



Generation of therapeutic antibody responses against IgE in dogs, an animal species with exceptionally high plasma IgE levels

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Abstract

Allergic diseases are common in dogs and the involvement of IgE in the pathogenesis of canine atopic dermatitis (CAD) and flea allergy dermatitis (FAD) is documented. However, many dogs do not achieve sufficient control of their allergic disease, and there is a great need for new treatment strategies. In order to address this issue we first needed to obtain a better picture of IgE-levels in dogs and how plasma IgE-levels are affected by breed, age and health status. IgE is normally present at diminutive concentrations in sera and detection by diagnostic methods has been a technical challenge. Here, we present a new in vitro assay for determining absolute levels of total IgE in sera from dogs. In a panel of 76 adult dogs diagnosed either as healthy or suffering from atopic dermatitis, autoimmunity or infection by skin parasites, we show that levels of IgE range from 1 to 41 µg/ml. This is almost 100 times the level observed in non-atopic humans. However, these exceptionally high IgE-levels in the dogs could not be correlated to either breed or health status. To address the issue of novel treatment strategies, the possibility of reducing the IgE-levels in nine Beagle dogs by immunization with a new therapeutic vaccine was investigated. High levels of anti-IgE antibodies were induced in all dogs, and the IgE-levels were subsequently decreased by a mean of 65%. This shows that the allergy vaccine is potent enough to break the tolerance against IgE, even when the initial IgE-levels are as high as those observed in dogs. Thus, the vaccination treatment may have the potential to serve as a future therapy for dogs with atopic diseases.

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1. Introduction

Allergic diseases are frequently occurring in dogs. The prevalence of canine atopic dermatitis (CAD) has been estimated to 3–15% in various dog populations [1,2], and in a study where more than 30,000 dogs were examined almost 9% were diagnosed with allergy, atopic/allergic dermatitis or atopy [3]. CAD thereby constitutes a serious medical problem in veterinary medicine and CAD is reported to be one of the most common causes of canine pruritus [2,4–6]. Many of these dogs experience side effects related to treatment, or do

not achieve sufficient control of the disease. Thus, there is a great need for new treatment strategies.

In order to address this issue we first needed to obtain a more detailed picture of the plasma levels of IgE in dogs and how these levels are affected by age and health status. The absolute IgE-levels reported in the literature for dogs show substantial variation and range from 140 ng up to milligram levels of IgE per milliliter [7,8]. This discrepancy in the reported IgE-levels clearly shows that there is a need for new and more reliable quantitative tests for determining serum IgE-levels in dogs.

IgE is normally present in diminutive concentrations in sera and detection of IgE by in vitro methods has therefore been a technical challenge. The amount of IgG in human sera

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is approximately 65,000 times higher than that of IgE. Hence, IgG constitutes an obvious contamination source when IgE is purified from sera and used to raise isotype-specific polyclonal antibodies for assay development. This has resulted in significant confusion in the field since few reliable measurements of absolute IgE-levels are available for dogs. The highly variable IgE-levels most likely depend on cross reactivity with other serum proteins, like IgG and serum albumin, resulting in erroneous measurements of the actual plasma concentration of IgE. Thus, the lack of reliable assays as well as pure IgE standards has been obstacles and consequently, the results from different studies are difficult to compare. The lack of reliable diagnostics has also hampered the use of dogs as a natural allergic animal model and the development of new treatment strategies for dogs.

The first objective of this study was therefore to develop a reliable total-IgE-ELISA for dogs and to determine the absolute IgE-levels in a cohort of adult dogs representing different breeds and autoimmune or dermatitis status. In addition, a number of juvenile dogs were included in this analysis to determine whether serum IgE concentrations change with age.

The second objective was to study the possibility to reduce plasma IgE-levels with a new therapeutic vaccine developed for dogs. Previously, we have shown that a therapeutic vaccine against IgE in rats induce high antibody titers against self IgE and that the response is so strong that it actually leads to a more than 90% reduction of IgE-levels in certain low and medium IgE-responder rat strains [9,10]. However, rodents are not naturally allergic and we therefore wanted to expand our studies and test the vaccine concept in dogs, a natural allergic species, which also may benefit from this potential new treatment strategy.

