

Allergenicity of cow milk proteins

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The allergenicity and antigenicity of cow milk proteins are age dependent. Because the nonspecific and specific factors inhibiting the passage of cow milk proteins through the epithelial layer of the intestine are deficient at birth, although developing during early infancy, allergy to cow milk may be acquired during the first year of life. Allergic reactivity to cow milk is lost during childhood in the vast majority of cases. This change may depend at least partly on the development of the local immune system of the gut producing antigen-specific IgA antibodies. Circulating IgG antibodies to cow milk proteins are always produced when an infant has cow milk in the diet but are not associated with allergy; their titer is reduced with age. Clinical challenge tests show that most cow milk-allergic patients react to several protein fractions of cow milk. A patient may have IgE antibodies to several fractions of cow milk, measured either by skin testing or by radioallergosorbent test. Likewise, various tests for cell-mediated immunity may show positive reactions to several fractions. No single major allergen is apparent in cow milk, according to either the challenge tests or laboratory procedures: casein, α -lactalbumin, and β -lactoglobulin all show a high proportion of positive reactions. (J PEDIATR 1992;121:S12-S20)

Before the turn of this century, the feeding of artificial food to newborn infants was hazardous, and the mortality rate associated with it was high. When the importance of pasteurization and of the purity of water added to cow milk was realized, the safety of artificial feeding was greatly increased. However, despite all precautions, some infants did not thrive on cow milk-based food; they had severe gastrointestinal symptoms and even collapse. It was realized that the foreign proteins in cow milk were responsible for the symptoms.¹ Still, the high nutritional value of cow milk proteins and their availability have secured their position as the major source of protein for infants in Western developed countries. The allergenicity of cow milk proteins has remained a major concern in infant nutrition.

PROTEINS IN COW MILK

Unprocessed cow milk. The protein concentration of unprocessed cow milk is more than three times that of human milk, about 33 gm/L²; nearly 80% belongs to the group of proteins called caseins. The common property of caseins is

low solubility at pH 4.6; at that pH value the whey proteins are left in solution. Whey proteins are 19% of the quantity of protein in unprocessed cow milk (6.3 gm/L).

The most abundant whey protein is β -lactoglobulin, which exists naturally as a dimer of two monomeric subunits. At least six genetic variants of BLG are known.³ α -Lactalbumin has two genetic variants differing in only one amino acid; the amino acid sequence of ALA has identical sequences with lysozymes. Its biologic activity is to

ALA	α -Lactalbumin
BLG	β -Lactoglobulin
BSA	Bovine serum albumin
CMA	Cow milk allergy

promote the transfer of galactose to glucose to form lactose. Bovine serum albumin is not synthesized in the mammary gland but enters the milk through secretory cells and comprises about 1.2% of the total milk proteins.²

There are numerous other fractions in the whey, such as orosomucoid and folate-binding protein. The fat globule in cow milk contains about 1% of the total protein of cow milk; several fractions can be separated from solubilized membranes. In addition, cow milk contains small amounts of β_2 -microglobulin (2 mg/L). Two iron-binding proteins are

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present in cow milk: transferrin, which is similar to that in serum; and lactoferrin, produced in the mammary gland. The concentration of transferrin is about 100 mg/L in mature milk.

Bovine milk contains immunoglobulins; in mature milk their proportion of the total protein is 2.1%. The major immunoglobulin in cow milk, in contrast to human milk, is IgG1, which is also transferred to the colostrum in high quantities. IgA in cow milk, as in human milk, exists in the secretory form; two monomeric IgA molecules are joined by a J chain and the secretory component. IgM is a pentamer joined by J chains and the secretory component.

Cow milk proteins in infant formulas. Most commonly used formulas for term infants contain 15 gm/L of cow milk proteins, whereas the concentration is higher (18 to 20 gm/L) in formulas for preterm infants. The cow milk proteins are processed to reduce the amount of caseins.⁴ In the so-called adapted formulas, the proportion of caseins is 40% (6 gm/L) of the total protein, which is close to that in human milk. This concentration may be achieved by mixing equal amounts of whey and skim milk protein. Commercial formulas are made of heat-treated cow milk proteins.

