

Zhikang Peng^a
Gilbert Arthur^b
Danuta Kierek-Jaszczuk^c
Elizabeth Thomas^a
Alec H. Sehon^c
Edward S. Rector^c
F. Estelle R. Simons^a
Allan B. Becker^{a, c}

^a Section of Allergy and Clinical
Immunology, Departments of Paediatrics,
^b Biochemistry and Molecular Biology, and
^c Immunology, University of Manitoba,
Winnipeg, Man., Canada

Purification and Identification of Polyclonal IgE Antibodies from Ragweed-Sensitized Dog Sera

75782

Abstract

We have purified and characterized polyclonal dog IgE. Serum IgE was precipitated by $(\text{NH}_4)_2\text{SO}_4$ and then purified by two different procedures. Ion exchange on DEAE-Sephacel, followed by HPLC using Tonen hydroxylapatite and then Protein G-Sepharose, produced a highly purified IgE fraction (No. 1) free of IgG, IgA and IgM as measured by ELISA, but recovery of IgE as measured by passive cutaneous anaphylaxis was low. Gel filtration on Sephacryl S-300, Con A-Sepharose and Protein G-Sepharose recovered 18% of initial IgE, 0.02% IgG, 0.4% IgM and 0.3% IgA. This IgE fraction (No. 2) was used to induce antibody production in rabbits. Western blot analysis was then performed for dog IgE fractions No. 1 and 2. Using the rabbit anti-dog IgE, a prominent IgE band with an apparent molecular mass of 226 kD was identified in fractions No. 1 and 2 subjected to nonreducing SDS-PAGE. This band also reacted with anti-human IgE, but not with anti-dog IgG or anti-dog IgA. Under reducing conditions the approximate molecular mass for the IgE ϵ chain, estimated by Western blot using rabbit anti-dog IgE, was 73 kD, providing a molecular mass of 196 kD for dog IgE.

Key Words

IgE, dog
Allergy
Purification
Ragweed

Introduction

We have established a dog model of allergy and airway hyperresponsiveness which demonstrates many features of human asthma [1]. We showed that neonatally sensitized dogs developed chronic, sustained airway hyperresponsiveness after repeated inhalation of a given allergen [1]. IgE antibodies are responsible for reagenic activity [2] and assessment of IgE is necessary for these studies. We have measured dog IgE in a semi quantitative fashion using the passive cutaneous anaphylaxis (PCA) method [3], a test which is extremely time-consuming and labor-intensive. Future studies would be greatly facilitated by the es-

tablishment of immunoassays for dog IgE measurement. In order to achieve this goal isolation of dog IgE for antibody production, especially for production of monoclonal anti-dog IgE, is required.

The availability of highly concentrated forms of monoclonal IgE from human [4] and rat [5] myelomas and mouse hybridomas [6] has made it possible to isolate sufficient quantities of the respective monoclonal IgE in a pure enough state for the subsequent production of monospecific antisera or monoclonal antibodies for the above species. The lack of a dog myeloma secreting IgE therefore leaves the isolation of polyclonal dog IgE as the only option for obtaining the required IgE antibody. However,

due to the extremely low concentration of polyclonal IgE in serum (0.0002% of the serum IgG in humans) [7], its purification has been exceedingly difficult. Schwartzman et al. [8] and Halliwell et al. [9–11], produced IgE-enriched fractions from allergic and roundworm-infected dog sera and used this for immunization of rabbits. Although the rabbit anti-dog IgE antisera were exhaustively adsorbed with dog serum low in IgE, the results from the immunoassay using the rabbit anti-dog IgE were variable with an agreement of between 12.5 and 82% for immunoassay and skin tests [12, 13]. Another study reported that immunoassays using this anti-dog IgE failed to demonstrate reaginic antibodies in sera from atopic dogs and from dogs with toxocariasis [14].

As a first step towards developing an immunoassay for dog IgE measurements, the current studies were undertaken to obtain IgE-enriched fractions essentially free of other immunoglobulins. The purification of polyclonal dog IgE, its identification and immunophysical properties are reported in this paper.

Materials and Methods

Short ragweed (*Ambrosia elatior*) extract was purchased from Hollister-Stier (Rexdale, Ont., Canada) and used for ELISA. Dog IgG, affinity purified rabbit antibodies to dog IgG Fc and to dog IgG F(ab')₂, alkaline phosphatase-conjugated rabbit anti-dog IgG Fc and conjugated goat anti-rabbit IgG (H+L) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, Pa., USA). Rabbit antisera to dog IgA and IgM were obtained from Nordic Immunological Laboratories (Tilburg, The Netherlands) and ICN Immunobiologicals (Lisle, Ill., USA), respectively, and were further purified by sequential chromatography on Protein A-Sepharose and dog IgG-Sepharose 4B columns. Purified rabbit anti-human IgE was purchased from Dakopatts (DK-2600 Glostrup, Denmark). Monoclonal anti-human IgE ϵ chain (ascites, No. 7.12, binding site CH2 domain) was a gift from Dr. A. Saxon (University of California, Los Angeles, Calif., USA). Alkaline phosphate-conjugated goat anti-mouse IgG was obtained from TAGO Inc. (Burlingame, Calif., USA).

Biotinylated SDS-PAGE standards, the Protein Assay Dye Reagent Kit, and the gold stain kit were purchased from Bio-Rad Laboratories (Richmond, Calif., USA). 5-Bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium were from Sigma Chemical Company (St. Louis, Mo., USA). DEAE-Sephacel, Protein G-Sepharose 4 Fast Flow, Sephacryl S-300, and Con A Sepharose were purchased from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). Tonen hydroxylapatite was a gift from Waters (Mississauga, Ont., Canada). Polystyrene microtiter plates and MR700 microplate reader were obtained from Gibco (Burlington, Ont., Canada) and Dynatech Laboratories (Alexandria, Va., USA), respectively. Centriprep-30 concentrators were purchased from Amicon Canada Ltd.

