

Possible role for rotavirus in the development of cows' milk enteropathy in infants

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Summary

In order to test the hypothesis that rotavirus infection leads to IgE hypersensitivity to cows' milk, evidence of past rotavirus infection, as reflected by the presence of antibodies, was sought in two groups of children with cows' milk allergy. The groups were defined according to the time to react to milk challenge. In group I there were fifteen children with an adverse reaction within 40 min of milk challenge. The twenty-one children in group II had gastrointestinal reactions between 1 hr and 24 hr after milk challenge. The results showed a higher incidence of antibodies to rotavirus in group II than group I ($P < 0.05$). Although group I patients had significantly higher levels of total and milk-specific IgE antibodies to cows' milk than those in group II, both patient groups had lower levels of serum IgG, IgA and IgM than a control population. No association between the presence of rotavirus antibodies and IgE hypersensitivity to cows' milk was found. However, an association between non-IgE milk enteropathy and previous rotavirus antibodies was demonstrated.

Introduction

The development of allergic diseases may be determined by many genetic and environmental factors. One environmental factor, viral infection, has been associated with enhanced IgE production in both humans [1] and animals [2]. It has been postulated that viral infections induce dysregulation of T-suppressor cell function, leading to increased IgE synthesis [1–3]. Rotavirus is a common cause of gastroenteritis in infancy and childhood [4]. Although naturally [5] and experimentally [6] induced inflammatory disorders of the gastrointestinal tract lead to increased absorption of antigenically intact dietary protein, the role of gastrointestinal infections in the pathogenesis of allergic gut disease is uncertain. Harrison *et al.* [7] and Iyngkaraen *et al.* [8] have reported that some children develop cows' milk allergy (CMA), manifesting

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as cows' milk protein enteropathy, following a gastroenteritis-like illness. However, they were unable to identify a causative organism. Collectively these studies suggest that IgE hypersensitivity to cows' milk might be triggered by a gastrointestinal infection that could induce disturbance in T-suppressor cell function, as well as altering gut permeability to dietary antigens.

In previous studies we identified three groups of infants and young children with CMA following a formal milk challenge [9]. They were defined as being milk allergic because they demonstrated a reproducible adverse clinical response to milk ingestion under controlled challenge conditions, and other causes of adverse response to milk ingestion, e.g. disaccharidase deficiency and milk aspiration, had been excluded. Subsequently, a cluster analysis of 100 infants and young children with CMA, using a computer data base, confirmed the existence of these three sub-groups of children with CMA [10] and their separate immunological features [11]. The first group showed an immediate allergic reaction to cows' milk; they were atopic in that they had high IgE levels, developed acute skin eruptions within minutes of milk ingestion, and demonstrated elevated IgE antibodies to cows' milk. The second group were non-atopic, in that milk-specific and total IgE levels were low, and symptoms confined to the gastrointestinal tract, consisting of vomiting and/or diarrhoea, developed several hours after milk ingestion; some have termed this disease 'cows' milk enteropathy' (CME). A third group of infants demonstrated a variety of gastroenteric symptoms with or without bronchitis and/or eczematous skin reactions evolving more than 24 hr after commencing cows' milk challenge; in this third group, only patients with eczematous reactions had raised IgE antibodies to cows' milk. One striking feature that emerged from a detailed analysis of the clinical and immunological findings in these three populations was the relatively homogeneous features of patients in group I with regard to their atopic features, and the uniform distribution of findings within the non-atopic group II patients with gastrointestinal reactions. These findings contrasted with the heterogeneous symptoms of group III patients where multi-system disease was common. Furthermore, patients in group I and group II were of similar age, whereas the group III patients were significantly older at the time that CMA was diagnosed [10].

