Video Article

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 interaction of IgE with its high-affinity Fc receptor (FcεRI) followed by an antigenic challenge is the principal pathway in IgE mediated allergic reactions. As a consequence of the high affinity binding between IgE and FcεRI, along with the continuous production of IgE by B cells, allergies usually persist throughout life, with currently no permanent cure available. Horses, especially race horses, which are commonly inbred, are a species of mammals that are very prone to the development of hypersensitivity responses, which can seriously affect their performance. Physiological responses to allergic sensitization in horses mirror that observed in humans and dogs. In this paper we describe the development of an *in situ* assay system for the quantitative assessment of the release of mediators of the allergic response pertaining to the equine system. To this end, the gene encoding equine FcεRIα was transfected into and expressed onto the surface of parental Rat Basophil Leukemia (RBL-2H3.1) cells. The gene product of the transfected equine α-chain formed a functional receptor complex with the endogenous rat β- and γ-chains [1]. The resultant assay system facilitated an assessment of the quantity of mediator secreted from equine FcεRIα transfected RBL-2H3.1 cells following sensitization with equine IgE and antigenic challenge using β-hexosaminidase release as a readout [2, 3]. Mediator release peaked at 36.68% ± 4.88% at 100 ng ml^{-1} of antigen. This assay was modified from previous assays used to study human and canine allergic responses [4, 5]. We have also shown that this type of assay system has multiple applications for the development of diagnostic tools and the safety assessment of potential therapeutic intervention strategies in allergic disease [6, 2, 3].

Video Link

The video component of this article can be found at https://www.jove.com/video/52222/

Introduction

Allergy has been known for millennia. An asthma treatment was described in the Ancient Egyptian medical text known as the Ebers Papyrus (~1550 BCE) and discussed herbal remedies to treat it [7].

Today allergy is classified as a type I hypersensitivity response, where the T helper cell type 2 (TH_2) arm of the immune system steers the production of immunoglobulin E (IgE) antibodies in response to environmental antigens called allergens. These are diverse substances that commonly interact with cells in the immune system and stimulate the synthesis and secretion of pro-inflammatory cytokines, including interleukin-4 and interleukin-13 [8, 9] as do particles in cigarette smoke or diesel exhaust particles which enhance IgE synthesis [10].

The rise in allergic manifestations in industrialized countries in the last 50 years has been attributed to a combination of the effect of environmental pollutants and a trend to a more sanitized environment, which combine to shift the immune response towards a profile predominated by TH_2 cytokines, as proposed by the 'Hygiene Hypothesis' [11].

As mentioned above, humans are not the only mammals afflicted by allergy. Notably horses and dogs can also develop classic allergic responses and a study by [12] has shown that, as in humans, equine allergy is attributed to genetic and environmental factors. As a consequence, these animals present good models for studying the interplay between genetic and environmental causes of allergy, its progression from sensitization to disease, and possible intervention strategies once clinical manifestations have set in

In 1887, Stömmer was the first person to describe the similarity between human and equine asthma [13], the effect of histamine on the equine cardiovascular system is very similar to that of humans [14]. Horses are also the cornerstone of the horse racing industry, which is worth US$72 billion with a betting turnover of US$115 billion annually [15].

Most contemporary racehorses are descendants of the small number of Arabian horses bred by Lady Anne Blunt from 1878 onwards. Modern racehorses are commonly inbred to select for performance abilities. They are prone to genetic disorders, one of which is their susceptibility to mount allergic responses. They also have 1000 times higher serum IgE levels than even the most severely allergic humans [16]. Horse allergic responses are usually manifested as insect bite hypersensitivity (IBH) [17, 18]. IBH results in dermatitis due to bites form insects in the genus

Culicoides. Another form of equine allergic disease is recurrent airway obstructions (RAO), this is manifested in the lungs and airways. It is characterized by wheezing and labored breathing. RAO commonly occurs in response to mould spores, and high allergen-specific IgE levels have been recorded in horses suffering from RAO in one study [19] although another investigation has not confirmed this [20].

