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Bovine milk fat enriched in conjugated linoleic and vaccenic acids attenuates allergic airway disease in mice

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Clinical and Experimental Allergy

Summary

Background It has been argued that a reduction in the Western diet of anti-inflammatory unsaturated lipids, such as n-3 polyunsaturated fatty acids, has contributed to the increase in the frequency and severity of allergic diseases.

Objective We investigated whether feeding milk fat enriched in conjugated linoleic acid and vaccenic acids (VAs) ('enriched' milk fat), produced by supplementing the diet of pasture-fed cows with fish and sunflower oil, will prevent development of allergic airway responses.

Methods C57BL/6 mice were fed a control diet containing soybean oil and diets supplemented with milk lipids. They were sensitized by intraperitoneal injection of ovalbumin (OVA) on days 14 and 28, and challenged intranasally with OVA on day 42. Bronchoalveolar lavage fluid, lung tissues and serum samples were collected 6 days after the intranasal challenge.

Results Feeding of enriched milk fat led to marked suppression of airway inflammation as evidenced by reductions in eosinophilia and lymphocytosis in the airways, compared with feeding of normal milk fat and control diet. Enriched milk fat significantly reduced circulating allergen-specific IgE and IgG1 levels, together with reductions in bronchoalveolar lavage fluid of IL-5 and CCL11. Treatment significantly inhibited changes in the airway including airway epithelial cell hypertrophy, goblet cell metaplasia and mucus hypersecretion. The two major components of enriched milk fat, *cis*-9, *trans*-11 conjugated linoleic acid and VA, inhibited airway inflammation when fed together to mice, whereas alone they were not effective.

Conclusion Milk fat enriched in conjugated linoleic and VAs suppresses inflammation and changes to the airways in an animal model of allergic airway disease.

Keywords airway disease, airway remodelling, allergen-specific Ig, allergy, CCL11, conjugated linoleic acid (CLA), eosinophils, IL-5, milk fat, vaccenic acid

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Introduction

The western lifestyle is believed to have contributed to the increased prevalence of asthma and other allergic diseases. Modern diets are characterized by high proportions of n-6 polyunsaturated fatty acids (PUFA), which are potentially pro-inflammatory, and low proportions of n-3 PUFA [1–3]. However, while some studies have suggested a beneficial effect of fish oil in asthma [4, 5], other studies have been negative [6]. Studies of the relationship between dietary fat

and allergic disease have also looked at the consumption of dairy products and/or margarine and the risk of asthma, hayfever or eczema. In a study from the Netherlands, the frequent consumption of products containing milk fat was associated with a reduced risk of asthma symptoms in pre-school children followed prospectively [7]. The consumption of milk has been associated with a lower risk of wheezing [8, 9], whereas the consumption of margarine has been linked to an increased risk of allergic sensitization, hayfever, eczema or asthma [10–13].

Milk fat contains a number of bioactive fatty acids that have potential anti-inflammatory actions. One of the most extensively studied fatty acids from milk is conjugated

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linoleic acid (CLA). Seed-derived CLA, synthesized from the linoleic acid of safflower and sunflower seeds, is usually produced as an approximately equal mixture of the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers of CLA, and has been proposed to have a number of health benefits [14]. Feeding of the mixture of synthetic *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers reduced allergen-induced release of histamine and prostaglandin E2 from guinea-pig tracheae, but did not affect tracheal contractions [15, 16]. *Cis*-9, *trans*-11-CLA inhibited the release of eosinophil cationic protein from activated eosinophils, and reduced the proliferation of human bronchial epithelial cells (HBECs) and their production of IL-8, suggesting it may have the potential to ameliorate airway inflammation by affecting epithelial and eosinophil functions [17]. Nonetheless, an exhaustive analysis of the published data on the effects of CLA on immune function came to the conclusion that supplementation of the diet with CLA could not be recommended at this time [18]. Most of the studies included in this review used CLA that was synthetically derived and contained equal amounts of the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers. In contrast, there is a paucity of information on the therapeutic properties of naturally occurring CLA. The *cis*-9, *trans*-11 isomer accounts for 75–90% of the CLA in milk fat. CLA from milk fat has been tested for its anti-cancer properties [19, 20], but has never been examined for its ability to influence the symptoms of asthma. In the present study, we employed a mouse model of allergen [ovalbumin (OVA)]-induced airway disease to study the potential anti-allergic properties of milk fat that had been enriched in CLA by feeding fish and sunflower oil to cows. Vaccenic acid (VA) was the only other major lipid in milk fat that was greatly elevated in the milk fat of cows-fed fish and sunflower oil, and was also examined for its therapeutic potential, alone and together with CLA.

Materials and methods

Mice

Eight to 9-week-old male and female C57BL/6 mice used in the study were bred and housed in the Animal Resource Unit, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand. They were kept in an air-conditioned room with controlled humidity, temperature and 12 h light : dark cycle. All experiments were approved by the Animal Ethics Committee, University of Auckland.

Diets

All mice were kept on an OVA-free normal mouse chow diet from weaning before assigning them to a particular experimental diet. The experimental diets were prepared

using as a base the powdered AIN-93G formulation, containing soybean oil as the fat source [21]. 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. Enriched milk fat was prepared by supplementing the feed of a herd of pasture-fed cows with fish and sunflower oil [22] to enrich the milk fat with CLA and VA. A single preparation of enriched milk fat, pooled from several cows in the herd, was used in the study. Mice were fed a control AIN93G diet, or normal and enriched milk fat diets containing 0.5%, 2%, 5% and 7% of either normal milk fat or enriched milk fat (representing 7.1%, 29%, 71% and 100% of the total fat content of the diet). The normal milk fat contained (as a percentage of milk fatty acids) 1.2% *cis*-9, *trans*-11 CLA and 4.7% VA, whereas these lipids were increased in enriched milk fat to 5.0% *cis*-9, *trans*-11 CLA and 22.9% VA as a result of feeding fish and sunflower oil to the donor cows (Table 1). *Cis*-9, *trans*-11 CLA (90%) was obtained from Larodan Fine Chemicals AB, Sweden; and VA (99%) from Nu-Chek Inc., Elysian, MN, USA. VA and the *cis*-9, *trans*-11 CLA isomer were tested for their ability to attenuate airway disease as they represent the two major components of enriched milk fat, being present at 5% and 22.9% (w/w), respectively (Table 1). Mice were fed diets containing 0.14% of the *cis*-9, *trans*-11 CLA isomer [$\sim$ 2% (w/w) of the fat content] in the free fatty acid form, 0.21% VA [$\sim$ 3% (w/w) of the fat content] or a combination of equivalent amounts of the latter supplements. Healthy control mice sensitized and challenged with phosphate-buffered saline (PBS) were maintained on the AIN-93G diet. Mice had free access to food and water throughout the study. Blood samples were collected via the tail vein, before assignment of mice to particular diets.

