

Characterization of two dog IgE-specific antibodies elicited by different recombinant fragments of the epsilon chain in hens

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Abstract

Two recombinant [His]₆-tagged fragments of the canine immunoglobulin E (IgE) heavy chain (second domain: IgEf2 and third and fourth domains: IgEf3/4) were cloned, expressed in *Escherichia coli* (*E. coli*) as [His]₆-tagged proteins, and affinity-purified over nickel-nitrilotriacetic acid columns. The recombinant proteins were used to immunize hens. The raised and affinity-purified chicken antibodies (Ab) isolated from egg yolk exhibited specific binding to the respective recombinant canine IgE fragment (IgEf) on immunoblots and displayed high titers against the IgEf in ELISA. Immunoblotting of canine serum separated by PAGE under native conditions with the IgEf2- and IgEf3/4-specific Ab resulted in staining of a protein of approximately 180 kilodaltons (kD). The IgEf3/4-specific Ab further recognized an 80 kD protein in IgEf3/4-specific Ab affinity-enriched dog serum separated under denaturing conditions. In an ELISA for the detection of

Abbreviations: AP, alkaline phosphatase; Ab, antibody; *E. coli*, *Escherichia coli*; ELISA, enzyme-linked immunosorbent assay; h, hour; IgE, immunoglobulin E; IgG, immunoglobulin G; kD, kilodalton; OD, optical density; PCR, polymerase chain reaction; PSG, porcine skin gelatin; IgEf, recombinant canine IgE fragment; IgEf2, recombinant canine IgE fragment of the second constant domain; IgEf3/4, recombinant canine IgE fragment of the third and fourth constant domains; RCA, reverse cutaneous anaphylaxis; RT, room temperature; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; T-20, Tween[®]-20; TBS, Tris buffered saline

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antigen-specific IgE in dog serum, reduced binding of the IgEf-specific Ab was observed after heat treatment of the dog serum. The reactivity of both of the raised chicken Ab was only present in postimmune reagents and could only be inhibited by preincubation with the IgEf used for immunization and not with dog immunoglobulin G, *E. coli* extract, or with a nonrelevant recombinant [His]₆-tagged protein. In immunohistochemistry, the IgEf3/4-specific Ab specifically recognized cells in paraffin-embedded tissue sections of lymph nodes. Furthermore, both of the IgEf-specific Ab elicited positive immediate type 1 skin reactions in dogs. Semiquantitative assessment of total serum IgE in dogs was developed using IgEf2-specific Ab as coating reagent and the biotinylated IgEf3/4-specific Ab as developing Ab in ELISA. In conclusion, both IgEf-specific Ab recognize native dog IgE with the advantages that they are directed against different and known constant domains of the IgE molecule, and that they can be used for immunohistochemistry on paraffin-embedded tissue. The two dog IgE-specific Ab could initiate clinical research on the involvement of immediate-type hypersensitivity reactions in dogs. © 1998 Elsevier Science B.V. All rights reserved

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1. Introduction

The role of immediate-type hypersensitivity reactions remains to be elucidated in many diseases of dogs involving the gastrointestinal or respiratory system. To analyze the involvement of immunoglobulin E (IgE)-mediated hypersensitivity (Ishizaka et al., 1966), well-characterized and highly specific antibodies (Ab) recognizing IgE are necessary. Several polyclonal and more recently monoclonal Ab to dog IgE have been described (Schwartzman et al., 1971; Halliwell et al., 1972; Halliwell, 1973 and Halliwell and Longino, 1985; deBoer et al., 1993; Peng et al., 1993a, b, 1996; de Weck et al., 1998); however, these reagents are not generally available. Inhibition studies to further prove specificity of the Ab have in part been hindered by the lack of pure dog IgE, because an IgE gammopathy has not yet been described in dogs. There are also no published reports on the usage of IgE-specific Ab in immunohistochemistry on paraffin-embedded tissue sections from dogs. Thus, there is still a lack of well-characterized reagents directed against distinct epitopes of dog IgE.

The complete DNA sequence of dog IgE has been published (Patel et al., 1995), which enabled us to apply recombinant technology for the expression of fragments of the canine epsilon chain. Furthermore, the successful production and characterization of horse IgE-specific Ab induced by a recombinant fragment of the equine IgE heavy chain in chickens in our laboratory has encouraged us to use a similar approach for the production of dog IgE-specific Ab (Marti et al., 1997).

The aim of this study was to produce dog IgE-specific Ab. We describe the cloning and expression of two different domains of the canine IgE heavy chain, that were used for the immunization of hens and the characterization of the raised Ab by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, enzyme-linked immunosorbent assay (ELISA), immunohistochemistry, and reverse cutaneous anaphylaxis (RCA).

2. Materials and methods

2.1. Cloning and expression of two recombinant fragments of the canine epsilon chain

Isolation of total RNA from the spleen of a dog and subsequent cDNA synthesis using oligo d-T primers were performed according to the manufacturer's instructions (Qiagen AG and Gibco BRL, Basel, Switzerland). The cDNA was used as a template to amplify two fragments of the canine epsilon chain (second domain: IgEf2 and third and fourth domains: IgEf3/4) by polymerase chain reaction (PCR). Primers were designed based on the published dog IgE sequence (Table 1; Patel et al., 1995). The IgEf2-fragment corresponded to nucleotides 300–620 and the IgEf3/4-fragment to nucleotides 927–1280. For unidirectional cloning two restriction sites were included, one into the forward (BamHI or BglII) and one into the reverse primer (HindIII). For cloning of the IgEf3/4, the PCR product was cleaved with HindIII and BglII to avoid internal restriction. Standard protocols were used for the PCR (Perkin Elmer, Branchberg, NJ, USA). The 321 bp (IgEf2) and 354 bp (IGEF3/4) PCR products were separated by a 1% agarose gel and purified with GenElute™ Agarose Spin Columns (Supelco, Bellefonte, PA, USA). The isolated PCR products were digested with the restriction endonucleases BamHI or BglII and HindIII (Boehringer Mannheim GmbH, Mannheim, Germany). The digested PCR products were ligated into the BamHI- and HindIII-cleaved vector pQE-30 with a [His]₆-affinity tag (Qiagen AG) using standard protocols (Sambrook et al., 1989). The plasmids were transformed into *Escherichia coli* (*E. coli*) M15[pREP4] cells (Qiagen AG) by electroporation. The transformants were selected on Luria Bertani plates containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml) and screened for the correct insert using PCR. Two colonies bearing the correct insert, IgEf2 or IgEf3/4, as shown by sequence analysis were subsequently used. Expression, purification, and refolding of both proteins were performed as described by Marti et al. (1997). The [His]₆-tagged recombinant protein fragments, IgEf2 and IgEf3/4, were analyzed by SDS–PAGE and immunoblotting.

