

# Comparison of intestinal anaphylactic reactions in sensitized mice challenged with untreated bovine milk and homogenized bovine milk

O. M. POULSEN, B. R. NIELSEN, A. BASSE & J. HAU

Department of Veterinary Pathology, Laboratory Animal Unit, the Royal Veterinary and Agricultural University, Copenhagen, Denmark

Outbred NMRI mice were sensitized for high IgE production either by subcutaneous injections of low doses of untreated bovine milk or homogenized bovine milk in combination with intraperitoneal injections of Freund's Complete Adjuvant or by oral administration of untreated or homogenized bovine milk without adjuvant. When analysed in murine passive cutaneous anaphylaxis test both types of milk resulted in production of reaginic antibodies against bovine milk proteins when given subcutaneously. When given orally, homogenized milk resulted in reagin production in 10 out of 14 mice, whereas untreated milk resulted in reagin production in only one out of 12 mice. The sensitized mice, and control mice, were orally challenged with either untreated milk, homogenized milk or 0.9% NaCl. Examination of the intestines 40 min after oral administration revealed that homogenized milk, contrary to untreated milk or 0.9% NaCl, resulted in a large increase in the mass of the proximal gut segment of mice sensitized orally with homogenized milk compared with control mice orally challenged with saline ( $P < 0.001$ ), and only mice both sensitized and challenged orally with homogenized milk showed degranulation of mast cells in the intestinal wall. By contrast, subcutaneously sensitized mice or mice sensitized orally with untreated bovine milk showed no significant intestinal reaction upon oral challenge with either homogenized or untreated bovine milk. These observations may indicate that the route of sensitization is of great importance when intestinal reactions are to be studied, and that homogenization of bovine milk may render the milk more aggressive with respect to its ability to induce intestinal reactions. The study indicates that mice may be an attractive experimental animal model for mimicking the intestinal anaphylactic reactions of cow milk-allergic humans.

*Key words:* animal model; homogenized milk; intestinal allergic reactions; oral sensitization.

*Accepted for publication 18 December 1989*

Laboratory animal models for human atopic cow milk allergy are widely employed (4, 8, 12-14). The validity of these models depends on the extent to which they simulate human cow milk allergy. A usable model should involve humoral and cellular immunological disorders similar to those in human disease and preferably, it should be possible to induce allergy by oral sensitization and the animals should exhibit intestinal allergic reactions simi-

lar to those occurring in allergic humans, i.e. decreased water uptake and degranulation of mast cells in the gut mucosa following oral challenge with the allergen (2).

It is well known that guinea pigs (4, 8), rats (6, 7) and mice (13) can be orally sensitized, and that high titres of reaginic antibodies are produced if low doses of antigen are used in the sensitization procedure (6, 7, 12, 16). However, little information exists on anaphy-

lactic reactions to cow's milk in the intestines of laboratory animals. Anaphylactic reactions, such as decreased water and ion uptake, degranulation of mast cells and accumulation of eosinophilic granulocytes in the gut mucosa have been described in sensitized rats upon oral challenge with ovalbumin (11). These results indicate that rats may simulate human intestinal allergic reactions.

We have previously demonstrated that homogenization of bovine milk increases its ability to sensitize mice (i.e. induce IgE production) when injected subcutaneously (9). Further, homogenization of milk increases the ability of milk to induce systemic anaphylactic reactions in sensitized mice challenged intravenously (12, 14) and this has later been confirmed in orally challenged children allergic to cow's milk (5).

To expand the study of the effect of homogenization, the present experiments were designed as a comparative study to analyse to what extent homogenization of bovine milk results in changes in the ability of milk to induce intestinal reactions in mice sensitized orally or subcutaneously with untreated or homogenized bovine milk.

