

Production and characterization of mouse monoclonal antibodies directed against canine IgE and IgG

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ABSTRACT

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Immediate-type hypersensitivity reactions have been studied infrequently in dogs, in part because of limited availability of antisera specific for canine IgE. A series of murine hybridoma cell lines were prepared, that produced monoclonal antibodies (MAb) with specificity for canine immunoglobulin E (IgE) and IgG. The MAb were tested for their ability to induce a reverse cutaneous anaphylaxis reaction in dog skin, to neutralize the Prausnitz–Küstner reactivity of atopic dog serum, to serve as a ligand in immunoaffinity chromatography, and to bind to IgE and other Ig subclasses in several ELISA systems. Some of the MAb produced were found to be specific for canine IgE. Other MAb recognized common or similar determinants on IgE and IgG, or on IgG and IgM, though with apparently differing affinities. Heat or acid treatment of canine IgE abolished most, but not all, of the reactivity with the anti-IgE MAb. These MAb will be useful for further study of IgE-mediated phenomena in the dog.

ABBREVIATIONS

BSA, bovine serum albumin; D_c2, epsilon-2 determinant; HAT, hypoxanthine-aminopterin-thymidine solution; Ig, immunoglobulin; MAb, monoclonal antibodies; PBS, phosphate buffered saline; PK, Prausnitz–Küstner; PPDS, pooled parasitized dog serum; RAST, radioallergosorbent test; RT, room temperature; S-DMEM, supplemented Dulbecco's minimal essential medium.

INTRODUCTION

Detection and quantitation of immunoglobulin E (IgE) antibodies is important in the diagnosis and study of Type I (immediate-type) hypersensitivity

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ity disease. Development of specific antisera and monoclonal antibodies (MAb) useful for detecting human IgE has enabled development of many standard assays used by physicians in allergy diagnosis, such as quantitation of total serum IgE and of allergen-specific IgE (Halpern, 1989). Likewise, these reagents have permitted a wealth of studies on pathomechanisms of allergic disease and the molecular events involved in immediate hypersensitivity reactions.

Diseases involving Type I hypersensitivity are common in dogs. For example, atopy (allergic inhalant dermatitis) has been estimated to affect between 3 and 15% of the canine population, and represents up to 30% of the patients encountered at veterinary dermatology referral practices (Reedy and Miller, 1989). Despite the prevalence of these diseases, canine IgE and IgE-mediated phenomena have received a minimum of study in the dog compared with other species such as human beings and rodents. Part of the reason has been a lack of availability of reagents to specifically detect canine IgE. Polyclonal antisera against canine IgE have been produced, but their use has been limited to commercial laboratories and a few investigators (Schwartzman et al., 1971; Halliwell et al., 1972; Peters et al., 1982; Schultz and Halliwell, 1985; Kleinbeck et al., 1989). Presently, the major application for detection of canine IgE is in the commercially-available *in vitro* allergy tests based on the radioallergosorbent test (RAST) or ELISA techniques. Greater availability of specific reagents to detect canine IgE would enhance our ability to study mechanisms of canine hypersensitivity disease and diagnose such diseases in clinical patients.

MAB have been produced against IgE of other species, including humans (Chandler et al., 1983; Sanchez-Madrid et al., 1984), mice (Hirano et al., 1988), rats (Rup, 1989), and cattle (Thatcher and Gershwin, 1988). We hypothesized that it should be possible to prepare MAB directed against canine IgE, that are specific for the IgE molecule (i.e. that do not react against other immunoglobulin subclasses). In the present study, we report production of mouse MAB against canine IgE and IgG, characterization of the specificities of these antibodies, and some of their immunologic properties.

MATERIALS AND METHODS

Animals and sera

Random-sex BALB/c mice (Charles River Laboratories, Wilmington, MA) at least 12 weeks of age were used for hybridoma generation and production of MAB as ascites fluid.

Healthy adult dogs used for the reverse cutaneous anaphylaxis and Prausnitz-Küstner (PK) testing experiments were from the teaching animal colony at the School of Veterinary Medicine. Pooled parasitized dog serum

(PPDS) was obtained from a group of laboratory dogs infested with *Sarcoptes scabiei* and *Dirofilaria immitis*. Sera from atopic dogs were obtained from the Veterinary Medical Teaching Hospital patient population. All sera were stored frozen at -30 to -70°C until used.

