



Attenuation of allergy to ovomucoid in pigs by neonatal treatment with heat-killed *Escherichia coli* or *E. coli* producing porcine IFN- γ

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ABSTRACT

Food allergy is epidemic and prompts investigation to reduce allergic predisposition. It was hypothesized that heat-killed *Escherichia coli* injected intramuscularly (im) with or without interferon gamma (IFN- γ), reduces neonatal susceptibility to experimental egg allergy. Two litters of Yorkshire pigs were assigned to three intramuscular treatment groups (four/group): control (PBS), heat-killed *E. coli* with or without IFN- γ -expressing plasmid. Pigs were sensitized to ovomucoid (Ovm) by intraperitoneal injection with cholera toxin. To assess induction of allergy, pigs were fed egg white in yoghurt and assigned scores for allergic signs. Significantly fewer pigs developed allergy and passive cutaneous anaphylaxis in *E. coli* and *E. coli* + IFN- γ vs control groups. *E. coli*-treated pigs also had significantly lower frequency of mean clinical scores. *E. coli* and *E. coli* + IFN- γ groups did not differ. Serum antibody associated with IgG (H & L), IgG₁, IgG₂ or IgE all correlated but did not differ by treatment groups. Thus, treatment of neonatal pigs with heat-killed *E. coli* by im injection reduced susceptibility to allergic sensitization with Ovm. Inclusion of the type-1 cytokine, IFN- γ , had no additional effect. Results indicate a method for prophylaxis of allergy and suggest support for the “hygiene hypothesis”.

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1. Introduction

In developed countries, human food-induced allergy has significantly increased in prevalence during the last decade (Helm and Burks, 2000). The “hygiene hypothesis” states that reduced exposure to microbial-derived stimuli in early life leads to increased susceptibility to allergy (Garn and Renz, 2007). It is proposed that protection from allergy is due to microbially induced immunological bias away from the allergy-conductive type-2 towards the non-conductive type-1 immune response (IR) phenotype. Molecular mechanisms related to the hygiene hypothesis are thought to involve microbial ligands (pathogen-associated molecular patterns, PAMPs) for membrane-bound pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and

the cytoplasmic NOD-like receptor (NLR) family, in modulating allergic inflammation (Vercelli, 2006). PAMPs also direct ontogeny of the immune system through activation of TLR signaling (O'Neill, 2006). Microbially derived PAMPs, such as bacterial endotoxin, peptidoglycan, muramyl dipeptide, CpG nucleotides and others, regulate production of interleukin 12 (IL-12) and interferon gamma (IFN- γ), to bias against type-2 IR and thus counteract allergic sensitization (Murosaki et al., 1998; Tulic et al., 2000). Treatment of cultured blood mononuclear cells (BMC) of grass pollen-allergic patients with inactivated *Lactobacillus acidophilus* and non-pathogenic *Escherichia coli* strain Nissle, biased the IFN- γ and IL-4 ratio towards IFN- γ (type-1 IR) (Rasche et al., 2007). Heat-killed lactic acid bacteria stimulate IL-12 (p70) production, a key cytokine in directing IR bias from proallergic type-2 to anti-allergic type-1 (Sashihara et al., 2006). Heat-killed *Listeria monocytogenes* administered together with specific allergen to dogs genetically predisposed to allergy, significantly reduced food-induced anaphylaxis (Frick et al., 2005).

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During pregnancy and shortly after parturition, less type-1 (IFN- γ and IL-2) and more type-2 cytokines (IL-4) are produced (Reinhard et al., 1998) biasing neonatal IR towards type-2 and predisposing to allergy-induction (Marodi, 2002; Siegrist, 2001). The type-1 cytokine, IFN- γ modulates antibody- and cell-mediated immune responses and activates T regulatory cell immunosuppression (Wood and Sawitzki, 2006). Killed *Pseudomonas fluorescens* cells producing recombinant bovine IFN- γ , when injected subcutaneously into cattle enhanced both innate and adaptive IR (Gaertner et al., 2005). This suggests neonatal treatment with IFN- γ to enhance anti-allergic, allergen-nonspecific type-1 IR.

Methods for inducing allergy in neonatal outbred pigs to the dominant allergen of egg white, ovomucoid (Ovm) (Rupa et al., 2008a) were used here to test the hypothesis that treating neonatal pigs either with putative type-1 IR-biasing heat-killed *E. coli* or *E. coli* + IFN- γ reduces allergic predisposition.