2.2. Immunization of hens with IgEf2 and IgEf3/4 and isolation of raised Ab

Sets of two and three laying Isabrown hybrid hens were immunized six times intramuscularly every three weeks with water-diluted IgEf2 and IgEf3/4, respectively. Hens immunized with IgEf2 received 1.2 mg of protein per immunization and those immunized with IgEf3/4 received 130 to 260 µg of the immunogen. The immunizations and isolation of immunoglobulin G (IgG) from the egg yolk were carried out as described previously (Marti et al., 1997).

Table 1
Primer sequences for PCR amplification of the two dog IgE heavy chain fragments (IgEf2 and IgEf3/4)

IgEf2	Forward	5'-GGCGGATCCTGTGCCTTAACTTCATTCCGC-3'
	Reverse	5'-AACAAGCTTTCATGAGCACTTGCGAGCCTCATC-3'
IgEf3/4	Forward	5'-GAAGATCTATTGCCAAGGCCCTTGGAAGCGTGCCCCCGGAT-3'
	Reverse	5'-GGGAAGCTTTCATTACCGGGGTTTGGACACCC-3'

2.3. ELISA for Ab screening

A capture ELISA as described by us previously (Marti et al., 1997) was applied with minor modifications. Reagents coated to the solid-phase included serial dilutions of IgEf2 and IgEf3/4 (0–100 ng/well), extract from *Parascaris equorum* (500 ng/well, own production), extract from peanut (100 ng/well, provided by one of our group), dog IgG (1–2 µg/well, Fluka Chemie AG, Buchs, Switzerland), preimmune chicken Ab (1–2 µg/well), and affinity-purified IgEf2-specific Ab (0.4–60 µg/well). After coating the plates with the antigens remaining, free sites were blocked with Tris-buffered saline solution (TBS), pH 7.2, containing 2% (wt/vol) porcine skin gelatin (PSG; Fluka Chemie AG). Sera were diluted in TBS containing 1% PSG and 2% Tween-20 (T-20, Fluka Chemie AG) and all developing Ab in TBS with 1% PSG and 1% T-20, respectively. For detection of antigen-specific canine IgE serum was applied in two-fold serial dilutions (1:5 to 1:40) and developed with affinity-purified IgEf2- or IgEf3/4-specific Ab followed by alkaline phosphatase (AP)-labeled goat anti-chicken IgG Ab (1:1000; Kirkegaard and Perry Laboratories, Gaithersburg, MA, USA). Determination of IgG titers was carried out as above but the serum was diluted 1:500 to 1:1000 followed by rabbit anti-dog IgG Ab (1:3000; Bethyl Laboratories, Montgomery, TX, USA) and AP-labeled mouse anti-rabbit Ab (1:10 000; Sigma Chemical, St. Louis, MO, USA). Both of the AP-labeled developing Ab had to be preincubated with 2.5% pooled dog sera to avoid nonspecific binding. The dog serum pool was obtained by pooling serum from approximately 100 dogs seen at our clinic at the University of Bern. In some ELISA experiments, dog sera were heated at 56°C and samples for analysis were taken at time points from 15 min to 4 h before application onto microtiter plates. ELISA results were expressed as the mean optical density (OD₄₀₅) of duplicate wells after subtraction of background controls measured at 405 nm (coated antigen without canine sera or primary developing Ab). Serum positive for *Parascaris*-specific IgE was detected by screening healthy and sick dogs from our clinic. Sera positive for peanut-specific IgE was provided by one of the co-authors, Prof. Dr. A.L. de Weck, which was raised at the Novartis® Dog Research Colony (Vienna, Austria). A positive ELISA result was defined as an OD₄₀₅ of at least three times above background (OD₄₀₅ obtained in the absence of serum).

2.4. Protein electrophoresis and immunoblotting

The [His]₆-tagged recombinant IgE fragments, ammonium sulfate precipitated dog sera, and IgEf3/4-specific Ab affinity-enriched serum fractions were analyzed by 5–15% PAGE (5–10 µg protein per lane or 10–100 µg per preparative gel) run under native or denaturing-reducing conditions using the buffer system of Laemmli (1970). The protein gels were stained with Coomassie brilliant blue (Merck, Darmstadt, Germany) or blotted to nitrocellulose by semi-dry electrophoretic transfer at 70 mA for one hour (h) according to the manufacturer's instructions (Multiphor II, Pharmacia, Uppsala, Sweden). The nitrocellulose membranes were blocked for 1 h with 5% non-fat dry milk (Bio-Rad, Glattbrugg, Switzerland) in washing buffer (0.02 M Tris-HCl, 0.5 M NaCl, pH 7.5, containing 0.25% T-20). The [His]₆-tagged IgEf2 and IgEf3/4 were detected by incubation of the blot with a nickel-nitrilotriacetic acid AP-labeled conjugate (Qiagen AG) diluted 1:1,000 in washing buffer. Serum immunoblots were analyzed with the same

Ab as described for ELISA. The AP-reactions were visualized using 5-bromo-4-chloro-indolyl-phosphate/4-nitro blue tetrazolium chloride (Boehringer Mannheim GmbH) in AP-buffer (0.1 M NaCl, 0.005 M MgCl₂, 0.1 M Tris-HCl, pH 9.5) and stopped by addition of distilled water.

