | 0                                        | ++++               |
| VI. HBSS                               | none                                  | 8                 | 0                                        | 0                  |

* Animals were sacrificed 48 h after the time of transfer.
† One animal died before the end of the experiment.
§ Donors had been immunized 7 days earlier with 100 μg of RGG or OVA. 400-500 × 10⁶ lymph node (LN) cells or 7 ml of serum were transferred. Serum from rats immunized with RGG contained 0.14 mg anti-RGG antibody/ml.

![Figure 1](image.jpg)

**Fig. 1.** Segmental increase in cellularity of a glomerulus from a recipient of 1.0 ml of anti-GBM antiserum and lymph node cells from donors sensitized to RGG (group I, experiment I). Hematoxylin and eosin used for staining. × 630.
FIG. 2. Segmental necrosis in a glomerulus from a recipient of 1.0 ml of anti-GBM antiserum and lymph node cells from donors sensitized to RGG (group I, experiment I). Hematoxylin and eosin used for staining. x 630.

the GBM (Table I). The animals in group III (anti-GBM antiserum plus serum from RGG donors) also showed, as anticipated, linear staining (2+) for rat IgG. Unexpectedly, the rats in group I, (anti-GBM antiserum followed by cells from RGG donors) also showed linear GBM staining for rat IgG (1+). This finding led to another group of experiments, as described below. In addition, there was mild, focal granular mesangial staining for rat IgG in animals in all groups; this appeared to be slightly more intense in the animals in group I. In none of the animals was there appreciable C3 staining in glomeruli. Irregular, generally mild staining with anti-fibrinogen serum was seen in the glomeruli of some of the animals in group I, apparently in areas of segmental necrosis.

AUTORADIOGRAPHIC STUDIES. Autoradiographic studies were also performed on renal tissue of the animals described above; the recipient rats received three injections of [3H]thymidine at 18, 27, and 44 h after the time of transfer. The results of these studies confirmed the histological observations by revealing increased numbers of labeled cells within glomeruli in the rats in group I; in addition, the autoradiographs revealed significant increases in other groups in which definite histologic changes had not been apparent (Table II). Thus, appreciable increases in labeled cells were found in the glomeruli of all animals that had received anti-GBM serum, as compared with rats injected only with HBSS (group VI). The animals given 1 ml of anti-GBM serum had about one and a half times as many labeled cells in glomeruli as those given 0.5 ml. For a given dose of anti-GBM antiserum, the rats in group I (anti-GBM antiserum plus lymph node cells from RGG donors) were found to have significantly
TABLE II
Experiment I: Number of Labeled Cells in Glomeruli*

| Amount of anti-GBM antiserum (Pool A) | Group I: Anti-GBM antiserum + LN cells (RGG) | Group II: Anti-GBM antiserum + LN cells (OVA) | Group III: Anti-GBM antiserum + serum (RGG) | Group IV: Anti-GBM antiserum + serum (OVA) | Group V: Anti-GBM antiserum + HBSS only | Pooled estimate of SD for all groups excluding VI |
|--------------------------------------|---------------------------------------------|---------------------------------------------|------------------------------------------|------------------------------------------|--------------------------------------|---------------------------------|
| 1 ml                                 | Number of labeled cells/100 glomeruli        | Mean in transformed scale†                  | Mean converted to original scale         | Mean converted to original scale         |                                      |                                 |
| 633                                  | 247                                         | 292                                         | 177                                      | 312                                      | 45                                   | ±2.62                           |
| 639                                  | 233                                         | 613                                         | 215                                      | 147                                      | 43                                   |                                 |
| 251                                  | 304                                         | 320                                         |                                          |                                          |                                      |                                 |
| Mean                                 | 25.63                                       | 16.01                                       | 19.43                                    | 13.95                                    | 14.42                                | 6.74 ± 2.62                     |
| Mean converted to original scale     | 657                                         | 256                                         | 378                                      | 195                                      | 208                                   | 45                              |
| 0.5 ml                               | Number of labeled cells/100 glomeruli        | Mean in transformed scale†                  | Mean converted to original scale         | Mean converted to original scale         |                                      |                                 |
| 348                                  | 144                                         | N.D.§                                       | N.D.                                      | 104                                      | 34                                   |                                 |
| 445                                  | 143                                         | N.D.                                        | N.D.                                      | 100                                      | 51                                   |                                 |
| 415                                  | 137                                         | N.D.                                        | N.D.                                      | 156                                      | 37                                   |                                 |
| 407                                  | 136                                         | N.D.                                        | N.D.                                      | 84                                       | 49                                   |                                 |
| Mean                                 | 20.07                                       | 11.83                                       | 10.46                                    | 6.51 ± 1.02                              |                                      |                                 |
| Mean converted to original scale     | 403                                         | 140                                         | 109                                      | 42                                       |                                      |                                 |

* 1 uCi of [3H]thymidine/g body weight was given at 18, 27, and 44 h after transfer of lymph node (LN) cells or serum. Autoradiographs were exposed for 2 wk.

