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Differences between normal and milk allergic subjects in their immune responses after milk ingestion

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SUMMARY In order to understand why non-atopic people do not have adverse symptoms to food antigens which enter the circulation after eating, 8 non-atopic and 10 atopic eczema- and milk-allergic subjects were challenged with milk, and the types of circulating immune complexes formed were analysed. Although the amount of β-lactoglobulin incorporated into complexes did not differ statistically between the groups, the type of immune complex did. Of the non-atopic individuals, 5 formed IgA and 2 IgG complexes. Of the milk-allergic group, all showed a rise in at least one type; 5 formed IgA, 7 IgG, 6 IgE, and 6 formed C1q-binding complexes. Our data suggest that serum IgA is concerned in safe food antigen handling in non-atopic people, and that the differences in the type of immune complexes formed in response to antigen challenge may underlie the systemic symptoms of food allergy.

Food antigen entry into the circulation across the gastrointestinal mucosa is now a well-known phenomenon[1 2]; about 10^{-4} to 10^{-7} of the amount ingested is absorbed in an antigenically-intact form.[3] This is sufficient to induce a systemic antibody response in most normal individuals.[4 5] However, antibody titres to dietary antigens do not increase despite continued antigenic stimulation throughout life[4], suggesting that a state of hyporesponsiveness prevails in healthy people. In mice, feeding an antigen gives rise to local secretory antibody production which limits antigen entry, and to the simultaneous induction of systemic tolerance,[6 7] the mechanisms of which are little known.

It is possible that food allergy results from a breakdown of either the immune exclusion mechanisms at the mucosal surface or this state of systemic hyporesponsiveness, or possibly both. After eating foods to which the individual is allergic local symptoms may arise in the gut, but systemic diseases—such as eczema,[8] asthma, or urticaria[9]—may be provoked.

In order to understand how the non-allergic individual handles the safe entry of food antigens into the circulation, we have studied the different types of circulating immune complexes containing the relevant antigen formed after oral challenge, and compared such responses between non-allergic and food-allergic individuals.

Methods

Subjects for the oral challenge. Eight non-atopic subjects were investigated. Four were healthy adults, and 4 were children (aged 6–12 years) who had had a steroid-responsive nephrotic syndrome, but who were now in remission and had been off all treatment for at least 6 months; they were studied as part of another investigation into the capacity to clear naturally-occurring immune complexes. None of these non-atopic subjects had a family history of allergy, nor did any react positively to skin prick testing with common allergens—such as house dust mite, grass pollen extract, cat fur, whole milk, and whole egg (Bencard Ltd). Twelve children (aged 5–13 years) with atopic eczema were investigated; 10 had had exacerbations of symptoms after milk ingestion given 'double blind' with placebo, and also had specific IgE antibodies to milk proteins. They constituted the group allergic to milk. The other 2 eczematous children were not allergic to milk according to these criteria, but reacted by skin testing to house dust mite and grass pollen extracts; they and the one adult with grass pollen rhinitis constituted the atopic group who were not allergic to milk proteins. Ethical committee approval had been obtained for the studies, and informed consent was obtained from parents. All the subjects received 10 ml/kg of fresh cows' milk to drink after an overnight fast, and blood samples were collected via an indwelling cannula before and at various times up to 5 hours after milk intake. All the specimens were clotted at room temperature for 2 hours and the sera stored at −70°C. Blood was also obtained from 11 non-atopic members of the laboratory staff

(age range 27–38 years) after an overnight fast to obtain a reference range for IgG and IgA antibodies to milk antigens.