Immunization of mice

IgE-rich and IgG-rich fractions of PPDS were prepared using a chromatographic procedure similar to one previously described (Halliwell et al., 1972). Briefly, whole serum was precipitated with 33% saturated ammonium sulfate, and the supernatant re-precipitated by raising the saturated ammonium sulfate concentration to 45%. The resulting precipitate was exhaustively dialyzed against 0.35 M sodium phosphate buffer, pH 8, and applied to a $2.5\text{ cm} \times 40\text{ cm}$ column of DEAE-cellulose beads (Cellex D, Amicon, Beverly, MA) equilibrated with the same buffer. The excluded protein was concentrated by ultrafiltration and applied to a $1\text{ cm} \times 100\text{ cm}$ gel filtration column (Bio-Gel P300, Bio-Rad, Richmond, CA) equilibrated with 0.01 M sodium phosphate-buffered 0.15 M NaCl, pH 7.4 (PBS). The first quarter of the major peak was collected and used as the IgE-rich fraction. It was later determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) that this fraction contained approximately 5% IgE and 95% IgG. The final quarter of the same peak was collected and used as an IgG-rich fraction. It contained no detectable IgE by SDS-PAGE.

Mice were immunized intraperitoneally with $250\text{ }\mu\text{g}$ of the IgE-rich fraction homogenized in Freund's complete adjuvant. One to 3 months later, the mice received a second immunization of $250\text{ }\mu\text{g}$ in Freund's incomplete adjuvant. Four days prior to the fusion procedure, each mouse received an intravenous injection of $250\text{ }\mu\text{g}$ of the same antigen in PBS.

Hybridoma production

Procedures for hybridoma production generally followed those detailed previously (Galfrè and Milstein, 1981). The non-secreting mouse myeloma cell line NS-1 was maintained in culture in Dulbecco's minimal essential medium (Gibco BRL, Grand Island, NY) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, and 15–20% fetal bovine serum (S-DMEM). All cell cultures were incubated at 37°C in 5% CO_2 .

Immunized mice were anesthetized with methoxyflurane and splenectomized. Splenic lymphocyte suspensions were prepared and adjusted to a concentration of $1 - 5 \times 10^7$ cells ml^{-1} in S-DMEM. The lymphocytes were combined with NS-1 cells at a 5:1 ratio and the combined cells were centrifuged at $200 \times g$ for 5 min. The pellet was gently resuspended in 1 ml 50% polyethylene glycol 4000 (Gibco BRL) with 5% DMSO for 60 s, then gently agitated for 90 s in a water bath at 37°C . Following this fusion step, the cell suspension

was diluted to 20 ml with S-DMEM, washed once, and adjusted to 2×10^6 cells ml^{-1} . Approximately 0.1 ml of cell suspension was pipetted into each well of 96-well tissue culture microplates (Falcon, Lincoln Park, NJ). After 1 week of cell growth, hybridomas were selected with S-DMEM to which was added hypoxanthine-aminopterin-thymidine solution (HAT supplement, Sigma, St. Louis, MO), according to standard techniques (Galfr  and Milstein, 1981).

Wells with antibody-producing cells were identified using an ELISA (see below). Producing cells were expanded to larger culture vessels, and when growing vigorously were subcloned by limiting dilution in S-DMEM a minimum of four times, until all cell-containing wells from the subcloning repeatedly produced antibody of the same specificity as judged by ELISA. The final antibody-producing cells were maintained in continuous culture in S-DMEM.

Production and purification of monoclonal antibody

The majority of the antibody used in experiments was produced as ascites fluid in BALB/c mice. Ten to 14 days after intraperitoneal injection of 0.1 ml pristane (Sigma), 10^6 hybridoma cells were injected intraperitoneally into each mouse. Ascites fluid was harvested from anesthetized mice 1–2 weeks later, centrifuged to pellet cellular debris, passed through a $0.2 \mu\text{m}$ filter, and stored at -30°C .

Monoclonal antibody was further purified by high-performance liquid chromatography of the ascites over a $1 \text{ cm} \times 25 \text{ cm}$ column of Bakerbond AbX (J.T. Baker, Phillipsburg, NJ), equilibrated with 0.04 M morpholino ethanesulfonate buffer, pH 5.8. The antibody was eluted with a 0–40% continuous gradient of 0.3 M ammonium sulfate in the same buffer, concentrated and exchanged into PBS by ultrafiltration, and stored frozen at -30°C . This chromatographic procedure produced MAb with less than 5% contaminant proteins, as determined by SDS-PAGE.

The subclass of each MAb was determined using a commercial isotyping kit (MonoAb ID EIA kit, Zymed, San Francisco, CA). Biotinylation of the purified antibodies with EACA-biotin was performed with an ImmunoSelect Biotinylation Kit (Gibco BRL).

