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Circulating Autoantibodies to Mammalian Tissue Kallikreins[1] (42671)

JULIE CHAO,*[,2] RONALD K. MAYFIELD,† AND LEE CHAO‡

*Departments of *Pharmacology, †Medicine, and ‡Biochemistry, Medical University of South Carolina, Charleston, South Carolina 29425*

Abstract. Autoantibodies to tissue kallikrein (EC 3.4.21.35) were discovered in normal human, rat, mouse, and guinea pig sera. Three independent methods—binding of iodolabeled antigen, enzyme-linked immunosorbent assay (ELISA), and immunoblotting—were used to demonstrate these kallikrein autoantibodies. Autoantibodies from rat and human sera were purified, using rat and human tissue kallikrein-affinity chromatography, respectively. Purified rat kallikrein autoantibody bound 50% of ^{125}I-labeled rat urinary kallikrein upon incubation of antibody at 2.5×10^{-10} *M*. The subtypes of rat and human kallikrein autoantibodies were determined by an ELISA, using antisera to immunoglobulin subclasses. In both species, autoantibody was predominantly IgG (~80%) and some IgM (~20%). Purified autoantibodies from rat and human sera were separated on sodium deodecyl sulfate–polyacrylamide gels, and their subunits were identified by Western blot analyses, using anti-rat and anti-human IgG antibodies, respectively. When primary cultures of mouse spleen cells were incubated for 1 to 5 days with lipopolysaccharide (1 to 5 μg/ml), the anti-kallikrein antibodies in the media increased up to seven-fold. We have demonstrated circulating autoantibodies that recognize and bind both autologous and heterologous kallikrein; however, their significance to the function of the tissue kallikrein–kinin system in normal and disease states remains to be explored.

Tissue kallikreins (EC 3.4.21.35) are serine proteases which were first identified in, and isolated from exocrine glands and their secretions (1). Kallikreins are also present in epithelia of the submaxillary gland, colon, kidney, and tracheobronchial tree, where they are released into the lumenal contents of these organs (2–5). Release of kallikrein into the circulation has also been demonstrated, and levels of tissue kallikrein in blood have been quantified (6–9). The enzymatic activity of circulating tissue kallikrein appears to be very low, and the presence of high-molecular-weight complexes of kallikrein in sera suggests that circulating kallikrein may be bound to inhibitors (6, 10). Geiger *et al.* (11) have shown that α_1-antitrypsin can form a complex with tissue kallikrein. However, the K_m of α_1-antitrypsin–kallikrein binding is very high, suggesting little physiological significance for α_1-antitrypsin as an inhibitor of circulating tissue kallikrein. Except for a recently identified novel serum protein (12), none of the other known serum protease inhibitors bind to tissue kallikrein.

In the course of using an enzyme-linked immunosorbent assay (ELISA) to screen monoclonal antibodies raised against purified tissue kallikrein (13), we discovered antibodies against tissue kallikrein in normal sera of mice. Further investigation revealed that normal rat, guinea pig, and human sera also contain similar antibodies. We have isolated these autoantibodies from rat and human sera, using rat and human kallikrein-affinity columns, respectively, and have characterized their titer and immunoglobulin subclasses in normal sera and their binding of kallikrein using ELISA and Western blot. Our studies have also demonstrated that primary cultures of mouse spleen cells release antibody that binds purified rat tissue kallikrein.

Materials and Methods. *Detection of autoantibodies by the ELISA method.* A microtiter plate assay for measuring human anti-kallikrein antibody was developed previously

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[2] To whom all correspondence and reprint requests should be addressed at Pharmacology Department, Medical University of South Carolina, 171 Ashley Avenue, Charleston, SC 29425.