In the current study we hypothesized that rotavirus infection would lead to IgE hypersensitivity to cow's milk. In order to examine this hypothesis we examined the frequency with which serological evidence of past rotavirus infection occurred in patients with CMA. The study was confined to our two relatively homogeneous populations of children who were of comparable age. The incidence of the presence of rotavirus antibodies in group I patients who developed acute skin reactions following milk ingestion, was compared with that of group II patients who had cows' milk enteropathy.

Subjects and methods

Patients studied were from a consecutive group of infants and young children being investigated for suspected CMA. The patients included in this investigation had CMA diagnosed by positive challenge according to a previous protocol [8,10]. They were regarded as being milk allergic in that their symptoms came into remission for periods of 6-12 weeks on a milk-free diet and relapsed when challenged with cows' milk in a specialized hospital unit. Disaccharidase deficiency as a cause of milk intolerance was excluded by jejunal biopsy and estimation of jejunal disaccharidase levels where relevant. Serum was taken immediately prior to commencement of the milk challenge

Table 1. Features of CMA patients

Group	Reaction time after challenge	No. patients	Age range (months)	Rotavirus antibodies	
				Present	Absent
I Skin reacting	40 min	15	4-16(8)*	5	10
II Gastrointestinal reacting	1-24 hrs	21	3-43(9)	16	5

* Median age in parentheses.

procedure when patients were asymptomatic and had been on a milk-free diet for periods of 6-12 weeks.

Thirty-six CMA patients between the ages of 3 months and 43 months were assigned to one of two groups (Table 1) according to the time of clinical reaction from the commencement of milk challenge. All the patients in group I developed perioral or generalized urticaria or angioedema and responded within 40 min of ingestion of up to 10 ml of cows' milk. The patients in group II developed vomiting and/or diarrhoea more than 1 hr, but less than 24 hr, after commencing cows' milk challenge; they had ingested a total of 30-240 ml of cows' milk at the time that reactions occurred [10]. The challenges were open but conducted in a specialized hospital unit. Patients were assigned to group I or group II according to the time of their clinical response to milk challenge. This was done before the results of their immunological studies were known. Patients were unable to be age matched, but there was no significant difference in the median ages between the two groups. (Mann-Whitney *U*-test, $P < 0.05$). Because of the disparate age range between the group I and group II patients (Table 1), despite similar median ages, the age distribution between the two groups was further assessed using the Wald-Wolfowitz Runs test. Using this test, the null hypothesis that the groups were drawn from the same age population was not rejected ($P = 0.36$).

Total serum immunoglobulins and specific antibodies

Total serum IgG, IgA and IgM levels were measured with a Technicon auto analyser fluoronephelometer and expressed as percentile values according to the age of the patient, and based on studies performed at the Royal Children's Hospital, Melbourne, Australia [12]. Serum IgE levels were measured by the Phadebas PRIST assay (Pharmacia, Uppsala, Sweden) and expressed as IU/ml.

IgG, IgA and IgM antibodies to cows' milk protein were measured using an ELISA [13]. Briefly, fresh cream-free pasteurized cows' milk was adsorbed to the wells of polystyrene microtitre trays. The patient's serum was diluted and added to the wells in duplicate. Cows' milk antibodies were identified by adding alkaline phosphatase labelled rabbit anti-human IgG, IgM or IgA antibodies (DAKOPATTS) followed by enzyme substrate (p-nitrophenyl phosphate). Optical densities at 405 nm were read on an automatic microplate reader (TITERTEK) and converted to IgG, IgA or IgM Milk Antibody Units (MAU)/ml by comparison with the dilution curve of a high titred standard serum. IgE antibodies to cows' milk were measured by the Phadebas RAST assay (Pharmacia, Uppsala, Sweden) in one batch and expressed as absolute counts per minute of isotope bound.

