Clinical Allergy, 1986, Volume 16, pages 451–458

Study of IgG sub-class antibodies in patients with milk intolerance

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(Received in original form 13 November 1985 and in revised form 17 April 1986; accepted for publication 17 April 1986)

Summary

An ELISA was applied to measure IgG sub-class antibodies to cow's milk beta-lactoglobulin (BLG), alpha-lactalbumin (ALA) and alpha-casein (AC) and to hen's egg ovalbumin (OA) in the sera of nineteen adult patients with milk intolerance causing either asthma, eczema or both. Results were compared with those of forty blood donors and twenty adult patients with either asthma or eczema due to inhalant allergy. Apart from one blood donor, high titres of IgG sub-class antibodies to all three milk proteins were found only in the milk intolerance group. The most frequently detected antibody was AC-specific IgG4; being high (i.e. > 9·98 μg/ml) in eight milk intolerance cases: six with eczema, one with asthma and one with both. A variable proportion of these eight patients also had high levels of IgG1, IgG2 and IgG3 antibodies to AC and IgG1, IgG2, IgG3 and IgG4 antibodies to BLG and ALA. In contrast, IgG antibody to the egg protein, OA, was remarkably restricted to IgG4 and was present in high titres in 68·4% of milk intolerant patients, 60% of inhalant allergy patients and 30% of blood donors. However, the greater incidence of high titres of IgG4 antibody to OA, compared to AC, was due to the superior coating efficiency of OA resulting in a more sensitive assay. We conclude that some adult cases of milk intolerance, particularly those with eczema, can be diagnosed by detecting raised serum levels of IgG sub-class antibodies to milk proteins.

Introduction

Two types of food sensitivity are recognized: 'immediate onset' and 'delayed onset' [1, 2]. In the 'immediate onset' type, usually provoked by egg, fish and nuts, symptoms occur within 1 hr and include angioedema, urticaria, asthma and anaphylaxis. The immunological mechanism underlying this type of reaction is IgE-dependent and IgE antibody specific to the food concerned is demonstrable by skin-prick test (SPT) and radio-allergosorbent test (RAST). In the 'delayed onset' type, which is most commonly

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induced by cow's milk, symptoms occur several hours to several days after ingestion of food and include asthma and eczema. The immunological basis for this type of reaction is not clear as the SPT and RAST are usually negative. Patients in this group are, therefore, referred to as 'food intolerant' to distinguish them from patients with IgE-mediated food allergy [1].

In seeking to explain the negative IgE tests in cases of milk intolerance some authors considered the role of digestion in the formation of *de novo* allergens, but reports on the diagnostic value of measuring IgE to such newly formed antigens are conflicting [3, 4]. We have investigated the relevance of antibodies directed against antigens present in the cream fraction of milk, but have failed to demonstrate any pathological significance [5]. Thus, the laboratory diagnosis of 'milk intolerance' still presents a frustrating problem because management is not difficult, particularly when milk is the only offending food [6].

Although IgG antibodies to milk have been detected in a large proportion of patients with 'delayed onset' food sensitivity [7], their pathological relevance remained uncertain. The problem is at least partly due to failure to appreciate the great heterogeneity of IgG and in particular differences in the biological and antibody properties of the four IgG sub-classes [8, 9]. Whole IgG assays are, therefore, bound to overlook small changes restricted to one or two sub-classes; and the pathological interpretation of a positive result is impossible without prior knowledge of the IgG sub-class being detected. However, highly specific monoclonal anti-IgG sub-class reagents have recently become available [10] and it is now possible to measure IgG subclass antibodies accurately. It seemed, therefore, of particular importance to re-consider the role of IgG in milk intolerance, using individual sub-class assays. In this publication we apply an enzyme-linked immunosorbent assay (ELISA) to measure IgG1, IgG2, IgG3 and IgG4 antibodies to three milk proteins and one egg protein (i.e., irrelevant food) in sera of patients with milk intolerance. Results are compared with those of blood donors and patients with inhalant allergy.

