

An oral screening procedure to determine the sensitizing capacity of infant feeding formulae

P. McLAUGHLAN, K. J. ANDERSON and R. R. A. COOMBS

Division of Immunology, Department of Pathology, Addenbrooke's Hospital, Cambridge

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Summary

Allergy to cows' milk is an important and frequent cause of cows' milk protein-intolerance in infants. Many infants within days of birth are fed cows' milk or cows' milk-based formula feeds. It is thought desirable to have a test available that would indicate the allergenic potency *per os* of milk formulae and of milk substitutes intended for infant feeding.

Such a test, based on the finding that guinea-pigs drinking cows' milk become anaphylactically sensitized to milk proteins, is described. After drinking pasteurized milk for only 4 days, 88% of guinea-pigs were susceptible to fatal anaphylaxis on intravenous challenge on the 22nd day; drinking milk for 8 days resulted in 100% of guinea-pigs becoming sensitive to fatal anaphylaxis. Following milk drinking for 7 days, a subsequent interval of 7 days was required before anaphylactic sensitivity could be shown in 100% of animals. With this information the effect of heat on the *per os* allergenic potency of cow's milk could be examined; likewise the allergenic potency of supposedly hypo-allergenic infant feed formulations.

Pregestimil induced no anaphylactic sensitivity even after 37 days of drinking, and no anaphylactic antibodies, detectable by PCA, were formed to the milk proteins β -lactoglobulin and casein. In contrast, AL110 likewise drunk by guinea-pigs for 37 days resulted in 90% fatal anaphylactic reactions in animals challenged with the same product. Anaphylactic antibodies were found to casein but not to β -lactoglobulin.

Comminuted Chicken and Prosobee (a soya milk), two alternative 'hypo-allergenic' cows' milk substitutes, rendered approximately 50% guinea-pigs anaphylactically sensitive to the product fed, but neither product stimulated cross-reactive anaphylactic antibodies to either casein or β -lactoglobulin as tested by PCA.

There is some indirect evidence that results of the guinea-pig test are relevant to responses in infants with cows' milk protein intolerance.

Correspondence: Professor R. R. A. Coombs, Division of Immunology, Department of Pathology, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge.

Introduction

Cows' milk protein intolerance (CMPI) and hypersensitivity to cows' milk are well recognized clinical conditions in human infants.

Although breast feeding is acknowledged to be the ideal method of nurturing infants, and is indeed encouraged, in western society most infants are fed cows' milk or infant formulae based on cows' milk from a very early age. Within the last few years most cows' milk-based infant formulae have been adapted to resemble human milk with respect to their protein concentration and casein to whey ratio. Immunologically, however, these cows' milk protein based preparations still remain 'foreign' proteins. Little has been done purposely to reduce their potential allergenicity and no effort has been made to test for this by an appropriate procedure.

We have recently shown, in guinea-pigs fed various cows' milk formulae *per os*, that the anaphylactic sensitizing capacity of these infant formulae varies considerably (Anderson *et al.*, 1979; McLaughlan *et al.*, 1981). This type of guinea-pig test may be an appropriate screening procedure for determining the sensitizing capacity of infant formula with respect to the human infant.

Anaphylactic sensitivity in the guinea-pig sensitized *per os* (or by intraperitoneal injection) may be assessed by (i) shock following intravenous injection with antigen, or (ii) formation of passive cutaneous anaphylactic (PCA) antibodies.

In a recent study (McLaughlan *et al.*, 1981) we reported on the varying sensitizing capacities *per os* of whole cows' milk and of infant formulae based on cows' milk proteins. We concluded that whilst unprocessed cows' milk was extremely sensitizing, heat-treatment of whole milk could greatly reduce its anaphylactic sensitizing capacity. The same was true of infant formulae based on cows' milk; the greater the degree of heat applied during the manufacturing process the less sensitizing was the final formula.

This approach has been extended and in the present report we first show the minimal period of feeding required to sensitize guinea-pigs with fully allergenic milk and the minimum time for development of the anaphylactic state following sensitization. Secondly, we investigated the supposedly hypoallergenic milks, Pregestimil (a predigested enzyme-treated, casein-based milk) and AL110 (a casein-based lactose-free milk), also two milk substitute feeding formulae—Comminuted Chicken and Prosobee (containing soya protein). These formulae have been designed for infants with cows' milk protein intolerance and milk hypersensitivity. Recently, Pregestimil and AL110 have been given to infants recovering from gastroenteritis and their effectiveness in preventing delayed recovery has been compared (Manuel & Walker-Smith, 1981). Our investigations were undertaken to see if these hypoallergenic products behaved as such in the guinea-pig screening model.