## MATERIAL AND METHODS

Outbred male Ico:NMRI mice, (IFFA-CREDO, Lyon France) were used in all experiments involving studies on intestinal anaphylactic reactions. This strain of mice was bred in France and maintained at our laboratory. Inbred female BALB/cBOM mice (Bomholtgård A/S, Denmark) were used as recipient mice in passive cutaneous anaphylaxis (PCA) test. The mice were fed pellets free from milk proteins (Avels-Tilväxtfoder R3, Ewos-Alab, Södertälje, Sweden) and drinking water *ad libitum*.

Untreated and homogenized bovine milk samples prepared from the same batch of milk were kindly donated on the day of experiment by the dairy Enighed, Copenhagen, Denmark. The milk samples contained 3.5% milk fat and 3.6% milk proteins. The milk samples were diluted with sterile 0.9% saline to produce preparations for sensitization.

### *Subcutaneous sensitization*

Three groups (A, B and C) of 20 inbred NMRI mice each were sensitized for high IgE production with low doses of milk samples as previously described (12). All mice received an intraperitoneal injection of 50 µl Freund's complete adjuvant. On the same morning, Group C received subcutaneous injections (100 µl) of sterile 0.9% NaCl (controls), Group A received subcutaneous injections (100 µl containing 10 µg protein) of a diluted sample of untreated milk, while the Group B received injections (100 µl containing 10 µg protein) of diluted homogenized-pasteurized milk.

After a period of 10 days the mice were boosted with 100 µl subcutaneous injections of 0.9% NaCl (Group C), 100 µl diluted untreated milk (Group A, 1 µg milk protein/animal), or 100 µl diluted homogenized-pasteurized milk (Group B, 1 µg milk protein/animal). The booster injections were repeated on Day 22.

On Day 29 blood samples (250 µl) were drawn from each mouse by puncture of the tail vein, and the titres of reaginic antibodies of the obtained sera were determined by murine PCA test. On the next day, the mice were orally challenged with milk samples and intestinal anaphylactic reactions recorded.

### *Oral sensitization:*

Two groups (D and E) of 15 inbred NMRI mice each were sensitized orally without use of adjuvant. On Day zero each animal of Group D was given 100 µl of a diluted sample of untreated milk containing 10 µg milk protein, while the animals of Group E received 100 µl diluted homogenized-pasteurized milk (10 µg milk protein/animal).

After a period of 10 days the mice were boosted orally with 100 µl diluted untreated milk (Group D, 1 µg milk protein/animal), or 100 µl diluted homogenized-pasteurized milk (Group E, 1 µg milk protein/animal). The booster administrations were repeated on Day 22.

On Day 29 blood samples (250 µl) were drawn from each mouse by puncture of the tail vein, and on the next day, the mice were orally challenged with milk samples.

*Homologue murine passive cutaneous anaphylaxis test (PCA)*

Non-sensitized BALB/cBOM mice were passively sensitized in the skin of the back by administering intradermal injections of three different dilutions of antiserum and one saline control. Two-fold dilution series of the tested sera were analysed, using a 4-fold dilution as the most concentrated preparation. After a passive sensitization period of 3 h, the animals were challenged intravenously with a mixture of 100 µg milk protein (untreated milk defatted by centrifugation at 4500 rpm for 10 min at 20°C) and 2.5 mg Evans blue (Sigma No. C1 23860, St. Louis, USA) in 250 µl sterile saline. After a 30-min reaction period the reactions were recorded and considered positive when the reaction zone on the inside of the skin appeared as a blue spot > 2 mm in diameter. The titre of serum expresses the

highest degree of dilution of serum producing a positive PCA-reaction.

*Intestinal anaphylaxis*

On Day 30 the NMRI mice of the three subcutaneously sensitized groups (A, B, C) were allocated to four subgroups and either not challenged or challenged orally with 1.5 ml undiluted milk samples of the two types or 1.5 ml 0.9% NaCl as outlined in Table 1. On the same day the mice of the two orally sensitized groups (D and E) were allocated to three groups and challenged with either 1.5 ml 0.9% NaCl or 1.5 ml undiluted milk samples of the two types (Table 1).

