

# Porcine IgE in the context of experimental food allergy: Purification and isotype-specific antibodies

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## Abstract

Measurement of allergen-specific immunoglobulin E (IgE) is a common practice in the investigation of allergy. It has not been possible to measure porcine IgE due to unavailability of anti-porcine IgE. This study was undertaken to purify and characterize porcine IgE from sera of allergic pigs, identify heterologous anti-IgE reactive with pig IgE and to use purified heavy (H) chain of porcine IgE to generate rabbit anti-IgE. A four-step process for the purification of porcine IgE is reported using ammonium sulphate precipitation, Protein G affinity chromatography, DEAE cellulose anion-exchange chromatography and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to obtain IgE H chain. The resultant IgE was evaluated for purity using SDS-PAGE and immunoreactivity was detected by Prausnitz–Küstner (PK) tests and passive cutaneous anaphylaxis with the allergen, crude peanut extract, used to induce experimental allergy. Cross-reactivity with anti-mouse and anti-human IgE antibodies were confirmed in western blot and enzyme-linked immunosorbent assays (ELISA). The H chain of IgE was excised from SDS-PAGE gels and used to develop rabbit anti-porcine IgE antisera. Antiserum obtained from rabbits immunized with porcine IgE, as well as heterologous murine and human-specific anti-IgE, induced reverse cutaneous anaphylaxis in pig skin and detected allergen-specific IgE in ELISA but did not react with IgG H chain in western blots. These results confirm allergy-associated bioactivity of porcine IgE and describe both homologous and heterologous anti-pig IgE suitable for use in allergen-specific and other assays. This will enhance utility of pig allergy models and provide an additional measure of type-2 immune response in pigs.

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## 1. Introduction

Recent increase in human immunoglobulin E (IgE)-mediated allergic diseases is associated with altered environmental risk factors in developed countries and susceptibility associated with genotype (Renz and Garn, 2007). Immunoglobulin E is a glycoprotein with a molecular weight (MW) of 190 kiloDaltons (kDa) which binds to Fc receptors on mast and other cells and

triggers degranulation when cross-linked by allergen, leading to release of mediators responsible for allergic reactions (Untersmayr and Jensen-Jarolim, 2006; Jane-way et al., 2001). Evaluation of allergen-mediated hypersensitivity can involve intradermal skin tests with specific allergens, Prausnitz–Küstner (PK) or passive cutaneous anaphylaxis (PCA) tests and allergen and immunoglobulin isotype-specific enzyme-linked immunosorbent assays (ELISA). Using these tools, development of phenotypically and genotypically defined large outbred animal models may contribute to better understanding of the complex immunopathogenesis of IgE-mediated allergic diseases.

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Outbred animal models that mimic allergic responses in humans are desirable to validate prophylactic and immunotherapeutic interventions that can be extrapolated to humans. To this end, pigs have been made allergic to peanut and the egg allergen ovomucoid (Ovm) (Helm et al., 2003; Rupa et al., 2007). Pigs may better reflect genetic and environmentally influenced variables affecting allergic predisposition in humans in contrast to inbred rodent-based models which have limited genetic and immunological phenotypic variation (Atkinson and Leitner, 1999; Mestas and Hughes, 2004). Pigs exhibit significant intra- and inter-litter individual variation in immune response phenotype (Mallard et al., 1992; Crawley et al., 2005; de Groot et al., 2005). Experimental food allergy in neonatal pigs resembles human food allergy with respect to acute gastrointestinal signs including diarrhea, emesis, bleeding, weight loss, cutaneous erythema and respiratory difficulty, as well as other immunologic functions (Helm et al., 2002; Rupa et al., 2007). Immunological correlates include direct skin test reactions and PCA, the latter mediated by heat-labile serum antibody consistent with IgE (Rupa et al., 2007). However, unavailability of anti-pig IgE precludes direct investigation of allergen-specific IgE antibodies in the context of porcine allergic disease.

Immunoglobulin E is present in human serum in a concentration range of nanograms per milliliter and has a short half-life of approximately 2 days, since IgE is removed from blood and tissue fluids by high-affinity Fc receptors on mast cells, basophils and eosinophils making it difficult to purify and characterize IgE. Although indication of immune response bias in pigs may be obtained by evaluating relative antibody activity associated with IgG1 and IgG2 (Furesz et al., 1998; Diaz et al., 2003; Crawley et al., 2003), this is not of direct relevance to allergic signs for which IgE may be more pertinent. Immunoglobulin E has been purified and characterized functionally for various mammals (Nielsen and Wilkie, 1977; Lehrer, 1979; Suter and Fey, 1983; Yilmaz et al., 1993; Peng et al., 1993a; Kleine-Tebbe et al., 1995; Gilbert and Halliwell, 1998). Although antibodies to porcine IgE have been reported (Roe et al., 1993), they are not now available from any source. Porcine IgE cDNA and predicted amino acid sequences indicate sequence homology to human IgE of >70% (Vernersson et al., 1997).

To address the unavailability of anti-porcine IgE and to associate IgE-related antibodies with allergy in pigs, the present report describes purification of IgE from anti-allergen-rich serum of pigs experimentally sensitized to peanut antigens. The IgE heavy (H) chain was

used to induce rabbit anti-pig IgE antibodies. Functional assays, molecular weight estimates and antigenic cross-reactivity were used to confirm that porcine IgE had been isolated. In addition, monoclonal anti-human and anti-mouse IgE antibodies reactive with porcine IgE were identified. Both homologous and heterologous anti-IgE antibodies induced reverse cutaneous anaphylaxis (RCA) and detected allergen-specific IgE antibodies in pig sera by ELISA as well as IgE or IgE H chain in western blots.

## 2. Materials and methods

### 2.1. Experimental design and animals

The study was designed to identify and purify IgE from allergic pig sera, to identify cross-reactive anti-IgE antibodies and to make rabbit anti-pig IgE. Two litters of Yorkshire pigs (10 days old), having 12 or more piglets/litter were maintained under specific pathogen-free conditions at the Arkel Swine Research Station, University of Guelph. Allergy was induced to crude peanut extracts (CPE) using cholera toxin (CT) as adjuvant and IgE was purified from allergic pig serum. Littermates were reared by the natural mother. The sow and piglet diets were devoid of peanut proteins. Equal numbers of animals within each litter were assigned randomly to four treatments in a split litter design ( $n = 3$  per treatment group). Animal use was approved by the local Animal Care Committee under guidelines of the Canadian Council for Animal Care.