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(13). This was modified for detection of species-specific anti-kallikrein antibodies. Briefly, 96-well polyvinyl microtiter plates (Falcon Becton Dickinson, Oxnard, CA) were coated with purified human or rat urinary kallikrein (0.5 μg protein per well). Free sites were blocked by incubating plates with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 hr at 37°C and plates were then washed with PBS containing 0.05% Tween 20. Normal sera or culture media were diluted serially in PBS containing 1% BSA and 0.05% polysorbate 20 (Tween 20). Aliquots (100 μl) were applied to each well and incubated at 37°C for 90 min. After the plate was washed three times with PBS containing 0.05% Tween 20 at room temperature, 100 μl of anti-IgG conjugated to peroxidase (Cappel Laboratories, West Chester, PA) diluted 1:500 in PBS containing 1% BSA, was added to each well and incubated for 1 hr at room temperature. Antibodies in rat, mouse, guinea pig, or human serum were detected using a species-specific anti-IgG conjugated to peroxidase. For typing the subclasses of tissue kallikrein autoantibodies in rat or human serum, we used goat anti-rat or anti-human IgG, IgM, or IgA conjugated to peroxidase (1:500 dilution in PBS containing 1% BSA). In all cases, following incubation with the anti-immunoglobulin conjugated to peroxidase, the plates were washed seven times with PBS containing 0.05% Tween 20. After washing, 100 μl of peroxidase substrate solution containing 0.03% 2,2′-azino-di(3-ethylbenzthiazoline sulfonic acid) (ABTS) and 0.003% H_2O_2 in 0.05 *M* citrate buffer (pH 4.0) was added to each well. After 10–30 min at room temperature product formation was detected by absorbance increases at 414 nm in a Titertek Multiscan (Flow Laboratories, Inc., McLean, VA).

Autoantibody detection in liquid-phase system. Normal sera from human, rat, mouse, or guinea pig were diluted serially in PBS containing 1% BSA; and 100 μl of these normal sera, or immune serum, was incubated with 0.1 ml of either ^{125}I-labeled rat urinary kallikrein (10,000 cpm, 71.8 μCi/μg) or ^{125}I-labeled human urinary kallikrein (10,000 cpm, 73.2 μCi/μg) in a final volume of 0.4 ml at 4°C for 24 hr. Bound kallikrein was separated from unbound with polyethylene glycol (Fisher Scientific Co., Fairlawn, NJ) (14, 15). Nonspecific binding determined in tubes containing no added serum was subtracted from each sample. It was not necessary to correct for nonspecific effects of serum proteins on binding as the binding curves were carried out in buffer containing 1% BSA. At least 3 individuals from each species were tested, except in humans, in which case more than 20 individuals were studied.

Purification of kallikreins and antibodies. Human and rat urinary kallikreins were purified to homogeneity, and antisera to the purified kallikreins were generated in rabbits or sheep, respectively, as described previously (15, 16). Human urinary kallikrein (2 mg/ml in 0.1 *M* Mops, pH 7.3) was mixed with 25 ml of Affi-Gel 15 (Bio-Rad, Rockville Center, NY), which was prewashed with cold distilled water on a sintered glass filter. The mixtures were incubated with gentle shaking for 4 hr at room temperature and then for 16 hr at 4°C. One-tenth volume of ethanolamine–HCl (1 *M*, pH 8.0) was added to block the remaining unbound sites, and following incubation for an additional 1 hr at 23°C, the conjugate was filtered on a Whatman filter paper (No. 40) (Clifton, NJ) and washed several times with water. Similarly, purified rat tissue kallikrein was coupled to Affi-Gel 15. Rat or human kallikrein-affinity columns (1.5 × 10 cm) were prepared from these enzymes immobilized to Affi-Gel and the columns were equilibrated with 0.1 *M* NaCl and 0.01 *M* sodium phosphate (pH 7.0) and were used for purification of anti-kallikrein antibodies, as described previously. Anti-rat kallikrein antibodies from pooled normal rat sera, normal guinea pig sera, or immune sheep serum were isolated with the rat kallikrein-affinity column; and the human kallikrein-affinity column was used for purifications of kallikrein antibodies from pooled normal human sera or immune rabbit serum. IgG from pooled normal human or rat sera was purified by using protein A–Sepharose-affinity chromatography (17). Purified kallikreins and protein A were labeled with ^{125}I, using the lactoperoxidase method according to Shimamoto *et al.* (15).

Autoantibody identification by Western blotting. The affinity-purified antibodies were separated by using SDS–polyacryl-

amide gel electrophoresis under reducing conditions in a 7.5–15% linear gradient gel containing 0.1% SDS (18). The proteins were then electrophoretically transferred to nitrocellulose as described by Burnette (19). The blot was incubated for 2 hr at room temperature with affinity-purified rabbit anti-human or anti-rat IgG antibody (Cappel Lab.) (10 μg/ml) in a buffer containing 0.15 *M* NaCl, 0.005 *M* EDTA, 0.05 *M* Tris/HCl (pH 7.4), 3% BSA, and 0.05% Nonidet-P40. The blots were washed and incubated for 2 hr at room temperature with ^{125}I-labeled protein A (10^6 cpm/ml), and the proteins recognized by specific antisera were then displayed visually by autoradiography (18).

