

## ORIGINAL ARTICLE

# Bovine milk fat enriched in conjugated linoleic and vaccenic acids attenuates allergic dermatitis in mice

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## Clinical & Experimental Allergy

### Summary

**Background** Orally administered milk fat enriched in conjugated linoleic acid (CLA) and *trans*-vaccenic acid (VA) ('enriched milk fat'), produced by supplementing the diet of pasture-fed cows with fish and sunflower oil, has been shown previously to suppress the development of allergic airway disease in mice.

**Objective** To investigate whether topical or oral application of enriched milk fat and its two major fatty acids *cis*-9, *trans*-11 CLA (c9,t11-CLA) and VA inhibit allergic dermatitis in mice.

**Methods** Allergic dermatitis was induced in C57BL/6 mice by epicutaneous sensitization of tape-stripped skin with ovalbumin (OVA). Enriched milk fat and its two major fatty acids were either topically applied to the OVA-sensitized skin, or orally fed to mice by supplementation of the diet. Blood and skin tissues were collected for analysis after the third skin sensitization.

**Results** Both topical and oral administration of enriched milk fat and its two major fatty acids led to significant suppression of allergic dermatitis as evidenced by reduced clinical and histological scores of affected skins, infiltration of inflammatory cells, and circulating allergen-specific IgE levels, compared with treatment with normal milk fat or the base control diet. C9,t11-CLA and VA individually inhibited multiple facets of allergic dermatitis when topically applied, and their combination produced a strong additive effect.

**Conclusion and Clinical Relevance** Enriched milk fat, and its two major fatty acids c9,t11-CLA and vaccenic acid attenuate allergic dermatitis in mice.

**Keywords** allergen-specific Ig, allergy, atopic dermatitis, conjugated linoleic acid, eosinophils, milk fat, vaccenic acid

*Submitted 14 October 2010; revised 19 December 2010; accepted 17 January 2011*

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### Introduction

Atopic dermatitis (AD) is a common chronic inflammatory skin disease primarily affecting infants and children, which is characterized by pruritic and eczematous skin lesions [1]. The incidence of AD is steadily increasing, especially in Western countries, where up to 15% of the population are affected during childhood [2]. The most common therapy for AD is topical application of corticosteroids, but AD often relapses when the treatment is suspended. Further, long-term application of corticosteroids can cause side effects including atrophy, thinning of the skin, skin stretch marks, easy bruising and tearing, telangiectasia, and susceptibility to skin infections. Therefore, it is important to investigate alternative therapeutic

strategies that exhibit fewer side effects or at least spare the use of corticosteroids.

In 1997, Black and Sharpe proposed that the increase in allergic disease was related to the decrease in the consumption of butter and the corresponding increase in use of margarine [3]. A series of epidemiological studies has since provided support for this notion [4–10]. Milk fat contains a number of bioactive fatty acids that have potential anti-inflammatory actions. One of the most extensively studied milk fatty acids is *cis*-9, *trans*-11 conjugated linoleic acid (c9,t11-CLA). Feeding of a mix of synthetic forms of c9,t11-CLA and the *trans*-10, *cis*-12 CLA isomer that is not present in milk reduced allergen-induced release of histamine and prostaglandin E2 from guinea-pig tracheae, but did not affect tracheal

contractions [11, 12]. C9,t11-CLA inhibited the release of eosinophil cationic proteins from activated eosinophils, and reduced the proliferation of human bronchial epithelial cells and their production of IL-8 [13]. Feeding of CLA mostly composed of c9,t11-CLA to 40 subjects with birch pollen allergy revealed that CLA had modest anti-inflammatory effects in humans, which led to a greater feeling of well being and less sneezing during the pollen season [14]. Dietary synthetic c9,t11-CLA reduced allergic airway inflammation in mice via a peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ )-related mechanism and by reducing eicosanoid precursors [15]. The c9,t11-CLA isomer accounts for 75–90% of the CLA in milk fat. This isomer and its precursor *trans*-vaccenic acid (VA) are greatly elevated (up to fivefold) in the milk fat of cows fed fish and sunflower oil [16, 17]. We have reported previously that feeding of milk fat enriched in CLA and VA ('enriched milk fat') suppressed the development of allergic airway disease in mice [17]. In accord, increased levels of ruminant fatty acids, which include low levels of c9,t11-CLA, and VA in human breast milk were associated with a lower risk of atopic eczema and allergic sensitization in a study of 310 mother–infant pairs [18]. Here, we investigated whether topical and oral administration of enriched milk fat and its two major fatty acids have the potential to attenuate AD in mice.

## Materials and methods

### Mice

Six- to 8-week-old female C57BL/6 mice were bred and housed in the Animal Resource Unit, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand. The mice were kept in an air-conditioned room with controlled humidity, temperature, and a 12 h light:dark cycle. All experiments were conducted under a protocol approved by the Animal Ethics Committee, University of Auckland. The mice were randomly assigned to groups where each group had six mice.

### Milk fats and synthetic fatty acids

Enriched milk fat was prepared, as described previously [17] by supplementing the feed of a herd of pasture-fed cows with fish and sunflower oil to enrich the milk fat with CLA and VA. A single preparation of enriched milk fat, pooled from a group of cows in the herd, was used in the study. Typical anhydrous milk fat was obtained from the Fonterra Co-operative Group Ltd. (Palmerston North, New Zealand). The fatty acid profiles of enriched milk fat and normal milk fat are described in Table 1. Fatty acids were derivatized with sodium methoxide and analysed by gas chromatography as FAMES (fatty acid methyl esters). Separations were carried out using a 30 m FFAP capillary

