



Generation of canine-human Fc IgE chimeric antibodies for the determination of the canine IgE domain of interaction with FcεRIα

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Abstract

Identification of the domain(s) of canine IgE that interact with FcεRIα may lead to novel therapeutic intervention strategies that inhibit the ability of canine IgE to engage FcεRIα. A panel of canine-human Fc IgE chimeric antibodies was constructed to investigate this interaction by replacing canine IgE-Fc domains with the corresponding human IgE-Fc domains since human IgE-Fc does not recognize canine FcεRIα. β-Hexosaminidase release assays were performed to assess the ability of the chimeric antibodies to bind to and sensitize a novel RBL cell line transfected with canine FcεRIα for antigen induced mediator release. Replacing canine Cε2 with human Cε2 resulted in similar levels of release as those elicited by canine Fc IgE from RBL-2H3 cells transfected with either canine FcεRIα or human FcεRIα. Substitution of canine Cε4 with human Cε4 resulted in approximately 10% lower levels of release compared to cells sensitized with canine Fc IgE. Receptor binding by flow cytometry and cell activation could not be detected when transfected RBL cells were incubated with chimeric constructs where canine Cε2 and Cε4 were substituted with human Cε2 and Cε4. However, when this construct was incubated with cognate antigen prior to cell challenge mediator release was observed, albeit at a 20% lower level, indicating that while canine Cε3 is the only domain essential for binding to canine or human FcεRIα, species specific residues in canine Cε2 and Cε4 inhibit dissociation of the ligand from the receptor.

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1. Introduction

Approximately 1 in 3 households in the United States own a dog and nearly US \$20 billion a year is spent on health-

care for these animals (AVMA, 2002; Sutter and Ostrander, 2004). According to the American Kennel Club Canine Health Foundation, more than half the dogs in the U.S. are purebreds with 17 breeds that are particularly susceptible to allergic disease. Understanding the relationship between canine IgE and its high-affinity receptor, FcεRI, on mast cells and basophils may contribute to our understanding of the molecular mechanisms of allergy in dogs as well as humans.

Investigations into the relationship between human IgE and FcεRIα has revealed that IgE binds to FcεRIα in a 1:1 ratio (Keown et al., 1997) with an unusually high affinity, $K_a \approx 10^{10} \text{ M}^{-1}$, as compared to 10^8 M^{-1} for IgG1 on FcγRI (Ravetch and Kinet, 1991). The binding sites of human IgE for FcεRIα are found within Cε3 (Nissim et al., 1991), although Cε2 is important for the high affinity of IgE for FcεRIα (Garman et al., 1998, 2000, 2001; McDonnell et al., 2001; Wurzburg and Jardtzy, 2002). IgE adopts a bent conformation which results in asymmetric positioning of the Cε2 domains which causes

Abbreviations: C2, canine mastocytoma cell line; CDS, cell dissociation solution; cFcεRIα, canine IgE high-affinity receptor alpha subunit; cIgE-Fc, canine Fc IgE antibody; DMEM, dulbecco's modified eagles medium; DNP-HAS, dinitrophenol coupled to human serum albumin; DTT, dithiothreitol; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; hFcεRIα, human IgE high-affinity receptor alpha subunit; hIgE-Fc, human Fc IgE antibody; IgE, immunoglobulin E; J558L, mouse myeloma cell line; mIgE, mouse IgE; NIP-HAS, 4-hydroxy-3-iodo-5-nitrophenylacetyl coupled to human serum albumin; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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one of the C ϵ 3 domains to adopt an open configuration due to residues in the AB loop in C ϵ 3 (Wurzburger and Jardetzky, 2002).

A comparison of the amino acid sequences between human, canine, rat, and mouse IgE heavy chains shows that the sequence identity between human and canine IgE (56%) is higher than rat (46%) or mouse (44%). Furthermore, a comparison of the amino acid sequences of human, canine, rat, and mouse Fc ϵ RI α showed that the sequence identity between human and canine (53%) was higher than rat (46%) or mouse (47%). Due to the higher homology between human and canine IgE and Fc ϵ RI α the study of a functional relationship between canine IgE and Fc ϵ RI α is likely to be more relevant to the human system than the current rodent model. To this end we aimed to establish an *in vitro* canine model system relevant to the study of allergies in dogs as well as humans.

