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Short communication

Cloning and expression of the extra-cellular part of the alpha chain of the equine high-affinity IgE receptor and its use in the detection of IgE

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Abstract

The high-affinity receptor for IgE (FcεRI) plays a central role in IgE-mediated allergic reactions. Cross-linking of FcεRI by IgE-antigen complexes results in the activation of mast cells and basophils and is thought to contribute to the immunopathology of Heaves, a chronic obstructive pulmonary disease of horses. Recombinant protein corresponding to the extra-cellular portion of the FcεRI alpha subunit, cloned and sequenced previously [McAleese, S.M., Halliwell, R.E.W., Miller, H.R.P., 2000. Cloning and sequencing of the horse and sheep high-affinity IgE receptor α chain cDNA. *Immunogenetics* 51, 878–881], was expressed using both mammalian cells and insect cells. The yield of expressed protein was considerably greater using insect cells and the baculovirus expression system. The recombinant proteins differed in size between the two systems, presumably due to differences in the extent of glycosylation. However, recombinant protein from both cell systems bound equine IgE present in bronchoalveolar lavage fluid from horses with Heaves. These results suggest that the recombinant extra-cellular part of FcεRI should be a useful tool with which to study equine IgE responses.

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1. Introduction

Domesticated animals such as horses are exposed to many of the same environmental allergens encountered by farmers and other individuals suffering from occupational asthma. Horses exposed to stable dust may suffer from a chronic obstructive

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pulmonary disease (Heaves) that involves mast cell activation and IgE responses to fungal allergens (Halliwell et al., 1993; McGorum et al., 1993; Schmallenbach et al., 1998). FcεRI, the high-affinity receptor for IgE, is expressed on the surface of mast cells and basophils. It plays an important role in IgE-mediated allergic reactions that are initiated by the binding of the Fc domain of IgE to this receptor and the cross-linking of the receptors in the presence of sensitising allergens. FcεRI is a tetrameric, transmembrane protein comprising one alpha, one beta and two gamma subunits. The extra-cellular portion of the alpha subunit is responsible for binding IgE (for review, see Kinet, 1999). The complete cDNA sequences for both the equine and ovine receptors have now been determined (McAleese et al., 2000; McAleese and Miller, 2003). The aim of the present study was to express the extra-cellular part of the alpha subunit of the equine receptor as a recombinant protein and determine its ability to bind equine IgE. This will enable functional analyses of the role of this high-affinity receptor during naturally induced allergic responses.

2. Material and methods

2.1. *FcεRIα* expression in mammalian cells

The extra-cellular part of the equine FcεRIα was amplified by PCR using a clone of the complete sequence as target; forward primer GGCGCCGCCA-CATGCCTGCTCCCATGGGAAGC which includes twelve bases (KOZAC sequence) 5' to the initiation codon ATG, reverse primer GGCTTTTTT-ACAGTAATGTTGAGGTA which corresponds to amino acids 192–200 (McAleese et al., 2000). The product was cloned into the vector pcDNA3.1TM/V5/His-TOPO (Invitrogen Life Technologies, Paisley, UK). This vector adds a V5 epitope and a six-histidine tag to the expressed protein that can then be identified with an anti-V5 antibody conjugate, and recovered via the C-terminal polyhistidine sequence using metal-affinity chromatography. This plasmid is designed for expression of proteins in eukaryotic cells. *Escherichia coli* cells (TOP10) were transfected and colonies containing the correct plasmid were identified by restriction enzyme analysis. The insert in the selected

clone was sequenced to confirm identity. Purified plasmid was then prepared using a QIAGEN Plasmid Maxi kit (QIAGEN Ltd., Crawley, UK). COS-7 cells were transfected by electroporation (Colosimeo et al., 2000), and stable cell lines created by selection using G418 antibiotic at 400 µg/ml. (Invitrogen Life Technologies). Recombinant protein was detected in the cell culture medium from several clones. The soluble recombinant protein was recovered from the culture medium with Ni-NTA Agarose (QIAGEN Ltd.), detected on a Western blot with an anti-V5-HRP conjugate (Invitrogen Life Technologies) and visualised using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences, Chalfont St. Giles).