Induction of airway disease

Groups of five to six mice that had been maintained on a chow diet were assigned to an experimental diet that they remained on until the completion of the experiment 6 weeks and 6 days later when they were killed. The latter regime was employed for all diets. After 2 weeks on the assigned diet mice were immunized by intraperitoneal (i.p.) injection of 20 μ g of OVA (chicken egg albumin grade V; Sigma Chemical Co., St Louis, MO, USA) complexed with 2 mg of inject alum [$\text{Al}(\text{OH})_3/\text{Mg}(\text{OH})_2$; Pierce, Rockford, IL, USA] in a total volume of 100 μ L of PBS] to induce disease. Two weeks following the first injection, they received a second injection of OVA, as above. After a further two weeks they were anaesthetized by i.p. injection of a mixture of ketamine and xylazine (Phoenix, Auckland, New Zealand), and challenged intranasally with 100 μ g of OVA in 50 μ L of PBS. Healthy control mice were immunized and challenged with PBS using a similar

Table 1. Content of milk fat diets

	Normal (% w/w)	Enriched (% w/w)	Fold increase
CLA component			
CLA (<i>cis</i> -9, <i>trans</i> -11)	1.17	5.04	3.3
CLA (<i>trans</i> -10, <i>cis</i> -12)	–	–	
CLA (minor c18:2 isomers)	0.55	1.30	1.4
TOTAL CLA (all forms)	1.72	6.34	2.7
Ratio <i>cis</i> -9, <i>trans</i> -11 CLA to total CLAs	68.0%	79.5%	
Fatty acid component			
c16:1 (palmitoleic acid)	1.8	3	0.7
c17:0 ante-iso br (methyl 14-methylhexadecanoate)	0.4	0.5	0.25
c18:1 n-7 (vaccenic acid)	4.7	22.9	3.9
c18:2 n-6 (linoleic acid)	1.4	2.1	0.5
c18:2 (CLA)	1.2	5.3	3.4
c20:1 n-9 (eicosenoic acid)	0	0.1	NA
c20:3 n-3 (eicosatrienoic acid)	0	0.1	NA
c20:4 n-3 (eicosatetraenoic acid)	0.1	0.2	1.0
c20:5 n-3 (eicosapentaenoic acid, EPA)	0.1	0.2	1.0
c22:1 n-13, n-11 (docosenoic acid)	0	0.1	NA
c22:5 n-3 (docosapentaenoic acid, DPA)	0.1	0	NA
c22:6 n-3 (docosahexaenoic acid, DHA)	0	0	NA

Fatty acids as determined by FAME (fatty acid methyl ester) analysis.

Fold increase refers to increase in lipid levels in enriched milk fat vs. normal milk fat. Only fatty acids that increase are shown, plus DPA and DHA.

c18:1 n-7 provides an estimate of the c18:1 trans fatty acid content.

CLA, conjugated linoleic acid; NA, not applicable.

regime. Blood, bronchoalveolar lavage (BAL) fluid and lung tissue samples were collected 6 days after the intranasal challenge, at which point the animal experiment ended. While the late-phase response is readily detectable 24–48 h after allergen challenge [23], the 6 day time-point was chosen to ensure that any inhibition of the late-phase response was sustained and was not just transient. Blood was collected by cardiac puncture, and serum stored at -80°C .

Bronchoalveolar lavage and cytology

Mice were euthanized with an overdose of ketamine and xylazine, and BAL was immediately performed by flushing 1 mL of PBS containing 1% heat-inactivated foetal calf serum (lavage buffer) thrice through the lungs and airways of mice via the cannulated trachea. The recovered BAL fluid was pooled for each animal, centrifuged at 365 g at 4°C , and the supernatant stored at -80°C . The cell pellets were resuspended in 1 mL of lavage buffer, and total cell numbers were counted using a haemocytometer. BAL fluid cytopspins were stained with Diff-Quik stain (Dade Behring Inc., Newark, DE, USA) and differential cell counts enumerated.

Histology and immunohistochemistry

The left lobes of the lungs were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned. Some sections were stained with a combined haematoxylin-Biebrich Scarlet solution by Luna's method for eosinophils, or with Alcian Blue-Periodic Acid Schiff to detect acid and neutral mucins and identify goblet cells. To detect apoptotic bodies, deparaffinized lung sections were permeabilized with 0.01% Triton-X100 and incubated with a rabbit anti-active caspase-3 antibody (Promega – G7481; 1:200 dilution). Immunoreactivity was detected with a goat anti-rabbit IgG-peroxidase conjugate (Sigma; 1:300) and diaminobenzidine (Sigma). Sections were counterstained with haematoxylin, examined by microscopy and digitally recorded.

Scoring of pathology

The level of Schiff's staining of airway epithelia was semi-quantitatively determined by microscopy to give a mucus index score [24]. A minimum of four to six representative transversely or sagittally sectioned airways were graded per animal using a scale of 0 (no staining), 1 (25% or less of the airway epithelium stained), 2 (26–50% of the airway epithelium stained), 3 (51–75% of the airway epithelium stained) and 4 (more than 75% of the airway epithelium stained). A histopathology score was determined from inspection of Alcian Blue-Periodic Acid Schiff stained paraffin-embedded sections of the left lung of each animal. Lung inflammation, perivascular/peribronchiolar infiltrates, airway epithelial hypertrophy, goblet-cell hyperplasia, constriction of bronchioles, mucin hypersecretion and beneficial presence of phagocytic macrophages were graded on a scale of 0 (no change) to 4 (marked change). Each animal received an overall histopathology score based on summation of individual scores for each criteria. All slides were scored in a blinded fashion (blinded to diet treatment/group assignment) and scores were presented as the mean $\pm$ SEM of four to six animals/group.

