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Comparison of the IgE Titers to Bovine Colostral G Immunoglobulins and their F(ab')₂ Fragments in Sera of Patients Allergic to Milk

Abstract

Colostral G immunoglobulins (IgGs) are described in many recent studies as having a beneficial effect for the treatment of viral, bacterial and parasitic diarrhea in animals and humans. The specific IgE titers to bovine colostrum IgG, to bovine serum IgG, and to F(ab')₂ fragments of IgG were immunoenzymatically quantified in sera of patients allergic to milk, to statistically evaluate and compare their relative immunoreactivity towards these purified antigens. The results clearly indicated that 36% of the population tested was potentially allergic to colostrum IgG, and serum IgG globally elicited significantly lower IgE titers. The F(ab')₂ fragments lead to a significantly decreased immunoreactivity as compared to colostrum IgG. This study shows the interesting use of peptic hydrolysis of IgG in producing fragments with preserved therapeutic immunoactivity and reduced potential allergenicity.

Key Words

Bovine colostrum
IgG
F(ab')₂
Allergenicity
Diarrhea

Introduction

Bovine colostrum imparts passive immunity to newborn calves, through absorption of intact immunoglobulins during the first 24 h of life [1]. Pooled or hyperimmune bovine colostrum, colostrum whey or milk protein concentrates have already been successfully used to orally treat or prevent diarrhea in calves or humans. These products can act on infections of viral [2, 3], bacterial [4, 5] or parasitic [6, 7] origin. In particular, the protozoan parasite *Cryptosporidium parvum* produces life-threatening diarrhea in immunocompromized persons, (e.g.) patients with acquired immunodeficiency syndrome. Pooled [8] or hyperimmune bovine colostrum [6] has often been the only effective treatment

among numerous chemotherapeutic, biological or other agents tested.

Colostrum antibodies, mainly IgG, present at a concentration 60–70 times higher than in milk, probably play the main role in the beneficial effect of these treatments, which IgGs are administered orally in significant quantities. However, colostrum is a lacteal product, and milk is one of the most common allergenic foods among young children [9]. Moreover, acute gastroenteritis has been reported to increase intestinal permeability, possibly caused by partial destruction of the intestinal mucosa [10]; this leads to an increased endocytosis of food proteins during [11] or after [12] infection. Many of the numerous proteins of milk are allergens, the most important ones being casein, α -lactalbumin,

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min, and especially β -lactoglobulin; the concentration of the latter is five times higher in colostrum than in milk. Until now these proteins are the only ones that have been tested; however, other milk proteins could sensitize babies and react with IgE antibodies of the sera of allergic children [13–15]. Bovine serum albumin and immunoglobulins are thus considered to be minor allergens. Compiled data [16] gave the following results: sensitivity to β -lactoglobulin occurred in 82%, to α -lactalbumin in 41% and to bovine serum albumin in 18% of patients allergic to milk, tested with the purified allergens. Sensitivity to bovine serum globulin was observed in 27% of these patients, but no indication was given for the potential allergenicity of bovine colostrum or milk immunoglobulins.

The essential aim of this study was to investigate whether bovine colostrum F(ab')₂ fragments instead of whole IgG would be of interest when used orally for the treatment of diarrhea, in order to reduce the incidence of allergic reactions and eliminate part of the harmful effects, which may be caused by the oral administration of foreign proteins. Indeed, the Fc fragment of IgG is often considered as the main immunogenic site of the molecule [17], carrying the most important part of the structural and antigenic differences between species [18], while the F(ab')₂ part carries the antibody activity. To have a concrete representation of the potential allergic risks, the potential immunoreactivity of a population of patients allergic to milk was examined and quantified towards bovine colostrum G immunoglobulins. The first goal was to examine whether this possible immunoreactivity was specific to bovine colostrum IgG or similar to that observed for serum IgG. Thus the IgE titers to bovine colostrum and serum IgG in sera of patients allergic to milk were determined and compared. Secondly, as the reduction of milk protein allergenicity through processing has been demonstrated [19], enzymatic hydrolysis was examined. As peptic hydrolysis leaves the F(ab')₂ portion of the molecule intact and breaks down the Fc fragment, the IgE titers to colostrum IgG were compared to those found to serum IgG and colostrum F(ab')₂ fragments of IgG. A sensitive enzyme immunoassay (EIA) was used, according to Wal et al. [15].

