

Heterogeneity of polyclonal IgE characterized by differential charge, affinity to protein A, and antigenicity

Zhikang Peng, MD,^{a, c} Gilbert Arthur, PhD,^b Edward S. Rector, PhD,^c
Danuta Kierek-Jaszczuk, PhD,^c F. Estelle R. Simons, MD,^a and
Allan B. Becker, MD^{a, c} Winnipeg, Manitoba, Canada

Functional and physical heterogeneity of polyclonal IgE has been reported. Extremely low serum concentrations of IgE have limited the study of these important differences. We have purified polyclonal dog IgE and developed polyclonal and monoclonal (mAb C2) anti-dog IgE antibodies. In this study chromatofocusing of dog IgE revealed two biologically active IgE fractions: IgE1 eluted at pH 5.0, and IgE2 eluted at pH 4.7. The two IgE subforms (IgEs) exhibited typical IgE characteristics: positive in the 48-hour passive cutaneous anaphylaxis response, heat-labile, identical molecular weight, and reactive to polyclonal anti-dog IgE. However, the two IgEs were found to be significantly heterogeneous. IgE1 bound to protein A and did not react with mAb C2 in ELISA and isoelectric focusing-immunoblotting, whereas IgE2 did not bind to protein A and reacted with mAb C2. Further, in sodium dodecylsulfate-polyacrylamide gel electrophoresis and immunoblotting, IgE2, but not IgE1, reacted with seven well-defined mAb anti-human IgE antibodies and an mAb anti-mouse IgE antibody, even though both IgE1 and IgE2 reacted with polyclonal anti-human and anti-mouse IgE. Neuraminidase or endoglycosidase treatment did not abolish the differential antigenicity and charge of IgE1 and IgE2, although the antigenicity of IgE2 was significantly reduced after incubation with endoglycosidase. These data suggest that carbohydrate moieties are not involved in the observed differences in antigenicity and charge and that the two IgE molecules represent distinct isotypes. In studies with seven purified IgE fractions obtained from different ragweed-allergic dogs, the distribution of ragweed IgE2 varied 200-fold, whereas ragweed total IgE levels varied only fourfold. This raises the possibility of a relationship between different IgEs and the allergic response. (*J Allergy Clin Immunol* 1997;100: 87-95.)

Key words: IgE, heterogeneity, cross-reactivity, dog, immunoglobulin

Abbreviations used

IEF:	Isoelectric focusing
IgEs:	IgE subforms
mAb:	Monoclonal antibody
PCA:	Passive cutaneous anaphylaxis
pI:	Isoelectric point
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis

Heterogeneity of polyclonal IgE has been reported on the basis of functional characteristics such as the response to a histamine releasing factor,¹⁻⁴ binding to IgE receptors in rats⁵ and human beings,⁶ and the capacity to induce histamine release.^{7,8} The partial binding of IgE to protein A^{9,10} and lectins,^{11,12} its response to heating at 56° C,¹³ and complement fixation¹⁴ have also provided evidence for IgE heterogeneity. However, the extremely low concentration of serum IgE has limited the study of these important differences. Recently, several forms of membrane and secreted IgE encoded by alternative RNA splicing of IgE heavy chain transcripts have been reported.^{15,16} To date, these differences in physicochemical or functional properties in IgE have not been related to any antigenic differences.

We have established a dog model of allergy and asthma.¹⁷ In our studies the measurement of dog IgE has been hampered by the lack of a dog myeloma-secreting IgE. To overcome this obstacle, we recently purified polyclonal IgE from ragweed-sensitized dog sera,¹⁸ developed both monoclonal (designated C2) and polyclonal anti-dog IgE, and established ELISAs for the measurement of dog ragweed-specific IgE.¹⁹ Although our monoclonal antibody (mAb) C2 and polyclonal antibodies were specifically directed against dog IgE, the ELISA values measured by the mAb C2 correlated less well with passive cutaneous anaphylaxis (PCA) titers ($r = 0.59, p < 0.0001$) compared with the ELISA values measured by the polyclonal antibody ($r = 0.84, p < 0.0001$).¹⁹ These observations suggest the possible existence of heterogeneous IgE subforms (IgEs) in dogs.

In this study we provide evidence of the existence of two physically distinct subforms of polyclonal dog IgE, and more importantly, evidence of differences in antigenicity between the two IgEs. This raises the possibility of

From the Departments of ^aPediatrics and Child Health, ^bBiochemistry and Molecular Biology, and ^cImmunology, University of Manitoba, Winnipeg.

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Reprint requests: Zhikang Peng, MD, MS746A-840 Sherbrook St., Winnipeg, Manitoba, Canada R3A 1S1.