† Mean obtained from square-root transformation of observed counts. Statistical significance (Tukey's t adjusted for making all possible comparisons): group I vs. II, IV, V, and VI = P < 0.05. Group I vs. III = not significant.

§ N.D., not determined.

greater numbers of labeled cells than any of the other groups. The animals with the next highest numbers were in group III (anti-GBM antiserum plus serum from RGG donors).

Autoradiographs of 1-μm thick plastic-embedded sections were examined at ×1,000 magnification to determine the precise location and nature of the labeled cells in glomeruli. 6–20 glomeruli from each of two animals from all of the five groups were studied. Cells lining the capillary lumen whose nuclei were toward the mesangial region were provisionally identified as endothelial cells (Fig. 3). Rats receiving anti-GBM antiserum followed by serum from donors immunized with RGG (group III) had the greatest increase in the number of labeled cells identified as endothelial cells. Except for the normal control rats, all animals had labeled cells present within the mesangium as well, and these
CELL-MEDIATED GLOMERULAR INJURY

FIG. 3. Autoradiograph of a glomerulus from a rat that had received 1.0 ml of anti-GBM antiserum and serum from donors sensitized to RGG (group III, experiment I). Many of the labeled cells are endothelial cells or are attached to endothelial cells (arrow heads). × 1,000.

were particularly numerous in group I, and to a lesser extent in group III (Fig. 4). A few labeled cells were seen within glomerular capillary lumens in all animals, including the normal controls. There were no labeled epithelial cells in glomeruli in any of the groups.

Electronmicroscopic observations. The most severe ultrastructural changes in glomeruli were seen in the animals from group I (anti-GBM antiserum plus RGG cells). The endothelial cells were prominent, and showed increased amounts of rough endoplasmic reticulum (RER). However, the endothelium was intact in all glomeruli examined, and fenestrae were readily identified. In several capillary loops, a neutrophil and/or a monocyte was wedged between the glomerular basement membrane and the overlying endothelium. Such cells were also seen free within capillary lumens. The mesangium contained increased numbers of cells, some of which had ultrastructural features of monocytes or macrophages, including an oval nucleus with peripherally clumped chromatin, and a moderate to a large amount of cytoplasm containing a few lysosomes. Also present were cells with a moderate amount of cytoplasm, an oval nucleus, and moderate amounts of RER; these cells could not be
FIG. 4. Autoradiograph of a glomerulus from a rat that had received 1.0 ml of anti-GBM antiserum and lymph node cells from donors immunized with RGG (group I, experiment I). Most of the labeled cells are in the mesangium (arrow heads). × 1,000.

classified. In some areas, mesangial cells were seen extending long processes to neighboring capillaries; the processes contained bundles of microfilaments (Fig. 5). Similar changes were seen in glomeruli in the other four experimental groups (groups II-V), although fewer cells were present in the mesangium, particularly in those animals receiving anti-GBM antiserum alone (group V). In the control animals (group VI), only a single cell resembling a macrophage was observed in the mesangium of one glomerulus. No mesangial deposits with features suggesting immune complex depositio were found in any of the groups.

Experiment II. To explore the possibility that T-cell preparations were capable of inducing glomerular abnormalities similar to those seen with whole lymph node cell preparations, an experiment was carried out employing lymph node cells enriched in T cells, prepared by the use of nylon wool columns (13). Lewis rats were given 0.5 ml of anti-GBM antiserum (pool B); 24 h later, the animals were divided into three groups. The rats in group IA were given 400 × 10⁶ lymph node cells from RGG donors; those in group IIA, 200 × 10⁶ of T-enriched cells from RGG donors (which we estimated to be less than the number of T cells present in the whole lymph node preparation); and rats in group IIIA received HBSS; the rats in group IVA were injected with HBSS alone. All
Fig. 5. Electronmicrograph of a portion of a glomerulus from a rat that had received anti-GBM antiserum and lymph node cells from donors immunized with RGG (group I, experiment I). The endothelial cell (E) is prominent and contains abundant endoplasmic reticulum (RER). Two monocytes (M) are present in the capillary lumen, and contain substantial RER and few granules. Two other cells with the morphological appearance of monocytes (M') are present immediately below the endothelium and in the mesangium. Another endothelial cell (E') borders a partially compressed capillary lumen. x 7,900.
### TABLE III

