

# Pasteurisation and Homogenisation of Milk Enhances the Immunogenicity of Milk Plasma Proteins in a Rat Model

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*Groups of rats were immunised intraperitoneally with 0.5 ml of raw milk, skim milk, pasteurised milk or pasteurised/homogenised milk. On day 14, animals were boosted with identical preparations, and serum samples were collected on day 28 for the measurement of IgG antibodies directed against milk plasma proteins. Samples were assayed for antibodies to casein,  $\beta$ -lactoglobulin, bovine gamma globulin and bovine serum albumin. Significantly higher concentrations of antibodies of all specificities were detected in the serum of animals immunised with pasteurised/homogenised milk compared with animals immunised with raw or skim milk. Higher concentrations of antibodies were also seen in the group immunised with pasteurised/homogenised milk than the group immunised with pasteurised milk, although significant differences were only seen in the response to casein ( $p < 0.01$ ). Additional animals were twice immunised with either raw milk fortified with keyhole limpet hemocyanin (KLH), or pasteurised/homogenised milk which had been fortified with KLH prior to processing. Such treatment significantly enhanced the immunogenicity of the KLH ( $p < 0.05$ ). These results raise the possibility that homogenisation may similarly effect the way in which the human immune system interacts with cows' milk proteins in the gastrointestinal tract, and suggest that milk processing may be an important variable to consider in studies of human allergy to cows' milk proteins.*

**Keywords:** Allergy, cows' milk, milk fat globule membrane, homogenisation, pasteurisation

## INTRODUCTION

Cows' milk stands out as probably the most common food allergen, with reports of the incidence of cows' milk protein allergy amongst young children ranging between 1.8 and 7.5% (Host, 1994). It has therefore been the focus of intensive study for many years. Most experimental models employed to study the immunogenicity and allergenicity of milk proteins have utilised purified proteins. Milk is, however, a complex fluid containing many components in different states of dispersion. Bovine milk typically contains approximately

3.3% protein, and between 3.5 and 5% total lipid, primarily in the form of emulsified globules having diameters which range from 0.1 to 20  $\mu\text{m}$ , with most being in the range 2–4  $\mu\text{m}$  (Valivullah *et al.*, 1988).

The extensive range of milk plasma proteins includes many which have been shown to be potentially allergenic, and milk allergic individuals frequently react to multiple milk proteins (Restani *et al.*, 1995; van Beresteijn *et al.*, 1995). The chemical diversity of these milk allergens argues against the view that allergenicity is necessarily a reflection of a biochemical property such as protease (Chua *et al.*, 1988; Shakib *et al.*, 1998; Yasueda *et al.*, 1993) or lipase activity (Dudler *et al.*, 1995). It rather suggests that the allergenicity of any milk protein may be the outcome of the overall chemical context in which antigens are encountered, and therefore of the association of particular milk proteins with other elements in milk. The associations which may be observed in milk between different proteins, and between proteins and lipids, are partly natural, but principally occur as a result of milk processing.

Significant changes to the properties of milk proteins occur as a consequence of heat treatment and homogenisation. It has been shown that aggregation of individual proteins, the formation of protein complexes between different milk proteins, and decreases in the solubility of whey proteins occur following heat treatment (deWit & Klarenbeek, 1984). An important outcome of homogenisation, on the other hand, is the association of milk plasma proteins with milk fat globules (MFG) (Melsen & Walstra, 1989).

The consequences of analogous lipid–protein interactions upon protein immunogenicity has been highlighted in recent years by the development of liposomes for the delivery of vaccines (Michalek *et al.*, 1992). This present study was undertaken as a result of the realisation that liposomes can be likened to MFG. Similarities are particularly evident after MFG have been homogenised, for this process reduces the size of the MFG to that of effective vaccine-delivering liposomes, with a consequent 4–6 fold increase in surface area that is accomplished by the incorporation of milk plasma proteins into the MFG membrane (McPherson & Kitchen, 1983).

