Different mediator systems in biphasic heterologous phase of anti-GBM nephritis in mice


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Abstract

Background. After the injection of rabbit anti-mouse glomerular basement membrane (GBM) antibody into normal C57BL/6J mice severe albuminuria develops, which reaches a peak at 24 h. This early albuminuria is dependent on polymorphonuclear granulocytes (PMN) and is completely absent in the congenic beige mutant strain (C57BL/6J, bg/bg), which is genetically deficient in leukocytic neutral proteinase activity. We now studied the development of anti-GBM nephritis in beige mice during the later heterologous phase.

Methods. In untreated beige mice we assessed the albuminuria and glomerular lesions on days 1–5 after i.v. injection of anti-GBM antibody. Secondly, effector mechanisms involved in the later days of the heterologous phase were studied by substitution of whole anti-GBM antibodies by F(ab’)_2 fragments, by leukocyte depletion (total body irradiation), scavenging of reactive oxygen metabolites (dimethylsulfoxide treatment), and complement depletion (cobra venom factor treatment).

Results. In the later part of the heterologous phase (days 2–5), when there is still no sign of autologous antibody formation, i.v. injection of anti-GBM antibodies in beige mice induces nephritis with gradually increasing albuminuria, that reaches levels similar to those in non-deficient, congenic controls by day 3. This late albuminuria did not occur after injection of F(ab’)_2 fragments of the antibody, could be prevented by leukocyte depletion, and was greatly reduced by treatment with dimethylsulfoxide, a scavenger of hydroxyl radicals. The late albuminuria was not influenced by complement depletion with cobra venom factor.

Conclusions. The heterologous phase in murine anti-GBM nephritis is a biphasic process, with sequential involvement of different and independent mediating systems: both phases are PMN-dependent, but only the early albuminuria depends on leukocytic neutral proteinase activity, whereas the albuminuria and the glomerular damage at later days are effected by reactive oxygen metabolites, most probably originating from PMN accumulating in the glomerulus.

Key words: albuminuria; anti-GBM nephritis; mice; monocytes; neutrophils

Introduction

The immunologic mediation of proteinuria in immune complex nephritis has been studied in a large number of experimental models [reviewed in 1 and 2]. Among these, the model of passive anti-glomerular basement membrane (GBM) nephritis, or nephrotoxic nephritis, has been most extensively studied. This nephritis, induced by i.v. injection of heterologous anti-GBM antibodies [1,3], has been studied in different species and has revealed that different immunological pathways may lead to glomerular damage. The increased glomerular permeability and the morphological changes can be induced by the binding of antibody to the GBM alone [4,5], by complement activation [6], or by polymorphonuclear granulocytes (PMN) with or without accessory involvement of complement [7–9]. In most species, the first histological reaction consists of glomerular accumulation of PMN, that is maximal at 2 h after the injection of the anti-GBM antibodies, and leads to endothelial damage, enhanced glomerular permeability, necrosis, and fibrin deposition [1,10]. The resulting albuminuria is maximal in the first 24 h, and persists, although to a lesser degree, in the following days. Six to 7 days after induction of the nephritis, the host response to the heterologous antibodies initiates the autologous phase, immunohistologically visible as linear deposition of mouse antibodies to the GBM-bound foreign immunoglobulin (Ig), and accompanied with histological damage, further increase
of the existing albuminuria, and eventually chronic renal disease.

We have described a murine model of passive anti-GBM nephritis with massive albuminuria (11), that is PMN dependent but complement independent [12,13]. In C57BL/6J, bg/bg (beige) mice, deficient for the leukocytic neutral proteinases elastase and cathepsin G [14], we found that the early albuminuria in the first 24 h was absent, despite unimpaired PMN influx at 2 h. After transplantation with normal bone marrow, leading to a circulating PMN pool with normal neutral proteinase activity, the early albuminuria in the beige mice became comparable to that in normal C57BL/6J mice [15,16]. In the present study we tested the effect of elastase and cathepsin G deficiency on the sequence of events in the later days of the heterologous phase, i.e. days 2–5.

