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Research paper

Development of an *in vitro* model system for studying the interaction of *Equus caballus* IgE with its high-affinity receptor FcεRI

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ABSTRACT

The binding of immunoglobulin E (IgE) to its high-affinity receptor (FcεRI) is the central protein interaction in IgE-mediated allergic reactions. The cross-linking of the IgE/FcεRI complex, through cognate allergens, on the surface of mast cells and basophil cells results in mediator release, and thus leads to the symptoms of type I hypersensitivity responses in mammals. To develop a baseline value for subsequent equine anti-allergy drug and vaccine research, the interaction of equine IgE with its high-affinity FcεRI receptor was investigated following the cloning and expression of equine IgE with specificity for NIP-HSA (4-hydroxy-5-iodo-3-nitrophenylacetic acid conjugated to human serum albumin). Receptor recognition and effector functions were assessed in Rat Basophil Leukemia (RBL-2H3.1) cells transfected with the α chain of equine and canine FcεRI. Results obtained showed that the equine FcεRI receptor recognizes both equine and canine IgE and supports similar β-hexosaminidase release levels from RBL cells transfected with equine FcεRI, peaking at 36.68% at 100 ng ml⁻¹ antigen and 32.00% at 100 ng ml⁻¹ antigen respectively. Furthermore, the binding kinetics of the equine IgE to the equine FcεRI receptor and the canine IgE to the same receptor was measured to be $K_A = 6.33 \times 10^9 \text{ M}^{-1}$ and $K_A = 1.84 \times 10^9 \text{ M}^{-1}$ respectively. This research established basic reagents and vitro assay systems to underpin the development of rational therapeutic intervention strategies to combat equine allergic manifestations.

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1. Introduction

Allergens are usually perceived as seemingly innocuous substances, although many common allergens have distinct biological activities enables them to interact with cells of the immune system. These substances include enzymes e.g. Phospholipase A₂, or house dust mite proteases, or lectins (Dudler et al., 1995; Machado et al., 1996) Furthermore, components in diesel exhausts and cigarette smoke,

acting as irritants, can enhance IgE synthesis by creating a cytokine environment favoring TH₂ responses (Smyth et al., 2000). Some studies suggest that allergy development is determined by genetic factors such as SNPs in the human genome (Akhabir and Sandford, 2010), but overwhelming evidence suggest that allergy development is dependent on a combination of environmental, lifestyle factors and an individual's overall genetic make-up (Romagnani, 1997; Ono, 2000).

A previous study (Hunter et al., 2008) has shown the similarity between canine and human allergic response mechanisms. The frequency of allergic disease in horses, as in other organisms, is largely attributed to genetic and environmental factors (Eder et al., 2001). Thus, these

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animals represent an excellent model system for the study of the interaction of genetic and environmental factors in the initiation and progression of allergic disease.

Horses (*Equus ferus caballus*) have been used as animal models due to the similarity of their physiological responses to humans (Eyre and Lewis, 1973), they are also the cornerstone of the horse racing industry which is of considerable economic importance. Horses have 1000 times higher serum IgE levels than humans (Wagner, 2009), and they frequently suffer from allergic reactions in the form of insect bite hypersensitivity (IBH) (Hellberg et al., 2006; Schaffartzik et al., 2012), which results in allergic dermatitis in response to bites of insects of the genus *Culicoides*, while horse lung inflammation is usually manifested in the form of recurrent airway obstructions (RAO), characterized by noticeable wheezing, repeated coughing and labored breathing commonly in response to mold spores. High allergen-specific IgE levels in horse serum have been associated with RAO in some studies (Künzle et al., 2007) but not in others (Tahon et al., 2009). There are not many systematic studies concerning horse allergies, systematic studies on equine allergies are thus still needed.