In this report we present the results of investigations in which groups of rats were immunised parenterally with skim milk (SM), raw milk (RM), pasteurised milk (PM) or pasteurised and homogenised milk (PHM). Their responsiveness to the major milk plasma proteins was measured by ELISA assay, and we show that homogenisation leads to a significant enhancement of the immunogenicity of milk plasma proteins.

## MATERIALS AND METHODS

### Animals

Female inbred Wistar rats were bred and housed in the animal house of the School of Microbiology and Immunology, UNSW. Animals were maintained on a bovine milk protein-free diet (Glen Forrest Stockfeeders, Glen Forrest, Australia), and all animals used in the experiments were from the second generation to have been so raised.

### Milk Processing

Fresh raw milk was centrifuged twice for 10 min at  $7500 \times g$  at room temperature to produce skim milk. Raw milk was heated at 74°C for 15 s to produce pasteurised milk. Pasteurised and homogenised milk was produced by passing pasteurised milk through a two-stage homogeniser (Gaulin Corp., Everett, MA, USA) with a first stage pressure of 500 psi and a second stage pressure of 2000 psi. Protein and lipid concentrations were measured spectrophotometrically, pre- and post-processing, using a Mijko Scan 133 (A/S N. Foss Electric), while mean fat globule size was determined by spectroturbidimetry using an Emulsion Quality Analyser (EQA<sup>TM</sup>; APV Baker). All processing and characterisation was conducted in the research laboratory of Australian Co-operative Foods (Wetherall Park, NSW, Australia), and was performed on the days of immunisation using a single batch of raw milk.

### Immunisation

Groups of six rats (10–12 week old) were immunised intraperitoneally on days 0 and 14 with 500  $\mu$ l of 1:9 dilutions of SM, RM, PM or PHM in phosphate-buffered saline (PBS). Serum samples were collected by tail bleeds on days 0 and 28. The ability of pasteurisation and homogenisation to affect protein antigenicity was further investigated by immunising two groups of five rats with either RM fortified with keyhole limpet hemocyanin (KLH), or PHM fortified with KLH prior to pasteurisation and homogenisation. The immunisation schedule and assay system was as described above. Each animal was exposed to 10  $\mu$ g KLH with each immunisation.

### ELISA Assay

The presence of serum IgG antibodies directed against casein,  $\alpha$ -lactalbumin ( $\alpha$ -LA),  $\beta$ -lactoglobulin ( $\beta$ -LG), bovine gamma globulin (BGG), bovine serum albumin (BSA), and KLH were measured by ELISA assays. Briefly, Nunc Maxisorp plates (Nunc A/S, Roskilde, Denmark) were coated with the various antigens, at concentrations of 10  $\mu$ g ml<sup>-1</sup> in carbonate buffer. All of the antigens were purchased from Sigma (St Louis, MO, USA). Plates were incubated overnight at 4°C, and were then blocked for 1 h using 1% gelatine (Bio-Rad; Richmond, CA, USA). Serum samples were then added to the plates at different dilutions and incubated for a further hour. The presence of specific antibody was detected using peroxidase labelled goat anti-rat IgG (Kirkegaard and Perry Laboratories; Gaithersburg, USA) and 3,3'-5,5'-tetramethylbenzidine (TMB) substrate (Sigma; St Louis, MO, USA). Results were quantified with respect to a series of laboratory standards produced by the repeated immunisation of rats with purified proteins in the presence, firstly, of Freund's Complete Adjuvant, and then of Freund's Incomplete Adjuvant. The standards were arbitrarily assigned concentrations such that the limit of detectability of antibodies of each specificity in test samples was 100 ELISA Units (EU) ml<sup>-1</sup>. Standards were assayed as doubling dilutions from initial dilutions of 1:1000, and the results were used to generate standard curves. Samples were assayed as duplicates at dilutions producing optical densities which fell on the near-linear slopes of the sigmoidal standard curves. Samples which failed to produce optical densities in this range at a minimum dilution of 1:9 were deemed antibody negative ( $\leq 100$  EU ml<sup>-1</sup>). Optical densities were read at 450 nm using a Bio-Rad 3550 Microplate reader (Bio-Rad Laboratories, Richmond, CA, USA) and calculations made using the Microplate Manager<sup>®</sup> software package.