**Experiment II: (T-Cell Transfer) Number of Labeled Cells in Glomeruli**

|                     | Group IA: anti-GBM antiserum* + LN cells (400 x 10⁶) | Group IIA: anti-GBM antiserum + T cells (200 x 10⁶) | Group IIIA: anti-GBM antiserum + HBSS | Group IVA: HBSS only |
|---------------------|------------------------------------------------------|--------------------------------------------------|-------------------------------------|---------------------|
| Number of labeled cells/100 glomeruli | 118                                                  | 115                                              | 60                                  | 37                  |
|                     | 149                                                  | 115                                              | 69                                  | 30                  |
|                     | 121                                                  | 84                                               | 46                                  | 36                  |
|                     | 230                                                  | 126                                              | 79                                  | 35                  |
| Mean in transformed scale† | 12.31                                                | 10.46                                            | 7.93                                | 5.87                |
| Mean converted to original scale‡ | 152                                                  | 109                                              | 63                                  | 34                  |

* Each animal received 0.5 ml of anti-GBM antiserum (pool B).
† Whole lymph node (LN) cell suspensions from RGG donors.
§ T-cell-enriched suspensions from RGG donors obtained by use of nylon wool columns.
¶ Each animal received [³H]thymidine at 18, 27, and 44 h after cell transfer. Autoradiographs were exposed for 2 wk.
† Mean obtained from square-root transformation of observed counts. Statistical significance (Tukey's t adjusted for making all possible comparisons): group IA vs. IIIA and IVA = P < 0.05.
Group IA vs. group IIA = not significant.

animals received [³H]thymidine at 18, 27, and 44 h after cell transfer. No definite histologic abnormalities were found (in comparison with experiment I in which all animals receiving 1.0 ml of anti-GBM antiserum showed histologic lesions, in experiment II, the animals received only 0.5 ml of anti-GBM antiserum; this was done to reduce the background injury from anti-GBM antibodies; furthermore, a different pool was used). Immunofluorescence revealed linear GBM staining for rabbit and rat IgG in groups IA and IIA, and for rabbit IgG only in group IIIA. In autoradiographs, it was found that animals given either lymph node cells or T-enriched cells showed appreciably more labeled cells than those given anti-GBM antiserum alone (Table III) and there was no significant difference between groups IA and IIA. It should be noted the pool B anti-GBM antiserum (group IIIA) did not result in as great an increase in labeled cells as pool A, which was used in experiment I.

**Experiment III.** As noted above, the rats in group I in experiment I (anti-GBM antiserum plus lymph node cells from RGG donors) were found to have linear GBM accumulation of rat IgG, indicating that they had produced antibodies against RGG. Since this group received an intravenous injection of a large number of lymph node cells, many of which were activated, we considered the possibility that the glomerular abnormalities resulted not entirely from a reaction of specifically sensitized cells with RGG fixed in glomeruli, but from a reaction of some of the injected lymph node cells of various specificities with glomerular basement membrane-bound immune complexes consisting of rat IgG and RGG. To explore this possibility, the following studies were performed. Preliminary observations showed that the injection of 0.5 ml of serum from RGG donors (containing 0.14 mg/ml antibody protein) into rats that had received rabbit anti-GBM antiserum 24 h earlier resulted in linear GBM...
CELL-MEDIATED GLOMERULAR INJURY

TABLE IV
Experiment III: Labeled Cells in Glomeruli

| Group | Description | Number of labeled cells/100 glomeruli | Mean in transformed scale | Mean converted to original scale |
|-------|-------------|--------------------------------------|--------------------------|----------------------------------|
| IB    | anti-GBM antiserum + LN cells (RGG) + anti-RGG antiserum | 377 | 14.70 | 216 |
| IIB   | Anti-GBM antiserum + LN cells (OVA) + anti-RGG antiserum | 127 | 10.28 | 106 |
| IIIB  | Anti-GBM antiserum + anti-RGG antiserum | 152 | 10.62 | 113 |
| IVB   | HBSS + anti-RGG antiserum | 52 | 6.66 | 44 |