The publication by (Wilson et al., 2006) discusses the production of monoclonal anti-equine IgE antibodies in an attempt to monitor total serum IgE responses, while the publication by (Wagner et al., 2003) describes the generation of mAbs in an attempt to neutralize equine serum IgE. On the other hand, the paper by (McAleese et al., 2006) discusses synthesis protocols of the extra cellular soluble domains of the horse FcεRIα receptor, this was in an attempt to use the specificity of ligand/receptor interaction to specifically detect horse serum IgE. The current study builds on these publications by investigating the relationship between equine IgE and its high-affinity Fc receptor (FcεRI). Furthermore, it studied the interaction of IgE from phylogenically related species with the equine receptor in order to establish a model system valid not only for the equine allergy but one that is also of potential relevance to other organisms.

It has been determined that human IgE binds with a 1:1 ration to its FcεRI receptor, i.e. one molecule of IgE to one molecule of receptor (Garman et al., 2000), with an affinity measured to be $K_A \approx 10^{10} \text{ M}^{-1}$ (Ravetch and Kinet, 1991). The actual site of the interaction between the two molecules has been shown to reside in the Cε3 domain of IgE which interact with residues of the 2nd extra cellular domain of the FcεRI receptor (Garman et al., 2000).

BLASTing the amino acid sequence between the ligand binding domain of human, canine and equine IgE receptors revealed a 56% similarity between the human and the canine receptors (Hunter et al., 2008), 64% between the human and equine receptors, and 55% between the canine and equine receptors. Furthermore the sequence of the human and equine IgE share 56% sequence similarity, the canine and equine IgE sequence show 66% homology. The Cε3 domains of the human, canine and equine IgEs display a sequence identity of 68% between the canine and the equine amino acid sequences, 62% between the human and canine sequences and 60% between the human and equine sequences. The high sequence homology shared

between human, canine and equine receptor suggests that the equine allergy model generated in this study may be of wider relevance to all these species.

We therefore studied the relationship between equine IgE and its FcεRI receptor, as well as the interaction of canine IgE with the equine FcεRI receptor. While the publication by (Wagner et al., 2002) discusses the production of equine IgE anti NP [4-(hydroxy-3-nitro-phenyl) acetyl] in an effort to examine equine allergy *in vivo*, which lead to the publication (Wagner et al., 2003) describing the production of mAbs against equine serum IgE. This study developed an *in vitro* analysis system for studying equine IgE mediated allergic reaction to develop a baseline value for subsequent equine anti-allergy drug and vaccine research. This was achieved by generating an equine IgE molecule with antigen specificity to NIP-HSA (4-hydroxy-3-iodo-5-nitrophenylacetic acid conjugated to human serum albumin), as well as using canine IgE with the same antigen specificity and mouse IgE with a specificity for DNP-HSA (2,4-dinitrophenol conjugated to human serum albumin) to engage the ligand binding domain of the equine receptor, the gene of which was transfected into RBL-2H3.1 cells where it coupled with the endogenous β and γ chains to effect cell surface expression and downstream signaling in response to an IgE-mediated cross-linking stimulus which was assessed by measuring β-hexosaminidase release (Wilson et al., 1993) and surface plasmon resonance (SPR) was used for the kinetic analysis.

2. Experimental procedure

2.1. Generating equine IgE

The equine IgE heavy chain sequence (GenBank: AJ305046.1 Navarro et al., 1995) was optimized for the mouse (*Mus musculus*) codon and synthesized chemically (GeneScript). The gene was cloned into the pSV-V_{NP} plasmid (Neuberger et al., 1985) where the IgE heavy chain was inserted downstream of a mouse λ chain NIP recognizing variable region. The recombinant plasmid was transfected into J558L cells (mouse B myeloma cells derived from BALB/c strain isolated by Morrison et al., 1984) using electroporation with the following parameters, 250 v 960 μF (Bio-Rad), and selected using 1 μg ml⁻¹ mycophenolic acid, 50 μg ml⁻¹ xanthine and 15 μg ml⁻¹ hypoxanthine. 48 colonies were obtained after 6 weeks of selection of which the highest IgE producing colony was isolated and expanded for IgE expression. Expression levels were assessed by SPR (Sayers et al., 1998).

Equine IgE was purified by hapten affinity chromatography (Sayers et al., 1998) and concentrated using a 10 kDa molecular filter (Millipore), which resulted in 1 ml IgE at 3.64 mg ml⁻¹.

