

The modified anaphylaxis hypothesis for cot death

ANAPHYLACTIC SENSITIZATION IN GUINEA-PIGS FED COW'S MILK

MADELEINE E. DEVEY, KAREN J. ANDERSON & R. R. A. COOMBS *Division of Immunology, Department of Pathology, University of Cambridge*
M. J. HENSCHEL & MARIE E. COATES *National Institute for Research in Dairying, Shinfield, Reading, Berkshire*

(Received 26 June 1976)

SUMMARY

Guinea-pigs on a normal diet, but given cow's milk to drink instead of water, very soon became anaphylactically sensitive to cow's milk and may be fatally shocked following either i.v. injection or intratracheal inhalation of cow's milk.

INTRODUCTION

In 1960 Parish *et al.* (1960a) put forward the hypothesis that cot death or sudden unexplained death in infancy could be explained by a modified anaphylactic reaction to the foreign antigens in cow's milk. The basis of the hypothesis is the development by the infant of an anaphylactic sensitization as a result of the alimentary intake of large amounts of cow's milk. There is no doubting the frequent allergic sensitization of infants to cow's milk; whether this is such as could lead to anaphylactic death is unknown as direct testing cannot be done. The hypothesis however is that a sleeping infant after a feed of cow's milk regurgitates some stomach contents and inhales a little of this material into the lungs—a probable shock organ in the human infant. Cow's milk antigens on absorption into the lungs set off an immediate anaphylactic reaction, which although resulting in death, is clinically modified due to the deep sleep of the infant.

Despite the high plausibility of this hypothesis it is extremely difficult to prove because cases are not seen alive and at present post-mortem findings afford little evidence either in support of or against the hypothesis.

There has been, however, considerable supporting evidence from investigations of a model situation in the guinea-pig (Parish *et al.*, 1964). Guinea-pigs can easily be anaphylactically sensitized to the protein antigens in cow's milk. Fatal anaphylaxis follows either i.v. injection or inhalation into the trachea. Agonal asphyxial death occurs with either route of challenge. The mode of death, however, can be completely changed if prior to challenge the animals are lightly anaesthetized, the nearest procedure we could invoke to represent the deep sleep of the infant.

In our previous investigations, although we challenged the guinea-pigs intratracheally to mimic inhalation in the infant, we used a parenteral route, mainly i.p., to achieve a uniform sensitization. We did not doubt the possibility that anaphylactic sensitization in the guinea-pig could occur by the alimentary route for there have been many reports in the older literature on this occurrence (see Laroche, Richet & Saint-Girons, 1930). We have also measured the rise of serum antibodies to milk proteins in infants on a cow's milk diet (Gunther *et al.*, 1962) but this was not a measure for anaphylactic sensitization—a direct *in vivo* test for this, of course, being impossible and indirect tests being very difficult to interpret.

The purpose of this present investigation was to elaborate on our earlier model experiments in the

Correspondence: Professor R. R. A. Coombs, Division of Immunology, Department of Pathology, Laboratories' Block, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QQ.

guinea-pig but using animals fed cow's milk (enteral sensitization—as in the human infant) instead of animals sensitized by parenteral injection.

MATERIALS AND METHODS

Animals. For the first experiment newborn guinea-pigs were obtained from the colony of the National Institute for Research in Dairying. The sows were housed in single cages about a week before parturition and allowed to remain with their young for fourteen days after littering. For the remainder of the experiments Duncan-Hartley guinea-pigs of both sexes weighing between 200 and 800 g were used unless otherwise stated.

Diets. For the first experiment a pelleted diet similar in composition to guinea-pig milk was compounded. It contained spray-dried whole cow's milk 312.5 g/kg; calcium caseinate 287.5 g/kg; maize oil 250 g/kg; sucrose 25 g/kg; solka floc 75.5 g/kg; salt mixture 35.7 g/kg; vitamin triturate 12.8 g/kg; l-ascorbic acid 1 g/kg. The animals accepted the diet if it was introduced over several days by mixing it in gradually increasing quantities with commercial guinea-pig pellets. Other animals received a normal commercial guinea-pig pellet diet, with water or cow's milk to drink as indicated.

Cow's milk and purified milk proteins. Pasteurized cow's milk was 'microfuged' and the middle layer used for the guinea-pig challenge injections. Total casein was prepared in the laboratory from raw cow's milk by two precipitations with 0.5 M HCl at pH 4.6. β -lactoglobulin (β LG) (containing only β -lactoglobulins A and B) was purchased from BDH Ltd., Poole, Dorset. Highly purified α -lactalbumin (α LA) was a gift from Dr T. E. Barman.