Studies on equine allergy revolved around the attempt at monitoring and neutralizing equine IgE by developing anti-equine IgE monoclonal antibodies (mAbs) [21, 22]. Furthermore the study by [23] discusses the production of the extracellular domains of the equine high-affinity Fc receptor's α chain (FcεRIα) receptor in an attempt to detect and quantitate equine serum IgE. A related study by Ledin[24] discusses a new approach aimed at neutralizing serum IgE by priming the immune system using a self/non-self immunogen. All these studies, however, lacked an effective assay to test the safety and efficacy of their protocols. In this article, we now present such an assay system applicable to the study of diagnostic and therapeutic strategies relevant to the equine system, where β-hexosaminidase release, as an indicator of cell mediator degranulation, was assessed on RBL-2H3.1 cells expressing equine FcεRIα. This protocol is based on previous publications [25, 4, 5, 2, 3] describing the engineering of RBL cells transfected with the gene encoding the IgE binding domain of the high-affinity receptor for IgE from different species. The protocol explains how to perform a β-hexosaminidase release assay, the results of which are presented as the mean ± standard deviation of triplicate experiments.

The release assay was first developed by Siraganian and Hook [25] to study human allergy. The lab group led by Dr. Reuben Siraganian also developed the RBL cell line. These RBL cells were developed to express the human FcεRIα and the protocol was published by [4]. The final piece of the assay came with the development of the pSV plasmid in the paper by Neuberger [26] which described the production of an IgE antibodies by cloning its heavy chain gene downstream of a mouse gene for an IgE variable region that targets the hapten 4-hydroxy-3-nitro-phenacetyl (NP), the resulting chimeric antibody was fully functional. The ability to develop any IgE targeting the same hapten, while also cloning its receptor on the surface of RBL cells resulted in the standardization of the assay making it a useful protocol to measure the degranulation of basophil cells.

The assay does have pros and cons. The pros of the assay is its adaptability to be used in any mammalian system, our lab has thus used it to test for the degranulation in the human, canine and equine systems, and this is achievable simply by synthesizing the organism's IgE and cloning its receptor onto the surface of the RBL cells.

On the other hand, the cons of the assay is that the RBL cells are very sensitive to thermal, mechanical and PH changes, making them give a variation of degranulation levels within the same assay. It is thus strongly advised that the assays are always repeated in triplicates and then an average is taken from them. Furthermore, the RBL cells tend to shift toward a non-releasing phenotype if they are left in tissue culture for an extended times (>10 weeks) [27], making their maintenance cumbersome. They are also prone to infections by mycoplasma bacteria, which are not visible from a light microscope and do not change the cell's morphology, but would drastically change their degranulation levels. Thus regular mycoplasma tests are needed.

Protocol

1) Preparation of Cell Line:

1. Developing the RBL-2H3.1 cell line expressing equine *FcεRIα:*
    1. Using basic tissue culture techniques for monolayer cell lines, transfect parental RBL-2H3.1 cells using the pEE6 plasmid, carrying the equine FcεRIα gene (GenBank: Y18204.1) [28]. Add 2 μg μl^{-1} of the plasmid DNA to 0.8 ml of cells at a density of 1.2 $x10^7$ cells ml^{-1}. Electroporate the cells at 250 V 960 μF using a 0.4 cm electrocuvette then immediately incubate on ice for 10 min.
    2. Select the transformed cells using media containing 0.4 g of geneticin G418 sulphate, then sort the remaining living cells through FACS by tagging them with a fluorescent IgE antibody. Use the resulting RBL-2H3.1 expressing equine FcεRIα cell line for the investigation [2, 3].

2. Pre-assay antibody sensitization:
    1. Harvest the RBL-2H3.1 expressing equine FcεRIα cells from a confluent Petri dish. Wash then re-suspend the cells in culture media to a cell density of $5x10^5$ cell ml^{-1}.
    2. Add the IgE of interest to the suspended cells to a final concentration of 1 ng ml^{-1}, then plate 100 μl of cells onto a 96 well plate at columns 1-6 and incubate at 37 °C + 5% CO_2 + 90% relative humidity for 16 hr. After the incubation time, and before performing the release assay, check the wells under a microscope for well confluency and cell adherence.