Table 1. Composition of experimental fats

| Fatty acid        | Control milk fat<br>(% w/w) | Enriched milk fat<br>(% w/w) |
|-------------------|-----------------------------|------------------------------|
| c4:0              | 3.2                         | 3.2                          |
| c6:0              | 2.3                         | 1.7                          |
| c8:0              | 1.3                         | 1                            |
| c10:0             | 2.8                         | 2.2                          |
| c10:1             | 0.3                         | 0.2                          |
| c12:0             | 3.2                         | 2.5                          |
| c12:1             | 0.2                         | 0.1                          |
| c13:0 br          | 0.1                         | 0                            |
| c13:0             | 0.1                         | 0.1                          |
| c14:0 br          | 0.2                         | 0.1                          |
| c14:0             | 10.9                        | 9.1                          |
| c14:1             | 0.9                         | 0.8                          |
| c15:0 iso br      | 0.4                         | 0.3                          |
| c15:0 ante-iso br | 0.6                         | 0.6                          |
| c15:0             | 1.3                         | 1.2                          |
| c16:0 br          | 0.2                         | 0.2                          |
| c16:0             | 30.6                        | 19.7                         |
| c16:1             | 1.8                         | 3                            |
| c17:0 iso br      | 0.6                         | 0.6                          |
| c17:0 ante-iso br | 0.4                         | 0.5                          |
| c17:0             | 0.8                         | 0.5                          |
| c17:1             | 0.3                         | 0.3                          |
| c18:0             | 10.5                        | 4.6                          |
| c18:1 n-9         | 16.6                        | 11.9                         |
| c18:1 n-7         | 4.7                         | 22.9                         |
| c18:2 n-6         | 1.4                         | 2.1                          |
| c18:3 n-3         | 0.8                         | 0.4                          |
| c18:2 conj        | 1.2                         | 5.3                          |
| c18:4 n-3         | 0                           | 0                            |
| c20:0             | 0.2                         | 0.1                          |
| c20:1 n-11        | 0.1                         | 0.1                          |
| c20:1 n-9         | 0                           | 0.1                          |
| c20:2 n-6         | 0                           | 0                            |
| c20:3 n-3         | 0.1                         | 0                            |
| c20:4 n-6 (AA)    | 0.1                         | 0                            |
| c20:3 n-3         | 0                           | 0.1                          |
| c20:3 n-3         | 0.1                         | 0.2                          |
| c20:3 n-3         | 0.1                         | 0.2                          |
| c20:5 n-3 (EPA)   | 0.1                         | 0.1                          |
| c22:0             | 0.1                         | 0.1                          |
| c22:1 n-13, n-11  | 0                           | 0.1                          |
| c22:2 n-9         | 0                           | 0                            |
| c22:4 n-6         | 0                           | 0                            |
| c22:5 n-3         | 0.1                         | 0                            |
| c24:0             | 0                           | 0                            |
| c22:6 n-3 (DHA)   | 0                           | 0                            |
| c24:1             | 0                           | 0                            |

Fatty acids as determined by FAME (fatty acid methyl ester) analysis. AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

column for standard FAME analysis and a polar 120 m BPX70 capillary column for CLA isomer separation. Synthetic c9,t11-CLA was obtained from Larodan Fine Chemicals AB (Malmo, Sweden) and synthetic VA from Nu-Chek Prep Inc. (Elysian, MN, USA).

### *Sensitization of skin to ovalbumin and topical application of milk fats and synthetic fatty acids*

The methodology of inducing allergic dermatitis has been described previously [19]. Briefly, the mice were shaved, and the dorsal skin was tape-stripped six times under anaesthesia. Ovalbumin [OVA; 100 µg in 100 µL of phosphate-buffered saline (PBS)] (Sigma, St Louis, MO, USA) was placed on a patch of gauze (1×1 cm), which was secured to the skin site with a transparent bio-occlusive dressing for a 1-week period and then removed. Two weeks later, the animals were randomly assigned to treatment groups. Each mouse received a total of three 1-week exposures to a patch separated by 2-week patch-free intervals. For the second and third sensitizations, patches were soaked in 100 µL of diluent containing 100 µg OVA and the treatment reagents including either 50 µg of the corticosteroid triamcinolone acetonide (Sigma), 2 mg of milk fat (containing ~23 µg of c9,t11-CLA and ~94 µg of VA), 2 mg of CLA-enriched milk fat (containing 100 µg of c9,t11-CLA and ~460 µg of VA), 500 µg of synthetic c9,t11-CLA, 2 mg of synthetic VA, or a combination of 500 µg of synthetic c9,t11-CLA and 2 mg of synthetic VA. Thus, the total amount of fat applied to the skin was similar for the synthetic fatty acids (2.5 mg) vs. the milk fats (2 mg). It is conceded that the levels of c9,t11-CLA and VA are elevated above that used in milk fat, but they are not protected by the presence of other major fatty acids as in the milk fats. The mice were monitored daily to ensure that the patches remained in place throughout the sensitization period. They were killed 24 h after removal of the third patch, and blood and skin tissue samples were collected. Serum was separated from blood samples, and stored at -80 °C.

### *Dietary treatments with milk fats and synthetic fatty acids*

The experimental diets were prepared by Crop and Food Research, Palmerston North, New Zealand using the AIN-93G formulation as a base, which contains soybean oil as the fat source at 7% of the diet [17, 20]. The final fat content of all diets was maintained at the same level as the fat content of the control AIN-93G diet by reducing the soybean oil (which contains no CLA and VA) content. The mice were randomly assigned to four diet groups, which included the control AIN-93G diet, or the AIN-93G diet containing either 1% enriched milk fat (representing 0.05% c9,t11-CLA and 0.23% VA), 1% milk fat (representing 0.012% c9,t11-CLA and 0.047% VA), or 0.14% synthetic c9,t11-CLA in combination with 0.5% VA. The latter amounts of c9,t11-CLA and VA were used, as we had shown previously that their combination was effective in inhibiting allergic airway disease in mice [17]. The ratio of VA to c9,t11-CLA was increased slightly from the previous study to be closer to the ratio found in enriched milk fat.