Materials and methods

Patients with Milk Intolerance

Serum samples were obtained from nineteen adult patients (16–57 years of age) in whom, despite a negative SPT and RAST for milk, the ingestion of milk produces repeatable and convincing delayed reactions including asthma (nine cases), eczema (nine cases) or both (one case). In two patients symptoms of eczema were brought about by egg as well as milk. None of these patients had an obvious non-immunological cause for milk intolerance, such as lactase deficiency or psychoneurotic illness. Total serum IgE level was > 100 i.u./ml in eleven patients, in six of whom levels were above 1000 i.u./ml.

Controls

Serum samples were obtained from twenty adult patients (16–57 years of age) with either asthma (nine cases) or eczema (eleven cases) caused by allergy to inhalants such as mite, pollen and dog and cat dander. Total serum IgE level was > 100 i.u./ml in eighteen patients, in eleven of whom levels were above 1000 i.u./ml. Sera were also obtained from forty blood donors.

Antibody reagents
Ascitic fluids containing mouse monoclonal antibody to IgG1 (NL16), IgG2 (GOM1), IgG3 (ZG4) and IgG4 (RJ4) were purchased from Seward Laboratory Limited (Bedford, U.K.). The production procedure and the specificity of these reagents have been discussed elsewhere [10].

Immunoglobulin classes and sub-classes
IgG representative of each subclass was prepared on DEAE-cellulose (Whatman DE 52) as described elsewhere [11]. IgM and IgA proteins were purchased from Serotec Limited (Bicester, U.K.).

Performance of the monoclonal anti-sub-class reagents
The specificity of the monoclonal antibodies was ascertained by allowing them to react with ELISA plates coated at 0·5 mg% [12] with a panel of isolated immunoglobulins representing IgG1, IgG2, IgG3, IgG4, IgM and IgA. The monoclonal antibodies were applied at optimal and equipotent dilutions: 1/3200 for anti-IgG1, 1/400 for anti-IgG2, 1/8000 for anti-IgG3 and 1/800 for anti-IgG4. This test had also served to assess the capacity of the monoclonal reagents to function in the ELISA system.

Milk- and egg-specific IgG sub-class assay
IgG sub-class antibodies to beta-lactoglobulin (BLG), alpha-lactalbumin (ALA), alpha-casein (AC) and ovalbumin (OA) were measured using a previously described ELISA for measuring IgG4 to the same food proteins [12]. The monoclonal anti-sub-class reagents were applied at equipotent dilutions, as indicated previously. Results of duplicate assays were expressed as optical density readings at 492 nm (OD492) using a Titertek Multiskan (Flow Laboratories, Herts, U.K.). Antibody titres were classified as either undetectable or low (OD492 < 0·1) or high (OD492 between 0·1 and 0·5); i.e., OD492 of 0·1 being the threshold above which antibody levels were considered raised.

Quantitation of AC-specific IgG4
A serum sample with threshold amount of IgG4 antibody to AC was absorbed with AC (1 mg/ml of serum) overnight at 4°C utilizing a rotator. This procedure resulted in complete removal of the AC-specific IgG4 activity. Total IgG4 content of the pre- and post-absorbed aliquots of serum was determined by an ELISA inhibition procedure [13], and the difference (9·98 μg/ml) was taken as the concentration of AC-specific IgG4 (or the quantitative threshold).

Investigation of the coating efficiency of AC and OA
Two sera, one with high titre of IgG4 antibody to AC and one with high titre of IgG4 antibody to OA, were employed in a depletion study aimed at comparing the coating efficiencies of the two proteins. An aliquot of each serum (diluted 1/80) was subjected to four consecutive incubations (2 hr each) in wells coated with the relevant protein. Following each incubation, parallel samples were tested by the ELISA [12] to assess antibody depletion.