Enzyme-linked immunosorbent assay

OVA-specific IgG1 and IgE in serum samples were measured by standard ELISA using horse-radish peroxidase-conjugated goat anti-mouse IgG1 (diluted 1:50 000; Bethyl Laboratories, USA) and biotin-conjugated rat anti-mouse IgE (diluted 1:200; Pharmingen, San Diego, CA, USA) antibodies. The levels of IL-5 and CCL11 (eotaxin-1) in the BAL fluid were quantitated by ELISA using Quantikine mouse IL-5 and CCL11 ELISA kits (R&D Systems, MN, USA). The detection limits were 3 pg/mL for CCL11 and 7 pg/mL for IL-5.

Statistical analysis

Data are expressed as the means + SEMs, where statistical significance was determined by the Student *t*-test to compare each treatment group with the control group. *P*-values <0.05 were considered statistically significant.

Results

Enriched milk fat attenuates lung inflammation

Allergen challenge led to a marked increase in the leukocyte content of the bronchial fluid of mice fed the control AIN93G diet ($P < 0.01$) and the normal milk fat diet ($P < 0.001$) (Fig. 1a). In contrast, the total leukocyte counts and eosinophil numbers in the bronchial fluid of mice fed an enriched milk fat diet were reduced by 72% and 88% (both $P < 0.01$), respectively (Figs 1a and b). The decrease in eosinophils was accompanied by 61% ($P < 0.01$) and 61% ($P < 0.05$) reductions in the numbers of monocytes/macrophages and lymphocytes, respectively (Fig. 1b). BAL fluid from healthy control mice had a low cellular content (Fig. 1a), which as expected was comprised almost entirely of monocytes/macrophages (Fig. 1b).

Enriched milk fat is effective over a broad dose range

The cellular content of the bronchial fluid increased with increasing doses (0.5%, 2% and 7%) of normal milk fat in the diet (Fig. 1c). In contrast, enriched milk fat at the latter doses reduced the total BAL cell counts by 38–56% ($P < 0.05$), compared with normal milk fat. The BAL fluid of mice fed the normal milk fat diet contained high numbers of monocytes/macrophages and eosinophils in almost equal proportions, and lesser but nevertheless high numbers of lymphocytes (Fig. 1d). In marked contrast, enriched milk fat skewed the leukocyte profile strongly in favour of monocytes/macrophages that were almost the only cell in lavage fluid from mice not challenged with allergen. While the macrophage content of the BAL fluid of mice-fed low doses of each milk fat diet was similar, the eosinophil and lymphocyte contents were markedly reduced by 90% ($P < 0.001$) and 56% ($P < 0.01$), respectively, after feeding enriched milk fat.

Feeding of enriched milk fat inhibits eosinophil survival

The abundant eosinophils in the bronchial fluid of OVA-challenged mice fed the control AIN93G and normal milk fat diets had intact nuclear, organellar and plasma membranes, and condensed peripheral chromatin (Fig. 2). In contrast, the small numbers of eosinophils in the bronchial fluid of mice-fed enriched milk fat had often perished, as evidenced by chromatolysis, loss of plasma

membrane integrity and a degenerated morphology (Fig. 2). Monocyte/macrophages had phagocytosed one or more eosinophils as they often contained clusters of free eosinophil granules (CFEGS), and were heavily vacuolated suggesting they had engulfed eosinophilic bodies.

Enriched milk fat suppresses the production of interleukin-5 and CCL11

IL-5 and CCL11 control eosinophil survival and recruitment to the asthmatic lung [25, 26]. Challenge with allergen led to significant 21- and fivefold (both $P = 0.003$) increases in IL-5 (Fig. 3a) and CCL11 (Fig. 3b) in the BAL fluid of control mice fed the AIN93G diet, respectively. The 0.5%, 2% and 5% enriched milk fat diets reduced the expression of IL-5 (Fig. 3a) below the level of detection, or by 70% ($P = 0.02$) and 71% ($P = 0.002$), respectively. Similarly, the expression of CCL11 (Fig. 3b) fell by 70% ($P = 0.01$) and 69% ($P = 0.005$) or was reduced below the limits of detection, respectively. The 0.5% and 2% normal milk fat diets reduced the levels of IL-5 by just 10% and 5.7%, whereas 5% milk fat slightly increased IL-5 levels. The 0.5% and 5% milk fat diets had negligible effect on the levels of CCL11, whereas 2% milk fat increased the levels by 25%.

Enriched milk fat inhibits other changes in the airways

As in humans, the airways of OVA-challenged mice underwent major pathologic changes, which included the presence of massive peribronchial and perivascular infiltrates of leukocytes, goblet cell metaplasia and mucus hypersecretion as evidenced by an abundance of neutral mucopolysaccharides that stained bright 'red' with Schiff's reagent (Fig. 4a). In marked contrast, these changes were reduced in the lungs of allergen-challenged mice fed the enriched milk fat diet (Fig. 4a). All three percentages of enriched milk fat significantly inhibited mucus production by 51–66% ($P < 0.01$ – 0.05), compared with the control AIN93G diet (Table 2, Fig. 4a). In contrast, of the three percentages of normal milk fat tested, only the lowest dose (0.5%) significantly inhibited (by 63%, $P < 0.05$) mucus production (Table 2). There were considerably less eosinophils in the lung tissue of mice fed enriched milk fat, and those present had degenerated (Fig. 4b, A, B). Sparse numbers of cells in the lung tissue stained positive for the cleaved active form of caspase-3 (Fig. 4b, C, D), and may represent apoptotic eosinophils, as such cells were not detected in the lung tissue of mice fed normal milk fat and control AIN-93 G diets (data not shown). Macrophages that had engulfed large numbers of eosinophilic bodies in common with their counterparts in the BAL fluid were detected in lung tissue (Fig. 4b, E). In contrast, inspection of blood smears (Fig. 4b, F) established that circulating eosinophils had a normal