2.2. Generation of an equine FcεRI expressing cell line

The equine FcεRIα gene sequence (GenBank: Y18204.1 McAleese et al., 2000) was optimized for rat codon (*Rattus norvegicus*) and yeast (*Pichia pastoris*) codons preference simultaneously before chemical synthesis (GeneScript). The gene was cloned into the pEE6 plasmid (Stephens

and Cockett, 1989). The plasmid encoding equine FcεRIα was transfected into RBL-2H3.1 cells (Wilson et al., 1993; Hunter et al., 2008) using electroporation with the following parameters, 250 v 960 μF (Bio-Rad), and clones were selected with Geneticin G418 Sulfate (Invitrogen). The equine FcεRIα chain was expressed and formed a fully functional chimeric receptor complex with the endogenous rat β and γ chains. After 2 weeks of selection the cells were harvested, sorted by FACS, where the cells were tagged with canine IgE conjugated with phycoerythrin (since at that time equine IgE was not available). Cells expressing equine FcεRIα were expanded leading to the development of an RBL-2H3.1 cell line expressing equine IgE receptor. Prior to assessment of ligand mediated, antigen induced β-hexosaminidase release, flow cytometry analysis confirmed the generation of a cell line expressing the equine receptor capable of engaging equine IgE.

2.3. β-Hexosaminidase release assays

β-hexosaminidase release, as an indicator of mediator degranulation, was assessed on RBL-2H3.1 cells expressing a functional equine FcεRIα chain as described previously (Siraganian and Hook, 1986; Wilson et al., 1993; Hunter et al., 2008). Results are presented as the mean ± standard deviation of experiments and were performed in triplicates.

2.4. Generation of a soluble equine FcεRIα

The gene encoding the soluble equine FcεRIα extra cellular domains 1 and 2 (sFcεRIαD1&2) were amplified by PCR from the FcεRIα gene as described in Section 2.2. The gene was cloned into the pPIC9k plasmid (Invitrogen). The plasmid encoding equine sFcεRIαD1&2 was transfected into *Pichia pastoris* following the protocol Multi-Copy Pichia Expression Kit Version F and the protocol Pichia EasyComp™ Kit (Invitrogen). Equine sFcεRIαD1&2 chain was expressed and purified using a chromatography column, using canine IgE as a ligand bound to Sepharose beads, by established procedures (Sayers et al., 1998). The eluate was dialyzed against PBS and concentrated using a 3 kDa molecular filter.

2.5. Kinetic binding analysis between equine IgE and equine sFcεRIαD1&2

Kinetic analysis of the equine IgE and the equine sFcεRIαD1&2 was measured by Surface Plasmon Resonance (SPR BiaCore 2000), using the Wizard BiaCore software. Equine IgE (suspended in 1x PBS) was passed over, and captured by the NIP-HSA antigen bound on to the surface of a sensor chip until it reached a response unit of ~1000. Subsequently increased concentrations of the equine sFcεRIαD1&2 (suspended in 1 × PBS) were passed over the chip surface to bind to the immobilized IgE, followed by a dissociation period. Kinetic constants were automatically generated by the software.

The binding signals from the association and dissociation curves were fitted onto three models, the distribution model, the discrete single site model, and

