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Monoclonal Antibodies Reactive with Dendritic Cells of Mongolian Gerbils

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Mongolian gerbils (*Meriones unguiculatus*) serve as an valuable model animal for several infectious diseases of medical and veterinary importance. Reagents available for characterization of the immune response of Mongolian gerbils are strictly limited. We describe three novel murine monoclonal antibodies (mAbs) to dendritic cells of Mongolian gerbils. These include HUSM-M.g.11 of IgG2b isotype, HUSM-M.g. 20 of IgG2a isotype, and HUSM-M.g.30 of IgG1 isotype. All of these mAbs had an identical profile of immunohistochemical reactions with various tissues taken from immune-naive Mongolian gerbils, and were intensively expressed on dendritic cells, including epidermal Langerhans cells, B-cell follicles, and the thymic reticulum. Positive reactions of the epidermis and intestinal mucosa with these mAbs were induced by cutaneous or intestinal infections with parasites. Competitive enzyme-linked immunosorbent assay and immunoblot analysis (western blotting) indicated that all of these mAbs recognize an identical peptide epitope on a molecule with approximate molecular mass of 29 kDa. These data suggest that the mAbs recognize major histocompatibility complex class-II molecules of gerbils. Use of described mAbs would facilitate characterization of immune responses as well as investigations on host responses to infections of medical and veterinary importance, using the gerbil model.

The Mongolian gerbil (*Meriones unguiculatus*) is a unique laboratory animal. It has high susceptibility to a wide range of infective agents of medical and veterinary importance (1-5). Owing to this characteristic, Mongolian gerbils are used as valuable laboratory models for varied infectious diseases of medical and veterinary importance (e.g., infections due to *Helicobacter pylori*, *Entamoeba histolytica*, varied filarial species, and the larval and adult stages of taeniid species including *Echinococcus multilocularis*). Immunologic reagents available for characterization of host responses to the infection are strictly limited in this animal species; thus, it is hard to explore immune responses in depth, and extrapolate the host-pathogen relationship in the natural infection from experimental data obtained by use of Mongolian gerbils.

We describe three novel murine monoclonal antibodies (mAbs) reactive putatively with major histocompatibility complex (MHC) class-II molecules that are expressed on the antigen-presenting cells playing a critical role in T-cell priming and proliferation.

Materials and Methods

Animals. Mongolian gerbils of a closed colony 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 was continuously available. The boxes were kept in a colony room maintained at 23°C on a 12/12-h light/dark cycle. Routine microbiological monitoring by the Institute for Animal Experiments for Sendai virus, mouse hepatitis virus, *Mycoplasma pulmonis*, and *Clostridium piliforme* has never indicated any problematic infective agents there. All animal experiments, including the method of euthanasia by use of an overdose of ether, were performed according to the Guidelines on Animal Experimentation as set out by Hirosaki University.

Hybridoma production. Three immunization protocols for BALB/c mice were done as follows. I) Four female mice, 8 weeks old, were injected intraperitoneally (i.p.) with 10^6 splenocytes from T cell-depleted Mongolian gerbils, and 29 days later intravenously (i.v.) with 2×10^6 splenocytes from similarly treated gerbils. The T-cell depletion of Mongolian gerbils was achieved using mAb HUSM-M.g.15 as described (6). II) Four female mice, 8 weeks old, were injected i.p. with 10^6 and 2×10^6 splenocytes from T cell-depleted Mongolian gerbils at a 29-day interval. Intravenous injection with 10^6 splenocytes from similarly treated gerbils was made on day 58 after the second injection. III) One female mouse, 5 weeks old, was injected i.p. with 5×10^6 low-buoyant cells taken from the spleen and lymph nodes of immune-naive Mongolian gerbils. Thereafter, intraperitoneal injection with $4\text{-}10 \times 10^6$ low-buoyant lymphoid cells or peripheral blood leukocytes from gerbils were repeated at 10- to 49-day intervals, and the last (sixth) intravenous injection was done on day 139 after the primary injection. Three days after the last injection, the spleen was carefully removed, and used for fusion with the X-63 mouse myeloma cell line. Briefly, splenocytes and myeloma cells were fused at a ratio of 5:1, using 50% polyethylene glycol (mol. wt. 1,300 to 1,600; Sigma Chemical Co., St. Louis, Mo.). The cells were suspended in RPMI 1640 medium (Nissui Pharmaceutical Co., Sugamo, Tokyo, Japan) supplemented with 0.3% L-glutamine, 100 U of penicillin/ml, 100 μg of streptomycin/ml, 0.25 μg of amphotericin B/ml, and 10% heat-inactivated fetal bovine serum (Filtron, Export Dve, Brooklyn, Australia), and plated on flat-bottomed 24-well plates at a concentration of 2×10^6 total cells/well. Hybridoma was selected by use of HAT medium (Sigma Chemical Co.) from the day of fusion under sterile conditions of 5% CO_2 at 37°C. Screening for antibody production was performed using an enzyme-linked immunosorbent assay

