

Prospective oral food challenge study of two soybean protein isolates in patients with possible milk or soy protein enterocolitis

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The soybean protein isolate used in powdered soybean formula is hydrolyzed more extensively than the isolate which is used in liquid soybean formula in most commercial soybean formulas. Previous *in vitro* studies have shown differences in human antibody response to these soybean protein isolates. Therefore, a prospective clinical study was undertaken to determine if there were differences in adverse reaction rates to these soybean protein isolates. Forty-three patients with possible milk- and/or soy-protein enterocolitis were enrolled in this study. Patients had 3 separate oral food challenges; using milk formula, soybean powder formula and soybean liquid formula. Ten (23%) patients challenged with milk had positive challenges. Fourteen (33%) patients challenged with powdered soy formula had positive challenges while thirteen (30%) challenged with liquid soy formula had positive challenges. In the 10 patients with positive milk challenges, 6 (60%) had a positive soy challenge. In the group with positive soy challenges, 5 reacted to the powdered soy challenge done first, but not the second challenge with the liquid soy formula, and 4 patients reacted to the liquid soy formula challenge done first, but not the second challenge with the powdered soy formula. These results indicate that a significant number of patients with milk protein enterocolitis have soy protein enterocolitis. In addition, an order effect can be demonstrated in the soy challenges because of the tendency to react to the first soy challenge regardless of the type of isolate. These results suggest that a local immune effect caused by the protein may be present.

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Milk- or soy-protein enterocolitis is a syndrome of adverse gastrointestinal reactions to either milk or soy protein. Adverse reactions to infant formulas are not uncommon in pediatric practice (1). Milk proteins have been implicated as the source of a number of these adverse reactions in infants and young children. It appears the incidence of cow's milk sensitivity is approximately 1% to 2% during

the first 2 years of life although estimates vary from 0.3% to 7.5% (2–5). Soybean-protein based formula have been used increasingly as alternatives for children thought to have adverse milk reactions.

Both immunologic and non-immunologic mechanisms have been examined to determine the cause of these adverse reactions in patients with milk- or soy-protein enterocolitis. Previous studies have produced conflicting results when the possible mechanisms of pathogenesis have been examined (2, 6–12). Many of these studies appear to have heterogeneous group of patients that have in-

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Table 1

Group 1 – (23 patients) milk formula, powdered soybean formula, liquid soybean formula

Pt.	Age	Challenges		
		Milk	Powdered soy	Liquid soy
1	7 m/o	+1,2,3	+1,3,4	+1,3,4
2	17 m/o	–	–	–
3	6 m/o	+1,3,5	+1,2,3	+1,2,3
4	3 m/o	–	+1,3,5	+2,3,5
5	4 m/o	–	+1,2,5	+1,2,5
6	12 m/o	+1,3,5	+2,3	+2,3
7	9 m/o	–	–	–
8	24 m/o	–	–	–
9	27 m/o	–	–	–
10	20 m/o	–	+3,5	+2,5
11	7 m/o	–	–	–
12	9 m/o	–	–	–
13	11 m/o	–	–	–
14	9 m/o	–	–	–
15	6 m/o	+1,2,3,4	+1,2,3,5	–
16	7 m/o	–	+1,2,3	+1,2,3
17	7 m/o	+1,2,3,4,5	–	–
18	11 m/o	–	–	–
19	3 m/o	+2,5	+1,2,3,5	–
20	7 m/o	–	+1,2	–
21	8 m/o	–	–	–
22	6 m/o	–	+1,2,5	–
23	14 m/o	+1,3,5	–	–

Group 2 – (12 patients) milk formula, liquid soybean formula, powdered soybean formula

24	6 m/o	–	–	–
25	4 m/o	+1,2,3,4	–	–
26	7 m/o	–	–	–
27	7 m/o	+1,2,3,4	–	–
28	8 m/o	–	–	+1,5
29	9 m/o	–	–	–
30	8 m/o	+1,3	+1,3,5	+1,2,3
31	7 m/o	–	–	–
32	8 m/o	–	+1,2,4	+1,2,4
33	3 m/o	–	–	–
34	9 m/o	–	–	–
35	4 m/o	–	–	–