Ragweed-Sensitized Dog Serum

The ragweed extract for immunization of the dogs and for PCA was prepared in our laboratory using the raw pollen purchased from Hollister-Stier (Rexdale, Ont., Canada). The lipids in the pollen were removed by ether distillation. The delipidated pollen was stirred in distilled water at 4°C overnight and then filtered through Whatman paper, centrifuged for 30 min at 12,000 rpm. The supernatant was dialyzed against distilled water until the color disappeared. The extract was lyophilized, and stored at -70°C.

For the production of ragweed-specific IgE, dogs were immunized according to the method of Kepron et al. [15]. Briefly, newborn mongrel dogs received intraperitoneal injections of 500 μ g ragweed pollen in 30 mg of aluminum hydroxide within 24 h of birth. The injections were repeated weekly for 8 weeks and then biweekly up to 16 weeks of age. Sensitization to ragweed was determined by PCA.

Passive Cutaneous Anaphylaxis

PCA was used throughout this study for semiquantitative measurement of ragweed-specific dog IgE [3]. For standardization of responses from recipient dogs between PCA tests, a 'reference IgE serum' from a ragweed-allergic dog, was used for each PCA test and was defined as a PCA titer of 1,024. Test samples were serially diluted 2-fold in 0.01 M phosphate-buffered saline (PBS), pH 7.2, and 0.15 ml of each diluted serum was injected intradermally into a prepared area along the flank of a recipient non-ragweed sensitized dog. Two fold serial dilutions of the reference serum (1:16 to 1:2,048) were injected to provide a standardized control between recipient dogs. PBS and dog IgG (0.2 mg/ml) were injected as negative controls. After 48 h, the dog was challenged by intravenous injection with 5 ml of ragweed pollen extract (5 mg/ml) and 10 ml of 1% (w/v) Evan's blue. After 30 min the largest and the orthogonal diameters of the blue area was measured. The reciprocal of the highest dilution which elicited a blue area of 4 mm or larger in diameter was taken as the PCA titer of each sample. PCA titres of test samples were then standardized by use of our reference serum.

Measurement of Dog Ragweed-Specific IgG, IgA and IgM by ELISA

Dog ragweed-specific IgG, IgA and IgM were measured by an indirect ELISA. For standardization of ELISA results between assays and estimation of relative amount of ragweed antibodies in each sample, a reference serum which was obtained from a ragweed-allergic dog was used and was defined as 1,000 U/ml for ragweed-specific IgG, IgA, and IgM antibodies. Polystyrene microtiter plates were coated overnight at 4°C (0.1 ml/well) with ragweed extract diluted in acetate buffer (0.1 M sodium acetate, 1.0 M sodium chloride, pH 6.5, 50 μ g/ml). After rinsing the plates three times with washing buffer (0.05% Tween 20 in 0.01 M PBS, pH 7.2), 0.1 ml of the test sample or of the reference serum diluted in washing buffer containing 0.2% bovine serum albumin (BSA) was added to each well and the plates were incubated for 1.5 h at 37°C. The wells were washed and 0.1 ml of rabbit antibodies to dog IgG Fc (1:10,000) dog IgA (1:1,000) or dog IgM (1:2,000) was added and incubated for 1.5 h at 37°C. After washing, 0.1 ml of enzyme-conjugated goat anti-rabbit IgG (1:10,000 for IgG and 1:2,000 for IgA and IgM) was added and incubated for 1.5 h at 37°C. After a final wash, 0.1 ml of the enzyme substrate (*p*-nitrophenylphosphate, 1mg/ml in diethanolamine buffer, pH 9.8) was added and incubated overnight at 4°C. The reaction was stopped by addition of 0.1 ml of 1 N NaOH. The optical absorbance at 410 nm was read with an automated MR700 microplate reader. The levels of the ragweed antibodies were calculated by interpolation from the dilu-

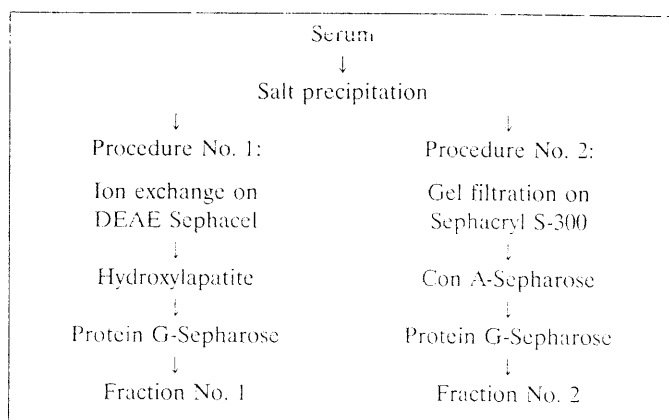


Fig. 1. Procedures of IgE purification.

tion curve of the reference serum which was mathematically fitted by a three-parameter spline function [16].

Chromatographic Purification of Dog IgE

Sera with a PCA titer of 1,024 or more were used for IgE purification. An IgE-containing fraction, obtained from the dog serum by precipitation with $(\text{NH}_4)_2\text{SO}_4$ at 30–55% saturation, was purified by two different procedures (fig. 1).

All fractions were assayed after each chromatographic step for the presence of ragweed-specific IgE by PCA and for ragweed-specific IgG, IgA and IgM by ELISA. The protein content of each column fraction was assayed by the optical density at 280 nm and that of the pooled fractions from each chromatographic step was measured by the Bio-Rad protein assay.