Circulating immune complexes. These were detected and quantitated by radiolabelled C1q precipitation,[10] inhibition of IgA-coated latex particle agglutination,[11] and 2·4% polyethylene glycol (PEG) precipitation followed by quantitation of IgG by radial immunodiffusion in agar.[12] IgE was measured by solid phase radioimmunoassay.[13]

Detection of bovine β-lactoglobulin within the complexes. The antigen within the complexes was measured after the complexes had been dissociated at low pH. Briefly, 100 μl of serum was mixed with an equal amount of 0·2 mmol/l EDTA pH 7·5 and 50 μl PEG (MW 6000, Sigma) 0·1 mmol/l in borate buffer pH 8·0 to a final concentration of 2·4%. After overnight incubation in the cold, the precipitates were washed in 2·4% PEG and resuspended in 0·2 mmol/l glycine HCl buffer pH 2·6 to the original volume in order to dissociate the complexes. This solution (50 μl of it) was absorbed on to polystyrene conical autoanalyser cups (18 hours' incubation at room temperature) and after saturation of the plastic surface with 1% human serum albumin, a ^{125}I-labelled immunoadsorbent purified rabbit anti-β-lactoglobulin was added for 18 hours at +4°C. After washing 4 times the radioactivity bound to the cups was measured in an LKB gamma counter, and expressed as net counts per minute (cpm) bound after background and control counts had been subtracted.[14]

Antibodies to bovine β-lactoglobulin. To assay IgG antibodies, 100 μl of sera were incubated overnight in polyvinyl microtitre plates (Cooke, Alexandra VA) which had previously been coated with β-lactoglobulin (Sigma Chemical Company) at a concentration of 10 μg/ml in 0·1 mmol/l bicarbonate buffer pH 9·2. After 3 washes the IgG bound to the plates was detected by a radiolabelled immunoadsorbent purified sheep antihuman IgG (Seward Laboratory) diluted in phosphate buffer saline +0·05% tween 20. A rabbit serum to β-lactoglobulin was used as a positive control.

To assay IgA antibodies, the IgG fraction of a sheep antihuman IgA antisera (Seward Laboratory) was coated on to the polyvinyl microtitre plates. Serum samples (100 μl) were incubated overnight at 4°C. After 3 washes, β-lactoglobulin was added in large excess (50 μg/ml) for 2 hours and after a further 3 washes, the amount bound was detected by a radiolabelled immunoadsorbent purified rabbit antibody to β-lactoglobulin. Results are expressed as cpm × 10^{-2} bound. Sera from 2 patients with X-linked agammaglobulinaemia were used in the IgG and IgA antibody assays as negative controls.

IgE antibodies were detected by radioallergosorbent test and results expressed in relation to cord serum (negative control).

Statistics. Non-parametric tests were used throughout. These were Fisher's exact test, Spearman rank correlation, and Mann-Whitney U test.

Results

Circulating immune complexes. Since assays for immune complexes are semi-quantitative only and peak levels of immune complexes occurred at a different time after milk ingestion in each subject; a significant rise in immune complexes was judged as a value more than twice the amount detected before challenge, occurring any time up to 5 hours after it. Using these criteria, 5 of 8 non-atopic subjects (3 children and 2 adults) showed an increase in IgA-containing complexes. Two (1 child and 1 adult) of the remaining 3 subjects had rises in IgG-containing complexes. None of the 8 subjects had a rise in IgE-containing or C1q-binding complexes (Table 1).

Of the 3 atopic subjects not symptomatically allergic to milk, the adult with allergy to grass pollen had raised IgA complexes and one of the 2 children with eczema had raised IgG- and IgE-containing complexes, although to a much lower extent than those present in the milk-allergic group. The other child with eczema showed no change in any parameter after milk ingestion.

A rise in IgA-containing complexes occurred after challenge in 5 of the 10 children with milk allergy and eczema, but 7 also formed IgG-containing complexes, 6 IgE-containing, and 6 C1q-binding complexes (Table 1). All 10 of the milk-allergic children showed a rise in at least one type of complex but not necessarily of the same combination.

Table 1 *Types of immune complexes formed after milk challenge*

Group	*IgG*	*IgA*	*IgE*	*C1q-binding*
Healthy subjects Children (n=4) Adults (n=4)	2	5	0	0
Atopic subjects not food allergic Children (n=2) Adult (n=1)	1	1	1	0
Eczema and milk allergy (n=10)	7	5	6*	6*

*P<0·025 compared with healthy children and adults, by Fisher's exact test.