### Statistical Analysis

The results were analysed using the Kruskal–Wallis test. A post-hoc evaluation of groups was performed using the Mann–Whitney U-test. A *p*-value <0.05 was considered significant.

## RESULTS

### Characterisation of Milk Post-processing

Total protein and fat concentrations of each milk type are presented as Table 1, and show that these characteristics were unaltered by processing. The mean size of MFG in each sample type are also presented in Table 1. The mean size of the laboratory-processed pasteurised and homogenised milk was indistinguishable from that of the commercially-processed product.

### Rat IgG Response to Milk Plasma Proteins

Previous time-course studies indicated that after priming intraperitoneally at day 0, and boosting on day 14, the rat IgG response to milk proteins peaked around day 28 (data not

TABLE 1. Analysis of laboratory-processed and commercially-processed milk samples

| Milk type                          | Fat (%) | Protein (%) | MFG mean size ( $\mu\text{m}$ ) |
|------------------------------------|---------|-------------|---------------------------------|
| Raw                                | 3.78    | 3.38        | 1.40                            |
| Pasteurised                        | 3.82    | 3.37        | 1.40                            |
| Pasteurised/homogenised            | 3.97    | 3.35        | 0.70                            |
| Commercial pasteurised/homogenised | 3.78    | 3.29        | 0.78                            |

shown). Antibody concentrations in the subsequent experiment reported here were therefore only measured at day 0 and day 28. Specific antibodies were undetectable in all samples at day 0. The results of the ELISA assays for IgG antibodies directed against BGG, casein, BSA and  $\beta$ -LG at day 28 are presented in Figure 1. Anti- $\alpha$ -LA antibodies were not detectable in animals from any of the four groups. With the exception of anti-BGG antibodies, little or no development of antigen-specific antibodies was seen in animals immunised with either raw