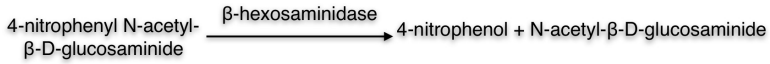
2) Release Assay:

1. Washing the cells:
    1. Warm release buffer (25 mM PIPES, 120 mM sodium chloride, 5 mM potassium chloride, 0.04 mM magnesium chloride, and 1 mM calcium chloride) at 37 °C to allow for gentle cell washing.
    2. Wash cells by flicking the plate to remove cell media and adding 100 μl warm, 37 °C, release buffer. Repeat twice.

2. Antigen challenge:
    1. Prepare a serial dilution of the antigen (NIP-HSA or DNP-HSA) of 0 ng ml^{-1}, 0.1 ng ml^{-1}, 1 ng ml^{-1}, 10 ng ml^{-1}, 100 ng ml^{-1}, 1,000 ng ml^{-1}, 10,000 ng ml^{-1} in release buffer and warm at 37 °C.
    2. After the second cell wash, discard the media and replaced with 100 μl of the antigen solutions. Ensure that wells in the same row (A1-6 for example) have the same antigen concentration added to them.
    3. Set up a negative control in row A by adding 0 ng ml^{-1} antigen. Add increasing antigen concentration down the rows (B-G) followed by triton-x buffer (5% Triton X-100) in row H cells to lyse the cells to be used as a positive control. Incubate at 37 °C for 20 min to allow the cells to release its mediators.

3. Setting up individual well controls:
    1. After the incubation, transfer 50 µl of cell supernatant to the other half of the plate (wells A1-6 to wells A7-12, etc.). Discard the remaining 50 µl of supernatant and replace with 50 µl of triton-x buffer to allow the measurement of the quantity of released mediators in each well in columns 7-12 as a percentage of the total mediators inside the cells in column 1-6.

4. Enzyme substrate:
5. Add 50 µl of β-hexosaminidase substrate (50 mM 4-nitrophenyl N-acetyl-β-D-glucosaminide prepared in DMSO diluted down to 2 mM by adding it to citrate buffer 0.2 M citric acid and 0.2 M sodium acetate, pH 4.5) to all the wells to facilitated the conversion of the substrate to 4-nitrophenol by the β-hexosaminidase enzyme. Incubate the plates at 37 °C for 2 hr.



5. Terminating the reaction:
    1. Stop the reaction by the adding 150 µl Tris buffer (1 M Tris-HCl, pH 9) to each well as the high pH of the buffer stops the reaction and turns the 4-nitrophenol into a yellow color.

6. Reading and analyzing the results:
    1. Read the plate using a plate spectrophotometer at 405 nm to measure the absorbance of the yellow color. Calculated the percentage of released β-hexosaminidase using the following formula:

$$\left(\frac{A7x100}{(A7x2)+A1}\right)x2$$

2. Apply this formula to each well, after which an average is taken for each row. A1 and A7 represent the location of the wells in the 96 well plate. Plot a graph of percentage of β-hexosaminidase release (which corresponds to total mediator release) against antigen concentration [2, 3].

Representative Results

The parental RBL-2H3.1 cells and those transfected with the equine FcεRIα receptor gene were first sensitized with mouse IgE anti DNP-HSA and challenged with the DNP-HSA antigen. Mouse IgE binds to the endogenous rat receptor in both cell lines and thus acts as a control to test the release viability of both cell lines to release mediators (**Figure 1 A**). This is an important check and should be carried out routinely since upon extended (> 10 weeks) passage in cell culture, RBL-2H3.1 cells drift towards the non-secreting phenotype [27]. The parental cells supported a peak mediator release of 51.54% ± 4.79%, while the cells expressing the equine FcεRIα receptor had a peak mediator release of 45.99% ± 5.76%. These same cell lines where then sensitized with equine IgE anti NIP-HSA and challenged with the NIP-HSA antigen (**Figure 1 B**). Parental cells did not undergo mediator release, since the equine IgE does not bind to the endogenous rat receptor. On the other hand, as expected, RBL-2H3.1 cells expressing the equine FcεRIα receptor underwent mediator release when sensitized with equine IgE anti NIP-HSA and challenged with the NIP-HSA antigen, giving a peak release of 36.68% ± 4.88%.