Methods

Animals

C57BL/6J, bg/bg (beige) mice were bought from the Jackson Laboratory (Bar Harbor, Maine, USA). Congenic C57BL/6J, +/- (control) mice were originally obtained from Harlan Olac (Biester, Oxon, UK) and were kept in our laboratory by continuous bro—sister matings. For all experiments we used 3–6 months old animals weighing 20–25 g, with age and sex matched controls. New Zealand White rabbits, used for the preparation of the anti-GBM antibodies, were bought from a local breeder.

Anti-mouse GBM antibodies and F(ab′)2 fragments

A first batch of RaMGBM (I), used for the study of the urinary albumin excretion and the glomerular damage, and for the quantitation of the binding of RaMGBM to the glomeruli in beige and control mice, was prepared, purified, and assessed for purity and specificity as described before [11,13]. The Ig concentration of the final preparation, determined by radial immunodiffusion [17] was 25 mg/ml. A second batch of RaMGBM (II), prepared in the same way, was used for the studies of leukocyte depletion, complement depletion, antioxidant treatment with DMSO, and the effects of injection of F(ab′)2 fragments in beige mice. The final Ig concentration of this antisera was 13 mg/ml.

F(ab′)2 fragments from whole anti-GBM antibodies were prepared by digestion with pepsin as described previously [18]. Briefly, RaMGBM, containing 15 mg Ig/ml, was dialysed for 4 h at room temperature against 0.1 M sodium acetate buffer, pH 4.5. Pepsin was added at a ratio of 4 mg/100 mg Ig. Digestion was allowed to proceed at 37°C for 20 h, and the precipitate was removed by centrifugation (600 g, 10 min). The supernatant was neutralized in TRIS buffer, pH 7.4, concentrated and fractionated by gel chromatography on a 80 x 2 cm Ultrogel ACA-44 column (Pharmacia, Uppsala, Sweden) with a separation range 10–130 kDa. The mol. wt of the collected F(ab′)2 fragments, was approximately half the mol. wt of the undigested Ig, as shown by SDS–PAGE analysis with simultaneous application of marker proteins. The fraction containing F(ab′)2 fragments was concentrated to a final concentration of 42 mg/ml as measured in a Lowry assay (19).

Glomerular antibody binding and quantitation

Glomerular binding of RaMGBM was assessed by indirect immunofluorescence (IF) using FITC-labeled swine anti-rabbit Ig (DAKO, Copenhagen, Denmark) as a second antibody. Both batches of RaMGBM and the F(ab′)2 fragments bound in a linear fashion to the GBM and tubulo-basement membrane of normal mouse kidneys. For quantitation of the glomerular binding undigested RaMGBM Ig, F(ab′)2 fragments, and normal rabbit Ig were labeled with 125I, using Iodo beads (Pierce Chemical Co., Rockford, Illinois, USA) as a coupling reagent (20) to a specific activity of 0.02–0.03 mCi/mg protein. Free 125I was removed by G25 Sephadex chromatography (Pharmacia, Uppsala, Sweden), and unlabeled Ig or F(ab′)2 fragments were added to obtain the desired concentration of 15 mg/ml. The specific activity of the preparations was measured immediately before i.v. injection. The in vivo glomerular binding of intact RaMGBM Ig, F(ab′)2 fragments, and normal rabbit Ig was measured according to the method of Gauthier and Mannik [21], with modifications as described previously [12]. Briefly, 1 h after i.v. injection of the 125I labeled antibodies, F(ab′)2 fragments, or normal rabbit Ig, the mice were anaesthesitized with sodium pentobarbital (Narcovet, Apharma B.V., Arnhem, The Netherlands). Via a cannula inserted in the abdominal aorta the kidneys were flushed with 0.01 M PBS, pH 7.4, for 3–5 min until they blanched and were immediately thereafter perfused in situ with Fe3O4 (BDH, Poole, UK), at a concentration of 1.2 mg/ml PBS. The kidneys were homogenized through a 90 µm sieve and tissue particles were allowed to sediment for 20 min. The supernatant was removed, and the pellet resuspended in 50 ml PBS in a polypropylene tube which was then placed against 1 pole of a strong permanent magnet (magnetic inductance of 0.3 Tesla) [22]. Iron-loaded glomeruli were allowed to accumulate against the wall of the tubing for 10–20 s, after which the supernatant with non-iron containing structures was removed. The glomeruli were resuspended in PBS, and the above described washing procedure was repeated three times. The final glomerular suspension was spread on 10 white 0.8 µm Sелеktron filters (Schleiger and Scull, Dassel, Germany) with a diameter of 25 mm which were placed on a glass slide and covered with a coverglass. The dark glomeruli were counted twice microscopically, using an ocular grid. All filters contained 150–300 glomeruli with minimal (<5%) contamination of non-glomerular structures, which were mostly tubuli or vascular fragments. Radioactivity of the counted glomeruli on 10 filters was measured in a gamma counter. Glomerular binding was expressed as pmol of bound antibody per kidney by using the equation: counts per minutes per glomerulus x 12000/number of glomeruli x specific activity (cpm/pmol) [21]. The cpm/glomerulus were calculated, using the mean of the counts of the 10 glomerular suspensions.