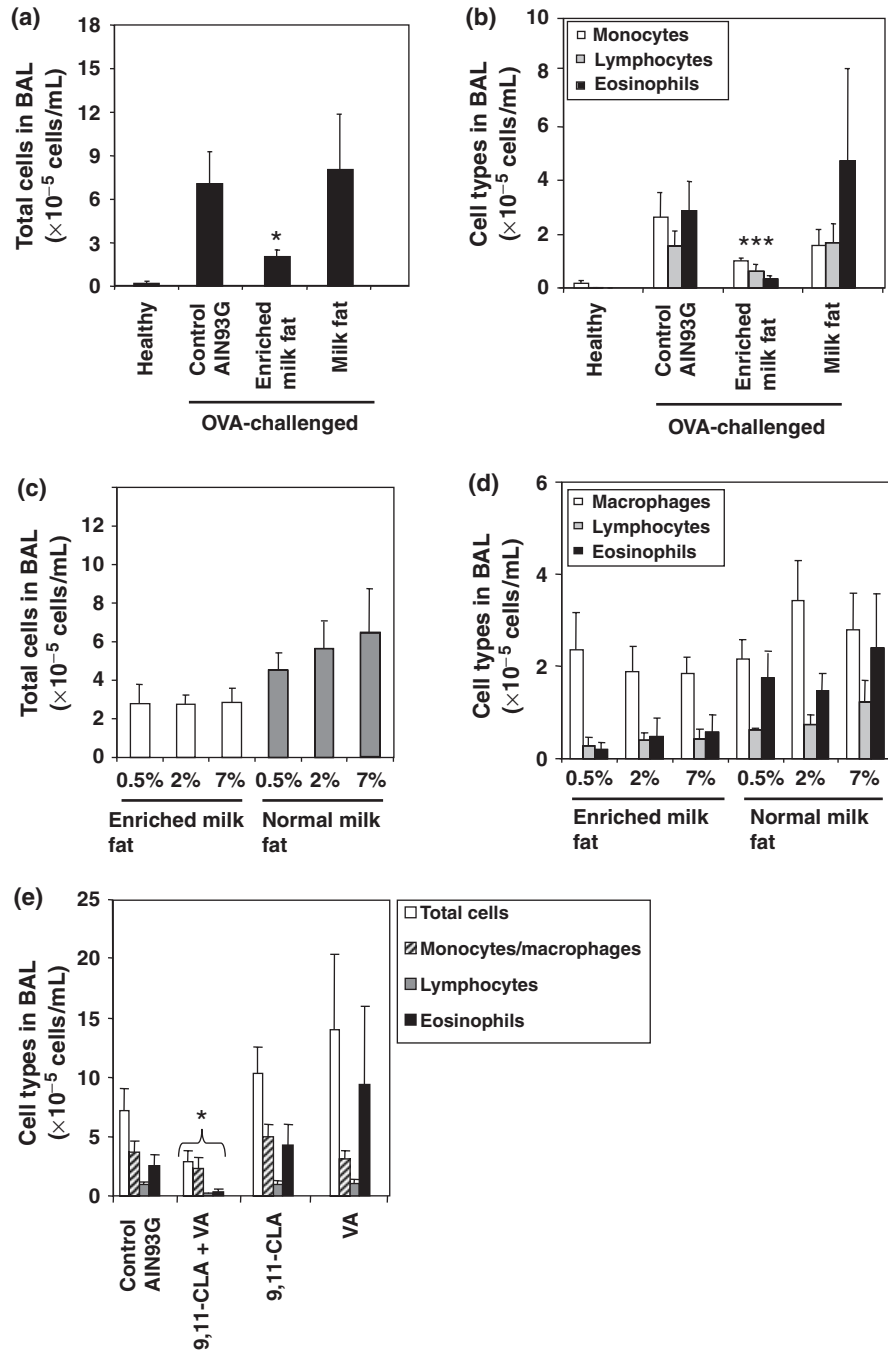


Fig. 1. Quantification of airway inflammation. (a) Total cell numbers in the bronchoalveolar lavage (BAL) fluid of mice fed the AIN93G diet, or the same diet containing enriched milk fat or normal milk fat. Mice were immunized and challenged intranasally with ovalbumin (OVA) or phosphate-buffered saline (PBS) (healthy control, designated 'Healthy'). The mean numbers of cells ($\pm$ SEM) in the BAL fluid is shown ($n = 5-6$ mice per group). *Statistically significant compared with OVA-challenged mice fed the control diet. (b) Cell types present in BAL fluid. Cytospins of cells in the BAL fluid (a) were stained with Diff-Quik and analysed microscopically to record the different cell types. Mean cell counts ($\pm$ SEM) are shown ($n = 5-6$ mice per group). (c) Total cell numbers in BAL fluid of mice fed diets in which the milk fat content ranged from 0.5% to 7%, as indicated. The mean numbers of cells ($\pm$ SEM) in the BAL fluid after OVA challenge is shown ($n = 5$ to 6 mice per group). (d) Enumeration of cell types present in BAL fluid (c). Mean cell counts ($\pm$ SEM) in the BAL fluid are shown ($n = 5-6$ mice per group). (e) Feeding a diet containing a combination of VA and *cis*-9, *trans*-11 conjugated linoleic acid (CLA) inhibits airway inflammation. Mice fed the AIN93G diet, or the same diet in which the soybean oil was partially substituted with Vaccenic acid (VA), *cis*-9, *trans*-11 CLA, or a combination of the latter supplements were immunized and challenged intranasally with OVA. BAL fluid was collected six days after the OVA challenge. The mean number of total BAL cells, monocytes/macrophages, lymphocytes and eosinophils ($\pm$ SEM) is shown ($n = 5-6$ mice per group). *Statistically significant compared with OVA-challenged mice fed the control diet.