Table 2. Immune features in group I patients

Patient	Immunoglobulin levels				Milk antibody				Rotavirus antibody (IgG titre)
	IgG (percentile)	IgA (percentile)	IgM (percentile)	IgE (IU/ml)	IgG (MAU/ml)	IgA (MAU/ml)	IgM (MAU/ml)	IgE (c.p.m.)	
1	23	33	43	500	29	130	105	49	< 300
2	10	10	9	164	27	100	1400	1954	< 300
3	10	17	35	76	33	< 10	525	202	< 300
4	20	20	25	260	17	32	92	4783	< 300
5	20	44	14	12	22	42	108	573	< 300
6	19	17	5	17	24	110	150	n.e.*	< 300
7	40	44	15	105	< 10	< 10	272	4212	< 300
8	14	Tr†	31	110	21	31	75	391	< 300
9	9	Tr	29	10	13	0	2050	1860	< 300
10	26	85	80	< 5	25	120	1050	2718	< 300
11	23	33	63	580	17	10	63	7088	328
12	17	40	7	30	20	65	800	7142	1043
13	33	6	7	4	0	45	200	1047	736
14	35	Tr	18	< 5	115	130	650	636	655
15	26	5	5	110	0	45	1600	579	521

* n.e.: Not examined

† Tr: Trace found

IgG antibodies to rotavirus were measured using a modification of an ELISA assay [14]. Based on longitudinal epidemiological data generated from this institution, a level of more than 300 rotavirus antibody units (RAU) was considered as evidence of past rotavirus infection [15]. Antibodies to rotavirus were determined in CMA patients in serum samples taken at the time of diagnosis.

Statistical analysis

The Mann-Whitney *U*-test, the Wald-Wolfowitz Runs test and the Chi-squared test were used where appropriate. The null hypothesis was rejected when *P* was less than 0.05. The Spearman rank correlation test was used to investigate the relation between various immunologic and rotavirus parameters; the null hypothesis was rejected when *P* was less than 0.01.

Results

Immunoglobulin levels

The immunoglobulin levels in group I and group II patients were relatively low. (Tables 2 and 3). The median percentile values for group I were IgG 15, IgA 17, and IgM 18, and in group II the median values were IgG 19, IgA 5, and IgM 35. There was no statistical difference in the level of these isotypes between group I and group II. However, IgE levels were higher in group I patients (median = 76 IU/ml) than group II (median = 9 IU/ml); this difference was statistically significant (*P* = 0.04). The distribution of IgA and IgE levels in the two populations is shown in Fig. 1. The levels of IgE antibodies to cows' milk were significantly higher in group I patients than those in group II (*P* = 0.01) (Fig. 2).

Table 3. Immune features in group II patients

Patient	Immunoglobulin levels				Milk antibody				Rotavirus antibody (IgG titre)
	IgG	IgA	IgM	IgE (IU/ml)	IgG (MAU/ml)	IgA (MAU/ml)	IgM (MAU/ml)	IgE (c.p.m.)	
16	23	0	3	<5	30	<10	350	201	<300
17	25	5	11	n.e.*	22	46	208	201	<300
18	13	<5	65	5	17	100	181	266	<300
19	15	33	35	80	10	150	700	812	<300
20	46	<5	19	36	16	40	1150	n.e.	<300
21	7	34	23	154	145	290	840	2010	495
22	12	n.e.	14	<5	20	170	1400	166	1203
23	n.e.	n.e.	n.e.	n.e.	34	35	208	223	373
24	10	0	10	<5	44	34	208	239	861
25	6	<5	40	12	20	40	525	230	1181
26	20	25	60	34	16	30	525	212	491
27	23	75	42	88	12	46	500	324	444
28	43	73	95	67	<10	36	625	524	1434
29	34	0	9	9	10	43	56	510	554
30	9	<5	>95	<5	<10	40	900	477	1594
31	17	10	62	20	<10	23	500	572	642
32	n.e.	n.e.	n.e.	<5	<10	72	0	536	342
33	10	48	66	25	<10	272	1500	331	326
34	24	4	49	4	23	163	201	157	671
35	12	12	5	<5	20	90	250	5782	360
36	19	5	21	58	10	20	400	309	461

*n.e.: Not examined

Rotavirus antibodies

There was a higher incidence of patients with elevated rotavirus antibodies in group II patients (16/21) than in group I (5/15); the association between the presence of rotavirus antibodies and the group II patients was statistically significant. ($\chi^2 = 11.37$; $P = 0.01$) (Table 1). In addition, as expected from this finding, the levels of IgG rotavirus antibodies in group II patients (median 461 RAU/ml) were higher than those in group I (median = 300 RAU/ml). These differences were statistically significant ($P = 0.036$).

