An adjuvant-free mouse model to evaluate the allergenicity of milk whey protein

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ABSTRACT

Milk allergy is the most common type of food allergy in humans with the potential for fatality. An adjuvant-free mouse model would be highly desirable as a pre-clinical research tool to develop novel hypoallergenic or nonallergenic milk products. Here we describe an adjuvant-free mouse model of milk allergy that uses transdermal sensitization followed by oral challenge with milk protein. Groups of BALB/c mice were exposed to milk whey protein via a transdermal route, without adjuvant. Systemic IgG1 and IgE antibody responses to transdermal exposure as well as systemic anaphylaxis and hypothermia response to oral protein challenge were studied. Transdermal exposure resulted in a time- and dose-dependent induction of significant IgE and IgG1 antibody responses. Furthermore, oral challenge of sensitized mice resulted in significant clinical symptoms of systemic anaphylaxis within 1 h and significant hypothermia at 30 min postchallenge. To study the underlying mechanism, we examined allergen-driven spleen cell T-helper 2 cytokine (IL-4) responses. There was a robust dose- and time-dependent activation of memory IL-4 responses in allergic mice but not in healthy control mice. These data demonstrate for the first time a novel transdermal sensitization followed by oral challenge mouse model of milk allergy that does not use adjuvant. It is expected that this model may be used not only to study mechanisms of milk allergy, but also to evaluate novel milk products for allergenic potential and aid in the production of hypo- or nonallergenic milk products.

Key words: milk protein, allergy, systemic anaphylaxis, immunoglobulin E

INTRODUCTION

Immediate hypersensitivity response to food, commonly called food allergy, affects 6% of children and

3 to 4% of adults in westernized countries including the United States (Sicherer and Sampson, 2008). Milk allergy is the most common food allergy with a prevalence of 2.5% among children and 0.3% among adults (Sicherer and Sampson, 2008). There is growing concern that food allergies are increasing at an alarming rate for reasons that are not well understood (Sicherer and Sampson, 2008). Furthermore, because food allergies are potentially fatal, they are considered clinically significant and very dangerous immune-mediated disorders.

The mechanism underlying milk allergy is not completely understood at present. In general, milk allergies are classified as IgE-mediated and non-IgE-mediated disorders (Sampson and Anderson, 2000). Whereas non-IgE-mediated milk allergy is generally not considered life threatening, IgE-mediated milk allergy is potentially fatal (Host, 1994). The IgE-mediated milk allergy involves production of IgE antibodies upon first exposure to milk protein (e.g., β -LG, α -LA, caseins) leading to sensitization of mast cells. Second and subsequent exposures to the same milk protein result in cross-linking of mast-cell-bound IgE, leading to activation and release of inflammatory mediators such as histamine. This results in clinical signs of disease such as hives, rashes, and in rare cases, potentially fatal systemic anaphylaxis (Sicherer and Sampson, 2008).

Many animal models to study immune and allergic responses to milk proteins are described in the literature (Li et al., 1999; Miller et al., 1999; Adel-Patient et al., 2005). However, most of the models use adjuvant to elicit an allergic response to milk proteins (Li et al., 1999; Miller et al., 1999; Adel-Patient et al., 2005). Although adjuvant-based models are very useful to study the immune response to milk protein in the context of an adjuvant as a co-factor, it has been suggested that use of adjuvant may interfere with evaluating the allergenic potential of novel proteins or chemicals because of enhanced risk of false positivity (Buehler, 1996). Consequently, adjuvant-free models might be more suitable for testing of novel proteins such as chemically or physically altered milk proteins to develop hypo- or

Received November 25, 2008.

Accepted July 7, 2009.

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nonallergenic milk products. Therefore, we focused our efforts to develop an adjuvant-free mouse model of milk allergy in this study—a critical research need in the area of dairy science.