Intravenous challenge. 0.2–0.5 ml middle layer cow's milk was injected into the interdigital or brachial vein using a gauge 27 hypodermic needle. Animals were observed for up to 15 min after injection for signs of adverse reaction. When anaphylactic death occurred it was usually within 4 min of challenge.

Intratracheal challenge. Guinea-pigs were lightly anaesthetized with 0.01 ml Nembutal/20 g body weight i.p. The trachea was exposed following a cutaneous incision and 0.25 ml cow's milk was injected directly into the trachea. Again anaphylactic death usually occurred within 4 min of challenge.

Measurement of specific antibodies. Just before challenge guinea-pigs were bled from the marginal ear veins. Serum was separated and stored at -20°C until tested for antibodies to the cow's milk proteins: casein, β LG and α LA. Specific antibodies were measured by two different methods: the passive haemagglutination test of Boyden (1951) using human red cells coated by tannic acid and by the red cell linked antigen antiglobulin reaction (Gell & Coombs, 1975) using a rabbit antiserum to guinea-pig 7S gammaglobulin.

The directly agglutinating antibodies that reacted in the first test were susceptible to 2-mercaptoethanol and were therefore presumed to be IgM whereas the second test detected antibodies of the IgG class.

Schultz-Dale experiments. Sensitization of guinea-pig ileum to cow's milk was measured in a Schultz-Dale bath using the automatic assay apparatus of Boura, Mongar & Schild (1954). Histamine-induced contractions were recorded using an isotonic transducer and a pen recorder.

RESULTS

Susceptibility to anaphylaxis of guinea-pigs given a diet soon after birth containing cow's milk solids, with liquid milk to drink

At the times after birth shown in Table 1 the guinea-pigs were bled for serum and then injected i.v. with 0.5 ml cow's milk and death from anaphylaxis recorded. Two guinea-pigs (not shown in Table 1), instead of being injected, were killed on days 68 and 100 and their ilea tested for contraction in a Schultz-Dale bath by the addition of milk; the guts proved to be anaphylactically sensitive.

The level of antibodies to milk proteins in the guinea-pig sera are also shown in Table 1.

Other guinea-pigs reared on a normal diet and killed over a comparable period showed no anaphylactic sensitivity to cow's milk when challenged, nor did the gut contract in a Schultz-Dale bath when challenged with milk.

Anaphylactic sensitization of guinea-pigs on normal diet but given ad lib cow's milk to drink instead of water

The anaphylactic sensitization in the previous experiment was produced by a diet containing cow's milk in both solid and fluid phases. The purpose of the present experiment was to investigate whether an anaphylactic sensitization would result also from feeding a normal guinea-pig pellet diet and offering fresh cow's milk to drink in the place of water.

Six- to eight-week-old guinea-pigs had their supply of water removed and cow's milk to drink offered instead. After varying intervals the guinea-pigs were bled for antibody testing and then injected i.v. with 0.5 ml cow's milk to test for general anaphylactic sensitization. The results (Table 2) show that after

TABLE 1. Susceptibility to anaphylaxis of guinea-pigs given a diet soon after birth containing cow's milk solids, with liquid milk to drink

Guinea-pig	Diet	Period on diet (days)	Intravenous challenge of 0.5 ml cow's milk	Serum antibodies to:					
				α LA		β LG		Casein	
				IgM*	IgG†	IgM	IgG	IgM	IgG
1	Milk pellets and milk	61	Death	2	0	4	32	256	512
2	Milk pellets and milk	61	Death	‡	n.t.	64	32	128	64
3	Milk pellets and milk	69	Death	0	0	8	16	64	128
4	Milk pellets and milk	74	Death	0	2	128	8	128	64
5	Milk pellets and milk	74	Death	‡	n.t.	‡	‡	128	256
6	Milk pellets and milk	74	Death	‡	n.t.	16	32	64	256
7	Normal diet	Control	Unaffected	0	0	0	0	4	0
8	Normal diet	Control	Unaffected	0	0	0	0	4	0
9	Normal diet	Control	Unaffected	0	n.t.	0	0	8	0
10	Normal diet	Control	Unaffected	0	n.t.	0	0	4	0
11	Normal diet	Control	Unaffected	0	n.t.	0	0	16	0
12	Normal diet	Control	Unaffected	0	n.t.	0	0	8	0
13	Normal diet	Control	Unaffected	0	n.t.	0	0	2	0

α LA = α lactalbumin; β LG = β lactoglobulin; n.t. = not tested.

* Measured by tanned cell test.

