

Efficacy of baked milk oral immunotherapy in baked milk-reactive allergic patients

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Background: Patients with IgE-mediated cow's milk allergy who are nonreactive to baked milk (BM) can be desensitized with BM to promote tolerance to unheated milk (UM).

Objective: We sought to test whether patients who are BM reactive can progress in BM oral immunotherapy (OIT) and become desensitized to UM as well.

Methods: Fifteen patients (>4 years) who previously failed to complete our milk OIT program were enrolled into the BM OIT protocol. A dose of BM (180°C for 30 minutes) which was less than the eliciting dose was increased 50% monthly while under medical supervision until the primary outcome dose of 1.3 g/d BM protein was achieved. Basophil reactivity and milk protein-specific IgE binding were analyzed at the first round of BM OIT therapy (T₀) and at 12 months of BM treatment.

Results: In terms of the primary outcome, only 3 (21%) of 14 patients tolerated the 1.3 g/d BM dose. Although some patients initially progressed in BM OIT, 8 of 11 failed because of IgE-mediated reactions. Three did not complete the program because of non-IgE-mediated factors. An increase in challenge threshold to UM was noted in patients continuing until 12 months ($P = .003$), including those among whom reactions precluded continuation in the program. Patients ($n = 3$) who successfully reached maintenance had decreased milk-specific IgE reactivity. Furthermore, the mean difference at T₀ between induced HM and UM percentages of CD203c expression was significantly lower in patients who successfully completed BM OIT than in those who did not (-11% vs 4.4% , $P = .0002$), which is consistent with their decreased clinical reactivity to BM.

Conclusions: Although use of hypoallergenic BM in OIT is a promising therapy, care must be taken before its administration in BM-reactive patients because of the risk for anaphylaxis and only limited increase in challenge threshold attained. (*J Allergy Clin Immunol* 2015;136:1601-6.)

Key words: Oral immunotherapy, cow's milk allergy, baked milk

An emerging treatment modality for patients with persistent IgE-mediated food allergy is the use of oral immunotherapy (OIT), which has been demonstrated to desensitize the majority of patients in observational and randomized controlled trials.¹⁻⁹ Despite OIT's overall success, it is not without adverse reactions.¹⁰ In a large milk OIT treatment program in Israel, 61.5% of patients reached full consumption of cow's milk protein (CMP; 7.2 g), and an additional 24% reached a partial level that might protect against incidental exposures.¹¹ About half of the remaining approximately 15% of patients who did not complete the program failed to do so because of IgE-mediated reactions. The high reaction rate, which was also reported in other studies,¹⁰ calls into question the risk/benefit ratio of these programs. The adverse effects are especially prevalent in highly sensitive patients. Therefore it is important to identify in advance those patients for whom OIT treatment is likely to fail. Furthermore, a small percentage of patients are too sensitive to enter an OIT program (sensitive to <12 mg of CMP) or have frequent side effects (initial tolerance <20 mg of CMP). Ironically, it is precisely this subset of patients for which a successful OIT program is most critical.

Previous studies have shown that some patients allergic to raw egg can tolerate cooked egg.¹²⁻¹⁴ It is likely that the heating process induces conformational changes to a significant proportion of the epitopes responsible for IgE binding. Of particular interest, furthermore, is the recent notion that hypoallergenic extracts can give rise to allergen-specific protective IgG responses to linear epitopes with T-cell help from a nonallergenic carrier molecule.¹⁵ Thus, although a heat-denatured allergen can have decreased unwanted side effects, it still might be efficacious to induce tolerance.^{16,17}

Regarding milk allergy, it was shown that children who eventually outgrew their milk allergy had milk-specific antibodies directed primarily against conformational epitopes.^{18,19} Therefore baked milk (BM) OIT might provide a viable alternative to unheated milk (UM) OIT for patients with IgE-mediated cow's milk allergy (IgE-CMA). Up to two thirds of patients with IgE-CMA tolerate BM (heat-denatured) products with minimal side effects.²⁰ Furthermore, up to 60% of BM-tolerant patients undergoing BM OIT (1.3-g maintenance dose) become desensitized to UM as well.^{20,21} However, the latter studies excluded patients who initially reacted to BM from BM OIT treatment.^{20,21} These patients have significantly higher casein- and milk-specific IgE levels, milk-specific basophil reactivity, and CMP-specific skin