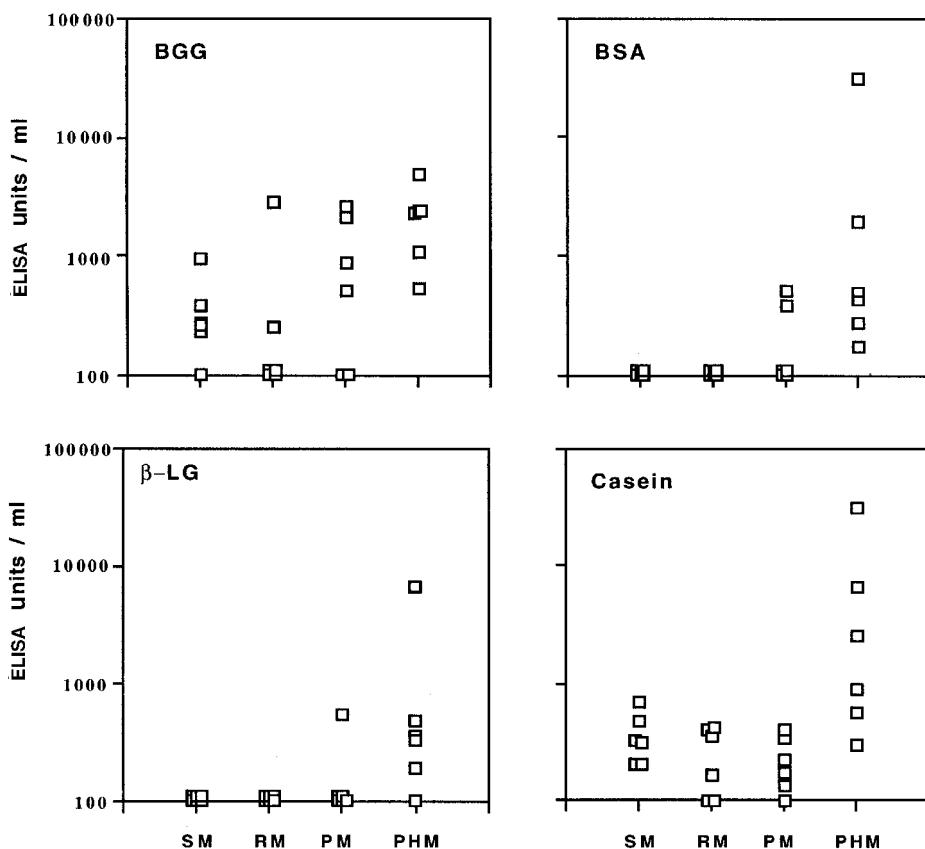


FIG. 1. Serum antigen-specific IgG antibody concentrations (ELISA Units  $\text{ml}^{-1}$ ) in rats immunised with raw milk (RM), skim milk (SM), pasteurised milk (PM) or homogenised and pasteurised milk (PHM), as determined by ELISA assay. Results are presented for assays of antibodies directed against casein,  $\beta$ -lactoglobulin ( $\beta$ -LG), bovine gamma globulin (BGG) and bovine serum albumin (BSA). Each group consisted of six rats.

TABLE 2. *p*-values for Kruskal–Wallis analysis of variance of IgG antibody concentrations of various specificities between groups of animals immunised with different milk preparations

|             | Kruskal–Wallis |
|-------------|----------------|
| Casein      | 0.02           |
| $\beta$ -LG | 0.05           |
| BGG         | 0.04           |
| BSA         | 0.007          |

or skim milk. The response to immunisation with pasteurised milk was highly variable. While an anti-casein response was barely evident, some animals in the group responded to one or all of BGG, BSA and  $\beta$ -LG. Although the response to homogenised milk was also highly variable, the magnitude of the responses stands in stark contrast to the other groups. The results of statistical comparisons between the groups are presented in Tables 2 and 3. Despite the small sizes of the groups, and the variability of responses seen within groups, significant differences were seen between the PHM group and the RM and SM groups for each of the specificities. Significant differences were only seen between the PM and PHM groups in the response to casein ( $p < 0.01$ ).

### Rat IgG Response to KLH

The results of the ELISA assay for anti-KLH antibodies in animals immunised with either KLH-fortified RM or PHM are presented as Figure 2. An enhanced IgG response was seen at day 28 in animals primed and boosted with KLH in PHM. A significant difference between the groups was demonstrated using the Mann-Whitney U-test ( $p < 0.05$ ).

## DISCUSSION

Homogenisation of milk is generally seen as a physical process leading simply to a reduction in the size of milk fat globules. As a consequence of homogenisation, cream rises very slowly through the aqueous phase, making homogenised milk a more attractive consumer product. The impact of this process on the immunogenicity of milk proteins has received little attention, despite the fact that most infant formulae are produced from homogenised milk. The few published immunological studies which have considered homogenisation have been prompted by anecdotal reports that milk allergic children tolerate raw milk better than they tolerate processed milk. The results of these studies have been equivocal, though suggestive.

TABLE 3. *p*-values for comparisons of IgG antibody concentrations of various specificities between groups of animals immunised with raw milk (RM), skim milk (SM), pasteurised milk (PM) or homogenised and pasteurised milk (PHM), using the Mann-Whitney U-test.

|                        | RM vs SM | RM vs PM | SM vs PM | RM vs PHM | SM vs PHM | PM vs PHM |
|------------------------|----------|----------|----------|-----------|-----------|-----------|
| Casein                 | n.s.     | n.s.     | n.s.     | 0.02      | 0.04      | 0.01      |
| $\beta$ -lactoglobulin | n.s.     | n.s.     | n.s.     | 0.03      | 0.02      | n.s.      |
| BGG                    | n.s.     | n.s.     | n.s.     | 0.04      | 0.007     | n.s.      |
| BSA                    | n.s.     | n.s.     | n.s.     | 0.003     | 0.003     | n.s.      |