† Measured by RCLAAR.

‡ Less than 16 or lowest dilution tested.

TABLE 2. Susceptibility to anaphylaxis of guinea-pigs given cow's milk to drink in place of water

Guinea pig*	Drinking fluid	Days after milk first offered	Intravenous challenge of 0.5 ml cow's milk	Serum antibodies to:					
				α LA		β LG		Casein	
				IgM†	IgG‡	IgM	IgG	IgM	IgG
1	Cow's milk	7	Unaffected	4	n.t.	4	§	128	0
2	Cow's milk	7	Unaffected	4	n.t.	2	§	128	0
3	Cow's milk	7	Very mild reaction	4	n.t.	2	§	256	0
4	Cow's milk	9	Very mild reaction	4	n.t.	4	§	128	2
5	Cow's milk	9	Mild reaction	8	n.t.	4	§	64	0
6	Cow's milk	9	Very mild reaction	4	n.t.	4	§	128	0
7	Cow's milk	13	Death	8	n.t.	16	§	256	8
8	Cow's milk	13	Death	16	n.t.	16	4	128	4
9	Cow's milk	13	Death	8	n.t.	8	0	64	4
10	Cow's milk	17	Death	0	n.t.	0	0	512	256
11	Cow's milk	18	Death	§	n.t.	§	§	64	64
12	Cow's milk	21	Death	0	n.t.	0	0	128	8
13	Cow's milk	21	Death	0	n.t.	0	0	128	16
24	Cow's milk	21	Death	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
15	Cow's milk	22	Death	§	n.t.	§	8	512	32
16	Cow's milk	31	Death	§	n.t.	§	0	8	0
17	Cow's milk	34	Death	0	n.t.	0	0	32	4
18	Cow's milk	40	Death	0	n.t.	0	8	512	32
19	Water		Unaffected	§	n.t.	§	§	§	§
20	Water		Unaffected	4	n.t.	8	§	64	§
21	Water		Unaffected	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
22	Water		Unaffected	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.

n.t. = Not tested.

* Six to eight weeks old.

† Measured by tanned cell test.

‡ Measured by RCLAAR.

§ Less than 8 or lowest dilution tested.

TABLE 3. The main anaphylactic sensitivity of guinea-pigs drinking cow's milk (over the period tested) is to casein and β lactoglobulin

Guinea-pig	Days after milk first offered	Protein used for i.v. challenge and dose (mg)		Result
1	17	Casein	1.5	Death
2	21	Casein	2.25	Death
3	29	Casein	1.5	Death
4	29	Casein	1.5	Death
5	21	β lactoglobulin	2.5	Death
6	29	β lactoglobulin	1.5	Death
7	17	α lactalbumin	1.6	Unaffected
8	21	α lactalbumin	2.7	Very mild reaction*
9	21	α lactalbumin	3.0	Mild reaction
10	29	α lactalbumin	1.5	Mild reaction
11	29	α lactalbumin	1.5	Very mild reaction

* After very mild reaction, injection of 0.3 ml cow's milk resulted in fatal anaphylaxis.

TABLE 4. Guinea-pigs which have been given cow's milk to drink die an anaphylactic death, if milk is injected under anaesthesia into the trachea

Guinea-pig	Days after milk first offered	Conditions of challenge	Results of challenge	Serum antibodies to:						
				α LA		β LG		Casein		
				IgM*	IgG†	IgM	IgG	IgM	IgG	
1	14	0.25 ml cow's milk	i.v.	Death	0	n.t.	0	‡	1024	32
2	14	0.25 ml cow's milk	i.v.	Death	2	n.t.	2	0	128	64
3	14	0.25 ml cow's milk	i.t.	Death	4	n.t.	4	4	64	4
4	14	0.25 ml cow's milk	i.t.	Death	2	n.t.	2	0	128	64
5	No milk	0.25 ml cow's milk	i.t.	Unaffected	0	n.t.	0	0	4	0
6	No milk	0.25 ml cow's milk	i.t.	Unaffected	0	n.t.	0	n.t.	4	n.t.
7	No milk	0.25 ml cow's milk	i.v.	Unaffected	2	n.t.	0	0	4	0
8	No milk	0.25 ml cow's milk	i.v.	Unaffected	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
9	17	0.25 ml cow's milk	i.t.	Death	2	n.t.	2	0	256	8
10	17	0.25 ml cow's milk	i.t.	Death	0	n.t.	0	0	512	2
11	17	1.5 mg casein	i.t.	Death	0	n.t.	0	0	1024	256
12	21	2.25 mg casein	i.t.	Mild reaction	0	n.t.	0	0	32	1024
13	21	3.0 mg casein	i.t.	Unaffected	4	0	2	2	> 1024	2
14	21	3.7 mg β lactoglobulin	i.t.	Death	0	n.t.	0	0	256	512
15	21	3.2 mg α lactalbumin	i.t.	Very mild reaction	0	n.t.	0	n.t.	32	n.t.
16	29	0.25 ml cow's milk	i.t.	Death	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
17	29	0.25 ml cow's milk	i.t.	Death	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
18	29	1.5 mg casein	i.t.	Death	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
19	29	1.5 mg casein	i.t.	Mild reaction	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
20	29	1.2 mg β lactoglobulin	i.t.	Very mild reaction	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.