2.2. *FcεRIα* expression in insect cells

To prepare recombinant baculovirus, the alpha chain sequence previously inserted into the vector pcDNA3.1, together with the V5 epitope and six-histidine tag, was cut out and ligated into the baculovirus transfer vector pVL1393 (BD Biosciences, Oxford, UK). Vectors containing the inserted sequence in the correct orientation were identified by restriction enzyme analysis, and correct identity was confirmed by DNA sequencing. This construct was mixed with linearised BacPAK6 viral DNA in the presence of LipofectinTM (Invitrogen Life Technologies) and the DNA–lipid complex formed used to co-transfect insect cells Sf21. Clonal recombinants were isolated by plaque purification. One positive clone was selected and used to infect Sf21 cells for expression studies. Supernatant from the cells was analysed by Western blot as detailed above, and high levels of recombinant protein production was observed. High FiveTM cells (Invitrogen Life Technologies) were used to produce larger quantities of purified recombinant protein, due to their ability to be cultured in serum-free medium.

2.3. Purification of soluble recombinant *FcεRIα*

Soluble recombinant protein (solFcεRIα) was recovered from cell culture medium using Ni-NTA Agarose (QIAGEN Ltd.) in 50 mM sodium phosphate buffer [pH8], containing 300 mM NaCl and 5 mM imidazole. Cell culture medium was centrifuged (400 × g for 10 min) to remove dead cells, dialysed overnight into the phosphate buffer and centrifuged

again ($10,000 \times g$ for 20 min) to remove any insoluble material. Ni-NTA Agarose (2 ml of 25% slurry to 50 ml) was added and mixed gently by inversion on a turntable for 2 h or overnight at 4 °C. The agarose was recovered by centrifugation ($400 \times g$ for 2 min) and washed with sodium phosphate buffer containing 500 mM NaCl and 20 mM imidazole. Recombinant protein was eluted using buffer containing 100 mM imidazole.

2.4. Detection of equine IgE using soluble recombinant FcεRIα

Bronchoalveolar lavage fluid (BALF) samples were obtained from three horses that exhibited Heaves symptoms after challenge with soluble hay dust extract (Pirie et al., 2002) and one clinically normal control. Samples were centrifuged for 5 min at $600 \times g$ to remove leucocytes before use. BALF heated at 56 °C for 4 h to denature IgE was used as a negative control for dot-blots and Western blots.

Dot-blots were prepared by spotting 5 µl BALF on to strips of Hybond C Extra (Amersham Biosciences). After air-drying, blots were blocked with 5% dried milk in Tris buffered saline (TBS), pH7.5. To demonstrate binding of solFcεRIα to IgE, blots were incubated with the recombinant protein in 5% dried milk in Tris buffered saline containing 0.5% Tween20 (TBST) for 2 h at room temperature. The blots were then probed with anti-V5-HRPO and visualised by ECL or for the control strip, incubation with solFcεRIα was omitted in order to detect non-specific binding of anti-V5-HRPO to the BALF.

Western blots of solFcεRIα were incubated with native and denatured BALF from Heaves and control horses, diluted 1:1 in TBST + 2% dried milk. Bound IgE was detected using a rabbit anti-equine IgE after cross-absorption against equine IgG (Halliwell et al., 1993) followed by anti-rabbit IgG–HRPO conjugate (Sigma–Aldrich) and visualised by ECL. Control strips were included in which the anti-equine IgE step was omitted. This would detect any non-specific binding of the anti-rabbit IgG–HRPO to bound BALF components.

3. Results and discussion

Human FcεRIα has seven N-linked glycosylation sites and is known to be highly glycosylated (40%

carbohydrate; Garman et al., 1998). The equine alpha chain has eight potential sites so may be glycosylated to a similar degree. FcεRIα also has four conserved cysteine residues that are thought to be involved in disulphide bridges. For these reasons, our initial intention was to express recombinant protein in mammalian cells to ensure correct processing of the product. Further expression studies used a recombinant baculovirus in insect cells. This method should also result in correctly processed recombinant protein and produce higher yields.