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(ELISA) of culture supernatants. For this purpose, ELISA plates were coated with splenocytes from immune-naive or T cell-depleted Mongolian gerbils 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 use of limiting dilution. To determine the isotype of mAbs produced by established clones, ImmunoType mouse monoclonal antibody isotyping kit (Sigma Chemical Co.) was used.

Parasite infection. A Puerto Rican strain of *Schistosoma mansoni* and a Japanese isolate of *Trichinella britovi* were maintained in our laboratory as described (7, 8). Male seven-week-old Mongolian gerbils were exposed to approximately 400 ultraviolet light-attenuated or normal cercariae as described (7), and 3 to 4 gerbils of each group were examined on days four and eight after exposure. Female eight-week-old Mongolian gerbils were inoculated orally with approximately 200 *T. britovi* muscle larvae as described (8), and five gerbils were sacrificed on day seven after infection. Cryostat-cut sections of the exposed epidermis or infected small intestine along with those taken from sex- and age-matched immune-naive gerbils were prepared for immunohistochemical examination.

Immunohistochemical analysis. Various tissue specimens were collected from immune-naive female Mongolian gerbils, 17 weeks old: spleen, thymus, lymph nodes, esophagus, trachea, kidney, liver, heart, small intestine, cerebrum, cerebellum, pons, and skin. Cryostat-cut sections, 12-µm thick from the skin and 6-µm thick from other tissues, were air-dried and fixed for 10 min in cold acetone. Details of the procedure have been described (7). Primary antibody was applied as culture supernatant for one hour, followed by a 1:200 dilution of peroxidase-conjugated goat $F(ab')_2$ fragment to mouse IgG (Fc) (Organon Teknika Co., Durham, N.C.) for one hour. Bound antibody was detected by addition of 3, 3'-diaminobenzidine (DAB) to develop color, followed by light counterstaining with hematoxylin.

Abdominal skin was collected from immune-naive male gerbils, 16 weeks old. With support of a synthetic adhesive (Aron Alpha; Toagosei Co., Ltd., Nishi-shinbashi, Tokyo, Japan) on the epidermal surface, epidermal sheets were separated from the underlying dermis after incubation of skin blocks in 20 m*M* ethylenediaminetetraacetic acid (EDTA)-phosphate-buffered saline (PBS), pH 7.3, at 37°C for two hours as described (9). After washing in 0.15*M* Dulbecco$^{(-)}$ PBS, pH 7.6, sheets were fixed for 10 min in cold acetone. After washing in PBS, sheets were processed for immunohistochemical analysis as described previously, except for three to four hours' incubation with antibodies and no counterstaining.

Labeling of antibody with biotin. A clone producing mAb HUSM-M.g.30 was injected i.p. into pristane-primed BALB/c mice to produce ascitic fluid, which was collected, then was centrifuged and precipitated by addition of saturated ammonium sulfate to a concentration of 50%. After dissolving and dialysis in PBS, a preparation was applied to a stirred ultrafiltration cell (Model 8200; Amicon Division, W. R. Grace & Co., Beverly, Mass.) 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, and was adjusted at 2.0 mg of protein/ml of PBS. The purity of the IgG was checked by use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and was found to be approximately 50%. The antibody was labeled with long-armed biotin according to the method of Holmes and co-workers (10). Briefly, after dialysis against a buffer (0.1*M* $NaHCO_3$, 0.1*M* NaCl, pH 8.4), 10 µl of biotin-AC_5-OSu (10 mg/ml; Dojindo Co., Masuki, Kumamoto, Japan) in anhydrous dimethylsulfoxide was added for each milligram of antibody followed by incubation for one hour. Unbound biotin was removed by dialysis at 4°C in 0.1*M* Tris buffer, pH 7.4.