The objective of this study was to investigate the structure/function relationship between canine IgE and its high-affinity receptor. This was achieved through the generation of several canine-human chimeric IgE antibodies. Canine IgE-Fc domains were replaced with the corresponding human IgE-Fc domains as previous work demonstrated that human IgE does not bind to canine Fc ϵ RI α (Stedman et al., 2001; Vratimos, 2002). This ensured that the homologous human IgE domains would maintain the overall conformation of the antibody. The first chimeric antibody designated canine C ϵ 3/C ϵ 4, consists of human IgE-Fc domains C ϵ 1–C ϵ 2 and canine IgE-Fc domains C ϵ 3–C ϵ 4. The second chimeric antibody named canine C ϵ 2/C ϵ 3, comprises human IgE-Fc domains C ϵ 1 and C ϵ 4, and canine IgE-Fc domains C ϵ 2–C ϵ 3. The final chimeric antibody designated canine C ϵ 3, consists of human IgE-Fc domains C ϵ 1, C ϵ 2, and C ϵ 4 and canine IgE-Fc domain C ϵ 3.

The canine-human chimeric antibodies were used to determine which domain of canine IgE interacts with the canine IgE high-affinity receptor, Fc ϵ RI α , assessing receptor binding using flow cytometry and measuring β -hexosaminidase release from rat basophilic leukemia cells, RBL-2H3, transfected with canine Fc ϵ RI α in response to challenge with IgE and cognate antigen. Although, receptor binding by flow cytometry could not be detected with chimeric constructs where canine C ϵ 2 and C ϵ 4 were replaced with human C ϵ 2 and C ϵ 4, cell challenge with chimeric IgE antigen complexes did induce mediator release, although at a 20% lower level compared to cIgE-Fc, indicating that canine C ϵ 3 is the only domain essential for binding to canine or human Fc ϵ RI α , while species specific residues in canine C ϵ 2 and C ϵ 4 contribute to the high affinity of interaction.

2. Experimental procedures

2.1. Generation of canine Fc IgE and canine-human IgE Fc constructs

Template canine DNA was obtained from mononuclear cells separated from canine blood samples by Ficoll gradient. Briefly, 20 ml canine blood was diluted in an equal volume of PBS. 5 ml samples were loaded onto 4 ml Ficoll density gradient centrifugation and centrifuged at 400 $\times$ g for 30 min. The mononuclear cell phase was formed between the plasma and gradient medium

phase. The PCR reaction was performed as described previously (Helm et al., 1996; Sayers et al., 1998) with an annealing temperature of 55 °C. The PCR product was blunt-cloned into the pPCR-TOPO vector and subsequently digested with BglII and subcloned into the BamHI sites in pSV-V_{NP}.

The chimeric canine-human Fc IgE constructs were PCR amplified (manufacturer's instructions, Abgene), from templates, canine Fc IgE in pPCR-TOPO and human Fc IgE in pSV-V_{NP}, using primers described in Table 1. Each canine or human IgE-Fc fragment was subcloned into pUC18, sequenced to verify the construct and ligated with the appropriate domains to form the three chimeric canine-human Fc IgE constructs subcloned into the mammalian expression vector, pSV-V_{NP}. The chimeric antibodies are designated for the canine IgE-Fc domains contained in the construct. The canine C ϵ 3/C ϵ 4 chimeric antibody consists of the human IgE-Fc domains C ϵ 1–C ϵ 2 and canine IgE-Fc domains C ϵ 3–C ϵ 4. The canine C ϵ 2/C ϵ 3 chimeric antibody consists of the human IgE-Fc domains C ϵ 1 and C ϵ 4 and canine IgE-Fc domains C ϵ 2–C ϵ 3. The canine C ϵ 3 chimeric antibody consists of the human IgE-Fc domains C ϵ 1, C ϵ 2, and C ϵ 4 and canine IgE-Fc domain C ϵ 3. Each chimeric antibody is specific for NIP, and contains the mouse λ light chain of IgE expressed by the J558L cell line.

Human IgE-Fc domains C ϵ 1–C ϵ 2 were PCR amplified using primers HV1 and HV2 and subcloned into pUC18. Canine IgE-Fc domains C ϵ 3–C ϵ 4 were PCR amplified using primers FK1 and FK3 and subcloned into pUC18. Human C ϵ 1–C ϵ 2 was ligated with canine C ϵ 3–C ϵ 4 and the human-canine C ϵ 1–C ϵ 4 construct was digested with BclI and ligated into the BamHI sites in pSV-V_{NP}. Human IgE-Fc domain C ϵ 1 was PCR amplified using primers LA1FB and LA1R and subcloned into pUC18. Canine C ϵ 2–C ϵ 3 domains were PCR amplified using primers LA2F and LA2R and subcloned into pUC18. Human C ϵ 4 was PCR amplified using primers LA3F and LA3R and subcloned into pUC18. Human C ϵ 1 was ligated with canine C ϵ 2–C ϵ 3 and human C ϵ 4 and digested with BclI and ligated into the BamHI sites in pSV-V_{NP}. Canine IgE-Fc domain, C ϵ 3, was PCR amplified with primers MH6 and MH7 and subcloned into pUC18. Human C ϵ 4 was PCR amplified using primers MH8 and MH9 and subcloned into pUC18. The human C ϵ 1–C ϵ 2 construct was ligated with canine C ϵ 3 and human C ϵ 4 and digested with BclI and subcloned into the BamHI sites in pSV-V_{NP}. XL1-blue competent cells (Stratagene) were used for the propagation of plasmid DNA. Each IgE Fc fragment cloned into pUC18 and each construct cloned into pSV-V_{NP} was sequenced to verify sequence authenticity.