ELISA assays for hybridoma screening

Several ELISA assays were used to monitor antibody production by the hybridoma cells and to characterize the final antibodies. All ELISA assays had the following features in common: polystyrene microtiter plates (Corning, Inc., Corning, NY) were coated with $100 \mu\text{l}$ of the desired protein in 0.1 M sodium carbonate, pH 9.6, overnight at 4°C . All washing steps were performed with an automated plate washing apparatus (Dynatech, Chantilly, VA), using six washes with 0.15 M NaCl containing 0.05% Tween-20. Un-

reacted protein-binding sites in the wells were blocked by incubation for 1 h at room temperature (RT; approximately 25°C) with 250 μ l of 0.4% bovine serum albumin (BSA; Sigma) in PBS. Dilutions of dog sera, mouse ascites, purified MAb, and peroxidase or biotin conjugates were made in 0.05 M Tris-buffered 0.15% NaCl, pH 7.4, containing 0.05% Tween-20 and 0.1% BSA. The volume in the wells was 100 μ l, except for the blocking reagent. The final step of each assay was incubation with ABTS substrate solution (Sigma) for 4–10 min. Color development was stopped with 50 μ l 0.2 M HF; color was measured as optical density at 405 nm in a microplate reader, with subtraction of the appropriate blank well value.

In one assay, used to screen growing hybridomas for antibody production, the IgE-rich and IgG-rich fractions from PPDS were used to coat microtiter plates at 10 μ g ml⁻¹. After blocking and washing, hybridoma tissue culture supernatant was added to the wells, and plates were incubated 1.5 h at RT. The wells were washed and incubated with 1:5000 peroxidase-conjugated goat anti-mouse IgG + IgM (Jackson ImmunoResearch, West Grove, PA) for 1.5 h at RT. Wells were washed, developed with substrate solution, and read on the microplate reader. Each clone or antibody was tested on both IgE-rich and IgG-rich coated wells. Optical densities of the wells were compared, and those clones giving a high reading on the IgE-rich fraction with a low reading on the IgG-rich fraction were selected as potential anti-IgE producers. Those clones giving strong positive reactions on both well types were considered to be potential producers of anti-IgG or anti-Ig light chain.

A second ELISA assay was used to further screen hybridoma clones. For this assay, the IgE-rich fraction of PPDS was first subjected to vertical slab SDS-PAGE under reducing conditions (Laemmli, 1970). A section of the gel containing the separated antibody and molecular weight standards was immediately removed from the edge, and stained with a rapid staining kit (Kodavue, Eastman Kodak, Rochester, NY). The positions of the bands corresponding to IgE epsilon-chain (approximately 85 000 Da), IgG gamma-chain (approximately 50 000 Da) and Ig light chain (approximately 25 000 Da) were determined, and the corresponding sections of the remainder of the gel were sliced out. Protein was eluted from the gel slices using an electroelution device (Elutrap, Schelicher and Schuell, Keene, NH), according to manufacturer instructions. The eluted protein was then diluted (1/30–1/500) and used to coat microplate wells. The remaining steps in the ELISA were identical to those described above. This assay allowed further identification and confirmation of antibodies with specificity towards epsilon-chain, gamma-chain, and Ig light chain.

Induction of reverse cutaneous anaphylaxis

Purified MAb solutions (anti-IgG and anti-IgE) were adjusted to a concentration of 10 μ g ml⁻¹ in PBS. This stock solution was then serially diluted

with PBS in five-fold increments (range, 2000 to 0.64 ng ml^{-1}). 0.05 ml of each dilution was injected intradermally into the clipped lateral thoracic skin of three healthy adult dogs. The quantity of antibody protein injected in each site thus varied from 0.03 to 500 ng. Injections of 0.05 ml PBS and 0.05 ml 1:100 000 histamine phosphate were administered along with the antibody injections, as negative and positive controls. Fifteen minutes later, wheal and flare reactions were noted and their horizontal and vertical diameters were recorded.

Neutralization of PK reactivity of atopic dog serum

Antibodies were tested for their ability to remove PK reactivity from atopic dog serum by absorbing the serum with MAb-coated discs. Each purified antibody (100–200 μg in 70 μl 0.5 M sodium carbonate, pH 9.3) was coupled to a 25 mm membrane-type activated affinity chromatography matrix disc (Nalge, Rochester, NY) according to the manufacturer's instructions. A control disc was prepared according to the same protocol, but using 0.5 M sodium carbonate only (without antibody protein). Coupled discs were placed in small, individual polypropylene beakers. A serum sample from a ragweed pollen-allergic dog, shown in pilot experiments to possess PK reactivity at a dilution of $> 1/200$, was diluted 1/10 in PBS. A 1 ml aliquot of the diluted serum was placed in each beaker. Diluted serum and discs were incubated 3 h at RT on a rotating platform, then the solution was recovered from each beaker. Serial two-fold dilutions of the recovered solutions were prepared (dilution range, 1/20 to 1/2560) and 0.1 ml of each dilution was injected intradermally into the clipped lateral thoracic skin of a healthy dog. Forty-eight hours later, the sites were challenged with 0.05 ml of ragweed pollen extract (Greer Laboratories, Lenoir, NC) at 1000 protein nitrogen units ml^{-1} . Control injections of histamine phosphate and PBS also were made. Fifteen minutes after challenge injection, the sites were observed and horizontal and vertical diameters of wheal and flare reactions were measured and averaged. The PK titer was defined as the highest serum dilution that produced a wheal at least 5 mm larger than the PBS control.