Competitive ELISA. To determine whether mAbs recognize the same epitope or not, competitive ELISA was performed, using unlabeled mAbs as potential competitors and labeled mAb HUSM-M.g.30. The ELISA plates were sensitized with 0.025% glutaraldehyde in 0.1*M* Na_2CO_3, pH 9.0, for two hours at 56°C, then were coated with lymph node cells from immune-naive gerbils at a concentration of 6×10^4 or 1.2×10^5 cells/well. Attached cells were fixed with 0.25% glutaraldehyde in 0.15*M* PBS, pH 7.6, for 3 min, and residual binding sites were blocked with 1% bovine serum albumin (fraction V; Sigma Chemical Co.) in PBS. Culture supernatant of unlabeled mAbs was applied for one hour, then was replaced by the labeled mAb without washing. After one hour of incubation, a 1:2,000 dilution of peroxidase-conjugated avidin D (Vector Laboratories, Inc., Burlingame, Calif.) was applied for one hour, followed by color development by addition of a substrate solution containing *o*-phenylenediamine. Before reading absorbance at 490 nm, the reaction was stopped by addition of 4*N* sulfuric acid solution.

Immunoblot analysis (western blotting). The thymus was collected from 10-week-old male and 18-week-old female gerbils, a total of 10 in number. After separation of single cells from residual cellular masses, three cycles of freezing and thawing of the latter were made. After washing twice in PBS, the sample was 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, followed by intermittent ultrasonication for five minutes. Supernatant was collected by centrifugation at $6{,}000 \times g$ for 30 min. Separation was performed by use of SDS-PAGE, using 15% gel under reducing conditions, followed by transfer onto a nitrocellulose membrane. Membrane strips with and without periodate treatment as described (11) were incubated with culture supernatant of each mAb, followed by a 1:500 dilution of conjugates. Similarly, other strips were incubated with a 1:200 dilution of biotin-labeled mAb HUSM-M.g.30, followed by addition of a 1:500 dilution of peroxidase-conjugated avidin D. All steps of incubation were made for 1 hour at a room temperature. Bound antibody was detected, using color development by addition of DAB. Periodate treatment was done to eliminate the carbohydrate epitopes of thymic antigens.

Statistical analysis. Data are expressed as mean ± SD. Differences between data from two groups were examined for significance, using the Student's *t* test. A value of $P < 0.05$ denoted statistical significance.

Results

Immunohistochemical analysis. Three immunization protocols produced different isotypes of mAbs (Table 1) with identical immunohistochemical reactivity. In the lymphoid tissues, extensive and intensive staining was observed, except for T cells. In the thymus, medullary epithelial and mesenchymal reticular cells were clearly positive as were cortical epithelial reticular cells (Fig. 1). In the lymph nodes, the cortex, including B-cell follicles and paracortical dendritic cells, was intensively stained but

Table 1. Murine monoclonal antibodies reactive with gerbil dendritic cells

Clone name	Isotype	Immunization protocol*
HUSM-M.g.11	IgG2b	I
HUSM-M.g.20	IgG2a	II
HUSM-M.g.30	IgG1	III

*Details are described in the Materials and Methods section.

with less intense staining of macrophages in the medulla (Fig. 2). A similar staining profile was observed in the spleen (Fig. 3) and Peyer's patches of the small intestine. Cells distributed in the lamina propria of intestine and in the epithelium covering Peyer's patches had positive reaction, but the rest of the intestinal epithelium did not. Cells diffusely distributed in the interstitium of all organs and sinusoidal lining cells in the liver were strongly positive. Epidermal Langerhans' cells were clearly defined by these mAb (Figs. 4 and 5). A few intraepithelial cells, probably dendritic cells, in the esophagus and trachea were similarly positive (Fig. 6). Endothelial cells and the brain did not have positive reaction to these mAbs. Percutaneous exposure to *S. mansoni* cercariae or oral inoculation with *T. britovi* induced signficant but not intense positive reaction of focal epidermis and intestinal epithelium by day seven after infection.