* Each animal in groups IB, IIB, and IIIB received 0.5 of anti-GBM antiserum (pool B), and animals in all groups received 0.5 ml of serum from animals immunized 7 days previously with RGG in CFA, 24 h after lymph node (LN) transfer and [3H]thymidine administration. Statistical significance (Tukey's t adjusted for making all possible comparisons): group IB vs. group IIB = P < 0.05.

staining for rat IgG with about the same intensity (1+) as had been observed in group I in experiment I. Additional rats were then injected intravenously with 0.5 ml of rabbit anti-GBM antiserum; 24 h later, two groups (IB and IIB) received 400 × 10⁶ lymph node cells from RGG or OVA donors, respectively; at 48 h, each group was given 0.5 ml of the serum containing anti-RGG antibodies that had been tested earlier; the animals were killed at 72 h. Rats in group IIIB received the same injections of antisera, but were not given lymph node cells. Rats in group IVB received no anti-GBM antiserum or lymph node cells, but were given 0.5 ml of rat anti-RGG antiserum. All the animals were injected with [3H]thymidine at 18, 27, and 44 h after the time of cell transfer. By immunofluorescence, linear GBM staining for rabbit and rat IgG was seen in animals in groups IB, IIB, and IIIB. In histologic preparations, no definite abnormalities were found, although there was equivocal glomerular hypercellularity in the rats in group IB. The results of the autoradiographic studies are shown in Table IV. As before, increased numbers of labeled cells were found in all animals that received anti-GBM antiserum, as compared with the controls (group IVB). However, the greatest increase was seen in animals in group IB (antisera plus RGG lymph node cells), and there were no significant differences between groups IIB (antisera plus OVA lymph node cells) and IIIB (antisera only).

Discussion

We have shown that the transfer of lymph node cells from rats sensitized to RGG into syngeneic recipients with RGG fixed in their glomeruli (present as anti-GBM antibodies in amounts too small to produce histologically detectable lesions) results in glomerular hypercellularity, sometimes together with seg-
mental necrosis. In support of the interpretation that these abnormalities were produced by a hypersensitivity reaction of the cell-mediated (delayed) type, are the following observations: (a) the reaction exhibited specificity, as shown by the finding that cells from donors sensitized to an unrelated antigen (OVA) failed to result in abnormalities; (b) T-cell-enriched cell suspensions from RGG donors were effective in producing the reaction; (c) both the donors and recipients of lymph node cells exhibited delayed-type hypersensitivity reactions after intradermal challenge with RGG; (d) the glomerular reactions were characterized by accumulation of mononuclear cells.

The recognition and characterization of the glomerular abnormalities were greatly facilitated by autoradiographic studies of renal tissue obtained from the recipients, which had been given $[^3H]$thymidine after cell or serum transfer. In many animals, significant increases in the number of labeled cells in glomeruli were demonstrated even though no definite histologic abnormalities were present. Aside from the fact that it is easier to count a relatively small number of labeled cells in glomeruli rather than the total number, autoradiographic studies permit detection of cells that have proliferated; some of these cells might have replaced destroyed cells, and this would not necessarily contribute to an increase in the overall number of glomerular cells. The labeled cells found in glomeruli could represent either glomerular cells that had proliferated, or cells that were labeled while proliferating in the bone marrow, lymphoid tissue, or circulation, which subsequently localized in glomeruli. However, the timing of injections of $[^3H]$thymidine used in the present experiments, which began after transfer, would probably favor the labeling of proliferating glomerular cells rather than infiltrating cells. Nevertheless, electron microscopic observations favor the interpretation that most of the increase in mesangial cells seen in group I resulted from an influx of mononuclear cells (probably monocytic) from the circulation, rather than from proliferation of mesangial cells. However, definite proof concerning the nature of these cells must await studies in which distinctive cell markers are identified.