Preparation of Rabbit Anti-Dog IgE Antibodies [17]

Rabbits were immunized three times at 2-week intervals. In each immunization, 0.4 mg of dog IgE fraction No. 2 in complete Freund's adjuvant (for the first immunization) or incomplete Freund's adjuvant was injected into 5 sites on the back and 1 site on each hind-quarter of each rabbit. Two weeks after the third immunization, anti-IgE activity was confirmed by reverse PCA. Before reverse PCA, the rabbit sera were mixed with Sepharose-4B beads coupled with control dog serum proteins and rotated at 4°C overnight to adsorb any possible anti-light chains which may elicit reverse PCA. The rabbits were then bled and the serum was sequentially applied onto the following columns for exhaustive adsorption and purification. (1) A Sepharose-4B column coupled with proteins from control dog serum which had been precipitated between 30 and 55% $(\text{NH}_4)_2\text{SO}_4$. (2) A Sepharose 4B column coupled with purified dog IgG; before coupling the commercial dog IgG was purified in our laboratory by a Protein G-Sepharose column to remove any possible IgE contamination, as dog IgE does not measurably bind to protein G [18]. (3) A Protein G-Sepharose column; rabbit IgG was eluted from the protein G column with 0.1 M glycine (pH 2.7), immediately neutralized with 0.5 M Tris buffer (pH 8.0) and passed through a Sephadex G-25 column equilibrated with 0.02 M PBS for buffer exchange. (4) Again, a Sepharose 4B column coupled with control dog serum proteins. The purified rabbit antibody was assayed for protein concentration (0.3 mg/ml), aliquoted, and stored at -70°C.

The anti-dog IgE activity in the antibody was confirmed by reverse PCA, PCA inhibition testing, and the loss of reaction to heat-inactivated dog IgE fractions. No detectable cross-reactivity of the antibody to either dog IgG or dog serum proteins was found as measured by ELISA.

Identification of Dog IgE by Western Blot

SDS-PAGE was performed under non reducing conditions in 5–15% gradient slab gels according to Laemmli [19]. Twenty micrograms of dog IgE fractions No. 1 and 2, or 10 µg of dog IgG were placed in each well. After electrophoresis the proteins were electrotransferred to nitrocellulose membranes using the Semi-dry Pharmacia Transblot system. Free binding sites on the membrane were blocked by incubation with 3% BSA dissolved in Tris-buffered saline for 2 h. The membrane was cut into strips which were incubated for 2 h with buffer or the following rabbit antibody to dog IgE (1:100), dog IgA (1:500), and human IgE (1:1,000) and washed three times with Tris-buffered saline containing 0.05% (v/v) Tween 20. Each strip was then incubated with either alkaline phosphatase-conjugated goat anti-rabbit IgG (1:1,000) or conjugated rabbit anti-dog IgG (1:1,000). The strips were washed and the color reaction was developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium dissolved in 0.1 M ammonium buffer (pH 9.6) at 0.25 and 0.50 mg/ml, respectively. Biotinylated standards were myosin, β-galactosidase, phosphorylase B, BSA, and ovalbumin. The molecular mass of the immunoreactive proteins was determined from the standard curve prepared on the basis of the measurements of the distance of migration of biotinylated standards separated in the same slab gel. Staining of total proteins transferred onto nitrocellulose membrane was achieved with the gold stain kit.

Dog IgE fraction No. 2 and dog IgG were also analyzed under reducing conditions by Western blot as mentioned above. Biotinylated standards were phosphorylase B, BSA, ovalbumin, anhydrase B, trypsin inhibitor, and lysozyme.

Two-dimensional electrophoresis followed by immunoblot was used to analyze the molecular nature of the immunoreactive proteins. The lane containing dog IgE (fraction No. 2) separated by non reduced SDS-PAGE as mentioned above was cut from the gel and incubated with a reducing sample buffer at room temperature for 1 h [19]. This 6-mm vertical gel strip was applied onto another slab gel for the second-dimension SDS-PAGE and followed by Western blot analysis using rabbit anti-dog IgE.

Results

Salt Precipitation with $(\text{NH}_4)_2\text{SO}_4$

Sixteen percent of the PCA activity was recovered in the serum fraction precipitating between 30 and 40% $(\text{NH}_4)_2\text{SO}_4$ saturation, 66% was recovered in the 40–50% fraction and 16% in the 50–55% fraction. Of the total IgE, 98% was therefore recovered in the fractions precipitating between 30 and 55% $(\text{NH}_4)_2\text{SO}_4$ saturation. Serum proteins precipitating in these fractions were then used for further purification of dog IgE. The recovery of immunoglobulins and protein in the various fractions are shown in tables 1 and 2.

Table 1 Recovery of dog serum immunoglobulins and protein during IgE purification by procedure 1 (% of original serum)

	IgE	IgA	IgM	IgG	Protein
Serum	100	100	100	100	100
Salt precipitation	79	92	41	68	15
Ion exchange	16	28	0.3	43	4.2
Hydroxylapatite	4	0	0	1.3	0.4
Protein G	4	0	0	0	0.3

Table 2. Recovery of dog serum immunoglobulins and protein during IgE purification by procedure 2 (% of original serum)

	IgE	IgA	IgM	IgG	Protein
Serum	100	100	100	100	100
Salt precipitation	79	92	41	68	15
Gel filtration	69	51	0.9	62	6.2
Con A	25	0.3	0.4	1.2	1.3
Protein G	18	0.3	0.4	0.0	0.2

Separation of Dog IgE by Procedure 1

Sixty milliliters of serum with a PCA titer of 1,024 from a single dog were used for salt precipitation. The fraction precipitated by 30–55% $(\text{NH}_4)_2\text{SO}_4$ saturation was diluted in 0.01 M Tris buffer (pH 8.0). After desalting on a Sephadex G-25 column (bed volume 25 ml), the fraction was applied onto a DEAE-Sephacel column (bed volume 64 ml, flow rate 48 ml/h) equilibrated with the Tris buffer. The column was washed with 3 bed volumes of the Tris buffer followed by two linear Tris buffer gradients (0.01–0.07 and 0.07–0.2 M, pH 8.0). As shown in figure 2, dog IgE was eluted at about 0.16 M Tris buffer. IgE-enriched fractions were pooled and concentrated with Centriprep-30 concentrators. The concentrated fraction was applied to a Sephadex G-25 column (bed volume 80 ml, flow rate 108 ml/h) equilibrated with 0.01 M phosphate, 0.3 mM CaCl_2 buffer (pH 6.8) to exchange buffer. Analysis of this pooled fraction indicated that ion exchange chromatography had removed more than 99% of serum IgM, but 43% of IgG and 28% of IgA still remained with the IgE fraction (table 1).