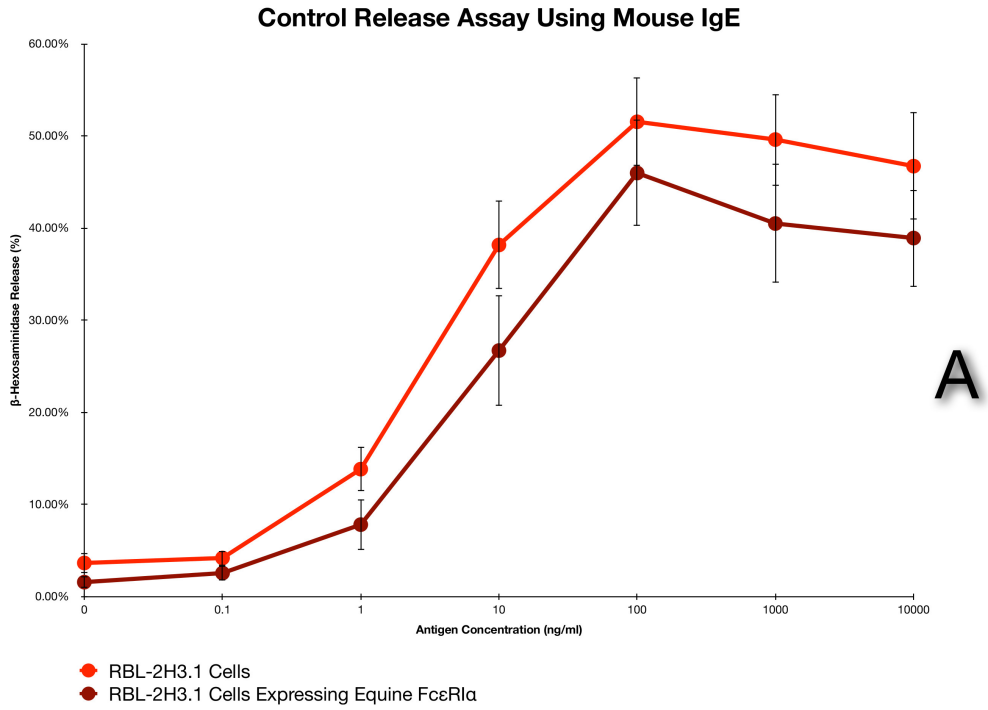
These results confirm the efficiency of this release assay protocol in the investigation of mediator release via equine IgE receptor. It shows that this cell line is a useful diagnostic tool in the quest to asses novel therapeutic intervention strategies in equine allergy *in vitro* reducing the need for animal experimentation. The same protocol is also applicable to RBL-2H3.1 cells transfected with the human and canine FcεRIα receptors.

This protocol was also used to test the safety of a potential immunization strategy that attempts to neutralize canine serum IgE antibodies [30]. From the results in (**Figure 2**) it can be determined that the immunization strategy is not safe. The anti-canine-IgE serum successfully bound to the canine IgE as was originally intended in order to neutralize serum IgE and prevent it from binding onto its receptor. But this binding was unspecific, and thus the anti-IgE serum cross linked the receptor on the cells' surface, resulting in mediator release, potentially resulting in an anaphylactic shock if this immunization strategy was used as an anti-allergy vaccine.