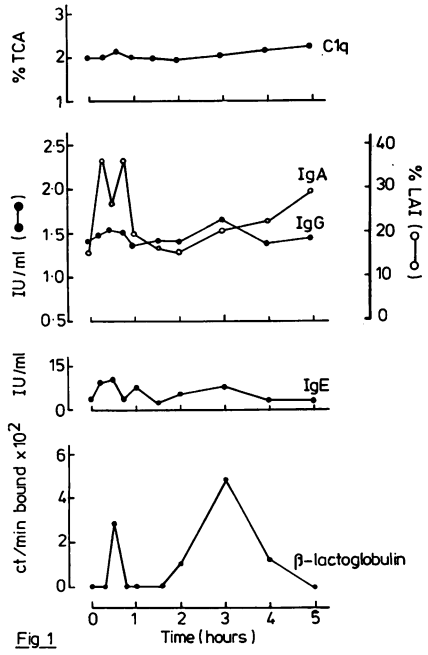

Fig 1

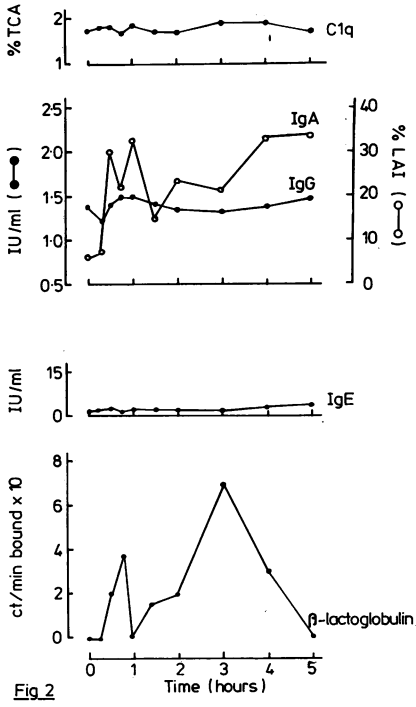

Fig 2

Figs 1 and 2 *Representative profiles of IgA immune complexes detected in sera after drinking milk in 2 healthy non-atopic subjects. The complexes contained β-lactoglobulin as antigen.*

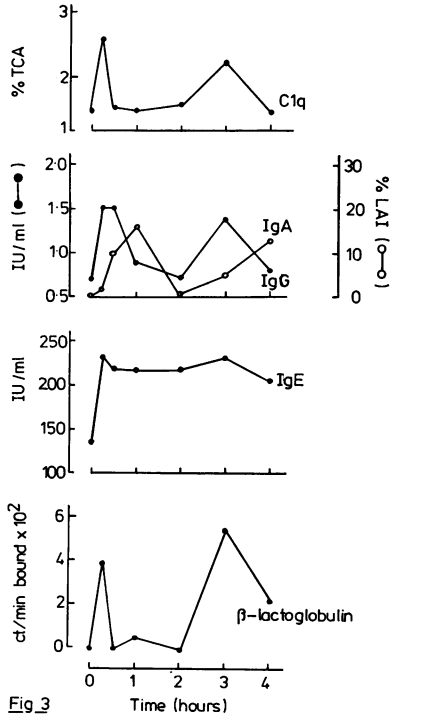

Fig 3

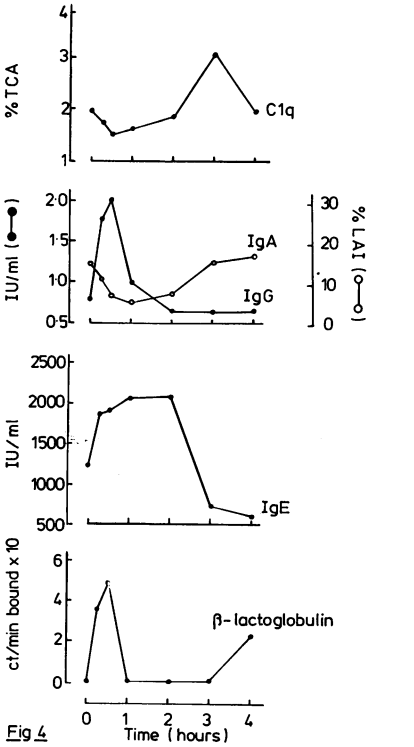

Fig 4

Figs 3 and 4 *Representative profiles of the IgG/IgE and C1q-binding immune complexes detected in sera after drinking milk in 2 subjects with eczema and milk allergy. These complexes also contained β-lactoglobulin as antigen.*

% TCA = per cent of radiolabelled C1q precipitated by 20% trichloroacetic acid, LAI = latex agglutination inhibition.