2. Materials and methods

2.1. Animals and design

Two litters of newborn Yorkshire pigs, 12 pigs per litter, four pigs/experimental group, were obtained at birth from the Arkell Swine Research Station (University of Guelph) and were maintained under normal husbandry conditions. The sow's diet was free of egg proteins. Animal use was approved by the Animal Care and Use Committee, University of Guelph under Canadian Council for Animal Care guidelines. Treatment days were equivalent to age in days.

Pre-treatment: Piglets were randomly assigned within two litters to three groups of four animals per treatment, as described below. Animals were given daily intramuscular (im) injections on days 1–7, of 1 ml of 10^9 colony-forming unit (CFU)/ml of heat-killed *E. coli* containing plasmid pET 28a (*E. coli* group; $n = 7$); 1 ml of 10^9 CFU/ml of heat-killed *E. coli* containing plasmid pET 28a expressing IFN- γ (*E. coli* + IFN- γ group; $n = 8$) and control animals ($n = 8$) were given 1 ml of phosphate-buffered saline (PBS).

Sensitization: On days 14, 21, and 35 all groups were given intraperitoneal (ip) injections of 100 μ g of purified Ovm adjuvanted with 10 μ g of cholera toxin (CT) (List Biologicals, CA, USA) as described (Rupa et al., 2008a). Blood was obtained from the retro-orbital sinus on days 14, 35, 45 and 46, and sera stored at -20°C .

Challenge: To induce hypersensitivity reactions to Ovm, pigs were fasted on day 45, challenged orally with egg white in yoghurt slurry (2:3, v/v, 45 ml) on day 46 and monitored for clinical signs of allergy as previously described (Rupa et al., 2008a).

2.2. Cloning, expression and purification of porcine interferon gamma (pIFN- γ)

First-strand cDNAs were prepared from 1 μ g of total RNA from pig blood mononuclear cells using the Cell Direct cDNA synthesis kit (Invitrogen, Burlington, Canada). The cDNA was amplified using Platinum Taq DNA-polymerase

(Invitrogen) and used directly in PCR amplification. Primers for PCR amplification of pIFN- γ were based on the pIFN- γ gene sequence (Dijkmans et al., 1990). The fragment containing pIFN- γ was introduced into TA cloning *E. coli* (pCR2.1, Invitrogen) and sequenced. The pIFN- γ -specific insert was then cloned into the commercial pET-28a expression *E. coli* which encodes a C-terminal His6-tag (Novagen, Madison, WI, USA) and constructs were transformed into BL-21 Star cells (Invitrogen), expressed and purified as recommended by the manufacturer (Novagen). Porcine IFN- γ was detected by western blot using rabbit anti-swine IFN- γ (Biosource, Camarillo, CA, USA) at 1:5000 dilution. The purified protein was quantified using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Richmond, CA, USA) with bovine serum albumin as standard. For im injections, *E. coli* cells (10^9 CFU/ml) with and without IFN- γ were heat-killed at 70°C for 30 min after induction and were stored at -80°C . Inactivation of the final suspension was confirmed by plating on LB agar.

2.3. Bioassay for purified IFN- γ

Biological activity of purified recombinant pIFN- γ was determined by upregulation of cell-surface MHC class II on porcine alveolar macrophage-derived myelomonocytic cells (line 3D4/31, Dr. Weingartl, NCFAD, CFIA, Winnipeg, Canada) (Weingartl et al., 2002) detected by ELISA using commercial rpIFN- γ (Biosource, Montreal) as positive control as previously described (Rupa et al., 2008).

2.4. Direct skin test

Immediate hypersensitivity reactions to Ovm were tested by intradermal (id) injection using sites on the inner thighs before (day 14) and after (days 21 and 35) sensitization. One hundred microliters of Ovm (100 μ g) in PBS was injected id using a tuberculin syringe and 28 gauge needle. The negative control was PBS. Skin tests were examined by at least three observers and consensus reached regarding positivity.

2.5. Clinical scores

Responses induced by oral challenge with egg white on day 46 were observed up to 2 h post challenge and scored by four observers in a blinded fashion. Scores were as follows: 0 = no signs; 0.5 = repeated scratching, repeated sneezing; 1 = isolation and immobility; focal cutaneous reddening, emesis (single incident); 2 = partially confluent cutaneous reddening, repeated emesis, respiratory difficulty; 3 = confluent cutaneous reddening. Total scores were obtained by adding individual scores.