Dilation of the intestine (and decreased water uptake) was monitored by measuring the intestinal weight gain in the following manner:

The mice were deprived of food but not drinking water for 24 h preceding oral challenge via the ventricle. 40 min after oral administration the mice were killed by cervical dislocation and a 5 cm intestinal segment immediately distal to the pylorus was ligated in both ends and surgically removed. Each of the intestinal segments were placed in weight equilibrated tubes containing 5 ml 0.9% NaCl solution and their mass was measured.

In a preliminary series of experiments the intestinal mass was measured at 30 min, 40 min and 50 min after oral challenge. These studies indicated that maximum increase in the gut mass occurred 40 min after challenge. Consequently, this reaction period was used routinely.

*Fixation and staining of the intestinal segment*

Fixation and staining of murine mast cells in the intestinal wall was done according to Eriksen (3). After measurements of mass of the intestinal segments, the segments were fixed in formalin-acetic acid for 48 h. and stained with HCl-toluidine blue to obtain staining of both connective tissue mast cells and subepithelial intestinal mast cells. A decreased number of granulae per mast cell (or a decreased number of mast cells) was indication of mast cell degranulation.

Table 1

Groups of mice for the study of intestinal anaphylactic reactions. Sensitization and oral challenge

| Group No. | No. animals | Sensitization route |    | Challenged with |
|-----------|-------------|---------------------|----|-----------------|
| A-1       | 5           | unt. milk           | sc | unchallenged    |
| A-2       | 5           | unt. milk           | sc | saline          |
| A-3       | 5           | unt. milk           | sc | unt. milk       |
| A-4       | 5           | unt. milk           | sc | hom. milk       |
| B-1       | 5           | hom. milk           | sc | unchallenged    |
| B-2       | 5           | hom. milk           | sc | saline          |
| B-3       | 5           | hom. milk           | sc | unt. milk       |
| B-4       | 5           | hom. milk           | sc | hom. milk       |
| C-1       | 5           | saline              | sc | unchallenged    |
| C-2       | 5           | saline              | sc | saline          |
| C-3       | 5           | saline              | sc | unt. milk       |
| C-4       | 5           | saline              | sc | hom. milk       |
| D-1       | 5           | unt. milk           | o  | saline          |
| D-2       | 5           | unt. milk           | o  | unt. milk       |
| D-3       | 5           | unt. milk           | o  | hom. milk       |
| E-1       | 5           | hom. milk           | o  | saline          |
| E-2       | 5           | hom. milk           | o  | unt. milk       |
| E-3       | 5           | hom. milk           | o  | hom. milk       |

Abbreviations: sc = subcutaneously; o = orally; unt. = untreated; hom. = homogenized.  
See Material and Methods for more details.

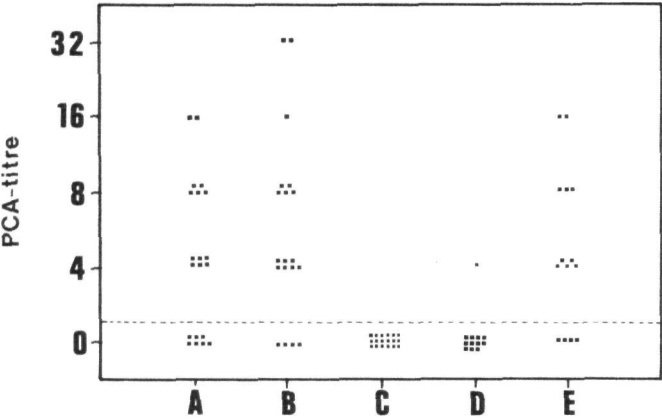


Fig. 1. PCA-titres of sera from NMRI mice orally or subcutaneously sensitized with saline, untreated milk or homogenized milk. The reaginic antibody titres of sera obtained from the five groups of mice (A-E, see Table 1) were determined by murine PCA-test as described in Material and Methods. Two-fold dilution series of the sera were tested starting with dilution 1:4. Undiluted sera were not tested since they tend to give non-specific skin reactions in the PCA-test. The titres were expressed as the reciprocal to the highest dilution producing a positive PCA-reaction. If a serum sample did not produce a positive PCA-reaction at dilution 1:4, the titre was assigned the value zero (samples below the dotted line). The number of sera tested were: Group A = 20, Group B = 19, Group C = 18, Group D = 12, Group E = 14.