2.2. Expression and purification of canine Fc IgE and canine-human IgE Fc constructs

Canine Fc IgE and canine-human Fc IgE chimeric constructs were transfected into J558L cells as described previously (Sayers et al., 1998). Expression of IgE variants was assessed by SPR and the highest expressing clones were sent to the Antibody Resource Centre, University of Sheffield, U.K., for large-scale production. NIP-specific antibodies were affinity purified from

Table 1

Designation of gene constructs and primers employed for the construction of canine-human IgE Fc chimeric antibodies

Canine Fc IgE		
AV10	Canine C ϵ 1–C ϵ 4	AATAATAGATCTCAGAGCAGATACCCAGGTCAACAGC
AV11	Canine C ϵ 1–C ϵ 4	GGCGGCAGATCTTCATTACCGGGGGTTTGGACACC
Canine C ϵ 3/C ϵ 4		
HV1	Human C ϵ 1–C ϵ 2	CAGACTGAATTCTGATCACTGCCACGGGGTCC
HV2	Human C ϵ 1–C ϵ 2	CGGACCTCTAGAGCAGGTGGGAACGTAC
FK1	Canine C ϵ 3–C ϵ 4	CTGTCCCTAGAAACCCAGATGCGCGAGGC
FK3	Canine C ϵ 3–C ϵ 4	TAGCTAAAGCTTGATCAATTACCGGGGGTTTGGAC
Canine C ϵ 2/C ϵ 3		
LA1FB	Human C ϵ 1	GTCAGCCATATGTGATCACTGCCACGGGGTCC
LA1R	Human C ϵ 1	CCCAGCGCATGCTGGGTGTTCCAC
LA2F	Canine C ϵ 2–C ϵ 3	TGCCGCGCATGCTGGTAACGCCACGAC
LA2R	Canine C ϵ 2–C ϵ 3	GCTCCCCCTGCAGGAGGCCCGCCACCTC
LA3F	Human C ϵ 4	GAGCGACCTGCAGGGGGCCCGGGCTGACC
LA3R	Human C ϵ 4	CATAGTCTAGATGATCA GCCCTGCCCGCTCCC
Canine C ϵ 3		
HV1	Human C ϵ 1–C ϵ 2	CAGACTGAATTCTGATCACTGCCACGGGGTCC
HV2	Human C ϵ 1–C ϵ 2	CGGACCTCTAGAGCAGGTGGGAACGTAC
MH6	Canine C ϵ 3	TAGCTGTCTAGAGATGAGGGCCAAGGCAC
MH7	Canine C ϵ 3	TGGATCGCATGCGGCTCCGGCTCAGGAGGC
MH8	Human C ϵ 4	TAGCTAGCATGCGGAGCGAGTGAGCGGGGC
MH9	Human C ϵ 4	ATACAGAAGCTTTGATCAAGCTGGATGGAGCCC
cFc ϵ RI α		
AV1	cFc ϵ RI α	AATAATCTCGAGGCCGCCATGCTGCTTCCATGGGA
AV2	cFc ϵ RI α	GGCGGCAGATTCAGTTCTTCCGGGTCTGG

J558L cell supernatant using a NIP-sepharose column. Purified antibodies were analyzed by 12.5% SDS-PAGE.

2.3. Generation of cFc ϵ RI α construct and transfection into RBL-2H3 cells

The gene encoding cFc ϵ RI α was generated by isolating the total RNA from C2 cells and RT-PCR reactions produced cDNA products. Primers AV1 and AV2 were used to PCR amplify the cDNA and subsequent products were digested with XhoI and EcoRI and subcloned into the expression vector, pIRES-EGFP. RBL-2H3 cells were cultured in DMEM, harvested and resuspended at 6×10^6 cells/0.8 ml in DMEM and incubated in electroporation cuvettes on ice for 10 min. 30 μ g plasmid DNA was added to the cuvettes and electroporated at 250 V with capacitance of 960 μ F. 24 h post-transfection selection media was added (800 μ g/ml Geneticin, G418-sulphate, Invitrogen) for 5 days, after which a concentration of 400 μ g/ml Geneticin was maintained.