Results

Validation of the milk- and egg-specific IgG sub-class assays
Using an ELISA test the monoclonal anti-IgG sub-class reagents were shown to be specific for the respective IgG sub-class proteins (Fig. 1). The slight cross-reactivity

noted in anti-IgG1 and anti-IgG2 was probably insignificant as we were looking for gross differences in antibody titres between the study groups. This test also confirmed that the monoclonal antibodies were applied at equipotent dilutions, as they scored similar OD492 readings when added to microtitre wells coated with the same concentration of the respective sub-class protein. The test also served to show that all four monoclonal anti-IgG subclass antibodies can function (i.e., bind to the respective sub-class on the solid-phase) in an ELISA system. These tests, however, do not allow for differences in the coating efficiency of the various IgG sub-class proteins, nor do they allow for differences in the affinity of the monoclonal antibodies. The specificity of the ELISA for measuring IgG antibodies against individual milk and egg proteins has been established previously using IgG4 as a model antibody [12].

Milk-specific IgG sub-class antibodies

Apart from one blood donor who had slightly high levels of IgG2 and IgG3 to ALA, high titres of IgG sub-class antibodies to all three milk proteins were found only in the milk intolerance group. In this group the incidence of high titres of IgG antibodies to milk varied according to the sub-class and the protein being tested. The IgG sub-class distribution was in the order of IgG4 > IgG3 > IgG2 and IgG1; while the protein specificity was in the order of AC > BLG > ALA. Thus, the most frequently detected antibody was AC-specific IgG4; being present in high titres (i.e. > 9·98 μg/ml) in eight out of nineteen milk intolerant cases. A reasonable proportion of these eight patients also had IgG1, IgG2 and IgG3 to AC and IgG1, IgG2, IgG3 and IgG4 to BLG and ALA (Table 1). Interestingly, six of these patients had eczema while one had asthma and one had both eczema and asthma. Titres of IgG sub-class antibodies to milk proteins were either undetectable or low (i.e. < 9·98 μg/ml in the case of AC-specific IgG4) in the rest of the milk intolerant patients, eight with asthma and three with eczema.

Statistical analysis of data obtained from the two eczema groups, ie milk intolerance (ten cases) and inhalant allergy (eleven cases), revealed significant difference in antibody titres (OD492). Thus, for instance the mean titre of AC-specific IgG4 was 0·169 for the milk intolerance group, and only 0·019 for the inhalant allergy group; the difference being highly significant ($P < 0·005$).

Egg-specific IgG sub-class antibodies

IgG antibodies to OA were present in high titres in twelve blood donors, twelve inhalant allergy patients and thirteen milk intolerant patients. Remarkably, however, the antibody was almost exclusively restricted to the IgG4 subclass (Table 1).

The frequent occurrence of high titres of IgG4 antibody to OA in the control groups (as well as the milk intolerance group) is in marked contrast to the pattern seen with IgG4 antibody to AC (and BLG and ALA). To find out if such discrepancy is real we compared the coating efficiencies of AC and OA using an antibody depletion experiment. This has shown that an OA-coated solid-phase is much more efficient at depleting IgG4 antibody than an AC-coated solid-phase (Table 2). The high titres of OA-specific IgG4 must, therefore, be due to the enhanced sensitivity of the IgG4 anti-OA ELISA. The slightly high incidence of IgG4 antibody to AC, compared to BLG and ALA, may also have been caused by a similar effect.