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Abbreviations used

BAT:	Basophil Activation Test
BM:	Baked milk
CMP:	Cow's milk protein
HM:	Heated milk
IgE-CMA:	IgE-mediated cow's milk allergy
OFC:	Oral food challenge
OIT:	Oral immunotherapy
UM:	Unheated milk

prick test responses compared with values in patients with IgE-CMA who can tolerate BM.²²⁻²⁴

All participants in heat-denatured food OIT trials thus far were patients tolerant to the heated form of the allergen, and its efficacy to desensitize to the native form was evaluated in this population. However, to date, none of these trials tested the feasibility of heat-denatured food OIT in the heat-denatured food-reactive population (see the summary of participants in heated egg and BM OIT trials in [Table E1](#) in this article's Online Repository at www.jacionline.org). Given the changes in conformational structure of the heated forms, we reasoned that it might provide a reasonable alternative to our patients who previously failed to complete our UM OIT program¹¹ and who, given their sensitivity, are likely to be BM reactive. The objective of this study was to determine whether BM-reactive patients in whom milk OIT previously failed would tolerate BM OIT. The primary outcome assessment was defined as attaining a maintenance BM dose of 1.3 g/d without adverse reactions at 1 year of treatment. In addition, the potential for concomitant desensitization to UM was evaluated.

METHODS

Study design

The study was conducted at a food allergy referral center in the Allergy, Pulmonary and Immunology unit of the Assaf Harofeh Medical Center (Beer Yaakov, Israel). The research protocol was approved by the Helsinki Review Board of the Assaf Harofeh Medical Center, and all patients signed written consent forms. Inclusion criteria included patients (>4 years) who reacted initially to 30 mg or less of UM protein during our milk OIT program and did not complete the program because of IgE-mediated reactions.¹¹ Patients with stable asthma being treated with daily controller inhaled steroid therapy or as-needed therapy were included.¹¹ Patients unable to tolerate 25 mg of BM were excluded from further treatment ($n = 1$). Patients received escalating doses of 3, 15, 25, 60, and 120 mg of BM (muffins) at 30-minute intervals (120 mg maximum) on the first day to determine their initial starting dose of BM OIT. This was performed before the baseline UM oral food challenge (OFC) to exclude patients with a high sensitivity to BM and thus forego the risk of the UM challenge performed 2 days later. In addition, the UM OFC was modified, taking into account the severity of the patient's milk allergy. Escalating doses of 6, 12, 21, 30, 45, 60, 90, 120, 240, and 480 mg were given at 20-minute intervals, except after the first dose, in which the interval was 15 minutes. After these baseline OFCs were completed, the following day the patients received a BM dose of less than their eliciting dose, which is defined as the last dose eliciting the reaction. After verification under medical supervision that this dose was tolerated, it was given daily for a month-long period of home therapy. The dose was then increased 50% monthly under medical supervision on subsequent visits. Reactions were defined by the involvement of the skin, ocular, nasal, digestive, or respiratory systems within 2 hours after administration of the dose. Anaphylaxis was defined as involvement of 2 or more organ systems during an allergic reaction. Those not reacting on the first day were given a maximum of 120 mg for the first month. Subsequent OFCs to UM were

performed after 6 (T_6) and 12 (T_{12}) months of BM treatment. Whole blood and sera were taken for the Basophil Activation Test (BAT) and immunoblotting, respectively, before or within the first round of BM OIT therapy (T_0) and subsequently at the T_6 and T_{12} time points.

BM and heated milk products

Low-fat dried milk powder (Adama, Airport City, Israel) containing 1 g of milk protein for each 2.837 g of milk powder was used to prepare BM doses. Preparation of BM products is described in the [Methods](#) section in this article's Online Repository at www.jacionline.org. For the preparation of heated milk (HM) for the BAT, nonfat dry milk powder was reconstituted at 2 mg/mL. One hundred eighty milliliters in a liter beaker was heated in a preheated oven at 180°C for 30 minutes. Samples were cooled and diluted to 1 mg/mL and then frozen at -20°C for future use.