It is estimated that mice fed enriched milk fat would have ingested 60 and 235 mg/kg/day of c9,t11-CLA and VA, respectively. Those fed the combination of c9,t11-CLA and VA would have ingested 168 and 600 mg/kg body weight/day, respectively. The skin sensitization protocol was the same as that used in the topical application experiment. Each mouse received a total of three 1-week exposures to a patch separated by 2-week patch-free intervals. The first patch was applied to the skin of mice 2 weeks following administration of the diets. Fresh diet was provided biweekly, and mice had free access to food and water throughout the study. The mice were killed 24 h after removal of the third patch, and blood and skin tissue samples were collected.

### *Assessment of dermatitis*

The assessment of the severity of dermatitis was performed on completion of the third sensitization, and was expressed as described previously [21] as the sum of the individual score grades of 0 (no symptoms), 1 (mild), 2 (moderate), to 3 (severe) for each of the following five symptoms: (1) erythema/haemorrhage, (2) oedema, (3) excoriation/erosion, (4) scaling/drying, and (5) lichenification.

### *Scoring of skin pathology by histological analysis*

Skin tissues were fixed in 10% formalin, embedded into paraffin, sectioned, stained with haematoxylin-eosin, and examined by light microscopy in a blinded fashion. The dermis was assessed for hypertrophy, hyperkeratosis, and both the dermis and hypodermis were examined for infiltration by inflammatory cells. The overall histology score was expressed as the sum of the individual score grades according to the following scoring system: 0 (no symptoms), 1 (mild), 2 (moderate), to 3 (severe) [21].

### *Enumeration of infiltrating inflammatory cells*

Skin sections were examined for infiltration by neutrophils and mononuclear cells. Eosinophils were identified in sections after staining with haematoxylin-Biebrich scarlet, and mast cells after staining with toluidine blue. The numbers of neutrophils, eosinophils, mononuclear cells, and mast cells were counted blindly in 20 high-power fields (HPFs) at ×1000 and expressed as the mean number of cells per HPF.

### *Enumeration of infiltrating leucocyte subsets by immunohistological analysis*

Cryosections (10 µm thickness) were prepared from skin tissues, and stained with monoclonal antibodies (mAbs) (Pharmingen, San Diego, CA, USA) against the leucocyte markers CD45, CD3, CD4, and CD8. Sections were

counterstained with haematoxylin, and individual inflammatory cells were counted.

#### *Enzyme-linked immunosorbent assay*

The methodology for measuring OVA-specific IgE and IgG1 has been described previously [17].

#### *Statistical analysis*

Results were expressed as mean  $\pm$  SD, and a one-way analysis of variance (ANOVA) followed by a Dunnett's test was used to evaluate statistical significance. One-way ANOVA was used by including all the groups except the healthy control. There was only one factor, i.e., the diet.  $P$ -values  $< 0.05$  were considered statistically significant.

### **Results**

#### *Topical application of enriched milk fat and its two major fatty acids attenuates allergic dermatitis*

Epicutaneous sensitization of mice with a patch impregnated with 100  $\mu$ g of OVA induced allergic dermatitis, as evidenced by significant increases in clinical and histological scores, compared with the skins of healthy control mice (Figs 1–6). In a preliminary study, there was no difference between the skins of healthy mice and those treated with PBS-treated mice; hence, the skins of untreated healthy control mice were used for comparison. The skins of diseased mice showed increased erythema/haemorrhage, oedema, excoriation/erosion, scaling/drying, lichenification/hypertrophy, hyperkeratosis, inflammatory cell infiltration of the dermis and hypodermis, and increased levels of allergen-specific Igs (Figs 1–6). The mice had skin lesions were infiltrated by neutrophils, eosinophils, mast cells, and mononuclear cells including CD3<sup>+</sup> T cells, and CD4<sup>+</sup>, and CD45<sup>+</sup> cells, whereas few CD8<sup>+</sup> cells were present. The potent corticosteroid triamcinolone acetonide was used to benchmark the therapeutic effectiveness of topically applied fatty acids in ameliorating allergic dermatitis. Triamcinolone acetonide (50  $\mu$ g) efficiently reduced the clinical signs of dermatitis by 80% ( $P < 0.001$ ) compared with the untreated diseased control mice (Fig. 1a). Synthetic forms of c9,t11-CLA and VA were used to tease out the individual effects of these two major fatty acids in milk fat. Enriched milk fat, c9,t11-CLA, VA, and the combination of c9,t11-CLA and VA significantly (all  $P < 0.05$ ) reduced the clinical score by 30%, 39%, 42%, and 53%, respectively, compared with the untreated diseased control mice (Fig. 1a). In contrast, the effects of milk fat did not reach significance.

The abilities of the agents to reduce the histological scores paralleled the clinical signs of disease such that enriched milk fat, c9,t11-CLA, VA, and the combination of

c9,t11-CLA and VA significantly (all  $P < 0.05$ ) reduced the clinical score by 20%, 34%, 40%, and 55%, respectively, compared with untreated diseased mice (Fig. 1a). Once again, milk fat was not found to have a significant effect. None of the bioactives displayed the high level of activity of triamcinolone acetonide, which significantly reduced the histologic score by 77% ( $P < 0.001$ ). Figure 1b illustrates the pathology of representative skin sections, where triamcinolone acetonide, and enriched milk fat and its major fatty acid components reduce hypertrophy, hyperkeratosis, and inflammatory cell infiltration of the skin to different extents.