RBL-2H3.1 Expressing Equine FcεRIα	-	-	Produced in the lab
Equine IgE anti NIP-HSA	-	-	Produced in the lab
96 Well Plate	Sigma	CLS3595	-
Multi Channel Pipette	Anachem	-	-
Incubator	Galaxy R	-	-
4Hydroxy-5-iodo-3-nitrophenylacetic acid	Cambridge Research Biochemicals	N-1070-1	NIP-OH was conjugated with Human Serum Albumin to make NIP-HSA in the lab
Dinitrophenyl Conjugated to Human Serum Albumin	Sigma	A6661	Abbreviated DNP-HSA
Plate Spectrophotometer	Anthos Labtec HT2	-	-
Pipes	Sigma	P1851	-

Sodium Chloride	Sigma	S7653	-
Potassium Chloride	Sigma	P9333	-
Magnesium Chloride	Sigma	M2670	-
Calcium Chloride	Sigma	C1016	-
Triton x100	Sigma	X100	-
4-nitrophenyl N-acetyl-β-D-glucosaminide	Sigma	N9376	Stock solution called β-hexosaminidase substrate was 50mM prepared in DMSO
Dimethyl Sulfoxide	Sigma	D2650	-
Citric Acid	Sigma	251275	-
Sodium Acetate	Sigma	S7670	-
Tris	Sigma	T5941	-

Table 1: Table of Material and Equipment:



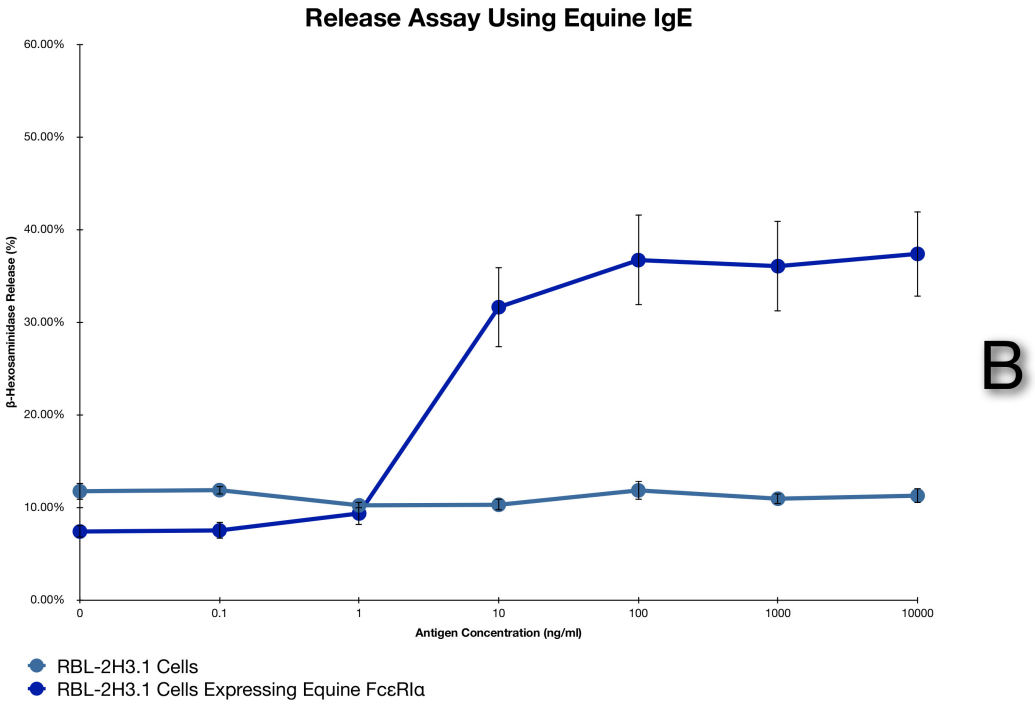


Figure 1: Release assay results: Graph **A** shows the release assay preformed on RBL-2H3.1 cells not expressing equine FcεRIα and expressing the receptor using mouse IgE anti DNP-HSA. The mouse IgE binds to the endogenous rat receptor resulting in mediator release when challenged with the DNP-HSA antigen, thus confirming that both cell lines are viable and support IgE-mediated antigen induced mediator release [2, 3]. Graph **B** shows the release assay preformed on RBL-2H3.1 parental cells which not express equine FcεRIα and those expressing equine FcεRIα, using equine IgE anti NIP-HSA for cell sensitization. The equine IgE binds to the equine receptor, but not the endogenous rat receptor, to result in mediator release, this is confirmed since the parental cells did not release mediator mediators, while the cells expressing the equine receptor support equine IgE-mediated, antigen induced, mediator release [2, 3].