Albuminuria

Albuminuria was measured in urine samples obtained by placing the animals in individual metabolic cages during 18 h [11] at the indicated day after i.v. injection of RaMGBM. The urinary albumin concentrations were measured by radial immunodiffusion using goat anti-mouse albumin antibodies [11,15].

Complement depletion

Complement depletion was induced by daily i.p. injections of 30 U of CoVF (Cordis Laboratories, Miami, Florida,
USA), starting 1 day before induction of the nephritis. Serum hemolytic complement activity (CH50) was assessed in a sensitive hemolytic assay as described previously [23].

Leukocyte depletion

Mice were depleted of leukocytes by TBI as described previously [15,24]. Irradiation was given in a single dose of 7.5 Gy/min, at a rate of 2 Gy/min, with a 13 MeV electron beam from a linear accelerator (CGR Saturne, Buc, France) 4 days before induction of the nephritis, as maximal leukocyte depletion is reached at day 4 after TBI [12,24]. Except for body weight loss for up to 20% after TBI, no adverse reactions were observed in the animals. They remained in excellent condition throughout the experimental period. The effectiveness of TBI was monitored by leukocyte counting in a H*T automated cell counter (Bayer-Technicon, Tarrytown, USA).

Dimethylsulfoxide treatment

The role of reactive oxygen species was studied by administering 100 μl of DMSO (Dimethylsulfoxide 100%; Merck, Darmstadt, Germany), a scavenger of hydroxyl (OH ) radicals, i.p. 30 min before and concomitantly (i.p.) with the i.v. injection of RaMGBM. Thereafter it was administered every 12 h throughout the entire experimental period.

Histology and immunohistology

After removal of the kidneys small fragments of renal cortex were fixed in Bouin’s solution, dehydrated, and embedded in paraplast (Amstelstad B.V., Amsterdam, The Netherlands). Four μm sections were stained with periodic acid Schiff (PAS) and silver methenamine [11]. For IF, kidney fragments were snap frozen in liquid nitrogen, and 2 μm cryostat sections were stained with FITC-labeled swine anti-rabbit Ig, rabbit anti-human fibrinogen that crossreacts with mouse fibrinogen (DAKO, Copenhagen, Denmark), sheep antimouse Ig, heavy and light chains (Cappel Laboratories, West Chester, USA) and goat anti-mouse C3 (Nordic, Tilburg, The Netherlands). Glomerular PMN were counted in 4 μm sections, stained with PAS. The number of glomerular macrophages was determined in 5 μm cryostat kidney sections using the rat anti-mouse macrophage monoclonal antibody FA/11 [25,26] (kindly donated by Dr G. L. E. Koch, MRC Laboratory of Molecular Biology, Cambridge, UK) as the first antibody in a 3-step avidin–biotin technique with biotinylated goat anti-rat IgG as secondary antibody (Vectastain ABC, Vector Laboratories, Burlingame, California, USA) [27]. The number of PMN and macrophages were counted in at least 40 glomeruli and expressed as the mean number per glomerular cross section. The number of CD4 or CD8 positive lymphocytes in the glomeruli was assessed in an indirect IF technique using rat monoclonal antibodies directed against mouse CD4 and CD8 (clone GK1.5 and 2.43 respectively, American Type Culture Collection, Rockville, MD, USA). Culture supernatants were received as a kind gift from Dr W. van Ewijk, Erasmus University, Rotterdam, The Netherlands) and FITC-labeled rabbit anti-rat Ig (DAKO) as a second layer. The staining intensity of glomerular depositions of rabbit Ig, mouse Ig, complement, and fibrinogen was recorded semiquantitatively on a scale from zero to 4+ as described before [11]. For EM small pieces of the renal cortex were fixed in 2.5% glutaraldehyde dissolved in 0.1 M sodium cacodylate buffer, pH 7.2, for 4 h at 4°C, and washed several times in the same buffer. The cortical fragments were postfixed in phosphate-buffered OsO4 for 2 h, dehydrated, and embedded in EPON 812. Ultrathin sections were cut in an Ultrotome (LKB, Bromma, Sweden) and stained with 5% uranyl acetate for 45 min and with lead citrate for 2 min at room temperature. The sections were examined in an electron microscope (JEOL 1200 EX2, JEOL, Tokyo, Japan).