2. Materials and methods

2.1. DNA-constructs

The entire C₂, C₃, and C₄ region of the dog IgE ϵ -heavy chain was produced as a recombinant protein denoted DDD. The C₂C₃C₄ region was obtained from full-length dog IgE ϵ -chain cDNA by PCR, and for purification purposes the 5' primer contained an 18-nucleotide region encoding a histidine hexapeptide (Fig. 1a). The initial primers for the isolation of the C₁–C₄ region of dog IgE were designed based on the published sequence of dog IgE [11]. The dog-specific allergy vaccine, ODO, consist of the C₃ domain of the dog ϵ -heavy chain flanked by the C₂ and C₄ domains of the opossum IgE ϵ -heavy chain [12]. An 18-nucleotide region encoding a histidine hexapeptide was added to the 5' primer of the opossum C₂ domain (Fig. 1a). Both the DDD- and the ODO-cDNA fragments obtained by PCR amplification were introduced into the pCEP-Pu2 vector by ligation, and the respective vector-inserts were then transfected into EBNA-positive human embryonic kidney (HEK-

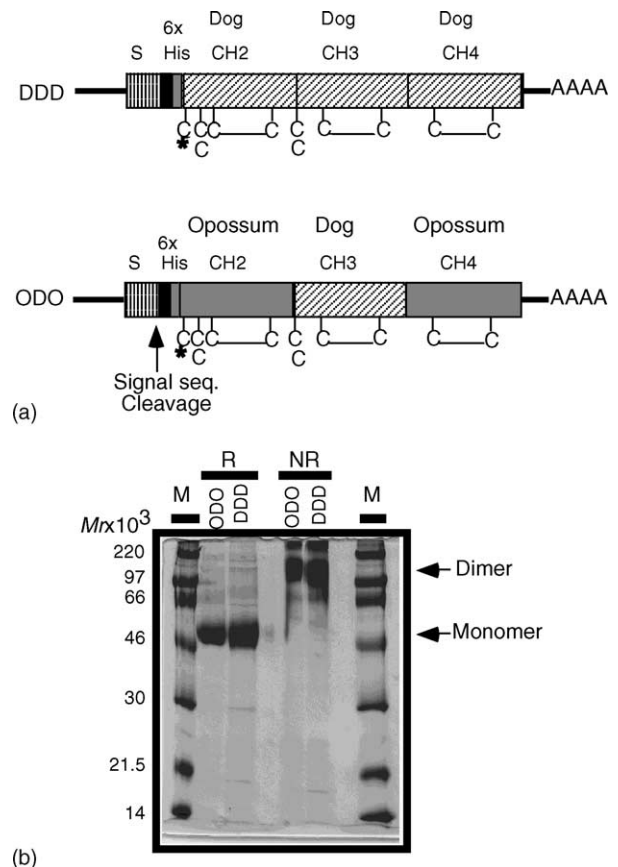


Fig. 1. (a) A schematic map of the DDD and the ODO DNA constructs. S indicates a signal sequence, and (*) an unpaired cysteine residue that was changed to serine by point mutagenesis. (b) SDS-PAGE analysis of ODO and DDD under reducing (R) and non-reducing (NR) conditions. Aliquots of approximately 5 μ g ODO and DDD, were loaded in each lane. Each band in the size marker (M) corresponds to $\sim$ 1.5 μ g protein.

293-EBNA) cells for expression and secretion into culture medium [10]. The recombinant proteins were purified from the culture medium on Ni-NTA Agarose (QIAGEN GmbH, Germany) as previously described. One and 2 mg/L of the recombinant DDD and ODO were obtained from the respective culture media. The purified proteins were analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). Under reducing conditions both ODO and DDD migrate as proteins with the apparent molecular weights of $\sim$ 55 kDa (Fig. 1b). However, under non-reducing conditions, the migration of the respective proteins corresponds to molecular weights of $\sim$ 115 kDa, suggesting that the recombinant proteins (DDD and ODO) are produced as disulfide-linked homodimers (Fig. 1b).

2.2. Total IgE-ELISA-reagents

To obtain reagents for the development of a sensitive and reliable quantitative ELISA assay to be used for determination of IgE-levels in dogs, two rabbits were immunized at up to eight occasions with 250 μ g of the recombinant protein DDD per injection. The collected polyclonal rabbit anti-dog

IgE serum was purified on protein A-Sepharose® (Pharmacia, Uppsala, Sweden) to obtain the IgG fraction. This purified rabbit IgG anti-dog IgE antisera served as the coating antibody in the ELISA. As a secondary antibody, a monoclonal antibody against dog IgE was generated as follows; mice were immunized with the recombinant DDD-protein, spleen cells from the immunized mice were fused with Sp2/0 cells according to standard procedures, and the supernatants from the resulting hybridoma cultures were tested for their reactivity against DDD. Positive clones were selected and expanded. Following several limiting dilution steps one hybridoma that appeared to have a high affinity for DDD was chosen. Antibodies from this hybridoma did not bind to the ODO protein why we conclude that the selected hybridoma produces an antibody that is directed against either dog Cε₂ or Cε₄. The supernatant from this cell line was passed over a protein G-Sepharose® column (Pharmacia Biotech, Uppsala, Sweden) to obtain a purified IgG fraction. The monoclonal mouse IgG anti-dog-IgE antibody was then biotinylated according to the instructions of the manufacturer of a biotinylation kit (Sigma–Aldrich, MI, USA).