Skin prick tests

Before the start of the BM OIT trial, skin prick tests to CMP (1:10 wt/vol; ALK-Abelló, Port Washington, NY) and the house dust mites *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* (5000 AU/mL) were performed. SPTs were performed on the volar surface of the forearm with single peak lancets (Heinz Herenz, Hamburg, Germany), and wheal and flare size was measured after 15 minutes, as previously described.¹¹ Histamine (1 mg/mL, ALK-Abelló) was used as the positive control, and saline was used as the negative control. A response was considered positive if the wheal was 3 mm or greater for 1 or more of the allergens.

BAT assay

Whole blood was taken from patients and diluted 1:1 in RPMI 1640 (Biological Industries, Beit HaEmek, Israel) containing 1 ng/mL recombinant human IL-3 (R&D Systems, Minneapolis, Minn) and UM or HM at a concentration of 100 ng/mL total protein. Baseline stimulation tubes were set up without the addition of milk. After stimulation, cells were stained for flow cytometric analysis, with the basophils defined as CD123⁺HLA-DR⁻ cells. Reactivity was assessed by the percentage of basophils expressing CD63 or CD203c above baseline levels. Further details on BAT methodology and antibody source are provided in the [Methods](#) section in this article's Online Repository.

Immunoblotting

CMP (Adama) and semipurified bovine-derived milk κ -casein (Sigma, St Louis, Mo) were mixed with sample loading buffer (1% SDS final) in the presence of 5% β -mercaptoethanol and heated for 5 minutes at 95°C. Proteins were run on a 15% SDS-PAGE gel at 200 V for 45 minutes and transferred to nitrocellulose membranes. Individual lane strips were cut after Ponceau S staining (Sigma) and blocked with 3% BSA in PBS overnight. Sera from patients taken at 0 and 12 months into the treatment program were used to probe the membranes overnight at a 1:6 dilution. After washing in PBS plus 0.1% Tween, the membranes were probed with horseradish peroxidase-conjugated mouse anti-human IgE-Fc (Southern Biotech, Birmingham, Ala) as a secondary antibody for 1 hour. After the final washes, the membranes were treated with chemiluminescent substrate (Thermo Scientific, Rockford, Ill), and images were acquired by using the MicroChemi Imager (DNR Bio-Imaging Systems, Jerusalem, Israel).

Statistical analyses

For comparisons between patients at T_0 and T_{12} , a 2-tailed Mann-Whitney U test was used because the data were not normally distributed. For the comparison of initial HM minus UM differential basophil reactivity between patients successfully completing BM OIT and those with IgE-mediated failures, an unpaired 1-tailed t test was used because the tested hypothesis was unidirectional. A P value of .05 was used for tests of significance.

RESULTS

The clinical background of the 15 patients entering our study is presented in [Table E2](#) in this article's Online Repository

TABLE I. Summary of OFC data and disposition in BM OIT

Patient no.	Sex (M/F)	Age (mo)	SPT to CMP	OFC UM ED (mg)					Reaction dose (mg)	Reaction description	BM OIT	
				T _{initial}	T ₀	T ₆	T ₁₂	Fold increase T ₁₂ /T ₀			Epinephrine use (intramuscular)	Disposition
1	F	79	15/25	6	—	—	—	—	3	2, 4	No	IgE-fail T ₀
2	M	130	13/22	30	—	—	—	—	30	2	No	IgE-fail T ₀
3	M	110	10/17	15	—	—	—	—	ND	—	ND	Fail-other-psych
4	F	92	8/35	6	21	—	—	—	120	2, 4	+ (Home)	IgE-fail
5	F	102	10/12	15	30	—	—	—	120	3, 4	+ (Induct)	IgE-fail
6	F	114	7/12	30	60	—	—	—	120	4	No	Fail-other-asthma exacerbation
7	F	153	7/35	15	30	—	—	—	120	4	No	Fail-other-relocation
8	F	78	9/22	30	12	90	>480	>40	60	2,4	No	1.3 g of BM
9	M	145	7/30	30	60	480	>900	>15	720	4	No	1.3 g of BM
10	F	134	8/10	15	12	120	480	40	120	3	No	IgE-fail
11	M	90	14/20	30	6	30	480	80	120	2	No	IgE-fail
12	M	143	6/18	15	12	60	90	7.5	120	3	+ (Home)	IgE-fail
13	F	132	5/18	15	45	120	720	16	270	3	No	1.3 g of BM
14	M	117	4/7	30	240	240	—	—	270	4	No	IgE-fail
15	F	96	5/9	15	30	480	1800	60	15*	3*	No	1.3 g of BM