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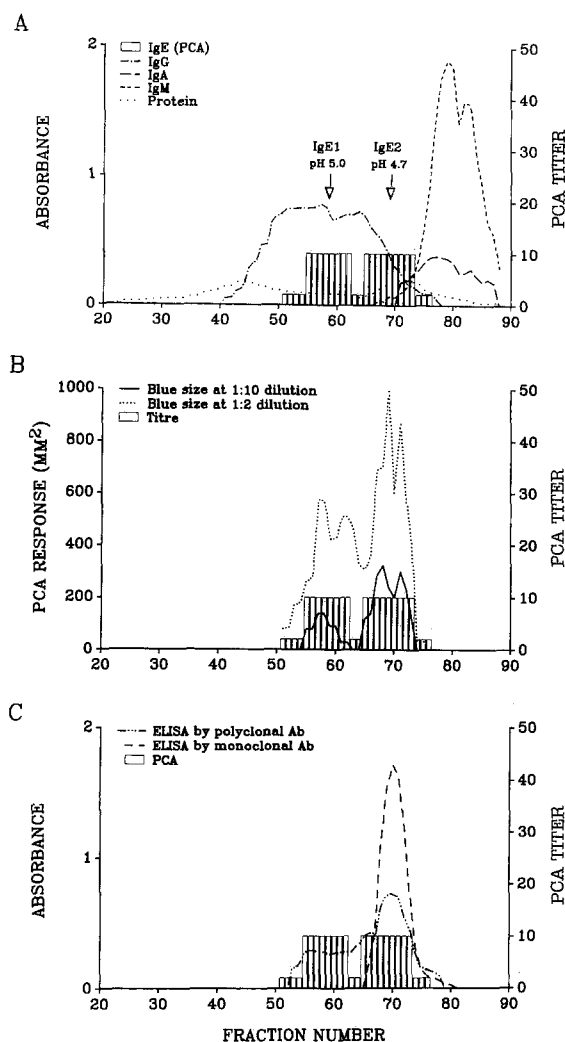


FIG. 1. Chromatofocusing of dog IgE (pH 4.0 to 7.4). The dog IgE fraction was loaded on a polybuffer exchanger column and eluted from the column with polybuffer, pH 4 (described in Methods). **A**, Dog IgE (blank bar, measured by PCA), IgG, IgA, IgM (measured by ELISA), and protein. **B**, Dog IgE measured by PCA with different calculations. Blank bar shows PCA titer; dotted line shows skin blue area at dilution of 1:2; solid line shows blue area at dilution of 1:10. **C**, Dog IgE measured by PCA (titer) and ELISA with polyclonal and mAb C2 anti-dog IgE antibodies. IgE1 (eluted at pH 5.0) reacted with only polyclonal anti-dog IgE antibodies, whereas IgE2 (eluted at pH 4.7) reacted with both polyclonal and mAb C2 antibodies.

a relationship between different IgEs and the allergic response.

METHODS

Reagents

Neuraminidase (*Arthrobacter ureafaciens*), endoglycosidase F/N-Glycosidase F (*Flavobacterium meningosepticum*), and DIG Glycan Detection kit were purchased from Boehringer Mannheim Canada (Laval, Quebec, Canada). IsoGel Agarose plates were purchased from FMC BioProducts (Rockland, Maine). The remaining reagents were obtained from either

Pharmacia LKB Biotechnology Inc. (Baie D'urfé, Quebec, Canada) or Bio-Rad Laboratories (Richmond, Calif.).

Dog IgE and anti-dog IgE antibodies

Dog ragweed IgE-enriched fractions were purified from ragweed-sensitized dog sera by using a previously established procedure.¹⁸ Polyclonal rabbit anti-dog IgE antiserum was developed, purified, and identified as described previously.¹⁸

Monoclonal anti-dog IgE antibodies were produced with the dog IgE-enriched fraction by the method described previously.²⁰ Hybridoma cultures were tested for anti-IgE antibody activity by ELISA. Polystyrene microtiter plates coated with ragweed extract (Hollister-Stier, Rexdale, Ontario, Canada) were sequentially incubated with dog IgE-enriched fraction, hybridoma supernatants, and peroxidase-conjugated goat anti-mouse IgG. The color was developed by the addition of substrate 2,2'-azino-di-(3-ethylbenzthiazoline)-6-sulfonic acid. The optical density at 405 nm was determined 30 minutes later. After the primary screening, several cultures were selected and expanded, and their corresponding supernatants were retested as described above. At this stage the selected supernatants were also tested with ragweed-specific dog IgG (purified from ragweed-sensitized dogs in our laboratory), heat-inactivated dog IgE-enriched fraction, and normal dog serum proteins (precipitated by 30% to 55% ammonium sulfate) substituting for IgE in this assay. Selection was based on supernatants, which reacted strongly with IgE and not with either IgG or heat-inactivated IgE and serum proteins. One culture, which met this criterion, was cloned and designated C2 (278.49.C2.6/73). The isotype of this mAb C2 was determined to be τ -1/kappa by a mouse Ig isotyping kit. The supernatant from this cell line was collected, and mouse IgG was purified by a Protein G-Sepharose column (Pharmacia).

Purified polyclonal¹⁸ and mAb C2 anti-dog IgE antibodies were demonstrated to be specific for dog IgE by inhibition of PCA response, reactions to heat-labile dog IgE, and absence of reactions to dog serum proteins as measured by ELISA.

A newly available mouse mAb anti-dog IgE, designated D9, was generously provided by Dr. Douglas J. DeBoer, University of Wisconsin,²¹ and was used for isoelectric focusing (IEF) studies of glycosidase treatment only.