2.3. Total IgE-ELISA-assay

Plates (Nunc-Immuno Plate, MaxiSorp Surface, NUNC Brand Products, A/S, Roskilde, Denmark) were coated with 10 µg/ml of the polyclonal rabbit IgG anti-DDD in carbonate buffer, pH 9.5, by incubation overnight at 4 °C. The plates were then washed and blocked overnight at 4 °C with blocking buffer (PBS/Tween-20 with 1% BSA). After washing, the diluted sera samples were added in duplicates. To obtain a standard curve, duplicates of the purified DDD protein, at concentrations of 240, 120, 60, 30, 15, 7.5, 3.75 and 1.9 ng/ml, were included on every plate. Blocking buffer was used as assay blank and diluent for the samples and the standard curve. Following a 60 min incubation of samples and standard, the plates were washed and the biotinylated monoclonal antibody against DDD, developed in the lab, was added at a concentration of 0.5 µg/ml. The plates were thereafter incubated at 37 °C for 60 min. After another washing step, the plates were incubated with alkaline-phosphatase-labeled streptavidin (DAKO A/S, Denmark) for an additional 60 min at 37 °C. After a final wash the substrate, *P*-nitrophenyl phosphate disodium tablets (Sigma 104® phosphatase substrate tablets, Sigma–Aldrich) in diethanolamine buffer, pH 9.8, was added to the plates and the absorbance at 405 nm was measured utilizing a multiscan photometer (Labsystems OY Helsinki, Finland, Multiskan MCC/340).

2.4. Total IgE-EEISA-calibration

The DDD protein, used as standard, contains six Ig-domains, whereas native dog IgE contains 14 such domains. Hence, the DDD-standard represents 7/3 times more moles Cs₃ domain per mg of protein compared to native dog IgE. Therefore, to calculate the concentrations of native IgE, the

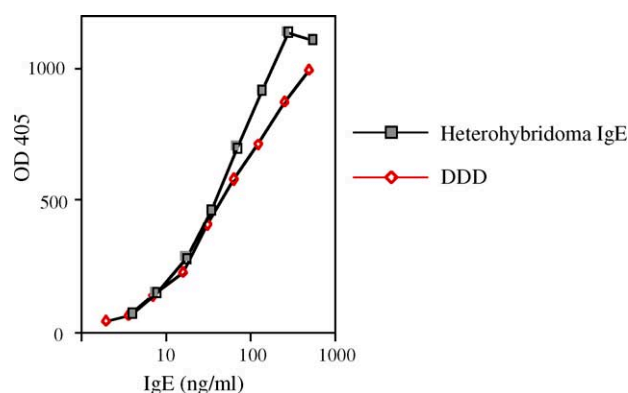


Fig. 2. A comparative analysis of two different IgE standards, the recombinant dog IgE C₂C₃C₄ (DDD) and the dog IgE heterohybridoma, in the new total IgE ELISA. The values were corrected for the difference in molecular weight as described in Section 2. The closed symbols (■) indicate the level of IgE from a heterohybridoma producing dog IgE, whereas the open symbols (◇) indicate DDD as standard.

obtained IgE-concentrations in the samples were multiplied by a factor of 7/3. In addition, the converted standard curve was calibrated against the commercially available heterohybridoma producing dog IgE (Bethyl Laboratories Inc., Montgomery, TX, USA) (see Fig. 2).

2.5. Anti IgE-ELISA

Plates, previously described, were coated overnight at 4 °C with 5 µg/ml of DDD in carbonate buffer, pH 9.6. After washing, the plates were blocked overnight at 4 °C with Blocking Buffer (PBS/Tween-20 with 1% BSA). Following a washing step, serial dilutions (1:5, 1:25, 1:125, 1:625, 1:3125) of the collected sera samples were prepared in blocking buffer and incubated in the plates overnight at 4 °C. The blocking buffer was also used as assay blank. After washing, the plates were incubated for 60 min at 37 °C with alkaline phosphatase-conjugated rabbit anti-dog IgG Fc-specific (Jackson ImmunoResearch Lab. Inc., PA, USA) diluted 4000 times in the blocking buffer. Following a final wash, the substrate *P*-nitrophenyl phosphate disodium tablets (Sigma 104® phosphatase substrate tablets, Sigma–Aldrich) in diethanolamine buffer, pH 9.8, was added to the plates and the absorbance was measured as previously described. The result for each individual is presented as the dilution where 50% of the maximal OD₄₀₅ was reached.