2.5. Purification of IgEf2- and IgEf3/4-specific Ab by antigen-affinity chromatography

Fifteen mg of the [His]-tagged IgEf2 or IgEf3/4 were loaded onto 3 ml nickel-nitrilotriacetic acid SuperflowTM columns (Qiagen AG) and incubated for 2 h at room temperature (RT) on a shaker. The columns were then washed with 0.025 M TBS, pH 7.2, and equilibrated with binding buffer (ImmunoPure gentle Ag/Ab binding buffer, Pierce, Rockford, IL, USA). Eight ml of IgEf2- or 10–12 ml of IgEf3/4-specific Ab were applied to the respective column and incubated for 2 h at RT. The columns were first washed twice with 0.025 M TBS, pH 7.2, containing 1% T-20, then once with 0.025 M TBS in 1 M NaCl, pH 7.2, followed by two washes with 0.025 M TBS, pH 7.2, containing 1% T-20 and finally once with 0.025 M TBS, pH 6.6. Bound Ab were eluted with ImmunoPure gentle Ag/Ab elution buffer (Pierce). From the IgEf2 column, 32 fractions of 1.5 ml volume each were collected and from the IgEf3/4-column only 6 fractions of 2 ml volume each were obtained. The protein content was determined in all fractions spectrophotometrically at 280 nm. All fractions were tested on peanut- or *Parascaris equorum*-positive dog serum by ELISA. Fractions from the IgEf2 or IgEf3/4 column displaying high specific activity were pooled, desalted, and concentrated by ultrafiltration with Centriplus concentrators[®] (Amicon, Beverly, MA, USA). The concentrated fraction pools were diluted in 0.1 M NaHCO₃ with 0.5 M NaCl, pH 8.3, and titrated on antigen-specific dog serum by ELISA. Unless indicated, affinity-purified IgEf3/4-specific Ab was used in all experiments, whereas the affinity-purified IgEf2-specific Ab was only used in ELISA for quantification of total serum IgE.

2.6. Specificity testing of raised Ab

To illustrate the specificity of the reactions on immunoblots and in ELISA, the IgEf2- and IgEf3/4-specific Ab were preincubated with either increasing amounts of IgEf2 (0.1–10 µg/ml), IgEf3/4 (0.001–10 µg/ml), dog IgG (10 µg/ml), *E. coli* lysate (10 µg/ml of M15 cells; Qiagen AG), or another [His]₆-tagged recombinant protein (recombinant equine IgE heavy chain fragment; Marti et al., 1997). In addition, any results of postimmune chicken Ab were compared with those of preimmune chicken Ab. In ELISA, reactivity of postimmune chicken Ab was further compared between reactions obtained with heated to non-heated dog sera.

2.7. Enrichment of dog serum IgE by affinity chromatography with IgEf3/4-specific Ab

Affinity-purified IgEf3/4-specific Ab (4.3 mg) was loaded onto 3 ml of Affi-Gel[®] Hz hydrazide gel according to the manufacturer's instructions (Bio-Rad). The column was then incubated for 2 h at RT with 8 ml of ammonium sulfate precipitated serum from a

dog with a high total serum IgE. After washing with 4 bed volumes of 1 M NaCl and 8 bed volumes of 0.025 M TBS, pH 7.2, bound proteins were eluted with 6 ml of 0.05 M Tris-HCl containing 0.3 M glycine, pH 2.6, into neutralizing buffer of 1 M Tris-OH, pH 11.2. Fractions were analyzed by SDS-PAGE and pooled for further characterization by immunoblotting.

2.8. Detection of IgE in canine serum

The same ELISA conditions as for antigen-specific Ab were applied with the exceptions that microtiter plates were coated with affinity-purified IgEf2-specific Ab (280 ng/well), incubated with two-fold serial dilutions of sera from 10 healthy dogs (1:50 to 1:6400), and developed with biotinylated, affinity-purified IgEf3/4-specific Ab (1:1000) followed by AP-labeled streptavidin (1:20 000, Sigma Chemical). Biotinylation was performed by incubation of 200 µg of affinity-purified IgEf3/4-specific Ab with 20 µg of biotin (ImmunoPure Sulfo-NHs-Biotin[®] Pierce) in 0.5 M NaHCO₃ at RT for 1 h. Unbound biotin was neutralized by addition of 10 ml of 1 M Tris-HCl, pH 8.0. The biotinylated IgEf3/4-specific Ab was stored at 4°C until further use.

2.9. Immunohistochemistry

Mesenteric, hilar, and popliteal lymph nodes were obtained from 3 dogs not affected with gastrointestinal disease within one h post mortem. Tissue specimens were fixed in 4% phosphate-buffered paraformaldehyde, pH 7.4, for 24 h, deparaffinized, and trypsin-treated for immunohistochemical evaluation (Vandeveldt et al., 1983). Nonspecific binding sites and the endogenous peroxidase were blocked with 2% PSG in TBS and with 1% hydrogen peroxide in methanol for 30 min at RT, respectively. The IgEf3/4-specific Ab was applied at dilutions ranging from 1:100 to 1:800 in TBS for 2 h at 37°C. The chicken Ab was followed by incubation with biotinylated monoclonal mouse anti-chicken light chains Ab (1:1000, 1 h at RT; Sigma Chemical) that had been preincubated with 2.5% pooled dog serum. To detect the immunoreactivity, a commercial kit with an avidin-biotinylated-horseradish-peroxidase complex was used according to the manufacturer's instructions (Vectastain ABC kit, Vector Lab., Burlingame, CA, USA). Enzyme activity was detected with diaminobenzidine (Sigma Chemical) and hydrogen peroxide. As negative control the horse IgE-specific Ab, which had been produced and processed in the same manner as the herein described chicken Ab (Marti et al., 1997), was applied at similar protein concentrations.

2.10. Reverse cutaneous anaphylaxis

Two dogs were prepared by sedation with medetomidine applied intramuscularly (0.05 mg/kg) and by clipping an area over the thorax for intracutaneous injections. Preimmune chicken Ab and affinity-purified IgEf2- and IgEf3/4-specific Ab were diluted in 0.9% NaCl to concentrations of 10 and 50 µg in a total volume of 50 µl. The Ab were applied intradermally in duplicate with a 26-gauge needle. The diluent was used as the negative control, and histamine dihydrochloride (8 µg/50 ml; Allergopharma J. Ganzer KG, Reinbek b. Hamburg, Germany) as the positive control.