Affinity chromatography for preparation of purified IgE

Purified MAb B3 (6 mg) was coupled to 2 ml of an activated beaded polymer chromatographic support (Affi-Prep Hz, Bio-Rad), according to manufacturer protocols. The coupled support was packed under unit gravity into a 4.6 mm $\times$ 100 mm chromatographic column and washed successively with PBS containing 0.5% Tween-20 (PBS-T); 1 M NaCl; and 0.5 M citric acid, pH 3.5. The column was then equilibrated with PBS-T. The canine serum proteins precipitating at between 33 and 45% saturated ammonium sulfate

concentration were prepared as above (using PPDS) and dialyzed against PBS-T. Ten ml of this solution was applied to the room-temperature column and slowly percolated through at a flow rate of 0.15 ml min^{-1} . Following this sample application, the column was washed with 1 M NaCl, then with PBS-T until the A_{280} of the column effluent reached baseline. Bound material was rapidly eluted with 0.5 M citric acid, pH 3.5, directly into an excess of ice-cold 1.5 M Tris, pH 8.8, containing 5% glycerol and 5 mM NaEDTA. Material recovered from repeated procedures was pooled, concentrated, and stored at -30°C prior to use. Purity of the recovered protein solution was assessed via SDS-PAGE and via its reactivity with monoclonal anti-canine IgG and IgE in an ELISA assay.

ELISA on immunoglobulin subfractions

Monoclonal anti-canine IgG and IgE antibodies were tested for their ELISA reactivity against various purified canine immunoglobulins and Ig subfractions, including whole IgM (Organon Teknika Cappell, Malvern, PA), whole IgE (affinity purified, as prepared above), heat-treated IgE (affinity purified, heated to 56°C for 4 h), acid-treated IgE (affinity purified, pH adjusted to 2.3 with 0.1 M glycine buffer for 4 h), IgG Fc, IgG Fab, and IgG F(ab')₂ (the latter three from Jackson ImmunoResearch). Each Ig or subfraction was diluted to $10 \mu\text{g ml}^{-1}$ and coated onto microtiter plates. Remaining steps were similar to the assays described above, including blocking with BSA, incubation with monoclonal anti-canine IgG or IgE, incubation with peroxidase-conjugated goat anti-mouse IgG, and development with ABTS substrate. Results were expressed as mean OD of duplicate wells after subtraction of blank well values.

Competitive ELISA

This experiment relied on competition between biotin-conjugated MAb and their unconjugated counterparts for antigen coated to microwells. Canine IgE was used to coat microtiter plates, and the plates were blocked with BSA as described above. Serial two-fold dilutions of each antibody-containing ascites ($1/50$ – $1/512\,000$) were prepared, and $100 \mu\text{l}$ of each dilution was pipetted sequentially into the wells of several rows. Dilution buffer only was placed in the last well of each row. Plates were incubated for 1.5 h at RT, then washed. To wells in one row was then added $100 \mu\text{l}$ of a biotin-conjugated MAb solution ($1/500$). A different biotin conjugated antibody was added to each row of the serial dilutions of ascites fluid. In this manner, each MAb was tested for its ability to compete for substrate with the other antibodies. After 1 h incubation at RT, plates were washed and $100 \mu\text{l}$ of $1/1000$ peroxidase-conjugated Avidin D (Vector, Burlingame, CA) was added to each well. Plates were incubated 1 h, washed, developed with ABTS substrate, and read in the

microplate reader. Absorbances in the wells containing no ascites were defined as 100% reactivity ($OD_{100\%}$), and the percentage inhibition by dilution Y of ascites was calculated as $(OD_{100\%} - OD_Y) / OD_{100\%} \times 100\%$.

Sandwich ELISA

These experiments were conducted to gain further insight into the molecular specificities of the anti-canine IgG and IgE antibodies, and to preliminarily evaluate them for use in a double antibody 'sandwich' ELISA for quantitation of canine IgE. Each purified MAb was coated onto microwells at $25 \mu\text{g ml}^{-1}$. Plates were washed and blocked with BSA as described above. PPDS was prepared in five-fold serial dilutions from 1/5 to 1/3125, and 100 μl of each dilution was added to the wells. After incubation for 1 h at RT, the plates were washed. Each purified, biotinylated monoclonal anti-canine IgG or IgE antibody was next diluted 1/500 and added to wells, and the plates were incubated 1 h at RT. Plates were washed, and 100 μl of peroxidase-conjugated avidin D was added to each well. After a final 1 h incubation at RT, plates were washed, developed with ABTS substrate, and read on a microplate reader. In this manner, each antibody was tested for its ability to 'capture' IgG or IgE from serum, and to 'detect' the captured material, in every pairwise combination.