2.6. Animals and sera handling

Sera from a cohort of 76 adult dogs of different breed were collected with their owners consent, when the dogs medical status were established at the Department of Small Animal Clinical Sciences, Swedish University of Agricultural Sciences (SLU), Uppsala. Samples from 34 privately owned Rhodesian Ridgeback dogs, one adult dam and 33 juvenile dogs, 7–8 weeks old, were available from the Department of Animal Breeding and Genetics, SLU, Uppsala. The assem-

blages of serum samples were performed in accordance with the animal ethics regulations of the Swedish National Board for Laboratory Animals and Swedish Board of Agriculture. Inclusion criteria for being classified as atopic dermatitis was as follows; the dogs should have skin problems according to Willemeses revised criteria for atopic dermatitis and this after that ectoparasites and skin infections (pyoderma and Malassezia) had been excluded [13]. This very strict criteria also includes positive intradermal test or the HESKA antigen specific IgE test. All dogs classified as having atopic dermatitis fulfilled these criteria. Healthy dogs in the study were clinically healthy privately owned dogs.

In the vaccination study, nine male Beagle dogs from Harlan CPG, The Netherlands, were used. They were all six months old at the beginning of the study and they were housed under controlled conditions at Scantox, Lille Skensved, Denmark. Blood samples were collected prior to the immunizations, at week 0, and at weeks 3, 5 and 11, post immunization. Venous blood was collected from the jugular vein and centrifuged at $1270 \times g$ for 10 min. Thereafter, the sera were transferred to new tubes and stored at -18°C until evaluation by ELISA.

2.7. Vaccination protocol

Nine Beagle dogs were immunized with the dog-specific anti-IgE vaccine, ODO. The response against the vaccine was estimated with the anti-IgE ELISA, in which the recombinant dog-IgE protein, DDD, was used as the coating antigen (as described above in section anti-IgE ELISA). Thus, nine Beagle dogs were randomly divided into three groups and immunized intramuscularly with 100 μg ODO at weeks 0 and 3. All groups received the vaccine mixed at a ratio of 50:50 with the respective mineral oil-based adjuvant; group 1 received Montanide 206, group 2 Montanide 51 and group 3 Montanide 50. All adjuvants are commercially available from Seppic, Paris, France. Montanide ISA 206 is a mineral oil adjuvant developed for veterinary use [14], which gives a water-in-oil-in-water emulsion, and has previously been used in vaccinations for pigs, cattle and guinea-pigs. Montanide ISA 51 is a mineral oil adjuvant, which is used to make water-in-oil emulsions intended primarily for human vaccines [15], whereas Montanide ISA 50 another mineral oil adjuvant used to make water-in-oil emulsions that was developed for veterinary use. In total, all injections amounted to 1 ml/dog. Local inflammatory reactions were seen in several dogs, an effect commonly seen with these relatively potent adjuvants.

3. Results

3.1. Establishment of a quantitative ELISA for measuring serum concentrations of dog IgE

To obtain pure ELISA reagents for the development of a quantitative IgE assay for dogs we needed pure dog IgE, and

this protein had to be free from all other dog serum proteins. This was obtained by producing the C₂, C₃, and C₄ region of the dog IgE ϵ -heavy chain (the DDD-protein) as a recombinant protein in a mammalian cell line (the human cell line 293 EBNA). Following secretion into the culture medium, the DDD protein was purified on Ni-NTA columns. SDS-PAGE analysis suggested that DDD was properly folded and that the surface epitopes were present in their native form. This assumption was based on the fact that the protein was secreted from the cell and that the majority of the protein was produced as disulfide-linked homodimers (Fig. 1b). However, a minor fraction of the protein appears to be aggregated or forming larger multimers (Fig. 1b). Rabbits were immunized with DDD and sera from these rabbits were passed over a protein G column to obtain the IgG fraction. These polyclonal IgG antibodies directed against dog IgE were used as coating antibody. As a secondary antibody, a monoclonal antibody against dog C $\epsilon_{2/4}$ was generated by immunizing mice with DDD. The monoclonal antibody was shown not to bind the C ϵ_3 region and thereby to recognize either dog C ϵ_2 or C ϵ_4 (see Section 2).

The DDD protein had never been in contact with any dog serum proteins and also lacks the V regions and the light chains that are common to other Ig isotypes. This recombinant form of dog IgE is thereby ideal as antigen for assay developments. However, two important questions remained: does this protein have the same structure as native dog IgE in the CH₂–CH₃–CH₄ region and can it be used as standard to obtain accurate values of plasma IgE? High quality monoclonal IgE of the desired species for comparison is rarely available. However, in this project we were very fortunate, Bethyl Laboratories had a commercially available monoclonal IgE from a dog heterohybridoma that could be used as standard. A comparative ELISA was performed with both standards and they were found to give almost fully co-linear curves from a few nanograms up to approximately 100 ng. At higher concentrations they only show a minor difference in efficacy (Fig. 2). This variation is well within a normal variation of ELISA experiments. The result from this comparative analysis shows that both standards can be used to obtain accurate estimates of the absolute concentrations of plasma IgE in dogs. The assay was also tested against protein A and protein G purified dog IgG and shown not to cross-react with dog IgG.