3. Results

3.1. Analyses of IgEf2 and IgEf3/4 and the raised Ab by SDS–PAGE and immunoblotting

Nucleotide sequence analysis of both inserts IgEf2 and IgEf3/4 in the vector pQE-30 was consistent with the published sequence of the epsilon heavy chain of dog IgE (Patel et al., 1995; data not shown). The yield of expressed recombinant [His]₆-tagged proteins was 10–100 mg per liter of induced M15 *E. coli* culture, which was measured after affinity purification over a Ni²⁺-chelate column and refolding. Analyses of the IgEf2 and IgEf3/4 by SDS–PAGE and staining by Coomassie blue revealed one major band at approximately 8.7 kD (Fig. 1, lane 1) and 12 kD (Fig. 2, lane 1), respectively. In both of the recombinant protein preparations, some minor protein bands could be detected. However, the histidine-specific conjugate reacted on immunoblots of the recombinant proteins with only the major bands (Figs. 1 and 2, lane 2).

Specificity of the raised chicken Ab against the immunogen was demonstrated by immunoblotting of the recombinant proteins separated by SDS–PAGE (Figs. 1 and 2). There was no binding of preimmune chicken Ab, but a strong binding reaction of the postimmune chicken Ab with the corresponding major protein (Figs. 1 and 2, lanes 3 and 4). Furthermore, binding was completely abolished by preincubation with the immunogen using protein concentrations up to 10 µg/ml but not by preincubation of the chicken Ab with similar concentrations of dog IgG or *E. coli* lysate (Fig. 1, lanes 5, 7, 8 and Fig. 2, lanes 6–8). Interestingly, binding of IgEf2-specific Ab was not influenced by preincubation with IgEf3/4 (Fig. 1, lane 6); however, partial inhibition was observed by addition of IgEf2 to IgEf3/4-specific Ab (Fig. 2, lane 5). Accordingly, there was a minor reaction of IgEf3/4-specific Ab on the immunoblot of IgEf2 (Fig. 1, lane 9).

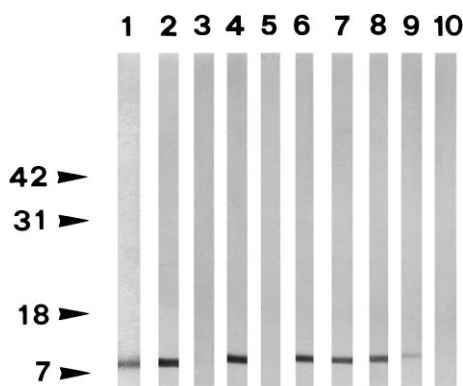


Fig. 1. Denaturing 15% SDS–PAGE and immunoblot of recombinant canine IgE fragment of the second domain (IgEf2). Lane 1: gel stained with Coomassie blue. Lanes 2–10 immunoblot was incubated with: lane 2, AP-labeled anti-histidine conjugate; lane 3, preimmune chicken antibodies (Ab); lane 4, IgEf2-specific Ab; lane 5, IgEf2-specific Ab preincubated with 10 µg/ml IgEf2; lane 6, IgEf2-specific Ab preincubated with 10 µg/ml IgEf3/4; lane 7, IgEf2-specific Ab preincubated with 10 µg/ml dog IgG; lane 8, IgEf2-specific Ab preincubated with 10 µg/ml *E. coli* lysate; lane 9, IgEf3/4-specific Ab; lane 10, conjugate control. Lanes 3–10 were developed with AP-labeled goat anti-chicken IgG antibody. Molecular weights are indicated to the left in kilodaltons.

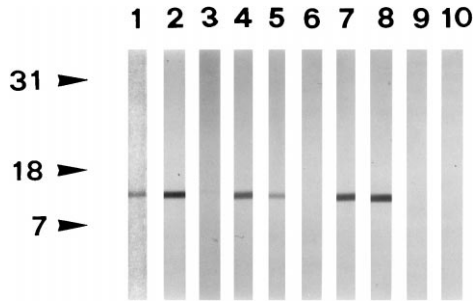


Fig. 2. Denaturing 15% SDS-PAGE and immunoblot of recombinant canine IgE fragment of the third/fourth domains (IgEf3/4). Lane 1: gel stained with Coomassie blue. Lanes 2–10 immunoblot was incubated with: lane 2, AP-labeled anti-histidine conjugate; lane 3, preimmune chicken Ab; lane 4, IgEf3/4-specific Ab; lane 5, IgEf3/4-specific Ab preincubated with 10 μ g/ml IgEf2; lane 6, IgEf3/4-specific Ab preincubated with 10 μ g/ml IgEf3/4; lane 7, IgEf3/4-specific Ab preincubated with 10 μ g/ml dog IgG; lane 8, IgEf3/4-specific Ab preincubated with 10 μ g/ml *E. coli* lysate; lane 9, IgEf2-specific Ab; lane 10, conjugate control. Lanes 3–10 were developed with AP-labeled goat anti-chicken IgG antibody. Molecular weights are indicated to the left in kilodaltons.

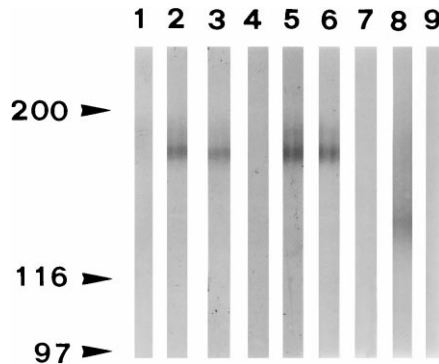


Fig. 3. Immunoblotting of native dog serum separated on a 5% PAGE gel with IgEf3/4-specific Ab and rabbit anti-dog IgG Ab. The blot was incubated with: lane 1, preimmune chicken Ab; lane 2, IgEf3/4-specific Ab; lane 3, IgEf3/4-specific Ab preincubated with 10 μ g/ml IgEf2; lane 4, IgEf3/4-specific Ab preincubated with 10 μ g/ml IgEf3/4; lane 5, IgEf3/4-specific Ab preincubated with 10 μ g/ml dog IgG; lane 6, IgEf3/4-specific Ab preincubated with 10 μ g/ml *E. coli* lysate; lane 7, conjugate control; lane 8, rabbit anti-dog IgG Ab; lane 9, conjugate control. Lanes 1–7 were developed with AP-labeled goat anti-chicken IgG Ab and lanes 8 and 9 with AP-labeled goat anti-rabbit IgG Ab, respectively. Molecular weights are indicated to the left in kilodaltons.

