Veterinary Immunology and Immunopathology 189 (2017) 43–52

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Dietary cinnamaldehyde enhances acquisition of specific antibodies following helminth infection in pigs

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ARTICLE INFO

Keywords:
Cinnamaldehyde
Pigs
Parasite
Ascaris suum
Antibody

ABSTRACT

Dietary phytonutrients such as cinnamaldehyde (CA) may contribute to immune function during pathogen infections, and CA has been reported to have positive effects on gut health when used as feed additive for livestock. Here, we investigated whether CA could enhance antibody production and specific immune responses during infection with an enteric pathogen. We examined the effect of dietary CA on plasma antibody levels in parasite-naïve pigs, and subsequently acquisition of humoral immune responses during infection with the parasitic nematode *Ascaris suum.* Parasite-naïve pigs fed diets supplemented with CA had higher levels of total IgA and IgG in plasma, and *A. suum*-infected pigs fed CA had higher levels of parasite-specific IgM and IgA in plasma 14 days post-infection. Moreover, dietary CA increased expression of genes encoding the B-cell marker *CD19*, sodium/glucose co-transporter1 (*SCA5L1*) and glucose transporter 2 (*SLC2A2*) in the jejunal mucosa of *A.suum*-infected pigs. Dietary CA induced only limited changes in the composition of the prokaryotic gut microbiota of *A. suum*-infected pigs, and *in vitro* experiments showed that CA did not directly induce proliferation or increase secretion of IgG and IgA from lymphocytes. Our results demonstrate that dietary CA can significantly enhance acquisition of specific immune responses in pigs. The underlying mechanism remains obscure, but apparently does not derive simply from direct contact between CA and host lymphocytes and appears to be independent of the gut microbiota.

1. Introduction

Immune function in animals can be influenced considerably by dietary components, either through manipulation of macronutrients such as protein and carbohydrates or micronutrients such as vitamins or phytonutrients (Dawson et al., 2009; Hoste et al., 2016). Phytonutrients can be defined as bioactive plant compounds which have health benefits when consumed in the diet. Such plant compounds include flavanols, carotenoids and essential oil components such as eugenol or cinnamaldehyde. Cinnamaldehyde (CA) has received recent attention for its potential use as a bioactive supplement in livestock production as it possesses antibacterial and possible immuno-stimulatory properties. For example, poultry fed a diet supplemented with CA have lower levels of infection with the bacteria *Brachypsperia intermedia* or the protozoan parasite *Eimeria acervulina* (Lee et al., 2011b; Verlinden et al., 2013). Moreover, pigs fed a diet supplemented with a blend of essential oil components containing CA have been shown to have increased levels of total serum IgG and IgA and circulating lymphocytes (Li et al., 2012; Yan and Kim, 2012), suggestive of a direct stimulatory effect on humoral immunity. The development of antibody-mediated immune responses are critical in young animals for protection against a variety of pathogenic microbes, therefore the ability of dietary additives such as CA to enhance the acquisition of pathogen-specific antibodies would be highly valuable in livestock production. However, the ability of CA to influence the development of specific immune responses in mammals remains to be established. and the mechanisms by which CA may influence immune function are unknown.

Ascaris suum is a common nematode parasite of pigs that resides in the small intestine and can cause significant productivity losses for pig producers (Hale et al., 1985 Jankowska-Mąkosa and Knecht, 2015; Vlaminck et al., 2015). Primary infections result in a high percentage of larval establishment, followed by rapid humoral and cellular immune responses in multiple tissue sites that results in expulsion of most of the infectious larvae from the intestine beginning around 17 days following

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http://dx.doi.org/10.1016/j.vetimm.2017.06.004
Received 2 December 2016; Received in revised form 20 March 2017; Accepted 16 June 2017

infection (Roepstorff et al., 1997). Thus, the *A. suum* model is particularly well-suited to studying the effect of dietary supplements on acquisition of specific immunity to pathogens.

Here, we hypothesised that antibody production would be enhanced in pigs fed pigs diets supplemented with pure CA. To this end, we first tested whether dietary CA increased total IgG and IgA in plasma from uninfected pigs. Subsequently, we investigated parasite-specific antibody levels in plasma from pigs that had been infected for 14 d with *A. suum*, thus addressing whether CA can enhance the acquisition of pathogen-specific immune responses. Finally, to provide mechanistic insights into the effect of CA on antibody levels, we examined the effects of dietary CA on intestinal gene expression and gut microbiota composition in *A. suum*–infected pigs, and investigated the in vitro effects of CA on porcine lymphocyte proliferation and antibody secretion.

2. Materials and methods

2.1. Materials

Cinnamaldehyde (> 99%) was obtained from Sigma-Aldrich (Stellenbosch, Germany).

2.2. Animals, diet and sampling

All animal experimentation was approved by the Experimental Animal Unit, University of Copenhagen, and carried out according to the guidelines of the Danish Animal Experimentation Inspectorate (Licence number 2010/561-1914).