RESULTS

Hybridoma production and antibody characterization

Fusion of immune splenic lymphocytes with the mouse myeloma cell line followed by culture in 96-well microplates yielded many antibody-producing hybridoma cell lines, as determined by ELISA of medium from the wells. After repeated subcloning, cell lines were found with apparent specificity to IgG, IgE, and Ig light chain. From these cell lines, six were selected for further characterization and confirmation of their specificities; these six cell lines were designated A5, B3, and D9 (tentatively identified as producing anti-IgE) and B7, C7, and E3 (tentatively identified as producing anti-IgG). All six antibodies were of the mouse IgG₁, kappa light chain isotype.

Reverse cutaneous anaphylaxis

As shown in Table 1, antibodies A5, B3, and D9 induced wheal-and-flare reactions when injected into the skin of normal dogs. These reactions were dramatic in appearance, being erythematous, indurated, and approaching or exceeding the size of the positive control injection (histamine) in many cases. Under the conditions tested, the minimum amount of monoclonal anti-IgE

TABLE 1

Summary of reverse cutaneous anaphylaxis experiments. Serially diluted monoclonal antibodies were injected intradermally into healthy dogs, and 15 min later the diameters of the reactions were measured. Values shown are means of horizontal and vertical diameters in three dogs (in mm)

Monoclonal antibody	Concentration of monoclonal antibody injected intradermally (ng/ml)						
	10 000	2000	400	80	16	3.2	0.64
A5	11.0**	10.7**	9.0**	7.2 ⁺	7.2	5.7	6.2
B3	10.7**	10.2**	9.2**	7.5 ⁺	6.5	6.3	6.8
B7	6.5	6.2	5.8	5.0	5.8	6.2	6.3
C7	5.2	7.0	6.3	6.5	6.3	6.8	6.8
D9	8.2 ⁺	9.7**	10.7**	6.8 ⁺	6.5	4.7	5.7
E3	7.5 ⁺	5.8	6.3	6.2	5.8	5.7	5.5

*, Value exceeds positive threshold, defined as the midpoint value between the saline control value (6.8) and the histamine control value (10.7).

⁺, Reaction sites were considered to be notably erythematous and indurated, as compared with saline control.

necessary to induce a wheal reaction was approximately 4 ng (0.05 ml at 80 ng ml⁻¹). Antibody E3 (apparently reacting primarily with IgG in all other experiments) induced a notably erythematous and indurated reaction when injected in high concentration. This whealing was especially prominent in one dog, but the overall mean diameter of these reactions in all three dogs was not higher than our defined positive threshold. The whealing reaction with E3 was not thought to be related to an irritant present in the preparation, as all antibodies were purified in an identical manner and all were diluted at least 50-fold in the same buffered saline prior to injection.

Inhibition of PK reactivity

Table 2 shows the results of PK testing, using atopic dog serum absorbed with MAb-coupled discs. It can be seen that the control disc (which was not coupled to any antibody) reduced the PK titer of the serum from 1/640 to 1/320, probably because of non-specific adsorption of serum proteins (including IgE) to the disc matrix. Discs coupled to all putative anti-canine IgG antibodies (B7, C7, and E3) and to one putative anti-canine IgE (antibody A5) did not reduce the PK titer of the atopic serum below control values. However, discs coupled to antibodies B3 and D9 (thought to be anti-canine IgE) reduced the titer to 1/160 and 1/40, respectively.

Affinity chromatography

Canine immunoglobulins eluted from the antibody B3 immunoaffinity column were subjected to SDS-PAGE under reducing conditions. The resulting

TABLE 2

Neutralization of Prausnitz-Küstner reactivity of atopic dog serum. Serum from a ragweed-allergic dog was absorbed by incubation with monoclonal antibody-coated discs, then serially diluted and assayed in the PK test

Atopic serum adsorbed with:	Atopic serum dilution							
	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
Unabsorbed serum control	+	+	+	+	+	+	-	-
Control disc (no antibody)	+	+	+	+	+	-	-	-
Antibody A5	+	+	+	+	+	-	-	-
Antibody B3	+	+	+	+	-	-	-	-
Antibody B7	+	+	+	+	+	-	-	-
Antibody C7	+	+	+	+	+	-	-	-
Antibody D9	+	+	-	-	-	-	-	-
Antibody E3	+	+	+	+	+	-	-	-

Positive result (+), wheal at least 5 mm larger in diameter than negative control site.

gel (Fig. 1) demonstrated that the eluted material resolved into two major bands, with molecular weights corresponding to those expected for the epsilon (heavy) chain of canine IgE and immunoglobulin light chain. A fainter contaminant band was present in approximately the position expected for IgG gamma (heavy) chain. However, when the eluted canine IgE was coated onto ELISA plates and probed with monoclonal anti-canine IgG, only background reactivity was detected (Table 3), possibly because the relative amount of contaminating IgG was too small to detect in the assay. Thus, canine Ig prepared by this procedure appear to contain principally IgE, with small amounts of contaminating IgG. In addition to providing a relatively easy technique for purification of canine IgE, this experiment also demonstrates the specificity of the MAb B3 for canine IgE and its tendency for crossreaction with canine IgG under some experimental conditions. The crossreactivity with IgG appeared to be slight: the starting material for affinity chromatography contained a vast excess of IgG over IgE, yet the final material contained mostly IgE and little IgG. This is evidence that the affinity of antibody B3 for IgE was much higher than its affinity for IgG.