Controls and statistical analysis

Since individual mice show a considerable variation in the albuminuric reaction to the administration of anti-GBM antibodies, no historical controls were used. Each experiment was performed with age and sex matched controls. For statistical analysis, Wilcoxon’s rank sum test was used. P values <0.05 were considered significant. All values are expressed as means ± SD.

Results

The heterologous phase of anti-GBM nephritis in beige mice and congenic controls

All animals tolerated the induction of the nephritis well, and underwent the placement in metabolic cages and the other experimental procedures without serious side effects. Figure 1 shows the effect of injection of 7.5 mg of RaMGBM (batch I) in groups of five beige mice and their congenic controls. At 24 h after injection the non-deficient control mice showed a massive albuminuria (12 024 ± 1680 μg/18 h) while the albumin excretion in the beige mice was 87 ± 47 μg/18 h, thus remaining within the physiologic range (49 ± 19 μg/18 h, n = 30), consistent with our previous results [15]. GMB binding of the RaMGBM, as assessed with radiolabeled antibody, was comparable in both strains (38 ± 6 pmol/glomerulus in C57BL/6J, +/+ mice and 43 ± 3 pmol/glomerulus in beige mutants). From days 2 to 5, the albuminuria in the beige mice...
mice increased gradually from 2356 ± 1365 at day 2, to 5700 ± 1294 at day 3, 9542 ± 3589 at day 4, and 11 763 ± 1061 µg/18 h at day 5 (Figure 1). By contrast, the early severe albuminuria on day 1 in the congenic controls did not further increase but remained at levels of 7904 ± 1236 at day 2, 5359 ± 2253 at day 3, 8829 ± 3448 at day 4, and 7698 ± 1262 µg/18 h at day 5. Surprisingly, these striking differences in albuminuria in both strains were not reflected in the histology or immunofluorescence of the kidneys: at each observation point the morphologic changes were comparable in both strains. The glomerular influx of PMN in beige mice was not inhibited and, like in the congenic controls, was maximal at 2 h after the injection of the antibody (Figure 2A). At 24, 48 and 72 h there was progressive endothelial swelling and cell necrosis accompanied by vascular thrombosis (Figure 2B). At day 5 epithelial cell proliferation was observed occasionally. By IF a strong linear binding of rabbit Ig to the GBM was found in both beige and normal control mice throughout the observation period (Figure 3A). From day 1, increasing deposition of fibrin was seen in the glomerular tufts in both strains (Figure 3B). Fine granular C3 deposits along the capillary wall, seen in both strains on day 1, had decreased at day 3 (Figure 3C), consistent with our earlier findings [15]. No binding of mouse Ig to the GBM was observed in this phase. Only small amounts of mouse Ig and C3 could be detected in mesangial areas in all mice (a normal finding in this species) and sparse granular deposits of mouse Ig were seen in areas of thrombosis. The number of glomerular macrophages on day 3, counted after immunostaining with FA/11 monoclonal antibody, was similar in beige and control mice (being 1.2 ± 0.2 and 1.1 ± 0.2 cells/glomerular cross section) and comparable to the values in untreated mice (1.1 ± 0.2 and 0.8 ± 0.3 cells/glomerular cross section respectively in beige mice and C57 B1/6J, +/+ controls). The numbers of CD4 and CD8 positive cells in the glomeruli, as determined in at least 40 glomeruli of 5 mice per group at day 3, was comparable in treated and untreated beige mice (being 0.4 ± 0.1 and 0.3 ± 0.1 cells/glomerular cross section for CD4 positive cells, and 0.2 ± 0.1 and 0.2 ± 0.1 cells/glomerular cross section for CD8 positive cells respectively, and not different from those in congenic controls.

