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Anti-Thy-1 Monoclonal Antibody-Induced Glomerulonephritis in Mongolian Gerbils

Hiroshi Sato, Moinuddin Chisty, and Haruo Kamiya*

Abstract | Two novel murine monoclonal antibodies (mAbs) were produced to the Thy-1 molecule of Mongolian gerbils (*Meriones unguiculatus*). These mAbs, HUSM-M.g.27 of IgG1 isotype and HUSM-M.g.40 of IgG2a isotype, immunohistochemically reacted with the thymus, nervous system, and glomerular mesangium in partially different manners, suggesting that they recognize distinct epitopes, although they reacted with Thy-1 antigen, with apparent molecular weight of about 25 kDa, on gerbil thymocytes. Mild and severe forms of mesangioproliferative nephritis after glomerular deposition of the antibody was observed in gerbils administered mAbs HUSM-M.g.27 and HUSM-M.g.40, respectively, intraperitoneally, with or without guinea-pig serum as supplementary complement. Distinct pathogenicity and requirement of guinea pig serum for pathologic sequels are discussed as they relate to the rat model of anti-Thy-1-induced glomerulonephritis.

Anti-Thy-1 glomerulonephritis (GN) is a rat model of mesangioproliferative nephritis induced by complement-dependent immune-mediated mesangial injury (1–3). The disease induced by a single injection of anti-Thy-1 antibody is characterized by an acute and self-resolving disease process, whereas repeated injection of the antibody induces irreversible glomerulosclerosis (4). Shimizu et al. (5) reported the tissue distribution of the Thy-1.1 molecule in Mongolian gerbils, *Meriones unguiculatus*, which appears similar to that in rats (i.e., in the thymus, brain, and glomerular mesangium of the kidney, but not on peripheral T cells). Using rabbit anti-rat thymocyte serum (ARTS), they observed anti-Thy-1 GN in gerbils that simultaneously received guinea pig serum as complement (6). The lack of GN in gerbils injected only with ARTS has been ascribed to deficiency of some factor of complement or its low functional activity in this rodent species (6).

We report two novel murine monoclonal antibodies (mAbs) to the gerbil Thy-1 molecule. The antigen reactive with these mAbs is distributed in the mesangium of the kidney as well as the thymus and brain. Repeated injection of one mAb along with guinea pig serum to gerbils induced mild GN, whereas a single injection of the other with or without guinea pig serum caused severe GN.

Materials and Methods

Animals: Mongolian gerbils and BALB/c mice were bred in the Institute for Animal Experiments, Hirosaki University School of Medicine. They were housed in plastic boxes, with commercial pellets (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and water continuously available. The boxes were kept in a clean colony room maintained at 23°C on a 12/12-hour light/dark cycle. Routine microbiological monitoring by the Institute for Animal Experiments on Sendai virus, mouse hepatitis virus, *Mycoplasma pulmonis* and *Clostridium piliforme* has never detected any problematic infective agents there. All animal experiments were performed according to the Guidelines on Animal Experimentation as set out by Hirosaki University.

Department of Parasitology, Hirosaki University School of Medicine, Hirosaki, Japan.
*Corresponding author: Dr. Haruo Kamiya, Department of Parasitology, Hirosaki University School of Medicine, Zaifu-cho 5, Hirosaki 036-8562, Japan.

Hybridoma production: Three female BALB/c mice, 8 weeks old, were injected intraperitoneally (i.p.) with 8 x 10^6 gerbil thymocytes. Immunization of animals was repeated at 6- to 31-day intervals for 10 weeks with 4 to 20 x 10^6 gerbil thymocytes. Four days after the last (5th) immunization, the spleen was carefully removed, and used for fusion with the mouse myeloma cell line X-63. Briefly, splenocytes and myeloma cells were fused at a ratio of 10:1, using 50% polyethylene glycol (mol. wt. 1,300 to 1,600; Sigma Chemical Co., St. Louis, MO). The cells were plated on flat-bottomed 24-well plates at 2 x 10^6 total cells/well. Hybridoma was selected with HAT medium (Sigma Chemical Co.) from the day of fusion. Screening for antibody production was performed, using an enzyme-linked immunosorbent assay (ELISA) of culture supernatants. For this purpose, ELISA plates were coated with gerbil thymocytes after sensitization of plates with 0.025% glutaraldehyde in 0.1*M* Na_2CO_3, pH 9.0. Immunohistochemically determined clones of interest were subcloned twice by limiting dilution. To determine the isotype of mAb produced by established clones, ImmunoType™ mouse monoclonal antibody isotyping kit (Sigma Chemical Co.) was used.