Materials and methods

Guinea-pigs

Female Dunkin-Hartley guinea-pigs aged 2–4 weeks on arrival from a commercial breeder (Bantin and Kingman) were used. At the start of each experiment the guinea-pigs were aged 4–6 weeks.

Diet

RGP-guinea-pig pelleted diet (Rank Hovis, McDougall) which had been shown to be

free of cows' milk by immunological testing was fed to the animals, they also received water to drink or milk *ad libitum* as indicated.

Milks and infant formulae fed

Whole cows' milks from three sources were examined, these were untreated cows' milk, pasteurized milk (Unigate) and evaporated milk (Carnation). Infant formulae fed to guinea-pigs were Ostermilk Complete (Glaxo), AL110 (Nestlé) a lactose-free purified casein formula, Pregestimil (Mead-Johnson) a 'hypoallergenic' feeding formula based on predigested casein. Two milk substitute diets were also included, these were Comminuted Chicken (Francis, 1978) and a soya-protein milk, namely Prosobee (Mead Johnson).

Infant formulae were fed to guinea-pigs at the concentrations recommended for infant feeding.

Challenge for anaphylactic sensitivity

Milk used for challenge was from the same source as that fed (this was centrifuged at $10\,000 \times g$ for 5 min and the middle layer used for injection), and was injected intravenously in 0.5 ml volume; the animals were observed for signs of anaphylactic sensitivity.

Passive cutaneous anaphylaxis reaction (PCA)

These tests were performed as described previously (Coombs, Devey & Anderson, 1978).

Tests to determine the interval required after drinking cows' milk before guinea-pigs become anaphylactically sensitive to cows' milk

Thirty female Dunkin-Hatley guinea-pigs aged 4–6 weeks were given pasteurized milk to drink *ad libitum* for the first 7 days of the experiment. Thereafter groups of five guinea-pigs were challenged by intravenous injection with 0.5 ml of pasteurized milk on days 8, 9, 11, 14, 18 and 22. On the day before intravenous challenge, i.e. on days 7, 8, 10, 13, 17 and 21 respectively the animals in each group were bled by cardiac puncture and the serum used for PCA reaction. A control group of 'water only' drinking guinea-pigs was included and these animals showed no reaction on intravenous injection with milk on day 22.

Results

Experiment 1: minimum period of drinking cows' milk required to sensitize guinea-pigs anaphylactically

Pasteurized cows' milk and evaporated cows' milk has previously been shown to induce anaphylactic sensitization in guinea-pigs after 37 days of drinking (McLaughlan *et al.*, 1981), evaporated milk was, however, less sensitizing than pasteurized. In order to determine the ease with which anaphylactic sensitization to cows' milk may be induced in the guinea-pig, these two milks were fed for varying periods. After completing their period of milk drinking the guinea-pigs were given water to drink, until 21 days had passed from the day when they were first given milk to drink; they were then challenged by intravenous injection with 0.5 ml of the milk with which they had been fed.

Animals in a control group (given water to drink) over the same 21-day period were also challenged by intravenous injection of either pasteurized or evaporated milk. On day 20 (before the systemic challenge) the animals were bled by cardiac puncture and the serum used for PCA testing. The results of *in vivo* anaphylactic challenge and PCA testing are shown in Table 1.

After only 4 days of drinking pasteurized milk, seven of eight animals subsequently developed anaphylactic sensitivity to milk which caused fatal anaphylactic reactions

Table 1. Anaphylactic status of guinea-pigs after drinking either pasteurized or evaporated milk for periods varying from 2 to 21 days

Number of days drinking milk		No. in group	Percentage anaphylaxis following i.v. challenge on day 22 with formula fed			Percentage PCA +ve to formula fed
Pasteurized	Evaporated		Fatal	Non-fatal	Total responding	
2	—	7	0	28	28	0
4	—	8	88	0	88	88
8	—	8	100	0	100	100
21	—	8	100	0	100	100
—	2	8	0	0	0	0
—	4	8	0	0	0	0
—	8	8	0	25	25	0
—	15	8	50	50	100	38
—	21	8	100	0	100	63
* Controls drinking water throughout		8	0	0	0	0

* Four animals from this group were challenged by i.v. injection with pasteurized milk and four were similarly challenged with evaporated milk. Sera from these control animals was tested for PCA activity by separate challenge with both pasteurized and evaporated milk.

following intravenous injection with milk. By comparison the guinea-pigs drinking evaporated milk for 4 days failed to develop any sign of anaphylactic sensitivity.