Statistical evaluation

Student's t-test was used for statistical evaluation.

RESULTS

Subcutaneous sensitization with both milk types in combination with intraperitoneally injected adjuvant (Groups A and B) resulted in production of reaginic antibodies measurable 7 days after the final booster injection (Fig. 1). No reaginic antibodies against bovine milk proteins could be measured in the sera of control mice (Group C). Oral sensitization with homogenized-pasteurized milk without the use of intraperitoneal injections of adjuvant resulted in measurable production of reaginic antibodies in 10 of 14 mice, whereas reaginic antibodies could be measured in only one of 12 mice orally sensitized with untreated milk (Fig. 1).

Intestinal reactions were recorded by mea-

Table 2  
Effects of oral challenge with untreated milk and homogenized-pasteurized milk on intestinal mass

| Group of mice | No. mice | Challenged with | Mass(g) |       |
|---------------|----------|-----------------|---------|-------|
|               |          |                 | Mean    | SD    |
| A-1           | 5        | unchallenged    | 0.206   | 0.016 |
| A-2           | 5        | saline          | 0.218   | 0.020 |
| A-3           | 5        | unt. milk       | 0.236   | 0.029 |
| A-4           | 5        | hom. milk       | 0.248   | 0.020 |
| B-1           | 4        | unchallenged    | 0.183   | 0.033 |
| B-2           | 5        | saline          | 0.208   | 0.043 |
| B-3           | 5        | unt. milk       | 0.240   | 0.021 |
| B-4           | 5        | hom. milk       | 0.250   | 0.026 |
| C-1           | 4        | unchallenged    | 0.185   | 0.038 |
| C-2           | 4        | saline          | 0.200   | 0.021 |
| C-3           | 5        | unt. milk       | 0.220   | 0.022 |
| C-4           | 5        | hom. milk       | 0.244   | 0.019 |
| D-1           | 4        | saline          | 0.213   | 0.033 |
| D-2           | 4        | unt. milk       | 0.232   | 0.028 |
| D-3           | 4        | hom. milk       | 0.235   | 0.016 |
| E-1           | 4        | saline          | 0.208   | 0.029 |
| E-2           | 5        | unt. milk       | 0.227   | 0.022 |
| E-3           | 5        | hom. milk       | 0.324   | 0.028 |

\**P* < 0.001 for E-3 compared with C-4.  
Mice showing signs of damage in the oral cavity or stomach after oral challenge were excluded from the experiment (see also Fig. 1).

suring the change in intestinal mass after oral challenge (Table 2). Oral challenge with both untreated and homogenized-pasteurized milk resulted in an increase in intestinal mass compared with unchallenged mice or mice challenge with saline (A-3 compared with A-2: *P* < 0.2, B-4 compared with B-2: *P* < 0.1). However, this increase in intestinal mass was also observed in non-sensitized mice (C-4 compared with C-2: *P* < 0.1).

Only mice sensitized orally with homogenized-pasteurized milk and challenged with homogenized-pasteurized milk showed a significant increase in intestinal mass compared with control mice challenged with milk (E-3 compared with C-4: *P* < 0.001) (Table 2), and only the mice of Group E-3 showed cytological changes in the mast cells indicating mast cell degranulation. Mice sensitized with untreated milk and challenged with untreated or homo-

genized-pasteurized milk showed no significant increase in intestinal mass compared with orally challenged control mice.

## DISCUSSION

Gastrointestinal reactions are the most frequent symptoms of human cow milk allergy (1). The gastrointestinal reactions include vomiting, diarrhoea, atrophy of intestinal villi, mast cell degranulation and accumulation of eosinophilic granulocytes in the gut mucosa (2, 10).