Antibody preparation: Clones producing mAb were injected i.p. into pristane-primed BALB/c mice to produce ascitic fluids. Ascitic fluids were centrifuged and precipitated by addition of ammonium sulfate to 50%. After dissolving and dialysis in 0.15*M* Dulbecco(-) phosphate-buffered saline (PBS), pH 7.6, these preparations were applied to a stirred ultrafiltration cell (model 8200; Amicon division, W. R. Grace & Co., Beverly, MA) equipped with a YM100 DIAFLO® ultrafiltration membrane (cutting point, 100 kDa; Amicon division). Final concentration of subpurified IgG was determined by use of a spectrophotometer set at 280 nm, adjusted at 2.0 mg of protein/ml of PBS, and sterilized by filtration. The purity of these preparations was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and was approximately 50%.

Labeling of antibody with biotin: According to Holmes et al. (7), mAbs were labeled with long-armed biotin. Briefly, after dialysis against a buffer (0.1*M* $NaHCO_3$, 0.1*M* NaCl, pH 8.4), 10 μl of a 10 mg of biotin/ml solution (biotin-AC5-OSu (Dojindo

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Co., Masuki, Kumamoto, Japan) in anhydrous dimethylsulfoxide was added for each milligram of antibody and incubated for 1 hour. Unbound biotin was removed by dialysis at 4°C in 0.1*M* Tris buffer, pH 7.4.

Immunoprecipitation and western blotting: The thymus was collected from 10-week-old male and 18-week-old female gerbils, total of 20 in number. The cells were washed thrice in PBS and immersed in 50 m*M* Tris buffer, pH 8.2, containing 1% Triton X-100, 150 m*M* NaCl, 10 m*M* EDTA and 0.5 m*M* phenylmethylsulfonyl fluoride. After 3 cycles of freezing and thawing, intermittent ultrasonication of the sample was made for 5 minutes. Supernatant collected by centrifugation at 6,000 $\times g$ for 30 minutes, 0.4 ml of a 2.5 mg/ml concentration, and 0.4 ml of subpurified ascitic preparation of each mAb were mixed overnight at 4°C, and the precipitate was collected by centrifugation at 13,000 $\times g$ for 20 minutes. Samples and prestained protein molecular weight standards (Life Technologies, Inc., Gaithersburg, MD) were separated by SDS-PAGE, using a 15% gel under reducing conditions, and transferred onto a nitrocellulose membrane. A 1:400 dilution of biotin-labeled mAb was added for 2 hours followed by addition of a 1:500 dilution of peroxidase-conjugated avidin D (Vector Laboratories, Inc., Burlingame, CA) for another 2 hours. Bound antibody was detected, using color development by addition of 3, 3'-diaminobenzidine.

In vivo administration of mAbs: Gerbils were injected i.p. with 0.5 ml of PBS containing 1.0 mg of protein from subpurified mAbs (Table 1). The mAbs HUSM-61 of IgG1 isotype and HUSM-M.g.10 of IgG2a isotype were used as control antibodies. The former recognizes an intracytoplasmic determinant of guinea pig macrophages and does not cross-react with any cells of gerbils, and the latter recognizes gerbil thymocytes and peripheral T cells (8). After antibody injection, intact or heat-inactivated (56°C, 30 minutes) pooled serum from 8-week-old female guinea pigs was injected i.p.

Table 1. Experimental protocol: Age and sex of gerbils, injected antibody and guinea pig serum, and day of examination

Exp. No. (Age of animals)	Animal group (No. of animals)	Antibody injected (1 mg/0.5 ml, i.p.)	Guinea pig serum injected i.p.*	Day of examination
1 (9-wk-old)	A (m 4; f 2)	HUSM-M.g.27	Intact	7
	B (m 3; f 2)	HUSM-M.g.27	Inactivated	7
	C (m 3; f 2)	HUSM-61	Intact	7
	D (m 3)	HUSM-61	Inactivated	7
	E (m 3; f 2)	None **	Intact	7
2 *** (8-wk-old)	A (m 6)	HUSM-M.g.27	Intact	14; 21
	B (m 3)	HUSM-M.g.27	None	21
	C (m 6)	HUSM-61	Intact	14; 21
3 (6-wk-old)	A (m 7)	HUSM-M.g.40	Intact	5; 14
	B (m 3)	HUSM-M.g.40	None	14
	C (m 6)	HUSM-M.g.10	Intact	5; 14
	D (m 3)	HUSM-M.g.10	None	14
	E (m 3)	None	Intact	14