Other antibodies

Dog IgG, affinity-purified rabbit anti-dog IgG Fc, alkaline phosphatase conjugates of rabbit anti-dog IgG (Fc fragment), goat anti-rabbit IgG (heavy and light chain), goat anti-mouse IgG, and rabbit anti-goat IgG Fc fragment were purchased from Jackson ImmunoResearch Labs, Inc. (West Grove, Pa.). Rabbit anti-dog IgA and IgM sera were obtained from Nordic Immunological Labs (Tilburg, The Netherlands) and ICN ImmunoBiologicals (Lisle, Ill.), respectively. Peroxidase-conjugated goat anti-mouse τ chain, used for the hybridoma screening assay, was purchased from Sigma Chemical Co. (St. Louis, Mo.). Rabbit anti-human IgE and goat anti-mouse IgE (Fc) were purchased from Dakopatts (Glostrup, Denmark) and Cedarlane Laboratories, respectively. For monoclonal anti-human IgE, clone CIA-E-7.12 (ascites), was provided by Dr. Andrew Saxon (University of California Los Angeles)²²; and the remaining seven clones were provided by Drs. William A. Hook and Reuben Siraganian (National Institutes of Health, Bethesda, Md.).²³ Monoclonal rat anti-mouse IgE ϵ chain (LO-ME-3) was purchased from Serotec Canada (Toronto, Ontario).

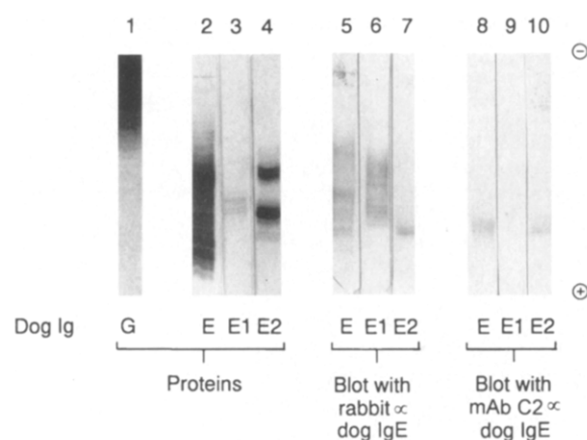


FIG. 2. IEF immunoblot for dog IgE, IgE1, and IgE2 fractions. The dog IgE fraction before chromatofocusing (strips 2, 5, and 8), the IgE1 (lanes 3, 6, and 9) and IgE2 (lanes 4, 7, and 10) fractions obtained from chromatofocusing, and dog IgG (lane 1) were separated by IEF. Separated proteins were either stained with Coomassie Brilliant Blue (lanes 1 to 4) or transferred onto nitrocellulose, followed by immunoblotting with polyclonal (lanes 5 to 7) or mAb C2 (lanes 8 to 10) anti-dog IgE. Lanes 5, 6, and 7 show that IgE, IgE1, and IgE2 fractions reacted with rabbit anti-dog IgE. Lanes 8, 9, and 10 show that IgE2 contained in the IgE fraction (lane 8) and the IgE2 fraction (lane 10) reacted with mAb C2 anti-dog IgE, whereas IgE1 fraction (lane 9) did not react with the mAb.

PCA

PCA was used throughout this study for the biologic measurement of dog ragweed-specific IgE.¹⁸ Dilutions of test samples and reference serum were injected intradermally into a dog not sensitized to ragweed. After 48 hours, the dog was challenged with intravenous injection of ragweed extract and Evan's blue dye. The blue skin area was measured 30 minutes later.

Measurement of dog ragweed-specific IgE, IgG, IgA, and IgM by ELISA

The levels of ragweed-specific antibodies were measured by indirect ELISA, as previously described.¹⁹ For standardization of ELISA results among assays and estimation of the relative amount of ragweed-specific antibodies in each sample, the reference serum that was obtained from a ragweed-sensitized dog was used and defined as 1000 U/ml for ragweed-specific IgG, IgA, and IgM antibodies. A dog IgE-enriched fraction was used as a reference and defined as 1000 U/ml for ragweed-specific IgE and 1000 U/ml for ragweed-specific IgE2. Briefly, polystyrene microtiter plates coated with ragweed extract were sequentially incubated with the following: (1) test sample or reference; (2) polyclonal or mAb C2 anti-dog IgE antibodies or rabbit antibodies to dog IgG, IgA, and IgM; (3) enzyme-conjugated goat antibodies to rabbit IgG or to mouse IgG; (4) substrate.

Chromatofocusing of dog IgE by polybuffer and polybuffer exchangers

The dog IgE-enriched fraction (3.5 ml) was loaded onto a Sephadex G-25 column (Pharmacia) equilibrated with 0.025 mol/L imidazole buffer, pH 7.4. The IgE fraction was then applied to a 25 ml column of polybuffer exchanger, which was equilibrated with the imidazole buffer. The column was eluted with polybuffer, pH 4.0. The pH of each fraction (2 ml) was

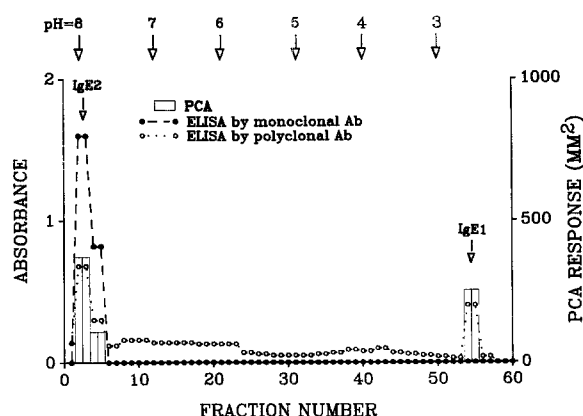


FIG. 3. Binding properties of dog IgE to protein A. Dog IgE fraction was loaded on a Protein A-Sepharose 4B column and then washed and eluted as described in Methods. Dog IgE was measured by PCA and ELISA with polyclonal and mAb C2 anti-dog IgE antibodies.