#### *Topical application of the fatty acids inhibits leucocyte infiltration of the skin*

Topical application of enriched milk fat significantly inhibited the infiltration of sensitized skin by all leucocyte types examined, including neutrophils and eosinophils (both  $P < 0.001$ ), mononuclear cells, and mast cells (both  $P < 0.05$ ) (Fig. 2a), CD45<sup>+</sup>, CD3<sup>+</sup>T, and CD4<sup>+</sup> cells (all  $P < 0.05$ ), but not CD8<sup>+</sup> cells (Fig. 2b). C9,t11-CLA and VA both significantly ( $P < 0.05$ ) inhibited the infiltration of neutrophils, eosinophils, mononuclear cells, and mast cells (Fig. 2a). They inhibited infiltration by CD3<sup>+</sup> T cells, and CD45<sup>+</sup> and CD4<sup>+</sup> cells (all  $P < 0.05$ ) (Fig. 2b). The combination of c9,t11-CLA and VA significantly inhibited the infiltration of sensitized skin by all leucocyte types examined, including neutrophils and eosinophils (both  $P < 0.001$ ), mononuclear cells and mast cells (both  $P < 0.05$ ) (Fig. 2a), CD3<sup>+</sup> T cells, and CD45<sup>+</sup> and CD4<sup>+</sup> cells (all  $P < 0.001$ ) (Fig. 2b). Representative photographs of skin sections stained by haematoxylin–Biebrich Scarlet for eosinophils are shown in Figs 2c–e. Relatively large numbers of eosinophils can be observed in the skin sections of untreated diseased control mice (Fig. 2c), whereas there is a paucity of such cells in the skin sections of mice treated with triamcinolone acetonide (Fig. 2d) or the combination of c9,t11-CLA and VA (Fig. 2e).

#### *Topical application of fatty acids diminishes allergen-specific immunoglobulin production*

Topical challenge with allergen led to high levels of OVA-specific IgE (Fig. 3) and OVA-specific IgG1 (Fig. 3) in the sera of untreated diseased mice, compared with healthy mice. Topical treatment of sensitized skin with enriched milk fat, c9,t11-CLA, VA, and the combination of c9,t11-CLA and VA significantly (all  $P < 0.05$ ) suppressed the increase in OVA-specific IgE by 36%, 42%, 49%, and 64%, respectively. In contrast, the effects of normal milk fat were not significant (Fig. 3). Topical application of c9,t11-CLA, VA, and the combination of c9,t11-CLA+VA also significantly (all  $P < 0.05$ ) suppressed the increase in OVA-specific IgG1 by 57%, 52%, and 73%, respectively,

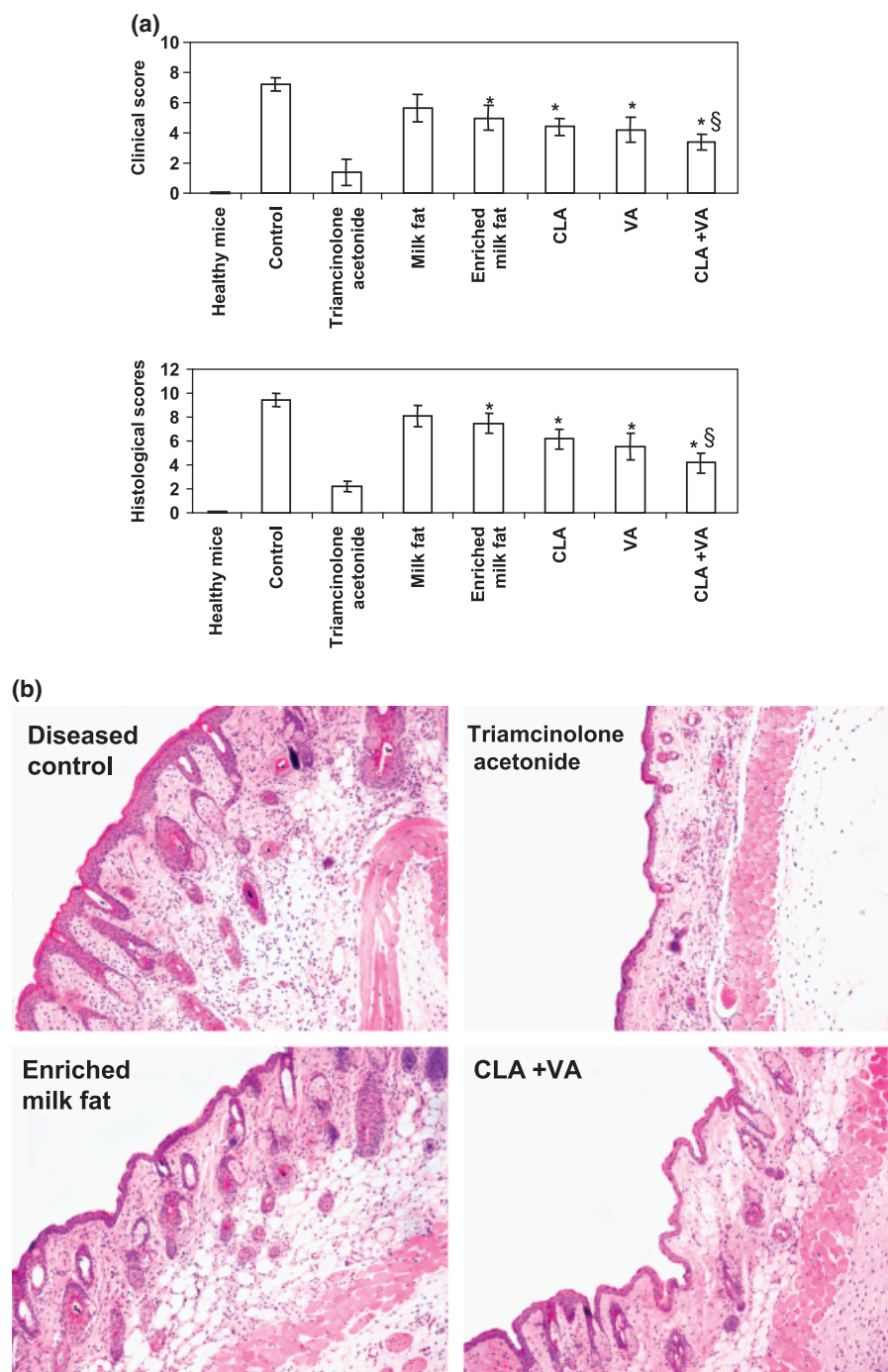


Fig. 1. Topical application of enriched milk fat and its two major lipids *cis*-9 *trans*-11 conjugated linoleic acid (c9,t11-CLA) and *trans*-vaccenic acid (VA) attenuates the symptoms of allergic dermatitis. (a) The severity of dermatitis was assessed based on overall clinical (upper figure) and histological (lower figure) scores. Data are presented as mean  $\pm$  SD. \* $P$  < 0.05, significantly lower than diseased control and § $P$  < 0.05, significantly lower than CLA and VA monotherapies. (b) Representative photographs ( $\times 400$  magnification) of skin sections stained with haematoxylin-eosin were taken from diseased control mice, and diseased mice treated with triamcinolone acetonide, enriched milk fat, and the combination of conjugated linoleic acid (CLA) and *trans*-vaccenic acid (VA).