Lipopolysaccharide-induced kallikrein autoantibody response. The spleen from a 12-week-old male Balb/c mouse was removed under sterile conditions and placed in a petri dish containing 5 ml of Dulbecco's modified Eagle's medium (DMEM). Spleen cells were dispersed by injecting the spleen with the same medium, using a 23-gauge needle. The dispersed cells were then pelleted by centrifugation at 200*g* for 7 min. Red blood cells were lysed by suspending the pellet in 5 ml of cold isotonic ammonium chloride solution on ice for 5 min. Lysis was stopped by adding 10 ml of DMEM containing 15% fetal calf serum (FCS). Cell suspensions were again centrifuged, and the cell pellet was resuspended in 30 ml of DMEM at room temperature. An aliquot of cells was counted in a hemocytometer and the final cell suspension was adjusted to contain 2×10^6 cells per milliliter of DMEM containing 10% FCS. One milliliter of the cell suspension was plated into each well of a 24-well plate. Sterile lipopolysaccharide in 0.9% NaCl was added in the following final concentrations: 0, 1, 2, 5 μg/ml. Plates were incubated at 37°C under 95% O_2, 5% CO_2. Samples (1 ml) from three wells at each concentration were taken on Days 0, 1, 2, 4, and 5. Cells were pelleted from the sample by centrifugation, as above, and kallikrein antibody levels were measured in 0.1 ml of the media supernatant, using the ELISA method described above.

Results. *Detection and identification of autoantibodies to tissue kallikreins.* Normal rat, mouse, and guinea pig sera, variously diluted in buffer (1:40 to $1:4 \times 10^4$) bound ^{125}I-labeled rat urinary kallikrein (Fig. 1A). Similar results were obtained from each serum within species, and therefore, representative titration curves are shown. Guinea pig serum showed greater binding of purified rat kallikrein than rat or mouse serum at comparable dilutions. Binding of ^{125}I-labeled human urinary kallikrein by rat, mouse, and guinea pig sera, as well as by human sera, was also demonstrated. Again, guinea pig serum exhibited greater binding capacity than rat, mouse, or human serum (Fig. 1B). Measurements in serially diluted sera using an ELISA, in which autoantibodies were detected with a species-specific second antibody, gave very similar results (data not shown).

To identify the kallikrein-binding components in sera, rat and human tissue kallikrein-affinity columns were prepared by immobilizing each enzyme to Affi-Gel 15. Normal rat or guinea pig sera were passed through the rat kallikrein-affinity column, and the kallikrein-binding components from rat or guinea pig sera were eluted from the column. The kallikrein-affinity-purified components from rat and guinea pig sera showed binding to rat urinary kallikrein and subsequent recognition by species-specific anti-IgG in an enzyme-linked immunosorbent assay (Fig. 2A). These purified kallikrein-binding proteins from normal rat or guinea pig sera also showed binding of ^{125}I-labeled rat urinary kallikrein in a liquid-phase system (Fig. 2B). At 2.5×10^{-10} *M*, the affinity-purified proteins from rat sera bound 50% of the ^{125}I-labeled rat urinary kallikrein. Similarly, when the kallikrein-binding component from normal human sera was purified with a human urinary kallikrein-affinity column, the purified protein bound human tissue kallikrein in both a solid-phase ELISA and a liquid-phase RIA system (data not shown). That these purified serum components bound rat or human kallikrein and were recognized by anti-immunoglobulin antisera in the ELISA suggests that a kallikrein-binding capacity in sera is due to immunoglobulins which recognize tissue kallikreins.