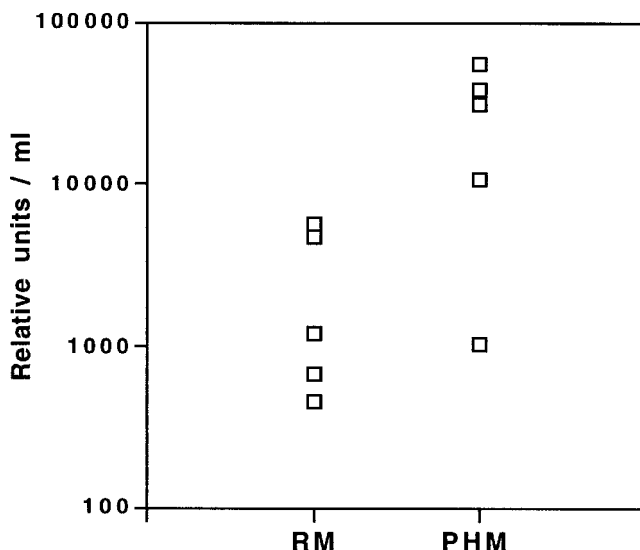


FIG. 2. Serum anti-KLH IgG antibody concentrations (ELISA Units ml<sup>-1</sup>) in rats immunised with KLH-fortified raw milk (RM), or homogenised and pasteurised milk (PHM), as determined by ELISA assay. Each group consisted of five rats.

A double-blind placebo-controlled study of milk allergic children reported no significant differences in their responsiveness to raw, pasteurised or homogenised and pasteurised milk (Host & Samuelsson, 1988). Reaction time and duration of symptoms after oral provocation, and wheal diameter after skin prick test were all reported. Differences seen upon exposure to the various milk preparations failed to reach statistical significance; however, the study was conducted with just five children.

A murine intestinal anaphylaxis model has also been reported (Poulsen *et al.*, 1987; Poulsen *et al.*, 1990), suggesting that homogenisation may enhance allergenicity. Serum tested by passive cutaneous anaphylaxis test showed the presence of reaginic antibodies in animals fed homogenised milk, but not in animals exposed to untreated, raw milk (Poulsen *et al.*, 1990). Further differences were shown when sensitised animals were subsequently challenged with different milk preparations, and their intestinal mass determined (Poulsen *et al.*, 1990).

In light of the growing interest in the adjuvant activity of lipids and amphipathic molecules such as synthetic copolymers and immune-stimulating immune complexes (ISCOMS), and as a consequence of the equivocal results of previous studies, the present study was designed to provide a straightforward test of the effect of milk processing upon milk protein antigenicity.

The response of animals challenged with raw and skim milk was poor, with no detectable response to  $\beta$ -LG or BSA. Antibodies against casein and BGG were, however, detectable in the sera of most animals. The pattern of responsiveness of animals challenged with pasteurised milk was similar, although some animals produced antibodies to BSA and  $\beta$ -LG. A significantly greater response was seen to each antigen in the case of animals challenged with homogenised and pasteurised milk. A significantly enhanced response to KLH was also seen when animals were challenged with milk which had been fortified with KLH prior to homogenisation.

The immunogenicity of casein highlighted by these experiments may be a consequence of the facility with which the immune system is able to respond to particulate antigen. Virtually all the casein proteins in milk are present as micelles having a molecular weight of around

10<sup>5</sup> kDa and having a mean diameter of about 100 nm (McMahon & Brown, 1984). Casein contributes up to 90% of the dry mass of the surface layer of globules post-homogenisation, and covers as much as 75% of the surface area (Leman & Kinsella, 1989).

The immunogenicity of BGG is harder to explain, but may reflect the fact that xenogeneic gamma globulins exhibit special immunological properties as a consequence of their homology with the immunised animals' own antibodies. The strength of the anti-BGG response reported here may have some bearing on our earlier hypothesis that the anti-BGG response in humans consuming cows' milk should include anti-idiotypic antibodies, and that such anti-idiotypic antibodies could play a role in the aetiology of some atopic disease (Collins, 1988).