ANTIGENICITY AND ALLERGENICITY OF COW MILK PROTEIN FRACTIONS AND TOLERANCE TO COW MILK

Antigenicity and allergenicity. Proteins of cow milk provoke an immune response, either humoral, cell mediated, or both, when they have passed the epithelium of the gastrointestinal tract. Antigenic structures, which are presented to B cells in association with major histocompatibility complex molecules of class II, consist of 11 to 13 amino acids. Recently the amino acid sequence for a peptide from BSA bound to HLA class II molecules was described.⁵ For protein antigens, interactions between T helper cells and B cells are needed to initiate the immune response. In an infant ingesting cow milk, the immune response to the proteins always takes place, as shown by the appearance of circulating antibodies of the IgG isotype and mucosal antibodies of the IgA isotype.⁶⁻⁸ The development of these responses may have a protective antiinflammatory role. If an infant has adverse clinical symptoms from ingesting cow milk proteins and at the same time has an abnormal immune response to cow milk proteins, the infant is allergic to cow milk.⁹

Whether a protective or an allergic type of immune response is provoked probably depends on the type of T helper cells activated by the antigen.¹⁰ Two types of T helper cells having different secretory profiles for lymphokine secretion were originally described in mice. T helper 2 (Th2) cells secrete interleukin-4 but no interferon gamma; this combination induces particularly IgE secretion by B cells. T helper

1 (Th1) cells secrete lymphokines inducing IgG and IgA secretion by B cells. In human beings, T-cell clones specific for house dust mites from atopic patients may be stimulated to a similar secretory pattern of lymphokines as Th2 cells from mice, and they induce IgE secretion of B cells in vitro.^{10, 11} In addition to IgE-mediated atopic reactions, allergic reactions may be provoked by cell-mediated immunity. However, stimulation of nickel-specific CD4⁺ cell clones from patients with contact dermatitis and from non-allergic persons results in clones with similar, Th1-like secretory patterns.¹² In mice, the type of major histocompatibility complex presenting the antigen determines which type of helper cells are activated.¹³

The lymphokines secreted by Th2 cells may also induce proliferation of precursors of various types of mast cells.¹⁴ IgE is fixed on these cells to a specific receptor; it is also fixed to another type of IgE receptor on eosinophils, macrophages, and platelets. When the exciting antigen (allergen) is bound to two molecules of fixed specific IgE, an immediate hypersensitivity reaction is initiated with liberation of several highly active mediators of inflammation.¹⁴ Much less is known about the pathogenesis of late allergic reactions.¹⁵ The intestine of an infant with a delayed reaction to cow milk protein has crypt hyperplasia and villous atrophy, increased density of the lymphocytes in the epithelium, and mononuclear cell infiltration of the lamina propria. The density of IgA- and IgM-containing cells in the intestine is greatly increased at the time of clinical relapse.¹⁶ Both immune complex-mediated (Arthus reaction) and delayed, cell-mediated reactions are compatible with these findings.

Oral tolerance. Many laboratory animals exhibit tolerance (another type of immune response) to protein antigen administered orally. Systemic unresponsiveness, both humoral and cell mediated, may be induced by protein antigens during feeding. Whether tolerance or priming develops depends on the animal, the age at feeding, and the amount of protein fed.¹⁷ A tolerant animal does not have a mucosal secretory immune response either. The development of oral tolerance has not been documented in human beings.