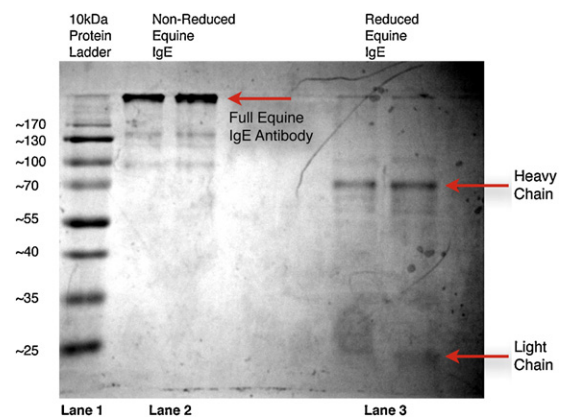


Fig. 1. SDS-PAGE gel showing bands of purified equine IgE anti NIP-HSA stained with Coomassie Brilliant Blue. Lane 2 shows the complete IgE protein ~192 kDa with minimal degraded protein impurity. Lane 3 shows equine IgE run in the presence of β-mercaptoethanol top, bands at ~72 kDa represents the heavy chains of the IgE, and the bands at ~25 kDa indicate the light chains of the IgE. Immunoblotting could not be carried out due to the unavailability of anti-equine antibodies. These results are consistent with results obtained for canine IgE expression and purification (Hongtu Ye, PhD Thesis, The University of Sheffield, 2010). Subsequent results (β-hexosaminidase release assays and kinetic analysis) supported the identity of the expressed protein as equine IgE.

the conformational change model, using the program EVILFIT - <https://sedfitsedphat.nibib.nih.gov/default.aspx> (Svitel et al., 2003, 2007; Gorshkova et al., 2008). The analysis generated in accord with the distribution model gave the best fit compared to the other the two models (Schuck and Zhao, 2010).

3. Results

3.1. Development of NIP recognizing equine IgE

The protocol in Section 2.1 led to the development of a J558L cell line that expresses equine IgE with anti NIP-HSA specificity. Purified equine IgE anti NIP-HSA was analyzed on a 12% SDS-PAGE in the presence and absence of the reducing agent β-mercaptoethanol (Fig. 1). The results show that molecular mass of the reduced and non reduced bands are consistent with the molecular weight for the IgE isotype.

3.2. Development of an RBL-2H3.1 cell line expressing equine FcεRIα

RBL-2H3.1 cells transfected with equine FcεRIα were tagged with the equine IgE anti NIP-HSA prepared as described in Section 2.2. The cells were sorted by FACS and then expanded, which resulted in an RBL-2H3.1 cell line expressing equine FcεRIα. Prior to performing the β-hexosaminidase release assays, the cells were tested by flow cytometry to confirm expression of the equine receptor (Fig. 2).

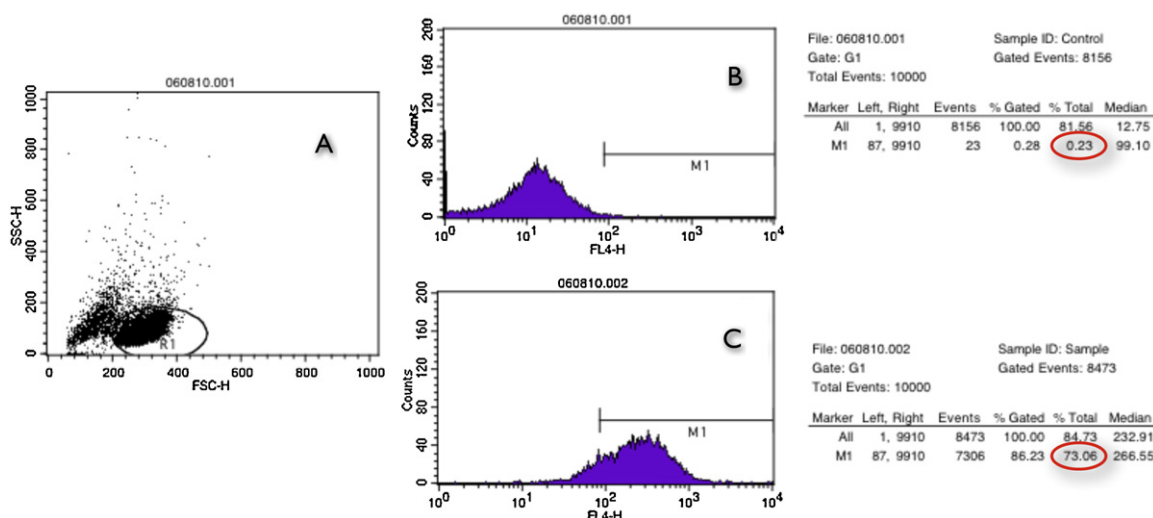


Fig. 2. Flow cytometry test on RBL-2H3.1 cell line expressing equine FcεRIα tagged with equine IgE conjugated with Allophycocyanin (APC). (A) Shows the cell population (live) used in the analysis (inside the circular gate), the cell population outside the gate represents dead cells. RBL-2H3.1 cells transfected with the gene encoding equine FcεRIα were selected and sorted by FACS and expanded (see Section 2.2). The final cell population demonstrated expression of equine FcεRIα. This cell population in C is computed to comprise 73.06% positive cells compared to the control in B (0.23%). Experiment was performed once.