### 2.2. Purification of crude peanut extract

One liter (1 L) of extraction buffer (1 mM Tris–EDTA, pH 8.3; 11.6 g of NaCl; 0.769 g of dithiothreitol; 0.5 mM of phenyl methane sulphonyl fluoride; 0.02% of sodium azide) was added to 60 g of ground Georgia red peanuts and the mixture was stirred at room temperature for 1 h. The mixture was filtered through cheese cloth and the filtrate centrifuged at  $3000 \times g$  for 30 min at 4 °C (Beckman J2-21M/E centrifuge; Beckman Instruments Inc., Palo Alto, CA, USA). The pellet was discarded and the liquid beneath the white fatty layer was removed and precipitated with 25% saturated ammonium sulphate by stirring for 30 min at 4 °C. After centrifugation ( $10,000 \times g$ , 1 h, 4 °C) the pellet was dissolved in phosphate-buffered saline (pH 7.2; PBS) and dialyzed against PBS overnight in 6–8 kDa cut-off tubing (Fisher Scientific, Pittsburg, USA). The resulting material designated CPE was analyzed by SDS-PAGE and protein concentration determined colorimetrically

(DC protein assay; Biorad Laboratories, Mississauga, Canada) with bovine serum albumin (BSA) as standard.

### 2.3. Sensitization and oral challenge with CPE

Peanut allergy was induced in pigs using CPE as antigen with CT as adjuvant (Helm et al., 2002). One hundred micrograms of CPE were injected intraperitoneally with various doses (10, 25 or 50 µg; Groups 1–3) of CT (List Biological Laboratories Inc., USA) in 200 µl of PBS on days 14, 21, and 35. A fourth group (adjuvant control) received 50 µg of CT alone. Blood was collected for serum on days 10 (pre-sensitization sera), 21, 35, 45 and 46 to detect anti-CPE antibodies. To test for allergy, on day 45, all animals were fasted overnight and challenged orally on day 46 with 10 g of peanut meal mixed in yoghurt at a ratio of 2:3 (w/v) administered using a 60 ml Monoject syringe with Toomey tip (Tyco Healthcare Group LP, Mansfield, MA, USA) and attached polypropylene tube. Pigs were monitored after the challenge (0–60 min) for signs of allergic hypersensitivity. Epinephrine was given as required to control severe allergic signs.

### 2.4. Intradermal skin testing

To test for immediate hypersensitivity reactions, on days 10 (baseline), 21 and 35, 0.1 ml of CPE (100 µg) or PBS were injected intradermally (ID) using a tuberculin syringe and 28-gauge needle. Marked injection sites were on each inner thigh. Injections were performed by one investigator and tests were considered positive if a weal and flare reaction was visible within 15 min of injection with consensus amongst three to four blinded observers.

### 2.5. Clinical scores

The response of individual pigs to oral challenge with CPE was graded using consensus clinical scores from at least three independent observers who were blinded to treatments (Rupa et al., 2007). Total scores were obtained by adding individual scores assigned as: 0 = no signs; 1 = immobility, lethargy, malaise, scratching, rash; 2 = diarrhea, emesis; 3 = increase in respiratory rate, neck extension; 4 = forced expiration; 5 = confluent cutaneous reddening, cyanosis, anaphylaxis.

### 2.6. Serum antibody to CPE

Sera collected on days 10, 21, 35, 45 and 46 were analyzed for anti-CPE IgG (whole molecule, Sigma,

Oakville, ON, Canada) antibody by ELISA. Polystyrene, high-binding 96-well flat-bottom plates (Immulon 2HB; VWR International, Mississauga, Canada) were coated overnight with 100 µl/well of 100 µg/ml of CPE in carbonate buffer (0.05 M; pH 9.6) at 4 °C. The plates were washed three times (Automatic plate washer, ELX405; BioTek Instruments Inc., Winooski, VT, USA) with PBS containing 0.05% Tween 20 (PBST; 200 µl/well) followed by blocking the wells with 200 µl/well of 3% Tween in PBS for 1.5 h at room temperature. Plates were washed with PBST three times and each test serum was diluted to 1:100 in PBST, added to triplicate wells (100 µl/well) and incubated for 2 h at room temperature. Plates were further washed three times with PBST and 100 µl of alkaline phosphatase-conjugated rabbit anti-pig IgG (whole molecule) diluted to 1:8000 was added to the plates (100 µl/well) and incubated for 1 h at room temperature. Subsequently, 1 mg/ml of *p*-nitrophenol phosphatase substrate (100 µl/well; Sigma) in 0.1 M diethanolamine (pH 9.8) was added and incubated in the dark at room temperature. Controls included wells without serum and with positive (provided by Dr. R. Helm) and negative (pool of day 10 pre-sensitization) sera. Optical density (OD) was measured using a 96-well plate reader at 405 nm (EL808; BioTek Instruments Inc.) when the OD of the positive control reached 1. The mean OD of each triplicate test serum was expressed as percentage (percent positivity) of the positive control as follows: % change OD = [sample OD / (positive control OD – negative control OD)] × 100.

### 2.7. Purification of serum IgE

Serum IgE-rich fractions were obtained by a protocol adapted from Peng et al. (1993a) and IgE heavy chain was obtained from the final chromatography step by excision from reducing PAGE gels. Sera of CPE-sensitized pigs (days 35, 45 and 46) were pooled (200 ml), diluted with equal volumes of sterile distilled water and precipitated with ammonium sulphate (50% saturation) to separate proteins by differential solubility. The mixture was incubated overnight and centrifuged at 5000 × *g* for 30 min at 4 °C. The pellet was dissolved in sterile PBS and a second precipitation performed (25% saturation) followed by centrifugation as above. The pellet was dissolved in PBS and dialyzed against PBS overnight using a 6–8 kDa cut-off membrane (Fisher Scientific) to remove ammonium sulphate before application to a Protein G affinity column (Sigma). The column was washed with PBS and adsorbed immunoglobulin eluted with 0.1 M glycine–HCl buffer (pH 2.7) into tubes