* Intact or heat-inactived guinea pig serum was injected at a dose of 0.05 ml for Exp. 1 and 2, or 0.1 ml for Exp. 3, after antibody injection. Intervals between these two injections were 2 hours for Exp. 1, and 24 hours for Exp. 2 and 3.
** Animals were injected with 0.5 ml of phosphate-buffered saline (PBS), the vehicle for the antibody.
*** Animals in Exp. 2 were repeatedly treated at 1-week intervals with the antibody with/without guinea pig serum. The rest of the animals of Exp. 1 and 3 were treated with a single combination of the antibody and guinea pig serum.
m = male; f = female.

Immunohistochemical analysis: Various tissue specimens were collected from an immune-naive male 6-week-old gerbil, an immune-naive male 8-week-old guinea pig of Hartley strain, and an immune-naive male 6-week-old BALB/c mouse as follows; the spleen, thymus, lymph nodes, esophagus, trachea, kidneys, liver, heart, small intestine, cerebrum, cerebellum, and pons. From gerbils used for experiments involving in vivo administration of mAbs, the spleen, thymus, and kidneys were collected. Cryostat-cut sections, 6-μm thick, were air-dried and fixed for 10 minutes in cold acetone. Details of the procedure have been described (8). Primary antibody was applied as culture supernatant, followed by a 1:200 dilution of peroxidase-conjugated goat $F(ab')_2$ fragment to mouse IgG (Fc) (Organon Teknika Co., Durham, NC). Bound antibody was detected, using color development by 3, 3'-diaminobenzidine, followed by light counterstaining with hematoxylin.

Histologic examination: Sections of the kidneys, spleen, thymus, liver, lungs, heart, cerebrum, cerebellum, and pons were stained with hematoxylin and eosin (H&E) according to a standard procedure.

Results

Characteristics of novel murine mAbs: Two novel murine mAbs, HUSM-M.g.27 and HUSM-M.g.40, were of different isotypes and partially different tissue distribution of reactive antigens in gerbils (Table 2). Positive immunohistochemical reactions were not seen with any tissues from a guinea pig and a BALB/c mouse. In addition to a positive reaction with thymocytes, particularly those in the cortex, both mAbs reacted strongly with the mesangium and distal urinary tubules in the renal cortex. The reactivity of the central nervous system and peripheral nerves with these two mAbs was different; mAb HUSM-M.g.27 evenly reacted with nervous tissues, including peripheral nerves, whereas mAb HUSM-M.g.40 reacted weakly with peripheral nerves and parts of the central nervous system. Western blot analysis of the immunoprecipitates of thymocyte extract with mAbs indicated that both antibodies reacted with an identical antigen with apparent molecular mass of about 25 kDa.

In vivo administration of mAbs to gerbils: Three experiments were carried out to determine the pathogenicity of in vivo administered mAbs in gerbils (Table 1). In experiment 1, gerbils were injected i.p. with mAb HUSM-M.g.27 or a control mAb, HUSM-61, and at a 2-hour interval with either intact or heat-inactivated guinea pig serum. Animals of group A became highly depressed or dyspneic within several hours after the treatment, and one male and two females died within 24 hours. Histologic examination revealed pulmonary edema. Gerbils of other groups did not manifest clinical signs of disease. Histologic examination of the rest of the animals sacrificed at day 7 of treatment revealed no histopathologic changes in any organs, including the kidneys. However, immunohistochemical analysis detected deposition of injected antibody in the glomerulus in gerbils of groups A and B (Figure 1). In experiment 2, injections of mAbs HUSM-M.g.27 and HUSM-61 with/without intact guinea pig serum were made weekly. Weekly follow-up of body weight revealed depressed weight gains in animals of group A (Figure 2). By histologic and immunohistochemical examinations, gerbils of group A sacrificed at day 21 had mesangial hypercellularity with mild infiltration of macrophages in the cortical interstitium (Figure 3). The glomerular change in gerbils of the same