Materials and Methods

Purification of Colostrum and Serum IgG

Colostrum whey was prepared by acid precipitation; colostrum was diluted sevenfold with water, and pH was adjusted to 3.8 with 1 M HCl during 10 min, then it was adjusted to 4.6 with 5 M NaOH during 2 h. The supernatant was concentrated by cross-flow ultrafiltration on spiral-wound membrane module with a nominal cutoff of 5,000 D (S4 MFK-613 VSV, Koch). Colostrum IgGs were first purified by affinity

chromatography on Avid Gel ALTM (Bioprobe International; Interchim; 10×2.5 cm). The elution was performed with sodium acetate buffer, 0.1 M, pH 3. The fractions, containing mostly IgG, were immediately adjusted to pH 6 with concentrated NaOH solution; they were diluted in Tris-HCl buffer and then concentrated on a Sartorius system with a cutoff of 10,000 D. The fractions obtained were then purified by fast-protein liquid chromatography (Pharmacia System) on an anion exchange column (Mono Q H/R 5/5, Pharmacia), following slightly modified conditions of Chen et al. [20]. Elution was performed using a gradient of NaCl in Tris-HCl buffer, 20 mM, pH 8, at a flow rate of 1 ml/min. The IgG elution was achieved between 120 and 150 mM NaCl, and fractions were collected, dialyzed and concentrated on a collodion filtration system, with a molecular cutoff of 10,000 D.

Serum IgGs were prepared in the same manner.

Purification of Colostrum F(ab')₂ Fragments

IgGs previously obtained on Avid gel were enzymatically hydrolyzed: pepsin digestion was performed according to slightly modified conditions of Fang and Mukkur [21]: pH 4.5, 37°C, 3.5 h, in 0.2 M sodium acetate, with a protein/enzyme ratio of 45:1. The reaction was stopped by adjusting pH to 8.0 with concentrated Tris solution. The product was concentrated on a collodion filtration system, with a cutoff of 10,000 D. F(ab')₂ fragments were then purified following slightly modified conditions of Akita and Nakai [22]. The pepsin-digested mixture containing F(ab')₂ fragments was first subjected to gel filtration on the Sephadex G 75 (Pharmacia; 43.5×2.3 cm), using a 20 mM Tris-HCl elution buffer pH 8, 150 mM NaCl at a flow rate of 0.5 ml/min. It was followed by fast-protein liquid chromatography in the same conditions as for IgG purification. F(ab')₂ fragments eluted at 50 mM NaCl. Each fraction was collected, dialyzed and concentrated on a collodion filtration system, with a molecular cutoff of 10,000 D.

Characterization of the Protein Composition and the Purity of the Samples

A spectrophotometric method, the Bicinchoninic Acid Protein Assay (BCA Kit, Pierce), was used to determine protein concentration. Bovine serum albumin was used as the standard.

Quantitative analysis was confirmed by radial immunodiffusion according to the method of Mancini et al. [23]. The antiserum used was antbovine IgG serum (Pharmacia), and pure antibodies (pure bovine IgGs (Sigma) or standard bovine F(ab')₂ fragments (Interchim) were used for standard curves. The concentrations obtained were compared to those found with the BCA assay, to ascertain their reciprocal identity.

The composition and purity of the samples was visualized by SDS-PAGE [24], in a 5–20% polyacrylamide linear gradient under non-reducing conditions. Reduced molecular-mass standards (Sigma) were used as markers. Electrophoresis was performed at a constant 35 mA until the dye front reached the bottom of the plate. The gel slabs were stained with Coomassie brilliant blue R 250.