The mechanism underlying the apparent enhancement of the immunogenicity of milk proteins by homogenisation is unknown. We believe the effect is most likely to be an outcome of the association of milk plasma proteins with MFG membranes during the homogenisation process, for it is well known that the immune system responds differently to particulate as opposed to soluble antigen. This seems to be particularly true in the case of lipid particles, and the recognition of this has led to the development of liposomes, lipid microspheres and lipophilic ISCOMS as vehicles for vaccine delivery.

Surprisingly, these lipid formulations have even proven effective for the delivery of antigen within the gastrointestinal tract. For example, it has been reported that antigen incorporated within liposomes is much more effective in inducing a mucosal immune response than free antigen (Jackson *et al.*, 1990). Furthermore, microspheres of a defined size range have been shown to be taken up by the Peyer's patches (Eldridge *et al.*, 1989). Liposomes may also be able to facilitate the transfer of a small proportion of their contents into the blood circulation, so stimulating a systemic response (Woodley & Prescott, 1988). The use of ISCOMS for oral immunisation can also result in both mucosal and systemic responses (Mowat & Donachie, 1991).

It may be that lipid-protein interactions are more generally relevant to an understanding of immunological responsiveness to dietary antigens introduced to the gastrointestinal mucosa. It is curious that a significant pathway for the delivery of lipid from the gastrointestinal tract to the liver is via the lymphatic system. The lacteals, so named for the milky appearance of the lipid-rich chyle which flows through them, are the minute lymphatic vessels of the villi of the small intestine. Lipid taken up from the gastrointestinal tract is carried away by the lacteals, ultimately reaching the circulation via the thoracic duct (Lowden & Heath, 1992; Nutting *et al.*, 1989). Absorbed lipid must therefore traverse the organised lymphoid tissue, and hence may promote the presentation of lipophilic protein to the immune system.

The present study has shown that homogenisation and pasteurisation of cows' milk enhances the humoral immune response of rats challenged intraperitoneally with milk in the absence of additional adjuvant. Although care must be taken in extrapolating from the rodent IgG response after parenteral challenge to the human IgE response after milk ingestion, it is reasonable to consider that commercial processing may contribute to the propensity for cows' milk to induce allergic reactivity in children.

## REFERENCES

- CHUA, K. Y., STEWART, G. A., THOMAS, W. R., SIMPSON, R. J., DILWORTH, R. J., PLOZZA, T. M. & TURNER, K. J. (1988) Sequence analysis of cDNA coding for a major house dust mite allergen, Der p 1. Homology with cysteine proteases, *Journal of Experimental Medicine*, **167**, 175–182.
- COLLINS, A. M. (1988) Xenogeneic antibodies and atopic disease, *Lancet*, **i**, 734–737.
- DEWIT, J. N. & KLARENBECK, G. (1984) Effects of various heat treatments on structure and solubility of whey proteins, *Journal of Dairy Science*, **67**, 2701–2710.
- DUDLER, T., MACHADO, D. C., KOLBE, L., ANNAND, R. R., RHODES, N., GELB, M. H., KOELSCH, K., SUTER, M. & HELM, B. A. (1995) A link between catalytic activity, IgE-independent mast cell activation, and allergenicity of bee venom phospholipase A2, *Journal of Immunology*, **155**, 2605–2613.