* Measured by tanned cell test.

† Measured by RCLAAR.

‡ Less than 8 or lowest dilution tested.

13 days with cow's milk to drink all animals may be fatally shocked with anaphylaxis if injected i.v. with 0.5 ml milk—a challenge which is without any overt effect on comparably aged guinea-pigs drinking water.

Again titres of milk antibodies as measured in the sera are recorded in Table 2.

A further batch of guinea-pigs (6–8 weeks old) on a normal solid diet, but given cow's milk to drink in the place of water, were tested for anaphylactic sensitivity to the purified constituent proteins of cow's milk by i.v. injection. The results, Table 3, show that the more potentially lethal sensitivity was to casein and β -lactoglobulin whereas only very mild reactions were produced by challenge with α -lactalbumin.

Guinea-pigs sensitized by the alimentary route may be anaphylactically shocked by intratracheal injection of milk

Having shown that 2–3 weeks of alimentary intake of cow's milk renders 6–8-week-old guinea-pigs anaphylactically sensitive and susceptible to death on i.v. injection of cow's milk (or its constituent proteins) it was important to see if such animals were also susceptible to anaphylactic death following inhalation of small amounts of milk.

To ensure that the small challenging dose reached the lungs the guinea-pigs, at the time of challenge, were lightly anaesthetized, the trachea exposed and the injection made directly into the trachea. In unsensitized guinea-pigs the procedure is without any untoward effect (see guinea-pigs 5–8, Table 4). The reader may be reminded (Parish, Barrett & Coombs, 1960b) that, as regards anaphylaxis in guinea-pigs sensitized by parenteral injection, death is just as rapid following intratracheal injection as with i.v. injection. Also light anaesthesia, although not influencing the outcome, greatly modifies the clinical picture which becomes one of simple cessation of respiration without the violent asphyxial struggle seen in the conscious animal. From Table 4 it may be seen that guinea-pigs sensitized by the alimentary route may suffer a generalized anaphylactic reaction following inhalation of milk into the lungs. Death could also be produced following inhalation of casein or β -lactoglobulin.

Development of anaphylactic sensitivity in very young guinea-pigs having access to cow's milk available in a bowl in their cage

Following parturition a bowl of cow's milk was placed in the cage making the milk available to both

TABLE 5. Sensitization of guinea-pigs offered cow's milk from birth

Guinea-pig	Treatment	Challenge (i.v.) with cow's milk (ml) (days after birth)*	Result	Serum antibodies to:					
				α LA		β LG		Casein	
				IgM†	IgG‡	IgM	IgG	IgM	IgG
1	Cow's	9th day 0.15	Very mild reaction	0	n.t.	§	§	§	n.t.
2	milk	17th day 0.2	Death	0	n.t.	0	0	64	0
3	available	17th day 0.2	Very mild reaction	0	n.t.	0	0	32	0
4	to drink	22nd day 0.2	Unaffected	0	n.t.	0	0	32	0
5	from day	22nd day 0.2	Death	0	n.t.	0	0	16	0
6	of birth	22nd day 0.2	Unaffected	§	n.t.	§	§	16	§
7	No	9th day 0.15	Unaffected	§	n.t.	§	§	§	§
8	cow's	17th day 0.2	Unaffected	0	n.t.	0	0	4	0
9	milk	22nd day 0.2	Unaffected	§	n.t.	§	§	8	§

n.t. = Not tested.

* Mother left in cage with young for first 13 days after birth.

† Measured by tanned cell test.

‡ Measured by RCLAAR.

§ Less than 8 or lowest dilution tested.

the mother and the newborn. The newborn guinea-pigs were also able to suck freely their mother's milk, until the 13th day when the mothers were removed.