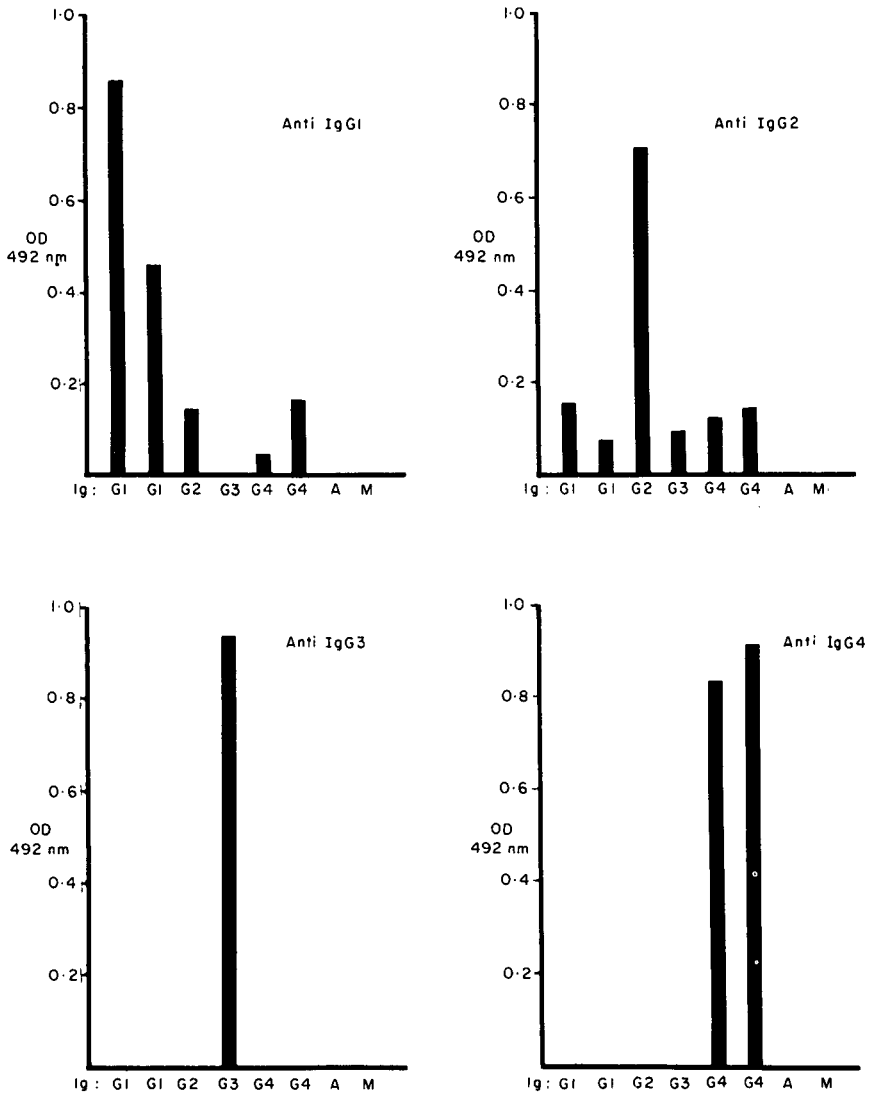

Fig. 1. Results of the ELISA test showing the reactivity of the monoclonal anti-IgG sub-class reagents with isolated IgG1, IgG2, IgG3, IgG4, IgA and IgM; two preparations of each of IgG1 and IgG4 were available for testing.

Discussion

In this study we examined the sera of adult patients with asthma and eczema due to milk intolerance for the presence of raised levels of IgG antibodies to milk. Children were not included in the study because of certain anomalies. Thus, during early infancy, the daily exposure to cow's milk is considerable and it is not surprising that the incidence and the concentration of IgG antibody to milk is comparable in healthy and milk intolerant children [14]. Levels of IgG antibody gradually decline during late childhood; and persistence of elevated levels in older children reflects a corresponding extension of the abnormal permeability of the gut in infancy [15]. It is probably the non-specific nature of such extra-permeability that may lead to the formation of

Table 1. Results of the ELISA for IgG sub-class antibodies to three milk proteins (AC, BLG and ALA) and one egg protein (OA) in nineteen milk intolerant patients

	Number of individuals with:							
	IgG1		IgG2		IgG3		IgG4	
Protein	U/L	H	U/L	H	U/L	H	U/L	H
AC*	14	5	15	4	14	5	11	8
BLG	15	4	15	4	14	5	14	5
ALA	16	3	16	3	16	3	16	3
OA	18	1	18	1	19	0	6	13

U/L = undetectable or low, H = high. *U/L levels of AC-specific IgG4 is $<9{\cdot}98$ μg/ml; while H Levels of AC-specific IgG4 is $>9{\cdot}98$ μg/ml.

antibodies to all sorts of foods, including those tolerated by the child. For instance, although IgG4 antibodies to milk- and egg-proteins are more frequently found in atopic children than in controls, the presence of these antibodies was found to be of no relevance to the child's food sensitivity [5, 12].