In immunoblots of native canine serum, both the raised Ab strongly bound to a protein of approximately 180 kD (illustrated for IgEf3/4-specific Ab in Fig. 3, lane 2). Immunoblotting with preimmune chicken Ab resulted in no staining (Fig. 3, lane 1). Binding of the IgEf3/4 Ab-specific was not inhibited by either preincubation with up to 10 μ g/ml of IgEf2, dog IgG, or *E. coli* lysate, but was totally inhibited by preincubation with IgEf3/4 (Fig. 3, lanes 3–6). Thus, in contrast to immunoblotting of denatured IgEf3/4 with IgEf3/4-specific Ab, preincubation of IgEf3/4-specific Ab with IgEf2 did not diminish binding to the 180 kD protein. The 180 kD protein band developed by

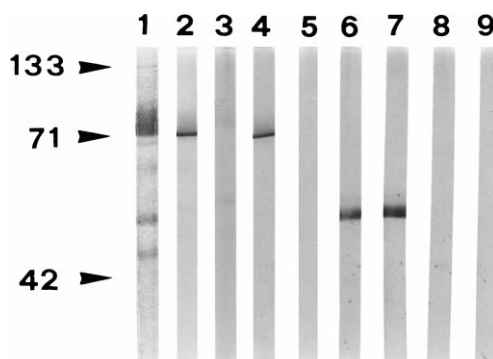


Fig. 4. Binding of IgEf3/4-specific Ab and rabbit anti-dog IgG Ab to an immunoblot of IgEf3/4-specific Ab affinity-enriched denatured dog serum separated on an SDS-10% PAGE gel. Lane 1: gel stained with Coomassie blue. Lanes 2–9 immunoblotting with: lane 2, IgEf3/4-specific Ab; lane 3, IgEf3/4-specific Ab preincubated with 10 μ g/ml IgEf3/4; lane 4, IgEf3/4-specific Ab preincubated with 10 μ g/ml dog IgG; lane 5, conjugate control for chicken-specific Ab; lane 6, rabbit anti-dog IgG Ab; lane 7, rabbit anti-dog IgG Ab preincubated with 10 μ g/ml of IgEf3/4; lane 8, rabbit anti-dog IgG Ab preincubated with 10 μ g/ml of dog IgG; lane 9, conjugate control for anti-rabbit Ab. Lanes 2–5 and lanes 6–9 were visualized with AP-labeled goat anti-chicken IgG Ab and AP-labeled goat anti-rabbit IgG Ab, respectively. Molecular weights are indicated to the left in kilodaltons.

staining with the raised chicken Ab was clearly distinct from the band at approximately 155 kD detected by staining for dog IgG (Fig. 3, lane 8). On immunoblots of denatured, IgEf3/4-specific Ab affinity-enriched canine serum, the IgEf3/4-specific Ab displayed specific binding to a protein of approximately 80 kD (Fig. 4, lanes 2–4), despite IgG contamination of the eluate of the IgE-enriched preparation (Fig. 4, lane 6).

3.2. ELISA reactivity of raised Ab with IgEf2, IgEf3/4, and antigen-specific dog sera

Preimmune chicken Ab did not react with the solid-phase bound IgE fragments (Table 2). However, 12 weeks after the first immunization, egg yolk Ab from the hen immunized with IgEf2 and the hen immunized with IgEf3/4 reacted to solid-phase bound recombinant IgE fragments used for immunization (Table 2). In addition, IgEf3/4-specific Ab did also recognize IgEf2 but to a lesser extent. Next, the produced Ab were compared in a native system with peanut- and *Parascaris*-specific dog sera. *Parascaris*-positive serum from one dog was found by screening 44 dogs on *Parascaris*-antigen bound to the solid-phase and developed with IgEf3/4-specific Ab. Both raised chicken Ab displayed OD₄₀₅ values well above background (Table 2). The background OD₄₀₅ values varied, however, which most likely represented naturally occurring Ab against peanut or *Parascaris* antigen. Accordingly, both preimmune chicken Ab showed a slightly increased OD₄₀₅ value. Affinity-purification of the chicken Ab resulted in a more distinct reaction with less protein required for a positive reaction and generally lower background values (Table 2). Both chicken Ab did not recognize IgG or preimmune chicken Ab bound to the solid-phase (OD₄₀₅ values below background).

Table 2
Comparison of ELISA reactivity of pre- to postimmune chicken antibodies (Ab) on solid-phase bound IgEf2 and IgEf3/4 and on peanut- and *parascaris*-positive dog sera. Binding of chicken Ab was developed with AP-labeled goat anti-chicken IgG Ab and the enzyme substrate *p*-nitrophenyl-phosphate. The *parascaris*-positive serum was diluted 1:10 and the peanut-positive serum 1:20, respectively. Results were expressed as mean OD₄₀₅ value of duplicate wells

Chicken Ab	µg Ab/well	Antigens bound to solid-phase					
		IgEf2 10 ng/well	IgEf3/4 10 ng/well	Peanut 100 ng/well		<i>Parascaris</i> equorum 500 ng/well	
				With serum	No serum	With serum	No serum
Preimmune IgEf2-specific Ab	50	0.072 ^a	0.070	0.343 ^b	0.209	0.562	0.535
Postimmune IgEf2-specific Ab	50	1.501	0.075	2.093	0.266	3.151	0.951
Affinity-purified postimmune IgEf2-specific Ab	0.5	0.818	0.074	1.347	0.500	1.929	0.422
Preimmune IgEf3/4-specific Ab	50	0.069	0.073	0.132	0.150	0.556	0.697
Postimmune IgEf3/4-specific Ab	50	2.311	2.706	0.693	0.212	2.575	1.002
Affinity-purified postimmune IgEf3/4-specific Ab	0.5	0.824	1.681	0.386	0.115	1.463	0.165

^a Background OD₄₀₅ values for solid-phase bound recombinant proteins were ≤0.1., reactions read after 15 min.