whereas the effects of the milk fats did not reach significance (Fig. 3). By comparison, triamcinolone acetonide reduced OVA-specific IgE and IgG1 levels by 85% and 82%, respectively.

In summary, significant attenuation of allergic dermatitis was achieved by topical application of enriched milk fat and its two major fatty acids, particularly when the two major fatty acids were administered in combination.

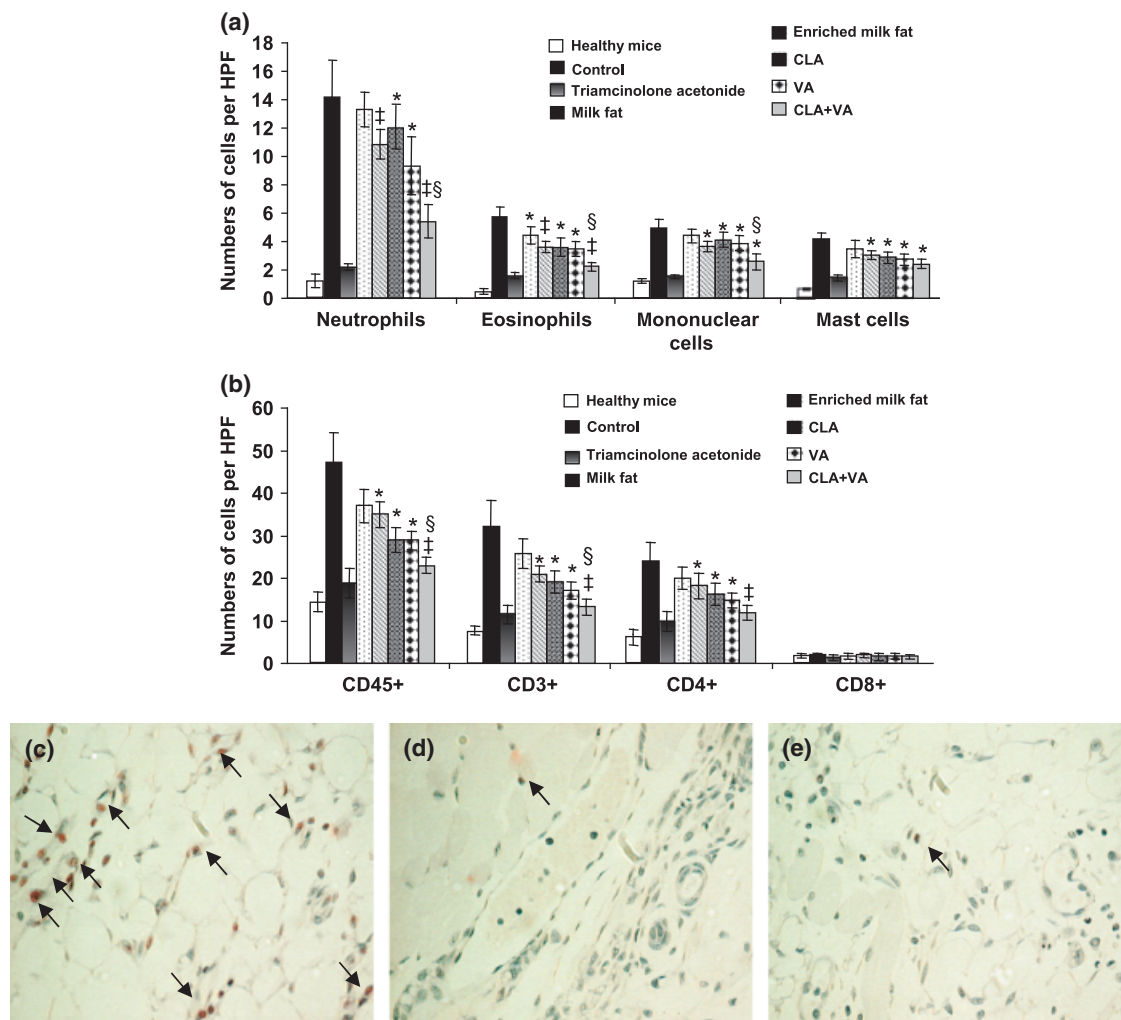


Fig. 2. Topical application of enriched milk fat and its two major fatty acids inhibits leucocyte infiltration of skin. (a) The number of neutrophils, eosinophils, mononuclear cells, and mast cells infiltrating the dermis and hypodermis of the skin was determined by randomly examining 20 high power fields (HPFs). (b) Identification of leucocyte subsets that infiltrate the skin. The numbers of leucocyte subsets in 20 randomly selected HPFs was determined after staining skin biopsies with antibodies against the leucocyte markers CD45, CD3, CD4, and CD8. Data are presented as mean  $\pm$  SD. \* $P < 0.05$ , significantly and  $^{\dagger}P < 0.001$ , highly significantly lower than diseased control.  $^{\S}P < 0.05$ , significantly lower than conjugated linoleic acid (CLA) and trans-vaccenic acid (VA) monotherapies. (c–e) Representative photographs ( $\times 400$  magnification) of skin sections stained with haematoxylin–Biebrich Scarlet for the presence of eosinophils were from diseased control mice (c), and diseased mice treated with triamcinolone acetone (d), and the combination of CLA and VA (e). Arrows point to eosinophils.