measured and, if required, carefully neutralized by 1 N NaOH to bring it up to pH 7. The IgE in each fraction was measured by PCA and ELISA with polyclonal and mAb C2 anti-dog IgE antibodies; and the IgG, IgA, and IgM were measured by ELISA. Protein elution from the column was monitored at an optical density of 280 nm. Fractions eliciting positive PCA responses in dogs (Fig. 1) were pooled and concentrated by using Centriprep-30 concentrators. IgE1 refers to the first PCA-positive pooled fraction, whereas IgE2 refers to the second PCA-positive pooled fraction. These fractions, IgE1 and IgE2, were used in IEF immunoblot, heat-lability tests by ELISA and PCA, and determination of molecular mass.

IEF immunoblot analysis for IgE1 and IgE2

The dog IgE-enriched fraction and its two subforms (IgE1 and IgE2) obtained by chromatofocusing were analyzed by IEF immunoblot with a horizontal, precast Isogel agarose plate (pH 3 to 10). The separated proteins were either stained with Coomassie Brilliant Blue or transferred onto nitrocellulose and immunoblotted. Before immunoblotting, free binding sites on the membrane were blocked by 3% (wt/vol) bovine serum albumin dissolved in Tris-buffered saline solution. The membrane was incubated with either rabbit anti-dog IgE, followed by alkaline phosphatase-conjugated goat anti-rabbit IgG, or the mAb C2 anti-dog IgE, followed by alkaline phosphatase-conjugated goat anti-mouse IgG. The color reaction was developed with a p-toluidine salt of 5-bromo-4-chloro-indolyl phosphate and nitro blue tetrazolium dissolved in 0.1 mol/L ammonium buffer, pH 9.6, at 0.25 mg/ml and 0.50 mg/ml, respectively.

Molecular mass of IgE1 and IgE2

The intact molecular mass of the two IgE molecules was determined as previously reported.¹⁸ The dog IgE-enriched fraction and dog IgE1 and IgE2 fractions obtained by chromatofocusing were separated on a 5% to 15% gel by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and nonreducing conditions, respectively, and then electrotransferred onto nitrocellulose membranes. The membranes were immunoblotted with rabbit anti-dog IgE, followed by incubation with goat anti-rabbit IgG conjugate.

TABLE 1. Ragweed-specific IgE, IgE2, IgG, IgA, and IgM values in unheated and heated (56° C, 2 hours) IgE1 and IgE2 fractions obtained by chromatofocusing

Sample	IgE activity			ELISA (U/ml)		
	PCA (titer)	ELISA (U/ml) (polyclonal antibody)	ELISA (U/ml) (mAb C2)	IgG	IgA	IgM
IgE1						
Unheated	32	>200	0	0.6	0	0
Heated	4	20	0	0.7	0	0
IgE2						
Unheated	64	>200	>200	0.3	14	0.2
Heated	8	17	<3	0.3	21	0.3

Affinity chromatography of dog IgE on protein A

The dog IgE-enriched fraction (2 ml) was loaded on a Protein A-Sepharose CL-4B column (bed volume, 6 ml) equilibrated with 0.1 mol/L phosphate buffer, pH 8.0. After loading, the fraction was allowed to stand in the column for 30 minutes. The column was then washed with phosphate buffer and eluted sequentially with pH 7 and 6 (0.1 mol/L phosphate) and pH 5, 4, and 3 (0.1 mol/L glycine) buffers. The fractions eluted with glycine buffers were neutralized by 0.5 mol/L Tris buffer, pH 8.0, to bring the pH up to 7.0 and dialyzed against phosphate buffer, pH 7.0, overnight. Each eluted fraction (2.5 ml) was assayed for dog IgE by PCA and by ELISA. The IgE1 and IgE2 fractions isolated by protein A were used in the experiments of cross-reactivity and neuraminidase and endoglycosidase treatment.

Cross-reactivity of IgE1 and IgE2 to human and mouse IgE

SDS-PAGE and immunoblot were performed according to our previous method.²⁴ The dog IgE-enriched fraction and dog IgE1 and IgE2 fractions obtained by chromatography on protein A, as described above, were separated on 10% SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were immunoblotted with anti-IgE antibodies, followed by incubation with the appropriate conjugated anti-IgG antibodies. The anti-IgE antibodies included a rabbit anti-human IgE, eight mouse mAb anti-human IgE, a goat anti-mouse IgE, and a rat mAb anti-mouse IgE.