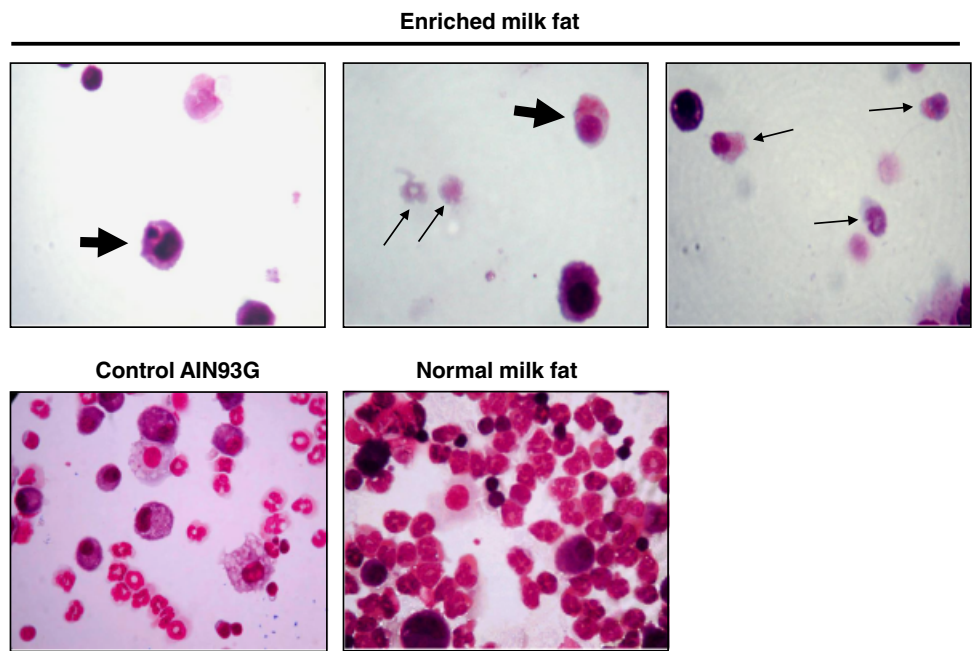


Fig. 2. Enriched milk fat inhibits the survival of eosinophils in the bronchial fluid. Cells in cytosmears of the bronchoalveolar lavage (BAL) fluid of mice fed the control AIN93G, enriched milk fat and normal milk fat diets, as indicated, were stained with Diff-Quick solution and analysed microscopically to record changes in cell morphology. Thin arrows indicate degenerated eosinophils; thick arrows indicate macrophages that have phagocytosed eosinophilic bodies.

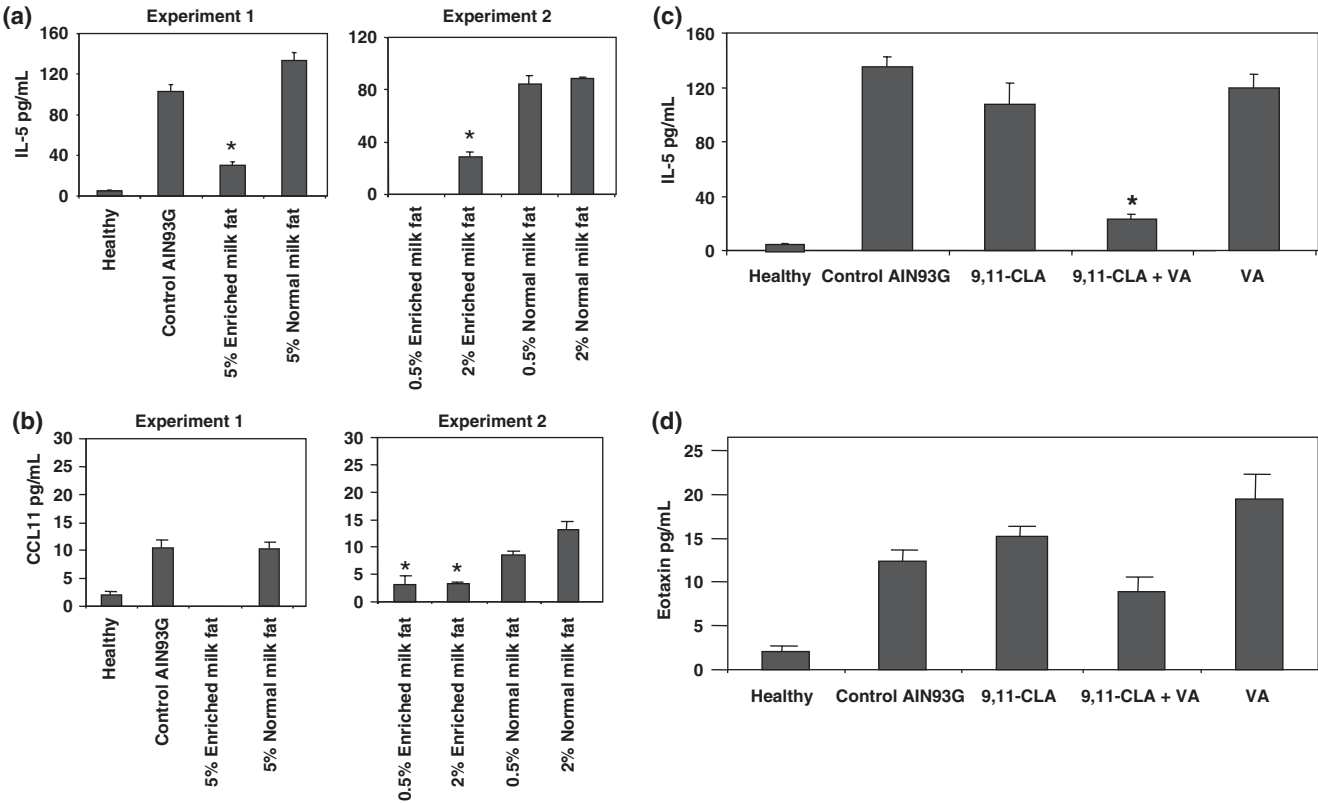


Fig. 3. Expression of IL-5 and CCL11 in bronchial fluid determined by ELISA. IL-5 (a, c) and CCL11 (b, d) levels in bronchial fluid after allergen challenge were determined by ELISA in two separate experiments, Experiments 1 and 2 (a, b), to determine the effect of enriched milk fat, or in a separate experiment to determine the effects of *cis*-9, *trans*-11 conjugated linolic acid (CLA) and vaccenic acid (VA) (c, d) on IL-5 and CCL11 expression. Data are expressed as mean $\pm$ SEM, $n = 5-6$ mice. *Statistical significance from control.