The immune reactivity to cow milk proteins is reduced with age. Circulating IgG cow milk antibodies start to decline after the first year of life, whereas IgA antibodies diminish only late during childhood.^{7, 18} The great majority of patients with cow milk allergy become clinically tolerant to cow milk during childhood. The delayed intestinal type of allergy is lost, usually before the age of 2 years¹⁹; IgE-mediated CMA, which causes mostly skin symptoms, disappears more slowly.²⁰ Circulating cow milk-specific IgE antibodies and positive skin prick reactions may be present in patients who clinically have become tolerant to cow milk.^{20, 21} The development of clinical tolerance may be

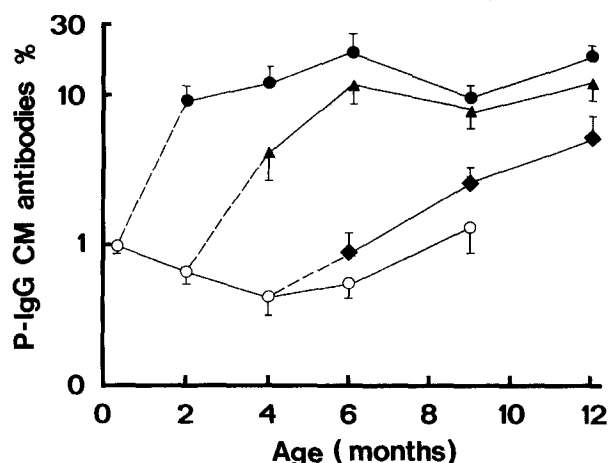


Fig. 1. Serum levels (mean $\pm$ SEM) of IgG antibodies to cow milk in infants exclusively fed breast milk (○), infants exposed to cow milk formula before the age of 30 days (●), infants first exposed to cow milk at an age of 31-90 days (▼), and infants first exposed to cow milk at an age of 151 to 210 days (◆). (From Tainio V-M, Savilahti E, Arjomaa P, Salmenperä L, Perheentupa J, Simmes MA. *Acta Paediatr Scand* 1988;77:807-11.)

linked to the maturation of the intestine and its ability to fully inhibit the passage of antigens in food.²²

ANTIGENICITY OF COW MILK PROTEIN FRACTIONS

Circulating antibodies of IgG and IgA isotypes. Maternal IgG cow milk antibodies are transported actively via the placenta during late pregnancy.²³ Passive BSA antibodies may inhibit the antibody response during the neonatal period.²⁴ We did not see any such effect in a large group of healthy term infants when IgG antibodies to whole cow milk and BLG were measured.⁷ IgG1 antibodies cross the placenta freely, whereas IgG4 antibodies to casein are much lower in infants than in mothers.²⁵ Feeding of cow milk or formulas based on cow milk elicits an IgG antibody response in all infants.⁶ The response is stronger when cow milk proteins are introduced early during the neonatal period (Fig. 1). The peak of the antibody response is reached 3 to 6 weeks after feeding with cow milk has started.^{7, 26, 27} Circulating antibodies of IgA isotype to cow milk develop more slowly and reach their peak only during late childhood. We observed the highest level of IgA antibodies to cow milk and BLG after the age of 7 years.

The antigenicity of various cow milk protein fractions has been measured by radioactive methods, which measure all precipitating antibodies together (Table I).^{18, 27, 28} Most of these antibodies are of the IgG isotype, but IgM and IgA antibodies contribute to the total amount of precipitating antibodies. The strongest antibody response, according to

studies using the precipitation method, is against BSA, followed by γ -globulin and casein.^{18, 27} With an antibody test in which protein fractions are attached to gel beads and the bound antibodies are detected with fluorescein-conjugated antibody active against all immunoglobulin isotypes,²⁸ positive titers are most frequently seen against casein (Table I). According to both methods, antibodies to ALA have the lowest titer. The contact with cow milk provokes antibody responses to several fractions. Although it has been claimed that this response is stronger among patients in whom CMA is verified with a positive reaction to a challenge test, compared with those not reacting,^{27, 29} recent studies have failed to show any difference in the level of IgG and IgA antibodies to cow milk among patients with positive or negative reactions to cow milk challenges.^{21, 30, 31} It is safe to conclude that because of great variability of the levels of these antibodies, they do not have diagnostic value for an individual patient.