One quarter of the pooled fraction from the DEAE column was then subjected to HPLC on two columns of Tonen hydroxylapatite (bed volume 3.8 ml, flow rate 60 ml/h) connected in series. After washing with 0.01 M phosphate, 0.3 mM CaCl_2 buffer (pH 6.8), a linear gradient from 0.01 to 0.25 M of the above buffer was applied. As shown in figure 3, IgE eluted from Tonen hydroxylapatite

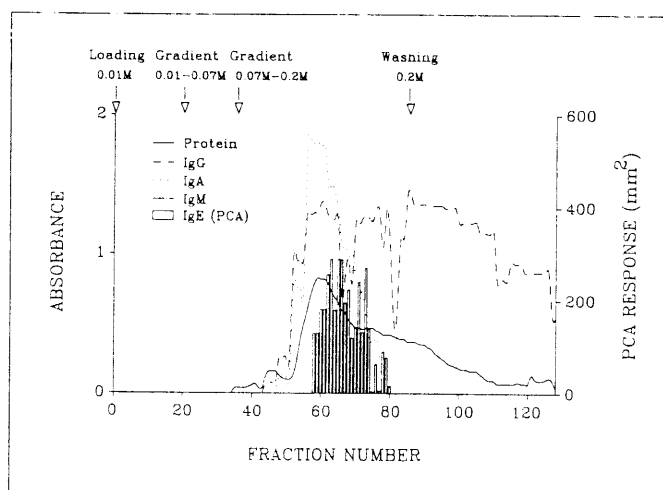


Fig. 2. Ion exchange chromatography of dog IgE fraction on DEAE-Sephacel. The IgE fraction obtained from $(\text{NH}_4)_2\text{SO}_4$ precipitation was loaded on a DEAE-Sephacel column as described in 'Results'. Elution was performed by using two linear gradients of 0.01–0.07 and 0.07–0.2 M Tris buffer (pH 8.0). IgE was eluted at about 0.16 M. Fraction values of 10 ml were collected.

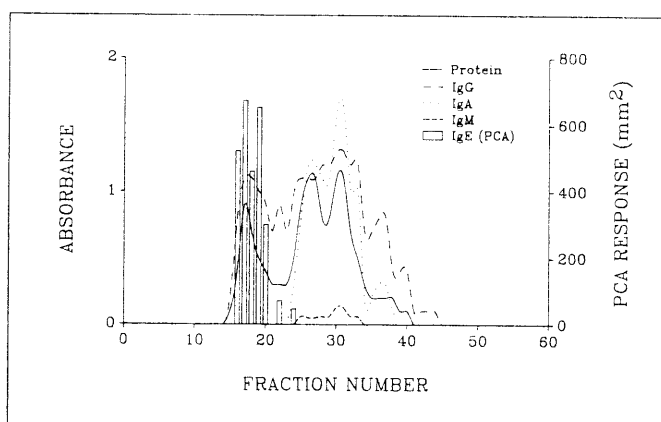


Fig. 3. HPLC chromatography of dog IgE fraction on Tonen hydroxylapatite. The IgE fraction obtained from ion exchange chromatography was loaded on a Tonen hydroxylapatite column as described in 'Results'. Elution was performed by a linear gradient of 0.1–0.25 M phosphate buffer (pH 6.8). IgE was eluted at about 0.1 M. Fraction values of 2 ml were collected.

at about 0.1 M before the peaks of IgA and IgM and before the main peaks of IgG and protein. The fractions from the IgE peak were pooled. This pooled fraction was free of IgM and IgA, and very low in IgG (1.3% of serum IgG; table 1).

Contaminating IgG associated with the above fraction was removed by passage through a Protein G-Sepharose 4B column (bed volume 11.8 ml, flow rate 30 ml/h) equi-

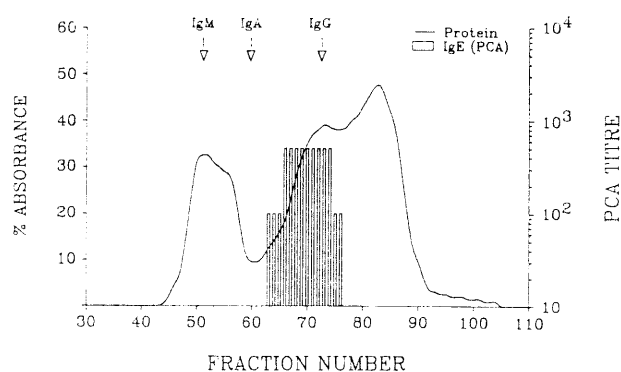


Fig. 4. Gel filtration chromatography on Sephacryl S-300 of the dog IgE fraction obtained from $(\text{NH}_4)_2\text{SO}_4$ precipitation. The position of IgG, IgA and IgM peak values are indicated by arrows. Fraction values of 13 ml were collected.

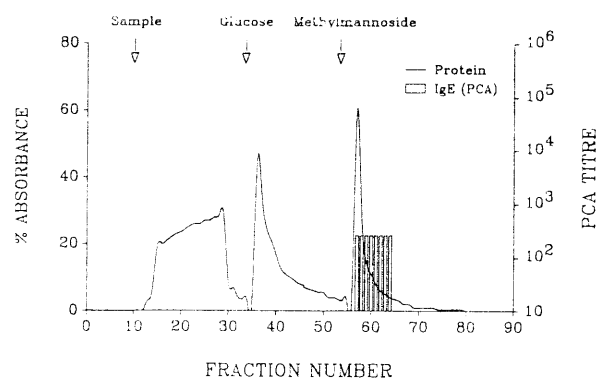


Fig. 5. Affinity chromatography on Con A-Sepharose of the dog IgE fraction obtained from gel filtration. Fraction values of 12 ml were collected.

brated with 0.02 M phosphate buffer (pH 7.2). The fractions were pooled and concentrated with Centriprep-30 concentrators. NaCl was added to a final concentration of 0.15 M and aliquots of the fractions were stored at -70°C . This fraction, IgE fraction No. 1, had a PCA titer of 128 and a protein concentration of 0.3 mg/ml, was free of IgG, IgA and IgM as measured by ELISA. The recovery of immunoglobulins and protein in procedure 1 is summarized in table 1.