Reaction description: 2, skin; 3, gastrointestinal; 4, upper respiratory.

ED, Last dose eliciting the reaction; Induct, induction; ND, not done; psych, psychological aversion to taking milk; T_{initial}, initial eliciting dose when patients underwent UM OIT.

*Resolved spontaneously and continued increasing BM doses.

at www.jacionline.org. The overwhelming majority had a history of anaphylaxis, past epinephrine use, and asthma. None completed our UM OIT program¹¹ because of the severity and frequency of adverse reactions. Their OFC eliciting dose at the start of UM OIT (T_{initial} in Table I) was 30 mg or less (range, 6–30 mg; median, 15 mg). Given their sensitivity to UM, we reasoned they would likely be BM reactive. Supporting this notion, HM increased basophil CD63 expression over baseline values (mean, 49%; range, 19% to 86%) for all 12 patients tested (patients 4–15).

A summary of patients' OFC data, the dose at which they reacted to BM, and their final disposition is presented in Table I. With 2 exceptions (patients 3 and 15), all patients experienced definitive clinical reactions to BM at some point in the program (dose range, 3–720 mg; median dose, 120 mg), with 9 reacting on the first day. Patient 3 withdrew from the BM OIT program because of psychological aversion. Patient 15 complained of only mild abdominal discomfort, which occurred on the first day of treatment. She successfully reached the maintenance dose but was not included in the analyses to ensure a homogeneous group of BM-reactive patients.

In evaluating the primary end point, an intention-to-treat analysis showed only 3 (21%) of 14 patients tolerated 1.3 g/d baked CMP (see Fig E1 in this article's Online Repository at www.jacionline.org). Eight did not successfully complete the program because of IgE-mediated reactions, and 3 did not complete the program for other reasons. The doses of BM reached by those still in the program beyond 3 months are presented in Fig 1, A. Not uncommonly, patients who tolerated a particular dose for an extended period of time (>1 month) subsequently had adverse reactions at that dose. Interestingly, 1 patient (patient 10; Fig 1, A) reached the maintenance dose at the end of the year and then regressed because of continued adverse reactions. This pattern of reactivity was reflected in her BAT results as well (data not shown).

To determine whether BM-reactive patients can be desensitized to UM, patients performed an OFC before treatment and again after 6 and 12 months to determine their threshold of reactivity.

There was a significant increase in the challenge threshold to UM during OFCs (last tolerated dose) for those patients continuing in the program up to 12 months (6 vs 360 mg of UM, medians for T₀ and T₁₂, respectively; n = 6; P = .005, Mann-Whitney U test; Fig 2), including the 3 patients who did not reach maintenance but remained in the program up to the T₁₂ time point (6 vs 240 mg of UM). Despite the increase in challenge threshold for these patients, frequent reactions to BM precluded continuation in the program.