The baby guinea-pigs were challenged by i.v. injection of very small doses of cow's milk on different days after birth. From Table 5 it may be seen that one guinea-pig exhibited a very mild reaction as early as the 9th day. Of five baby guinea-pigs aged 17-22 days—two died, one suffered a mild reaction, and two were unaffected.

DISCUSSION

The experiments reported in this paper were undertaken to forge a further link in the chain establishing the hypothesis that cot-death in infancy may be due to anaphylaxis brought about by inhalation of cow's milk in an infant sensitized by the alimentary route against the antigenic proteins in cow's milk.

In 1960 and again in 1964 (Parish *et al.*, 1960a; Parish *et al.*, 1964) we reported that guinea-pigs sensitized by i.p. injection of cow's milk suffered fatal anaphylaxis if challenged intratracheally and further that if the challenge was performed under light anaesthesia the mode of death and post-mortem findings were very similar to those occurring in infant cot death.

The model guinea-pig experiments would have been even more close had the guinea-pigs been sensitized to cow's milk by the oral route; the parenteral route had been used in the earlier experiments to achieve what we thought would be a more uniform state of sensitivity.

The present experiments were designed to look into the question of anaphylactic sensitization by the oral route in relation to this hypothesis. The experiments show that within 13 days (Table 2) of drinking cow's milk (together with a normal diet) guinea-pigs will die of anaphylaxis should milk be injected either i.v. or intratracheally (to simulate inhalation).

The somewhat unexpected finding was the ease and rapidity with which the simple replacement of drinking water with milk lead to this sensitized state. We had thought that a total milk diet (see Table 1) in the form of both solids and fluids might be necessary right from birth; but as the experiments have shown sensitization occurred very much more easily. Further, and also unexpectedly, the sensitization following replacement of water with cow's milk was found to be very constant in the present schedule. We feel that this finding further strengthens the plausibility of this hypothesis for cot death and that it should be taken more seriously.

The guinea-pigs fed cow's milk nearly always developed higher titres of specific antibodies to cow's milk than did the controls. High titres of IgM antibodies developed during the first week of cow's milk feeding but these titres showed no correlation with susceptibility to milk challenge. IgG antibodies were first detected at about day 13 of feeding when the animals first became susceptible to challenge, but again, particularly in animals fed from birth, there appeared to be little correlation between these titres and susceptibility to anaphylaxis.

From other unpublished experiments we anticipate that the type of sensitization (not excluding tolerance itself) and the overt manifestations of effector or shock reactions may display interesting and very important variations depending on the age when cow's milk is first introduced into the diet and on the period and constancy over which it is maintained. These are now some of the considerations under investigation.

We should like to thank Mr R. H. Matthews and Mr B. Burgess for excellent technical assistance.

The work was supported by a grant from The Foundation for the Study of Infant Deaths.

REFERENCES

- BOURA, A., MONGAR, J.L. & SCHILD, H.O. (1954) Improved automatic apparatus for pharmacological assays on isolated preparations. *Brit. J. Pharmacol.* **9**, 24.
- BOYDEN, S.V. (1951) Adsorption of proteins on erythrocytes treated with tannic acid and subsequent haemagglutination by antiprotein sera. *J. exp. Med.* **93**, 107.
- GELL, P.G.H. & COOMBS, R.R.A. (1975) Basic immunological methods. *Clinical Aspects of Immunology*, 3rd edn (ed. by P.G.H. Gell, R.R.A. Coombs and P.J. Lachmann) p. 16. Blackwell Scientific Publications, Oxford.
- GUNTHER, M., CHEEK, E., MATTHEWS, R.H. & COOMBS, R.R.A. (1962) Immune responses in infants to cow's milk proteins taken by mouth. *Int. Arch. Allergy*, **21**, 257.
- LAROCHE, G., RICHEL FILS, C. & SAINT-GIRONS, F. (1930)

Alimentary Anaphylaxis (Translated by Rowe & Rowe).
University of California Press, California.

PARISH, W.E., BARRETT, A.M., COOMBS, R.R.A., GUNTHER,
M. & CAMPS, F.E. (1960a) Hypersensitivity to milk and
sudden death in infancy. *Lancet*, ii, 1106.

PARISH, W.E., BARRETT, A.M. & COOMBS, R.R.A. (1960b)

Inhalation of cow's milk by sensitized guinea-pigs in the
conscious and anaesthetized state. *Immunology*, 3, 307.

PARISH, W.E., RICHARDS, C.B., FRANCE, N.E. & COOMBS,
R.R.A. (1964) Further investigations on the hypothesis
that some cases of cot-death are due to a modified anaphyl-
actic reaction to cow's milk. *Int. Arch. Allergy*, 24, 215.