In accordance with our previous report (12) the present study shows that intraperitoneal sensitization of mice with low doses of untreated milk or homogenized milk in the presence of Freund's complete adjuvant resulted in measurable titres of reagenic antibodies indicating sensitization. In the present study the measured titres of reagenic antibodies were low compared with those in the previous study. The difference in titres of reagenic antibodies may be explained by the difference in the employed strains of mice (e.g. NMRI mice in the present study and BALB/c in the previous study (12)). In the present study blood samples were collected 7 days after the final booster injection, whereas blood samples were collected 4 days after the final booster injections in the previous study (12). IgE antibodies in the circulation are known to be labile and, consequently, this may also explain the difference in titres measured.

Oral sensitization with homogenized-pasteurized milk without adjuvant resulted in production of reagenic antibodies in 10 of 14 mice (Group E) whereas only one out of 12 mice (Group D) produced a measurable titre of reagenic antibodies as a result of oral sensitization with untreated milk. These results are in accordance with our earlier findings that sensitization with homogenized but not untreated bovine milk resulted in reagin production in mice (9). We have previously suggested that homogenized milk may possess some adjuvant-like properties which may increase the ability of homogenized milk to induce reagin production (9).

Oral challenge of sensitized rats with ovalbumin (allergen) resulted in intestinal reactions

including decreased water uptake from the gut and degranulation of mast cells in the intestinal mucosa (11). In the present study the anaphylactic intestinal reactions such as dilatation of the intestine (and perhaps decreased water uptake) were recorded simply by measuring the increase in intestinal mass. Oral challenge of non-sensitized mice with untreated or homogenized milk resulted in an increase in the intestinal mass compared with oral challenge with saline (Table 2, Group C). Due to the high content of lactose, minerals, lipids and proteins in the milk samples these samples have a higher osmolarity than saline, and the difference in osmolarity may explain the difference in intestinal mass of non-sensitized mice challenged with milk versus saline. The large increase in intestinal mass induced by oral challenge with homogenized-pasteurised milk (Group E-3, Table 2) was associated with mast cell degranulation in the intestinal mucosa. Consequently, the present study indicates that measurements of intestinal weight gain may be an easy method for assessment of intestinal anaphylactic reactions in laboratory animal models, and that mice may be a useful model for studying anaphylactic intestinal reactions.

When using a murine systemic anaphylactic shock model and intravenous challenge with various milk preparations, we have recently demonstrated that homogenized bovine milk induced systemic anaphylactic reactions in sensitized mice at doses significantly lower than those needed with untreated bovine milk (12, 14). We suggested that the lipid-protein complexes in homogenized milk represent a more aggressive presentation of the antigenic proteins compared with untreated milk (14). In these studies (12, 14) the route of sensitization had no impact on the subsequent induction of anaphylactic reactions, and orally sensitized mice reacted in the same manner and with the same sensitivity as subcutaneously sensitized mice. By contrast, the present study demonstrated that the route of sensitization is of importance if intestinal reactions are to be studied. Only mice orally sensitized with homogenized-pasteurized milk, but not with untreated milk, react with intestinal reactions, e.g. dilatation of intestine

and mast cell degranulation, upon oral challenge with homogenized milk, but not with untreated bovine milk. Mice sensitized subcutaneously with milk of both types showed no intestinal reaction upon oral challenge with either homogenized or untreated milk. Thus, the present study indicates that murine intestinal anaphylactic reactions can only be induced if oral sensitization is used to obtain a selective sensitization of mast cells in the intestinal wall.

The present study may add weight to the hypothesis that homogenization renders the milk both more allergenic (9) and also more capable of inducing anaphylactic reactions (12, 14). However, it is well known that untreated milk produces a tight coagulum in the human ventricle, whereas homogenized milk is less efficiently precipitated and a "soft curd" is produced (15). It is thus possible that more native, unhydrolysed milk proteins reach the gut segment studied when homogenized milk is administered.