MATERIALS AND METHODS

Milk whey protein extract was purchased from Greer Labs (Lenoir, NC), and the protein content was measured by the Lowry-Folin assay. Briefly, protein solution in different dilutions was first mixed with copper sulfate and then Folin-Ciocalteu's phenol reagent was added. The color reaction was read for absorbance at 750 nm. Bovine serum albumin in different amounts was used to obtain the data to generate the standard curve (Lowry et al., 1951). The LPS content was tested and found to be <0.5 pg/mg of protein as measured by the Limulus amebocyte assay. Briefly, samples were mixed with the Limulus amebocyte assay reagent and chromogenic substrate reagent (Cambrex Bio Science Walkersville Inc., Walkersville, MD). After an incubation period (16 min), absorbance was measured at 405 to 410 nm. Biotin-conjugated rat anti-mouse IgG1 and IgE antibodies and paired antibodies and recombinant standards for mouse IL-4 were purchased from BD PharMingen (San Diego, CA). Purified β -LG, α -LA, and *p*-nitro phenyl phosphate were purchased from Sigma Chemical (St. Louis, MO). Streptavidin alkaline phosphatase was purchased from Jackson ImmunoResearch (West Grove, PA) and protein-G was purchased from GE Healthcare (Piscataway, NJ). Adult BALB/c female mice were purchased from The Jackson Lab (Bar Harbor, ME). The animal procedures used were approved by the Institutional Animal Care and Use Committee (Michigan State University, East Lansing).

Transdermal Sensitization and Bleeding

Adult female animals (6–8 wk of age) were used in the study and they were on a casein-free JL Rat & Mouse/Auto 6F 5K52 lab diet (PMI Nutrition International, Brentwood, MO). Transdermal exposure experiments were performed using a modified method described previously (Birmingham et al., 2005). Groups of mice ($n = 5$ –10 per group) were exposed to saline (100 μ L per mouse per application) or milk whey protein (1 mg and 2.5 mg per mouse per application); each mouse had the reagent applied to an area of skin of the back that had the hair clipped off, and the area was covered with a nonlatex, nonocclusive bandage for 1 d. Mice were rested for 4 d. Then, the cycle of exposure to saline or milk whey protein was continued for 6 wk, with mice exposed once a week. Blood samples were

collected from the saphenous vein into heparin-coated microvette collecting tubes (CB300, Sarstedt AG & Co., Numbrecht, Germany) and plasma was used in the antibody analysis.

Measurement of Milk Protein–Specific IgE and IgG1 Antibody Levels

We described previously the optimization of ELISA for food-specific IgG1 and IgE antibody analyses (Birmingham et al., 2003). The ELISA procedure used in this study was essentially as described previously (Birmingham et al., 2003).

Induction of Systemic Anaphylaxis, Clinical Scoring, and Measurement of Rectal Temperature

Groups of milk protein-sensitized versus saline-exposed mice were orally challenged with milk protein (15 mg/mouse) or saline (500 μ L/mouse) on d 13 following the sixth exposure using mouse feeding needles (22-gauge, Popper and Sons Inc., New Hyde Park, NY). Mice were then observed for signs of systemic anaphylaxis during the next 60 min. Clinical scoring (on a scale of 0 to 5) was performed by 2 individuals according to the method described previously (Li et al., 2000). A score of 0 indicates no symptoms; 1 indicates scratching and rubbing around the nose and head; 2 indicates puffiness around the eyes and mouth, diarrhea, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate; 3 indicates wheezing, labored respiration, cyanosis around the mouth and the tail; 4 indicates no activity after prodding, or tremor and convulsion; and 5 indicates death. Rectal temperature was measured using a temperature probe (Physitemp Instruments, Inc., Clifton, NJ) before and 30 min after oral challenge.

Spleen Cell Culture and Cytokine Analyses

Spleen cells were harvested and standard cell cultures were established as described previously (Birmingham et al., 2007; Parvataneni et al., 2009). Briefly, spleen cells were cultured (7.5 million cells/mL) in the absence and presence of milk protein (100 and 500 μ g/mL). Cell culture supernatants were harvested for use in cytokine analyses using a preoptimized ultrasensitive assay (assay sensitivity: IL-4: 3.1 pg/mL).

Statistical Analysis

The Wilcoxon nonparametric test was used to compare treatment versus control for clinical scores. The

IgE antibody titer data were log-transformed and subsequently analyzed using one sample *t*-test. Analysis of variance was used to analyze rectal temperature, IL-4, and purified milk protein data; SAS software was used for all statistical analysis (SAS Institute Inc., Cary, NC). The statistical significance level was set at 0.05.