In order to confirm the reliability of our ELISA, known amounts of the dog heterohybridoma IgE or DDD was added to dog serum samples and assayed for the increase in OD values. The expected increases in OD values upon addition of DDD or the heterohybridoma IgE were obtained in all samples tested, which further confirmed the reliability of the assay (data not shown). The same samples were also tested repeatedly, and the assay was found to be very reliable with a maximum variation of 20% (data not shown). Although this assay gives highly reliable quantitative data of free IgE this assay cannot discriminate between free IgE and IgE in immune complexes, and measures therefore all IgE present in the plasma of the animal.

3.2. IgE-levels in adult dogs

Using our newly developed ELISA, sera obtained from 76 adult dogs, representing different breeds and immunological status, were analyzed with regards to their total IgE-levels. As shown in Table 1, the median levels of IgE in the different dog breeds ranged between 4 and 35 µg/ml. No difference was observed in the IgE-levels irrespective whether the dogs had been diagnosed as healthy, suffering from autoimmune disease, parasite infection or diagnosed with atopic dermatitis. For example, among the 23 German Shepherds, 18 dogs that were diagnosed as healthy had IgE-levels ranging from 1 to 22 µg/ml. Four dogs with antinuclear antibodies (ANA-positive) had IgE concentrations at 1, 15, 22 and 41 µg/ml, and one German Shepherd with *Sarcoptes* infestation and a positive *Sarcoptes* antibody test had 12 µg IgE/ml. Hence, levels of IgE were not lower in the ANA-positive dogs than in the healthy dogs or in the parasite infected dog. Furthermore, we did not observe any elevated IgE-levels in the six dogs diagnosed with atopic dermatitis, in which the IgE-levels were found to range between 4 and 20 µg/ml. The 70 dogs in

the cohort not diagnosed with atopy, had IgE-levels ranging from 1 to 41 µg/ml. Thus, the levels of IgE were generally very high in adult dogs, and did not correlate to the clinical manifestations of atopic dermatitis, autoimmune disease or skin infection caused by certain parasites.

3.3. IgE-levels in juvenile dogs

To study whether the IgE-levels in dogs correlate with age, we analyzed IgE in sera from three litters of juvenile Rhodesian Ridgeback dogs and in sera from the dam of one of the litters. The IgE-levels were generally low in the 33 young dogs, except for one individual that had more than 4 µg IgE/ml in sera. Four juvenile dogs showed undetectable levels of IgE (below 5 ng/ml) and the median IgE values in the three litters ranged from 0 to 2 µg/ml (Table 2). The dam of one of the litters, with 14 offspring, had 20 µg IgE/ml in sera (Table 2), i.e. in the same range as observed in the other adult dogs included in this study. None of the Rhodesian Ridgeback dogs showed any symptoms of atopic dermatitis, autoimmune disease or parasite skin infection.

Table 1

Total IgE concentrations in sera from 76 adult dogs representing different breeds and health status

Dog breed	Number of dogs	Health status	IgE (µg/ml)	
			Value range	Median value
Beagle	9	Healthy	19–31	20
German shepherd	18	Healthy	1–22	13
Irish Setter	2	Healthy	8–16	12
American Cocker Spaniel	2	Healthy	2–17	9
English Cocker Spaniel	1	Healthy	15	15
Nova Scotia Duck Tolling Retriever	3	Healthy	11–19	17
Poodle	1	Healthy	35	35
Pug	1	Healthy	20	20
Danish–Swedish Farmdog	1	Healthy	25	25
Tervueren	2	Healthy	17–20	18
Beauceron	1	Healthy	20	20
Rottweiler	1	Healthy	22	22
Border Collie	1	Healthy	22	22
Cairn Terrier	1	SLE	32	32
Nova Scotia Duck Tolling Retriever	9	Systemic autoimmune disease	6–29	13
German shepherd	4	ANA+	1–41	19
Poodle	3	Addison	5–18	17
Dachshund	1	Sarcoptes + skrap (before treatment)		16
Rottweiler	1	Cheyletiella + (before treatment)		17
Tibetan Terrier	1	Sarcoptes +, ab in sera		18
German shepherd	1	Sarcoptes +, ab in sera		12
Riesenschнауzer	1	Sarcoptes +, ab in sera		17
Cairn Terrier	1	Sarcoptes +		21
Boxer	1	Sarcoptes +		12
Schanuzer	1	Sarcoptes +		12
Japanese chin	1	Sarcoptes +		25
Labrador Retriever	1	Lice		10
Boxer	1	Atopic dermatitis		18
West Highland White terrier	1	Atopic dermatitis		5
Great Dane	1	Atopic dermatitis		20
Fox terrier	1	Atopic dermatitis		14
Chinese Crested	1	Atopic dermatitis		15
Miniature Bullterrier	1	Atopic dermatitis		4

Dogs were either diagnosed as healthy or suffering from autoimmunity, parasite infections or atopic dermatitis.