The subtypes of these kallikrein autoantibodies in rat and human sera were determined by an ELISA, using goat anti-rat or anti-human IgG, IgM, or IgA antisera, re-

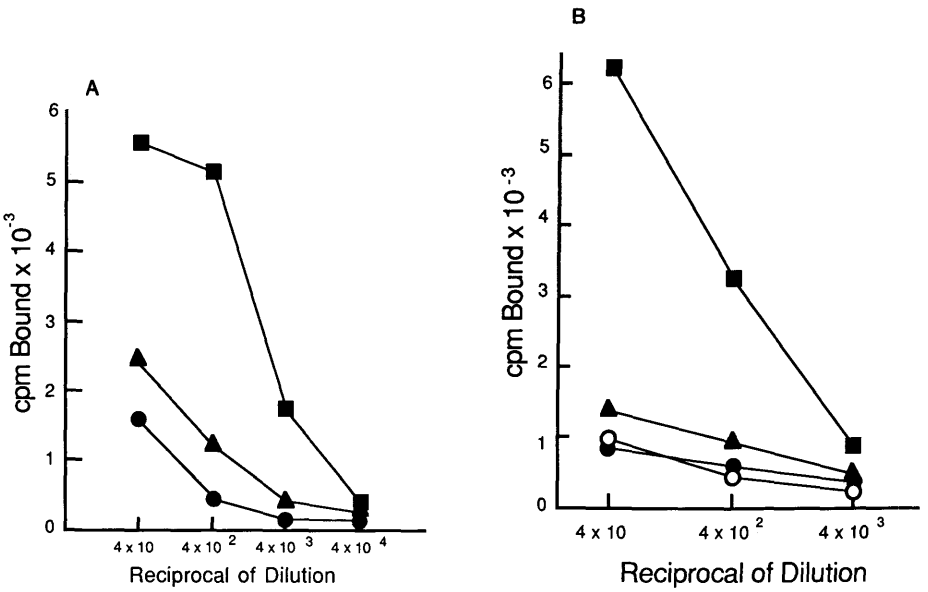

FIG. 1. Kallikrein antibody titration curves by RIA. (A) One-tenth milliliter of the serum or antiserum was diluted and incubated in the indicated final concentrations with 0.1 ml of ^{125}I-labeled rat urinary kallikrein (10,000 cpm, 71.8 μCi/μg) in a final volume 0.4 ml at 4°C for 24 hr. Antibody-bound tracer was separated from the free form with a polyethylene glycol separation technique. (B) Kallikrein antibody titration curves by RIA. The procedures are identical to those described in (A) except ^{125}I-labeled human urinary kallikrein was used. Normal serum from human (○); mouse (▲); rat (●); and guinea pig (■).

spectively. In either crude sera or affinity-purified autoantibodies, the antibody subtypes from rat and human sera were shown to be predominantly IgG (~80%) and some IgM (~20%), with a negligible amount of IgA.

Direct identification of kallikrein autoantibodies by Western blotting. The affinity-purified kallikrein autoantibodies from rat and human kallikrein were further analyzed by Western blotting, using rabbit anti-rat or anti-human immunoglobulin antiserum followed by ^{125}I-labeled protein A binding. Anti-immunoglobulin binding was then displayed visually by autoradiography. The purified proteins were recognized by anti-immunoglobulin antiserum, and the molecular weights of anti-kallikrein immunoglobulin subunits from rat or human serum were similar to those of heavy and light chains purified by protein A-affinity column from normal rat (Fig. 3A) or human sera (Fig. 3B).

Lipopolysaccharide-induced kallikrein antibody response of spleen cells. Lipopolysaccharide (LPS) at 0, 1, 2, and 5 μg/ml was added to the mouse spleen cell suspensions in triplicate and incubated in nutrient culture media at 37°C for 1, 2, 4, and 5 days, respectively. Antibodies which bound rat kallikrein were secreted into the media, as measured by ELISA, and the concentration in the media increased up to sevenfold in response to addition of LPS (Fig. 4). From daily microscopic examination, LPS showed no effect on the morphology of the spleen cells under these conditions.

Discussion. We have found that the kallikrein-binding capacity of normal mammalian sera is due in part to autoantibodies which recognize tissue kallikrein. The presence of these antibodies was demonstrated in several ways. After purification of the binding components from guinea pig, rat, and human sera by kallikrein-affinity chromatography, these proteins showed the same binding characteristics as crude sera. In an ELISA, the binding components were recognized by species-specific anti-immunoglobulins (IgG, IgM). Furthermore, in Western blotting, the heavy and light chains of these antibodies were displayed visually by autoradiography after binding to anti-IgG and subsequent labeling with ^{125}I-labeled protein A.