Separation of IgE by Procedure 2

Serum (140 ml) with a PCA titer of 1,024 from a single dog was used in this procedure. The fraction precipitating between 30 and 55% saturation of $(\text{NH}_4)_2\text{SO}_4$ was dissolved in 0.02 M PBS (pH 7.2) and applied to a Sephacryl S-300 column (bed volume 1,800 ml, flow rate 132 ml/h) equilibrated with PBS. A typical elution pattern is shown in figure 4. IgE-containing fractions were pooled and applied to a Con A-Sepharose column (bed volume 46 ml, flow rate 30 ml/h). After washing with 6–7 column volumes of PBS followed by 6–7 bed volumes of 0.1 M glucose in PBS, dog IgE was eluted with 0.4 M methylmannoside (fig. 5). The IgE-containing fractions were pooled and applied to a Protein G-Sepharose column (bed volume 11.8 ml, flow rate 30 ml/h). The fractions were pooled, concentrated, aliquoted and stored at -70°C .

Analysis of the pooled fractions from each step indicated that gel filtration had removed 99% of serum IgM, but 62% of serum IgG and 51% of IgA remained in the IgE-enriched fraction. However, Con A chromatography removed almost all of the remaining IgG and IgA. The last

1.2% of serum IgG after Con A column was removed by Protein G-Sepharose. This procedure recovered 18% of initial IgE, 0.02% IgG, 0.4% IgM and 0.3% IgA (table 2). The final fraction, IgE fraction No. 2, had a PCA titer of 1,024 and a protein concentration of 2 mg/ml. The recovery of immunoglobulins and protein in procedure 2 is summarized in table 2.

Heat-Lability Studies

IgE is unique among immunoglobulins in being heat-labile. After heating at 56°C for 2 h, the PCA activity of dog IgE fraction No. 2 was significantly reduced from 1,024 to 64 (6% of the titer before heating), while the trace amounts of IgG, IgA and IgM in the fraction were not changed.

Identification of Dog IgE by Western Blot

Although the purified IgE fractions were essentially free of other immunoglobulins, gold-staining of the nitrocellulose membrane subsequent to electrotransfer of the proteins from SDS gels, revealed the presence of a large number of protein bands. Identification of the IgE band therefore utilized Western blot analysis with specific anti-dog IgE developed in our laboratory as well as anti-human IgE which cross-reacts with dog IgE [9].

Separation of dog IgE fractions under non reducing conditions, followed by immunoblotting with rabbit anti-dog IgE, revealed a prominent band with a molecular mass that was 40 kD greater than that for dog IgG in dog IgE fractions No. 1 and 2 (fig. 6, lanes B and C). This IgE band also reacted with rabbit anti-human IgE (lane E), but did

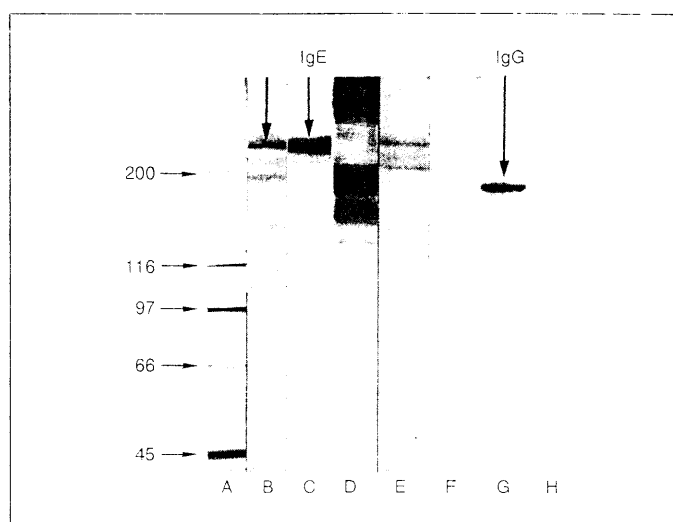


Fig. 6. Immunoreactivity of dog IgE fractions No. 1 and 2 under nonreducing conditions in Western blot. The proteins were separated by nonreducing SDS-PAGE, transferred onto nitrocellulose membranes, and incubated with different antibodies. Lane A (biotinylated high molecular weight markers) was incubated with conjugated avidin. Lanes B (dog IgE fraction No. 1) and C (dog IgE fraction No. 2) were incubated with rabbit anti-dog IgE followed by conjugated anti-rabbit IgG. Lane D (dog IgE fraction No. 2) was incubated with anti-dog IgA followed by conjugated anti-rabbit IgG. Lane E (dog IgE fraction No. 2) was incubated with rabbit anti-human IgE followed by conjugated anti-rabbit IgG. Lanes F (dog IgE fraction No. 2) and G (dog IgG) were incubated with conjugated rabbit anti-dog IgG. Lane H (dog IgE fraction No. 2) was incubated with buffer as a control. A prominent band with a molecular weight of about 40 kD higher than dog IgG, was identified in dog IgE fractions No. 1 (lane B) and 2 (lane C) using rabbit anti-dog IgE. This IgE band also reacted with rabbit anti-human IgE (lane E), but did not react with anti-dog IgA (lane D) and anti-dog IgG (lane F). The estimation of molecular weight was 226 kD for dog IgE and 186 kD for dog IgG as indicated.