containing 0.25 ml of phosphate buffer (2 M, pH 7.2). Fractions were pooled, filtered (0.22  $\mu$ m filter, Millex-GV, Millipore Corp., Bedford, MA, USA), and stored at 4 °C. The protein was further purified using diethylaminoethyl (DEAE) cellulose high performance liquid chromatography (HPLC). Immunoglobulin E was eluted using a gradient of 0–1 M NaCl in 20 mM Tris–HCl pH 8.0 over 40 min with a flow rate of 1 ml min<sup>-1</sup> using a Bio-Rad Biologic HPLC system (Bio-Rad Laboratories, Hercules, CA, USA). Fractions (unbound and eluates) were collected in 1 ml aliquots and analyzed by SDS-PAGE with mouse IgE (BD BioScience, California, USA) as control. The concentration of purified protein was determined by the DC protein assay (Bio-Rad) with BSA as the standard. Immunoglobulin heavy chain (70 kDa) was cut from PAGE gels of the IgE-rich DEAE cellulose purified protein, protein content estimated by PAGE gel densitometry with BSA standards and used to immunize rabbits to produce anti-pig IgE. Each separation step was monitored by SDS-PAGE for IgE size-compatible bands with reference to standard mouse IgE and IgE-related anti-CPE activity confirmed by PCA or PK tests.

## 2.8. Western blot assays

The ability of anti-IgE (human, murine and porcine) to detect pig IgE was compared by western immunoblotting PAGE (reducing and non-reducing) purified IgE fractions (DEAE cellulose and Protein G eluates) transferred onto a nitrocellulose membrane (Millipore). The blots were washed in TBS (0.1 M Tris–HCl, pH 7.4, 0.1 M NaCl, 2.5 mM MgCl<sub>2</sub>) containing 0.05% Tween (TBST) for 15 min and were incubated for 1 h with blocking buffer (1% BSA in TBST) at room temperature. For blots of Protein G eluate, 1% ovalbumin (Sigma) in TBST was used as blocking agent. Biotinylated rat anti-mouse IgE (Invitrogen; diluted 1:2000 in TBST), mouse anti-human IgE conjugated with alkaline phosphatase (Sigma; 1:5000) and rabbit anti-pig IgE sera (1:500) were added to separate blots and incubated for 2 h. All blots were washed three times with TBST buffer and as detection ligands, alkaline phosphatase conjugates of avidin (diluted 1:1000; Vector Laboratories, Burlington, ON, Canada) or alkaline phosphatase-conjugated goat anti-rabbit IgG (whole molecule; 1:8000 dilution; Sigma) were added. Binding was visualized by incubating with the substrate/chromogen mixture (5-bromo-4-chloro-indolyl-phosphate disodium salt and nitroblue tetrazolium chloride (Sigma)). The reaction was stopped with deionized water.

## 2.9. Passive cutaneous anaphylaxis (PCA) and Prausnitz–Küstner (PK) tests

To test for pig anti-CPE associated with IgE, PCA and PK tests were performed using fractions (load, unbound, and eluates) obtained from DEAE cellulose chromatography. Sera were pooled from treatment groups (CT-treated controls and sensitized) and aliquots heated at 56 °C for 4 h to inactivate putative IgE-anti-Ovm PCA-mediating antibodies. For PCA tests, pooled sera and chromatography fractions were injected ID (100  $\mu$ l) into marked sites on the inner thighs of naïve pigs and after 24 h, 5 mg of CPE in 1 ml of PBS was injected intravenously. For PK tests, 0.1 ml of chromatography fractions from various stages of purification were injected as for PCA. After 24 h CPE (0.1 ml; 100  $\mu$ g) was injected ID into the same sites which were then observed (0–20 min) for weal and flare reactions.

## 2.10. Immunization of rabbits

The porcine IgE H chain excised from SDS-PAGE gel of IgE-rich DEAE cellulose chromatography fractions was used to produce rabbit anti-porcine IgE. Protein concentration was estimated by densitometric analysis using as reference standard, data derived from bands developed by known amounts of BSA (1–4  $\mu$ g). Digitalized images of the SDS-PAGE patterns were acquired using a Gel Doc 2000 (Bio-Rad) and analyzed with Image Pro software (Media Cybernetics Inc., Bethesda, MD, USA) to construct a standard curve for estimation of protein concentration. Two New Zealand White rabbits were immunized by Pacific Immunology Corp., Ramona, CA, USA (approved by NIH OLAW Assurance # A4182-01 and USDA Registration # 93-R-283, USA) using the gel slice-derived pig IgE heavy chain (2 mg total). Each rabbit received a primary immunization in complete Freund's adjuvant (CFA in 1:1 emulsion with immunogen) and after 20 days, three immunizations using incomplete Freund's adjuvant at intervals of 20–30 days. Immunized and preimmunization (negative control) rabbit sera were screened by western blot and ELISA for anti-pig IgE activity. Mouse anti-human IgE and rat anti-mouse IgE shown here to detect pig IgE in western blots were used as cross-reactive positive controls.

## 2.11. Reverse cutaneous anaphylaxis (RCA)

Antisera were assessed for binding to pig IgE *in vivo* by their ability to induce RCA due to release of

allergic mediators after cross-linking FcεRI-bound IgE. Rabbit anti-porcine IgE was injected ID at 0.1 ml into a naïve pig. As positive and negative controls, anti-mouse and anti-human IgE (diluted in PBS at 1:5) and PBS were injected into adjacent sites. Preimmunization rabbit sera were also used as negative controls. Injection sites were evaluated over 10–15 min for reactivity.