2.4. Assessing expression of cFc ϵ RI α in the RBL-2H3 cell line through flow cytometry

RBL-2H3 cells transfected with cFc ϵ RI α were cultured, harvested with CDS (Sigma–Aldrich) and resuspended at 5×10^6 cells/sample. Samples were incubated on ice for 30 min with 1 μ g canine Fc IgE, 1:400 dilution of biotinylated goat anti-mouse lambda light chain (Amersham) for 30 min on ice, followed by incubation with 1:25 dilution of streptavidin R-phycoerythrin (Vector) on ice for 30 min. The highest expressing

cells were sorted using the CyanTM ADP or MoFlo[®] Flow cytometers using Summit software (Dako).

2.5. Binding analysis of chimeric antibodies by flow cytometry

Binding analysis of chimeric antibodies: canine C ϵ 3/C ϵ 4, canine C ϵ 2/C ϵ 3, and canine C ϵ 3 with RBL-2H3 cells transfected with cFc ϵ RI α was performed using flow cytometry. Canine Fc IgE was used as a positive reference control. Briefly, 1×10^7 cells/ml were washed in PBS and harvested with cell dissociation solution (Sigma–Aldrich) and resuspended in wash buffer (PBS containing 1% FBS). Cells were incubated with 1 μ g of cIgE-Fc, canine C ϵ 3/C ϵ 4, canine C ϵ 2/C ϵ 3, or canine C ϵ 3 for 30 min on ice. This was followed by incubation with 1:400 dilution of goat anti-canine IgE for 30 min on ice, followed by 1:2000 dilution of an anti-goat FITC-labeled antibody (Sigma–Aldrich) for 30 min on ice. Data was collected using CyanTM ADP Flow Cytometer using Summit software (Dako).

2.6. β -Hexosaminidase release assays

Degranulation of preformed mediators was observed through cell activation via endogenous rodent and transfected cFc ϵ RI α and hFc ϵ RI α receptors in the RBL-2H3 cell line using β -hexosaminidase release assays as a readout. A confluent 100 mm plate of cells was harvested and resuspended at a cell density of 0.5×10^6 cells/ml. 1 μ g of control or chimeric IgE was added to the cell suspension before return to the cell incubator.