2.6. Serum antibody to Ovm

Ovm-specific porcine IgG (H and L chain specific) and IgE (H chain specific) antibodies were detected by ELISA as previously described (Rupa et al., 2008a,b). Antibody associated with IgG₁ and IgG₂ was detected using biotinylated murine monoclonal antibodies (Dr. K. Nielsen,

ADRI, Nepean, Canada) (Crawley et al., 2003). Briefly, 96-well plates (Immulon 2HB, VWR International, Mississauga, ON) were coated overnight at 4 °C with 10 µg/well of Ovm in 0.05 M carbonate bicarbonate buffer pH 9.6. Plates were washed (ELX405, Bio-Tek Instruments Inc., Winooski, VT, USA) with PBS+0.5% Tween-20 (PBST), blocked with 3% Tween in PBS, washed and serum (1:100, 100 µl/well) was added. Pooled day 14 and day 45 pre- and post-sensitization sera were used as negative and positive controls. Samples were tested in triplicate and means calculated. One hundred microliters of alkaline phosphatase (Alk-phos)-conjugated rabbit anti-pig IgG (H and L chain specific, Sigma, Oakville, Ontario), rabbit anti-pig IgE (Rupa et al., 2008b) or, for anti-IgG₁ and IgG₂, streptavidin-Alk-phos (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) were added to the wells. Plates were washed and chromogen (*p*-nitrophenyl-phosphatase (pNPP)) (Sigma) in diethanolamine buffer, pH 9.8 was added and incubated in the dark at room temperature. Reaction product and background optical densities (OD) were quantified at 405 nm and 630 nm, respectively (96-well plate reader, EL808, Bio-Tek Instruments Inc.) when the OD of the positive control reached 1.0. Results were expressed as percentage of the positive control as follows: sample %OD of positive control = [(sample OD)/(positive control OD – negative control OD)] × 100. All serum samples from one litter were analyzed on the same day.

2.7. Passive cutaneous anaphylaxis

Passive cutaneous anaphylaxis (PCA) was induced as described (Rupa et al., 2008a). Sera (100 µl) from sensitized pigs were injected id into marked sites on the inner thighs of naïve pigs. After 24 h, 5 mg of Ovm in 1.0 ml of sterile PBS was injected via a dorsal ear vein using a 23-gauge needle. Cutaneous injection sites were examined for reaction by at least 3 blinded observers. A positive immediate reaction at the cutaneous injection site indicated Ovm-specific IgE in the sensitized pig serum. Each site was arbitrarily graded (1, 2, 3 or 4) on the basis of reaction intensity.

2.8. Statistical analysis

Data were analyzed using GraphPad InStat Version 3.05 and GraphPad Prism Version 4.0 (GraphPad, San Diego, CA, USA). Difference between means was evaluated by Student's *t*-test. Linear regression and Chi-square test were used to evaluate relationships between data sets. Results were taken to be significant at $p \leq 0.05$.

3. Results

3.1. Expression and purification of rpIFN-γ

His-tagged-purified rpIFN-γ full-size protein, estimated molecular weight (MW) 19 kDa, was detected on sodium dodecyl sulphate acrylamide gel electrophoresis with Coomassie staining and the concentration of purified rpIFN-γ was estimated at 38 µg in 10⁹ CFU/ml of *E. coli*. The purified pIFN-γ was identified on western blot with

rabbit anti-swine IFN-γ and the estimated MW was similar to that observed with Coomassie staining (data not shown).

3.2. Bioactivity of pIFN-γ

Surface expression of class II MHC on porcine alveolar macrophage 3D4/31 cells was determined after rpIFN-γ stimulation for 24 h at 1/7.5 to 1/250 dilutions. The mouse Ig isotype negative control provided baseline OD values (data not shown). Expression of MHC class II was increased ($p \leq 0.05$) after treatment with *E. coli* + IFN-γ-derived samples prior to heat treatment. After treatment with heat-killed *E. coli* + IFN-γ, MHC II expression was also increased relative to heat-killed *E. coli* and to unstimulated cells. Expression was significantly enhanced ($p \leq 0.05$) in the cells treated with pIFN-γ-purified fractions at all dilutions and with commercial rpIFN-γ. The control sample, *E. coli* containing pET28a alone did not alter expression. Commercial rpIFN-γ, heat-killed *E. coli* or non-heat-killed *E. coli*-derived IFN-γ increased MHCII expression from background to maximum values at the lowest added estimated doses, 0–0.5 ng/ml of pIFN-γ (data not shown).

3.3. Antibody response and sensitization to Ovm

Sensitization to Ovm was confirmed by direct skin test. Positive skin reactions to Ovm were observed on day 45 in all but one animal in each litter. Skin reactions were not observed at PBS injection sites. In all animals in both litters there was a significant increase ($p < 0.001$) in serum Ovm-specific IgG (H and L chain specific), IgG₁, IgG₂ and IgE antibody activity from day 14 to day 45. No significant difference in antibody activity was detected between groups and there was positive correlation ($p \leq 0.05$) between Ovm-specific antibody of all Ig isotypes (data not shown).