### 2.12. Anti-IgE and allergen-specific ELISA

Rabbit anti-pig IgE was used to detect allergen-specific IgE antibody activity in the sera of CPE-sensitized and control pigs by ELISA. Briefly 96-well plates (Immulon 2HB, Alexandria, VA) were coated either with porcine IgE-rich fraction (DEAE cellulose purified) at 1 µg/well or with CPE (100 µg/ml) in 100 µl of 0.05 M carbonate/bicarbonate buffer at pH 9.6. The plates were incubated overnight at 4 °C, washed with PBST and blocked for 1 h at room temperature with 200 µl/well of 3% Tween in wash buffer. The plates were washed three times with PBST and for CPE-specific ELISA, 100 µl of pooled serum from pigs (pre- and post-sensitization to CPE) was added to replicates of three wells as a 1:100 dilution in PBST and incubated overnight at 4 °C followed by washing three times with PBST. One hundred microliters of serum from immunized and pre-immunized rabbits were added to both plates as a 1:500 dilution in PBST and incubated for 1 h at room temperature, followed by washing three times. Goat anti-rabbit IgG (Sigma; whole molecule), 100 µl of a 1:10,000 dilution, was added to each well. After 1 h incubation at room temperature, the plates were washed as before. Substrate buffer (polynitrophenol phosphatase (pNPP) tablets, Sigma, St. Louis, MO in 0.1 M diethanolamine buffer) was added at 100 µl/well and incubated for 30 min. Plates were read at a wavelength of 405 nm (EL808; BioTek Instruments Inc.) at 30 min and expressed as the mean OD of triplicate samples.

### 2.13. Data analysis

Data were tested for significance of difference between positive and negative controls in ELISA using GraphPad Prism software (Version 4.02 for windows, GraphPad Software, San Diego, CA, USA). Significance of difference between the groups for each ELISA was assessed by one-way analysis of variance (ANOVA). A value of  $P \leq 0.05$  was taken to indicate statistical significance.

## 3. Results

### 3.1. Direct skin test with allergen

Prior to sensitization, all piglets were nonreactive to intradermally injected CPE. After sensitization, all pigs were reactive to ID injected CPE on day 35. The test sites developed a weal and flare at about 15 min post-injection with diameter usually >1 cm. Skin reactions in response to CPE varied from mild to severe between individuals within each litter. Reactions were not observed at the PBS injection sites.

### 3.2. Oral challenge with CPE

Response to oral challenge with CPE is summarized in Table 1. Clinical signs scored 0–60 min after oral challenge with CPE ranged from mild erythematous skin reactions, facial edema to more severe signs such as confluent cutaneous erythema over the entire body surface and dyspnoea. Seven of nine CPE-sensitized pigs in one litter and 5/6 pigs in the other expressed clinical signs. Cholera toxin-treated control pigs did not develop allergic signs. Individual and litter differences in clinical signs were obvious (Table 1).

### 3.3. Serum antibody to CPE

Pre-sensitization (day 10) and post-sensitization (day 45) sera were analyzed by ELISA to verify CPE-specific antibody. Analysis of variance confirmed significant increase of serum antibodies in both litters of CPE-sensitized pigs from day 10 to day 45 ( $P \leq 0.05$ ). Variation was observed between pigs in CPE-specific antibodies between and within litters. Sera of CT-treated negative control pigs had significantly lower ( $P \leq 0.05$ ) ELISA-OD values than did sera of CPE-sensitized pigs (Table 1).

### 3.4. Purification of porcine IgE

Peanut protein-specific IgE antibodies from 200 ml of pooled serum of pigs sensitized by IP injection of CPE with CT were isolated by sequential ammonium sulphate precipitation, Protein G affinity chromatography, anion-exchange chromatography and PAGE to obtain porcine IgE H chain. Efficacy of separation was monitored by SDS-PAGE and western immunoblot after each step. By SDS-PAGE analysis, several proteins were detected in the fraction obtained by precipitation of pooled serum by ammonium sulphate with molecular weight ranging from 6 to 220 kDa.

Table 1  
Peanut-specific allergy in pigs

| Treatment             | CPE-Sp-IgG, day 45<br>(ELISA-OD values) | Skin test,<br>day 35 | Clinical score,<br>day 46 | Clinical signs, day 46                                                                          |
|-----------------------|-----------------------------------------|----------------------|---------------------------|-------------------------------------------------------------------------------------------------|
| <b>A</b>              |                                         |                      |                           |                                                                                                 |
| 50 µg CT-control      | 23.1214                                 | —                    | 0                         | No response                                                                                     |
| 50 µg CT-control      | 42.2455                                 | —                    | 0                         | No response                                                                                     |
| 10 µg CT + 100 µg CPE | 124.9339                                | +                    | 6                         | Malaise, rash, scratching,<br>increased respiratory rate                                        |
| 10 µg CT + 100 µg CPE | 153.2674                                | +                    | 0                         | No response                                                                                     |
| 10 µg CT + 100 µg CPE | 125.7961                                | +                    | 1                         | Rash                                                                                            |
| 25 µg CT + 100 µg CPE | 123.7583                                | +                    | 10                        | Malaise, rash, scratching, emesis, increased<br>respiratory rate, treated once with epinephrine |
| 25 µg CT + 100 µg CPE | 119.7218                                | +                    | 0                         | No response                                                                                     |
| 25 µg CT + 100 µg CPE | 151.3864                                | +                    | 1                         | Rash                                                                                            |
| 50 µg CT + 100 µg CPE | 110.6692                                | +                    | 2                         | Scratching, rash                                                                                |
| 50 µg CT + 100 µg CPE | 111.1395                                | +                    | 1                         | Rash                                                                                            |
| 50 µg CT + 100 µg CPE | 119.9961                                | +                    | 11                        | Rash, scratching, emesis, increased<br>respiratory rate, treated with epinephrine twice         |
| <b>B</b>              |                                         |                      |                           |                                                                                                 |
| 50 µg CT-control      | 40.34392                                | —                    | 0                         | No response                                                                                     |
| 50 µg CT-control      | 67.23986                                | —                    | 0                         | No response                                                                                     |
| 50 µg CT-control      | 40.12346                                | —                    | 0                         | No response                                                                                     |
| 10 µg CT + 100 µg CPE | 104.7178                                | +                    | 3                         | Malaise, rash scratching                                                                        |
| 10 µg CT + 100 µg CPE | 168.739                                 | +                    | 1                         | Rash                                                                                            |
| 25 µg CT + 100 µg CPE | 182.3633                                | +                    | 5                         | Malaise, rash, scratching, emesis                                                               |
| 25 µg CT + 100 µg CPE | 161.5961                                | +                    | 0                         | No response                                                                                     |
| 50 µg CT + 100 µg CPE | 189.2416                                | +                    | 5                         | Malaise, scratching, rash, emesis                                                               |
| 50 µg CT + 100 µg CPE | 163.0071                                | +                    | 1                         | Rash                                                                                            |

Crude peanut extract (CPE) and cholera toxin (CT) were used to induce allergy in neonatal pigs. Serum antibody to CPE was measured by ELISA using alkaline phosphatase-conjugated rabbit anti-pig IgG (whole molecule) and antibody activity is reported as percentage of positive control serum pool optical density. All CPE-immunized pigs had significantly greater ELISA OD values than negative controls. A and B represent two litters.