The purity of isolated immunoglobulins and F(ab')₂ fragments was confirmed by immunoelectrophoresis as described by Scheidegger [25]. It was performed at room temperature with a constant voltage of 30 V, using pure bovine IgG (Sigma) or standard bovine F(ab')₂ fragments (Interchim) as check samples, versus anti-IgG serum, antbovine whey serum or antbovine whole serum (Sigma). The arcs of precipitate obtained with purified fractions were compared to those identified by Fang and Mukkur [21].

Population Tested

Ninety-two human sera samples from patients with milk allergies, presenting clinical symptoms and a positive 'Rast' test (Pharmacia) to whole milk, were tested for their IgE titers towards bovine IgG of colostrum origin. Sera presenting IgE titers to IgG were then tested towards bovine serum IgG and colostrum F(ab)₂ fragments.

Enzyme Immunoassay

To determine IgE specific to bovine colostrum IgG or F(ab)₂ fragments, a direct solid-phase EIA was performed, according to the method of Wal et al. [15], by using Titertek microtitration equipment from Flow Laboratories. Ninety-six-well microtiter plates (Immunonunc I 96F) were filled with 200 µl of a 5-µg/ml solution of each of the purified allergens in coating buffer, left overnight at room temperature, then washed extensively with washing buffer. Plates were saturated by adding 300 µl of EIA buffer in each well, sealed, and stored at 4°C. At least 8 h later, and just before use, plates were washed. For revealing human specific IgE bound to these coated proteins, 50 µl of individual allergic serum at convenient dilutions were distributed in each well and left 24 h at room temperature. Plates were then extensively washed, and 50 µl of a solution of antihuman IgE monoclonal antibodies labelled with acetylcholinesterase [26] (3 Ellman-units/ml) were added and left 18 h at room temperature. After washing, 200 µl of Ellman's reagent [27] was added and absorbance measured at 414 nm after 1–3 h of enzymatic reaction. Negative controls were performed by using nonallergic sera on allergen-coated plates and some allergic sera on plates only saturated with human serum albumin. Results were expressed in terms of absorbance units.

Statistical Analysis

The statistical correlation between results of the dosages was examined using Pearson's *r* correlation coefficient. In order to examine differences of allergenicity between the molecules, measured by the IgE titers detected in patients to serum IgG, colostrum IgG and F(ab)₂ fragments of IgG, two tests were used: Student's *t* test for paired data, and the equivalent nonparametric test, the Wilcoxon rank sum test for paired data [28]. The significance level *p* < 0.01 was used (critical value *t* = 2.779 for Student's *t* test).

Results

Purity of the Samples

SDS-PAGE of the samples obtained after purification exhibited a single band each time, with a molecular mass corresponding to either IgG or the F(ab)₂ fragments (results not shown). Moreover, the BCA and Mancini assays gave identical concentrations for each sample tested. Finally, immunoelectrophoretic analysis of the IgG and F(ab)₂ fragments attested the purity of the fractions: a single arc, characteristic of the molecule tested, developed each time with specific antiserum (fig. 1).

Human Sera IgE Titers to Bovine Colostrum IgG

The quantitative results of the EIA are presented in table 1. Serum IgE antibodies against bovine colostrum IgG were