Table 3. Molecular mass of dog IgE and IgG in Western blot analysis (kD)

SDS-PAGE	IgE		IgG		Light chain
	ϵ chain	whole molecule	γ chain	whole molecule	
Reduced	73	196	51.5	153	25
Nonreduced		226		186	

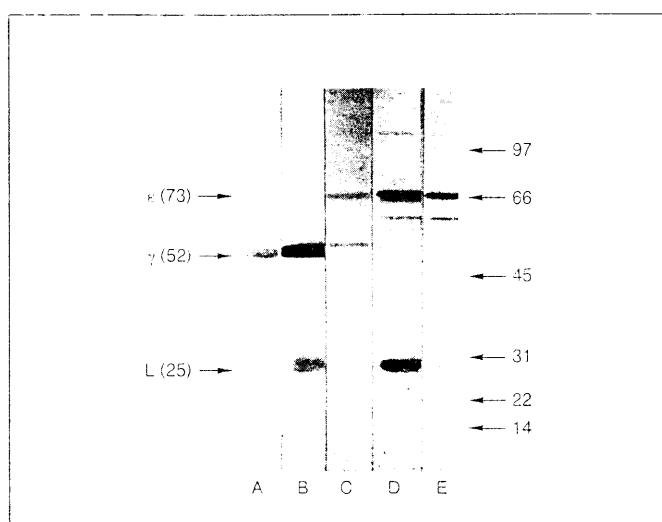


Fig. 7. Immunoreactivity of the dog IgE fraction under reducing conditions in Western blot. The proteins (lanes A and B, dog IgG; lanes C, D, E, dog IgE fraction No. 2) were separated by reduced SDS-PAGE and transferred onto nitrocellulose membranes. Incubations were as follows: lane A with buffer followed by enzyme-conjugated rabbit anti-dog IgG Fc; lane B with rabbit anti-dog IgG F(ab')₂ and followed by goat anti-rabbit IgG conjugate; lanes C and D with rabbit antibodies to dog IgE, and to human IgE, respectively, followed by goat anti-rabbit IgG conjugate; lane E with monoclonal anti-human IgE followed by goat anti-mouse IgG conjugate. The IgE ϵ chain reacted with anti-dog IgE (lane C), polyclonal (lane D) and monoclonal (lane E) anti-human IgE antibodies. The molecular weight was 73 kD for the ϵ chain, 52 kD for the γ chain, and 25 kD for the light (L) chain.

not react with anti-dog IgG (lane F) and anti-dog IgA (lane D). The molecular mass of dog IgM precludes it from entering the gel, hence the dog IgE fraction was not incubated with anti-dog IgM. The approximate molecular mass estimated by Western blot under these non reducing conditions was 226 kD for dog IgE and 186 kD for dog IgG (table 3).

Furthermore, using two-dimensional electrophoresis followed by immunoblot with rabbit anti-dog IgE, the 226-kD dog IgE band dissociated into subunits below the diagonal under reducing conditions in the second dimensional electrophoresis. This suggests that the molecular nature of the 226-kD immunoreactive protein is an immunoglobulin consisting of subunits.

Separation of dog IgE fraction No. 2 by SDS-PAGE under reducing conditions, followed by immunoblotting with anti-dog IgE, revealed two distinct bands (fig. 7, lane C). The higher band, but not the lower band, also reacted with both polyclonal (lane D) and monoclonal anti-human IgE (lane E), thus identifying it as the dog IgE ϵ chain.

When the dog IgE was incubated with the polyclonal anti-human IgE, another major band with the same molecular mass as the dog light chain identified in lane B (25 kD) was found (lane D), but when incubated with the monoclonal anti-human IgE specific to the ϵ chain (CH2 domain) only, the 25-kD band was not recognized (lane E). This suggested that the polyclonal anti-human IgE was not specific to the ϵ chain and also cross-reacted with dog light chain. Dog IgG reacted with rabbit antibodies to dog IgG Fc (lane A) and dog IgG F (ab')₂ (lane B). The approximate molecular mass for the dog IgE ϵ chain, the IgG γ chain, the light chain, and the IgE and IgG calculated from the data on the heavy and the light chains are shown in table 3.

Discussion

In our initial separation procedure, ion exchange on DEAE-Sephacel removed almost all of the IgM from the material precipitated by (NH₄)₂SO₄. Hydroxylapatite chromatography separated IgE from IgA, IgM and most of the IgG. We have previously shown that dog IgE does not bind measurably to Protein G-Sepharose, while 100% of IgG binds to the column [18]. Thus, affinity chromatography using Protein G-Sepharose removed all detectable IgG from the IgE fraction. This procedure produced highly purified dog IgE free of IgG, IgA and IgM, but the IgE recovery was low, representing only 4% of that from the original sample. Therefore, another procedure was designed for the purification of IgE.

Our subsequent approach was rather more simplified, as it required only a single buffer, recovered 18% of the original serum IgE, and could be used for a large sample of serum. Gel filtration on Sephacryl S-300 recovered more IgE (69%) than ion exchange on DEAE Sephacel (19%). On the Sephacryl S-300 column, IgE eluted between the IgG and IgA peaks and on the ascending slope of the second protein peak. This pattern was very similar to that reported by Schwartzman et al. [8] on Sephadex G-200 gel filtration. Gel filtration separated IgE from IgM, the highest molecular weight immunoglobulin, but at this stage the pooled IgE-enriched fraction still contained more than 50% of original serum IgG and IgA.

The use of Con A to separate proteins on the basis of their carbohydrate content was a very helpful step in the isolation of IgE. The carbohydrate content of human immunoglobulins ranges from 3% for IgG, 8% for IgA to 12% for IgM and IgE [7]. Our previous study has shown that all dog IgE binds to Con A, while only 60% of IgG and

58% of IgA bind to Con A [20]. Furthermore, IgG could be eluted by glucose, while IgE could be eluted by mannose, methylglucose, and methylmannoside, but not glucose [20]. Therefore, gel filtration, followed by Con A-Sepharose with methylmannoside elution, eliminated almost all of the remaining IgG and IgA.