RESULTS

Transdermal Exposure to Milk Whey Protein Elicits Dose-Dependent IgE Antibody Response

We performed dose-response and time-course experiments and analyzed antibody responses in mice following transdermal exposure to milk protein. As is evident in Figure 1A, significant milk protein-specific IgE antibody responses were observed at a dose of 1 mg/mouse after the sixth exposure (Figure 1A). No IgE antibodies were detectable in the samples collected before allergen exposure or at any time point in saline control mice. A dose of 2.5 mg/mouse also elicited significant IgE antibody responses by the fourth exposure (IgE titer $1,066.6 \pm 213.3$, $n = 10$ mice). The IgE induction was confirmed by analyzing the plasma samples after depleting IgG1 and IgG2a with protein-G treatment (data not shown). Furthermore, using purified β -LG, α -LA, and caseins in ELISA coating, we found that IgE was directed against β -LG and α -LA (Figure 1B). In addition to IgE responses, significant IgG1 responses were detectable in milk protein-exposed mice but not in saline control mice (Figure 2).

Transdermal Exposure to Milk Whey Protein Is Sufficient to Sensitize BALB/c Mice for Clinical Signs of Systemic Anaphylaxis in Response to Oral Challenge with Milk Protein

After confirming IgE responses, mice were orally challenged with milk whey protein and observed for clinical reactions. Only transdermally sensitized mice, not saline-exposed mice, exhibited immediate and significant clinical symptoms of systemic anaphylaxis (Figure 3).

Clinical Symptoms of Systemic Anaphylaxis Are Associated with Significant Hypothermia

We tested the rectal temperature of mice before and 30 min after oral challenge with milk protein. Following oral challenge, only mice that had been transdermally sensitized to cow's milk protein showed a significant decrease in rectal temperature (Figure 3B).

Milk-Allergic Mice but not Healthy Control Mice Exhibit Dose-Dependent Milk Protein-Driven Memory IL-4 Response

We studied dose-response and time-course of IL-4 response in healthy versus milk-allergic mice using spleen cell culture. Significant IL-4 responses were observed on d 3 of culture at both doses of milk protein in milk-allergic mice but not in control healthy mice (Table 1).

DISCUSSION

There are 3 important and novel findings from this study: 1) transdermal exposure of BALB/c mice to milk whey protein (in the absence of adjuvant) results in a significant systemic allergic (IgE) response; 2) exposure to milk protein via the skin is sufficient to clinically sensitize mice for immediate hypersensitivity reactions such as systemic anaphylaxis and hypothermia in response to oral challenge with the milk protein; and 3) the mechanism underlying allergic response to transdermal milk protein exposure involves activation of the prototypic type-2 cytokine, IL-4, response.

We chose the BALB/c strain of mice in this study to examine allergic responses to milk protein because this strain has been used in many earlier allergy studies including our previous studies. However, it was unknown whether BALB/c mice develop allergic responses to transdermal exposure to milk protein in the absence of adjuvant (Navuluri et al., 2006; Birmingham et al., 2007; Parvataneni et al., 2009). Moreover, because gene knockout mice are available with the BALB/c genetic background, this strain is desirable for conducting mechanistic studies on milk allergy.

It is very common for humans, especially children, to be exposed to milk proteins via the skin. However, the immune and clinical consequence of such transdermal exposure is not completely clear at this time. Emerging evidence indicates that transdermal exposure to allergenic food proteins can have clinical consequence, at least in mice. Thus, transdermal exposure to other allergenic food proteins such as hazelnut, cashew nut, and sesame seed can result in both immune activation and clinical sensitization for immediate hypersensitivity reaction including systemic anaphylaxis (Navuluri et al., 2006; Birmingham et al., 2007; Parvataneni et al., 2009). Others have reported a delayed hypersensitivity response to peanut via skin exposure in the ear using a tape-stripping method (Strid et al., 2005). Those researchers reported that mice also suffered from signs of anaphylaxis (Strid et al., 2005). Thus, in addition to the above food proteins, we demonstrate here

Figure 1. BALB/c mice exhibit systemic allergic response to transdermal exposure with cow's milk whey protein. Groups of mice ($n = 10$ per group) were exposed to cow's milk protein (1 mg/mouse) via the transdermal route 6 times over a period of 6 wk. Plasma samples were collected before transdermal exposure (Pre) and after 6 exposures (6R). A) Cow's milk protein-specific IgE (sIgE) titers were measured using an optimized indirect ELISA; ANOVA, 6R versus Pre: $P < 0.0001$; B) binding of IgE from 6R sample to different purified milk proteins. ^{a,b}Bars labeled with different letters are significantly different. OD = optical density.

that transdermal exposure to milk whey protein can activate the immune system, especially IL-4 and IgE responses, leading to clinical sensitization for systemic anaphylaxis in mice.