2.2.1. Experiment 1

Twelve pigs (Danish landrace x Yorkshire; mean weight 20 kg; females and castrated males) were purchased from a specific-pathogen free farm and stratified on the basis of weight and sex to two groups (n = 6). After 1 week acclimatization, Group 1 was fed a basal diet based on ground barley and soybean meal (Table 1), whilst Group 2 was fed the same diet supplemented with 1000 mg CA/kg of feed, mixed thoroughly into their food immediately prior to feeding. The experimental feeding period was 16 days. Pigs were initially fed 1 kg of food per day, rising to 1.4 kg of food per day by the end of the experiment, resulting in a daily CA intake of around 45 mg/kg body weight, fed to each pig in two equal meals per day. Pigs were weighed weekly, and before and 16 days after feeding commenced blood was collected by venipuncture of the jugular vein into vacutainers containing sodium heparin. Plasma was separated by centrifugation and stored at −20 °C.

2.2.2. Experiment 2

The design of the *A. suum* experiment has been described previously (Williams et al., 2015). Briefly, 15 helminth-naïve pigs (mean weight 26 kg; females and castrated males) were stratified on the basis of weight and sex to three groups (each n = 5). The housing of the pigs, acclimatization and feeding of the basal diet was as described for Experiment 1, except that animals were fed restrictively with 1.0 kg of food/pig/day throughout the experiment. The experimental feeding commenced five days prior to infection, when all pigs were infected orally with 5000 embryonated *A. suum* eggs. Group 1 received only the basal diet as control. Group 2 (CA – supplement 'CAS') received a daily dietary supplement of 1000 mg cinnamaldehyde/per kg of food, as described above. Group 3 (CA dose – 'CAD') received no supplement but instead was dosed orally by stomach tube with 1000 mg CA placed in acid-resistant capsules on days 11 and 13 post-infection (p.i.), in order to deliver a more concentrated dose to the small intestine. On day 14 p.i., all pigs were euthanised by captive bolt pistol and exsanguination and parasite burdens determined. Blood was collected by venepuncture before infection and before slaughter, and plasma stored as above. A ~5 cm section of the jejunum was removed at the midgut, washed in ice-cold phosphate-buffered saline (PBS) and the mucosa removed using a glass slide. Samples of the proximal colon digesta were taken approximately 15–20 cm distal to the ileal-caecal junction. Mucosal and digesta samples were then placed into cyrovials, snap-frozen in liquid nitrogen and stored at −80 °C until needed.

2.3. Parasites and antigenic material

Ascaris suum eggs were collected from the uteri of adult female worms obtained from the intestine of pigs at a local slaughterhouse (Danish Crown, Ringsted). Eggs were embryonated at room temperature for at least two months before being used for infection. *Ascaris suum* body fluid (AsBF) was obtained from the pseudocoelom of freshly collected adult worms, and centrifuged at 10,000 *g* for 15 min. The supernatant was filtered through a 0.2 µm syringe filter and stored at −80 °C before use. Protein content was determined by the BCA assay (Pierce).

2.4. ELISA

In Experiment 1, total IgA and IgG in plasma was measured using ELISA kits (Bethyl Labs, Montgomery, TX) according to the manufacturer's instructions. For *A. suum*-specific responses in Experiment 2, Maxisorb ELISA plates (Nunc, Denmark) were coated overnight at 4 °C with AsBF (5 µg/mL). Between all steps, plates were washed 4 times with phosphate buffered saline (PBS) containing 0.01% Tween 20. Plates were blocked with 1% bovine serum albumin (BSA) in PBS, followed by the addition of plasma and then detection antibodies; goat anti-swine IgM or IgA conjugated to horseradish peroxidase (HRP), or mouse anti-swine IgG1 (clone K139 3C8) or mouse anti-swine IgG_2 (K68 1G2). All antibodies were from AbD serotec. In the case of IgG_1 and IgG_2, a secondary antibody incubation (goat anti-mouse IgG:HRP conjugate; AbD serotec) was included. All incubations were for one hour at 37 °C. Plates were developed with TMB substrate (Sigma-Aldrich). For each antibody a standard curve was prepared using a hyper-immune plasma sample. Samples were then quantified by interpolation of dilutions that fell within the linear portion of this standard curve, and expressed as a percentage of the positive sample (Miquel et al., 2005).

2.5. Proliferation of peripheral lymphocytes (PBL)

Blood was collected from either healthy, parasite-free pigs or pigs infected with a dose of 5000 *A. suum* eggs 14 days previously. Peripheral blood mononuclear cells (PBMC) were isolated using histopaque 1.077 (Sigma-Aldrich), washed and suspended in complete culture media (RPMI 1640 with 10% inactivated foetal porcine serum, 2 mM L-glutamine, 100 µg/mL streptomycin and 100 U/mL penicillin). For proliferation assays, cells were labelled with CFSE (Sigma-Aldrich; 1 µM) and then incubated either in CA (ranging from 150 to 1000 ng/mL) and/or phytohaemagglutinin (PHA; Sigma-Aldrich, 5 µg/mL). Parasite-specific proliferation was measured by addition of 20 µg/mL *A. suum* ABF in the presence or absence of CA. After 5 days, proliferation was determined by flow cytometry.

2.6. ELISPOT and antibody secretion assays

PBMC were isolated from either healthy, parasite-free pigs or pigs infected with a dose of 5000 *A. suum* eggs 14 days previously. For ELISPOT assays, PBMC (5×10^5) were cultured with AsBF (20 µg/mL) for 72 h in complete media as described above, in the presence of either