Effect of leukocyte depletion

Four days after TBI the leukocyte count was less than 200 cells/µl. As previously shown, 20–25% of these cells are PMN (12). These low levels were maintained during the whole experimental period (data not shown). At day 4 after TBI, RaMGBM was administered i.v. Similar to our findings in normal mice, TBI prevented the albuminuria also in the beige mice. At 3 days after injection the albuminuria did not exceed the physiologic level (Table 2). TBI by itself did not influence the albumin excretion. In IF a strong linear binding of rabbit Ig was observed along the GBM, but there was no C3 deposition in the capillary wall and no deposition of fibrin in the capillary tuft (Table 2). Light microscopy did not reveal glomerular damage in the TBI group. PMN were absent and macrophages were extremely scarce in the glomeruli at 2 h, while their numbers at day 3 were lower than in the non-irradiated control animals (Table 1).

F(ab')2 fragments

I.v. administration of F(ab')2 fragments of anti-GBM antibody did not cause albuminuria (Table 3). The glomerular binding of an equimolar dose and an equal protein dose respectively of radiolabeled F(ab')2 fragments was uninhibited, and even slightly higher than the binding of intact RaMGBM (P < 0.01), as assessed with radiolabeled antibodies (Figure 5). Histological examination of the kidneys showed no abnormalities. IF showed a linear binding of the F(ab')2 fragments to the GBM, and absence of C3 or fibrin deposits. The number of glomerular PMN at 2 h was identical to that in untreated controls, while no macrophages were seen at that time (Table 1). At day 3 PMN could not be detected in glomeruli, and glomerular macrophage counts were comparable to those in control mice (Table 1).
Biphasic heterologous anti-GBM nephritis in mice

Fig. 3. Immunofluorescence of kidneys of beige mice at day 3 after induction of the nephritis. (A) Linear binding of rabbit anti-mouse GBM Ig to the glomerular capillary wall; (B) Segmental depositions of fibrin in the capillary tuft; (C) Deposits of C3 along the capillary wall and, in the mesangium in non-CoVF treated mice; (D) Absence of C3 deposits in the capillary wall in CoVF treated mice (x 300).

Table 1. Glomerular polymorphonuclear granulocyte (PMN) and macrophage (MØ) counts in kidney sections of C57BL/6J, bg/bg (beige) mice at 2 h and day 3 after injection of anti-GBM antibodies in different treatment protocols.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glomerular PMN^a</th>
<th>Glomerular MØ^b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 hours</td>
<td>day 3</td>
</tr>
<tr>
<td>RaMGBM</td>
<td>4.6±1.3 (3)^d</td>
<td>0.6±0.4 (8)</td>
</tr>
<tr>
<td>RaMGBM + CoVF</td>
<td>5.9±1.3 (3)</td>
<td>0.3±0.1 (3)</td>
</tr>
<tr>
<td>RaMGBM</td>
<td>6.0±0.5 (3)</td>
<td>0.2±0.1 (3)</td>
</tr>
<tr>
<td>RaMGBM + TBI</td>
<td>0.0±0.0 (3)</td>
<td>0.1±0.1 (7)</td>
</tr>
<tr>
<td>RaMGBM/F(ab')2</td>
<td>0.6±0.5 (3)</td>
<td>0.0±0.0 (5)</td>
</tr>
<tr>
<td>RaMGBM</td>
<td>9.1±1.9 (3)</td>
<td>0.3±0.2 (6)</td>
</tr>
<tr>
<td>RaMGBM + DMSO</td>
<td>6.6±1.8 (3)</td>
<td>0.2±0.1 (6)</td>
</tr>
</tbody>
</table>

^a RaMGBM, rabbit anti-mouse glomerular basement membrane antibody; CoVF, Cobra venom factor; TBI, total body irradiation; DMSO, dimethylsulfoxide.

^b PMN were counted in at least 40 glomeruli in PAS stained kidney sections (mean±SD).

^c MØ were immunohistologically stained with mAb FA/11 and counted in at least 40 glomeruli (mean±SD).

^d Number of mice between parentheses.