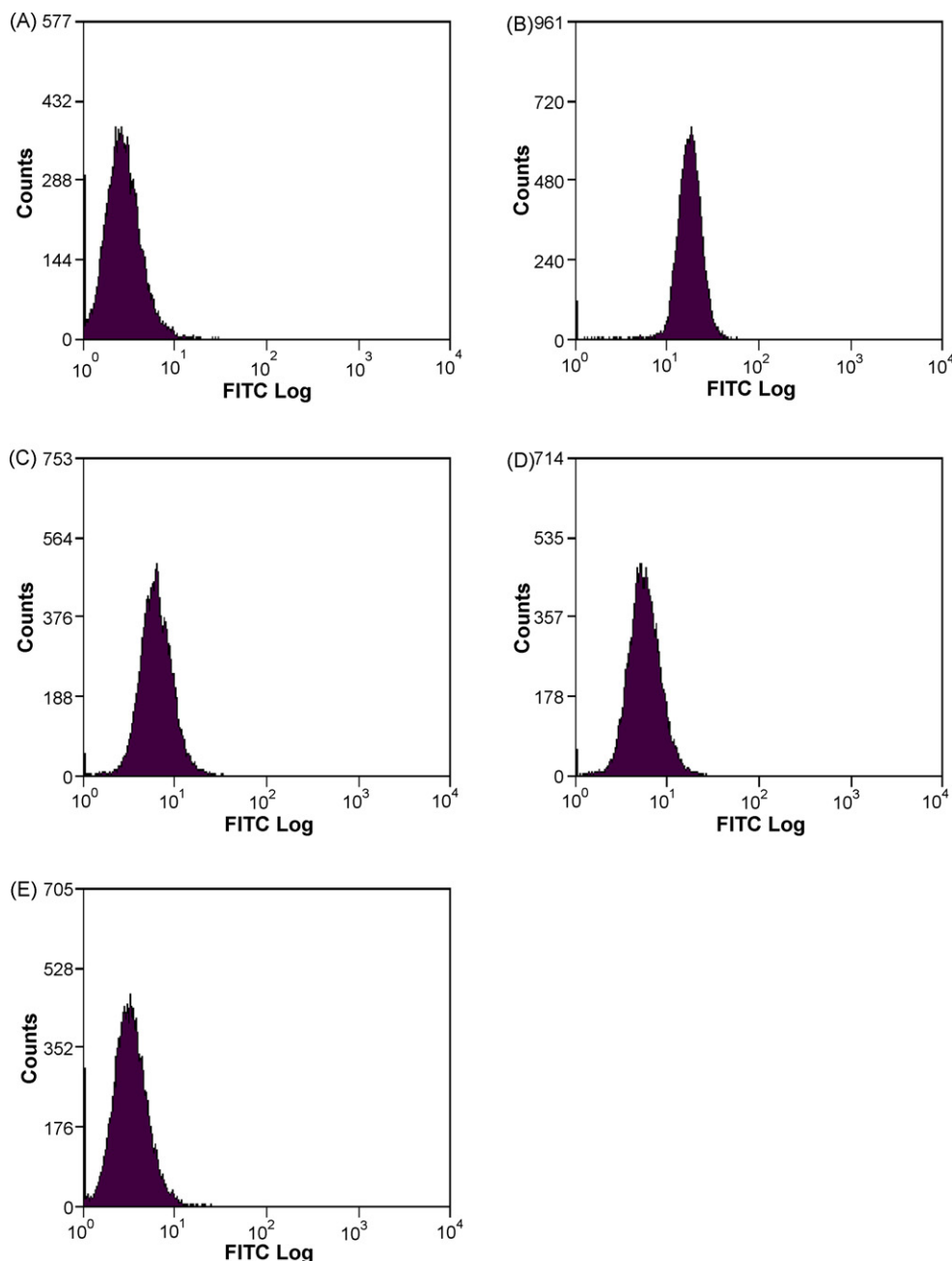


Fig. 1. Binding analysis of cIgE-Fc and canine-human chimeric antibodies with RBL-2H3 cells transfected with cFcεRIα by flow cytometry. Cells were harvested and an aliquot was removed for background fluorescence (A), and labeled with cIgE-Fc (B), canine Cε3/Cε4 (C), canine Cε2/Cε3 (D), and canine Cε3 (E), followed by goat anti-canine IgE and an anti-goat FITC-labeled antibody (each for 30 min on ice).

Following a 16 h incubation period, cells were washed twice with PBS (37 °C) and challenged with increasing concentrations (1–10⁴ ng/ml) of cognate antigen as described previously (Sayers et al., 1998). Alternatively, preformed antigen-IgE complexes were prepared by incubating cIgE-Fc and canine-human chimeric antibodies with increasing concentrations of NIP-HSA for 1 h prior to the challenge of target cells. Following 30 min cell challenge, 50 μl cell supernatant was transferred to new wells followed by the addition of 50 μl β-hexosaminidase substrate (50 mM *p*-nitrophenyl *N*-acetyl β-D-glucosaminide in DMSO, Sigma) for 3 h. The reaction was stopped with 150 μl of 1 M Tris–HCl, pH 9 and read spectrophotometrically at 405 nm. Data

is presented as the mean ± S.D. representative of at least three experiments performed in triplicate.

3. Results

3.1. Binding analysis of canine-human IgE Fc antibodies through flow cytometry

Canine-human IgE chimeric antibodies were expressed in the J558L cell line (Sayers et al., 1998; Carroll et al., 2001). Expression levels were in the range of 1.0–8.0 μg/ml. The aim of the study was to determine which domain(s) of canine IgE is

responsible for the interaction with the canine IgE high-affinity receptor. In the human system, Cε3 is the only domain necessary for binding to the receptor (Nissim et al., 1991). Data in Fig. 1 shows that canine Cε3/Cε4 and canine Cε2/Cε3 binding to cFcεRIα can be demonstrated although no binding could be detected with the canine Cε3 chimeric antibody under equivalent conditions.

3.2. β-Hexosaminidase release assays

Antigen-mediated cross-linking of IgE receptors on mast cells and basophils leads to the release of inflammatory mediators including β-hexosaminidase, thus providing a reliable method for assessing IgE-mediated, antigen induced cell activation. RBL-2H3 cell lines with endogenous rat receptors or transfected with cFcεRIα or hFcεRIα were assessed for their ability to release β-hexosaminidase after sensitization for 16 h with DNP-specific mIgE, NIP-specific cIgE-Fc, NIP-specific hIgE-Fc, or NIP-specific canine-human Fc IgE antibody constructs: canine Cε3/Cε4, canine Cε2/Cε3, and canine Cε3. Following the 16 h incubation step with IgE variants, receptors were activated with either DNP-HSA or NIP-HSA as appropriate.

The parental RBL-2H3 cell line demonstrated a maximal release of ($46.6 \pm 5.5\%$) following mIgE mediated receptor activation. Negative reference controls showed that DNP-HSA, NIP-HSA, the latter in the presence or absence of cIgE-Fc, did not stimulate any significant levels of release confirming that canine IgE does not engage rat receptors as shown in Fig. 2A. The RBL-2H3 cell line transfected with cFcεRIα, following a 16 h incubation period supported maximal release with cIgE-Fc ($59.9 \pm 4.5\%$), canine Cε3/Cε4 ($55.4 \pm 6.1\%$), canine Cε2/Cε3 ($46.4 \pm 3.6\%$), and canine Cε3 ($2.8 \pm 1.2\%$) as shown in Fig. 2B. To determine if the failure to activate target cells with constructs comprising Cε3 is due to loss of species specific effector residues or might be attributed to increased dissociation of ligand from the receptor during the sensitization period, the chimeric antibodies were pre-incubated with NIP-HSA for 1 h before cell challenge by chimeric IgE antigen complexes. The subsequent assay demonstrated maximal release with cIgE-Fc ($72.8 \pm 5.4\%$), canine Cε3/Cε4 ($63.9 \pm 7.0\%$), canine Cε2/Cε3 ($47.3 \pm 5.4\%$), and canine Cε3 ($49.9 \pm 6.1\%$) as shown in Fig. 3.