The PCA test, derived from the Prausnitz-Küstner test, is a classic method to measure reagenic antibody. Sensitivity of the Prausnitz-Küstner test has been shown to be 0.2 ng/ml in humans and 2–3 ng/ml in mice [21]. IgE antibodies have a uniquely long sensitizing period with an optimal latent time of 1–2 days and the sensitization persists for 2–3 weeks. IgG-sensitizing antibodies have a short optimal sensitizing period of 1–6 h which disappears within 1–2 days [21]. In our studies, recipient dogs were sensitized for 48 h before challenging with ragweed extract. This 48-hour sensitizing period in PCA testing excludes any possible response which may have been elicited by ragweed-specific IgG-sensitizing antibodies. In addition, dog IgG containing all IgG subclasses was used as a negative control in each PCA testing.

Dogs sensitized to ragweed not only have increased ragweed-specific IgE levels but also have increased ragweed-specific IgG, IgA and IgM levels [Z. Peng and A. Becker, unpublished data]. We therefore closely monitored dog ragweed-specific IgG, IgA and IgM using the ELISA similar to these with reported sensitivities for human ragweed-specific antibodies (1 ng/ml for IgG and IgG1 and 0.1 ng/ml for IgG4) [22] to ensure that the purified dog IgE fraction No. 1 had no detectable levels of IgG, IgA and IgM. Unlike most mammals, IgD has not been identified in dogs [23]. In previous studies, the purity of dog IgE was assayed by less sensitive and semi quantitative techniques such as immunoelectrophoresis and immunodiffusion [8].

Knowledge of IgE structure in humans, mice, and rats has been obtained from monoclonal IgE proteins. As listed in table 4, the molecular mass of these monoclonal IgE proteins ranges from 184 to 196 kD in humans [4, 21, 24, 25], 183 to 187 kD in rats [5, 26], and 184 to 200 kD in mice [6, 27, 28]. Estimation of molecular mass for these monoclonal immunoglobulins was based on ultracentrifugation analysis [4, 26], sedimentation equilibrium techniques [24, 25, 27], sedimentation and diffusion data [24], electrophoresis [6], or SDS-PAGE under reducing conditions [5, 28]. Attempts to isolate polyclonal IgE antibodies have utilized sera from dogs [11], horses [29], cows [30], and guinea pigs [31], but the molecular mass has only been reported for horse IgE [29]. Estimation of its molecular mass by non reducing SDS-PAGE revealed a value of

Table 4 Estimation of molecular mass (kD) for IgE in different species

Species	Source	Method	ϵ chain	IgE molecule	Author [year]	Reference No.
Human	Myeloma (ND)	Archibald ¹		196,000	Johansson and Bennich [1976]	4
	Myeloma (ND)	Sedimentation and diffusion		193,000	Bennich and Johansson [1971]	24
	Myeloma (ND)	Yphantis ²	72,500	188,100	Bennich and Johansson [1971]	24
	Myeloma (PS)	Yphantis ²	69,800	184,500	Kochwa et al. [1972]	25
	Myeloma		72,300	190,000	Ishizaka [1988]	21
Rat	Immunocytoma	SDA-PAGE	68,000	183,000	Bazin et al. [1974]	5
	Immunocytoma	Archibald ¹		187,000	Bennich et al. [1978]	26
Mouse	Hybridoma	Sepharose-6B electrophoresis		200,000	Böttcher and Hämmerling [1978]	6
	Hybridoma	Yphantis ²		184,000	Liu et al. [1980]	27
	Hybridoma	SDS-PAGE	75,000		Bose et al. [1984]	28
Horse	Parasite infected horse serum	SDS-PAGE (non-reducing)		200,000	Suter and Fey [1983]	29
Dog	Allergic dog serum	SDS-PAGE and immunoblot	73,000	196,000	Peng et al. [1993]	Present study

¹ Ultracentrifugation analysis.

² Sedimentation equilibrium techniques.

200 kD (table 4) [29]. In the present study the estimated molecular mass of dog IgE under reducing conditions was 196 kD. This closely resembles the molecular mass of the monoclonal and polyclonal IgE proteins shown for other mammalian species (table 4).

The intact dog IgE (fig. 6) and the IgE ϵ chain (fig. 7) also showed cross-reactivity with anti-human IgE in Western blot analysis. Halliwell et al. [9] reported that absorption of dog reaginic serum with anti-human IgE resulted in a substantial reduction in Prausnitz-Küstner titers to specific antigen and that anti-human IgE was effective in eliciting a reversed PCA response in the dog. In the present study, the cross-reactivity of dog IgE with anti-human IgE was directly demonstrated by Western blot analysis (fig. 6, 7).

Based on the PCA response, the heat lability and the purity of our dog IgE fractions, the specificity of the rabbit anti-dog IgE, the apparent molecular weight, the immunological reactivities, and the dissociation in two-dimensional electrophoresis of the dog IgE band, we believe this measurement of molecular mass for dog IgE to be the first identification for any polyclonal IgE.

Acknowledgements

This work was supported in part by the Manitoba Health Research Council, Inspiraplex (the Respiratory Health Network of the Centres of Excellence), and the Children's Hospital of Winnipeg Research Foundation. Dr. Z. Peng was a fellow of the Canadian Lung Association and Dr. G. Arthur is a Scholar of the Medical Research Council of Canada.

We thank Mrs. Marilyn K. Lilley for her excellent technical assistance.