^e P<0.01 compared to untreated animals.

^f P<0.01 compared to non-irradiated controls.

Complement depletion

Consistent with our previous findings (13), daily i.p. administration of 30 U of CoVF resulted in unmeasurable CH50 titers throughout the experimental period (data not shown). The effects of complement depletion on albumin excretion and IF findings are summarized in Table 2. Complement depletion did not affect the level of albuminuria in beige mice. Non-CoVF treated control mice showed granular deposits of C3 in the capillary wall and in the mesangium (Figure 3C). In the CoVF treated group, C3 deposits could not be detected by IF, except for a faint diffuse staining in areas of glomerular necrosis (Figure 3D). The remaining morphologic and IF findings were similar to those described above for the non-CoVF treated groups (Table 2), with the exception of the number of glomerular macrophages, that proved to be
Table 2. Effect of total body irradiation, Cobra Venom Factor or DMSO treatment on albuminuria and immunofluorescence findings in C57BL/6J, bg/bg mice at day 3 after i.v. injection of anti-GBM antibody

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals</th>
<th>Albuminuria (µg/18 hours)</th>
<th>Immunofluorescencea</th>
<th>RaMGBM</th>
<th>C3</th>
<th>mlg</th>
<th>Fibrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>RaMGBM + TBIb</td>
<td>5</td>
<td>16±10</td>
<td>++/++e</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>TBI</td>
<td>5</td>
<td>43±50</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RaMGBM</td>
<td>8</td>
<td>5880±1387</td>
<td>++/++e</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>RaMGBM + CoVF*</td>
<td>8</td>
<td>13476±4393</td>
<td>++/++e</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>RaMGBM + 0.9% NaCl</td>
<td>8</td>
<td>10504±3680</td>
<td>++/++e</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>RaMGBM + DMSOf</td>
<td>7</td>
<td>383±338b</td>
<td>++/++e</td>
<td>d</td>
<td>-</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>RaMGBM + 0.9% NaCl</td>
<td>7</td>
<td>11729±10421</td>
<td>++/++e</td>
<td>c</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

a RaMGBM: rabbit anti-mouse GBM antibodies; mlg: mouse immunoglobulin.
b TBI: total body irradiation (7.5 Gy) was given at day −4 before injection of RaMGBM.
c CoVF: Cobra Venom Factor was given in daily i.p. injections of 30 U from day −1 to +3 after injection of RaMGBM.
d Linear staining along the capillary wall.
e Granular deposits in the capillary wall.
° 100 µg of DMSO (dimethylsulfoxide) was given i.p. before induction of the nephritis and every 12 h thereafter during 3 days.
h P<0.01 compared to controls.

Table 3. Effect of i.v. administration of RaMGBM F(ab')2 fragments to C57BL/6J, bg/bg mice on albuminuria on day 3

<table>
<thead>
<tr>
<th>F(ab')2 dose</th>
<th>Albuminuria (µg/18 h)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mg</td>
<td>36±20</td>
</tr>
<tr>
<td>1.0 mg</td>
<td>35±33</td>
</tr>
<tr>
<td>2.0 mg</td>
<td>31±13</td>
</tr>
<tr>
<td>4.0 mg</td>
<td>34±31</td>
</tr>
<tr>
<td>8.0 mg</td>
<td>24±8</td>
</tr>
</tbody>
</table>

a n=4, mean ±SD.

Fig. 4. Quantitation of glomerular binding of 125I labeled normal rabbit Ig, intact RaMGBM Ig, and F(ab')2 fragments of RaMGBM, at 1 h after i.v. injection in C57BL/6J, bg/bg. *P<0.01, as compared with each previous observation.