Immunologic parameters were monitored at the start and at 12 months of the program. Immunoblot analysis revealed that patients who reached the maintenance dose of 1.3 g/d had clearly decreased CMP-specific IgE binding at 12 months (Fig 1, B, solid line boxes). Decreases in reactivity to α -lactalbumin and casein-specific IgE (solid white circles in patient 13) were noted. In contrast, in 2 patients who did not successfully complete the protocol because of IgE-mediated reactions (patients 11 and 12), an increase in CMP-specific IgE binding was detected. A third patient (patient 10) is of interest because she initially reached 1.3 g of BM by 12 months and the immunoblot demonstrates a considerable decrease in reactivity of antibody against κ -casein (hatched white circle, patient 10). However, she still had considerable IgE to the casein fraction and α -lactalbumin, although the latter was decreased compared with that at T₀.

We further investigated whether the BAT could prospectively distinguish between patients who did not and those who did successfully complete BM OIT, with the hypothesis that the latter would react less to HM relative to their respective reactions to UM. At T₀, the mean difference between HM- and UM-driven CD203c percentage expression was significantly lower in the patients who completed BM OIT than in those who did not successfully complete the protocol (−11% vs 4.4%, P = .0002, n = 9, Fig 3). A trend for a lower HM minus UM values was also noted for CD63 percentage expression in successful patients (−9% vs 0.5%, P = .06, n = 9, Fig 3). This difference was particularly evident in patients 8 and 9 (see Fig E2 in this article's Online Repository at www.jacionline.org).