#### Oral administration of enriched milk fat and its two major fatty acids attenuates allergic dermatitis

The above experiments established that localized topical application of enriched milk fat and its two major fatty acids attenuates allergic dermatitis. Here, we tested whether oral administration of enriched milk fat and the combination of c9,t11-CLA and VA would have a systemic effect on allergic dermatitis. The amount of enriched milk fat added to the diet (1%) was selected based on our previous study, which showed that enriched milk fat was effective over a broad dose range (0.5–7%) in attenuating allergic airway disease in mice [17]. The amount of

synthetic c9,t11-CLA and VA selected to add to the diet (0.14% and 0.5%, respectively) was more than twice the amount of the respective fatty acids present in enriched milk fat, as these amounts had been shown previously to inhibit allergic airway disease in mice [17]. The ratio ( $\sim 1 : 4$ ) of c9,t11-CLA to VA reflects the ratio of these fatty acids in enriched milk fat. CLA-enriched milk fat and the combination of c9,t11-CLA and VA fed to OVA-sensitized mice significantly ( $P < 0.05$  and  $P < 0.001$ , respectively) reduced the clinical score by 34% and 63%, respectively, compared with that of untreated diseased mice (Fig. 4). In contrast, the effects of normal milk fat did not reach significance. The histological scores correlated with the

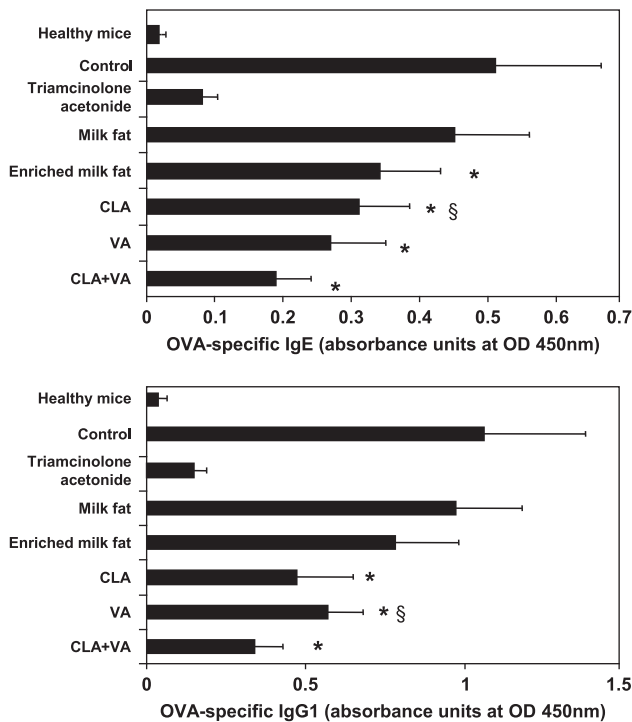


Fig. 3. Topical application of enriched milk fat and its two major fatty acids inhibits the production of allergen-specific antibodies. Serum levels of ovalbumin (OVA)-specific IgE (upper figure) and IgG1 (lower figure) antibodies determined by ELISA. \* $P < 0.05$ , significantly lower than diseased control and § $P < 0.05$ , significantly different from combinational treatment with conjugated linoleic acid (CLA) and trans-vaccenic acid (VA).

clinical signs of disease, where significant (both  $P < 0.05$ ) levels of inhibition were obtained with enriched milk fat (33% inhibition) and the combination of c9,t11-CLA and VA (46%) (Fig. 4). Once again, milk fat was found to be ineffective.

#### Oral administration of fatty acids inhibits leucocyte infiltration of the skin

Oral enriched milk fat significantly ( $P < 0.05$ ) inhibited the infiltration of sensitized skin by all leucocyte types, except mononuclear cells (Figs 5a and b). The diet containing a combination of c9,t11-CLA and VA highly significantly inhibited infiltration of sensitized skin by neutrophils, eosinophils, mast cells, CD45<sup>+</sup>, CD3<sup>+</sup>, and CD4<sup>+</sup> cells ( $P < 0.001$ ), and significantly inhibited infiltration by mononuclear cells ( $P < 0.05$ ) (Figs 5a and b). Milk fat only significantly ( $P < 0.05$ ) inhibited the infiltration of eosinophils and CD45<sup>+</sup> cells (Figs 5a and b).

#### Oral administration of fatty acids diminishes allergen-specific immunoglobulin production

Oral delivery of enriched milk fat and the combination of c9,t11-CLA and VA suppressed the increase in OVA-

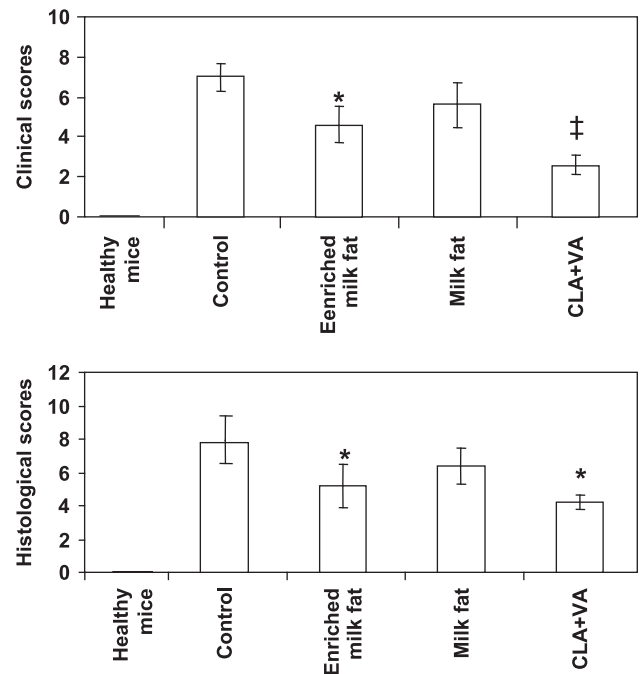


Fig. 4. Oral delivery of enriched milk fat and its two major fatty acids attenuates the symptoms of allergic dermatitis. The severity of dermatitis was assessed based on overall clinical (upper figure) and histological (lower figure) scores. Data are presented as mean  $\pm$  SD. \* $P < 0.05$ , significantly lower than diseased control and † $P < 0.001$ , highly significantly lower than diseased control.

specific IgE in response to allergen by 40% and 50% (both  $P < 0.05$ ), respectively, whereas milk fat was ineffective (Fig. 6). The combination of c9,t11-CLA and VA suppressed the increase in OVA-specific IgG1 by 59% ( $P < 0.001$ ), whereas the effects of the milk fats did not reach significance (Fig. 6).