To complement this approach, canine-human chimeric antibodies were also assessed for their ability to recognize and cross-link hFcεRIα when challenged with antigen using the RBL-2H3 cell line transfected with hFcεRIα (Gilfillan et al., 1992; Wilson et al., 1993). Fig. 4 shows maximal release elicited via the positive reference control, hIgE-Fc ($46.6 \pm 5.1\%$), cIgE-Fc ($38.2 \pm 0.6\%$), canine Cε3/Cε4 ($42.8 \pm 4.8\%$), canine Cε2/Cε3 ($36.5 \pm 1.6\%$), and canine Cε3 ($3.6 \pm 1.5\%$). Again, there was no significant level of degranulation observed with canine Cε3, so the chimeric antibodies were incubated with NIP-HSA for 1 h prior to addition to the cells when the maximal observed release was hIgE-Fc ($27.0 \pm 3.5\%$), cIgE-Fc ($27.3 \pm 1.4\%$), canine Cε3/Cε4 ($16.7 \pm 2.3\%$), canine Cε2/Cε3 ($10.5 \pm 2.8\%$), and canine Cε3 ($14.0 \pm 3.1\%$) as shown in Fig. 5.

To confirm that the formation of antigen-IgE complexes did not initiate activation of the RBL-2H3 cells by-passing

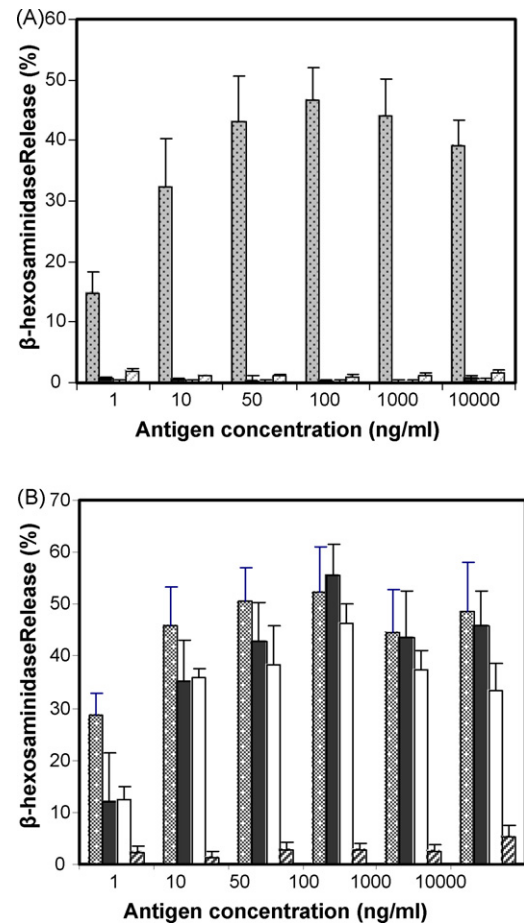


Fig. 2. Assessing the secretory output of RBL-2H3 and RBL-2H3 cells transfected with cFcεRIα by β-hexosaminidase release assays. (A) RBL-2H3 cells were incubated with mIgE (hatched) or cIgE-Fc (solid black) for 16 h and activated with DNP-HSA or NIP-HSA ($1-10^4$ ng/ml) or incubated with DNP-HSA (white) or NIP-HSA (diagonal lines) in the absence of IgE. (B) RBL-2H3 cells transfected with cFcεRIα were incubated with cIgE-Fc (hatched), canine Cε3/Cε4 (solid black), canine Cε2/Cε3 (white), and canine Cε3 (diagonal lines) for 16 h and activated with NIP-HSA ($1-10^4$ ng/ml). β-Hexosaminidase release is represented as a percentage of the total β-hexosaminidase within the cell.

receptor-mediated events, control experiments were performed by incubating mIgE and DNP-HSA for 1 h and cIgE-Fc and canine Cε3 with NIP-HSA for 1 h prior to cell challenge. Mouse IgE-DNP-HSA exhibited a maximal release of $62.2 \pm 6.8\%$, whereas, canine Fc IgE- and canine Cε3-NIP-HSA complexes failed to activate the RBL cells demonstrating that the observed release was a result of activating IgE receptor signaling as shown in Fig. 5.