Interrelation of rotavirus infection and immunoglobulins

There was a higher incidence of rotavirus antibodies in group II patients but, as discussed above, there was no significant difference in the percentile levels of serum IgG, IgA or IgM between the groups. In addition, there was no difference in these immunoglobulin levels within either group I or group II patients according to their rotavirus antibody status. In particular there was no correlation between low serum IgA levels in the group I or group II patients and rotavirus antibody levels.

Although total IgE levels were higher in group I than in group II patients, there was no statistical difference in the levels of total IgE between rotavirus seropositive and seronegative patients either within each group or when the total population was analysed (Fig. 1).

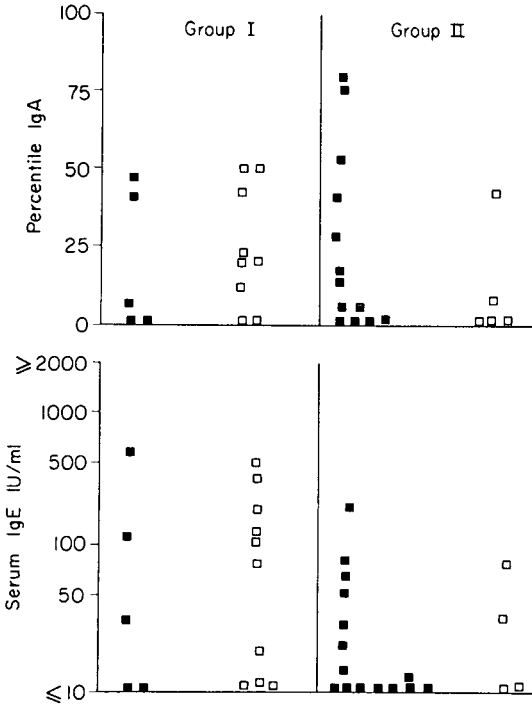


Fig. 1. The distribution of IgA percentile values ($n=33$) and total IgE levels IU/ml ($n=34$) in group I and in group II patients in relation to presence (■) or absence (□) of rotavirus antibody.

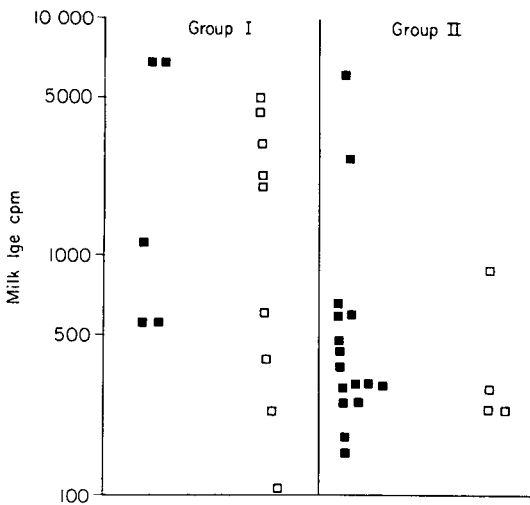


Fig. 2. Levels of IgE antibodies to cows' milk in group I and group II patients according to rotavirus IgG antibody status ($n=34$). (■) Rotavirus present, (□) rotavirus absent.

The levels of IgE antibodies to cows' milk were higher in group I than group II ($P=0.01$), but there was no significant difference in the level of IgE antibodies to cows' milk in patients with evidence of rotavirus antibodies compared with those without such evidence. (Fig. 2). Furthermore, there was no significant difference in the levels of IgE antibodies to cows' milk in patients within group I or group II, regardless of their rotavirus antibody status. No association between IgG, IgA or IgM milk antibodies and rotavirus antibodies could be demonstrated (data not shown).