CLINICAL SENSITIVITY TO COW MILK PROTEIN FRACTIONS

In the extensive study by Goldman et al.,³² 45 patients were first challenged with cow milk, and three positive reactions were required for the diagnosis of CMA. They were then challenged with the amount of cow milk protein present in 100 ml of native cow milk. Positive reactions to challenges with BLG were most common, but the differences in reactions to other fractions were not great (Table II).^{19, 33} Most patients reacted to more than one fraction, and symptoms varied. Some patients reacted rapidly with skin and gastrointestinal symptoms (40% in less than an hour), but 30% reacted only after 12 hours from the beginning of the challenge. No association was observed between the symptoms and the fraction of cow milk provoking the symptoms. Davidson et al.³⁴ demonstrated that BLG induced chronic diarrhea and malabsorption syndrome in a child. Eight patients who had similar symptoms and frequent damage in the jejunal mucosa were challenged with cow milk protein fractions. BLG caused symptoms most frequently, but the majority reacted to other fractions as well.^{19, 35} (Table II). In another study of similar patients,³³ most reacted only to BLG.

These studies show that several cow milk fractions provoke symptoms in patients with CMA. Patients allergic to only one fraction are not common. It is apparent that no single fraction in cow milk may be designated as the major allergen on clinical grounds.

Clinical sensitivity to other food proteins of patients with CMA. Because of the cross-reactivity of cow milk and goat milk proteins, most patients with CMA do not tolerate goat milk, which causes symptoms in 57% to 73% of patients allergic to cow milk.^{36, 37} Infants with CMA and intestinal

Table I. Circulating antibody response to cow milk protein fractions in healthy infants

Study	Age of group (mo)	Casein (%)	ALA (%)	BLG (%)	BSA (%)	γ -Globulin (%)
May et al., ¹⁸ 1977	5-6	26*	10	18	36	29
May et al., ²⁷ 1982	6½	32*	9	16	32	31
Burgin-Wolff et al., ²⁸ 1980	1½-4	75 (positive)	38 (positive)	65 (positive)	49 (positive)	NT

NT, Not tested.

*Percentage of radiolabeled milk proteins bound by serum dilution.

Table II. Positive clinical reactions to challenge tests with purified protein fractions of cow milk in cow milk-allergic patients

Study	Clinical reaction to protein fraction								γ -Globulin	Positive to 1 fraction
	Casein		ALA		BLG		BSA			
	No.	%	No.	%	No.	%	No.	%		
Goldman et al., ^{32, 38} 1963	27/45	60	18/34	53	23/37	62	23/44	52	NT	19/45
Kuitunen et al., ¹⁹ 1975	5/6		1/4		6/7		1/7		1/5	2/8
Freier et al., ³³ 1969	0/6		1/6		5/6		2/6		1/6	4/6

NT, Not tested.

damage frequently acquire clinical hypersensitivity to other proteins used in an elimination diet; reactions to soy, wheat, egg, lamb, and chicken have been described.¹⁶

IgE ANTIBODIES TO COW MILK PROTEIN FRACTIONS

Skin test reactivity. Goldman et al.³⁸ performed intradermal skin tests. Among patients with CMA, 59% had a positive reaction to a skin test with one or more fractions of cow milk (Table III).³⁹⁻⁴² This frequency, however, was smaller than that observed in allergic children not clinically sensitive to cow milk. The skin reaction to a fraction did not correlate with the clinical reaction to the same fraction in the whole study group. When only strong skin reactions were compared with the clinical reaction, a clear association was seen.³⁸

A well-standardized skin prick test with cow milk extract is a sensitive and fairly specific predictor of IgE-mediated CMA.^{43, 44} Most patients with CMA react to several cow milk fractions when tested with skin prick tests^{40, 42} (Table III). In recent studies with standardized skin prick techniques, ALA most frequently caused skin reactivity, but the difference in reactions to other fractions was small.^{40, 42}

IgE antibodies to cow milk protein fractions. The increase of specific IgE for cow milk protein fractions, measured by the radioallergosorbent test, and the skin reactivity to the same fraction are closely associated, but the radioallergosorbent test is less sensitive, particularly to casein and BSA.⁴⁰ This agrees with the finding that skin testing with

cow milk extract is more sensitive than measuring specific IgE to all cow milk proteins.⁴³ When the serum from 17 cow milk-allergic children was tested for IgE antibodies against various forms of casein, the alpha form was most frequently positive, but several patients had antibodies to many forms of caseins.⁴¹ The basophil degranulation test revealed antibodies most frequently against BLG.³⁹