Competitive ELISA and western blotting. As indicated in Fig. 7, unlabeled mAbs HUSM-M.g.11, M.g.20 and M.g.30, disturbed the reaction of labeled mAb HUSM-M.g.30 with gerbil lymphoid cells, suggesting that all three mAbs recognize the same epitope. The reaction of each mAb associated with SDS-PAGE-separated thymic antigens indicated a specific band at about 29 kDa under reducing conditions, even after periodate treatment (Fig. 8).

Discussion

We report three novel murine mAbs of different isotypes to an identical peptide epitope of a thymic molecule with approximate molecular mass of 29 kDa under reducing conditions. The reactive molecule was expressed intensively by dendritic cells, B cells, and thymic reticulum, along with less intense expression by macrophages. Although the epidermal cells and intestinal epithelial cells in immune-naive Mongolian gerbils stained negatively with the mAbs, positive reaction of these epithelia was induced by percutaneous or oral infection with parasites. Observed tissue distribution was typical of MHC class-II antigens as reported (12-14). Furthermore, the expression was found to be inducible in the keratinocytes and intestinal epithelium by the parasitic infection, in accordance with the feature of MHC class-II molecules (7, 15-17).

Mongolian gerbils are known to have notably high susceptibility to a wide range of pathogens, from viruses to helminth parasites, including those of medical and veterinary importance (1, 3-5). Despite their high possibility as an experimental model animal of infectious diseases, reagents available for characterization of immune responses to the infection is strictly limited in Mongolian gerbils. To overcome this situation, we attempted to make mAbs reactive with gerbil leukocytes, and have described HUSM-M.g.15 reactive with gerbil T cells and HUSM-M.g.27 or M.g.40 reactive with the gerbil Thy-1.1 molecule in previous reports (6, 18). The reason(s) why Mongolian gerbils are highly susceptible to pathogens is still uncertain. Some defects or poor activity relative to that in other animals in the effector cells/factors, such as complement (19, 20), mucosal mast cells (21, 22), macrophages (23, 24), and natural killer (NK) cells (25, 26) has been suggested.

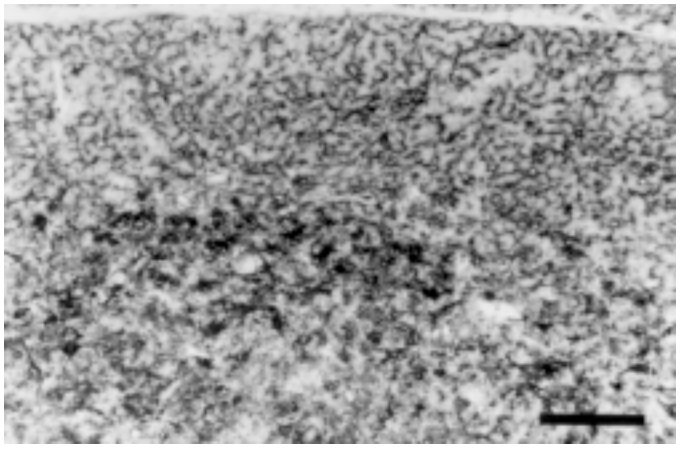

Figure 1. Photomicrograph of a section of the thymus from a gerbil. Notice positive staining of reticular cells in the cortex (upper half of this photograph) and medulla (lower half). Immunohistochemical analysis, using mAb HUSM-M.g.11; bar = 100 µm.

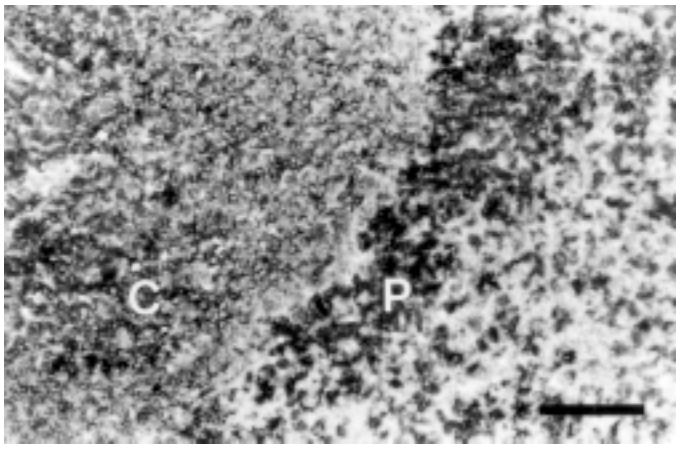

Figure 2. Photomicrograph of a section of the lymph node from a gerbil. Notice intensive staining of a lymphoid follicle (C) and dendritic cells in the paracortex (P). The T-cells in the paracortex are apparently negative. Immunohistochemical analysis, using mAb HUSM-M.g.20; bar = 100 µm.