Table 2
Total IgE concentrations in sera from three litters of Rhodesian Ridgeback dogs and the dam of the litter with 14 offspring

Number of dogs	Age	IgE (μg/ml)	
		Value range	Median value
Dog breed: Rhodesian Ridgeback			
14	8	0–4	1
9	7	0–19	0.2
10	8	0.6–3.6	0.8
1	Adult		20

3.4. Production of the new allergy vaccine for dogs

Allergies to common allergens constitutes a serious medical problem in veterinary medicine and many dogs experience side effects related to treatment, or do not achieve sufficient control of the disease. New treatment strategies are therefore needed. In order to address this important issue we have initiated the development of an allergy-vaccine for dogs. We have during the initial studies concentrated our efforts on investigating the possibility to reduce IgE-levels when the levels of IgE are as high as those observed in the dogs in this study. The new vaccine, ODO, is a recombinant protein consisting of the third constant domain from the dog ε-heavy chain, flanked by the second and fourth constant domains of the American opossum IgE heavy chain (Fig. 1a). The flanking domains of the vaccine, opossum Cε₂ and Cε₄, serve both as structural support for the self-component, dog Cε₃, and provide foreign T-cell epitopes that are required to break the T-cell tolerance against IgE. The ODO vaccine was expressed in 293-EBNA human embryonic kidney cells [10]. Based on SDS–PAGE, the polypeptide has an estimated purity of approximately 95% and is expressed as a dimer (Fig. 1b).

3.5. Antibody response against IgE after vaccination against IgE

Following immunization with 100 μg of ODO at week 0, high titers of IgG anti-IgE antibodies were observed in all dogs, and the response increased further following the booster injection, with 100 μg ODO at week 3 (Fig. 3A). In total, the dogs received two vaccinations, i.e. at weeks 0 and 3. The highest titer of antibodies against IgE was observed at week 5, two weeks after the booster injection. Thereafter, the anti-IgE antibody titers declined in all dogs between weeks 5 and 11 showing that the anti IgE response is reversible also in dogs [10].

3.6. IgE-levels after vaccination against IgE

Even though the total IgE-levels in sera were surprisingly high with initial values between 7 and 21 μg/ml, a decrease in IgE was observed in six out of nine treated dogs after the first vaccination (Fig. 3B). Following the booster injection at week 3 the decrease was verified and after vaccination the newly

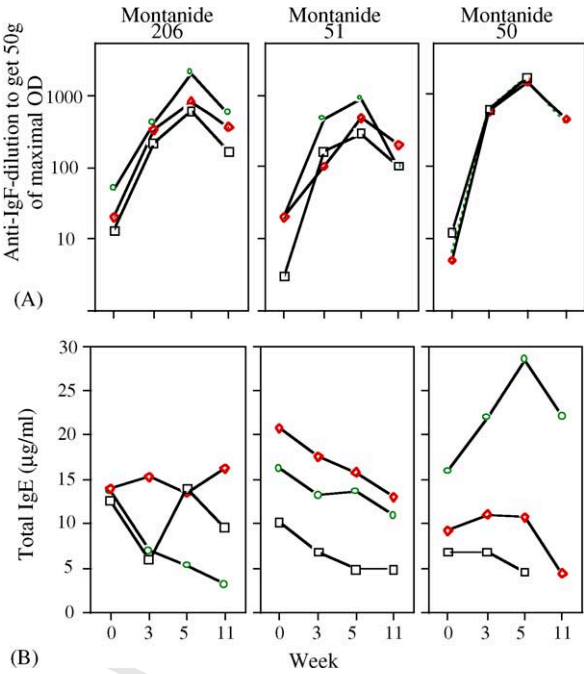


Fig. 3. Induction of a therapeutic antibody response against IgE and its effect on total IgE-levels. Nine Beagle dogs were injected with 100 μg of ODO at day 0 in one of three different adjuvants, Montanide 206, Montanide 51 and Montanide 50. Three weeks later the animals were given a booster dose consisting of 100 μg of ODO protein in the same adjuvant as used during the first injection. Serum was sampled at day 0, and weeks 3, 5 and 11. (A) Anti IgE-levels presented as the dilution at which 50% of the maximum absorbance OD₄₀₅ was reached. (B) Total IgE concentrations. The same symbol is used for the same animal in both the (A) anti-IgE and the (B) total IgE assays.

formed antibodies against IgE reduced the total IgE-levels in seven, out of the nine, dogs. Among the eight dogs that were bled all four times, the median reduction in the IgE-levels was 65% between weeks 0 and 11 of the treatment program.