In a system in which cow milk proteins were first precipitated with rabbit antiserum, and binding of patient serum IgE to the precipitates was tested subsequently, all 21 patients had IgE antibodies to casein, ALA, BLG, and BSA. The same rabbit antiserum precipitated proteins in goat milk and patients' IgE bound to these precipitates.⁴⁵

COW MILK PROTEIN FRACTIONS IN TESTS FOR CELL-MEDIATED IMMUNITY

Stimulation of lymphocytes in vitro. The occurrence of positive lymphocyte stimulation reactions to cow milk proteins has varied greatly among patients with CMA; frequencies from 38% to 79% have been reported.^{31, 46, 47} In a series of 34 patients, casein was the most frequent stimulator of lymphocytes (Table IV).⁴⁷ Stimulation of lymphocytes was often seen, however, in patients with chronic gastrointestinal complaints who did not react to cow milk clinically.⁴⁷ All studies agree that the increased stimulation index of lymphocytes is not associated with the type of clinical symptoms; positive reactions are seen both in patients reacting immediately and in those with delayed reactions. However, a positive stimulation test result is often seen in

Table III. Positive skin test reactions and IgE antibodies to purified protein fractions of cow milk in cow milk-allergic patients

Study	Method	Casein		ALA		BLG		BSA	
		No.	%	No.	%	No.	%	No.	%
Goldman et al., ^{32,38} 1963	Intradermal skin tests	31/86	36	23/86	27	20/86	23	21/86	24
Moneret-Vautrin et al., ³⁹ 1982	Basophil degranulation test		23		31		46		0
Vanto et al., ⁴⁰ 1987	Skin prick test	6/11	55	9/10	90	7/11	64	8/11	73
Vanto et al., ⁴⁰ 1987	RAST	4/12	33	10/12	83	7/12	58	3/12	25
Otani et al., ⁴¹ 1989	RAST			1/17	6	5/17	29	0/17	
	α	13/17	76						
	β	6/17	35						
	κ	7/17	41						
Kaiser et al., ⁴² 1990	Skin prick test	9/13	69	11/13	85	10/13	77	NT	

RAST, Radioallergosorbent test; NT, not tested.

Table IV. Positive results on tests for cell-mediated immunity in cow milk-allergic patients using purified protein fractions of cow milk

Study	Method	Casein		ALA		BLG		BSA	
		No.	%	No.	%	No.	%	No.	%
Scheinmann et al., ⁴⁶ 1976	Lymphocyte transformation test	NT		9/45	20	10/45	22	NT	
Baudon et al., ⁴⁷ 1987	Lymphocyte stimulation test	26/32	81	7/33	21	20/34	59	NT	
Tainio and Savilahti, ³¹ 1990	Lymphocyte stimulation test	NT		NT		6/15	40	NT	
Ashkenazi et al., ⁴⁹ 1980	Leukocyte migration inhibition	NT		NT		24/24	100	NT	
Vanto et al., ⁴⁰ 1987	Leukocyte migration inhibition	9/12	75	12/12	100	9/12	75	6/12	50

NT, Not tested.

patients with CMA who have no specific IgE cow milk antibodies; the test may therefore be helpful in the diagnostic evaluation.^{31, 48}

Secretion of leukocyte migration inhibition factor. When lymphocytes from patients with CMA were incubated with BLG, they produced a lymphokine that inhibited the migration of leukocytes.⁴⁹ The test was specific and results became negative or weaker when clinical sensitivity was lost. When similar patients with predominantly chronic gastrointestinal symptoms were studied by Khoshoo et al.,⁵⁰

a positive reaction was also found among 4 of 78 patients not reacting to a cow milk challenge test; BLG induced the secretion of leukocyte migration inhibiting factor in all specimens from 12 patients reacting to the cow milk challenge test. In a patient group with predominantly skin symptoms in the cow milk challenge tests, the production of a leukocyte migration inhibiting factor was also frequently found when lymphocytes from patients with CMA were stimulated with various cow milk protein fractions (Table IV); however, the test result was often positive in patients