Group 3 – (8 patients) milk formula, soybean formula challenges randomized

36-b	4 m/o	–	–	+1,4,5
37-b	8 m/o	–	–	+3,4
38-a	8 m/o	–	+1,4	–
39-a	7 m/o	–	–	–
40-a	4 m/o	–	–	–
41-b	5 m/o	–	–	+1,3,4
42-b	5 m/o	–	–	–
43-b	5 m/o	–	–	–

Symptoms during positive challenges

- 1 – symptoms of vomiting or diarrhea
- 2 – an increase in the absolute neutrophil count $> 3500/\text{mm}^3$ between blood drawn immediately before the challenge and 6 to 8 hours after challenge
- 3 – the appearance of blood in the stool
- 4 – the appearance of leukocytes in Wright's stained fecal mucus
- 5 – the appearance of Charcot-Leyden crystals or eosinophilic debris in the stool by Hansel's stain

Results

- + – positive challenge
 – – negative challenge

Order of Group 3 challenges

- a – milk formula, soybean powdered formula, soybean liquid formula
 b – milk formula, soybean liquid formula, soybean powdered formula

cluded children in many different age groups with gastrointestinal, as well as respiratory and cutaneous symptoms. Several studies, however, have examined the mechanism of this reaction in a well-defined pediatric population. Various immunologic parameters have been studied including serum-specific immunoglobulin levels (13, 14) and specific cellular immune responses to the food proteins (11).

The soybean protein isolates used to produce the liquid and powdered soy protein formulas are different in their final soybean protein composition (15). The soybean protein isolate used to make the powdered soy formula is a mildly hydrolyzed form of the soybean isolate used in the liquid soy formula. In a previous study we examined the difference in IgE-specific and IgG-specific antibody responses to the different soybean protein isolates used in liquid and powdered soybean formula (15). The serum IgE- and IgG-specific antibody response *in vitro* to the liquid soybean protein isolate was significantly greater in comparison with the same antibody response to the powdered soybean protein isolate. Because we found significant differences in the antibody responses between the 2 different soybean isolates, we chose to undertake a prospective clinical challenge study to determine if there were also differences in adverse reactions in patients with possible soy enterocolitis.

Methods

Patients

Patients referred to the Arkansas Children's Hospital Pediatric Gastroenterology Clinic over an 18-month period suspected of having milk- or soy-protein gastrointestinal enterocolitis were hospitalized for a standardized oral challenge to milk, powdered soy protein formula, and liquid soy protein formula. At diagnosis patients were younger than 18 months of age and suspected of having milk- or soy-protein enterocolitis because of a history of either vomiting and/or chronic diarrhea with stools containing mucus, blood, and leukocytes. Patients with suspected IgE-mediated adverse food reactions were excluded from this study. Most patients in the study presented to their primary care physician within the first several months of life for gastrointestinal symptoms. Patients with suspected milk- or soy-protein enterocolitis received a commercially-available, hydrolyzed protein formula for 4 to 12 weeks before enrollment in the study. Informed consent was obtained for each patient. All studies were approved by the Human Research Advisory Committee of the University of Arkansas for Medical Sciences.

Table 2. Results of milk and soybean challenges

	Total	(mean age)
Negative milk and soybean (both) challenge	21	7.05 m/o
Positive soybean challenge study	12	7.33 m/o
Positive milk and soybean challenge	6	7.00 m/o
Positive milk challenge only	4	8.00 m/o

Challenges

Patients were considered in a baseline state on hospital admission if they had no evidence of diarrhea, occult blood in the stools, vomiting, or other symptoms indicative of milk- or soy-enterocolitis. The first 23 patients (Group 1) had challenges done in the following order: milk, powdered soy and liquid soy (Table 1). The order of these initial challenges was designed based on previous *in vitro* studies so that if there were differences in adverse reaction rates, the patients would be more likely to react on the last day of the challenges. In a similar group of patients there was significant increase in the antibody response to the protein isolate used on the last day of the challenges (15). Because several patients had positive challenges to the first soy challenge (powdered soybean isolate) and not the second soy challenge, the subsequent 12 challenges (Group 2) were done in the order of milk, liquid soy and powdered soy. The last 8 challenges (Group 3) were done with the milk challenge first and then the 2 soy challenges randomized.