3.7. The response against the vaccine between the adjuvant groups

Neither the response against IgE nor the total level of IgE varied among the groups that received the three different mineral oil-based adjuvants: Montanide ISA 206, 51 and 50. Based on the data presented here, we cannot distinguish differences in potency among the three different adjuvants.

4. Discussion

A major obstacle in studying the significance of IgE in various dog diseases has been the lack of pure reagents specific for dog IgE. Consequently many investigators have measured and presented IgE as a relative value to reference serum, developed in their own laboratory. This has made it very difficult to compare different studies, and thus to draw any conclusions regarding, if and how, IgE is involved in the pathogenesis of various diseases in dogs. In order to solve

this important issue we here present a study with reliable and reproducible measurements of absolute IgE-levels in dogs.

The recombinant IgE from dogs, DDD, enabled us to develop an assay without contaminating isotypes, and to generate a standard-curve with absolute IgE-levels. With this new assay we show that in a cohort of 76 dogs representing different breeds and health status, the plasma IgE-levels were surprisingly high, ranging from 1 to 41 μg IgE/ml (Table 1). However, we could not detect any significant difference in the IgE-levels among the various dog breeds or among dogs that were diagnosed as healthy, showed signs of autoimmune disease, were infected by parasites or diagnosed with atopic dermatitis (Table 1). Considering that the presence of ANA is characteristic for certain systemic autoimmune diseases, in both humans and dogs [16], it is notable that we do not observe lower levels of IgE in the ANA-positive dogs than in both the healthy and the parasite infected dog with antibodies against sarcoptes. In fact, two of the lowest IgE-levels were found in the West Highland White terrier and the Miniature Bullterrier, both diagnosed with atopic dermatitis (Table 1). Thus, based on these data, total levels of plasma IgE cannot be used as an inclusion criterion for the pathogenesis of autoimmune diseases, parasite infections or atopic dermatitis in dogs.

In non-atopic humans without parasitic infections, IgE-levels are normally approximately 0,15 μg /ml, whereas atopic humans, who have a higher risk of developing allergies, can have up to 1,5 μg /ml of IgE in their sera. Hence, compared to the situation in humans, IgE-levels in dogs presented here, between 1 and 41 μg /ml, are significantly higher. Values in the same range were recently obtained by Jackson and Hammerberg where total plasma IgE-levels between 7 and 34 μg /ml in a Maltese $\times$ Beagle colony were observed [17]. A plausible explanation for the extraordinary high IgE-levels observed in dogs, is related to the fact that the natural environment for dogs contains a vast variety of parasites. The parasite load, which is incomparable to the situation in humans, should inevitably increase IgE-production in dogs. Jackson et al. postulated that in contrast to humans, there is no direct correlation between circulating and cell bound IgE in dogs [8]. They furthermore hypothesize that the number of IgE producing B-cells may be higher in dogs than in humans [8]. This is an appealing hypothesis, but whether the bias for IgE production in dogs is due to their environment, or to their genetic background, is unknown and remains to be elucidated.

Moreover, we show that serum IgE-levels are low in 33 juvenile dogs from three different Rhodesian Ridgeback-litters. Median IgE-levels ranged from 0, 2 to 1 μg /ml, and that four of the dogs had undetectable levels of IgE, whereas the dam of one of the litters had 20 μg IgE/ml (Table 2). This implicates that IgE-levels in dogs increase with age from low concentrations at birth, and our data thereby support the results of Racine et al. [18]. They showed that sera levels of IgE varied with age and gender in a colony of 147 healthy Beagles; the older the dogs were, the higher were

their IgE-values, up to the age of four years. It is tempting to hypothesize that in dogs, the IgE response against parasites accumulates during the first 4 years. However, in contradiction to Racine's and our findings, a recent study by Fraser et al. did not find any correlation between the age of non-atopic guide dogs and their total IgE concentrations in sera [19]. This apparent discrepancy indicate that other factors, than only age, may be involved in the timing of the increase in IgE-levels over age in these populations.