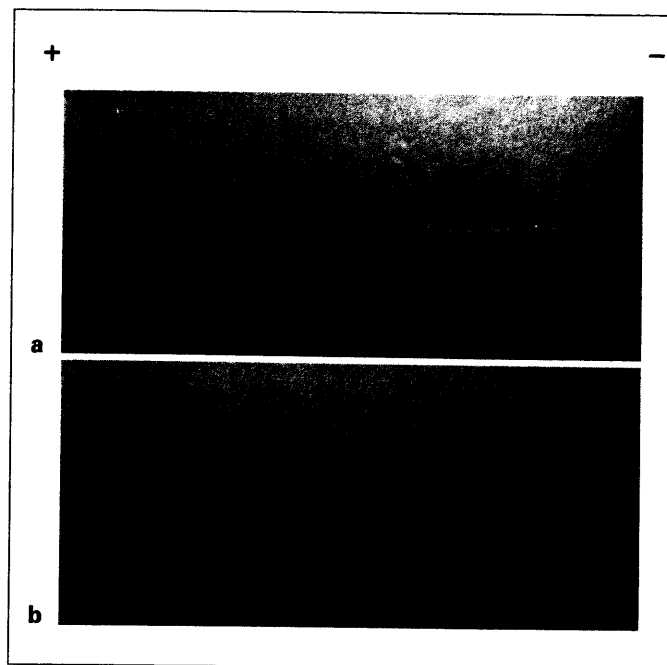


Fig. 1. Immunoelectrophoretic patterns of purified bovine serum and colostrum IgG, and colostrum F(ab)₂ fragments. **a** Colostrum IgG (top) and total bovine whey serum (bottom). **b** Serum IgG (top) and F(ab)₂ fragments (bottom). The central troughs contained antbovine whole serum.

found in 33 of the 92 sera tested, which represents 36% of the population tested. Six sera whose available volumes were not sufficient to be tested towards each of the other molecules were excluded from the paired statistical test, in order not to distort the final result. Only 27 sera were thus tested towards serum IgG and colostrum F(ab)₂ fragments.

Comparison of Observed Human Sera IgE Titers to Bovine Colostrum and to Serum IgG

A strong correlation was observed between IgE titers to bovine colostrum and to serum IgG (*r* = 0.96; table 1).

Figure 2 shows IgE responses to bovine IgG of colostrum and of serum origin, classified from the lowest to the highest titers to bovine colostrum IgG. Significantly increased IgE titers to bovine colostrum IgG were found, compared to titers to serum IgG (Student's *t* test: *t* = 2.93; Wilcoxon test: *p* = 0.006).

Comparison of Observed Human Sera IgE Titers to Bovine Colostrum IgG and to F(ab)₂ Fragments

A strong correlation was observed between IgE titers to bovine colostrum IgG and to F(ab)₂ fragments (*r* = 0.96), in particular for the highest values observed (table 1).

Fig. 2. IgE titers (UI/ml) to bovine colostrum IgG (black column) and bovine serum IgG (white column) determined in human sera of patients allergic to milk.

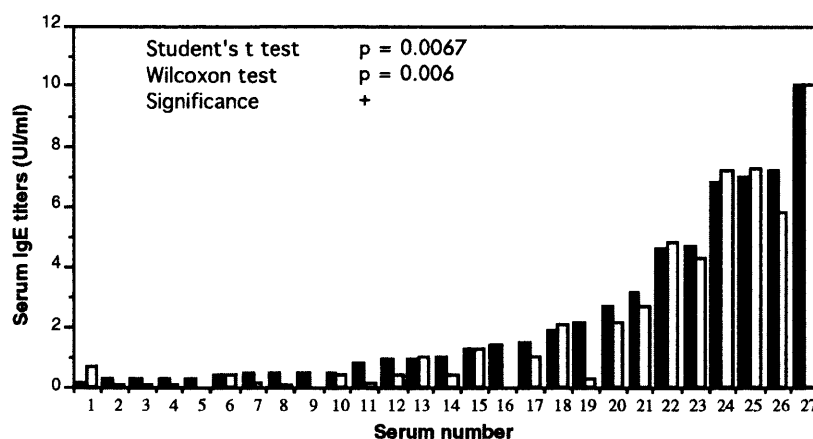


Fig. 3. IgE titers (UI/ml) to bovine colostrum IgG (black column) and to F(ab')₂ fragments (white column) determined in human sera of patients allergic to milk.