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Table 2. Characteristics of murine monoclonal antibodies reactive with Thy-1 antigen of gerbils

Clone	Subclass	Molecular mass of reactive antigen	Immunohistochemical distribution of antigen
HUSM-M.g.27	IgG1	25 kDa	Thymocytes (particularly stronger in the cortex), the central nervous system and peripheral nerves, kidney (the mesangium and distal urinary tubules in the cortex, the interstitium of the inner zone of the medulla, and connective tissue in the pelvis renalis), the capsule of the spleen and lymph nodes, trabeculae lienis, splenic mega karyocytes, lamina propria of the small intestine, and certain vascular walls.
HUSM-M.g.40	IgG2a	25 kDa	Almost identical tissue distribution as described above, except for lower positivity of peripheral nerves, certain area of the central nervous system (e.g., the cortex of cerebellum), lamina propria of the intestine, vascular walls, and trabeculae lienis.

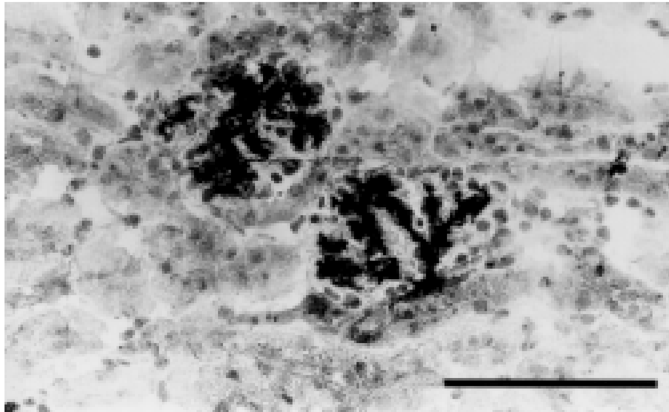

Figure 1. Photomicrograph of a section of the kidney from a gerbil injected 7 days before with monoclonal antibody (mAb) HUSM-M.g.27 (experiment 1, group A). Notice deposited antibody on the glomerular mesangium. Immunohistochemical analysis; bar = 100 μm.

group sacrificed at day 14 was unclear, although some glomeruli had mesangial hypercellularity. The rest of the animals had no histopathologic changes in the kidneys.

In experiment 3, the pathogenicity of a single injection of mAb HUSM-M.g.40 was examined similarly to that in experiments 1 and 2 for mAb HUSM-M.g.27. Three gerbils of group A were highly depressed within a few days of treatment and became moribund at day 5. Loss of body weight was marked in these animals; mean ± SD of relative body weight of moribund animals was 0.76 ± 0.14 (n = 3), and that of the rest of the animals from group A was 1.05 ± 0.10 (n = 4) at day 5. Similarly, animals of group B had depressed weight gain at this time (Figure 2). Moribund gerbils of group A were sacrificed at day 5, and grossly had severe atrophy of lymphoid organs and marbled kidneys with irregular surface. Histologically, most of the renal cortex contained pathologic changes; glomeruli were highly atrophic with proteinaceous fluid in the space of Bowman's capsule (Figure 4), and most of the urinary tubules contained protein casts with calcium deposition, frequently causing loss of lining cells. Immunohistochemical analysis confirmed disappearance of the mesangium in the glomerulus (Figure 5). At day 14, pathologic changes were found in the kidney of gerbils of groups A and B. Gross appearance of the kidney resembled that at day 5, albeit milder. The mesangium had remarkable expansion (Figures 6 and 7), and regenerative changes of urinary tubules were observed mainly in the upper half of the cortex. These changes were milder in group-B than group-A animals.