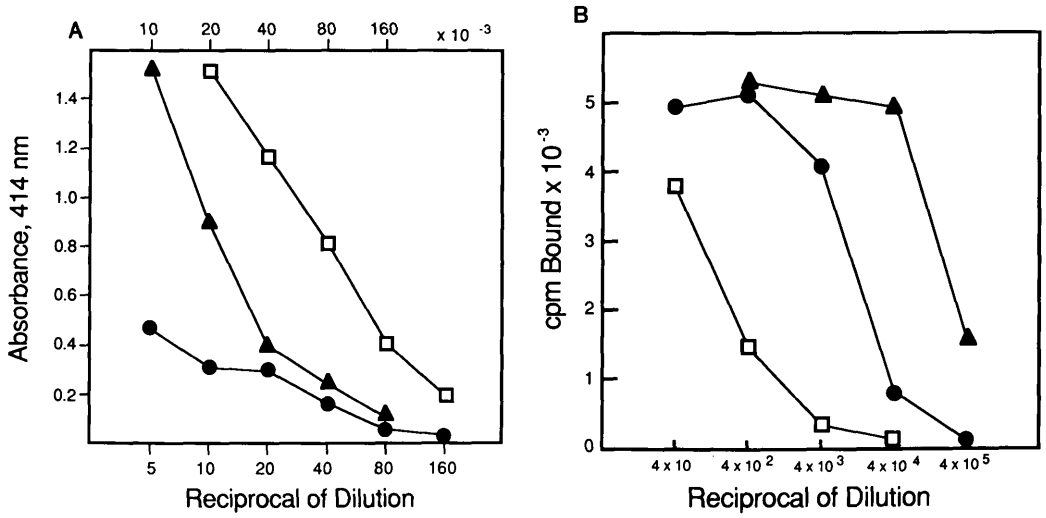

FIG. 2. (A) Kallikrein antibody titration curves by ELISA. The microtiter plate was coated with 0.5 μg of purified rat urinary kallikrein in 0.1 ml of PBS for 2–3 hr at 37°C and blocked with 5% BSA in PBS for 1 hr at 37°C. One-tenth milliliter of affinity-purified kallikrein antibodies to normal serum components was applied to each well and incubated at 37°C for 90 min. The wells were washed with PBS containing 0.05% Tween 20. Peroxidase-conjugated anti-IgG to rabbit (▲), rat (●), or guinea pig (□) (1:500) in PBS containing 1% BSA was added. Absorbance at 414 nm was determined after the addition of 0.003% of H_2O_2 and 0.03% 2,2'-azino-di(3-ethylbenzthiazoline sulfonic acid) (ATBS) in 0.05 *M* citrate buffer, pH 4, and incubation for 30 min. Affinity-purified rabbit anti-rat kallikrein antibodies (1.5 mg/ml, ▲); rat serum proteins (0.34 mg/ml, ●); guinea pig serum proteins (0.67 mg/ml, □). (B) Kallikrein antibody titration curves by RIA. The experimental conditions are as described in the legend to Fig. 1. Proteins and symbols are identical to those shown in Fig. 2A. Rabbit anti-kallikrein antibody (▲) dilutions in Fig. 2A are shown on the upper panel.

The finding of autoantibodies to tissue kallikrein raises several questions about their origin and significance. Guinea pig sera demonstrated greater binding of both rat and human kallikrein than other sera. It is interesting to speculate about the possibility that autoantibody production is greater in the guinea pig than in other species, as the immune system of the guinea pig is easily sensitized to antigens. The presence of anti-kallikrein antibodies from mammalian species, and in virtually all human subjects tested (data not shown), suggests that their origin is probably not the result of an abnormal immune response. It is possible that *in vivo* modification of tissue kallikrein results in an antigenic protein or that tissue kallikrein is recognized by an antibody generated against an epitope which is shared by a foreign protein or another modified autologous protein and kallikrein.

There are no reports of tissue kallikrein being modified to an antigenic state; however, such a process has been demonstrated for other circulating and structural proteins. Low-density lipoprotein (LDL) can be modified through oxidation by superoxides when the lipoprotein is incubated in cultures of endothelial or vascular smooth muscle cells (20, 21). Modified LDL is recognized and taken up more avidly by macrophages than is natural LDL (21, 22). The recent demonstration that tissue kallikrein circulates makes it plausible that the enzyme could make contact with endothelial cells and become modified (6–9). The isolation of a kininogenase from vascular tissue further supports the possibility of such an interaction (23).