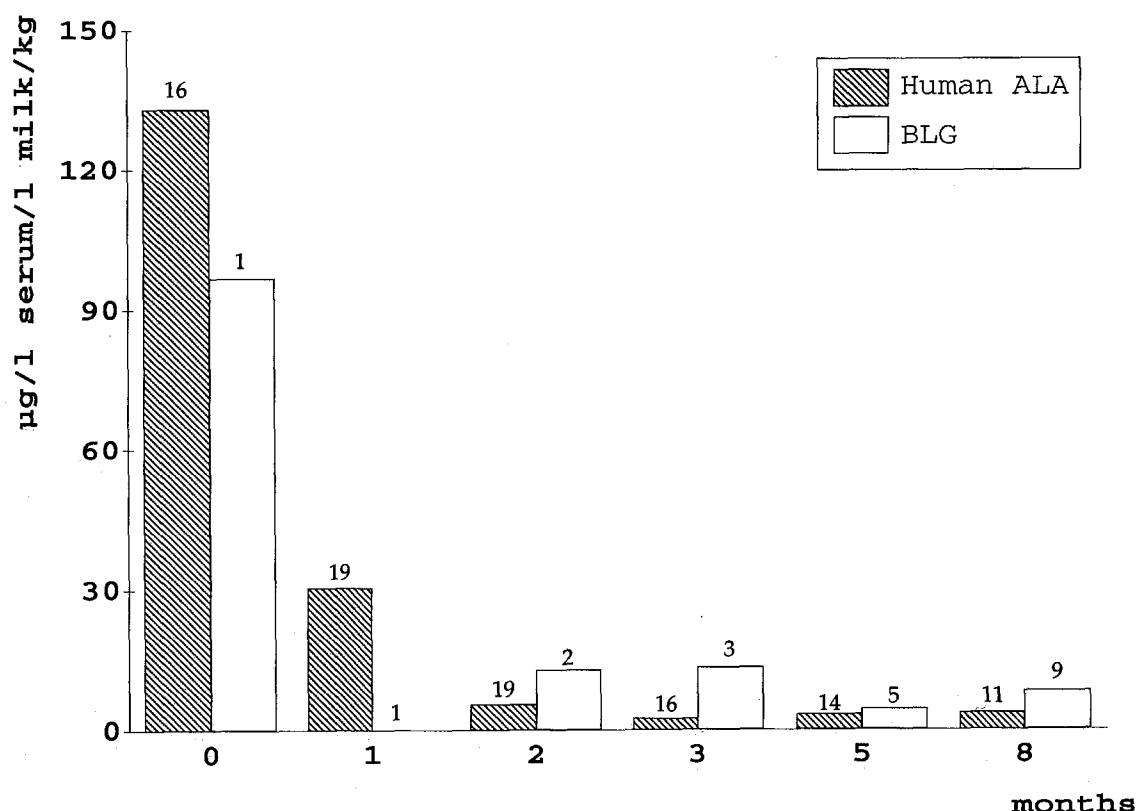


Fig. 2. Mean concentrations of human ALA and BLG in serum of 20 healthy infants. Number of infants studied indicated by numbers at top of columns.

not reacting to cow milk.⁴⁰ It was positive with ALA not only in all 12 patients reacting to a cow milk challenge but also in 15 of 25 patients who had no reaction.

FACTORS DETERMINING THE ALLERGENICITY AND ANTIGENICITY OF COW MILK PROTEIN FRACTIONS

Permeability of the intestine. Allergy to cow milk proteins develops only during the first year of life; in fact, in most cases CMA appears during the first 3 months of life.¹⁶ In infants who were breast fed for a long time, allergic symptoms appeared late. We have seen initial symptoms as late as the age of 10 months.⁵¹ In the late-appearing cases, sensitization may have taken place much earlier, often in the hospital shortly after birth.⁵² The possibility of the development of CMA, like other food allergies, is associated with the permeability of the intestine to proteins during infancy. In the human infant the intestine is "closed" at term; the amount of maternal IgA antibodies absorbed from the colostrum or milk is less than 0.1%, even in a premature infant,⁵³ whereas many animals, such as rodents and ruminants, absorb most of their circulating antibodies from the

colostrum.⁵⁴ Small quantities of food protein, enough to provoke allergy, are still absorbed through the intestine during infancy.