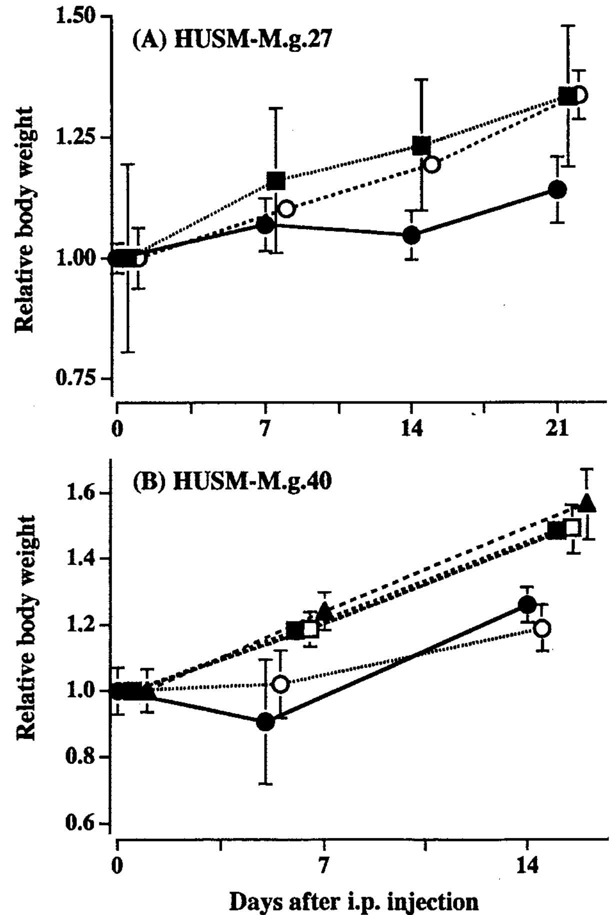

Figure 2. Temporal changes in the body weight of gerbils of experiments 2 **(A)** and 3 **(B)**. Groups: **(A)** (closed circles), **(B)** (open circles), **(C)** (closed squares), **(D)** (open squares), and **(E)** (closed triangles). Changes in the body weight are expressed as values relative to mean body weight of each group at the commencement of the experiment.

Discussion

The tissue distribution of the Thy-1 molecule is remarkably variable among animal species (9, 10). In mice and dogs, Thy-1 is expressed on peripheral T cells as well as thymocytes, particularly in the cortex, brain, and kidneys (9, 11). In rats, Thy-1 is absent on peripheral T cells, but is expressed on bone marrow cells, including immature B cells and hematopoietic cells, as well as in the brain, thymus, glomerular mesangium, and certain connective tissues, such as the dermis, basement membranes of the urinary collecting tubules, certain blood vessels, and the lamina propria of the intestine (12–17). In Mongolian gerbils, the tissue distribution of Thy-1 (Thy-1.1) has the closest resemblance to that in rats (5, 18). As summarized in Table 2, the antigen reactive with our two mAbs was distributed in various organs, and the pattern of distribution was akin to that of Thy-1 molecule. Furthermore, an apparent molecular mass of about 25 kDa from the thymocyte extract corresponded to the reported molecular

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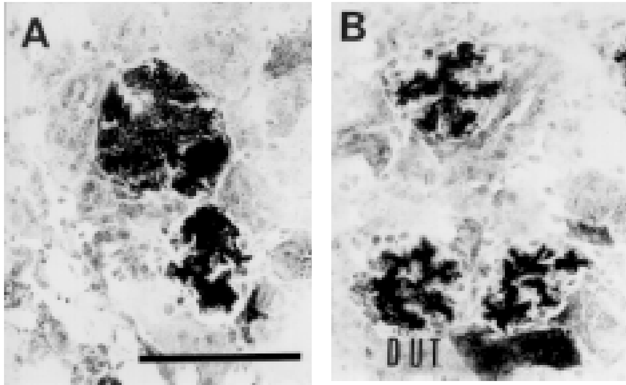

Figure 3. Photomicrographs of sections of the kidney from gerbils injected repeatedly with mAbs HUSM-M.g.27 (A; experiment 2, group A) or HUSM-61 (B; experiment 2, group B) and sacrificed at day 21. Notice remarkably expanded mesangial area in the glomerulus of (A). Positive reaction with the distal urinary tubule is indicated by DUT. Immunohistochemical analysis, using mAb HUSM-M.g.27; bar = 100 μm.