The apparent molecular mass of the recombinant protein produced in mammalian cells was 40–45 kDa (Fig. 1). This is about 50% higher than the predicted value for the peptide including the tag (28 kDa). The difference was probably due to extensive glycosylation. A Western blot of proteins from the cell pellet showed multiple bands when probed with anti-V5-HRPO, possibly indicating protein at different stages of processing/glycosylation (data not shown). The yield of soluble expressed protein from stably transfected COS-7 cell lines was low and although transient expression (harvesting after 48 h) produced higher concentrations, this method was impracticable for preparing sufficient protein for use in IgE-binding studies. An insect expression system was, therefore,

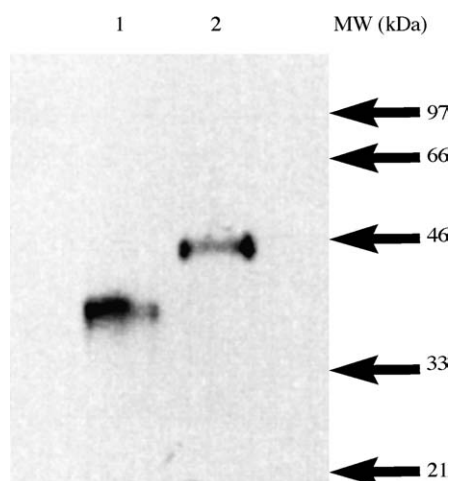


Fig. 1. Western blot analysis of purified solFcεRIα. The blot was probed with anti-V5-HRPO and visualised by ECL. Molecular weights are given in kilodaltons on the right side. Lane 1, solFcεRIα expressed by baculovirus in High Five™ insect cells. Lane 2, solFcεRIα expressed in COS-7 cells.

investigated to increase yield, while maintaining glycosylation and functional activity.

Protein produced in insect cells using recombinant baculovirus was of a smaller apparent molecular mass than that produced in mammalian cells. A 35 kDa band corresponding to recombinant solFcεRIα, expressed in High Five™ cells using the baculovirus system, was faintly detected by SDS-PAGE when stained with Coomassie Blue. Fig. 1 shows a Western blot of solFcεRIα detected with anti-V5-HRPO and visualised using ECL. The difference between the molecular masses of the solFcεRIα protein expressed in insect (Fig. 1: lane 1) and mammalian cells (Fig. 1: lane 2) is clearly evident. Differences between glycoprotein products of insect cells and those expressed in mammalian cells have been described previously (Whalley et al., 1995) and are likely to be due to differences in post-translational processing, particularly glycosylation.

Two methods were used to demonstrate that solFcεRIα can bind to equine IgE. Fig. 2A shows dot blots of BALF probed with solFcεRIα followed by anti-V5-HRPO. Low-level non-specific staining was observed in dot-blots of BALF from both “Heaves” and control horses probed with anti-V5-HRPO only (Fig. 2A: strip 1). This may be due to non-specific binding of the anti-V5-HRPO conjugate or to the presence of endogenous peroxidase activity in the BALF. Generally, solFcεRIα from both baculovirus (Fig. 2A: strip 2) and mammalian (Fig. 2A: strip 3) expression systems gave similar results, although labelling of BALF from control horse L (lane 1) was more intense with mammalian solFcεRIα. Dot-blots of BALF from Heaves horses were labelled more intensely with solFcεRIα (lanes 2 and 4) than dot-blots of BALF from control horse L (lane 1). Heating BALF for 4 h at 56 °C, to denature IgE, reduced the labelling intensity obtained with both forms of solFcεRIα (lanes 3 and 5). Fig. 2B shows Western blots of solFcεRIα incubated with BALF to allow IgE to bind, then developed with rabbit anti-equine IgE (Halliwell et al., 1993), after cross-absorption against equine IgG. Results from three Heaves horses indicate strong binding of equine IgE by solFcεRIα (lanes 1, 5 and 9), which was substantially abrogated when BALF was heated for 4 h at 56 °C to denature IgE (lanes 2, 6 and 10). Minimal non-specific binding was seen with a sample from the control “non-Heaves” horse (lane