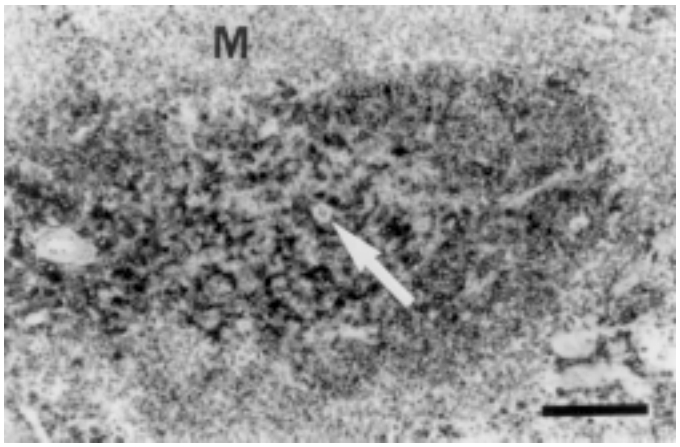

Figure 3. Photomicrograph of a section of a splenic lymphoid follicle from a gerbil. Notice intensive staining of dendritic cells in the periarteriolar sheath around the central artery (arrow) and B cells in the germinal center. The T-cells in the periarteriolar sheath and cells distributed in the marginal zone (M) are apparently negative. Immunohistochemical analysis, using mAb HUSM-M.g.11; bar = 100 µm.

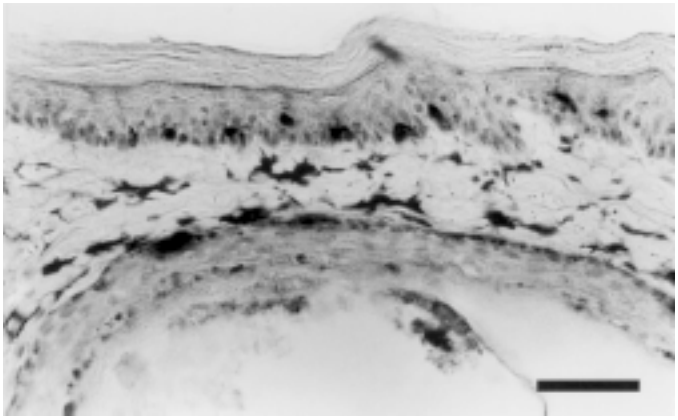

Figure 4. Photograph of a section of the skin over the abdominal scent gland (lower half of this photograph) from a gerbil. Notice intensive staining of epidermal Langerhans' cells located in the suprabasal layer and dendritic cells in the dermis. Immunohistochemical analysis, using mAb HUSM-M.g.20; bar = 50 μm.

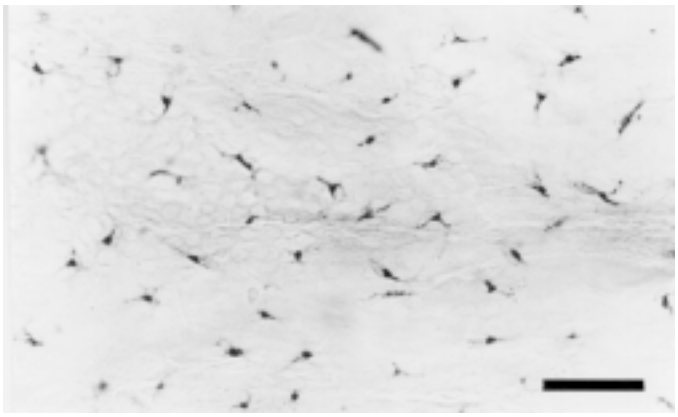

Figure 5. Photomicrograph of an epidermal sheet taken from the flank of a gerbil. Notice intensive staining of epidermal Langerhans' cells with dendrites. Immunohistochemical analysis, using mAb HUSM-M.g.30; bar = 50 μm.