Absorption and elution of this material from Protein G yielded multiple bands, including H chains of IgG (~50 kDa) and IgE (~70 kDa), indicating need for additional chromatography. To remove other proteins from affinity-purified Protein G eluates, DEAE cellulose ion exchange chromatography was used. A distinct major peak towards the mid range of the 0–1 M eluting salt gradient contained proteins identified as H (~70 kDa) and light chains (L ~ 23 kDa) of IgE when compared to standard mouse IgE in SDS-PAGE (Fig. 1A) and as whole IgE (H and L chains; 190 kDa) when separated on non-reducing PAGE (Fig. 1B). The majority of IgG was in the unbound fraction from DEAE cellulose chromatography. The putative porcine IgE H chain had an estimated molecular weight of 70 kDa consistent with the standard mouse IgE while the L chain was 23 kDa. The IgE H chain was excised from preparative SDS-PAGE gels and used in immunizing rabbits to produce anti-pig IgE. This procedure yielded from 40 ml of starting pig serum, approximately 4000 µg of pure

porcine IgE H chain protein as estimated by densitometry analysis of PAGE gels using BSA standards (data not shown).

### 3.5. PK and PCA tests

To test the ability of purified pig IgE to mediate allergic reactions, pooled sera from sensitized and control pigs and fractions from DEAE cellulose, confirmed by western blotting to contain IgE (see above) were tested. Only sera of CPE-sensitized pigs induced PCA which was inactivated by heat confirming mediation by IgE-anti-CPE antibodies (data not shown). Control (CT) sera (native and heat inactivated) as well as pre-sensitized sera did not elicit PCA. Both PCA (Fig. 2A) and PK (Fig. 2B) tests were positive with the major DEAE cellulose-eluted peak (at the mid-molarity range) and with the unbound fraction representing overloaded starting material. The positive control serum was positive in both tests.

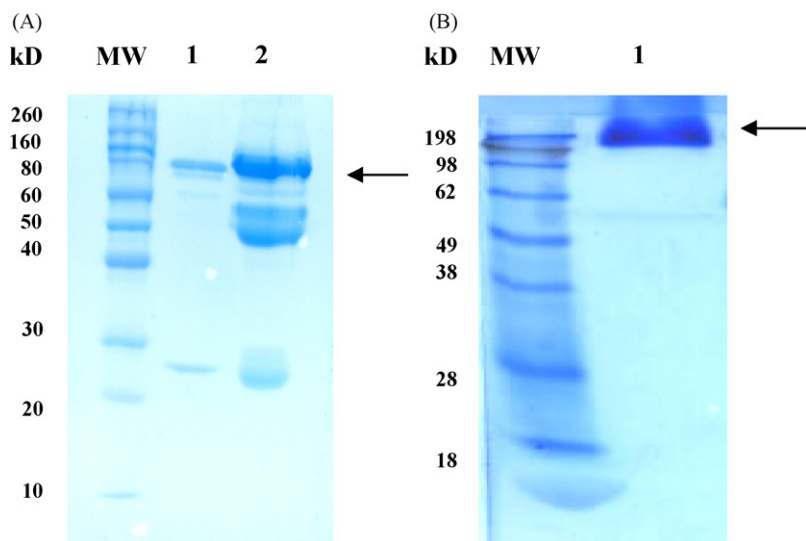


Fig. 1. Polyacrylamide gel electrophoresis (PAGE) of a PCA positive fraction obtained from DEAE cellulose chromatography as the second step in purification of pig IgE from serum of CPE-sensitized pigs. The DEAE cellulose eluate was run on PAGE gel with standard mouse IgE and the gels were stained with Coomassie blue. Molecular size standards (in kDa) are indicated at the left. (A) Reducing gel (SDS-PAGE). MW: molecular weight markers (Invitrogen; Novex Sharp Prestained markers). Lane 1: standard mouse IgE (BD Biosciences); lane 2: DEAE cellulose purified pig IgE eluate fraction. (B) Native gel. MW: molecular weight markers (Invitrogen; SeeBlue Plus2 marker). Lane 1: DEAE cellulose purified pig IgE. Arrows indicate the whole IgE molecule on the native gel and the heavy chain of IgE on the reducing gel.

### 3.6. RCA using rabbit anti-porcine IgE

Reverse cutaneous anaphylaxis was induced by ID injections of anti-porcine IgE from both IgE H chain-immunized rabbits with one having better apparent anti-IgE activity. Anti-mouse and anti-human IgE antibodies also induced RCA (Fig. 3). Neither PBS nor pre-immunization rabbit serum induced reactions.

### 3.7. Binding of rabbit anti-pig IgE, rat anti-mouse IgE and mouse anti-human IgE to purified pig IgE in western blot and ELISA

#### 3.7.1. ELISA

ELISA was performed to test binding of rabbit anti-pig IgE, rat anti-mouse IgE and mouse anti-human IgE to DEAE cellulose purified pig IgE. Sera of both immunized rabbits produced significantly greater OD values by ELISA than did preimmune rabbit sera ( $P \leq 0.001$ ). Both rat anti-mouse IgE and mouse anti-human IgE bound to pig IgE at OD values significantly higher ( $P \leq 0.001$ ) than blank (no sera) using dilutions of antisera as for western blot.