^b Antigen-specific reactions read after 60 min.

Table 3

ELISA results of *parascaris*-specific serum IgE and IgG including inhibition studies of the primary developing Ab and effect of heat treatment of the dog serum on the titers. The serum was diluted 1:20 for IgE and 1:500 for IgG detection, respectively

		IgEf2-specific Ab	IgEf3/4-specific Ab	Rabbit anti-dog IgG Ab
No inhibition		100 ^a (OD ₄₀₅ 1.34)	100 (OD ₄₀₅ 1.48)	100 (OD ₄₀₅ 1.35)
Inhibition with	µg/ml			
IgEf3/4	0.001	ND	87	ND
	0.01	ND	66	ND
	0.1	ND	0	ND
	1	ND	0	102
	10	103	0	98
IgEf2	0.1	40	ND	ND
	1	26	ND	ND
	10	12	104	95
Recombinant equine IgE	10	95	101	95
heavy chain fragment				
Dog IgG	0.1	ND	ND	31
	1	103	85	1
	10	97	85	0
Heating (56°C)	15 min	54	160	ND
	30 min	50	135	ND
	45 min	40	136	ND
	1 h	37	119	257
	2 h	28	82	266
	3 h	24	68	263
	4 h	23	51	268

ND=not done

^a Results were expressed as mean of duplicate OD₄₀₅ values after subtraction of background values and expressed as % absorbance of the values obtained with no inhibition.

To further prove that the raised Ab are specific for the recombinant fragment used for immunization and that they indeed recognize native IgE, inhibition ELISA and heat treatment of dog sera were performed (Table 3). Preincubation of the IgEf2- and IgEf3/4-specific Ab with different concentrations of the corresponding immunogen, IgEf2 or IgEf3/4 (0.001–10 µg/ml), resulted in a dose-dependent inhibition of binding. In contrast, preincubation of Ab with a range of 0.1–10 µg/ml of either the recombinant IgE fragment not used for immunization, dog IgG, or the non-relevant [His]₆-tagged protein fragment of horse IgE did not influence the ELISA results. IgG detection was in a dose-dependent manner completely abolished by preincubation of the rabbit anti-dog IgG Ab with dog IgG. Heating canine sera at time points from 15 min to 4 h 56°C before application onto microtiter plates resulted in a decrease of binding of both of the raised chicken Ab. However, during the first hour of heating there was an initial consistent increase in detected IgE levels when using IgEf3/4-specific Ab, which was not apparent in ELISA with IgEf2-specific; the epitope recognized by IgEf2-specific Ab appeared to be more heat labile. As expected, heating did not influence IgG titers.

Table 4

Summary of reverse cutaneous anaphylaxis tests in two dogs after intradermal injections of affinity-purified IgEf2- and IgEf3/4-specific Ab

Substance injected	μg per injection	Wheal diameter (mm)		A Erythem (yes or no)	
		dog 1	dog 2	dog 1	dog 2
Pooled preimmune chicken Ab	50	0 ^a	0	no	no
Affinity-purified IgEf2-specific Ab	50	4	12	$\pm$	yes
	10	6	11	$\pm$	yes
Affinity-purified IgEf3/4-specific Ab	50	8	11	yes	yes
	10	4	10	no	yes
Diluent	NA	0	0	no	no
Histamine	8.5	13	17	yes	yes

NA = not applicable.

^a Reactions were expressed as mean of duplicates values read 15 min after injection.

3.3. Reverse cutaneous anaphylaxis

Both of the affinity-purified chicken Ab induced wheal-and-flare reactions when injected into the dermis of two normal dogs, whereas the pooled preimmune Ab preparation and the diluent alone elicited no response (Table 4). The reactions were most pronounced 15 min post application and diminished within 30 min. Dog # 2 exhibited a more prominent reaction to the immune chicken Ab than dog # 1.

3.4. Measurement of total serum IgE by ELISA

Semi-quantitative assessment of serum total IgE revealed that there was a serum dilution-dependent decrease of OD₄₀₅ values (Fig. 5). Heat treatment of the sera at time points from 1 to 4 h at 56°C caused reduced binding of the two chicken Ab of up to 85% compared with the OD₄₀₅ values (100%) obtained before heating treatment. As outlined for antigen-specific IgE, similar results were obtained for the ELISA for total serum IgE with inhibition of the developing IgEf3/4-specific Ab. Furthermore, preimmune chicken Ab bound to the solid-phase revealed OD₄₀₅ values that did not exceed background values obtained with IgEf2-specific without addition of dog serum.

3.5. Immunohistochemistry

Distinct binding of the IgEf3/4-specific Ab was evident on paraffin-embedded lymphoid tissue and spleen and liver as illustrated for the mesenteric lymph node (Fig. 6(a)). The chicken Ab specifically recognized cells in the medulla and paracortical area of the mesenteric lymph node. The number of positive cells appears to vary among the mesenteric lymph nodes from the three dogs but also among hilar, popliteal, and mesenteric lymph nodes. No staining was observed with the horse IgE-specific Ab used as negative control (Fig. 6(c)).