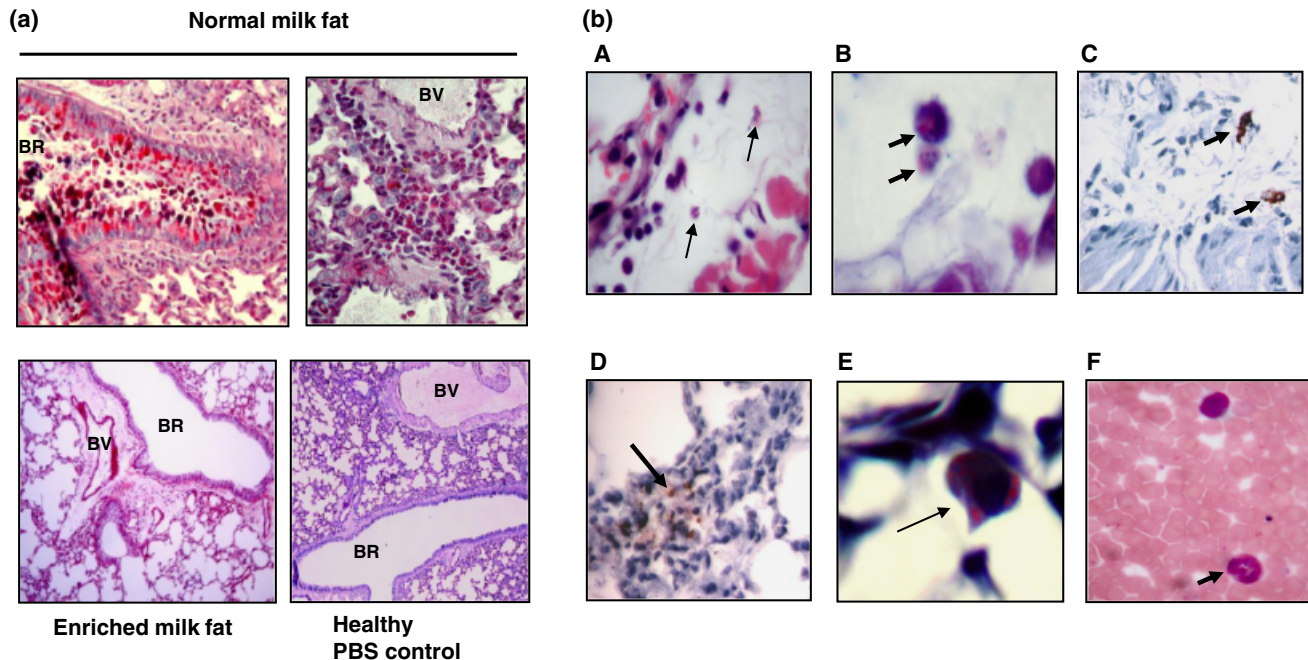


Fig. 4. Effects of diets on airway remodelling and eosinophil survival in the lung tissue. (a) Representative lung sections from mice fed 7% normal milk fat and 0.5% enriched milk fat, and from undiseased mice sensitized and challenged with phosphate-buffered saline (PBS) (healthy control, designated 'Healthy'), as indicated. Sections were stained with Alcian Blue-Periodic Acid Schiff reagent. Note the extensive perivascular infiltrates of leukocytes (top right-hand image) and red-stained neutral mucins (top left-hand image) that line the bronchial airways of diseased mice fed high doses of normal milk fat. BR, bronchial airway; BV, bronchial venule. (b) Lung sections (A-E) and a blood smear (F) prepared from ovalbumin (OVA)-challenged mice fed enriched milk fat. Sections were stained with Alcian Blue-Periodic Acid Schiff reagent (A, B) and haematoxylin-Biebrich Scarlet solution (E), and the smear was stained with Diff-Quick (F). Apoptotic eosinophils containing active caspase-3 were stained with an anti-caspase-3 antibody (C, D). Arrows in (A, B) indicate degenerated eosinophils in peribronchial spaces, and the arrow in (E) denotes a tissue macrophage that appears to have engulfed large numbers of eosinophilic bodies/cfegs. The arrow in F denotes a morphologically normal eosinophil in the peripheral blood.

morphology, indicating that eosinophil degeneration in response to enriched milk fat was confined to the lung. Changes to the lung were scored for inflammation, perivascular/peribronchiolar infiltrates, beneficial presence of phagocytic macrophages, airway epithelial hypertrophy, goblet-cell hyperplasia, constriction of bronchioles and mucus hypersecretion to give an overall histopathology score (Table 2). Enriched milk fat at 0.5% of the diet reduced the histopathology score by 64% ($P < 0.001$), whereas normal milk fat at any of the tested percentages tested had no significant effect, compared with the control diet.

Enriched milk fat diminishes allergen-specific immunoglobulin production

Challenge with allergen led to large increases ($P < 0.001$) in the levels of OVA-specific IgE and IgG1 in the sera of mice fed the control AIN93G diet, and the 5% normal milk fat diet (Table 3). In contrast, the 5% enriched milk fat diet significantly suppressed the increase in OVA-specific IgE by 30% ($P < 0.05$) and OVA-specific IgG1 by 45% ($P < 0.05$), compared with levels in the sera of mice fed the control AIN93G diet. In a separate experiment, diets

Table 2. Mucus and histopathology scores of lung airway changes

Diet Group	Mucus		Histopathology	
	score	<i>P</i> -value	score	<i>P</i> -value
PBS control (undiseased)	<0.02		0.05 ± 0.04	
Control AIN93G	3.5 ± 0.2		3.82 ± 0.18	
Enriched milk fat				
0.5	1.7 ± 1.4	<0.05	1.36 ± 0.76	<0.001
2	1.2 ± 1.0	<0.01	1.85 ± 0.52	<0.001
7	1.5 ± 1.4	<0.05	1.68 ± 0.72	<0.001
Normal milk fat				
0.5	1.3 ± 1.0	<0.05	3.25 ± 0.73	>0.05
2	2.5 ± 1.1	>0.05	3.35 ± 0.40	>0.05
7	3.6 ± 0.5	>0.05	3.87 ± 0.15	>0.05

P-values compared to control AIN93G diet.

PBS, phosphate-buffered saline

containing 0.5%, 2% and 7% enriched milk fat suppressed the increase in OVA-specific IgE by 60%, 50% and 55%, and OVA-specific IgG1 by 31%, 31% and 38% (Table 3), respectively, compared with levels in the sera of mice fed the corresponding amounts of normal milk fat.

Table 3. Allergen-specific Ig responses

Diet group	IgE	<i>P</i> -value ^a	IgG1	<i>P</i> -value
Healthy	0.002 ± 0.001		0.003 ± 0.00	
Control AIN93G	0.32 ± 0.04		0.79 ± 0.30	
Enriched milk fat 5	0.22 ± 0.07	<0.05	0.44 ± 0.07	<0.05
Normal milk fat 5	0.48 ± 0.06	>0.05	0.83 ± 0.21	>0.05
Diet group	IgE	<i>P</i> -value ^b	IgG1	<i>P</i> -value
Enriched milk fat				
0.5	0.47 ± 0.11	<0.05	1.62 ± 0.20	<0.001
2	0.55 ± 0.09	<0.01	1.63 ± 0.48	<0.05
7	0.59 ± 0.11	<0.001	1.73 ± 0.26	<0.01
Normal milk fat				
0.5	1.17 ± 0.35		2.36 ± 0.10	
2	1.10 ± 0.12		2.36 ± 0.19	
7	1.31 ± 0.23		2.78 ± 0.60	

Ig values represented as OD490 nm. *P*-value^a compared with control AIN93G diet. *P*-value^b compared to corresponding normal milk fat diet.