In the model employed, there was an underlying component of glomerular injury produced by the rabbit anti-GBM antiserum, even though this was not detected in histologic sections; thus, increased numbers of labeled cells were found in glomeruli in all groups given anti-GBM antiserum, as compared with normal control rats. Furthermore, in two groups there also occurred the combination of rat anti-RGG antibodies with glomerular-bound RGG, as evidenced by linear GBM staining for rat IgG. In group III this was anticipated, since anti-RGG antibodies were transferred. Unexpectedly, rats in group I (anti-GBM antiserum plus RGG cells) also exhibited linear GBM staining for rat IgG. However, it does not seem that the antibodies found in these animals could have accounted for all the glomerular abnormalities, since rats in group III which received large amounts of rat anti-RGG antisera (and which showed very intense GBM staining for rat IgG) failed to develop abnormalities of comparable severity and appearance. The possibility that the increased glomerular damage seen in group I could have been initiated by interaction of glomerular basement membrane-bound rat IgG anti-RGG complexes with lymphocytes sensitized to irrelevant antigens was considered, because of the large number of cells (many of which were activated) injected into these
animals. However, this explanation seems unlikely for the following reasons:
(a) most of the injected cells had probably disappeared from the circulation before the time rat anti-RGG was produced and fixed in glomeruli; (b) the administration of rat anti-RGG to recipients of rabbit anti-GBM antiserum and lymph node cells from OVA donors (group IIB) failed to produce comparable glomerular alterations.

There remains one other mechanism by which the heightened glomerular damage seen in animals in group I might have been antibody-mediated rather than cell-mediated; namely through the glomerular deposition of circulating immune complexes containing RGG and rat anti-RGG antibodies. Thus, it is probable that the amount of rat anti-RGG antibodies formed in the animals in group I was sufficiently small to lead to the formation of soluble complexes with the RGG still present in the circulation. In contrast, in the animals in group III, which received a large amount of rat anti-RGG, large insoluble complexes were probably formed, with rapid reticuloendothelial system clearance. Despite these considerations, it seems highly unlikely that there was appreciable glomerular deposition of immune complexes in the rats in group I, since there was only faint irregular mesangial staining for the rat IgG in these animals, and this was only equivocally greater than in the other groups. Furthermore, there were no electron-dense deposits in glomeruli with features of immune complexes. Further evidence against the possibility that the changes in group I were due to circulating immune complexes is provided by the finding that rats in group IIB (experiment III), which received small amounts of rat anti-RGG antibodies after having received rabbit anti-GBM antiserum, failed to develop similar changes.

The mechanisms by which the cell-mediated reaction mediated the glomerular abnormalities are not clear. Based on studies of delayed hypersensitivity reactions elicited in the skin, it is reasonable to assume that contact between antigen (RGG) and a small number of specifically sensitized cells initiated the reaction, which led to the accumulation of mononuclear phagocytes and of lymphocytes of other specificities (19). In addition, it appears that proliferation of glomerular cells was stimulated, possibly through the release of lymphokines. Proliferation of endothelial cells has been shown to be a feature of cutaneous delayed reactions (20, 21). Although it appears most likely that the glomerular changes observed in the present experiments were initiated by contact between sensitized cells and antigen (RGG) fixed in glomeruli, it is also possible that systemic release of lymphokines, resulting from interaction of sensitized cells and RGG in the circulation, may have contributed to the glomerular reaction.

The relevance of the present observations to other forms of experimental or human glomerular disease is uncertain. The possibility that cell-mediated mechanisms may participate in human anti-GBM disease is worth considering, in view of the observation that evidence has been obtained from in vitro studies indicating that such patients develop cell-mediated reactivity to GBM constituents (1, 22). In addition, it may be that cell-mediated reactivity against exogenous antigens fixed in glomeruli, as in the form of immune complexes, could participate in the production of glomerular injury. However, even if they occur, definite identification of cell-mediated reactions in human glomeruli may not be possible at present, since there are no reliable morphologic criteria for
identifying such reactions, and since transfer experiments obviously cannot be performed in man.

Summary

Lewis rats were injected intravenously with rabbit anti-rat glomerular basement membrane (GBM) antiserum in doses that were sufficient to cause glomerular fixation of rabbit gamma globulin (RGG) detectable by immunofluorescence, but which failed to induce histologically detectable lesions. 24 h later, groups of rats received lymph node cells or serum from syngeneic donors that had been immunized with either RGG or ovalbumin; they were injected with $[^3]H$thymidine three times during the next 2 days, and sacrificed 48 or 96 h after transfer. Only the rats given anti-GBM antiserum plus lymph node cells from donors sensitized to RGG showed histological glomerular lesions, in the form of segmental hypercellularity and necrosis. Autoradiographs revealed the greatest number of labeled cells in glomeruli in the same group. In analogous experiments, it was shown that T-cell-enriched populations could induce hypercellular glomerular reactions. On the basis of electronmicroscopic and autoradiographic observations, it appears that the glomerular hypercellularity resulted from both infiltration of mononuclear cells and proliferation of endothelial cells. The findings indicate that interaction of specifically sensitized lymphocytes with glomerular-bound antigen can induce a cell-mediated (delayed-type) reaction in glomeruli.