Many milk allergy models reported in the literature use adjuvant to demonstrate robust allergic or immune responses in mice. Thus, C3H/HeJ mice were shown to develop clinical milk allergy disease (such as atopic dermatitis and systemic anaphylaxis) following oral exposure to milk protein along with cholera toxin adjuvant (Li et al., 1999). Other models have used IgE response but not clinical disease features (Miller et al., 1999; Adel-Patient et al., 2005). In this study we demonstrate, for the first time, that it is possible to use an adjuvant-free transdermal approach to develop a mouse model of milk allergy that includes not only immune responses (IgE and IL-4) but also clinical findings of

systemic anaphylaxis and a physiological response of hypothermia.

One previous study using BALB/c mice and a cholera toxin adjuvant approach reported that BALB/c mice are "genetically resistant" to milk allergy (Morafo et al., 2003). In contrast, others reported that BALB/c mice could develop an allergic response to milk following oral exposure to milk protein along with cholera toxin adjuvant (Adel-Patient et al., 2005). Using a different approach (i.e., transdermal exposure), we demonstrate that BALB/c mice are indeed susceptible to milk allergy even in the absence of adjuvant as a co-factor.

Development of novel milk products containing hypo- or nonallergenic milk proteins is an area of significant interest in the dairy science and functional food fields (Businco et al., 1993; Giampietro et al., 2001). Testing such novel products in adjuvant-based milk allergy

Figure 2. BALB/c mice exhibit robust IgG1 response to milk whey protein upon transdermal exposure. Groups of mice (n = 10 per group) were exposed to cow’s milk protein (1 mg/mouse) via the transdermal route 6 times over a period of 6 wk. Plasma samples were collected before transdermal exposure (Pre), after 3 exposures (3R), and after 6 exposures (6R). Cow’s milk protein-specific IgG1 (sIgG1) levels were measured using an optimized indirect ELISA (optical density, OD, at 450–690 nm).

models pose problems of interpretation. For example, a chemically or physically altered whey protein might be intrinsically hypoallergenic, but when used in an adjuvant-based model, may test positive for allergenicity because of the adjuvant effect. With the adjuvant-free model described here, we now provide an improved opportunity to study the intrinsic allergenicity of chemically or physically altered milk proteins in the absence of adjuvant effect.

It is largely unclear whether exposure to allergenic foods such as milk whey proteins via skin might lead to food allergy in humans. There is extensive discussion on this topic in the recent literature (Hayday and Shannon, 2003; Lack et al., 2003). One epidemiological study suggested the possibility of peanut allergy in children following skin exposure to peanut (Lack et al., 2003). We are not aware of such a study in milk allergy. Here we directly demonstrate this possibility

Table 1. Interleukin-4 responses (pg/mL) to cow’s milk protein challenge in healthy control mice and milk-allergic BALB/c mice¹

Cow’s milk protein used in cell culture (mg/mL)	IL-4 levels in cell culture supernatant (d 3)	
	Saline control mice (n = 5)	Milk-allergic mice (n = 5)
0	0.50 ^c	28.99 ± 0.43 ^b
0.1	3.90 ± 1.64 ^c	251.38 ± 8.6 ^a
0.5	0.28 ^c	261.86 ± 11.3 ^a

^{a-c} ANOVA: numbers with different letters within a row and column are significantly different (*P* < 0.05).
¹ Data shown are average ± SE of triplicate analysis.

In conclusion, we demonstrate, for the first time, a novel transdermal sensitization followed by oral challenge mouse model of milk allergy that does not use adjuvant. This model might be useful to study mechanisms of milk allergy and is expected to serve as a research tool to evaluate novel milk products for allergenic potential and aid in the production of hypo- or nonallergenic milk products and development of new preventive and therapeutic methods for milk allergy.

This work was supported by a grant from the United States Environmental Protection Agency

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