#### Discussion

The present study has demonstrated that topical and oral administration of milk fat enriched in c9,t11-CLA and VA suppress allergic inflammation in a mouse model of AD. The findings are in accord with our recent study, which revealed that the same product attenuated the development of allergic airway disease in mice [17]. In contrast, these therapeutic properties are not shared with normal milk fat, at the level used here. In general, the various disease parameters were consistently reduced by milk fat, but without reaching significance. We cannot discount the possibility of a type-2 error i.e. sample size was too small to detect the effect of normal milk fat at the level used. Note, however, a milk fat diet did not reduce disease symptoms in our earlier study of allergic airway disease [17]. Inspection of the clinical and histological scores suggested that oral delivery of enriched milk fat and synthetic c9,t11-CLA and VA was more effective than topical delivery. At some future time-point, it would be

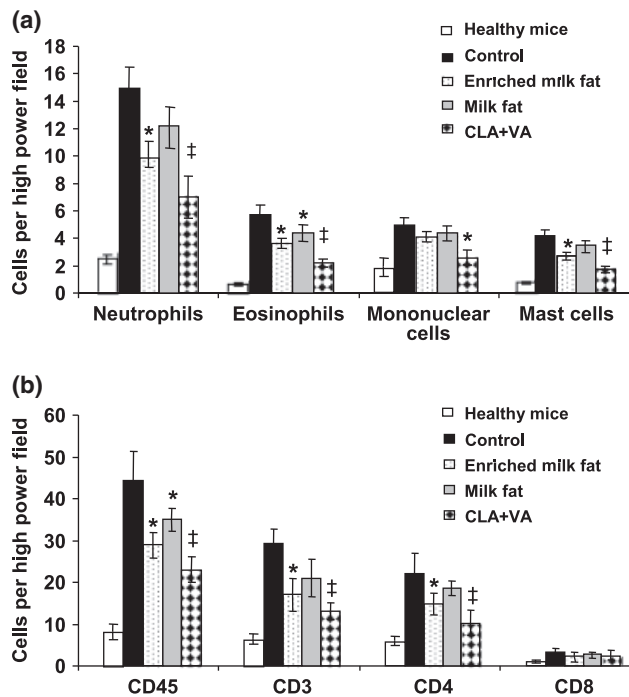


Fig. 5. Oral application of enriched milk fat and its two major fatty acids inhibits leucocyte infiltration of skin. (a) The number of neutrophils, eosinophils, mononuclear cells, and mast cells infiltrating the dermis and hypodermis of the skin was determined by randomly examining 20 high power fields (HPFs). (b) Identification of leucocyte subsets that infiltrate the skin. The numbers of leucocyte subsets in 20 randomly selected HPFs was enumerated after staining skin biopsies with antibodies against the leucocyte markers CD45, CD3, CD4, and CD8. Data are presented as mean  $\pm$  SD. \* $P$  < 0.05, significantly and  $^{\dagger}P$  < 0.001, highly significantly lower than diseased control.

of interest to simultaneously deliver enriched milk fat and/or synthetic c9,t11-CLA and VA via both routes.

The c9,t11-CLA isomer and VA are major lipids that are markedly elevated in enriched milk fat vs. normal milk fat [17], and presumably account for at least some of the therapeutic activity. Both topical and oral administration of c9,t11-CLA and VA suppressed allergic inflammation, but the fatty acids were much more effective in combination as reported previously in a mouse model of allergic airway disease [17]. The mechanisms responsible for the additive effect displayed by VA and c9,t11-CLA are unknown. Only ~12% of dietary VA is converted into c9,t11-CLA, and would not be enough to account for the increased activity [22]. Synergy between c9,t11-CLA and VA is evident in treatment of the metabolic syndrome. Thus, VA, which has lipid-lowering properties independent from CLA, enhanced the hypolipidaemic effects of chronic c9,t11-CLA supplementation on circulating dyslipidaemia and hepatic steatosis in rats [23].

Enriched milk fat and its two major fatty acids inhibited many of the facets of allergic dermatitis including pathological changes to the skin, leucocyte infiltration, and aller-

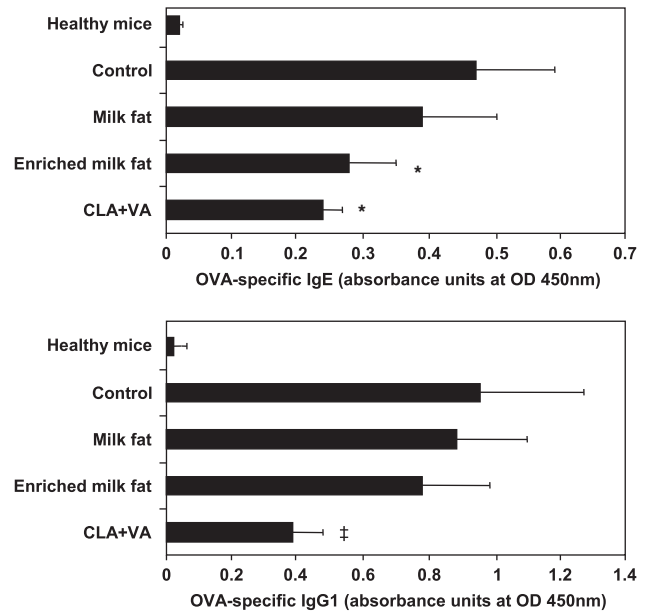


Fig. 6. Oral application of enriched milk fat and its two major fatty acids inhibits the production of allergen-specific antibodies. Serum levels of ovalbumin (OVA)-specific IgE (upper figure) and IgG1 (lower figure) antibodies determined by ELISA. \* $P$  < 0.05, significantly lower than diseased control and  $^{\dagger}P$  < 0.001, highly significantly lower than diseased control.