The expert technical assistance of Margaret Davis, Patricia LaFrazia, George Stavrakis, and Bruce Kaynor is gratefully acknowledged. The authors wish to thank Doctors James H. Warram, Jr. and Ray E. Gleason for their expert assistance with the statistical analysis.

Received for publication 27 March 1978.

References

1. Rocklin, R. E., E. J. Lewis, and J. R. David. 1970. In vitro evidence for cellular hypersensitivity to glomerular basement membrane antigens in human glomerulonephritis. N. Engl. J. Med. 283:497.
2. Zabriskie, J. B. 1974. New concepts in post-streptococcal glomerulonephritis. In The Immune System and Infectious Diseases. E. Neter and F. Milgrom, editors. S. Karger, White Plains, N.Y. 282.
3. Andres, G. A., F. Cerra, G. Elti, C. Casciani, R. Cortesini, and K. C. Hsu. 1974. Lymphocyte and blast cell glomerulonephritis in renal allografts of rats injected with phytohaemagglutinin. Cell. Immunol. 13:146.
4. Dixon, F. J. 1970. What are sensitized cells doing in glomerulonephritis? Editorial. N. Engl. J. Med. 283:536.
5. Johnston, W. H., and H. Latta. 1972. Acute focal glomerulonephritis in the rabbit induced by injection of saccharomyces cerevisiae: an electron microscopic study. Lab. Invest. 26:741.
6. Kondo, Y., H. Shigematsu, and Y. Kobayashi. 1972. Cellular aspects of rabbit Masugi nephritis. II. Progressive glomerular injuries with crescent formation. Lab. Invest. 27:620.
7. Shigematsu, H. 1976. Glomerular events during the initial phase of rat Masugi nephritis. Virchows Arch. Abt. B. Zellpathol. 5:187.
8. Kuhn, K., G. B. Ryan, S. J. Hein, R. G. Galaske, and M. J. Karnovsky. 1977. An ultrastructural study of the mechanisms of proteinuria in rat nephrotoxic nephritis. Lab. Invest. 36:375.
9. Schneeberger, E. E., A. B. Collins, H. Latta, and R. T. McCluskey. 1977. Diminished glomerular accumulation of colloidal carbon in autologous immune complex nephritis. Lab. Invest. 37:9.
10. Schreiner, G. F., R. S. Cotran, V. Pardo, and E. R. Unanue. 1978. A mononuclear cell component in experimental immunological glomerulonephritis. J. Exp. Med. 147:369.
11. Unanue, E. R., and F. J. Dixon. 1965. Experimental Glomerulonephritis. VI. The autologous phase of nephrotoxic serum nephritis. J. Exp. Med. 121:715.
12. Krakower, C. A., and S. Greenspon. 1951. Localization of the nephrotoxic antigen within the isolated renal glomerulus. Arch. Pathol. 51:629.
13. Julius, M., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived lymphocytes. Eur. J. Immunol. 112:420.
14. Williams, C. A., and M. W. Chase. 1971. Methods in Immunology and Immunochemistry. Academic Press, Inc., New York. 31.
15. Shantz, A., and A. Schecter. 1965. Iron-hematoxylin and safranin as a polychrome stain for Epon sections. Stain Technol. 40:279.
16. Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell. Biol. 27:137 (Abstr.).
17. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. Iowa State University Press, Ames, Iowa. 6th edition. 325.
18. Li, C. C. 1964. Introduction to Experimental Statistics. McGraw-Hill, Inc., New York. 425.
19. McCluskey, R. T., and A. K. Bhan. 1977. Cell-mediated reactions in vivo. In Mechanisms of Tumor Immunity. I. Green, S. Cohen, and R. T. McCluskey, editors. John Wiley & Sons, Inc., New York. 1.
20. Dvorak, A. M., M. C. Mihm, and H. F. Dvorak. 1976. Morphology of delayed type hypersensitivity reactions in man. II. Ultrastructural alterations affecting the microvasculature and the tissue mast cells. Lab. Invest. 34:179.
21. Polverini, P. J., R. S. Cotran, and M. M. Sholley. 1977. Endothelial proliferation in the delayed hypersensitivity reaction: an autoradiographic study. J. Immunol. 118:329.
22. Mahieu, P., M. Dardenne, J. F. Bach. 1972. Detection of humoral and cell-mediated immunity to kidney basement membranes in human renal diseases. Am. J. Med. 53:185.