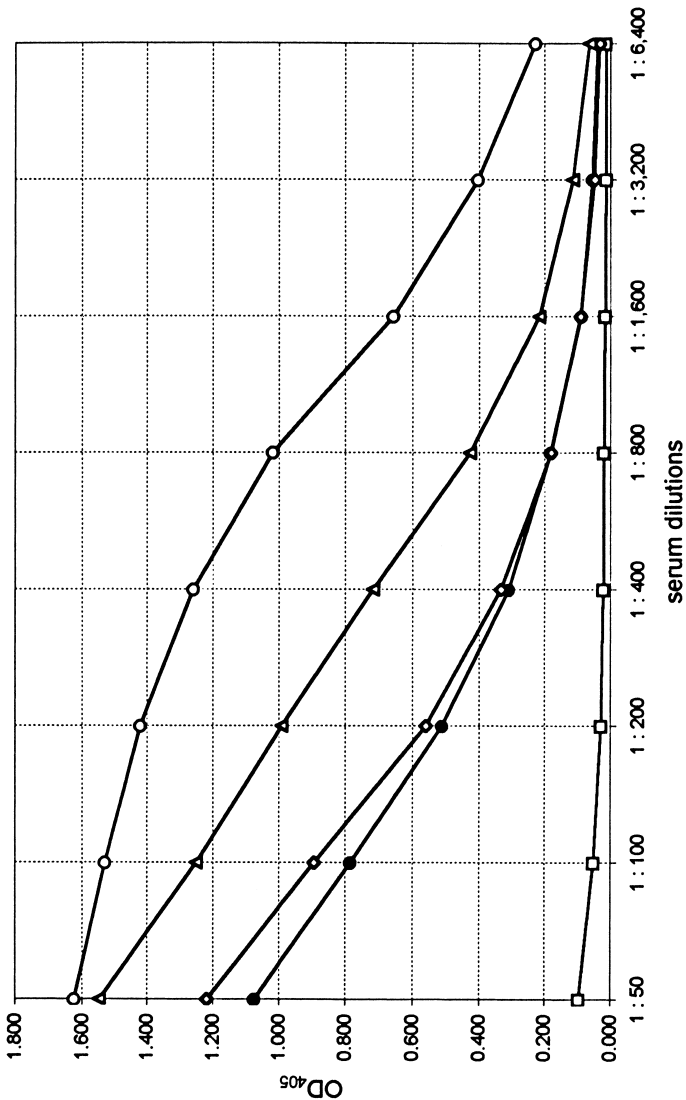


Fig. 5. ELISA results of semiquantitative assessment of total serum IgE concentrations of two-fold serial dilution curves of reference dog serum pool (●) and four representative serum samples (open symbols). IgE2-specific Ab was bound to the solid-phase and biotinylated IgE3/4-specific Ab was used as developing Ab. Results are expressed as the mean OD₄₀₅ of duplicate values.

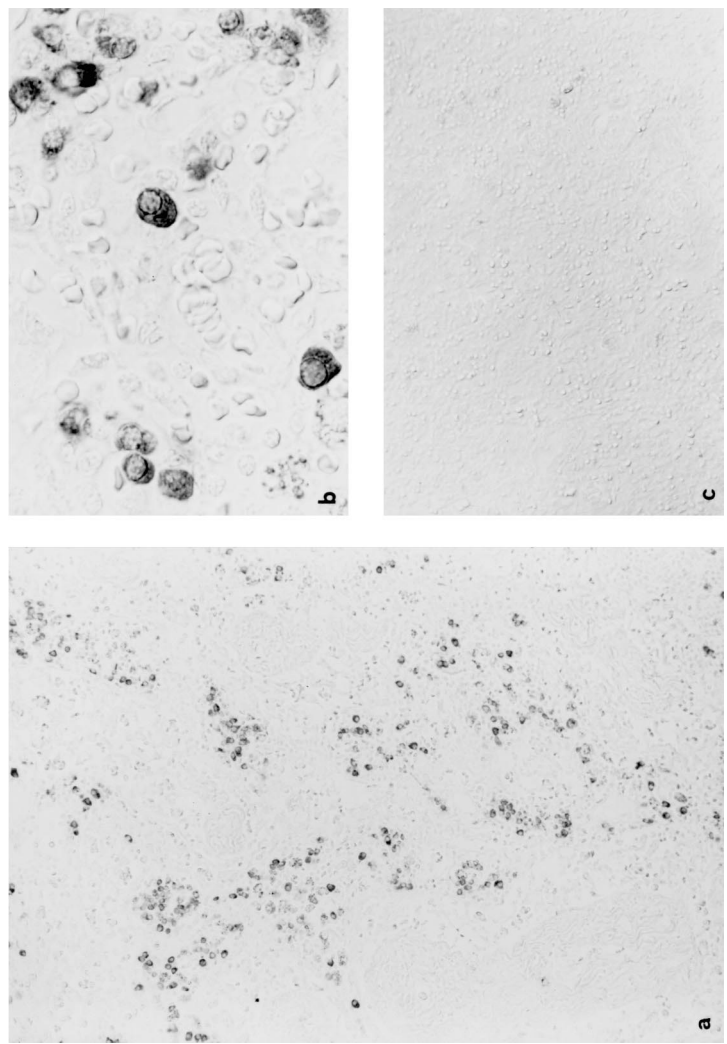


Fig. 6. Immunostaining for dog IgE with chicken IgEf3/4-specific Ab and developed with biotinylated goat anti-chicken IgG Ab in a tissue section of paraffin-embedded mesenteric lymph node (Fig. 6(a); low power magnification). The lymphocytes show intense membrane associated reaction as shown by high power magnification (Fig. 6(b)). Conjugate control is shown in Fig. 6(c), low power magnification.

4. Discussion

The production and characterization of two polyclonal Ab specific for dog IgE are reported here which will facilitate the elucidation of hypersensitivity reactions in dogs. The Ab were raised in hens and are directed against two different recombinant fragments of the canine epsilon chain. The [His]₆-tagged recombinant proteins of the canine IgE heavy chain, namely IgEf2 and IgEf3/4, induced high titers of specific Ab (Table 2) as described for the horse (Marti et al., 1997). The raised Ab reacted solely with the major protein band of the corresponding recombinant protein (Figs. 1 and 2). Thus, the minor contamination of the recombinant protein extracts with *E. coli* did not influence the immune response. Specificity of the postimmune Ab was further illustrated by the absence of any binding reaction of the preimmune chicken Ab, and abolishment of binding only by preincubation of the postimmune chicken Ab with the immunogen and not by preincubation with *E. coli* extract or dog IgG. The IgEf3/4-specific Ab reacted on immunoblots of IgEf3/4 as well as IgEf2, which suggested shared epitopes of IgEf2 and IgEf3/4 (Figs. 1 and 2). Cross-reactivity of IgEf3/4-specific Ab to IgEf3/4 and IgEf2 was also evident in the ELISA with IgEf2 coated to the solid-phase (Table 2).