Neuraminidase- and endoglycosidase-treated IgE1 and IgE2

To determine whether sialic acid residues or oligosaccharide chains were involved in the differential response of IgE1 and IgE2 to the mAb anti-dog IgE C2 or D9, dog IgE1 and IgE2 fractions were digested with neuraminidase or endoglycosidase F/N-Glycosidase F and subsequently subjected to IEF immunoblot analysis. The IgE1 and IgE2 fractions used in this study were obtained by chromatography of the dog IgE-enriched fraction on protein A-Sepharose. Fifty microliters of IgE1 (0.4 mg/ml) or IgE2 (1 mg/ml) was incubated with 0.5 U of neuraminidase F in 10 mmol/L Tris acetate buffer, pH 6.0, containing 1 mg/ml cholic acid²⁵ for 18 hours at 37° C. Identical incubations without neuraminidase served as controls. Endoglycosidase F/N-Glycosidase F digestion of IgE1 and IgE2 was conducted by incubation of the two IgE fractions (100 µl for each) with 1 U of the enzyme in 0.25 mol/L sodium acetate buffer, pH 7.0, with 20 mmol/L ethylenediaminetetraacetic acid in the presence of protease inhibitors (leupeptin, aprotinin,

phenylmethylsulfonyl fluoride) for 18 hours at 37° C. At the end of the incubations, the fractions were subjected to IEF immunoblot analysis with the anti-dog IgE mAb C2 and D9.

To determine the extent of removal of carbohydrates after the digestion, dog IgE fractions treated with neuraminidase or endoglycosidase F/N-Glycosidase F under conditions identical to those described above were subjected to SDS-PAGE on 10% gels under reducing conditions.²⁴ The proteins were transferred to nitrocellulose membranes and either stained for glycoproteins with a DIG Glycan Detection kit or immunoblotted with rabbit anti-dog IgE for identification of the IgE ϵ chains.

RESULTS

Chromatofocusing and IEF immunoblot analysis for dog IgE

Two fractions, IgE1 and IgE2, were obtained from the chromatofocusing column by pooling fractions from the column that produced a positive PCA response. The first fraction, IgE1, was eluted at pH 5.0. The second fraction, IgE2, was eluted at pH 4.7. These two fractions were different from those of IgG, IgA, and IgM, which were eluted at pH 4.9, 4.3, and 4.2, respectively (Fig. 1, A). The separation was observed regardless of whether the PCA results were expressed as the titer or the blue area of skin response at different dilutions (Fig. 1, B). Both IgE1 and IgE2 were recognized by the polyclonal anti-dog IgE, whereas only IgE2 was recognized by mAb C2 (Fig. 1, C).

The above results were further confirmed by IEF immunoblot analysis. As shown in Fig. 2, the isoelectric spectrum of IgE1 had higher isoelectric points (pIs) and reacted with the polyclonal anti-dog IgE but not with the mAb C2. The spectrum of IgE2 had a lower pI and reacted with both polyclonal and mAb C2 anti-dog IgE antibodies.

Heat lability of IgE1 and IgE2

IgE1 and IgE2 fractions separated by chromatofocusing were heated at 56° C for 2 hours. Fractions were tested before and after heating for ragweed-specific IgE by PCA and by ELISA with the polyclonal or the mAb C2 anti-dog IgE, and for ragweed-specific IgG, IgA, and IgM by ELISA. As shown in Table 1, IgE activity in the IgE1 and IgE2 fractions was significantly reduced after heating. In contrast to IgE and its subforms, IgG, IgA,

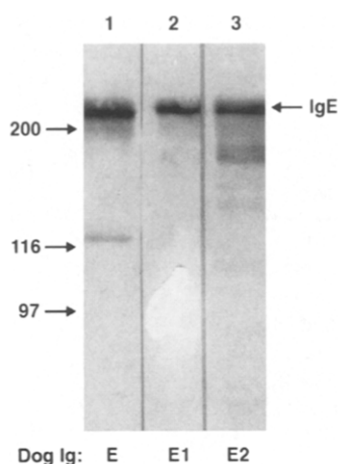


FIG. 4. Estimation of molecular mass for dog IgE, IgE1, and IgE2. Dog IgE (lane 1), IgE1 (lane 2), and IgE2 (lane 3) were separated by 5% to 15% gel SDS-PAGE under nonreducing conditions and electrotransferred onto nitrocellulose membranes. Membranes were incubated with rabbit anti-dog IgE, followed by incubation with enzyme-conjugated goat anti-rabbit IgG. Molecular mass of dog IgE was 226 kd, as indicated. Ab, Antibody.

and IgM activities in the two fractions were unchanged after heating.

Molecular mass for IgE1 and IgE2

As shown in Fig. 3, the approximate intact molecular weight estimated by Western blot, under nonreducing conditions, was 226 kd for dog IgE, IgE1, and IgE2. The molecular mass of the ϵ chains was 75 kd for dog IgE, IgE1, and IgE2 (data not shown).

Affinity of dog IgE to protein A

As described in the section on results of chromatofocusing, the IgE that did not react with mAb C2 was designated as IgE1, whereas the IgE that reacted with mAb C2 was designated as IgE2. Chromatography of the IgE fraction on protein A revealed that IgE1 bound to protein A and was eluted at pH 3, whereas IgE2 did not bind to protein A and was found in the effluent (Fig. 4).