Vaccenic acids and cis-9, trans-11 conjugated linoleic acid in combination attenuate airway inflammation

The *cis-9, trans-11* isomer and VA diets increased the total BAL fluid cell counts on average by 43% ($P < 0.05$) and 94% ($P = 0.06$), and the accumulation of eosinophils by 69% ($P = 0.08$) and 270% ($P = 0.07$), respectively (Fig. 1e), though the latter increases did not reach statistical significance. The increase in leukocyte infiltration from feeding *cis-9, trans-11* CLA was partly due to an increase (35%, $P = 0.05$) in monocytes/macrophages. Neither supplement had a significant effect on infiltration by lymphocytes. In contrast, the combination of *cis-9, trans-11* CLA and VA on average reduced the total BAL fluid cell counts by 60% ($P < 0.01$), and suppressed the accumulation of eosinophils by 86% ($P < 0.01$), compared with the control diet (Fig. 1e). The decrease in eosinophils was accompanied by a 38% ($P < 0.05$) reduction in the numbers of monocytes/macrophages and a 79% ($P = 0.001$) reduction in the numbers of lymphocytes.

Vaccenic acids and cis-9, trans-11 conjugated linoleic acid in combination inhibit changes to the airways, including interleukin-5 and CCL11 expression

The histopathology score and mucus index used to denote changes to the airways were reduced in mice fed the combination of VA and *cis-9, trans-11* CLA by 55% ($P < 0.001$) and 44% ($P < 0.05$), respectively, compared with the scores of mice fed the control diet (Fig. 5). In contrast, diets containing either VA or *cis-9, trans-11* CLA did not significantly reduce the histopathology score and mucus index. The combination of VA and *cis-9, trans-11* CLA markedly reduced the expression of IL-5 in the BAL fluid by 83% ($P = 0.0005$). In contrast, diets containing either VA or *cis-9, trans-11* CLA only slightly reduced IL-5

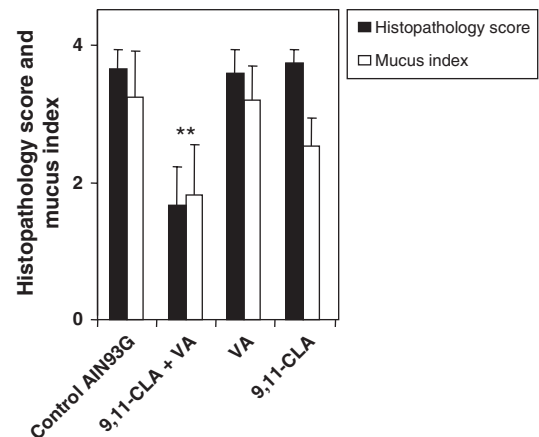


Fig. 5. Feeding a diet containing a combination of Vaccenic acid (VA) and *cis-9, trans-11* Conjugated linoleic acid (CLA) inhibits changes to the airways. Sections of the left lung were examined histologically and given an overall histopathology score for inflammation, perivascular/peribronchiolar infiltration, airway epithelial hypertrophy, goblet-cell hyperplasia and presence of phagocytic macrophages. Sections were also scored for mucus hypersecretion to give a mucus index. All slides were scored in a blinded fashion (blinded to diet treatment/group assignment) and scores were presented as the mean ± SEM of 5–6 animals/group.

levels by 11% and 21%, respectively (Fig. 3c). The level of CCL11 was similarly reduced by the combination of VA and *cis-9, trans-11* CLA, but did not reach statistical significance, whereas the individual bioactives were not effective (Fig. 3d).

Discussion

This study has demonstrated that the enriched milk fat, produced by supplementing the diets of cows with fish and sunflower oil, suppresses the development of OVA-induced inflammation in a mouse model of allergic airway

disease. The results indicated that the activity of enriched milk fat is largely attributable to its two major components, *cis*-9, *trans*-11 CLA and VA. In accord, normal milk fat which contains ~5-fold lower concentrations of CLA and VA had no discernible effect at moderate doses. At very high doses normal milk fat slightly exacerbated disease symptoms, but this is unlikely to be relevant to man because such high doses are not consumed in the normal human diet.

Allergen-specific IgE is believed to be intricately linked to the induction of allergic airway disease [27]. In the present study, low-dose enriched milk fat reduced allergen-specific IgE and IgG1 levels by at least 30% and 45%, respectively. Pulmonary eosinophilia induced in response to IL-5 is a hallmark of most types of asthma [28–30]. The numbers of eosinophils have been correlated broadly with disease severity [31], where the release by eosinophils of proinflammatory cationic proteins contributes to airway hyperresponsiveness and epithelial damage, and promotes oedema [32]. In the present study, enriched milk fat and the combination of *cis*-9, *trans*-11 CLA and VA were potent inhibitors of lung eosinophilia and lymphocytosis, whereas normal milk fat with lower amounts of *cis*-9, *trans*-11 CLA and VA had no discernible effect at low to moderate doses. Enriched milk fat and the combination of *cis*-9, *trans*-11 CLA and VA appeared to reduce eosinophil survival in the lung. The decreased recruitment of eosinophils to the lungs and their failure to survive correlated with the ability of enriched milk fat and the combination of *cis*-9, *trans*-11 CLA and VA to markedly decrease the levels of IL-5 and CCL11. Eosinophils may have undergone apoptosis, as suggested by the appearance of active caspase-3-positive cell bodies in lung tissue. There was evidence that eosinophilic cellular debris and apoptotic bodies were rapidly cleared by macrophages in both the airway lumen and tissue of the lung, thereby returning the lung to a state of normality. Reduced apoptosis of eosinophils in induced sputum has been found to correlate with asthma severity in human patients [33]. It has been proposed that removal of eosinophilic infiltrates by apoptosis and concomitant phagocytosis offers the potential to ameliorate inflammation [34], an outcome which enriched milk fat may promote.