The presence of LPS from *Escherichia coli* at 1 to 5 μg/ml in the mouse spleen cell cultures showed a dose- and time-dependent increase of autoantibodies which bound purified rat kallikrein, as measured by ELISA (Fig. 4). The morphology of spleen cells was not changed under these conditions. As LPS has been shown to stimulate spleen cell differentiation *in vitro* (24), the increase of anti-

bodies to tissue kallikrein may be ascribed to the increase of antibody synthesis but not the cell leakage of the antibodies.

We do not know the significance of kallikrein autoantibodies to the function of the kallikrein–kinin system, but can speculate as to some possibilities. Antibody-bound kallikrein may contribute to the circulating immunoreactive kallikrein (6–9). In this regard, we have some evidence that these antibodies do bind kallikrein *in vivo*. In the course of isolating human kallikrein from plasma, using a kallikrein antibody-affinity column, we eluted kallikrein antibody that was of human origin (data not shown). Kallikrein autoantibody might also contribute to the clearance of active kallikrein from the circulation. One study found that inactivated kallikrein is cleared more rapidly than active enzyme, and that kallikrein bound in high-molecular-weight complex is cleared primarily by organs with active reticuloendothelial systems (25). Whatever their role, the finding that kallikrein antibodies are rather ubiquitous among individuals of several species suggests that they may have fundamental importance to the function of the tissue kallikrein–kinin system. Studies to examine this can now be carried out.

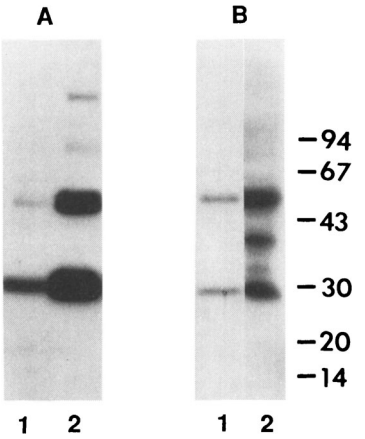

FIG. 3. Western blot analyses of rat and human tissue kallikrein antibodies. (A) Immunoblotting with rabbit anti-rat IgG antiserum antibodies. (1) Rat kallikrein-affinity-purified rat serum; (2) protein A-affinity-purified rat serum. (B) Immunoblotting with rabbit anti-human IgG antiserum. (1) Human kallikrein-affinity-purified human serum; (2) protein A-affinity-purified human serum. Protein molecular-weight markers are phosphorylase *b* (94,000); bovine serum albumin (67,000); ovalbumin (43,000); carbonic anhydrase (30,000); soybean trypsin inhibitor (20,000); and α-lactalbumin (14,000).

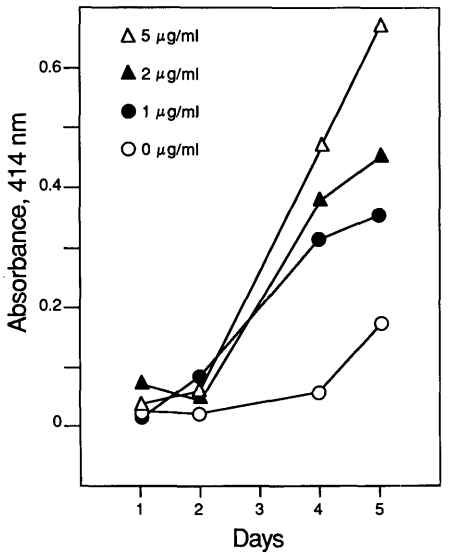

FIG. 4. Lipopolysaccharide-induced autoantibody response. The spleen cells were obtained from Balb/c mice, and red blood cells were lysed in cold isotonic ammonium chloride. One milliliter of spleen cells in suspension at 2×10^6/ml was applied to each well of a 24-well plate. Sterile lipopolysaccharide in 0.9% NaCl was added to each well in triplicate at the concentration of 0 μg/ml (○), 1 μg/ml (●), 2 μg/ml (▲), or 5 μg/ml (Δ). One milliliter of sample from each well was withdrawn at the indicated time intervals. The cells were removed by centrifugation and 0.1-ml aliquots of the media were used to measure the production of kallikrein autoantibodies using an ELISA as described in the legend to Fig. 2A.

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