Based on results from this and several other studies, the levels of total IgE does apparently not correlate to atopic conditions in dogs. However, it is well documented that dogs frequently have antigen-specific IgE and that the presence of this antigen-specific IgE correlate with allergic symptoms [20–22]. It is also well documented that allergies to common allergens constitutes a serious medical problem in veterinary medicine and that many dogs do not achieve sufficient control of the disease [23]. Therefore, new treatment strategies are needed. We have for several years been working on a new treatment strategy based on the vaccination against IgE and wanted to study the effects of a dog-specific vaccine in dogs, a naturally allergic species. In rats we have previously been able to show that a corresponding IgE-vaccine often reduce circulating IgE-levels by more than 90% and also result in significant reduction in allergen-induced skin reactivity [9,10]. Our hypothesis was that the extremely high total IgE-levels in dogs might conceal a pool of allergen-specific IgE that can be reduced by vaccination. The vaccine was designed as a therapeutic vaccine to induce an autoimmune response against IgE and thereby to reduce the total levels of IgE in sera. The assumption behind the concept is that a reduction of total IgE, and thereby also of allergen-specific IgE, will serve to reduce allergic symptoms. The aim of the vaccine is to break the T cell tolerance against IgE and since the vaccine is a pure recombinant protein, it was administered together with an adjuvant. In this study, we used three different adjuvants, all based on mineral oil. Blood samples were collected at four occasions and sera were analyzed focusing on the response against IgE as well as the total level of IgE. The data, Fig. 3A, show a marked increase in the levels of anti-IgE in all adjuvant groups after the first vaccination at week 0. The levels of anti-IgE had increased even further after the second immunization, at week 3, and the highest anti-IgE-levels were reached at week 5, i.e. two weeks after the final immunization. These data are indeed promising, especially considering the initial high total IgE-levels in the dog sera, Fig. 3B. Given that the nine Beagle dogs had initial IgE-levels between 7 and 21 μg /ml, it is truly remarkable that the ODO-vaccine can break the tolerance and induce such a strong response against IgE. Interestingly, Xolair, the humanized monoclonal antibody against IgE from Novartis, is not prescribed to human patients with serum IgE-levels exceeding 1.5 μg IgE/ml (700 IU/ml) due to dosing considerations [24]. However, our results in dogs suggest that vaccination could serve to decrease IgE-levels even in these patients.

A reduction in IgE was observed in almost all dogs after the first vaccination, and the median reduction of IgE between weeks 0 and 11 of the treatment program was as much as 65%. Surprisingly, we could not identify any significant differences among the three mineral oils that were used as adjuvants: Montanide ISA 206, 51, and 50. In a previous study conducted in rats, Montanide ISA 51 induced the highest antibody titers against self-IgE, as compared to other adjuvants [25]. This encouraged us to further explore different mineral oils administered together with the vaccine, but this time in dogs. It is conceivable that all three mineral oils are equally efficient given that they all provide an antigenic depot effect. The lack of difference could also be attributed to the small numbers of individuals monitored in the experiment. Both Montanide ISA 206 and Montanide ISA50 were developed for veterinarian use. Montanide ISA 206 is a water-in-oil-in-water emulsion, whereas Montanide ISA 50 is a water-in-oil emulsion. However, we cannot discern any difference between these adjuvants in efficacy in these dogs.

Recently Wang et al. presented an analysis of a peptide-based vaccine directed against dog IgE [26]. In this article, the authors show that a 23 amino acids long peptide from the CH3 domain of dog IgE can elicit an anti-IgE response in dogs when coupled to a foreign carrier peptide. However, the authors do only present anti-IgE data from 4 out of 16 vaccinated animals and they only present the effect on IgE levels in 6 out of 16 animals. Therefore, based only on the published data, no conclusions can be made concerning the efficacy of this variant of an anti-IgE vaccine. It would, however, be of great interest to have complete data for both of these vaccines to compare their efficacy and safety profiles. Peptide based vaccines are usually much less potent due to their smaller size and thereby smaller number of epitopes. The structure of the peptide also often differs in structure from the corresponding peptide region in the native protein, which may dramatically affect the efficacy of the vaccine. However, peptide based vaccines are often more easy to produce and may thereby be an interesting alternative to fusion protein based vaccines, if efficacy data are convincing.

In summary, using our new dog-specific total IgE assay we show that, in comparison with serum IgE-levels in humans, the levels of IgE in dogs are remarkably high, ranging from 1 to 41 $\mu\text{g/ml}$. Further, we show that IgE-levels are significantly lower in juvenile dogs, in which the median levels ranged between 0, 2, and 1 $\mu\text{g/ml}$ in the three litters. In addition, nine Beagle dogs received a new allergy-vaccine for dogs, ODO, which was administered together with one of three different mineral-oil adjuvants. Despite the initially high IgE-levels, a response against IgE was induced in all dogs, which resulted in a decrease in their IgE-levels. The results indicate that the allergy-vaccine ODO is potent and can induce a response against the self-molecule IgE, even when the initial levels of IgE are as high as those observed in dogs. Thus, the vaccination treatment may have the potential to serve as a future therapy for dogs with atopic diseases. However, due to the

high IgE-levels and its strong tolerizing effect on the anti-IgE response in dogs, efficacy increasing modifications of the vaccine antigen and optimizing the adjuvant formulation may be needed to obtain sufficient clinical effects.

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