#### 3.7.2. Western blot

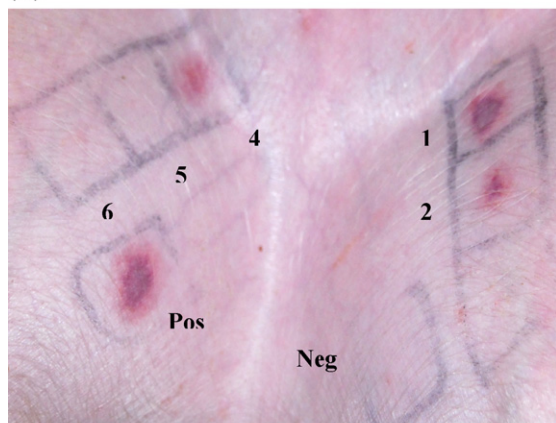
Purified pig IgE was electrophoresed on SDS-PAGE gel (Fig. 4A) and pooled immunized rabbit serum reacted with the purified 70 kDa porcine IgE H chain

protein in western blot as well as the putative L chain at 23 kDa (Fig. 4B). The antisera did not react with the IgG H chain (~50 kDa) in the blots of the Protein G eluate fraction (data not shown). Similarly, post-immunization rabbit sera bound to a 70 kDa band in ammonium sulphate precipitate of sera from pigs sensitized with CPE but not in sera of pre-sensitized pigs. Also, both rat anti-mouse and mouse anti-human IgE monoclonal antibodies cross-reacted with pig IgE and detected a strong band at 190 kDa, the expected MW of the unreduced IgE molecule in native PAGE gels (Fig. 5A and B). Purified standard mouse IgE (BD Bioscience) was used as a reference to compare the MW of mouse with pig IgE. Both heterologous anti-IgE antibodies detected H chain of porcine IgE with a MW of approximately 70 kDa and the L chain of 23 kDa under reducing conditions (Fig. 5C and D) corresponding to the expected molecular weight of porcine IgE H chain. The heterologous antibodies did not bind the IgG H chain (~50 kDa) in the blots of the Protein G eluate (data not shown).

### 3.8. Ability of rabbit anti-pig IgE, rat anti-mouse IgE and mouse anti-human IgE to detect IgE-related pig anti-CPE in ELISA

Anti-CPE-specific IgE was detected using ELISA with CPE-coated wells. Rabbit anti-pig IgE was

(A)



(B)

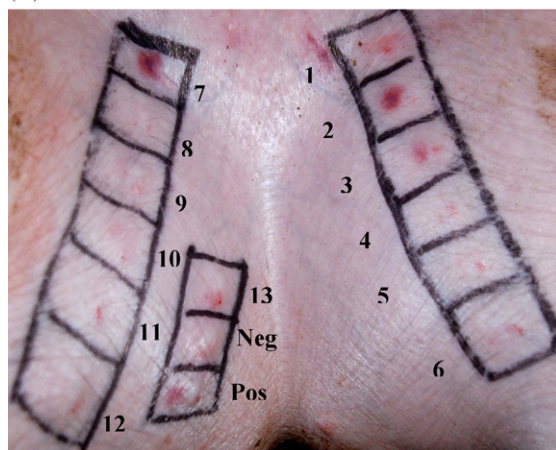


Fig. 2. (A) Passive cutaneous anaphylaxis (PCA) mediated by porcine IgE in materials loaded onto; unbound; and eluted from DEAE cellulose column chromatography. The fractions (0.1 ml) were injected intradermally (ID) into a naïve pig and after 24 h, crude peanut extract (CPE; 5 mg) was injected intravenously to induce PCA. 1: Protein G-eluted sample that was loaded onto the DEAE cellulose column; 2: unbound fraction; 3–6: sequential eluates from increasing molarity salt gradient; Pos: positive control serum (donated by Dr. Helm) and Neg: negative control (pre-sensitized sera). (B) PK reaction mediated by pig IgE. Putative IgE containing materials were injected ID (0.1 ml) into a naïve pig, followed 24 h later by ID injection of CPE (0.1 ml; 100 µg) into the same site. Injection sites were examined for reaction in the period 0–15 min post-injection. 1: Protein G-eluted sample that was loaded onto the DEAE cellulose column; 2: unbound fraction; 3–13: sequential eluates from increasing molarity salt gradient; Pos: positive control (Dr. Helm) and Neg: negative control (pre-sensitized sera).

positive in this assay with sera of CPE-allergic pigs but not of pre-sensitization pigs ( $P \leq 0.001$ ). Cross-reactivity of monoclonal anti-mouse and anti-human IgE with pig IgE was also confirmed in this system by their significantly greater reactivity to CPE-sensitized than to pre-sensitized pig sera ( $P \leq 0.001$ ).

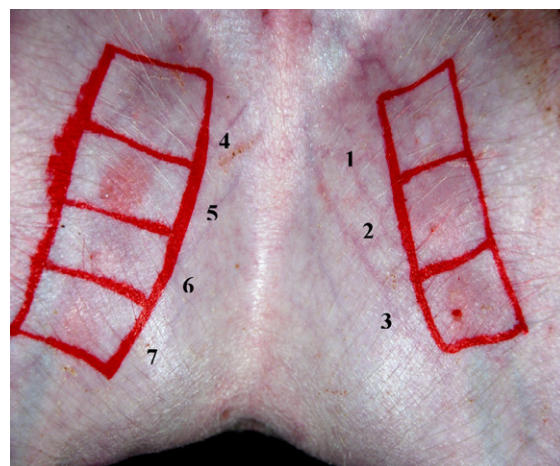


Fig. 3. Reverse cutaneous anaphylaxis (RCA) induced by rabbit anti-porcine IgE, rat anti-mouse IgE or mouse anti-human IgE. Anti-pig IgE or heterologous anti-IgE were injected ID into a naïve pig (0.1 ml) and injection sites were monitored for reaction. Sites were treated as follows—1: PBS (negative control); 2: rat anti-mouse IgE (1:5 dilution); 3: mouse anti-human IgE (1:5 dilution); 4: pre-sensitization serum (rabbit #1); 5: post-sensitization serum (rabbit #1); 6: pre-sensitization serum (rabbit #2); 7: post-sensitization serum (rabbit #2).

#### 4. Discussion

Allergen-specific serum IgE-related antibody may be an important correlate of clinical and experimental allergy (Peng et al., 1993b; Morgan et al., 2007), which has not been exploited in studies of pig allergy due to unavailability of anti-pig IgE and pure IgE protein. To enhance pig allergy research and to correct a deficit in knowledge of pig immunobiology, the present study was undertaken to purify IgE from serum of allergic pigs, test heterologous anti-IgE for cross-reactivity with pig IgE and to produce rabbit antibodies specific for pig IgE.