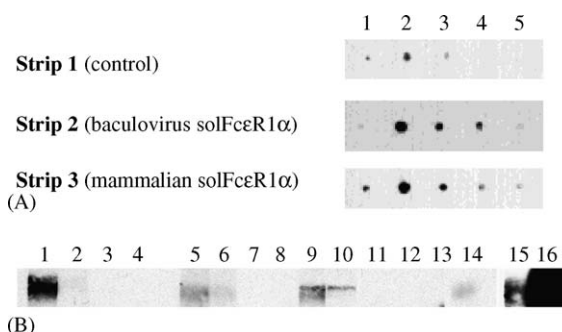


Fig. 2. Demonstration of solFcεRIα binding to equine IgE by immunoblotting. (A) Dot-blots of BALF from three horses before and after heating at 56°C to denature IgE. Lane 1, BALF from control horse (L); lane 2, BALF from heaves horse (H); lane 3, heat-treated BALF from heaves horse (H); lanes 4, BALF from heaves horse (M); lanes 5, heat-treated BALF from heaves horse (M). Strip 1 (control) was incubated with buffer alone, strip 2 was probed with baculovirus solFcεRIα and strip 3 was probed with mammalian solFcεRIα. Bound solFcεRIα was subsequently probed with anti-V5-HRPO and visualised by ECL. (B) Western blots of baculovirus solFcεRIα cut into strips and incubated with: BALF from heaves horse H (lanes 1 and 3); heat-treated BALF from heaves horse H (lanes 2 and 4); BALF from heaves horse N (lanes 5 and 7); heat-treated BALF from heaves horse N (lanes 6 and 8); BALF from heaves horse M (lanes 9 and 11); heat-treated BALF from heaves horse M (lanes 10 and 12); BALF from control horse L (lane 13); recombinant equi-murine IgE (lane 15); or buffer alone (lanes 14 and 16). Lanes 1, 2, 5, 6, 9, 10, 13 and 15 were subsequently probed with rabbit anti-equine IgE, this step was omitted for lanes 3, 4, 7, 8, 11, 12, 14 and 16. All lanes were then probed with anti-rabbit IgG-HRPO, with the exception of lanes 14 and 16, which were probed with anti-V5-HRPO to detect solFcεRIα directly. HRPO activity was visualised by ECL, exposed to film for 5 (lanes 1–14) or 30 min (lanes 15 and 16).

13) or when incubation with rabbit anti-equine IgE was omitted (lane 3, 4, 7, 8, 11 and 12). Lane 14 shows solFcεRIα detected directly with anti-V5-HRPO. Both the dot-blot and Western blotting results indicate that solFcεRIα binds to a component in equine BALF which is most likely to be IgE. This was confirmed using a sample of recombinant equi-murine IgE (Fig. 2B: lane 15), gifted by Dr. B. Wagner (Wagner et al., 2002).

Attempts to develop reagents for studying IgE responses in the horse have, until recently, been hampered by a combination of the difficulty of obtaining purified equine IgE and its lack of homology with IgE from other species. Two different approaches to solving this problem have been adopted. Polyclonal antiserum, raised against a peptide antigen corre-

sponding to amino acids 186–300 in the C2 region of the IgE heavy chain, has recently been shown to recognise equine IgE and has been adapted for use in a semi-quantitative enzyme linked immunosorbent assay (ELISA) and Western blot analysis (Kalina et al., 2003). Using an alternative approach, Wagner et al. (2003) recently generated a panel of monoclonal antibodies against a recombinant chimeric equi-murine IgE, a sample of which was used to confirm binding of solFcεRIα to IgE in the current study (Fig. 2B: lane 15). Several monoclonals from this panel are specific for native equine IgE and binding inhibition studies have identified at least two different epitope specificities within the panel, allowing their use in ELISA as well as flowcytometry and Western blot assays (Wagner et al., 2003).

In the current manuscript, we show that the extra-cellular part of FcεRIα can be expressed as a soluble recombinant protein that retains the ability to bind equine IgE. One advantage of solFcεRIα, over IgE specific antibodies, is that it was and can be produced without use of laboratory animals. Secondly, it should be possible to use solFcεRIα as a competitive “decoy receptor” with which to block IgE mediated allergic responses. Whilst this may be impractical with respect to clinical disease management, it could be used to confirm the presence of allergen specific IgE in the serum of horses using the Prausnitz-Kustner reaction. In addition, it should also be possible to use solFcεRIα to generate antibodies. As such, solFcεRIα provides both a novel and specific alternative to anti-equine IgE antibodies and a potential candidate for raising FcεRIα specific antibodies, with which to study allergic inflammation in horses.

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University of Durham, did DNA sequencing. The experiments described in this report were conducted according to UK legislation.

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