Cross-reactivity of IgE1 and IgE2

As shown in Fig. 5, although both IgE1 and IgE2 reacted with polyclonal anti-human and anti-mouse IgE (lanes 2 and 3), differential cross-reactivity was found between the two IgEs when monoclonal anti-human or anti-mouse IgE was used. IgE2 reacted with both mAb anti-human IgE (clone 7.12) (Fig. 5, A, lane 6) and mAb anti-mouse IgE (Fig. 5, B, lane 6), whereas IgE1 did not react with the two mAbs at all (lane 5). Identical results were found when six of the remaining seven mAb anti-human IgE antibodies (clones 3, 8, 9, 10, 13, and 15) were used (data not shown). Only one mAb anti-human IgE (clone 6) reacted with both IgE1 and IgE2. The results are summarized in Table III.

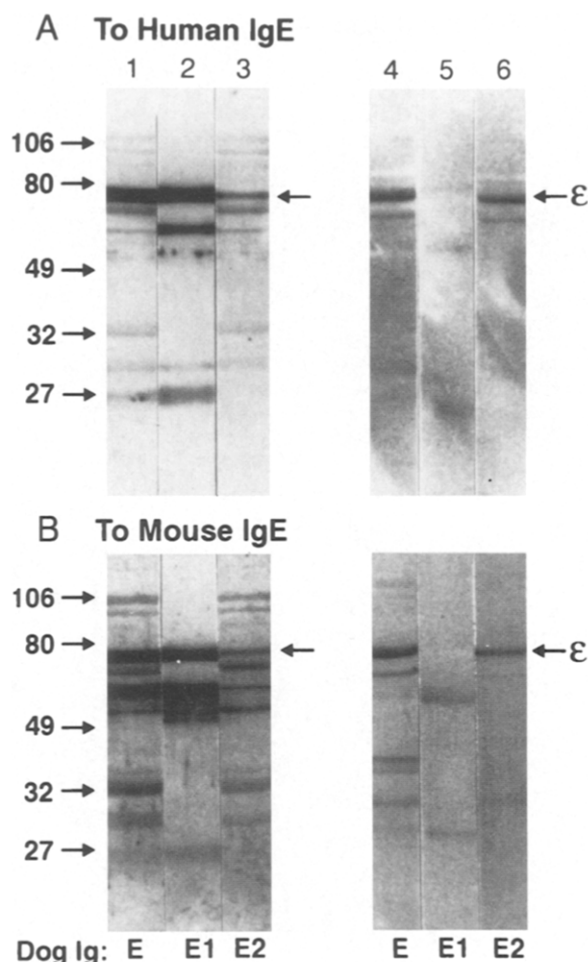


FIG. 5. SDS-PAGE and immunoblot analysis of cross-reactivity of dog IgE1 and IgE2 to human and mouse IgE. Proteins of dog IgE-enriched fraction (lanes 1 and 4) and dog IgE1 (lanes 2 and 5) and IgE2 (lanes 3 and 6) fractions, as indicated at bottoms of lanes, were separated by SDS-PAGE on 10% gel and then transferred to nitrocellulose membranes. Membranes were immunoblotted with anti-human IgE (A) or anti-mouse IgE (B), followed by incubation with relevant conjugated anti-IgG antibodies. Lanes 1, 2, and 3 were incubated with polyclonal anti-IgE; and lanes 4, 5, and 6 were incubated with mAb anti-IgE.

Neuraminidase- and endoglycosidase-treated IgE1 and IgE2

Glycoprotein staining showed that digestion of dog IgE with neuraminidase caused only a slight decrease in the carbohydrate content, whereas endoglycosidase digestion removed most of the carbohydrate moieties from the IgE, resulting in a reduction of the molecular mass of the ϵ chains. Because only one ϵ band was found in both glycoprotein staining and immunoblotting, this suggests that IgE1 and IgE2 have the same molecular weights after glycosidase treatments (data not shown). IEF immunoblot analysis of neuraminidase- and endoglycosidase-treated IgE1 and IgE2 (Fig. 6) showed that before digestion with neuraminidase and endoglycosidase, mAb