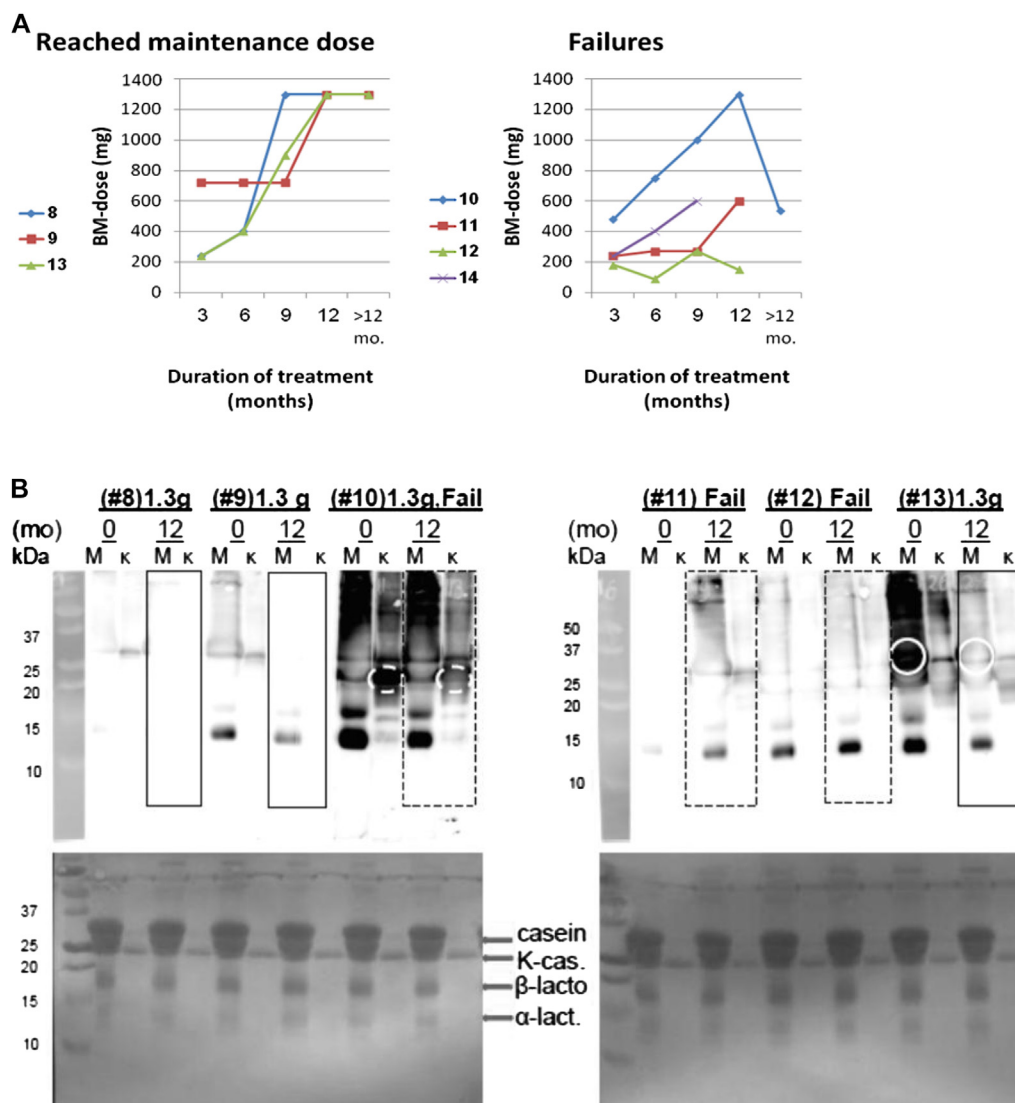


FIG 1. Clinical progression and biological parameters in patients undergoing BM OIT. **A**, BM dose (in milligrams) progression for patients greater than 3 months in treatment. **B**, Immunoblot analysis (*top panels*) against CMP using patients' sera at 0 and 12 months and corresponding membrane Ponceau S staining (*bottom panels*) are presented. Patients 8 to 13 are labeled with their final disposition in the BM OIT program. *Solid box*, Completed BM OIT; *hatched box*, failed to complete the program. κ , 5 μ g of semipurified bovine-derived milk κ -casein; *kDa*, molecular weight markers in kilodaltons; *M*, CMP (28 μ g).

DISCUSSION

BM products hold great promise for use in milk OIT programs by virtue of their relatively lower allergenicity and retained potential to promote desensitization in patients with IgE-CMA.²¹ However, to date, the data have been restricted to patients with IgE-CMA with no reactivity to BM products. Ironically, it is the most sensitive and persistent patients, who are likely to be BM reactive,²²⁻²⁵ who could benefit most from such therapy. Here, we report on the utility of BM OIT in BM-reactive patients. Only 21% of patients were able to reach the goal of a maintenance dose of 1.3 g of BM protein, a dose previously used by investigators for home maintenance in BM OIT.^{20,21} Failure to complete the protocol was mainly (8/11) caused by IgE-mediated adverse reactions, including 2 patients with anaphylactic episodes requiring intramuscular epinephrine during home maintenance.

It should be noted that during previous UM OIT, 7 of these patients required intramuscular epinephrine. However, a direct comparison of adverse reactions between our UM OIT and BM OIT programs is complicated by the purposeful slow escalation used in the latter.

In terms of the ability of BM to increase the reaction threshold for UM exposure in these patients, the success of this program must be viewed in terms of the risk/benefit ratio of such treatment. The maximum tolerated dose of UM achieved, even among the 3 patients who reached the 1.3-g BM maintenance dose, was only 900 mg of unheated CMP. It is conceivable that either a higher maintenance dose or a 1.3-g maintenance dose given for a longer period of time²¹ would allow for greater desensitization to UM. Furthermore, it remains to be determined whether BM OIT can promote the transition of patients to UM OIT. Alternatively, it

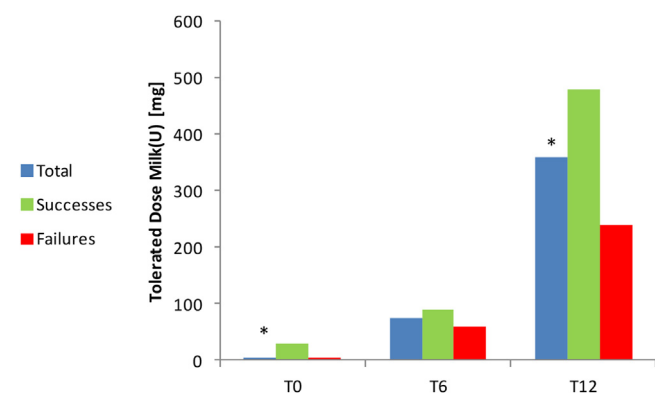


FIG 2. Desensitization to UM. The median maximum tolerated dose from OFC of UM at T₀, T₆, and T₁₂ grouped according to disposition in BM OIT after 12 months of treatment is shown. Blue column, n = 6 for all patients reaching the 12-month mark; green column, n = 3 for those successfully reaching the maintenance goal; red column, n = 3 for failures. *P = .003, Mann-Whitney U test. z = -2.8, comparing the T₁₂ and T₀ tolerated dose. Milk(U), Unheated milk.