3.4. Oral challenge with egg white

Clinical signs in both litters were most frequent and severe in the control groups which were pretreated with PBS alone before sensitization with Ovm (Table 1, Fig. 1). Clinical scores pooled by treatment groups indicate significantly more pigs showing clinical signs in the control group as compared to the *E. coli* and *E. coli* + IFN-γ groups (Fig. 1) ($p < 0.0001$). There was no significant difference between the *E. coli* and *E. coli* + IFN-γ group. Mean clinical scores of the pigs pretreated with *E. coli* ($p < 0.004$) or *E. coli* + IFN-γ ($p < 0.04$) were significantly less than controls. There was no significant difference between mean clinical scores of *E. coli* and *E. coli* + IFN-γ ($p < 0.47$).

3.5. Passive cutaneous anaphylaxis

Sera from 3 of 8 pigs in the control group, 1 of 7 pigs in the *E. coli* group and 1 of 8 pigs in the *E. coli* + IFN-γ group mediated positive PCA reactions. The PCA results pooled across both litters (day 45) indicate a significantly more

Table 1

Clinical signs and scores following oral challenge of ovomucoid sensitized piglets.

Treatment	Pig number	Clinical scores	Clinical signs
Control	1	7	Erythema, emesis, scratching, II ^a
	2	4	Emesis, erythema
	3	3.5	Erythema, scratching, II ^a
	4	1.5	Scratching, erythema
	5	1	Erythema
	6	1	Retching and scratching
	7	1	Erythema
	8	0	No signs
<i>E. coli</i>	1	3	Scratching, sneezing, emesis
	2	1.5	Erythema, scratching
	3	0.5	Scratching
	4	0	No signs
	5	0	No signs
	6	0	No signs
	7	0	No signs
<i>E. coli</i> + IFN- γ	1	2.5	Emesis, sneezing, erythema
	2	2	Erythema
	3	1	Erythema
	4	0.5	Scratching
	5	0	No signs
	6	0	No signs
	7	0	No signs
	8	0	No signs

^a Isolation and immobility.

control pigs with positive PCA than in the *E. coli* and *E. coli* + IFN- γ groups ($p = 0.0002$ and $p < 0.0001$, respectively). The frequency of PCA-positive pigs did not differ significantly between the *E. coli* and the *E. coli* + IFN- γ treatment groups. Individual clinical scores and graded PCA responses were positively correlated ($p = 0.008$; $r^2 = 0.2836$). There was marked individual variation such that in some pigs high PCA score was associated with low allergic signs and vice versa.

4. Discussion

Treatment of neonates with bacteria to alter IR bias towards type-1 may reduce overall allergic predisposition. This seeks general, not allergen-specific protection. It was hypothesized that im injections of *E. coli* or *E. coli* + IFN- γ , given prior to experimental allergic sensitization would reduce allergy in piglets subsequently sensitized and orally challenged with egg white (Rupa et al., 2008a). Results indicate significant reduction in allergy.

As expected, sensitization with Ovm and CT, variably induced allergen-specific positive direct skin tests, clinical signs, serum antibody and positive PCA reactions. No significant difference in Ovm-specific IgG (H + L), IgG₁, IgG₂ and IgE-associated antibody activity was detected between groups. Similar observations were made in groups of children developing atopic eczema and treated with probiotics, in which serum IgE antibody activity did not differ between groups which did differ in clinical signs (Kalliomäki et al., 2003). Measurement of serum IgE-related anti-allergen was not in the present study a better predictor of allergic clinical signs. Positive correlations are however reported between human and murine serum IgE