Antibody characteristics by ELISA

Reactivity of each monoclonal anti-canine IgG or anti-canine IgE against various immunoglobulins and Ig subfractions, as measured using an ELISA assay, is shown in Table 3. Antibodies A5 and D9 appeared to be specific for canine IgE, and did not appear to bind to any other Ig class tested. Heat or acid treatment of the IgE prior to coating abolished much of the reactivity of these antibodies, suggesting that they are directed against a heat and acid-

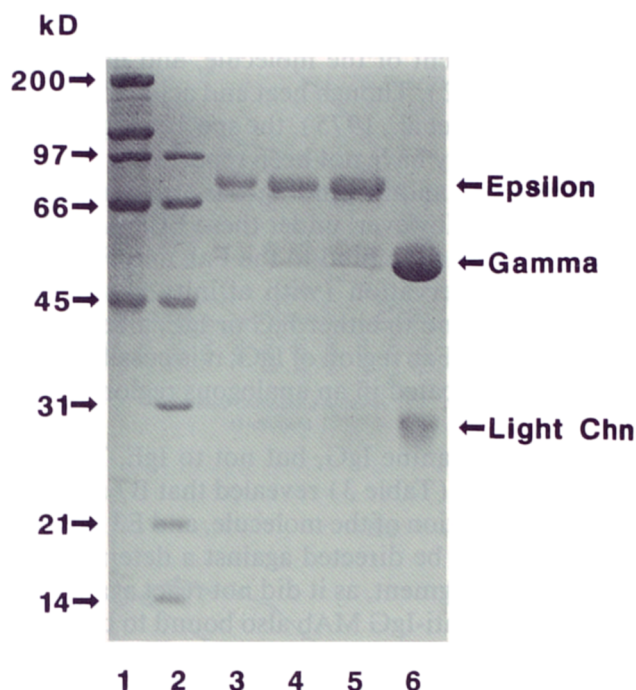


Fig. 1. Affinity chromatographic purification of canine IgE. SDS-polyacrylamide gel electrophoresis under reducing conditions, with Coomassie Blue stain. Lanes 1 and 2, molecular weight standards (weight in kilodaltons indicated at left). Lanes 3–5, canine IgE eluted from the affinity chromatography column; note predominant heavy (epsilon) and light chain bands and apparent IgG (gamma) contaminating band. Lane 6, commercially-obtained canine IgG; note heavy (gamma) and light chain bands.

TABLE 3

Reactivity of monoclonal antibodies against immunoglobulins and IgG fractions in an ELISA. Values shown in table are mean OD_{405} after subtraction of blank well value

Ig coated on microwells	Monoclonal antibody tested					
	A5	B3	B7	C7	D9	E3
IgE	0.288	0.283	0.027	0.034	0.324	0.001
IgE (HT)	0.108	0.118	0.045	0.018	0.144	0.054
IgE (AT)	0.166	0.181	0.003	0.041	0.218	0.066
IgG-Fc	0.002	0.008	0.952	1.197	0.041	0.108
IgG-Fab	-0.007	0.317	0.100	0.104	0.023	1.067
IgG-F(ab') ₂	0.002	0.178	0.030	0.016	0.027	1.004
IgM	0.006	0.038	0.841	0.635	0.028	0.620

HT, heat-treated Ig (56°C, 4 h); AT, acid-treated Ig (pH 2.3, 4 h).

labile portion of the canine IgE molecule. In the case of human IgE, the heat-labile portion is found on the CH₂ segment of the molecule, and has been designated the epsilon-2 determinant (D_ε2). Though heat and acid lability of canine IgE is well documented (Halliwell et al., 1975), the specific region(s) on the molecule responsible for this lability have not been reported.

Antibody B3 also bound to canine IgE, and its binding was also decreased by heat or acid-denaturation of the IgE. However, under these ELISA conditions, B3 also showed a consistent tendency to bind to the Fab and F(ab')₂ fractions of IgG. This supports our observation (with affinity chromatography, above) that MAb B3 is able to bind to either IgG or IgE, albeit with differing affinity. Because B3 binds to the Fab region of IgG, it is possible that the determinant it recognizes on IgE is located in an analogous region of the molecule.

Antibodies B7, C7, and E3 bound to canine IgG, but not to IgE. Testing these antibodies against IgG subfractions (Table 3) revealed that B7 and C7 appeared to be directed against the Fc portion of the molecule, and E3 against the Fab portion. Antibody E3 appears to be directed against a determinant on the heavy chain segment of the Fab fragment, as it did not react against Ig light chain (data not shown). All of the anti-IgG MAb also bound to the IgM preparation coated to the microplate, though with lesser color development than with the IgG coating. This result demonstrates that canine IgG and IgM share some common or similar determinants.