The next question was whether the raised Ab would indeed recognize native dog IgE. To answer this question, an ELISA with antigen-positive dog sera was used to screen the chicken Ab preparations. As seen in Table 2, Ab from both of the hens immunized with either IgEf2 or IgEf3/4 displayed distinct results in an ELISA comparing pre- and postimmune Ab. Antigen-affinity chromatography enhanced the specific ELISA reactions of the induced Ab with less protein required for positive results and with generally lower background values (Table 2). The specificity of the raised chicken Ab for the respective immunogen was further underlined by the inhibition studies carried out in antigen-specific ELISA with dog serum and immunoblotting of native serum: only the immunogen was able to abolish binding of the postimmune chicken Ab. Interestingly, in contrast to immunoblotting of IgEf3/4, binding of IgEf3/4-specific Ab did not appear to be diminished by preincubation with IgEf2 (Table 3) suggesting that the shared epitopes are only accessible on the denatured recombinant protein fragments in immunoblots or when IgEf2 is coated to the solid-phase in an ELISA (Table 2).

Heat treatment of serum substantially decreased ELISA reactions with both postimmune chicken Ab further supporting the specificity for IgE. Not all epitopes on IgE are heat labile and there appear to be species-specific differences. The herein described two chicken Ab only recognize partially heat-labile epitopes as do all other currently described dog IgE-specific Ab (Halliwell et al., 1975; deBoer et al., 1993; Peng et al., 1993a). In contrast to dogs, heating horse serum for only 10 min at 54°C completely prevents any detection of IgE by the horse IgE-specific Ab (Marti et al., 1997), whereas inactivation is described after a 10 min to 4 h incubation at 56°C for human IgE (Ishizaka et al., 1967). It was interesting that the IgEf2-specific Ab recognized a more heat-labile epitope than the IgEf3/4-specific Ab (Table 3). Heating of canine serum caused consistently increased OD₄₀₅ values when developed with IgEf3/4-specific Ab, which could have been due to the recognition of an epitope more accessible only through disruption of immune complexes of IgG anti-IgE auto-Ab as described for human IgE (Jensen-Jarolim et al., 1992).

Immunoblotting of native dog serum with both of the raised chicken Ab revealed a strong reaction with an approximately 180 kD protein, which corresponds to the expected size of IgE (Fig. 3). Binding of the IgEf3/4-specific Ab to a 80 kD protein, the expected size of the epsilon chain, was only apparent in the IgEf3/4-specific Ab affinity-enriched immunoglobulin fraction from denatured dog serum (Fig. 4). The dog IgG contamination of the IgE affinity-enriched serum fraction could be due to either concurrent enrichment of IgG-IgE auto-Ab complexes or to a low titer of dog IgG against chicken. Immunoblots run under denaturing-reducing conditions of non-affinity enriched serum revealed several bands most likely due to the presence of IgE heavy chain fragments (data not shown).

Finally, the induction of wheal-and-flare reactions by intradermal injections of both of the chicken IgEf2- and IgEf3/4-specific Ab further documented the *in vivo* recognition of native dog IgE. Thus, the hypothesis that the herein described IgEf2- and IgEf3/4-specific Ab indeed recognize native dog IgE is strongly supported by the inhibition experiments with the raised Ab, the reduced binding of the Ab after heat treatment of serum in ELISA, and the recognition of 180 and 80 kD proteins on immunoblots, as well as the induction of RCA.

The significance of *in vitro* allergy tests including ELISA and radioallergosorbent tests for determination of allergy-specific IgE in dogs is under dispute (Bond et al., 1994; Codner and Griffin, 1996). A high false-positive rate of test results was found when comparing results of atopic with non-atopic dogs, effect of hyposensitization with allergens selected based on *in vitro* results or skin tests, or when comparing results between two different ELISA test kits with similar antigens. In view of these studies, it was surprising that we found only one positive dog with *Parascaris*-specific IgE out of 44 dogs screened; the dog had an OD₄₀₅ value of more than ten times above background. Similarly, screening for various other antigens, including house dust mites, detected very few positive dogs (data not shown). This finding of a low incidence of positive dogs is interesting and might be explained by the usage of different antigen preparations and concentrations, and developing Ab. It underlines the difficulties in interpretation and comparison of different *in vitro* tests for antigen-specific IgE. However, as more IgE-specific reagents become widely available and used in *in vitro* test systems including the recently described IgE-specific monoclonal Ab, the specificity of *in vitro* test results of antigen-specific IgE is expected to increase (de Weck et al., 1998).

The availability of two dog IgE-specific Ab directed against different epitope regions is a great tool to quantify total serum IgE. It will be interesting to compare the different published ranges of total serum IgE (Vriesendorp et al., 1975; Schwartzman, 1984; Halliwell and Longino, 1985; Wilkie et al., 1990; Hill and deBoer, 1994; Hill et al., 1995) after evaluation of a larger dog population by our ELISA in comparison with a reference dog pool and inhibition ELISA.

The distinct immunolabeling of cells by the raised IgEf3/4-specific Ab in paraffin-embedded tissue is a major advantage. As of yet, immunohistochemistry has only been described for frozen sections which makes clinical investigations more labor intensive and difficult, as frozen material is not commonly available from clinical cases. There were surprisingly many IgE-positive cells localized in mesenteric lymph nodes. These cells had the morphologic appearance of plasma cells rather than other IgE-bearing cells, such as mast cells.

In conclusion, both of the polyclonal chicken Ab elicited by immunizations with two recombinant fragments of the epsilon chain specifically recognize native dog IgE. These chicken Ab directed against different and known constant domains of the dog IgE heavy chain could initiate clinical research of diseases with type 1 hypersensitivity reactions, including, for example, the involvement of allergic reactions in chronic idiopathic gastroenteropathy. The major advantage of our Ab include that they can be used for immunohistochemistry of paraffin-embedded tissue, as well as the availability of recombinant IgE fragments for further characterization of dog IgE and for inhibition studies.

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