4. Discussion

The higher amino acid sequence homology between human and canine IgE compared to rat and mouse IgE suggests that a study of canine IgE-receptor interaction represents a more relevant model than the rodent system. The current study attempts to compare the effector domains of canine IgE and human IgE and to develop molecular tools for investigating intervention strategies in canine allergic disorders with practical applications to analogous studies of human allergies.

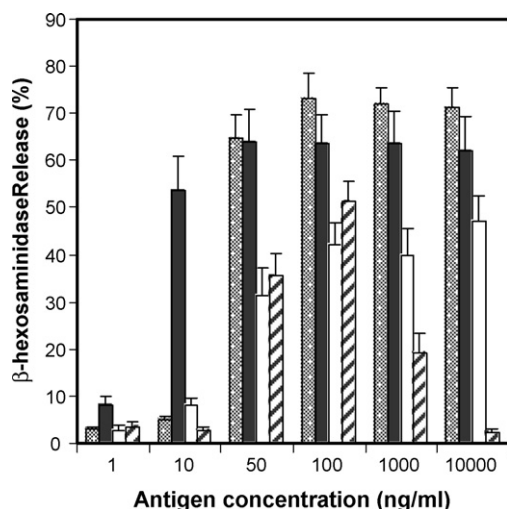


Fig. 3. Assessing the secretory output of RBL-2H3 cells transfected with cFcεRIα and antigen-IgE complexes. Canine Fc IgE (hatched), canine Cε3/Cε4 (solid black), canine Cε2/Cε3 (white), and canine Cε3 (diagonal lines) were incubated with NIP-HSA (1–10⁴ ng/ml) for 1 h prior to addition to the cells to form antigen-IgE complexes. β-Hexosaminidase release is represented as a percentage of the total β-hexosaminidase within the cell.

Previous experiments by us and others (Sayers et al., 1998; Carroll et al., 2001) confirmed that human IgE does not recognize cFcεRIα or endogenous rat receptors on the RBL-2H3 cell line making the human IgE-Fc domains suitable for replacing the homologous canine IgE-Fc domains. Earlier investigations have reported that the human IgE-Fc domain, Cε3, is sufficient to bind hFcεRIα (Nissim et al., 1991) and crystal structure analyses confirmed that the sites of interaction between human IgE and hFcεRIα are located in human Cε3 (Garman et al., 1998; Garman et al., 2000; Garman et al., 2001). Based on these observations, we expected to find analogous results for the interaction of canine IgE with its cognate high-affinity receptor.

When the chimeric antibodies, detailed in Table 1, were assessed for their ability to bind to cFcεRIα using flow cytometry and assessing β-hexosaminidase release from RBL-2H3 cells transfected with c- or h- FcεRIα, the outcome suggested that canine Cε3 did not recognize either the canine or human receptor subunit. Further investigations, facilitated through our generation of a functional receptor expressing cell line, showed that pre-incubation of canine Cε3 with antigen prior to cell challenge facilitates cell degranulation via activation of the transfected receptors, confirming that the canine IgE-Cε3 domain, like its homologous human counterpart, is sufficient to bind to and activate cells expressing c- and h- FcεRIα.

In view of the fact that cells incubated with the canine Cε3 construct for the 16 h period prior to antigenic challenge, commonly used in protocols for the study of sensitization of cells of mast cell lineage (Sayers et al., 1998; Carroll et al., 2001) do not support detectable levels of mediator release, we conclude that chimeric IgE antibodies comprising only canine Cε3 dissociate faster from the receptor complex than the native canine IgE-Fc. The high affinity of IgE for the α-chain of its receptor complex has been attributed to the Cε2 domain, which is thought to act as a molecular clamp, inhibiting IgE dissociation from the receptor

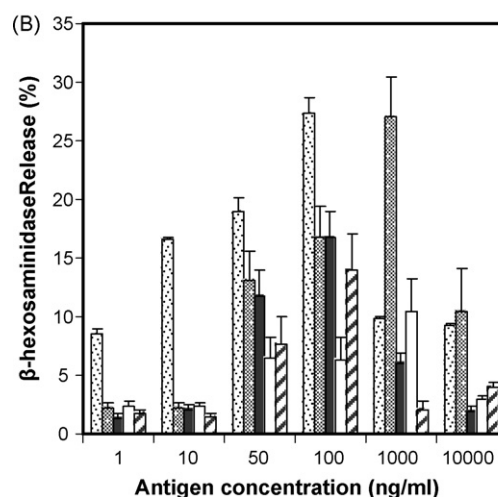
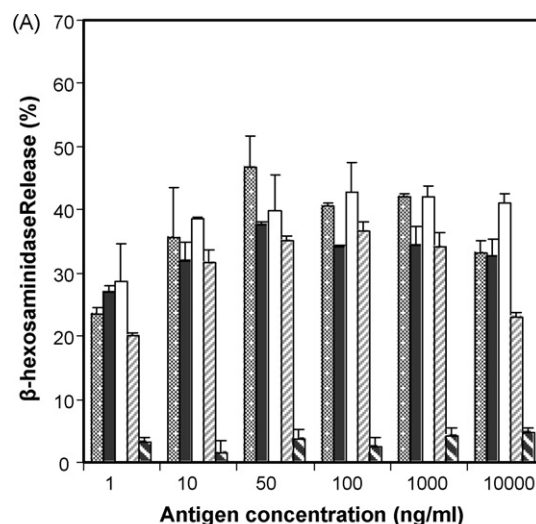