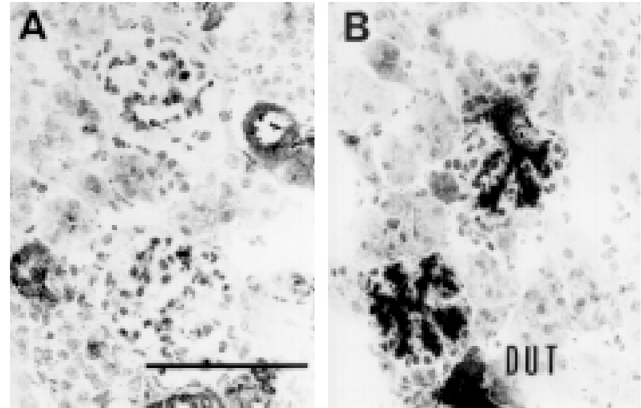

Figure 5. Photomicrograph of a section of the kidney from gerbils injected 5 days before with mAbs HUSM-M.g.40 (A; experiment 3, group A) or HUSM-M.g.10 (B; experiment 3, group C). Notice disappearance of the mesangium in the glomerulus of (A). DUT = distal urinary tubule. Immunohistochemical analysis, using mAb HUSM-M.g.27; bar = 100 μm.

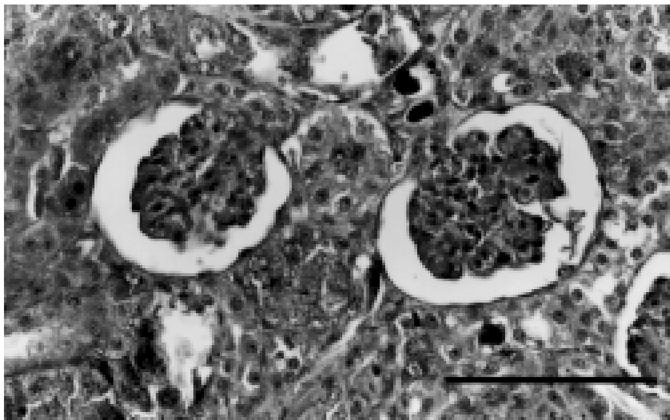

Figure 4. Photomicrograph of a section of the kidney from a gerbil injected 5 days before with mAb HUSM-M.g.40 (experiment 3, group A). Notice atrophic glomeruli. H&E stain; bar = 100 μm.

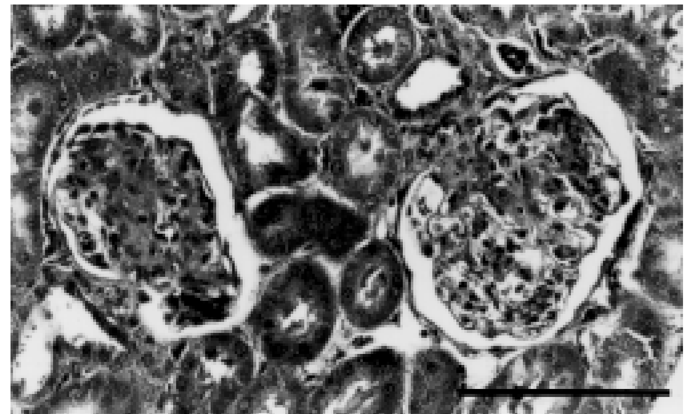

Figure 6. Photomicrograph of a section of the kidney from a gerbil injected 14 days before with mAb HUSM-M.g.40 (experiment 3, group A). Notice mesangioproliferation with capillary aneurysm in the glomerulus. H&E stain; bar = 100 μm.

mass of rat Thy-1.1 antigen (18, 19). Partial differences in reactivity of two mAbs might be ascribed to tissue-specific modification of the molecule, since tissue-specific N-glycosylation, site-specific oligosaccharides patterns and site-specific lentil lectin recognition of the Thy-1 molecule have been documented in rats (20).