After 8 days of drinking pasteurized milk all guinea-pigs in that group became anaphylactically sensitive, whereas only two of eight guinea-pigs drinking evaporated milk became anaphylactically sensitive and their reactions were not fatal. Following 15 days of drinking evaporated milk all of the group developed anaphylactic sensitivity but in only seven of eight were the reactions fatal. Twenty-one days of drinking evaporated milk were required to achieve 100% fatal anaphylaxis compared with only 8 days of drinking pasteurized milk. The PCA reactions show a similar pattern of sensitization.

It would appear that animals given a milder allergenic form of milk (i.e. evaporated) to drink require a longer exposure period in order to induce maximum sensitization. Also, if the milk is in a 'strongly' allergenic form only a trivial exposure is enough to produce an anaphylactic sensitivity.

Experiment 2: interval required after drinking cows' milk before guinea-pigs become anaphylactically sensitive to cows' milk

Having determined the minimum period of drinking milk required to induce subsequent development of anaphylactic sensitivity, a further experiment was done to determine the minimum interval between cessation of drinking cows' milk and induction of an anaphylactic reaction on intravenous challenge with cows' milk as described in 'Materials and methods'.

The results show (Table 2) that not until day 14 (i.e. 7 days after the milk-drinking period finished) were all the animals in the group rendered anaphylactically sensitive, although by day 11 (i.e. only 4 days after cessation of milk drinking) four of the five animals challenged showed some anaphylactic sensitization. The PCA reactions were consistent with the *in vivo* challenge.

Table 2. Development of anaphylactic sensitivity to cows' milk after drinking pasteurized milk for seven days

Day number	No. in group	Percentage anaphylaxis following i.v. challenge with milk fed			Percentage PCA positive to milk fed
		Fatal	Non-fatal	Total responding	
8 (7+1)*	5	0	0	0	0
9 (7+2)	5	0	0	0	0
11 (7+4)	5	40	40	80	40
14 (7+7)	5	100	0	100	100
18 (7+11)	5	100	0	100	80
22 (7+15)	5	100	0	100	100

* Seven days drinking milk and *n* days drinking water.

Experiment 3: the anaphylactic status of guinea-pigs after drinking 'hypoallergenic' milk formulae or milk substitutes for 37 days

The results obtained in experiment 1 indicate that when milk of low sensitizing potency is fed to guinea-pigs, a prolonged period of feeding is required in order to induce anaphylactic sensitization. In order therefore to test whether 'hypoallergenic' milk formulae (viz. Pregestimil and AL110) designed for infants with milk intolerance problems are of lower sensitizing capacity than ordinary infant formulae, groups of guinea-pigs were given milk formulae and milk substitutes to drink *ad libitum* for 37 days. The anaphylactic status of the guinea-pigs was then assessed by injecting the animals intravenously with the formula fed and observing the animals for signs of anaphylaxis. On day 35 the animals were bled by cardiac puncture and the serum was tested for PCA activity.

As 'positive controls', fresh untreated whole cows' milk and a commonly used infant formula (Ostermilk Complete) were used. Two milk substitute diets based on soya-bean (Prosobee) and chicken meat (Comminuted Chicken) were also investigated.

With the exception of one guinea-pig, the animals drinking AL110 suffered fatal anaphylactic shocks on intravenous challenge. The PCA reactions, however, show that this formula (which is casein based) did not sensitize to β -lactoglobulin.

Most interesting were the findings on the guinea-pigs drinking Pregestimil; these animals were completely unaffected on intravenous challenge, and PCA tests on their sera were completely negative.

The milk substitutes induced some anaphylactic sensitization but this was not as severe as that induced by some of the milk formulae, and PCA reactions to β -lactoglobulin and casein were negative. However, on challenging five of the Comminuted Chicken fed guinea-pigs intravenously with whole cows' milk, three gave weak but definite anaphylactic reactions suggesting some degree of cross-reactivity. When Prosobee fed guinea-pigs were likewise injected with whole cows' milk no reaction was observed.

Experiment 4: a further experiment with Pregestimil, Prosobee and Comminuted Chicken; attempted anaphylactic sensitization by intraperitoneal injection

Since Pregestimil was shown to be completely non-sensitizing via the oral route in guinea-pigs, an attempt was made to sensitize by intraperitoneal injection, followed 37

Table 3. Anaphylactic status of guinea-pigs after drinking milk or milk substitutes *ad libitum* for 37 days

Formula fed	No. in group	Percentage anaphylaxis following i.v. challenge with formula fed			Formula fed	Percentage PCA positive to	
		Fatal	Non-fatal	Total responding		β -LG†	Casein
Untreated* whole cows' milk	10	100	0	100	100	100	100
Ostermilk* (Complete)	9	67	33	100	89	33	89
Pregestimil	10	0	0	0	0	0	0
AL110	10	90	0	90	90	0	80
Comminuted Chicken	10	20	50	70	60	0	0
Prosobee	9	22	33	55	33	0	0
Water only† to drink	5	0	0	0	0	0	0

* Positive controls for 'hypoallergenic' formulae.