The challenges were done by standard protocol (16). After a 4–6 h fast, patients were fed either skim milk, powdered soybean formulas, or liquid soybean formula in a volume calculated to deliver a standard protein load (0.6 g of protein per kg of body weight). Infants were observed and stools were collected for the next 24 h. Five parameters were monitored: 1) symptoms of vomiting or diarrhea, 2) an increase in the absolute neutrophil count of $3500/\text{mm}^3$ between blood drawn immediately before oral challenge and 6 to 8 h after challenge, 3) the appearance of blood in the stool, 4) the appearance of leukocytes in Wright's stained fecal mucus, and 5) the appearance of Charcot-Leyden crystals or eosinophilic debris in the stool by Hansel's stain. The laboratory personnel were blinded to the clinical challenge. For this study, the challenge reaction was considered positive if two or more manifestations were present and negative if one or fewer were observed. There was 48 h between each food challenge.

Statistics

Statistical comparisons were done by multiple parametric and non-parametric tests (17).

Results

Forty-three patients were enrolled in the study (Table 1). The demographics of the population included 29 (67% Caucasians, 14 (33%) African-American, and 26 (60%) males. The mean age of the patients at the time of challenge was 8.37 months (range 3 to 27 months). Patients 8, 9, 10, 11 and 22 were admitted for re-challenge after a previous positive milk and soy challenge (Table 1). Twenty-one patients (49%) had negative challenges to both milk and soybean formula (Table 2). Twelve patients (28%) had a positive soybean challenge only (Table 2). Ten (23%) patients had a positive milk challenge with six of these having positive soybean challenges also (Table 2). The mean age of the patients in these groups were not significantly different ($p < 0.05$). In the soy challenges, 13 (30%) had a positive challenge to the liquid soy formula and 14 (33%) had a positive challenge to the powdered soy formula (Table 3).

In the initial group, 23 patients (Group 1) were challenged with the order: milk, powdered soy formula and liquid soy formula. In this group 4 had positive challenges to the powdered soy formula, but negative challenges to the liquid soy formula (Table 3).

Because of the discrepancy in findings from our original hypothesis we changed the order of the next 12 challenges to milk, liquid soy formula and powdered soy formula. In this group (Group 2), 1 patient had a positive challenge to the first soy challenge (liquid soy), but not the second challenge (powdered soy) (Tables 1, 3).

Table 3. Results of positive soybean formula challenges

	Total	
Positive soybean formula challenge (both)	9	
Positive powdered soybean formula challenge (only)	5	
Positive liquid soybean formula challenge (only)	4	
Positive challenge to soybean formula done first		
	Liquid soybean formula	Powdered soybean formula
Group 1 (powdered first)		4
Group 2 (liquid first)	1	
Group 3 (randomized)	3	1

The last 8 patients enrolled in the study had the milk challenge done first with the first 2 soy challenges randomized (Group 3). In this group, 4 had a positive soy challenge the first day of the soy challenges, but a negative challenge the second day. Three of these received liquid soy formula first, while one received the powdered soy formula first (Tables 1, 3).

In summary, there were 9 patients who had positive soy challenges the first day of the soy challenges, but not the second day. In these 9, 5 had the powdered soy challenge done first and 4 had the liquid soy challenge done first (Table 3). There were 8 who had consecutive positive challenges to both the liquid and powdered soybean formula.

Discussion

Adverse reactions to various food products are common problems that primary care physicians encounter each day. Numerous reactions have been ascribed to milk and soy products (1). Unfortunately, many of the adverse reactions have not been proven any type of blinded food-challenge studies. A recent publication by the National Institutes of Health has denoted specific terms to describe certain types of adverse food reactions (1,18). Intolerance to a particular food is described as an abnormal physiologic response to an ingested food that is not proved to be immunologic in nature. Hypersensitivity (allergy), in contrast, is described as a reaction in which an immunologic mechanism is primarily responsible. Enterocolitis is a spectrum of adverse gastrointestinal reactions secondary to the ingestion of a food thought to be immunologic in nature but not systemically mediated by IgE. The majority of previous studies have pooled these distinctly different types of patients. This grouping of patients has led to confusion in the literature regarding the pathogenic mechanism. Estimates of milk protein reactions vary between 0.3% to 7.5% because of the above confusion (2). Based on a birth rate of 3 million infants per year in the United States, a prevalence rate of 1% would add an additional 30,000 patients with adverse reactions to cow's milk (19).