However, a rise in C1q-binding complexes invariably occurred in children who showed a rise in IgG-containing complexes. In 4 children, substantial amounts of IgE precipitable by PEG before the challenge were found. This suggests that initially they had IgE complexes which contained other antigens and the rise which occurred after oral challenge was due to the addition of the milk antigen-containing complexes. The children with eczema and milk allergy differed significantly from the healthy non-atopic subjects with regard to the rise in IgE-containing and C1q-binding complexes (Fisher's exact test $P<0.025$). A similar trend was shown for IgG-containing complexes but this was not statistically significant.

A criticism which has been levelled at the latex agglutination inhibition assay for IgA complexes is that it cannot distinguish dimeric IgA from complexed IgA.[15] Dimeric IgA is not precipitated by PEG in concentrations up to 3%, whereas IgA complexes >19S in size are.[15] We therefore investigated whether the IgA complexes could be precipitated by 3% PEG. In 2 of 3 non-atopic and in all 3 milk-allergic children, the PEG precipitates retained their ability to inhibit the IgA-latex agglutination, whereas the PEG-depleted sera were devoid of such activity.

Representative profiles of 2 non-atopic subjects (Figs 1 and 2) and 2 subjects with eczema and allergy to milk (Figs 3 and 4) are shown. Both the rise in IgA-containing complexes in the non-atopic subjects and that in the IgG and C1q-binding ones in the patients with milk allergy and eczema had early peaks within 1 hour after drinking the milk. In most of the subjects challenged, a second peak occurred between 3 and 5 hours after challenge but this did not always occur for all the different types of immune complexes (Fig. 4).

A similar biphasic formation of β-lactoglobulin-containing complexes was found in the sera of most of the non-atopic and allergic subjects. However, where both IgG and IgE complexes were detected in the same individual, the techniques did not allow the quantitation of β-lactoglobulin within each class of complex individually, nor could we determine whether they were distinct entities or mixed complexes.

The technique of measuring β-lactoglobulin within the complex is semi-quantitative.[14] Therefore, because of the dynamic nature of immune complexes continually being formed and cleared from the circulation, it is difficult to compare amounts in different individuals during a 5-hour time span. However, when peak heights of complexed β-lactoglobulin were measured, there was a trend for them to be higher in the milk-allergic group compared with the non-atopic group, although the results were not statistically significant ($P>0.05$, <0.1 Mann-Whitney U test).

Table 2 *Antibodies to β-lactoglobulin*

Group	*IgG*	*IgA*	*IgE*
Non-atopics (n=8)	11·1 (6·9–16·7)	13·4 (11·3–15·6)	1·1 (0·8–1·3)
Atopic (not milk allergic) (n=3)	8·8 (6·7–10·7)	11·6 (11·3–13·4)	1·1 (0·8–1·3)
Eczema and milk allergic (n=10)	10·3 (5·2–17·5)	14·1 (10·6–19·1)	2·2* (1·3–15·2)

IgE results expressed as binding relative to cord serum (=1); IgG and IgA results as net cpm bound × 10^{-2}. Median and range (in parentheses) are shown for each group. *$P<0.005$ compared with non-atopics. All other comparisons P=NS.

Antibodies to β-lactoglobulin. IgE antibodies, determined by radioallergosorbent test, were in the normal range (<1·7 relative to cord serum) in all the non-atopic subjects, including the children who previously had had steroid-responsive nephrotic syndrome. Eight of 10 patients with eczema and milk allergy had raised levels of IgE antibodies to β-lactoglobulin (>1·7 relative to cord serum). The other 2 children had been included in this group because of severe clinical exacerbations of eczema after double-blind administration of milk products for 2 months and disappearance of symptoms while on placebo.[8]

None of the atopic but not milk allergic or non-atopic groups had raised levels of IgE antibodies to β-lactoglobulin ($P<0.0005$ Mann-Whitney U test compared with milk allergic).