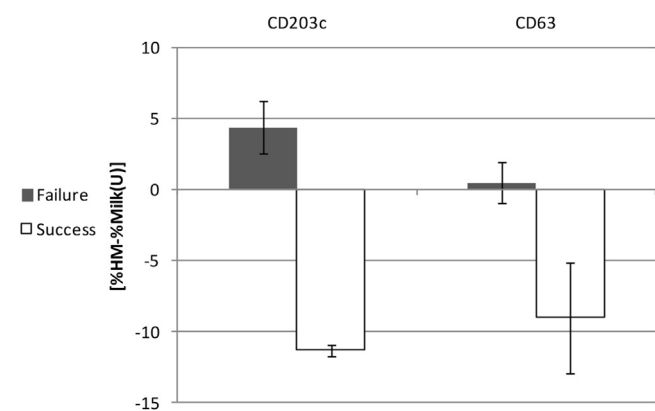


FIG 3. Relationship between disposition in BM OIT and initial differential basophil reactivity to HM and UM. T₀ basophil reactivity induced by 100 ng/mL HM subtracted by that induced by 100 ng/mL UM is presented as the mean $\pm$ SE (bars) of CD203c and CD63 expression in those successfully completing the program (Success, n = 3) and in those who stopped because of IgE-mediated reactions (Failure, n = 6). Milk(U), Unheated milk.

is quite possible that these patients represent a unique cohort of patients not amenable to any form of desensitization with whole protein.

BM is likely to be hypoallergenic in part because of changes in the higher-order structure of conformational epitopes. We noted, as have others, that for the total patient population, there were no clear differences between reactivity on the BAT assay for heated and unheated proteins.²⁶ Interestingly, however, looking only at patients who succeeded in reaching the maintenance goal of 1.3 g/d BM, a modest but significant differential response on the initial BAT assay to UM versus milk heated to 180°C for 30 minutes was noted. It is quite possible that heating for a longer duration would accentuate this difference, which is consistent with the low heat stability of whey proteins, as has been previously described.²⁷ Furthermore, a recent report demonstrates an additional matrix effect with wheat components in BM products²⁶ that was not evaluated in our HM sample, which might provide a further explanation for the low reactivity of BM products. The HM-UM BAT

difference for the patients who successfully completed the program was particularly pronounced for CD203c, a marker of piecemeal basophil degranulation, in contrast to CD63, which reflects anaphylactic degranulation.²⁸ Validation of these findings in a larger cohort of patients could provide a predictive parameter to identify BM-reactive patients who might successfully complete BM OIT.

Casein-specific IgE levels have been demonstrated to predict reactivity to BM.²² Although levels were not evaluated in this study, immunoblot analysis revealed striking decreases in IgE reactivity against casein and α -lactalbumin, which were noted in patients reaching the BM maintenance goal, and, surprisingly, an increase in some who did not reach the goal. The fact that an increase in reaction threshold was attained even for the patients with increased IgE reactivity might indicate that other physiologic compensatory mechanisms are at play. These findings support the notion that treatment with anti-IgE should provide a significant benefit for these patients to proceed in milk OIT.^{29,30} Many aspects of OIT are still under intense investigation. BM products hold great promise for use in milk OIT programs by virtue of their relatively lower allergenicity and retained potential to promote desensitization in patients with IgE-CMA.²¹ However, caution must be exercised, and further studies are warranted before BM OIT is used freely in BM-reactive subjects. The studies presented here demonstrate that BM-reactive subjects are at high risk of adverse allergic reactions throughout their course of therapy. Furthermore, even in the few selected patients who reached the BM maintenance dose, only a limited, although for these sensitive patients perhaps significant, increase in challenge threshold to UM was achieved.

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