Discussion

Our results show that, in spite of preventing the albuminuria at day 1 after injection of RaMGBM, the leukocytic neutral proteinase deficiency of the beige mutant does not inhibit or delay the development of glomerular lesions, and from day 2 albuminuria develops, rising to levels comparable to those of congenic nephritic controls from day 3, i.e. clearly within the time limits of the heterologous phase. Both this late albuminuria and the glomerular lesions can be prevented by depletion of PMN and removal of Fc fragments in the RaMGBM, and can be considerably reduced by ROM scavenging. As shown by the binding studies with radiolabeled antibodies, the absence of a nephritic effect of the F(ab')2 fragments could not be attributed to loss of binding to the GBM. The glomerular accumulation of PMN, from 30 min after injection of antibody to a maximum at 2 h, was similar in beige mice and congenic controls. These findings indicate also reduced the glomerular damage at day 3, i.e. the deposition of fibrin and the necrosis in the capillary tufts, as assessed by light microscopy and IF (Table 2). At 2 h after injection of RaMGBM the kidneys of DMSO treated mice showed a moderately reduced influx of PMN, and glomerular macrophage counts in DMSO treated and non DMSO treated mice were identical (Table 1). EM examination at 2 h showed glomerular damage both in DMSO treated mice and controls, i.e. PMN that attached to the GBM between the moderately swollen endothelial cells. At day 3, the glomeruli of control mice showed severe endothelial cell damage, with swelling, lysis, and detachment from the GBM, while at many sites cellular debris and fibrin fibrils could be observed between the damaged endothelial cells and the GBM. In the DMSO treated mice the glomerular damage was less prominent with only moderate endothelial cell damage (not shown).

Dimethylsulfoxide treatment

Treatment of mice with DMSO caused a considerable reduction in albuminuria at day 3, to levels of less than 5% of those of non-DMSO treated controls. It significantly increased in the CoVF treated group (Table 1).
that the heterologous phase in murine anti-GBM nephritis can be divided in at least two subphases with different Fc-mediated pathogenic pathways: a first albuminuric episode predominantly mediated by the action of leukocytic neutral proteinases, and a second phase with glomerular damage and albuminuria that are dependent on other Fc-mediated effector systems, such as the production of ROM.

Our data strongly point to PMN as the essential mediators not only for the first, but also for the second phase. Apart from PMN, other cells bearing one of the three known receptors for the Fc part of Ig are monocytes/macrophages, natural killer cells (NK), and platelets. The characteristics of the Fc receptor have been extensively studied in human leukocytes, but data on the murine system are incomplete [28]. It is generally assumed that there are many analogies between the systems in man and mice, with the exception that on mouse platelets no Fc receptors have been demonstrated [29-31]. With regard to a possible involvement of macrophages, in our experimental groups only the CoVF treated beige mice showed an increase of FA/11 positive cells in the glomeruli (Table 1). This somewhat unexpected finding will require further study. In all other groups the number of macrophages, counted at day 3 after RaMGBM injection, did not differ from the number seen in normal beige controls. Consistent with these findings, Holdsworth et al. found no increase of glomerular macrophages during the heterologous phase of anti-GBM nephritis in rats and rabbits [32]. In these species they demonstrated that macrophages only start to accumulate in the glomerular tuft when the animal produces antibodies to the heterologous Ig, indicating that macrophages do not mediate damage prior to the onset of the autologous phase. Holdsworth et al. attributed the sequential glomerular accumulation of PMN and macrophages to different affinities of these cells for the Fc part of the heterologous and autologous antibodies respectively [33]. We have tried to induce a highly selective depletion of PMN without an effect on monocytes by treatment with a rat monoclonal anti-mouse PMN antibody (NIMP-R14), kindly provided by M. Strath (National Institute for Medical Research, Mill Hill, London, UK) [34]. In a pilot experiment we found indeed a severe depletion of PMN accompanied with inhibition of the albuminuria (data not shown). However, like the TBI, the treatment with NIMP-R14 did not only induce PMN depletion, but also reduced the levels of monocytes by more than 90%, thus giving no essential additional information about a potential influence of monocytes.

With regard to lymphocytes, a possible role for these cells in the development of anti-GBM nephritis has only been postulated in a WKY rat model [35-37], in which increase of CD8 positive cells preceded a glomerular increase of macrophages and induction of glomerular lesions. Depletion of CD8 positive cells by injection of monoclonal antibodies abolished the glomerular macrophage invasion and also the glomerular lesions [37]. The authors suggest that the CD8 positive cells may be NK cells, that are involved in ADCC activity, and influence the increase of macrophages with resulting immune injury by factors released after binding of their Fc receptor to the GBM fixed antibodies. In our model it is very unlikely that CD4 or CD8 positive lymphocytes are involved in the induction of glomerular damage in the heterologous phase, since we did not find any differences in CD4 and CD8 positive cell counts between nephritic beige mice and normal controls. Moreover, the role of CD8 positive lymphocytes in the WKY rat model concerns proteinuria that appears at day 7, a time at which the autologous phase has already started. A possible role for NK cells is also unlikely in our model, since beige mice are known to have decreased numbers of circulating NK cells [38].