## ACKNOWLEDGEMENTS

The authors thank the dairy Enighedden, Copenhagen, for the donation of various well-defined milk samples, Mr. Kenneth Worm for handling and care of laboratory animals, and Direktør Jakob Madsen og Hustru Olga Madsens Fond for financial support.

## REFERENCES

1. Bahna, S. L.: Pathogenesis of milk hypersensitivity. *Immunology Today* 6, 153-154, 1985.
2. Eastham, E. J. & Walker, W. A.: Adverse effects of milk formula on the gastrointestinal tract. An update. *Gastroenterology* 76, 365-369, 1979.
3. Eriksen, L.: Host parasite relations in *Ascaris suum* infection in pigs and mice. DVM-thesis, Copenhagen 1981, Commissioned by D. Fr. Mortensen, Bülowsvej 5C, DK-1870, Frederiksberg C, Denmark.
4. Heppell, L. M., Cant, A. J. & Kilshaw, P. J.: Reduction in the antigenicity of whey proteins by heat treatment: a possible strategy for producing a hypoallergenic infant milk formulae. *Br. J. Nutr.* 51, 29-36, 1988.
5. Høst, A. & Samuelsson, E.-G.: Allergic reactions to raw, pasteurized and homogenized and pasteurized cow's milk: a comparison. A double-blind placebo-controlled study in milk allergic children. *Allergy* 43, 113-118, 1988.
6. Jarrett, E. E. E., Haig, D. M., McDougall, W. & McNulty, E.: Rat IgE production. II. Primary and booster reagenic antibody response following intradermal or oral immunization. *Immunology* 30, 671-677, 1976.
7. Jarrett, E. E. E.: Stimuli for the production and control of IgE in rats. *Immunol. Rev.* 41, 52-76, 1978.
8. McLaughlan, P., Anderson, K. J., Widdowsson, E. M. & Coombs, R. R. A.: Effect of heat on the anaphylactic-sensitising capacity of cow's milk, goat's milk, and various infant formulae fed to guinea pigs. *Arch. Dis. Child.* 56, 165-171, 1981.
9. Nielsen, B. R., Poulsen, O. M. & Hau, J.: Reagin production in mice: Effect of sensitization with untreated bovine milk and homogenized bovine milk. *In Vivo* 3, 271-274, 1989.
10. Pearson, J. R., Kingston, D. & Shiner, M.: Antibody production to milk proteins in the jejunal mucosa of children with cow's milk protein intolerance. *Pediatr. Res.* 17, 406-411, 1983.
11. Perdue, M. H., Chung, M. & Gall, D. G.: Effect of intestinal anaphylaxis on gut function in the rat. *Gastroenterology* 86, 391-396, 1984.
12. Poulsen, O. M., Hau, J. & Kollerup, J.: Effect of homogenization and pasteurization on the allergenicity of bovine milk analysed by a murine anaphylactic shock model. *Clin. Allergy* 17, 449-458, 1987.
13. Poulsen, O. M. & Hau, J.: Murine passive cutaneous anaphylaxis test (PCA) for the "all or none" determination of allergenicity of bovine whey proteins and peptides. *Clin. Allergy* 17, 75-83, 1987.
14. Poulsen, O. M. & Hau, J.: Application of a murine anaphylactic shock model. A colloid chemical approach to cow's milk allergy. *Proceedings of the IX ICLAS Symposium, Bangkok, January 12-14, 1988.*
15. Spur, B.: Metoder til fremstilling af "blødt koagulerende" (soft curd) mælk og dens fordøjelighed. *Nordisk Mejeritidskr.* 14, 3-12, 1948.
16. Vaz, E. M., Vaz, N. M. & Levine, B. B.: Persistent formation of reagins in mice injected with low doses of ovalbumin. *Immunology* 30, j11-15, 1971.

Address:

Dr. J. Hau

The Royal Veterinary and Agricultural University

Bülowsvej 13

DK 1870 Frederiksberg

Denmark

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.