Rat anti-Thy-1 GN is induced by a single injection of ARTS or anti-Thy-1 mAb, and the disease is complement dependent, since absence of mesangiolysis was evident in rats deficient in complement by treatment with cobra venom factor (3), or using a noncomplement binding anti-Thy 1.1 mAb of the IgG1 isotype (1). The prominence of C9, as well as the presence of C3, was observed before mesangiolysis in rat glomeruli after deposition of anti-Thy-1 mAb (2). Furthermore, Johnson et al. (21, 22) and Mosley et al. (23) have documented an important role for complement in mediating glomerular localization of platelets or generating reactive oxygen species during anti-Thy-1 GN in rats. These observations indicate an essential role for complement in the mesangiolysis of anti-Thy-1 GN by formation of the membrane attack complex or by complement-mediated recruitment of platelets. Using ARTS, Shichinohe et al. (6) observed anti-Thy-1 GN in gerbils simultaneously injected with guinea pig serum as supplementary complement, but not in gerbils treated only with ARTS. They compared the complement activity of gerbils with that of guinea pigs and rats by detection of complement-dependent hemolysis of sheep red blood cells, and found no hemolytic activity of gerbil complement. Accordingly, they and others suggested that gerbils might be deficient in complement factor(s) or have low functional activity of complement in the classical and alternative pathway of its activation (6, 24). Our results coincide with their suggestion, because simultaneous injection of guinea pig serum with mAb HUSM-M.g.27 was needed to cause renal lesions, or the injection of guinea-pig serum with mAb HUSM-M.g.40 caused more severe clinical manifestations and histologic lesions of acute GN.

What is responsible for the distinct pathogenicity of our two mAbs, low pathogenicity of mAbHUSM-M.g.27 or high pathogenicity of mAb HUSM-M.g.40? The first possibility is the difference in IgG isotype. In the rat model of anti-Thy-1 GN, Bagchus et al. (1) reported that acute glomerular injury was induced by intravenous administration of complement-fixing IgG2a anti-Thy-1 mAb, but not by noncomplement-fixing IgG1 anti-Thy-1 mAb. Accordingly, we could explain our observations as follows: mAb HUSM-40 of IgG2a isotype might efficiently utilize a limited

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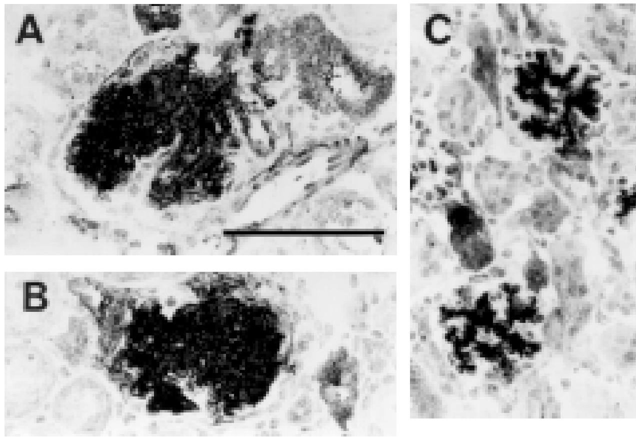

Figure 7. Photomicrograph of a section of the kidney from gerbils injected 14 days before with mAbs HUSM-M.g.40 (A & B; experiment 3, group A) or HUSM-M.g.10 (C; experiment 3, group C). Notice remarkably expanded mesangial area in the glomerulus of **(A)** and **(B)**. Immunohistochemical analysis, using mAb HUSM-M.g.27; bar = 100 µm.

quantity of gerbil complement to induce GN, whereas the other IgG1 isotype might not utilize it. The second possibility is a difference in the epitopes on the mesangium recognized by the two mAbs. Distinct GN pathogenicity of two anti-Thy-1 mAbs, 1-22-3 and OX-7, has been reported in rats, and is ascribed to differences in the distribution pattern of recognized epitopes on the mesangial cells (19, 25, 26). As indicated previously, the reactivity of our two mAbs with certain tissues was different, indicating their recognition of distinct epitopes.

The third possibility is a difference in induction of apoptosis by the two mAbs. Sato et al. (27) reported apoptosis of rat mesangial cells exposed to anti-Thy-1 mAb of IgG2a isotype in vitro, and Morita et al. (28) suggested that complement-dependent cell lysis and Thy-1-mediated apoptosis may coexist in the initial injury of the mesangium in rat anti-Thy-1 GN. Enhanced mAb-mediated apoptosis of rat mesangial cells in vitro was detected in the presence of C1q, a subunit of the first component of complement (29), indicating again that IgG isotype of anti-Thy-1 mAb might determine the level of mesangial apoptosis associated with anti-Thy-1 GN. To further delineate the differences in pathogenicity of our two mAbs and detailed disease process in gerbils injected with them, electron microscopic observations on the kidney are in progress.

The gerbil model of GN reported here is valuable for comparing the pathogenesis and progress of mAb-induced mesangiolysis with that seen in the rat GN model. In addition it may provide further insight into the mechanism of disease.

Acknowledgments

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