Overall, 23% of our patients with symptoms suggesting protein enterocolitis had positive milk challenges. Interestingly 60% of these also had positive soy challenges, which is a percentage significantly higher than previously documented (20). There were no variables such as age, race, sex, etc., which identified patients who reacted to both milk and soy.

The overall incidence of positive soy challenges was higher than that for milk challenges. In the total group 30% reacted to the liquid soy product

while 33% reacted to the powdered soy product. In the 9 patients who reacted to the first soy challenge, 5 of these challenges were done with the powdered soy challenge first and the liquid soy challenge second. The remaining 4 patients had positive challenges to the liquid soy challenge first, but not the powdered soy challenge. These results were obviously somewhat unexpected since the challenges were not randomized from the beginning. But with the challenge results being based on objective information there appears to be no significant bias introduced by changing the challenge order. The introduction of the milk challenge first did not introduce any bias into the study as 6 patients had positive soy challenges after a positive milk challenge and 4 had a negative soy challenge after a positive milk challenge.

The intention of the study was to determine if the processing which makes the soybean isolate appropriate for use in a powdered formula was sufficient to render the product less likely to cause adverse reactions in this group. In patients with documented IgE-mediated reactions (hypersensitivity) to milk, the protein has to be significantly digested to render it hypoallergenic (21). Even a moderately hydrolyzed milk protein (Good Start) has been shown to have significant amounts of intact milk protein which could cause IgE-mediated reactions (22).

The mechanism of milk or soy protein enterocolitis remains unknown. Multiple articles have examined the possible immunologic parameters involved in this disease (6-12). Recent studies which have identified children with documented milk and/or soy protein enterocolitis have found no differences in the challenge-positive and challenge-negative groups with regard to serum IgG- or IgE-specific antibodies to any of the individual milk or soy proteins (14,15).

Aside from antibody production, other immunologic factors have been thought to play a role in food protein enterocolitis. One study found evidence for lymphocyte sensitization in children who have documented milk and soy protein enterocolitis (11). Their results indicated a significant difference in the mean geometric stimulation index to various milk and soy proteins when comparing the challenge positive and challenge negative groups. Another mechanism was postulated when an investigator found evidence for an increase in nonspecific concanavalin A-induced suppressor cell activity in patients who have milk protein enterocolitis (23). They postulated that the delayed maturation of a suppressor cell population might be the cause of the development of enterocolitis. Depressed neutrophil chemotaxis was noted in one group with milk or soy-protein ente-

rocolitis (12). Increased production of a lymphokine, leukocyte-migration inhibition factor has been linked to a cause of food protein enterocolitis (24). A recent study by Isolauri examined a number of immunologic parameters in a group with adverse reactions to cow's milk protein. They found on rechallenge a change in antibody-secreting cells of the IgA isotype in those who acquired tolerance (25).

The pathology of food protein enterocolitis often includes mucosal eosinophilia, epithelial damage (from subtle to total villous atrophy), and disruption of the mucosal barrier with blood or protein loss in the intestinal lumen (26). In animal models using GI parasitic infection and graft-versus-host disease, a linkage between cell-mediated immune reactions and enterocyte damage has been postulated (27). The changes noted in these animal models include the finding that increased numbers of intraepithelial lymphocytes were associated with increased crypt mitotic activity and with decreased villous enterocyte height. A clinical study did note that in children with apparent food protein enterocolitis the relative number of intraepithelial lymphocytes is increased (28). A separate conflicting study found that patients who had apparent soybean protein enterocolitis responded not to changes in the protein source of the formulas but to changes in the type of carbohydrate used in the formula (29).

This study detailing an effect from the order of the challenges suggests that a local immune mechanism may be present in milk- and soy-protein enterocolitis. One plausible explanation is the depletion of gastrointestinal immune mediators after the first challenge. Future studies should take this information into account in the experimental design of the challenges and in the search for pathogenic mechanisms.

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