The abilities of the MAb to compete for binding to IgE was examined (Fig. 2). When all six antibodies were tested for their ability to inhibit binding of A5, B3, or D9 antibodies to IgE, it was found that any of the anti-IgE antibodies would inhibit the binding of any other anti-IgE antibody in a concentration-dependent fashion. In contrast, none of the anti-IgG antibodies would similarly inhibit this binding. The maximum degree to which the unlabelled antibody would block binding of labelled antibody varied. Unlabelled MAbs B3 and D9 were able to block up to 80–90% of the binding of biotinylated anti-IgEs; MAb A5 consistently revealed a lesser ability (40–60%) to inhibit such binding, perhaps because of lesser affinity.

Table 4 shows the results of the sandwich ELISA assays, where MAb were tested pairwise for their ability to capture and to detect immunoglobulin in a serum sample. Several antibody pairs were useful in this assay, both for IgE and for IgG detection. Among the anti-IgE antibodies A5, B3, and D9, nearly any pair could serve as capture and detection antibodies, though with apparently differing efficiencies. It was generally possible to use the same antibody to both capture and detect the same Ig, demonstrating that the IgE and IgG molecules are polyvalent with respect to the determinant to which these MAb bind. An interesting observation was that the assay result for a particular sandwich configuration (e.g. C7 capture, D9 detection) was not always the same as for the reversed configuration (e.g. D9 capture, C7 detection). In

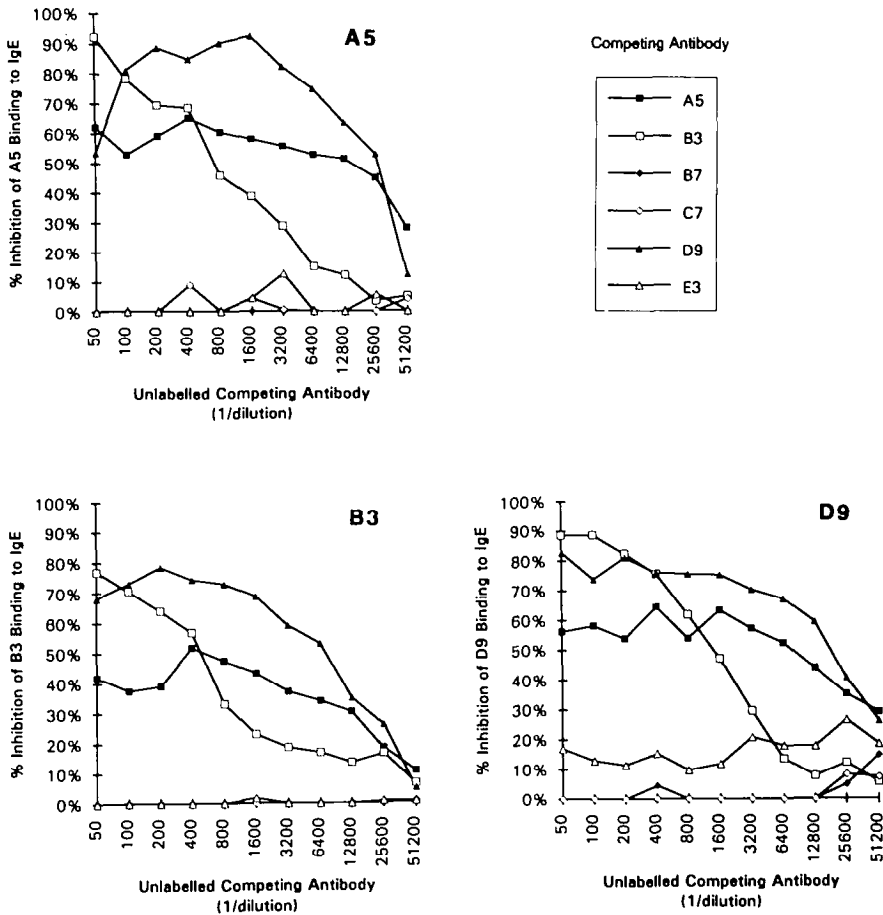


Fig. 2. Competitive ELISA assay using anti-canine IgE antibodies. Microtiter plates were coated with canine IgE. Graphs show percent inhibition of binding of biotin-labelled monoclonal anti-IgEs A5 (top left), B3 (bottom left), and D9 (bottom right) by serial dilutions of unlabelled antibodies (see legend, top right).

particular, MAbs C7 and E3 seemed to be able to function to detect either IgG or IgE, yet apparently would principally capture IgG. This result contradicts the result of the direct-binding ELISA (Table 3); the differences in results between the two assays may reflect differing inherent sensitivities of the two assay systems, or different stabilities of the complexes built up on the ELISA plate between the two assays. These experiments formed the basis for a quantitative sandwich ELISA assay to measure IgE concentrations in canine serum samples, now in operation in our laboratory.