Although a role for platelets and the coagulation system has been demonstrated in several models of experimental nephritis [30], such an effect could not be demonstrated in our model, since platelet aggregation and fibrin deposition were not seen before PMN accumulation and endothelial cell swelling were already evident. The coagulation is probably initiated by the endothelial cell damage with concomitant upregulation of procoagulant activity, or by mediators, released by the PMN after their adherence to the endothelial cell. Preliminary data show that platelet depletion has no influence on the albuminuria (manuscript in preparation).

When concentrating on PMN as the most likely inducers of the glomerular lesions in the heterologous phase of murine anti-GBM nephritis, the more specific effector mechanism that inflicts the damage to the endothelial cells and the albuminuria in the late heterologous phase remains to be defined further. From the results in the beige mice elastase and cathepsin C can be excluded as essential mediators in the late albuminuria of days 2-5, leaving other systems such as ROM, acid-, basic-, or metalloproteinases as effector systems, either apart or in cooperation. PMN are the principal source of ROM in inflammation [39,40], and in our model of glomerulonephritis PMN accumulate in the glomerular tuft soon after the i.v. administration of RaMGBM [11]. In beige mice this influx of PMN also occurs, and ultrastructurally there is evidence of endothelial cell damage in both strains from 2 h after the i.v. injection of the anti-GBM antibodies [15]. PMN from beige mice can produce ROM in amounts equal to those of congenic controls when properly stimulated [15]. Fc receptor mediated stimuli have been shown to trigger an oxidative burst from leukocytes with the generation of ROM that may play a role in the cellular injury and albuminuria which accompany PMN-dependent glomerulonephritis [31,39-41]. The generated ROM can also inhibit ADP-ase activity and ADP-ase mediated anti-thrombotic activity, as was recently shown in a passive anti-GBM nephritis in the rat [42]. The glomerular endothelial cells, damaged by leukocytic ROM, may in turn generate potent proinflammatory agents [43], causing activation of coagulation system, platelets [31], and complement system. The finding that DMSO treatment, with only moderate
reduction of PMN accumulation, almost completely suppressed the late albuminuria, also suggests a role for ROM, or, more specifically, hydroxyl radicals in the pathogenesis of the albuminuria and morphological damage in the late heterologous phase. In passive anti-GBM nephritis in rats and rabbits the mediation of glomerular injury by ROM was demonstrated by Rehan et al. [44] and Boyce et al. [45], and ROM have been identified as mediators in the pathogenesis of other models of glomerulonephritis [reviewed in 41]. In earlier experiments we could not demonstrate a protective effect of ROM scavengers for the early albuminuria of the first day in normal C57Bl/6J mice [15]. We therefore assume that ROM as such have no direct effect on the permeability of the glomerular filter. One could speculate that ROM, generated by PMN, act by stimulating or damaging endothelial cells or other resident glomerular cells, that in turn release mediators that are responsible for the albuminuria and the intravascular coagulation seen in the late heterologous phase. This hypothesis is, however, not supported by our finding that scavenging of hydroxyl radicals did only partly reduce the endothelial changes that are already visible from 2 h after injection of RaMGBM.

At this moment it is not clear whether ROM can induce albuminuria by a direct, but slowly developing injury to the GBM, or by triggering of other mediator systems.

In conclusion, our studies in normal C57Bl/6J mice and the beige mutants show that in the heterologous phase of murine anti-GBM nephritis a short initial subphase can be recognized, characterized by an early severe albuminuria that is dependent on the presence of leukocytic neutral proteinases. The glomerular lesions and the more gradually developing albuminuria of the later days depend on other, most likely also PMN dependent effector systems. One of the mediator systems that play a role in this neutral proteinase independent damage is that of ROM, derived from the activated PMN, and most likely acting indirectly by damaging endothelial cells or other resident glomerular cells. Further studies are necessary to confirm this hypothesis and to delineate the possible involvement of other mediator systems, including those effected by resident glomerular cells that may play a role in the late heterologous phase.

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