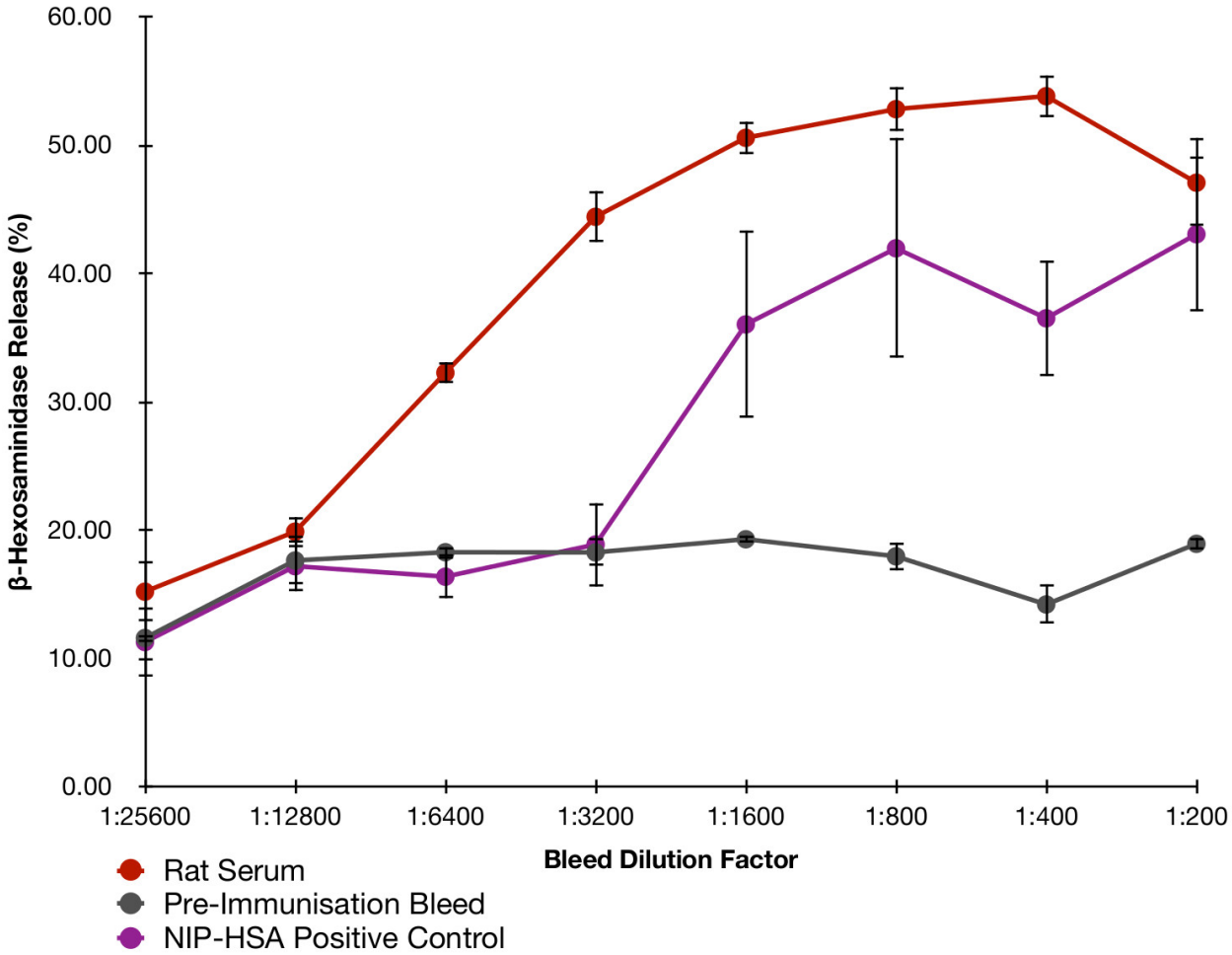


Figure 2: Immunization release assay results: The graph shows the release assay preformed on RBL-2H3.1 cells expressing canine FcεRIα, using canine IgE anti NIP-HSA for cell sensitisation, and immunized rat anti-canine-IgE serum as the antigen. The control used was pre-immunized serum, and the positive control was NIP-HSA antigen. The canine IgE binds to the canine receptor. Furthermore, the anti-IgE serum binds to the receptor bound canine IgE and cross links it, and the receptor, on the cells' surface resulting in mediator release. This, in effect, proves that this particular immunization strategy has the potential to cause an anaphylactic shock reaction, and is thus considered not safe [30].

Discussion

In summary the results of this investigation showed that when RBL-2H3.1 cells expressing equine FcεRIα are sensitized with equine IgE and challenged by an antigen, they give a peak mediator release of 36.68% ± 4.88% of the total amount of mediator inside the cells, compared to the RBL-2H3.1 parental cells not expressing equine FcεRIα.

Thus this assay provides a useful tool for investigating and studying equine allergic responses *in vitro*. Its allows the determination of the quantity of mediator released by cells of mast cell/basophil lineage and thus can be expected to advance research in equine allergy, whether assessing allergy causing agents, or researching IgE/receptor blocking agents.

This assay was modified to study equine allergy from previous publications that used it to study human and canine allergies [4529] for the same purposes. The engineered cell lines expressing human and canine FcεRIα were employed to study the efficacy and safety of allergy blocking agents such as vaccines targeting the inhibition of IgE/receptor interaction [30]. The investigation revealed an anaphylactogenic side effect of immunogens comprising the entire Cε3 domain, which was also proposed by others as a vaccine for attenuating IgE-mediated allergic responses in dogs [24]. Based on our investigation, the molecular basis for this potentially serious complication could be rationalized on the basis of recognition of epitopes in the IgE antibody by the anti-IgE immune serum containing anti-IgE antibodies which target epitopes in Cε3 and are not obscured