3.3. β-Hexosaminidase release assays

RBL-2H3.1 cells expressing equine FcεRIα were harvested from a confluent 100 mm Petri dish, cells were diluted to a final concentration of 5×10^5 cell ml⁻¹ and sensitized with IgE from the designated species to a final concentration of 1 μg ml⁻¹ for 16 h. Mediator release was determined as outlined in Section 2.3. The data shown represent the mean of three experiments (Fig. 3). The transfected cells were sensitized with mouse, canine and equine IgEs with non-transfected RBL-2H3.1 cells acting as a reference control, maximum release occurred at 100 ng ml⁻¹ of antigen. RBL-2H3.1 cells expressing canine FcεRIα were also assessed for stimulus secretion coupling with equine IgE. Mouse IgE binds to the endogenous rat FcεRI receptor and the transfected receptor, hence is used as a positive reference control.

The results showed both the non-transfected and transfected cells expressing equine FcεRIα support mediator release when sensitized with mouse IgE, with a maximum release of 45.9% and 51.5% at 100 ng ml⁻¹ DNP-HSA respectively (Fig. 3A). When cells were sensitized with equine IgE they showed a maximum release of 36.6% at 100 ng ml⁻¹ NIP-HSA. Non-transfected cells showed no release. Thus it can be concluded that canine IgE does recognize and sensitizes the equine FcεRIα to support mediator secretion (Fig. 3B). Non-transfected cells showed no release, indicating that equine IgE binds selectively to equine FcεRIα and supports antigen-induced mediator release (Fig. 3C). Cells sensitized with canine IgE showed a maximum release of 31.9% at 100 ng ml⁻¹ NIP-HSA.

3.4. Kinetic analysis

Equine IgE was tested for the kinetics of binding to the soluble extracellular domains of the equine

(sFcεRIαD1&2) receptor using Surface Plasmon Resonance (SPR) as described in Section 2.5 (Fig. 4). The kinetic results shown (Fig. 5) indicated that equine IgE binds to the equine sFcεRIαD1&2 receptor with high affinity, $K_A = 6.33 \times 10^9$ M⁻¹. Assessment of canine IgE binding to the equine sFcεRIαD1&2 showed similar binding affinity $K_A = 1.84 \times 10^9$ M⁻¹. The kinetic binding constants are summarized in Table 1 using the distribution model to counter the potential errors caused by the heterogeneity of the surface sites and mass transport limitations on the SPR biosensor surface.

4. Discussion

The equine IgE heavy chain formed a chimeric molecule with the λ light chain of the mouse variable region with specificity for NIP to give rise to an equine IgE antibody capable of binding to NIP-HSA, the protocol has facilitated the production of adequate quantities of protein for research purposes.

The development of the RBL-2H3.1 cell line expressing a chimeric receptor complex, where equine FcεRIα combined with the endogenous rat β and γ chains, led to the development of a model system that can be employed to assess the release of the mediators of allergy relevant to the equine system and should allow the assessment of individual horse allergies by sensitizing cells with horse serum from allergic horses and challenge of the transfected sensitized cells with a panel of potential allergens (Rashid et al., 2012). Mediator release assays showed that the transfected ligand binding domain of the equine receptor was functional and, when sensitized with equine IgE followed by antigenic challenge, induced cells to release mediators peaking at 36.6% at 100 ng ml⁻¹ NIP-HSA antigen. The release assays have also demonstrated that canine IgE recognizes and sensitizes the equine FcεRIα