In addition to immunoglobulins, the most widely used markers for permeability are human milk ALA, cow milk BLG and casein, BSA, and ovalbumin. Human proteins do not elicit any mucosal immune response; changes in their permeability reflect the development of nonimmunologic factors. The permeability to human ALA is higher in premature than in term infants, and the absorption decreases with increasing maturity and with increasing postnatal age, both in preterm and term infants.^{55, 56} Premature infants, with gestational age between 26 and 32 weeks, had 10-fold human ALA levels in their sera compared with those in term infants.⁵⁶ Concentrations of BLG were higher in the sera of premature infants than in those of term infants.⁵⁷

We investigated the changes in permeability with age to both human ALA and BLG by following 20 healthy infants from birth to 8 months of age or until weaning, before which they were on a strictly cow milk-free diet. By studying both human and bovine proteins, we wanted to distinguish the effects of nonspecific factors⁵⁴ on human ALA from the

combined effects of both specific and nonspecific factors affecting the permeability of BLG. A sensitive solid-phase, double-sandwich immunofluorometric assay was used to measure the proteins. The absorption of human ALA and BLG varied considerably among individuals. High levels of human ALA and BLG were seen if the child was weaned during the first weeks of life (Fig. 2). At 1 and 2 months of age, smaller but still detectable amounts appeared in the sera. After the age of 3 months, most infants had no detectable human ALA or BLG in their blood (Fig. 2). One week after introduction of cow milk formula, BLG was found in one third of the infants, and 2 weeks after weaning BLG was absorbed in a few infants. Circulating antibodies of the IgA and IgG isotypes appeared even during exclusive breastfeeding, which agrees with our earlier findings.⁷ The rise was accelerated after introduction of cow milk formula in the infants' diet.

The degree of permeability of the intestine is not, however, directly related to the risk of developing CMA or food allergy in general. When we followed a group of premature infants to the age of 12 years, they had similar cumulative risks for all types of allergy; in fact, those fed cow milk formula early had symptoms of food allergy less frequently.⁵⁸ The permeability of the intestine to horseradish peroxidase in children with CMA during an elimination diet was similar to that in control subjects, but increased during positive reactions to challenge.⁵⁹

Development of the local mucosal immune system of the gut. The major factor inhibiting the passage of undigested protein molecules through the epithelium of the intestine is the specific mucosal antibody response. Even though the human fetus is capable of producing IgA and IgM early in gestation,⁶⁰ the lamina propria of the intestine of a newborn child is devoid of immunoglobulin-containing cells.⁶¹ IgM-containing cells appear within 2 weeks after birth, and the number of IgA-containing cells reaches that of IgM cells at the age of 6 weeks.⁶¹ Quantitatively, the IgA-producing system is growing even after the age of 2 years.⁶² The specific IgA response to bacterial antigens in saliva is already significant in infants 2 to 3 weeks of age,⁶³ although the adult level is reached at the age of 12 months and IgM antibodies predominate during the first few months of life.⁶⁴ The specific mucosal antibodies inhibit the absorption of antigens.⁶⁵

During infancy, the gastric acidity, the proteolytic activity, and the secretion of mucus in the jejunum mature gradually.⁵⁴ In concert with the developed local immune system, they may render the intestine so impermeable to cow milk protein that clinical reaction to cow milk no longer takes place, even though the effectors of the allergic reaction, such as IgE antibodies, are still present.^{20, 21} The de-

velopment of local specific IgA response to cow milk proteins may determine when the clinical hypersensitivity to cow milk is lost.²² No evidence for the development of factors blocking the allergic reaction to cow milk has been found.

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