Discussion

This study failed to confirm our initial hypothesis and an association could not be demonstrated between past rotavirus infection, indicated by elevated IgG rotavirus antibodies, and IgE hypersensitivity to cows' milk. Instead, rotavirus antibodies occurred more frequently in group II infants with cows' milk enteropathy (CME), than in the group I patients with IgE-mediated immediate type hypersensitivity reactions. Several explanations could account for these findings. Firstly, it is possible this is an epi-phenomenon due to rotavirus infection in children whose gut mucosa has been previously damaged by milk-protein enteropathy. Secondly, it could simply reflect the distribution of patients of different ages with the slightly older group II patients having a greater opportunity to acquire rotavirus infection irrespective of CME. Thirdly, rotavirus infection during a period of relative immune vulnerability leads to milk-protein enteropathy.

The first explanation cannot be excluded by this study. Although gut mucosal abnormalities have been demonstrated in patients with either IgE or non-IgE cows' milk hypersensitivity [16,17], the possibility that these abnormalities render the group II patients more susceptible than group I patients to secondary rotavirus infection must be acknowledged. The second explanation seems unlikely because the patients in groups I and II were of similar median age, and the Wald-Wolfowitz Runs test did not suggest that they were drawn from populations with different age distributions.

We favour the third explanation and suggest that rotavirus infection may play a role in subsequent development of milk enteropathy, for the following reasons.

Acute infective gastroenteritis has previously been reported to precede the onset of milk allergy [5,6], but an infective agent was not identified. In studies of experimental gastroenteritis, Hamilton documented increased absorption of dietary macromolecules in piglets after infection with transmissible gastroenteritis (TGE) [18]. We propose that the mucosal damage induced by rotavirus [19] causes a similar change in gut permeability and that increased absorption of milk antigen follows. The observation that IgE [20] and non-IgE [21] antibodies to cows' milk are present in most children ingesting cows' milk, suggests that immunological priming to milk antigens is a physiological event. It is possible this normal course of events is disturbed as a result of an increased milk antigen load secondary to mucosal damage caused by rotavirus. This disturbance could induce changes in mucosal immunity subsequently manifest clinically as milk enteropathy.

As not all infants who suffer rotavirus infection develop CME, other factors must play a role in the development of the disease. We envisage a transient immune vulnerability during infancy as one of these factors. This is supported by the finding of Harrison *et al.* [7] who reported a high incidence of IgA deficiency in children developing CME after gastroenteritis. Our patients with CME showed not only low levels of IgA but also of IgG and IgM, findings consistent with immune vulnerability.

However, low levels of these immunoglobulins were also seen in our IgE-sensitized atopic group I patients. Indeed, the only immune factor that we measured which distinguished the two patient groups was the presence high serum IgE levels. Possibly high IgE levels confer some protective benefit against rotavirus in infancy, although we are not aware of any experimental or clinical data to support this notion. It is likely that the development of CME is determined by other factors operating during infancy. Genetic factors, the age at the time of exposure to milk antigen [21] and other factors [22] are likely to modify the immune response to cows' milk and the clinical manifestations of CMA. Recently we have shown that the intensity of exposure to milk antigen in infancy is one factor determining the development of IgE-mediated milk hypersensitivity [23].

Our previous studies have shown that patients with milk enteropathy are frequently intolerant to other foods such as soybean milk, egg and wheat, and may develop chronic diarrhoea and failure to thrive [10]. Rotavirus and other gastrointestinal infections could cause sensitization to multiple dietary antigens by the mechanisms described above. Alternatively, and subsequent to the initial damage, milk enteropathy itself could lead to sensitization to 'innocent' bystander antigens as described by Bloch [24].

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