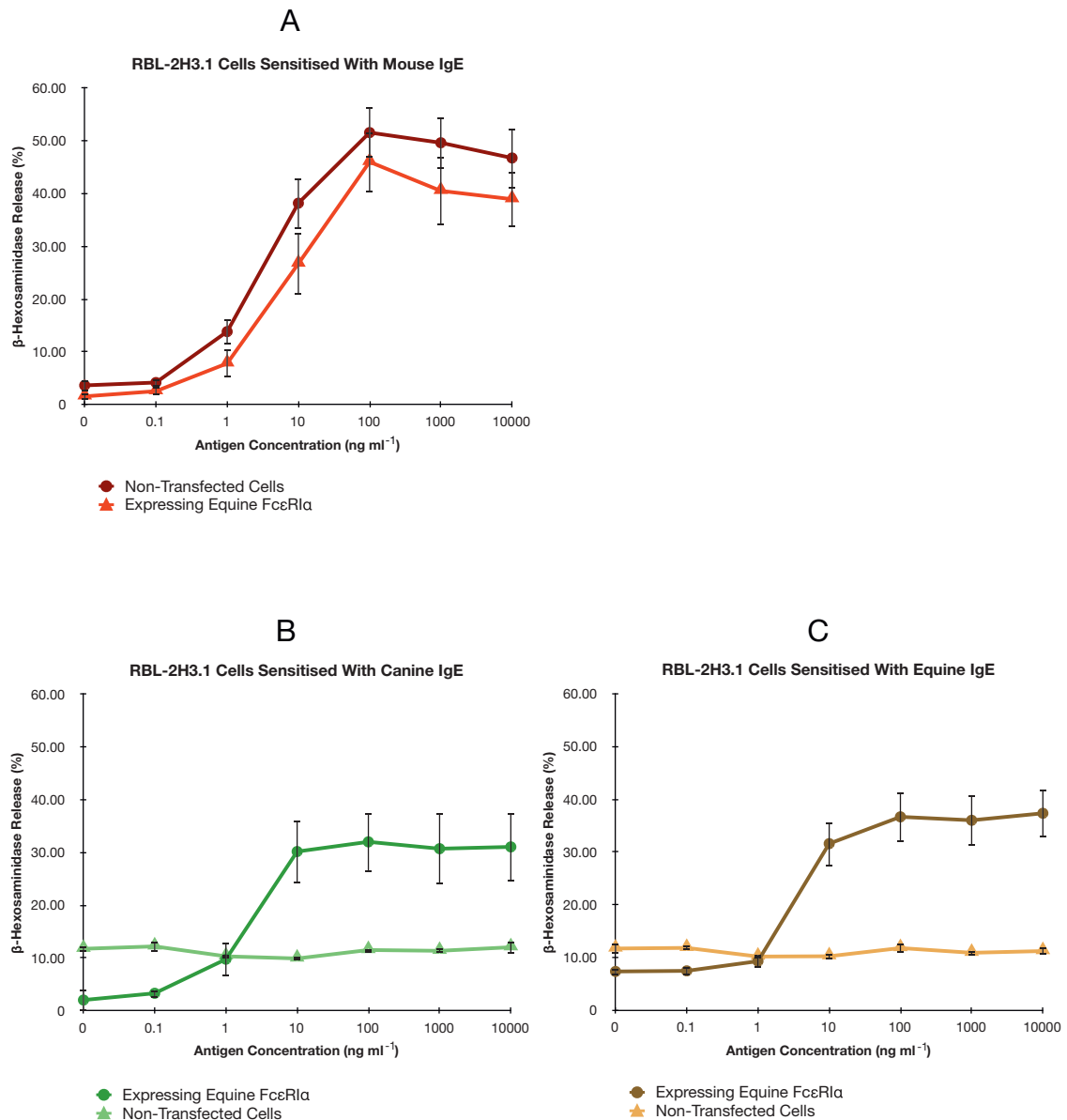


Fig. 3. (A) Release of β -hexosaminidase from non-transfected and transfected RBL-2H3.1 cells expressing equine Fc ϵ RI α sensitized with mouse IgE. Mediator release from non-transfected cells peak at 51.55% at 100 ng ml⁻¹ antigen while the transfected cells have a peak release of 45.99% at 100 ng ml⁻¹ antigen. (B) Graph shows mediator release from non-transfected and transfected RBL-2H3.1 cells expressing equine Fc ϵ RI α when sensitized with canine IgE. The dark green line (transfected RBL-2H3.1 cells expressing equine Fc ϵ RI α) shows mediator release with a peak of 32.00% at 100 ng ml⁻¹ antigen compared to 11.50% from non-transfected cells (light green line). (C) Mediator release from non-transfected and transfected RBL-2H3.1 cells expressing equine Fc ϵ RI α following sensitization with equine IgE. The dark orange line (transfected RBL-2H3.1 cells expressing equine Fc ϵ RI α) shows peak mediator release at 36.68% at 100 ng ml⁻¹ antigen compared to 11.80% mediator release from non-transfected cells (orange line). Each experiment was performed in triplicates. Routinely cells were sensitized for 16 h with IgE of desired species specificity before the release assay was tested.

Table 1