The application of an ELISA test for IgG antibodies to milk in adults has identified milk intolerance causing eczema in six patients, asthma in one and both asthma and eczema in one. The test has failed, however, to identify milk intolerance in a further three patients with eczema and eight with asthma due to milk. The presence of high levels. (i.e. $>9{\cdot}98$ μg/ml in the case of AC-specific IgG4) of IgG antibody to the three milk proteins was diagnostic of milk-intolerance because such levels were not observed in twenty patients with inhalant allergy (nine asthmatics and eleven eczematous) and in thirty-nine out of forty blood donors. Thus, it may be reasonable to assume that in those milk intolerant patients in whom raised levels of IgG antibody to milk was detectable, IgG antibodies may play an important pathological role. The pathological mechanism involved in IgG-mediated tissue injury would to a large extent be determined by the sub-class distribution. For instance, IgG1 and IgG3 are efficient complement fixing antibodies [8] and complexes containing milk antigens and IgG of these sub-classes may initiate tissue damage through complement activation. Thus,

Table 2. Results of IgG4 antibody depletion study showing the higher coating efficiency of OA compared to AC

		Titre (OD492) of:	
		IgG4 Anti-OA	IgG4 Anti-AC
Before depletion		0·326	0·373
After depletion step	−1	0·081 (75)	0·211 (43)
	−2	0·026 (92)	0·174 (53)
	−3	0·007 (98)	0·133 (64)
	−4	0·000 (100)	0·127 (66)

Figures in brackets represent % depletion of IgG4 antibody.

complement-fixing IgG immune complexes detected in the sera of eczema patients are thought to be responsible for the low grade persistent inflammatory mediator release that causes the characteristic chronic pruritis [16]. The IgG4 subclass, on the otherhand, can apparently bind to basophils, and presumably mast cells, and its participation in allergen-triggered release of mediators has been implied but not demonstrated [17]. It is interesting to note, in this connection, that a large proportion of asthma and eczema patients have a rheumatoid factor-like antiglobulin capable of releasing mediators from (presumably IgG4 bearing) leucocytes [18].

It is equally possible, however, that the raised levels of IgG antibody to milk found in milk intolerant patients represent a protective secondary phenomenon. For instance, IgG2 and IgG4 are poor complement fixing sub-classes and could, therefore, interfere with complement fixation by other antibodies.

Our data present a case for adopting individual sub-class ELISAs in the search for IgG antibodies to milk. Taking the AC-specific IgG sub-class assays as an example, the test for IgG4 (represents 3% of IgG) identified milk intolerance in eight patients; while the tests for IgG3, IgG2 and IgG1 (adding up to 97% of IgG) identified milk intolerance in 5, 4 and 5 patients respectively. Thus antibodies belonging to a minor sub-class (e.g. IgG4 and IgG3) may not be detected with tests designed to measure whole IgG.

Eczema was a characteristic feature of milk intolerant patients showing raised IgG antibodies to the three milk proteins tested. Perhaps milk intolerant patients who did not show raised IgG to AC, BLG and ALA, have antibodies to other more relevant proteins in milk. Thus, cow's milk is known to contain more than thirty antigenic components [19]. Antibodies to minor components of milk may not be detectable in tests employing whole milk.

In contrast, high levels of IgG antibodies to the egg protein, OA, were found in milk intolerant patients (68·4%), inhalant allergy patients (60%) and in blood donors (30%). However, such discrepancy is artefactual because of the enhanced sensitivity of the IgG4 anti-OA ELISA. In keeping with other studies [20], we have found that IgG antibodies to OA were remarkably restricted to IgG4. It is rather intriguing, however, that the antibody restriction to IgG4 occurs with one dietary protein (i.e. OA) and not with others (i.e. BLG, AC and ALA). Thus, the fact that some OA (but not BLG) is absorbed intact [21] might be relevant here in that there might be a fundamental difference in the immunogenic requirements (e.g. molecular size) between a selective IgG4 response and a diffuse sub-class response.

We conclude that the detection of raised levels of IgG sub-class antibodies to milk proteins might be useful in the diagnosis of milk intolerance in adults, particularly those with skin manifestation.

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