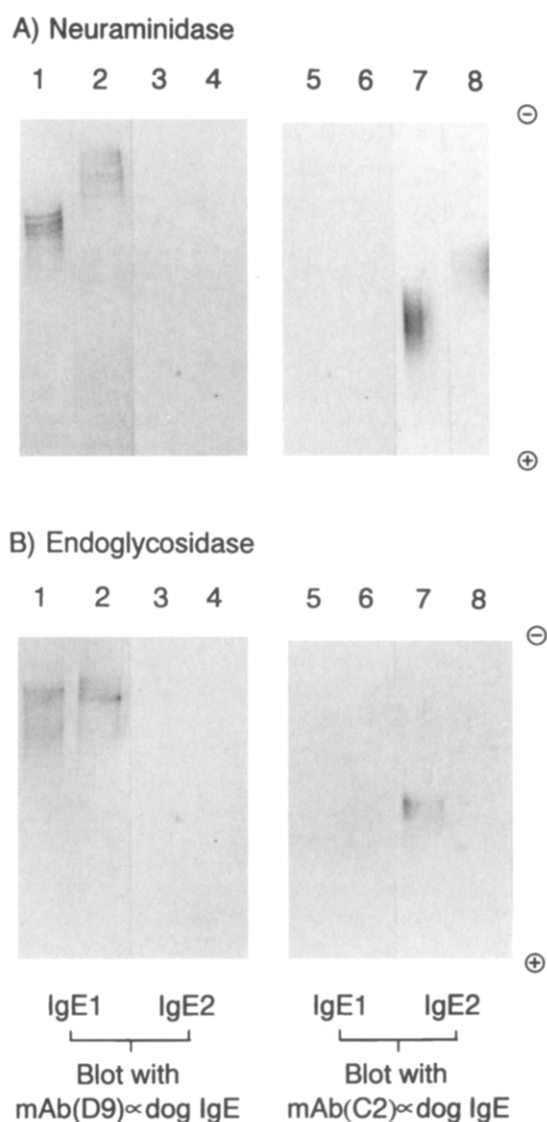


FIG. 6. IEF immunoblot analysis of neuraminidase- and endoglycosidase-treated IgE1 and IgE2 fractions. Dog IgE1 (lanes 1, 2, 5, and 6) and IgE2 (lanes 3, 4, 7, and 8) obtained by chromatography on Protein A-Sepharose were incubated with indicated enzymes or relevant buffers as controls. **A**, Lanes 2, 4, 6, and 8 were incubated with neuraminidase; lanes 1, 3, 5, and 7 were incubated with buffer. **B**, Lanes 2, 4, 6, 8 were incubated with endoglycosidase F/N-Glycosidase F; lanes 1, 3, 5, and 7 were incubated with buffer. These incubations were then subjected to IEF immunoblot with mAb D9 (lanes 1 to 4) or mAb C2 anti-dog IgE (lanes 5 to 8), as described in Methods.

D9 reacted with IgE1 (lane 1) but not IgE2 (lane 3), whereas mAb C2 reacted with IgE2 (lane 7) but not IgE1 (lane 5). After neuraminidase and endoglycosidase digestion, mAb D9 was still only able to recognize IgE1 (lane 2) but not IgE2 (lane 4). Similarly, the ability of the mAb C2 to react with IgE2 (Fig. 6, *A*, lane 8) but not IgE1 (Fig. 6, *A*, lane 6) was unchanged by neuraminidase treatment. However, the antigenicity of IgE2 was abol-

TABLE II. Ragweed-specific IgE and IgE2 values in IgE-enriched fractions from ragweed-sensitized dogs

Dog No.	Ragweed IgE		Ragweed IgE2
	PCA titer	ELISA (u/ml)* (polyclonal Ab)	ELISA (U/ml)* (mAb C2)
1	1024	1775	835
2	1024	584	1242
3	1024	2164	35
4	2048	1000	1000
5	2048	1388	3575
6	2048	2232	28
7	256	634	18

Ab, Antibody.

* The values (in units per milliliter) of ragweed IgE and IgE2 in samples were calculated by interpolation from a reference dilution curve. This reference was a purified dog fraction (dog no. 4) and was defined as 1000 U/ml for ragweed IgE and 1000 U/ml for ragweed IgE2.

ished after endoglycosidase treatment (Fig. 6, *B*, lane 8) compared with the control (Fig. 6, *B*, lane 7).

Fig. 6 also shows that after glycosidase treatment, IgE1 and IgE2 still show different pIs, although the pIs of both IgEs are reduced after the neuraminidase treatment. This strongly suggests that the differential pIs of IgE1 and IgE2 are not dependent on glycosylation.

Distribution of ragweed-specific IgE and IgE2 in allergic dogs measured by ELISA

Dog IgE fractions purified separately from seven ragweed-sensitized dogs were tested for ragweed-specific IgE by PCA and ELISA with the polyclonal anti-dog IgE, and for ragweed-specific IgE2, by ELISA with the mAb C2 (Table II). In the ELISA each IgE determination was performed on two dilutions of the fraction with each dilution duplicated. The ELISA standard curve was diluted in parallel with all of the seven dog IgE fractions, as shown by the very small coefficients of variation between dilutions (mean coefficient of variation < 0.20),¹⁹ indicating accurate measurement of the IgE levels. Although the values in units per milliliter obtained by ELISA with the polyclonal and the mAb C2 anti-dog IgE antibodies are not the same and cannot be compared, valid comparisons can be made within the same assay. The ELISA results obtained with the mAb C2 among dogs with the same PCA titer (dogs 1, 2, and 3 or dogs 4, 5, and 6) clearly demonstrate a differential distribution of the IgE2 levels. In general, there was a much greater variation in the IgE2 levels measured by ELISA with the mAb C2 (200-fold) compared with the IgE levels obtained by PCA and ELISA with the polyclonal antibody (fourfold).

Summary of the characteristics of IgE1 and IgE2

The characteristics of the two subforms of dog IgE are summarized in Table III.