Respiratory mucus is normally produced to protect the alveoli from dehydration and damage from inhaled particles, pathogens and chemical irritants, but it is produced in excessive amounts in asthma and contributes to airway obstruction. Enriched milk fat at the three doses tested, and the combination of *cis*-9, *trans*-11 CLA and VA, inhibited goblet cell metaplasia and the overproduction of airway mucus. The airways of mice-fed enriched milk fat, and the combination of *cis*-9, *trans*-11 CLA and VA, were relatively free of mucus, whereas those of mice fed the two highest doses of normal milk fat or the control diet were occluded with mucus plugs. Surprisingly, low doses

of normal milk fat were as effective as enriched milk fat in inhibiting mucus overproduction. In summary, the results indicate that enriched milk fat and the combination of *cis*-9, *trans*-11 CLA and VA are able to inhibit both allergen-induced lung inflammation and remodelling. In contrast, normal milk fat does not inhibit lung inflammation, but at low doses is capable of inhibiting mucus production.

Of note, the present study has demonstrated a synergy between *cis*-9, *trans*-11 CLA and VA in attenuating the symptoms of allergic airway disease. VA is produced in the rumen of cows, and normally constitutes ~0.4–4% of the total fatty acid content [35]. VA is converted in the tissues of mice [36] and humans [37] to *cis*-9, *trans*-11 CLA by the enzyme delta(9)-desaturase. While the effects of *cis*-9, *trans*-11 CLA on immune cell function have been widely investigated [reviewed in O'Shea et al.[38]], those of VA are as yet unexplored. The *cis*-9, *trans*-11 CLA isomer is able to induce the apoptosis of Jurkat T cells, whereas VA is without effect [39], suggesting *cis*-9, *trans*-11 CLA and VA exert distinct effects on immune cells. The relationship between VA and CLA has been studied in animal models of cancer. Banni et al. [40] demonstrated that feeding rats VA increased the tissue concentrations of *cis*-9, *trans*-11 CLA, which has anti-carcinogenic properties, leading to a corresponding reduction in the number of premalignant mammary lesions after exposure to a chemical carcinogen. Corl et al. [41] extended these findings by demonstrating an additive effect for dietary CLA and VA. The combination caused a dose-dependent increase in the accumulation of CLA in the mammary fat pad and a parallel reduction in tumour number and incidence. Although VA may just act by increasing the level of *cis*-9, *trans*-11 CLA in the tissues, it was suggested that VA may also exert anti-carcinogenic effects independent of *cis*-9, *trans*-11 CLA [42]. Santora et al. [36] determined that ~12% of the VA consumed by mice during a 2-week feeding period was retained as *cis*-9, *trans*-11 CLA in its triacylglycerol form. In the current study, mice were fed 3 g of VA together with 2 g of *cis*-9, *trans*-11 CLA per 1.4 kg of diet. Given the findings of Santora et al. the VA fed to mice would elevate the level of dietary *cis*-9, *trans*-11 CLA by just 18% to 2.36 g. It is improbable that an 18% increase in the level of *cis*-9, *trans*-11 CLA can explain the synergy displayed by the combination diet. Unraveling the mechanism for the synergy between *cis*-9, *trans*-11 CLA and VA will require further study. Total 18:1 *trans* fatty acid intake has been associated with adverse effects in humans, including proinflammatory effects (increases in CRP and IL-6) and adverse effects on endothelial function and plasma lipids [43]. This is probably not the case with VA which is not an industrially produced *trans* fatty acid. A double blind controlled trial of the effects of a test butter that was high in VA found that it reduced total cholesterol and did not lead to a change in the ratio of total to HDL cholesterol or in CRP compared with a

control butter [44]. Nonetheless further studies in this area are warranted.

Enriched milk fat attenuates several major facets of asthma including leukocyte infiltration, B-cell responses, eosinophil survival, mucus gland hyperplasia and mucus secretion. *Cis*-9, *trans*-11 CLA and VA appear to be responsible for many of these effects of enriched milk fat. The *cis*-9, *trans*-11 CLA isomer and VA were the only major lipids that were markedly elevated (~three-fourfold) in enriched milk fat vs. normal milk fat. Palmitoleic acid and linoleic acid were slightly increased (0.5–0.7-fold). The latter increases were at the expense of the major lipids of normal milk fat including palmitic (c16:0), stearic (c18:0) and oleic (c18:1 n-9) acids, which were all reduced (data not shown). Bovine milk fat contains principally (75–90%) the *cis*-9, *trans*-11 isomer of CLA. The second most prevalent CLA isomer in milk fat is the *trans*-7, *cis*-9 isomer, present at about 10% of the level of the *cis*-9, *trans*-11 isomer [45], but milk fat contains traces of many additional isomers of CLA which together with the *trans*-7, *cis*-9 isomer comprise ~1% of milk fat components. Another fatty acid in milk fat which may have important beneficial effects on human health is butyric acid (C4:0) [46], however, the level of this bioactive was identical in enriched milk fat and normal milk fat. We cannot discount the possibility that the therapeutic activity of our enriched milk fat preparation results from the effects of a number of additional bioactive milk fat components acting in concert with *cis*-9, *trans*-11 CLA and VA.

It is important to consider whether the doses of enriched milk fat used in the present study would be logistically feasible in humans. Enriched milk fat was most effective at its lowest dose, representing 0.5% of the overall diet and 7% of the fat content of the diet. In the present study, the lowest effective dose of enriched milk fat was not ascertained. Nevertheless, normal milk fat provides another dosage point for the active ingredients *cis*-9, *trans*-11 CLA and VA. The amount of *cis*-9, *trans*-11 CLA and VA in normal milk fat is one-fifth of that in CLA-enriched milk fat, and this amount was not effective at attenuating the symptoms of allergic airway disease in the mouse model. Hence, the lowest dose of enriched milk fat used in the current study is a good starting point for consideration of the dosage to be fed to humans. Assuming that enriched milk fat was as effective in humans as in mice, and given that the recommended daily intake of fat (30% of calories) for an adult human male is 100 g, then 7 g of enriched milk fat would have to be consumed each day. By comparison, a single serving of full-fat ice-cream contains 25–40 g of fat, suggesting that an effective amount of enriched milk fat could be readily consumed.

In conclusion, enriched milk fat could potentially be employed to attenuate the symptoms of asthma in human patients. It was most effective at the lowest dose tested,

representing just 0.5% of the overall diet (7% of the fat content) and this suggests that it may be a therapeutic option in man. In future studies, it would be worthwhile to determine whether feeding enriched milk fat is effective in preventing allergy in the newborn, whose immune system, though fully developed, is albeit immature. In addition, enriched milk fat could possibly be taken by lactating mothers to influence the composition of their breast milk. The potential of enriched milk fat in treating other allergic diseases also deserves consideration. Clearly clinical studies will need to be undertaken in man to determine whether or not this is the case.

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