References

- 1 Becker AB, Hershkovich J, Simons FER, Simons KJ, Killey MK, Kepron MW: Development of chronic airway hyperresponsiveness in ragweed-sensitized dogs. *J Appl Physiol* 1989; 66:2691-2697.
- 2 Ishizaka K, Ishizaka T, Hornbrook MM: Physicochemical properties of human reaginic antibody. IV. Presence of a unique immunoglobulin as a carrier of reaginic activity. *J Immunol* 1966;97:75-85.
- 3 Ovary Z: Immunological methods; in Ackroyd JF (ed): CIOMS Symposium. Oxford, Blackwell Scientific, 1964, pp 259-283.
- 4 Johansson SGO, Bennich H: Immunological studies of an atypical (myeloma) immunoglobulin. *Immunology* 1967;13:381-394.
- 5 Bazin H, Querinjean P, Becker A, Heremans JF, Dessy F: Transplantable immunoglobulin-secreting tumours in rats. IV. Sixty three IgE-secreting immunocytoma tumours. *Immunology* 1974;26:713-723.
- 6 Böttcher I, Hammerling G: Continuous production of monoclonal mouse IgE antibodies with known allergenic specificity by a hybrid cell line. *Nature* 1978;275:761-762.
- 7 Roitt I: Immunoglobulin classes and subclasses; in Roitt I (ed): *Essential Immunology*. London, Blackwell Scientific, 1991, p 45.
- 8 Schwartzman RM, Rockey JH, Halliwell REW: Canine reaginic antibody. Characterization of the spontaneous anti-ragweed and induced anti-dinitrophenyl reaginic antibodies of the atopic dog. *Clin Exp Immunol* 1971;9:549-569.
- 9 Halliwell REW, Schwartzman RM, Rockey JH: Antigenic relationship between human IgE and canine IgE. *Clin Exp Immunol* 1972;10: 399-407.
- 10 Halliwell REW: The localization of IgE in canine skin: An immunofluorescent study. *J Immunol* 1973;110:422-430.
- 11 Halliwell REW, Schwartzman RM, Montgomery PC, Rockey JH: Physicochemical properties of canine IgE. *Transplant Proc* 1975;7:537-543.
- 12 Halliwell REW, Kunkle GA: The radioallergosorbent test in the diagnosis of canine atopic disease. *J Allergy Clin Immunol* 1978;62:236-242.
- 13 Kleinbeck ML, Hites MJ, Loker JL, Halliwell RE, Lee KW: Enzyme-linked immunosorbent assay for measurement of allergen-specific IgE antibodies in canine serum. *Am J Vet Res* 1989; 50:1831-1839.
- 14 Willemse A, Noordzij A, Rutten VPMG, Bernadina WE: Induction of non-IgE anaphylactic antibodies in dogs. *Clin Exp Immunol* 1985;59: 351-358.
- 15 Kepron W, James JM, Kirk B, Schon AH, Tse KS: A canine model for reaginic hypersensitivity and allergic bronchoconstriction. *J Allergy Clin Immunol* 1977;59:64-69.
- 16 Rogers DF, Adams JA: Space curve; in Rogers DF, Adams JA (eds): *Mathematical Elements for Computer Graphics*. New York, McGraw Hill, 1976, pp 133-138.
- 17 Peng Z, Thomas E, Lilley MK, Rector ES, Schon AH, Simons FER, Becker AB: ELISA assays for measurement of ragweed specific IgE in dog serum (Abstract). *J Allergy Clin Immunol* 1992;89:337.
- 18 Peng Z, Simons FER, Becker AB: Differential binding properties of protein A and protein G for dog immunoglobulins. *J Immunol Methods* 1991;145:225-258.
- 19 Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-685.
- 20 Peng Z, Arthur G, Simons FER, Becker AB: Binding of dog immunoglobulin G, A, M, and E to concanavalin A. *Vet Immunol Immunopathol* 1993;36:83-88.
- 21 Ishizaka T: Mechanisms of IgE-mediated hypersensitivity; in Middleton E Jr, Reed CE, Ellis EF, Adkinson NF Jr, Yunginger JW (eds): *Allergy Principles and Practice*. St. Louis, Mosby, 1988, pp 71-93.
- 22 Peng Z, Naclerio RM, Norman PS, Adkinson NF Jr: Quantitative IgE and IgG subclass responses during and after long-term ragweed immunotherapy. *J Allergy Clin Immunol* 1992; 89:519-529.
- 23 Bankert RB, Mazzaferro PK: Immunoglobulins; in Leob WF, Quimby FW (eds): *The Clinical Chemistry of Laboratory Animals*. Toronto, Pergamon Press, 1989, pp 118-140.
- 24 Bennich H, Johansson SGO: Structure and function of human immunoglobulin E. *Adv Immunol* 1971;13:1-55.
- 25 Kochwa S, Terry WD, Capra JD, Yang NL: Structural studies of immunoglobulin E. I. Physicochemical studies of the IgE molecule. *Ann NY Acad Sci* 1972;189:49-70.
- 26 Bennich HH, Ellerson JR, Karlsson T: Evaluation of basic serum IgE levels and the IgE antibody response in the rat by radioimmunoassays. *Immunol Rev* 1978;41:261-287.
- 27 Liu F, Bohn JW, Ferry EL, Yamamoto H, Molinaro CA, Sherman LA, Klinman NR, Katz DH: Monoclonal dinitrophenyl-specific murine IgE antibody preparation, isolation, and characterization. *J Immunol* 1980;124:2728-2737.
- 28 Bose R, Bundesen PG, Holford-Stevens V, Stefura WP, Kelly KA, Jeffrey JC, Rector ES, Fischer J, Schon AH, Schwenk RJ: Immunochemical properties of some monoclonal IgE antibodies to 4-hydroxy-3-nitrophenylacetyl (NP). *Immunology* 1984;53:801-809.
- 29 Suter M, Fey H: Further purification and characterization of horse IgE. *Vet Immunol Immunopathol* 1988;4:545-553.
- 30 Thatcher EF, Gershwin LJ: Generation and characterization of murine monoclonal antibodies specific for bovine immunoglobulin E. *Vet Immunol Immunopathol* 1988;18:53-66.
- 31 Karol M, Jin R, Bennedsen M, Vaughan F: Production and isolation of guinea pig IgE antibody. *J Immunol Methods* 1991;139:123-134.