- ELDRIDGE, J. H., GILLEY, R. M., STAAS, J. K., MOLDOVEANU, Z., MEULBROEK, J. A. & TICE, T. R. (1989) Biodegradable microspheres: vaccine delivery systems for oral immunization, *Current Topics in Microbiology and Immunology*, **146**, 59–66.
- HOST, A. (1994) Cow's milk protein allergy and intolerance in infancy. Some clinical, epidemiological and immunological aspects, *Pediatric Allergy and Immunology*, **5** (Suppl.), 1–36.
- HOST, A. & SAMUELSSON, E. G. (1988) Allergic reactions to raw, pasteurized, and homogenized/pasteurized cow milk: a comparison. A double-blind placebo-controlled study in milk allergic children, *Allergy*, **43**, 113–118.
- JACKSON, S., MESTECKY, J., CHILDERS, N. K. & MICHALEK, S. M. (1990) Liposomes containing anti-idiotypic antibodies: an oral vaccine to induce protective secretory immune responses specific for pathogens of mucosal surfaces, *Infection and Immunity*, **58**, 1932–1936.
- LEMAN, J. & KINSELLA, J. E. (1989) Surface activity, film formation, and emulsifying properties of milk proteins, *Critical Reviews in Food Science and Nutrition*, **28**, 115–138.
- LOWDEN, S. & HEATH, T. (1992) Lymph pathways associated with Peyer's patches in sheep, *Journal of Anatomy*, **181**, 209–217.
- McMAHON, D. J. & BROWN, R. J. (1984) Composition, structure, and integrity of casein micelles: a review, *Journal of Dairy Science*, **67**, 499–512.
- McPHERSON, A. V. & KITCHEN, B. J. (1983) Reviews of the progress of Dairy Science: The bovine milk fat globule membrane – its formation, structure and behaviour in milk and dairy products, *Journal of Dairy Research*, **50**, 107–133.
- MELSEN, J. P. & WALSTRA, P. (1989) Stability of recombined milk fat globules, *Netherlands Milk Dairy Journal*, **43**, 63–78.
- MICHALEK, S. M., CHILDERS, N. K., KATZ, J., DERTZBAUGH, M., ZHANG, S., RUSSELL, M. W., MACRINA, F. L., JACKSON, S. & MESTECKY, J. (1992) Liposomes and conjugate vaccines for antigen delivery and induction of mucosal immune responses, *Advances in Experimental Medicine and Biology*, **327**, 191–198.
- MOWAT, A. M. & DONACHIE, A. M. (1991) ISCOMS – a novel strategy for mucosal immunization, *Immunology Today*, **12**, 383–385.
- NUTTING, D., HALL, J., BARROWMAN, J. A. & TSO, P. (1989) Further studies on the mechanism of inhibition of intestinal chylomicron transport by Pluronic L-81, *Biochimica et Biophysica Acta*, **1004**, 357–362.
- POULSEN, O. M., HAU, J. & KOLLERUP, J. (1987) Effect of homogenization and pasteurization on the allergenicity of bovine milk analysed by a murine anaphylactic shock model, *Clinical Allergy*, **17**, 449–458.
- POULSEN, O. M., NIELSEN, B. R., BASSE, A. & HAU, J. (1990) Comparison of intestinal anaphylactic reactions in sensitized mice challenged with untreated bovine milk and homogenized bovine milk, *Allergy*, **45**, 321–326.
- RESTANI, P., VELONA, T., PLEBANI, A., UGAZIO, A. G., POIESI, C., MURARO, A. & GALLI, C. L. (1995) Evaluation by SDS-PAGE and immunoblotting of residual antigenicity in hydrolysed protein formulas, *Clinical and Experimental Allergy*, **25**, 651–658.
- SHAKIB, F., SCHULZ, O. & SEWELL, H. (1998) A mite subversive: cleavage of CD23 and CD25 by Der p 1 enhances allergenicity, *Immunology Today*, **19**, 313–316.
- VALIVULLAH, H. M., BEVAN, D. R., PEAT, A. & KEENAN, T. W. (1988) Milk lipid globules: control of their size distribution, *Proceedings of the National Academy of Sciences of the United States of America*, **85**, 8775–8779.
- VAN BERESTELIJN, E. C., MEIJER, R. J. & SCHMIDT, D. G. (1995) Residual antigenicity of hypoallergenic infant formulas and the occurrence of milk-specific IgE antibodies in patients with clinical allergy, *Journal of Allergy and Clinical Immunology*, **96**, 365–374.
- WOODLEY, J. F. & PRESCOTT, A. R. (1988) Body distribution of liposome-entrapped <sup>125</sup>I labelled insulin after oral administration to rats, *Biochemical Society Transactions*, **16**, 343–344.
- YASUEDA, H., MITA, H., AKIYAMA, K., SHIDA, T., ANDO, T., SUGIYAMA, S. & YAMAKAWA, H. (1993) Allergens from dermatophagoides mites with chymotryptic activity, *Clinical and Experimental Allergy*, **23**, 384–390.