when IgE is complexed to its receptor. This observation could not have been made in the absence of this assay system and is in striking contrast to the investigation by [24], who also investigated the anti-allergy intervention strategy based on *in vivo* immunization with Cε3 domains, but have not discussed the safety of the strategy due to the absence of such an assay.

The critical steps of this protocol is to ensure that the majority of the RBL-2H3.1 cells are of the secreting phenotype; having the cells in culture greater than 10 weeks causes them to start to drift towards the non-secreting phenotype [27] regardless of whether they expressed the cloned FcεRIα of interest or not. Therefore it is always essential to run mouse IgE positive controls alongside the experiment (**Figure 1**), and to maintain a large frozen stock of secreting cells.

Furthermore, the pH of all buffers must be accurate or they risk giving false negative results; extending the shelf-life of buffers alters their pH, and thus any buffer older than 2 months needs to be discarded.

The technique shows the interaction of a chemical with the RBL-2H3.1 cells, and how it promotes, or suppresses, their mediator release *in vitro.* This is a good indication of what might happen *in vivo,* thus this protocol is an essential step when researching potential anti-allergy therapeutic strategies. The technique does not, however, indicate which part of the antibody/receptor complex the molecule of interest interacts with.

Other research work, such as [20] have indicated that equine RAO is not related to IgE mediation, which is why some discrepancy can be seen with other publishers such as Moran *et al.*[31] when using total equine serum to test to Ca^{2+} influx from RAO suffering horses using unspecific reaginic antibodies, for which different antibodies would be responsible for the cellular Ca^{2+} influx.

This assay is specific for IgE mediated hypersensitivities, which can also be applied to other organisms, such as humans and dogs [29], and specifically used to test the safety of anti-allergy intervention strategies, such as synthetic/chimeric anti-allergy antibodies [30].

Disclosures

The authors declare that they have no competing financial interests in this paper.

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