TABLE III. Characteristics of dog IgE1 and IgE2

	IgE1	IgE2
IgE characteristics of the 2 subforms		
1. PCA response (48 hr)	Yes	Yes
2. Reaction with rabbit anti-dog IgE	Yes	Yes
3. Heat lability	Yes	Yes
4. Molecular mass		
Intact	226 kd	226 kd
ϵ Chain	75 kd	75 kd
Heterogeneity of the 2 subforms		
1. pI	4.7	5.0
2. Binding to protein A	Yes	No
3. Reaction with mAb anti-dog IgE		
mAb C2 (IEF immunoblot)	No	Yes
mAb D9 (IEF immunoblot)	Yes	No
4. Cross-reactivity to the IgE of other species (Western blot)		
Human: Rabbit anti-human IgE, to whole molecule	Yes	Yes
Human: Mouse mAb anti-human IgE:		
Clone 7.12, to C _H 2 aa 218-301	No	Yes
Clone 3, to C _H 1	No	Yes
Clone 6, binding sites not identified	Yes	Yes
Clone 8, to C _H 1	No	Yes
Clone 9, to Fc	No	Yes
Clone 10, to Fc	No	Yes
Clone 13, to Fc	No	Yes
Clone 15, to C _H 2	No	Yes
Mouse: Goat anti-mouse IgE, to Fc	Yes	Yes
Mouse: Rat mAb anti-mouse IgE, to ϵ	No	Yes
5. Reduction of antigenicity after incubation with		
Neuraminidase	No	No
Endoglycosidase	Low	High

DISCUSSION

In this study two subforms of polyclonal dog IgE were physically separated by chromatofocusing, IEF, and chromatography on protein A-Sepharose. Evidence that these two fractions were immunoglobulins of the IgE class includes the following: (1) PCA response; (2) loss of IgE activity after heating at 56°C for 2 hours; (3) specific reactions with the polyclonal anti-dog IgE, but not with anti-dog IgG, IgA, and IgM; and (4) a molecular mass of 226 kd, identical to that recently established for dog IgE.¹⁸ Both IgE1 and IgE2 elicited a positive response in a 48-hour PCA test. This long sensitizing period (48 hours) in the PCA test limited the possibility of the response being elicited by IgG sensitizing antibodies, which have a short sensitizing period²⁶ and which have been shown to induce PCA response at 3 to 4 hours, but not at 48 hours, in dogs.²⁷ The 226 kd band, identical to that of dog IgE, was present in both IgE1 and IgE2 fractions and was the only band that reacted with the polyclonal anti-dog IgE. This suggests that one of the IgE subforms was not derived from the other by proteolysis and also that, in spite of their different pIs and binding properties to protein A, the two IgEs have an identical molecular mass.

Protein A binding property has separated human IgG1, IgG2, and IgG4 from IgG3²⁸ and human IgA2

from IgA1.²⁹ Studies of human IgG and IgE suggest that the binding sites to protein A are located on the heavy chains, but not on the light chains.^{30, 31}

The most significant and novel differences were the reaction of IgE2, but not IgE1, with the mAb C2 anti-dog IgE and the reaction of IgE1, but not IgE2, with the newly available mAb D9 anti-dog IgE,²¹ even though both IgE1 and IgE2 fractions reacted with the polyclonal anti-dog IgE and elicited positive 48-hour PCA responses. Although heterogeneity of IgE has been proposed in human beings and rodents,¹⁻¹⁶ as far as we are aware, our study is the first to demonstrate that specific mAbs recognize subforms of biologically active polyclonal IgE that are physically and immunologically different, suggesting that there may be distinct isotypes for the two IgEs.

This is further supported by differential cross-reactivity to human and mouse IgE. As shown in Table II and Fig. 5, IgE2 is closer to human and mouse IgE in cross-reacting with anti-human and anti-mouse IgE. IgE2, not IgE1, reacted with seven mAb anti-human IgE antibodies including the well-defined mAb clone 7.12, which was specific to CH2 aa 218-301.²² The epitopes recognized by the remaining six mAb anti-human IgE were also well-defined to be on the human IgE epsilon CH1, CH2, or the Fc.²³ The lack of the binding of IgE1 to the mAbs suggests that either IgE1 lacks the epitopes

recognized by the mAbs or the epitopes recognized by the mAbs are masked or sterically hindered by the carbohydrates.

Early suggestions that functional heterogeneity of IgE¹⁻⁸ might be due to posttranslational events and also that heterogeneous sialylation might be responsible for the differential binding of polyclonal human IgE to lectins^{11,12} led to the question of involvement of carbohydrates in this process. Our study with neuraminidase indicated that although sialic acid residues contribute to the charge of IgE1 and IgE2, clearly they are not involved in differential antigenic responses to the mAbs and differential pIs. Digestion of the IgE fractions with endoglycosidase removed most of the carbohydrates and resulted in the loss of the antigenicity of IgE2, recognized by mAb C2. Thus carbohydrate moieties are important in recognition of antigenic determinants by the mAb C2. On the other hand, the same degree of endoglycosidase digestion of IgE1 did not significantly affect its recognition by mAb D9. However, mAb D9 was still unable to recognize endoglycosidase-treated IgE2. Thus the antigenic determinants recognized by mAb D9 do not directly or indirectly depend on the carbohydrates tested. A recent report that lectins did not distinguish functional heterogeneous IgE molecules is consistent with our observations.³²

The available evidence therefore indicates that the heterogeneity of the two IgEs is unlikely to be due to carbohydrates, but rather, is due to differences in the primary protein structure, which may be caused by the existence of a second functional ϵ -immunoglobulin gene in dogs or by alternative RNA splicing of IgE heavy chain transcripts as reported in human beings.^{15,16}

Interestingly, we were able to demonstrate that these two IgE subforms participate to a different extent in total ragweed-specific IgE level of allergic dogs. This raises the possibility of a relationship between the levels of different IgEs and the pathophysiology of the IgE-mediated hypersensitivity response.

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