Kinetic analysis of IgE/Fc ϵ RI α interaction: values of kinetic binding constants based on the results in Fig. 5 are adjusted according to the distribution model to counter the errors caused by the heterogeneity of the surface sites and mass transport limitations on the SPR biosensor surface.

	K_D (M)	K_A (M ⁻¹)	k_d (s ⁻¹)	Signal (RU)	Root mean square deviation (RU)
Horse IgE – Horse Fc ϵ RI α	1.58×10^{-10}	6.33×10^9	2.44×10^{-5}	124.64	0.74
Dog IgE – Horse Fc ϵ RI α	5.43×10^{-10}	1.84×10^9	4.75×10^{-5}	161.09	0.63

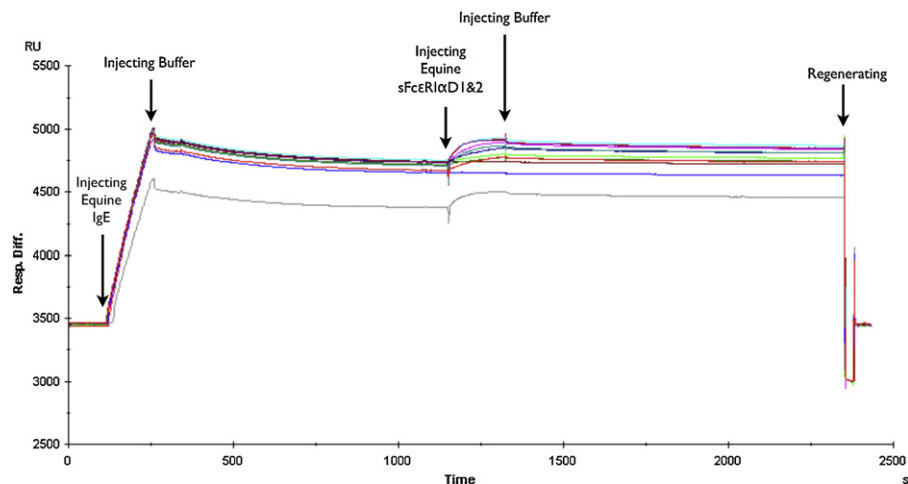


Fig. 4. Graph of the SPR cycle used to measure the binding of equine sFcεRIαD1&2 to equine IgE. Equine IgE anti NIP-HSA was first injected onto a CM5 SPR chip surface coated with NIP-HSA. This was followed by injecting a buffer to remove all non-bound IgE from the surface of the chip. Subsequent injections of equine sFcεRIαD1&2 showed that it bound to the equine IgE on the chip with a characteristic dose response curve. Several cycles were carried out at increasing concentrations of equine sFcεRIαD1&2 while maintaining a constant concentration of equine IgE.

receptor for antigen-induced mediator release. Our observations showed that the equine and canine IgEs were capable of binding to the equine FcεRI receptor generating similar levels of mediator release.