Purification of IgE from serum is challenging due to its low concentration. A useful strategy involves using serum of allergic individuals to allow confirmation that putative IgE-rich fractions mediate *in vivo* (PCA or PK) or *in vitro* (allergen-specific ELISA or western blotting) effects expected of IgE-related, allergen-specific antibody. Hence peanut allergy was induced experimentally as reported by others (Helm et al., 2002) in pigs sensitized with CPE using CT as an adjuvant. Allergic pigs developed antibody to CPE and their sera mediated heat-sensitive PCA reactions to CPE confirming presence of CPE-specific IgE.

Strategies for obtaining anti-IgE include use of heterologous mast cells to adsorb the isotype due to cross-reactivity of the FcεRI receptor, followed by immunizing the mast cell-donor species with the

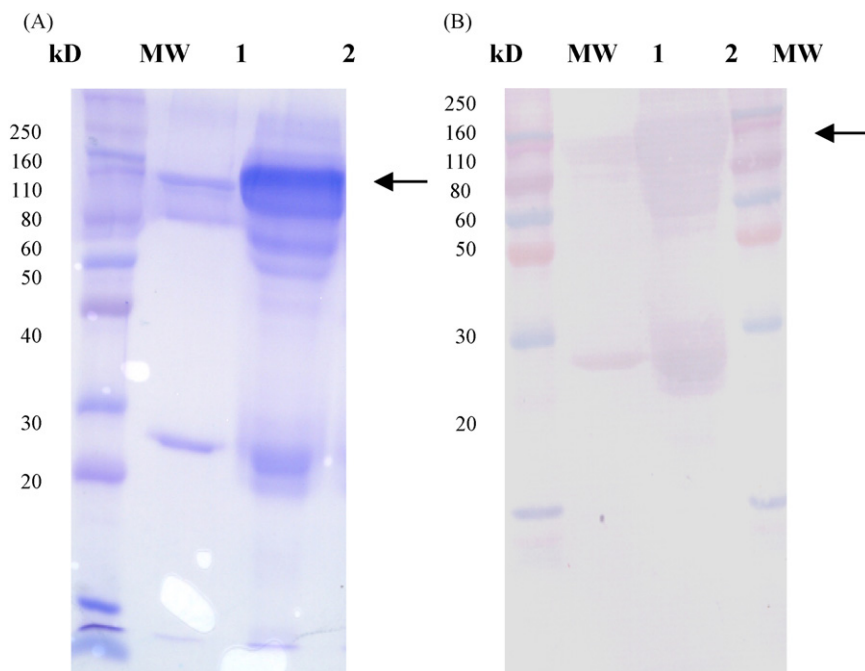


Fig. 4. SDS-PAGE and western immunoblot for rabbit anti-IgE binding to pig IgE. (A) Pig IgE (DEAE cellulose eluate) and standard mouse IgE were electrophoresed on 12% SDS-PAGE and stained with Coomassie blue. (B) The electrophoresed proteins were transferred to nitrocellulose and incubated with sera pooled from two pig IgE-immunized rabbits (1:500) and assayed for ability to bind IgE using a second antibody goat anti-rabbit IgG (whole molecule; 1:10,000) conjugated to alkaline phosphatase. In both the gel and the blot MW indicates molecular weight markers; lane 1 standard mouse IgE and lane 2 pig IgE (DEAE cellulose eluate). Arrow indicates the heavy chain of IgE.

homologous cells carrying the heterologous target IgE (Nielsen and Wilkie, 1977). Alternatively, synthetic peptide-carrier conjugates with sequence predicted from the target IgE may induce IgE-specific antibodies (Kalina et al., 2003). The latter approach has been used to induce autoantibody inhibiting IgE binding to the FcεRI receptor and to reduce serum IgE (Peng et al., 2007). The recombinant FcεRI alpha chain can be used as surrogate anti-IgE in ELISA to detect IgE or IgE-related antibodies (Stedman et al., 2001). Cross-species reactivity of the FcεRI alpha chain (Nielsen and Wilkie, 1977; Stedman et al., 2001) makes this a versatile approach. Similarly, cross-species reactivity of anti-IgE as demonstrated in the present study and by others (Halliwell et al., 1972; Roe et al., 1993; Peng et al., 2007) provides the possibility to use readily available heterologous anti-IgE to detect or affinity-purify IgE of other species. A combined chromatographic approach involving ammonium sulphate precipitation, Protein G affinity chromatography, anion-exchange chromatography and PAGE to obtain IgE H chain was used here for its simplicity and potential to produce both IgE-rich sources for *in vivo* and *in vitro* confirmation of biological, antigenic and physiochemical attributes of IgE as well as pure H chain for induction of anti-pig IgE.

Similarly, serum of pigs sensitized to *Ascaris suum* was used as a source of PCA-reactive IgE H chain to produce rabbit anti-pig IgE (Roe et al., 1993) that bound in western blot to 73 kDa protein. This antiserum inhibited PCA but did not induce RCA in pig skin.

Ammonium sulphate fractionation, which utilizes the relative hydrophobicity of immunoglobulin molecules compared to other serum proteins for their precipitation, increased the relative content of IgE in serum protein. Further purification of IgE was attempted using protein A affinity chromatography however, pig IgE did not bind to Protein A. It did however bind to Protein G and was eluted with other proteins, including IgG. Subsequent DEAE cellulose anion-exchange chromatography yielded a fraction confirmed by PAGE with mouse IgE standard, to contain IgE at the expected molecular weight in reducing and non-reducing gels, while separating IgG and other proteins eluted with IgE from the Protein G column. Contaminating proteins were few and the IgE H chain was readily excised from reducing PAGE in quantities sufficient for immunization of two rabbits. These results are in agreement with the previous report of a similar protocol used to prepare dog serum IgE-enriched fractions (Peng et al., 1993a).