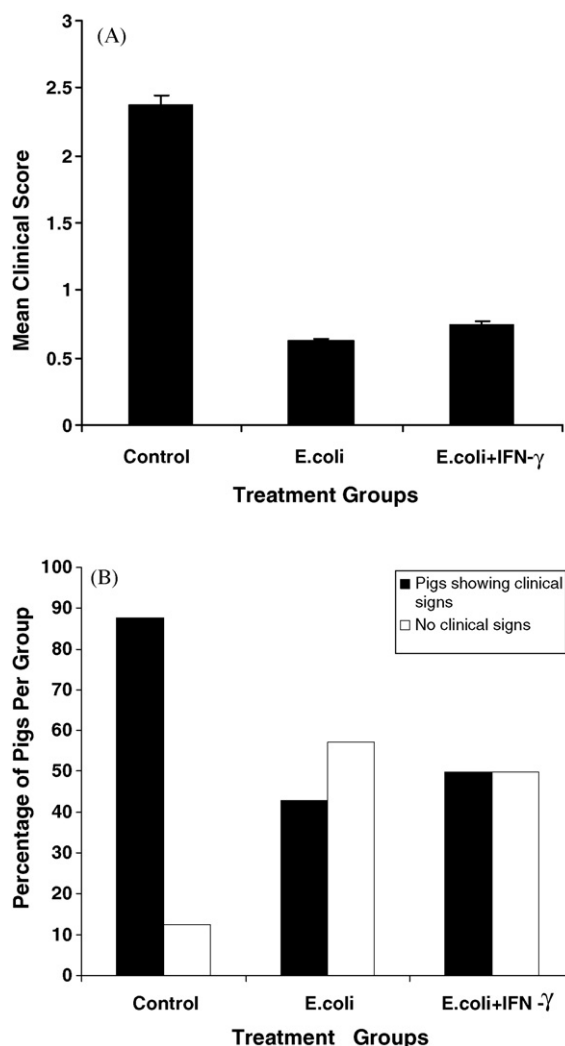


Fig. 1. Response to oral challenge with ovomucoid. (A) Mean clinical scores of the pigs pretreated with *E. coli* ($p < 0.004$) or *E. coli* + IFN- γ ($p < 0.04$) were significantly less than controls. There was no significant difference between mean clinical scores of *E. coli* and *E. coli* + IFN- γ ($p < 0.47$). (B) Percentage of pigs that showed clinical signs observed post-Ovm challenge in the three different treatment groups. Treatment and control groups differed significantly ($p \leq 0.0001$) in frequency of pigs expressing allergic signs.

antibody and allergic signs (Sampson, 2001; Rupa and Mine, 2006). A possible explanation for this may be that quantification of allergen-specific serum IgE may not reflect allergy-mediating activity of leucocyte Fc ϵ R1-bound antibody. However, in the present study, frequency of sera mediating PCA reactions was significantly less in groups treated with *E. coli* with or without IFN- γ , than in positive controls and individual PCA grades correlated with severity of clinical signs, suggesting a functional role of IgE anti-Ovm. While the IgE-mediated PCA reaction was overall significantly and positively correlated with clinical scores, individual variation compromised the predictive value of the PCA reaction there being a high frequency of clinical sign-negative, PCA-positive pigs. This individual variation complicates experimentation but likely renders

this large outbred animal model more relevant to human allergy than studies using inbred mice (Atkinson and Leitner, 1999; Mestas and Hughes, 2004).

The present study demonstrates that systemically administered, heat-killed *E. coli* significantly reduces predisposition to allergic sensitization with Ovm. While the mechanism was not investigated here, it appears likely that it is mediated by bacterial ligands for innate IR receptors such as TLRs, NODs and others (Vandenbulcke et al., 2006). Sustained LPS exposure initiated in mice prenatally and continued postnatally, increased susceptibility to endotoxin and prevented sensitization to allergen through inhibition of type-2 IRs (Gerhold et al., 2006). However, inclusion here of biologically active IFN- γ had no additional effect, although others report positive effects of IFN- γ in several other systems. Recombinant pIFN- γ enhanced IL-1 secretion by porcine monocytic cells *in vitro* (Charlie et al., 1990) and modulates leukocyte function of pigs *in vivo* (Saulnier et al., 1991). Addition of IFN- γ to inactivated porcine herpesvirus-1 potentiates secondary IgG and IgA antibody responses and may enhance mucosal and systemic IR (Vandenbroeck et al., 1998). Also, heat-killed recombinant *P. fluorescens* expressing bovine IFN- γ was a potent immunological adjuvant when injected systemically (Gaertner et al., 2005). In the present study, treatment effect of IFN- γ may have been obscured by effects of the *E. coli* delivery vehicle. However, we have confirmed that rpIFN- γ (Biosource) at 1 μ g/kg bodyweight given by daily im injection to piglets 1–7 days of age had no whole animal immunomodulating effects but variably upregulated blood mononuclear cell MHCII expression (Hamilton, 2008). While the dose of heat-killed *E. coli* + IFN- γ may have been inappropriate, dose titration, given the association of bacterium and cytokine, would be confounded by the confirmed bioactivity of the bacterium itself. However, in the present experiments there was no attempt to ascertain a dose of pIFN- γ that induced a measurable *in vivo* response, such as upregulation of MHC II.

We report that treatments of piglets with heat-killed *E. coli* attenuated allergy, suggesting prophylactic and therapeutic methods for beneficially reducing type-2 IR bias in neonates.

Conflict of interest

None.

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