The release assays have confirmed the establishment of a basic assay system for the assessment of equine allergies, and as such, this work presents a step forward in the quest for the development of assay systems to test

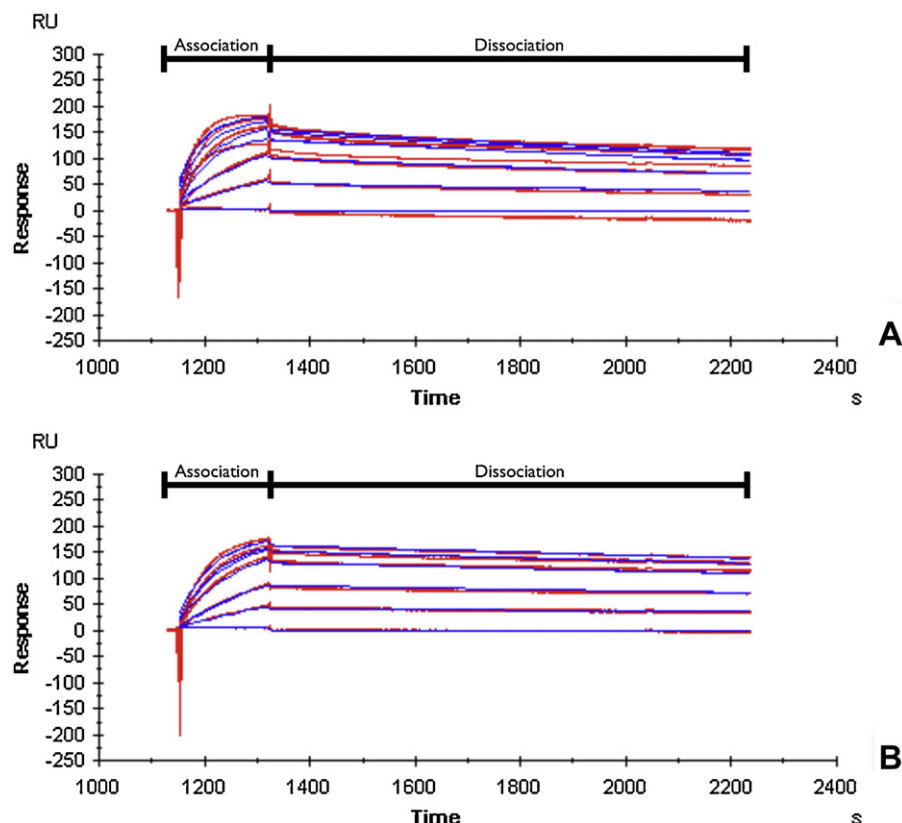


Fig. 5. Kinetic analysis assessing the binding of the equine sFcεRIαD1&2 to equine IgE. The red lines representing the actual cycle measurements from Fig. 4 while the blue lines show the results fitted in accordance with the 1:1 Langmuir binding model. (A) Graph of the binding between equine IgE and equine sFcεRIαD1&2 show a fast association curve followed by slow dissociation curve. (B) Graph of the binding between canine IgE and equine sFcεRIαD1&2 again showing a fast association curve followed by slow dissociation curve.

rational anti-allergic therapeutic intervention strategies. Our results also suggest that common modes of investigation may be applicable for assessing the underlying causes of allergic responses in horses and dogs with potential applications for the use of this system for assessment of rational intervention strategies for dog, horse and human allergic manifestations (Rashid et al., 2012).

The publication by (McAleese et al., 2006) discussed expression of the extra cellular part of the horse FcεRI receptor using the baculovirus system, we found that using the *Pichia pastoris* system was more economical. The development of the soluble form of the equine FcεRIα (sFcεRIαD1&2) enabled the assessment of the kinetics of binding between equine IgE and its high affinity receptor. A dissociation constant value of $K_D = 1.58 \times 10^{-10}$ M ($K_A = 6.33 \times 10^9$ M⁻¹) was observed, corrected for SPR errors. Information regarding the complementary affinity between ligand and receptor is crucial for vaccine and drug research where a binding affinity higher than $K_A = 6.33 \times 10^9$ M⁻¹ needs to be achieved in order to prohibit or reverse receptor sensitization with IgE.

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