† Negative control.

‡ β -lactoglobulin.

days later by intravenous challenge to assess anaphylactic sensitization. Prosobee and Comminuted Chicken were also examined in this experiment. Only one animal (injected with 1 ml of Pregestimil intraperitoneally) had a very mild anaphylactic reaction on intravenous challenge (Table 4). Prosobee and Comminuted Chicken induced anaphylactic sensitization in more than 50% of the guinea-pigs sensitized via the intraperitoneal route. This experiment shows that Pregestimil in guinea-pigs possesses extremely low allergenicity indeed.

Table 4. A further experiment with Pregestimil, Prosobee and Comminuted Chicken: anaphylactic sensitivity* 35–37 days after primary intraperitoneal injection

Formula and volume injected i.p.	No. of animals showing anaphylaxis after i.v. challenge		Total responding	No. of animals PCA positive
	Fatal	Non-fatal		
Pregestimil (1 ml)	0/3	1/3	1/3	0/3
Pregestimil (2 ml)	0/3	0/3	0/3	1/3
Prosobee (2 ml)	2/8	6/8	8/8	4/8
Comminuted Chicken (2 ml)	2/6	2/6	4/6	4/6

* Anaphylactic status judged by either PCA tests or direct intravenous challenge.

Discussion

We have shown (experiment 1) that guinea-pigs given pasteurized cows' milk to drink become anaphylactically sensitized after very short periods of drinking (4–8 days), whereas guinea-pigs given evaporated cows' milk to drink were not so readily sensitized; they required 21 days of drinking to achieve an equivalent degree of anaphylaxis on challenge (Table 1). Following a 7-day period of drinking cows' milk, a further 7 days, with or without milk drinking (see Table 2) had to elapse before all animals exhibited PCA antibodies in their sera and died on intravenous challenge with milk.

To screen supposedly 'hypo-allergenic' infant formulae, guinea-pigs were examined after a more prolonged 'feeding period' of 37 days. Pregestimil *per os* (Table 3) appeared to be completely non-sensitizing. However formula AL110 did sensitize, not to β -lactoglobulin but to casein.

Comminuted Chicken and Prosobee are alternative cows' milk-free liquid diets suitable for infants with cows' milk protein intolerance and cows' milk hypersensitivity. From the PCA reactions (Table 3) it can be seen that guinea-pigs fed these two formulae did not form any tissue-sensitizing antibodies which cross-reacted with cows' milk. On direct challenge however three of five guinea-pigs fed Comminuted Chicken did show mild shock reactions when injected intravenously with cows' milk. These two formulae (Comminuted Chicken and Prosobee) however were sensitizing to themselves giving 70% and 55% anaphylactic reactions, though not all fatal, on challenge.

In view of current trends to introduce soya-milk feeding for normal healthy human infants as an alternative to cows' milk based formulae, it is worth noting that in our guinea-pig feeding experiments, soya-milk was not non-sensitizing. Maintaining cows' milk-sensitive infants and young children on cows' milk-free diets is not easy; however, to maintain soya-sensitive infants and children on soya-free diets may, in the future, be even more difficult.

Recently Manuel & Walker-Smith (1981) compared the effects of three infant feeds (namely, Pregestimil, AL110 and SMA Gold Cap Powder) given to infants recovering from gastroenteritis. They found that delayed recovery occurred in 26% of infants fed on AL110, while of the infants fed Pregestimil and SMA only 5% and 2.5% respectively

showed signs of delayed recovery. If delayed recovery is associated with cows' milk protein intolerance as these authors suggest, then our present findings in the guinea-pig would seem to be relevant. The non-sensitizing Pregestimil would be expected to be bland and non-aggravating, while with the sensitizing capacity of AL110 one would anticipate this product being less satisfactory. Again, since these authors report good clinical results with SMA Gold Cap Powder it could be anticipated (see McLaughlan *et al.*, 1981) that even better results would have been obtained if SMA Liquid Concentrate had been fed.

Only further investigations in infants themselves will assure us that this guinea-pig screening system reflects the likely findings in infants. But, in the meantime, the guinea-pig test is probably the best that can be done.

In discussing these results we would again stress that with special attention to the manufacturing process, especially with regard to heat-treatment, it is likely that a whole cows' milk product could be produced which was non-sensitizing *per os*, yet which was nevertheless still fully nutritious. The 'evaporated milks' and 'liquid concentrates', as presently produced, have already lost much of the sensitizing capacity of whole cows' milk. Further attention to the heat-process could well lead to an ideal product in this regard.

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