Fig. 4. Assessing the ability of canine-human IgE chimeric antibodies to activate RBL-2H3 cells transfected with hFcεRIα when stimulated with antigen. (A) Cells were incubated with hIgE-Fc (hatched), cIgE-Fc (solid black), canine Cε3/Cε4 (white), canine Cε2/Cε3 (diagonal lines), and canine Cε3 (diagonal lines) for 16 h and activated with NIP-HSA (1–10⁴ ng/ml). (B) hIgE-Fc (hatched), cIgE-Fc (solid black), canine Cε3/Cε4 (white), canine Cε2/Cε3 (diagonal lines), and canine Cε3 (diagonal lines) were incubated with NIP-HSA (1–10⁴ ng/ml) for 1 h prior to addition to the cells to form antigen-IgE complexes. β-Hexosaminidase release is represented as a percentage of the total β-hexosaminidase within the cell.

(McDonnell et al., 2001). It is interesting to note in this context, that the presence of hCε2 domain associated with the cCε3 construct is not a functional substitute for the canine homologue to prevent dissociation of the chimeric ligand from the receptor.

The increased secretory output observed in the current study with constructs comprising canine Cε3–Cε4 domains compared to chimeras encompassing Cε2–Cε3 seems therefore unexpected. It is however important to note that in our study we correlate secretory output with receptor recognition. This parameter, as pointed out by a number of researchers (Ortega et al., 1988; Schweitzer-Stenner et al., 1994; Holowka et al., 2007) appears to be dependent on orientational constraints imposed by the ligand on the receptor rather than by the affinity of the ligand for the receptor. Since comparable studies assessing secretory

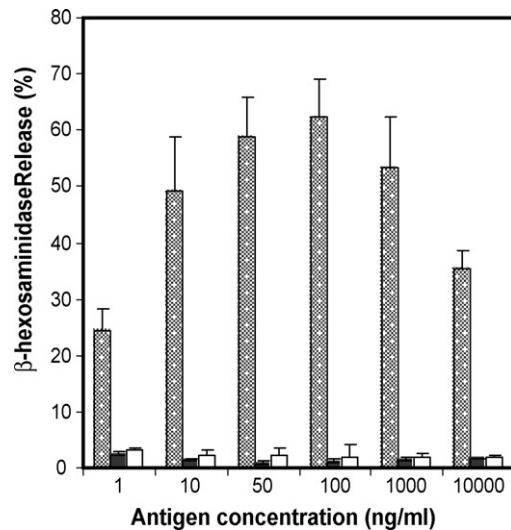


Fig. 5. Assessing the ability of antigen-IgE complexes to activate the parental RBL-2H3 cell line. Antibodies were incubated with either, DNP-HSA or NIP-HSA ($1-10^4$ ng/ml) as appropriate for 1 h prior to incubation with the cells mIgE (▨), cIgE-Fc (■), and canine Cε3 (□). β-Hexosaminidase release is represented as a percentage of the total β-hexosaminidase within the cell.

output of human/mouse chimaeras have, to the best of our knowledge, never been published, and no information is available concerning the structural consequences of domain swapping on IgE-mediated receptor activation, the nature of the contribution, at the molecular level, of Cε2 and Cε4 to canine IgE-induced cell degranulation, must at present remain speculative.

Expressing the ligand binding domain of the canine high-affinity receptor complex in RBL cells, where the transfected ligand binding domain couples with the endogenous rodent subunits of the receptor complex, has allowed us to demonstrate, that analogous to hIgE:hFcεRIα interaction, the canine IgE-Fc derived Cε3 domain contains the recognition sites for both c- and h- FcεRIα and is the only canine IgE-Fc domain essential for receptor activation. Furthermore, our study has provided the foundation for the testing of the safety of anti-Cε3-derived peptide antibodies, which, if shown to be non-anaphylactogenic, could provide through active immunization, protection of dogs

against all forms of IgE-mediated hypersensitivity responses (Ledin et al., 2006) and have wider implication as a safety screen for the testing of human anti-Cε3 derived anti-peptide antibodies.

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