Levels of IgG and IgA antibodies to β-lactoglobulin were not significantly different for the three groups (Table 2). In the eczema- and milk-allergic group, levels of IgG or IgA antibodies to β-lactoglobulin did not correlate significantly with IgE antibodies to β-lactoglobulin (IgG/IGE $r_s=0.128$; IgA/IgE $r_s=0.343$; IgG/IgA $r_s=0.251$; P=NS all three).

Clinical correlations. Although the children with eczema had been designated milk allergic because of exacerbation of symptoms after prolonged daily administration of milk products, the single challenge with milk did not provoke any immediate clinical reaction. However, several of the children complained of increased skin itching 2–3 days after challenge.

It was not possible to correlate such subjective and mild symptoms with the different types of immune complexes generated.

Discussion

For ethical reasons it was not possible to perform this type of challenge on healthy non-atopic children who

were not part of a hospital population. The control non-atopic group therefore consisted of 4 healthy adults and 4 children who had had steroid-responsive nephrotic syndrome but who had been in remission and off treatment with steroids for at least 6 months. They were being studied as part of an investigation on the clearance of naturally-occurring immune complexes and the intention was to compare them when in remission and during relapse. The children did not differ from the adults in the types of immune complexes formed after drinking milk, and for this reason we believe that they are appropriate controls.

We have previously reported that atopic people absorb more antigenically-intact food proteins into the circulation after oral challenge than non-atopic individuals,[9] but this is not necessarily the causative mechanism of food allergy. We now report another difference in food antigen handling between non-atopic and atopic individuals allergic to a particular food; that they are incorporated into immune complexes of different immunoglobulin classes. The different biological properties of these may influence whether the immune complexes are cleared physiologically or cause systemic reactions. IgA in its secretory form (SIgA) is known to protect mucosal surfaces[16] but the role of serum IgA is less clear. Our data suggest that in healthy individuals serum IgA is concerned in handling antigens which enter via the gastrointestinal tract. We do not know how these IgA complexes are cleared from the circulation but the liver route is likely. In the rat, dimeric IgA- and IgA-containing complexes are transported across the hepatocyte after binding to secretory piece on the sinusoidal surface of the hepatocyte.[17 18] Some antigen degradation (and possibly processing) occurs intracellularly and SIgA is secreted intact into the bile and then into the gut where it probably functions as a mucosal protective layer. This mechanism has not yet been shown to be true for man but there is some evidence that here too IgA complexes are selectively cleared by the liver.[19] This would therefore provide a safe way of handling enterically-absorbed antigens since complexed IgA is not thought to activate complement or elicit damaging reactions.

The milk allergic subjects showed clear differences in the immune complexes formed after food challenge. An increase in IgA complexes was found in only half of them, but most also produced IgG-, IgE-, and C1q-binding complexes. In all children, the milk protein β-lactoglobulin was demonstrated to be a constituent of the complexes. In fact, the quantity of antigen detected within the complexes was not significantly greater in the milk-allergic group than in the non-atopic individuals, which suggests that the symptoms produced in food allergy after antigen exposure are not merely a reflection of the amount absorbed.

The two groups did not differ in the levels of IgA and IgG antibodies to β-lactoglobulin, but the milk-allergic group had significantly higher levels of IgE antibodies to β-lactoglobulin.

The systemic symptoms of food allergy—such as eczema—may well be due to these mixed IgG/IgE immune complexes which bind C1q *in vitro* and presumably are capable of activating the classical pathway of complement *in vivo*. Hence the mechanisms of damage in food allergy may be due to both a type I (immediate) and type III (immune complex) hypersensitivity reaction.

The formation of IgA complexes after food challenge was not exclusive to the healthy non-atopic population. It is the formation of the other varieties that provides the major distinction between the two groups. Our results do not explain why these differences in immune response to ingested antigens occur, but they do provide a plausible explanation for how food proteins absorbed into the circulation intact are safely cleared in healthy subjects, yet cause debilitating disease in allergic individuals.

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Annual meetings

1983	12–16 April	York University
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