TABLE 4

Summary of sandwich ELISA to detect immunoglobulins in canine serum. Microtiter plates were coated with the capture (first) antibody, then serum was added, followed by the biotinylated detection (second) antibody, avidin-peroxidase, and substrate

Capture (first) antibody	Detection (second) antibody					
	A5	B3	B7	C7	D9	E3
A5	2+	3+	0	4+	2+	3+
B3	4+	4+	0	4+	4+	3+
B7	0	0	2+	4+	0	3+
C7	0	0	4+	4+	0	3+
D9	3+	4+	0	3+	3+	3+
E3	1+	3+	3+	4+	0	3+

Results are expressed as the optical densities typically obtained in an experiment using pooled parasitized dog serum as the serum source at a 1/25 dilution, as follows: 0 = OD < 0.1; 1+ = OD 0.1–0.2; 2+ = OD 0.2–0.4; 3+ = 0.4–1; 4+ = OD > 1.

DISCUSSION

In this study, we were able to produce and identify MAb specific for canine IgE. It was clear from our results that canine immunoglobulin subclasses (IgG, IgE, IgM) contain some common or closely-related antigenic determinants, as several of the MAb revealed affinity for more than one Ig subclass. When a MAb did show binding to multiple Ig subclasses, there was generally evidence that the affinity of the antibody was greatest to one subclass, with variably less binding seen to other subclasses.

The monoclonal anti-IgE antibodies produced appeared to differ in their affinities for canine IgE. MAb A5 seemed to have the lowest affinity, as it was unable to reduce the PCA titer of canine serum (Table 2), was less able to compete with MAbs D9 and B3 for binding to IgE (Fig. 2), and consistently yielded less color development than other antibodies in the indirect sandwich ELISA (Table 4).

Heat and acid treatment of canine IgE did not completely abolish the binding of our MAb. There are several possible explanations for this observation. First, it is possible that the determinant to which the MAb bind is only partially heat- and acid-labile. Second, heat or acid inactivation of IgE may have been incomplete. A previous study demonstrated that treatment of canine IgE for 4 h at 56°C or at pH 2.3 was adequate to abolish binding of a polyclonal antiserum (Halliwell et al., 1975). This study, however, used an immunodiffusion method to measure IgE activity; the ELISA in our study may have been more sensitive and able to measure smaller amounts of remaining IgE activity. Finally, it is possible that canine IgE exists in several forms or subclasses with varying heat and acid lability.

Antibody E3 (anti-IgG-Fab) appeared to induce a wheal reaction in reverse cutaneous anaphylaxis experiments. Although we could not detect binding of E3 to IgE in a direct ELISA (Table 3), it was capable of slight inhibition of binding of other anti-IgE antibodies to IgE (Fig. 2). It is therefore possible that E3 recognizes a crossreacting epitope in canine IgE with very low affinity, and thus is able to trigger IgE-mediated mast cell degranulation when used at sufficient concentration. Alternatively, E3 may actually recognize a subclass of IgG molecules that can serve as mast cell sensitizing antibodies in the dog. IgG-subclass sensitization of mast cells or basophils is documented in other species; both IgE and IgG₄ can sensitize human mast cells (Fagan et al., 1982), and in the guinea pig, IgG₁ can be the predominant mast cell-sensitizing antibody (Ovary et al., 1963). Additionally, non-IgE skin-sensitizing antibodies, termed 'IgG_d' have been reported in the dog (Willemse et al., 1985).

Several aspects of our observations demonstrate that there are common or similar antigenic determinants present on canine IgG and IgE, and on canine IgG and IgM. Affinity chromatography and ELISA data revealed that MAb B3 is capable of binding both IgE and IgG, though with very low affinity to IgG. Direct ELISA demonstrated that the anti-Ig antibodies could also bind to IgM. These observations suggest that it is critical that any MAb against canine Ig be tested for reactivity against all other canine Ig to insure specificity; also, polyclonal antisera against canine IgE must be absorbed exhaustively against other Ig subclasses to eliminate unwanted crossreactivities.

Development of MAb with specificity for canine IgE will facilitate study of many aspects of hypersensitivity disease in dogs. We are finding our MAb useful for antigen-specific IgE ELISA, for quantitation of canine IgE, for immunostaining of Western Blot assays to evaluate the specificity of IgE antibodies at the molecular level, and for *in vitro* studies on degranulation of mast cells. In addition, at least one of the antibodies shows binding to a feline serum protein with physicochemical characteristics of feline IgE (DeBoer et al., 1992). Many additional studies will be necessary to bridge the gap between our knowledge of human IgE and that of IgE in domestic animal species.

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