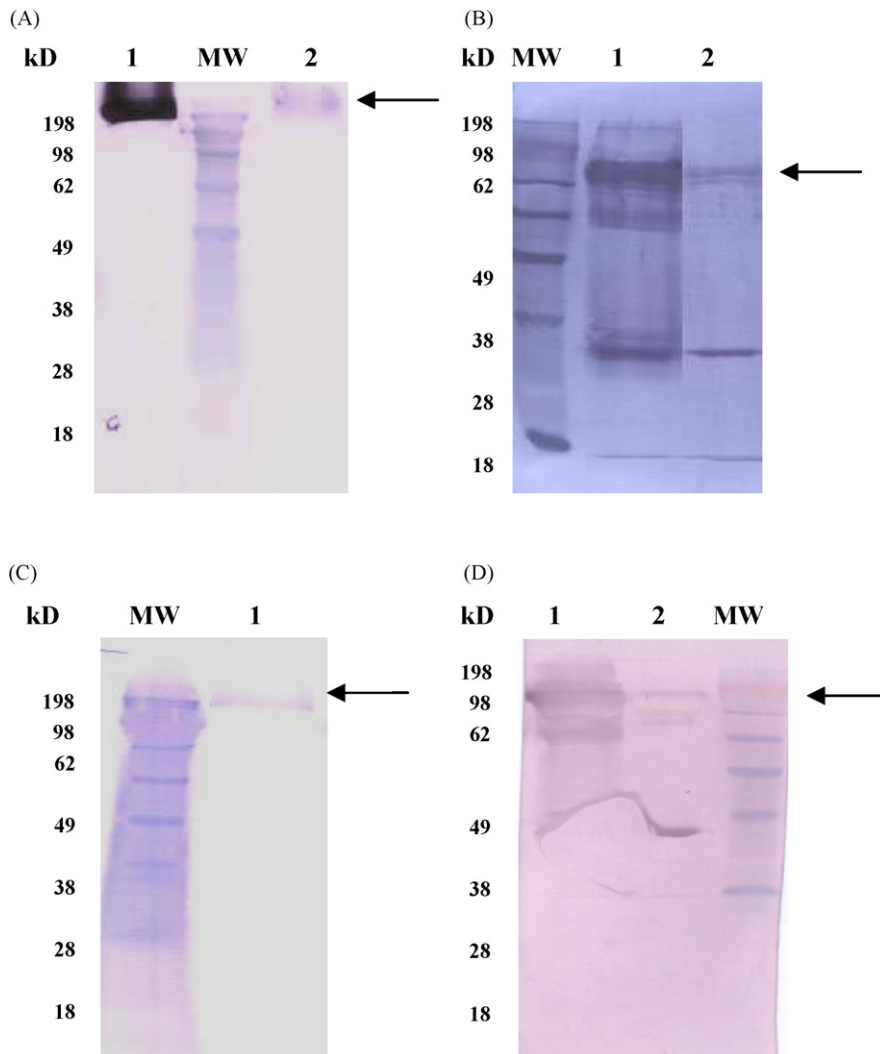


Fig. 5. Reactivity of anti-mouse (A and B) and anti-human IgE (C and D) monoclonal antibodies with purified porcine IgE in western blot. Porcine IgE fraction (DEAE cellulose eluate) was resolved under native (A and C) and reducing (B and D) PAGE conditions and reactivity was assessed by western immunoblotting. (A) Native gel (MW indicates molecular weight markers (Invitrogen, SeeBlue Plus2); lane 1: standard mouse IgE; lane 2: pig IgE) and (B) SDS-PAGE (lane 1: pig IgE; lane 2: standard mouse IgE); A and B blots were developed using biotinylated rat anti-mouse IgE and detected using avidin-conjugated to alkaline phosphatase. The H chain is ~70 kDa on the reducing gel and the whole IgE molecule is ~190 kDa on the native gel. (C) Native gel (lane 1: porcine IgE) and (D) SDS-PAGE (lane 1: pig IgE; lane 2: standard mouse IgE). Blots C and D were developed using monoclonal mouse anti-human IgE alkaline phosphatase-conjugated antibody. Arrow indicates pig IgE with a molecular weight of 190 kDa on native for the whole IgE molecule and 70 kDa on reducing gels for the heavy chain of IgE.

Cross-reactivity of anti-mouse and anti-human monoclonal antibodies with porcine IgE was described here. Others have reported binding of anti-pig IgE to human IgE (Roe et al., 1993). Similarly, rabbit anti-human IgE was reported to bind dog IgE (Halliwell et al., 1972; Peng et al., 1993a) and rat-anti-human IgE H chain peptides reacted with mouse, rat and dog IgE (Peng et al., 2007). Hence pig IgE heavy chain shares epitopes with mouse and human IgE H as also demonstrated here by immunoblot and ELISA developed with heterologous

anti-IgE. A search of porcine IgE H chain using the blast program ([www.expasy.ch](http://www.expasy.ch)) revealed 48% homology to murine and 49% to human IgE H chain. An expedient source of functional anti-pig IgE would therefore be a screen, as reported here, of available heterologous anti-IgEs for ability to detect pig IgE. However, in western blots the polyclonal rabbit and monoclonal mouse and rat antibodies confirmed here to bind pig IgE H chain also bound a 23 kDa band assumed to be L chain both of porcine IgE and standard murine IgE. This was true under

both conditions of blocking. It is not known if this represents specific or nonspecific binding but it may indicate that absorption of the antisera with a non-IgE pig immunoglobulin would enhance specificity.

The present results confirm that rabbit anti-porcine IgE and heterologous antibodies binding to pig IgE are functionally active in RCA. This is in contrast to a previous report (Roe et al., 1993) in which RCA was negative. Ability to cross-link FcεRI receptor-bound IgE reassures that the anti-IgE binds epitopes exposed on adjacent IgE H chains, a condition that may not be met due to specificity, quantity or steric inhibition inadequacies of the anti-IgE. While sera of rats immunized with synthetic IgE peptides bound soluble or plate-bound IgE, they did not bind IgE associated with FcεRI receptor (Peng et al., 2007).

These studies have confirmed that pig IgE can be purified from allergic serum by simple multi-step chromatography and PAGE and that the resulting IgE H chain induced anti-pig IgE that detects IgE bound to 96-well plates, in western blots or bound as antibody to allergens. It was also confirmed that some mouse or rat monoclonal antibodies reactive with heterologous IgE bind usefully to pig IgE. These reagents may enhance ongoing investigation of experimental food allergies in pigs (Rupa et al., 2007) and provide a needed addition to the pig immunological toolbox for diverse applications.

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