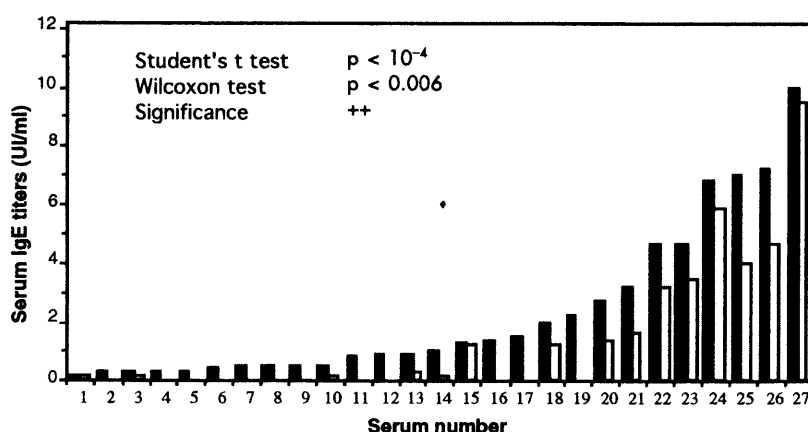


Figure 3 shows IgE responses to IgG and F(ab')₂ fragments from bovine colostrum origins, classified from the lowest to the highest titers to bovine colostrum IgG. Very significantly higher levels of IgE antibodies to colostrum IgG were found compared to those observed to F(ab')₂ fragments (Student's t test: $t = 6.26$; Wilcoxon test: $p < 0.006$).

Discussion

Diarrhea of various microbial origins are common diseases. They can be life-threatening for very young or for immunodeficient patients. Colostrum IgGs have often been useful in treating such affections; but our aim was to investigate whether these bioactive molecules administered orally in large quantities could produce allergic reactions in humans with increased intestinal mucosal permeability.

The purity of the antigens was a necessary condition for testing them. We needed to determine whether these molecules could be potential allergens, and to be sure that no impurity present in the sample could interfere with the results of the analysis. The sought purity was only obtained after successive chromatographic steps. Various methods of IgG purification have been previously published, but mainly concerning serum and not colostrum IgG. However, strong interactions may exist between the different colostrum proteins; this made them particularly difficult to purify. Moreover, F(ab')₂ fragments were especially difficult to separate from residual IgGs or from their hydrolytic fragments.

In this trial, results indicated that 36% of the population allergic to milk exhibited an immunoreactivity towards bovine colostrum IgG; they also showed that these sera presented, by comparison, a decreased immunoreactivity towards F(ab')₂ fragments.

Table 1. Direct EIA; IgE titers (UI/ml) to bovine colostral IgG, serum IgG and colostral F(ab')₂ fragments of IgG determined in human sera of patients allergic to milk

Serum No.	IgE, UI/ml		
	AC IgG	AS IgG	AC F(ab') ₂
1	0.2	0.7	0.1
2	0.3	0.1	0.0
3	0.3	0.1	0.2
4	0.3	0.1	0.0
5	0.3	0.0	0.0
6	0.4	0.4	0.0
7	0.5	0.2	0.0
8	0.5	0.1	0.0
9	0.5	0.0	0.0
10	0.5	0.4	0.2
11	0.8	0.2	0.0
12	0.9	0.4	0.0
13	0.9	0.1	0.3
14	1.0	0.4	0.1
15	1.3	1.3	1.2
16	1.4	0.0	0.0
17	1.5	1.0	0.0
18	1.9	2.1	1.2
19	2.2	0.3	0.0
20	2.7	2.2	1.4
21	3.1	2.7	1.7
22	4.6	4.8	3.2
23	4.7	4.3	3.4
24	6.8	7.2	5.9
25	7.0	7.3	4.0
26	7.2	5.8	4.7
27	10.0	10.0	9.5
Mean ± SD	2.289 ± 2.667	1.967 ± 2.756	1.374 ± 2.336

AC = Anticolostral; AS = antiserum.