gen-specific IgE production. Similar levels of effectiveness were seen with oral vs. topical administration, indicating that enriched milk fat, c9,t11-CLA isomer and/or VA have systemic effects. In all measures of efficacy, the combination of c9,t11-CLA and VA displayed greater activity than that of enriched milk fat. However, it is important to consider the doses of the individual fatty acids used. For topical application, the amount of CLA-enriched milk fat used contained about one-fifth of the amount of c9,t11-CLA and VA that was used for the synthetic fatty acids. For oral administration, the enriched milk fat diet contained less than half the amount of c9,t11-CLA and VA that was used for the diet containing the combination of synthetic fatty acids. Presumably, the effectiveness of enriched milk fat in combating allergic dermatitis relates not only to the presence of c9,t11-CLA and VA but also to minor fatty acids and other components in the enriched milk fat. Normal milk fat which contains ~5-fold lower concentrations of CLA and VA, was not effective, at the level used here.

In our previous study of allergic airway disease, it was found that supplementing the diet with enriched milk fat suppressed eosinophilic inflammation, and decreased the production of allergen-specific IgE, IL-5, and eotaxin-1 [17]. In the present study, enriched milk fat and its two major fatty acids inhibited several facets of dermatitis including skin leukocytosis and eosinophilia, and allergen-specific IgE production, whereas normal milk fat was not effective. There was increased infiltration of CD4<sup>+</sup> cells but not CD8<sup>+</sup> cells into skin lesions in the mouse

model of OVA-induced dermatitis, in accord with a previous report [24]. Oral-enriched milk fat and its two major fatty acids inhibited infiltration by CD3<sup>+</sup> T cells, CD4<sup>+</sup> cells, and CD45<sup>+</sup> cells.

There are a number of mechanisms by which polyunsaturated fatty acids (PUFAs) might influence allergic inflammation including exerting changes in cell membrane fluidity and composition, acting as substrates and/or inhibitors of cyclo-oxygenases (COXs) or lipoxygenases (LOXs), and as ligands for nuclear receptors [25–27]. The cellular uptake of c9,t11-CLA has been reported to cause a decrease in eicosanoid release, which is thought to be due to inhibition of the incorporation of arachidonic acid into membrane phospholipids [25, 28]. C9,t11-CLA inhibits COX-2 [29, 30] and LOX [31] activity and/or expression, and thereby blocks the rate-limiting step of prostaglandin, thromboxane, and leukotriene synthesis. The three PPARs, PPAR $\alpha$ ,  $\beta/\delta$ , and  $\gamma$ , are the best characterized nuclear receptors for PUFAs [32–34], although PUFAs also bind to liver X receptors (LXR) and hepatocyte nuclear factor  $\alpha$  (NHF-4 $\alpha$ ) [35, 36]. Nuclear receptors bind to DNA when activated and promote transcription of their target genes. CLA binds to both PPAR $\alpha$  and PPAR $\gamma$  [36], and *cis*-vaccenic acid binds PPAR- $\beta/\delta$  [37]. Activation of PPAR $\alpha$  and PPAR $\gamma$  dramatically inhibits cellular immune responses and production of inflammatory mediators, in part by inhibiting activation of NF- $\kappa$ B [32–34]. Dietary synthetic c9,t11-CLA was shown to reduce allergic airway inflammation in mice via a PPAR $\gamma$ -related mechanism and by reducing eicosanoid precursors [15]. In the only previous experimental study of the effects of CLA on dermatitis, oral CLA (90 mg/kg body weight/day) did not improve the clinical signs of canine AD [38], but in this report the authors used a mixture of CLA isomers (80% purity, c9, t11 and t10, c12 ratio 1:1) rather than pure c9,t11-CLA isomer. Whereas the c9,t11-CLA isomer has anti-inflammatory effects, the *trans*-10 *cis*-12-CLA iso-

mer has been reported to induce inflammation (at least in white adipose tissue), and to accelerate inflammation in atherosclerosis [39, 40]. The anti-inflammatory properties of VA have not been extensively investigated. VA protects against the development of atherosclerosis in mice, as does c9,t11-CLA [40, 41], and it synergizes with c9,t11-CLA to inhibit allergic airway disease in mice [17]. As mentioned previously, increased levels of ruminant fatty acids including c9,t11-CLA, and VA in human breast milk were associated with a lower risk of atopic eczema and allergic sensitization in a study of mother–infant pairs [18].

As mentioned earlier, Black and Sharpe [3] proposed that the increase in allergic disease was related to the decrease in the consumption of butter and the corresponding increase in use of margarine. In the present study, the various signs of disease were consistently reduced by milk fat, but without reaching significance. A human clinical trial will be required to address the validity of the latter hypothesis.

In conclusion, the results demonstrate that enriched milk fat inhibits the development of allergic dermatitis in mice, where its two major fatty acids c9,t11-CLA and VA individually inhibit multiple facets of the disease and display additive effects when applied in combination. Therefore, enriched milk fat is a natural product that may warrant consideration as a functional food and/or topical cream to spare the usage of corticosteroids and prevent AD, depending on the outcome of clinical studies in humans.

## Acknowledgements

This work was supported by a grant from the Foundation for Research, Science and Technology, New Zealand, and supported by the Fonterra Co-operative Group Ltd., New Zealand, and Auckland Uniservices Ltd. We thank Yvonne van der Does (Fonterra) for help in preparing the diets.

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