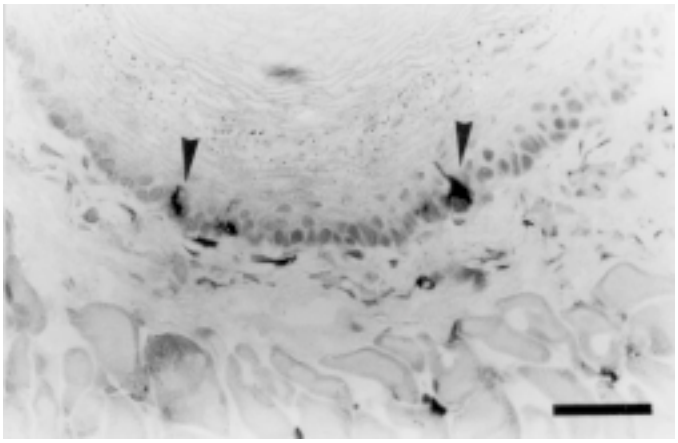

Figure 6. Photomicrograph of a section of the esophagus from a gerbil. Notice positive staining of a few intra-epithelial cells with dendrites (arrow heads) and cells distributed in the lamina propria. Immunohistochemical analysis, using mAb HUSM-M.g.30; bar = 50 μm.

Another possible explanation for the high susceptibility of Mongolian gerbils might be some defects in the induction phase of immunity (e.g., dendritic cells playing a central role in the initiation of T-cell responses [27]). For the present, the mAbs described here

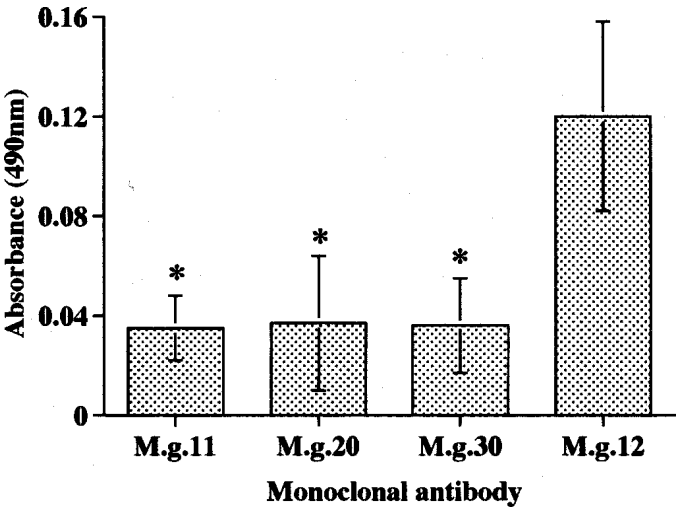

Figure 7. Results of competitive ELISA between unlabeled mAbs shown in X-axis and labeled mAb HUSM-M.g.30. The MAb HUSM-M.g.12 of IgG1 isotype is an unrelated control antibody specific to a gerbil serum factor. Statistically significant interference of the reaction is indicated by asterisks (n = 16). Experiments were repeated twice, and the results were consistent.

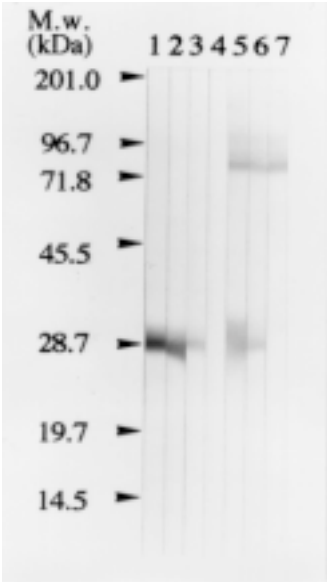

Figure 8. Western blotting of SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis)-separated thymic antigens with mAbs (lanes: 1 to 4, mAbs HUSM-M.g.11, M.g.20, M.g.30, and an unrelated control antibody HUSM-M.g.12, respectively, without periodate treatment; 5, labeled mAb HUSM-M.g.30 without periodate treatment; 6, labeled mAb HUSM-M.g.30 with periodate treatment; and 7, peroxidase-conjugated avidin D only). Notice a specific band appearing at about 29 kDa irrespective of periodate treatment.

are the only available markers of dendritic cells in Mongolian gerbils, and studies using the mAbs will provide invaluable information on unique features of this animal's immune responses.

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