The IgE titers to bovine colostral IgG were found significantly higher than those to bovine serum IgG. Epitopic and structural differences between serum and colostral bovine IgG were described, mainly located on the Fc fragment [29]. Colostral IgG1, which are more numerous in colostrum, have a higher molecular weight than serum IgG1, probably due in part to the difference in total amino acid composition on the heavy chain, and to the presence of a larger quantity of carbohydrates [30]. In fact, they contain more isoleucine and proline than serum IgG1. On the other hand, colostral IgG2 contain more proline, glutamic acid, lysine, isoleucine and methionine than serum IgG2. Colostral immunoglobulins also have a notably higher content of sialic acid in comparison with serum immunoglobulins

[31]. Our percentage of patients reactive to colostral IgG (36%) is higher than that of Lebenthal [16] for subjects reactive to bovine serum IgG (27%), and very near to that found for patients reactive to one of the major milk allergens: α -lactalbumin (41%). Immunoglobulins should thus be considered as important potential allergens, likely to elicit a pathological immune response when orally administered to predisposed patients.

The IgE titers found towards bovine colostral F(ab')₂ fragments were significantly lower than those found towards intact molecules. This tendency supports the decreased immunoreactivity induced by F(ab')₂ fragments of colostral IgG.

The patients with the lowest titers to bovine IgG often presented no reactivity to F(ab')₂ fragments; thus one might attribute the global potential allergenicity of the IgG molecule to the Fc fragment, which bears the allotypic markers. But patients with the highest IgE titers to colostral IgG showed serum IgE titers to F(ab')₂ fragments as well, even if they were always lower than the former. Moreover the results of the two assays for this subgroup of the population tested were strongly correlated, leading us to attribute the decrease of immunoreactivity to the size reduction of the IgG molecule. Further experiments are necessary to correctly understand these results and to determine the exact origin of the lower immunoreactivity observed with F(ab')₂ fragments in our study.

The loss of the biological activities associated with the Fc part of IgG is of no consequence, since the molecules are orally and not parenterally administered. The Fc fragment does not seem to be involved in the mechanisms implied in the prevention or treatment of diarrhea by colostral immunoglobulins. In fact, these mechanisms are related to the specific fixation of IgG on bacterial or viral antigens in the intestinal lumen [32], inhibiting the penetration of the infectious agents in the digestive mucosa and leading to their elimination with the intestinal peristaltism. They also sometimes involve in competitive occupation of receptors on the intestinal mucosa, inhibiting the autoinfectious cycles of parasites [6].

The final colostral product contained a polyclonal population of F(ab')₂ fragments. The present study has been conducted on pooled colostrum, in which a broad spectrum of antibodies was guaranteed, but the titer of antibody specific to each antigen was low. Thus, the *in vivo* efficiency of our product against diarrhea has not been tested. Nevertheless many studies have been carried out on the antigen-binding capacity of F(ab')₂ fragment obtained from monoclonal antibodies. Variation in susceptibility towards enzymatic treatment can, in fact, occur among IgG isotypes and among

individual monoclonal antibodies of the same isotype [33]. Similar binding results have been found among different immunoreactive fractions [IgG and F(ab')₂; ref. 34], while other authors have shown that the hydrolytic conditions could considerably affect the yield of F(ab')₂ fragments or their immunoreactivity [35]. It would now be interesting to compare the concrete binding capacity of whole IgGs and their F(ab')₂ fragments, obtained from hyperimmune colostrum towards specific bacterial or viral antigens, in a further study.

This work was performed on a very large quantity of allergic human sera, collected at the time of clinical allergic episodes. A further test of a greater number of sera should confirm these results. Molecular studies could allow us to understand the potential allergenic epitopes of the mole-

cule, eliciting production of IgE to IgG or to F(ab')₂ fragments.

The present results lead us to conclude that bovine colostrum IgG could induce allergic reactions when orally administered to humans. Moreover they show the significance of peptic hydrolysis for the preparation of immunoactive F(ab')₂ fragments, which would probably reduce the incidence of these reactions and preserve the potential therapeutic action against diarrhea.

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Announcement

Stress of Life

Stress and Adaptation from Molecules to Man

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July 1-5, 1997, Budapest, Hungary

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