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The safety and efficacy of sublingual and oral immunotherapy for milk allergy

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Abstract

Background—Oral immunotherapy (OIT) and sublingual immunotherapy (SLIT) are potential therapies for food allergy, but the optimal method of administration, mechanism of action, and duration of response remain unknown.

Objective—We sought to explore the safety and efficacy of OIT and SLIT for the treatment of cow's milk (CM) allergy.

Methods—We randomized children with CM allergy to SLIT alone or SLIT followed by OIT. After screening double-blind, placebo-controlled food challenges and initial SLIT escalation, subjects either continued SLIT escalation to 7 mg daily or began OIT to either 1000 mg (the OITB group) or 2000 mg (the OITA group) of milk protein. They were challenged with 8 g of milk protein after 12 and 60 weeks of maintenance. If they passed the 60-week challenge, therapy was withdrawn, with challenges repeated 1 and 6 weeks later. Mechanistic correlates included end point titration skin prick testing and measurement of CM-specific IgE and IgG_4 levels, basophil histamine release, constitutive CD63 expression, CD203c expression, and intracellular spleen tyrosine kinase levels.

Results—Thirty subjects with CM allergy aged 6 to 17 years were enrolled. After therapy, 1 of 10 subjects in the SLIT group, 6 of 10 subjects in the SLIT/OITB group, and 8 of 10 subjects in the OITA group passed the 8-g challenge ($P = .002$, SLIT vs OIT). After avoidance, 6 of 15

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subjects (3 of 6 subjects in the OITB group and 3 of 8 subjects in the OITA group) regained reactivity, 2 after only 1 week. Although the overall reaction rate was similar, systemic reactions were more common during OIT than during SLIT. By the end of therapy, titrated CM skin prick test results and CD63 and CD203c expression decreased and CM-specific IgG_4 levels increased in all groups, whereas CM-specific IgE and spontaneous histamine release values decreased in only the OIT group.

Conclusion—OIT was more efficacious for desensitization to CM than SLIT alone but was accompanied by more systemic side effects. Clinical desensitization was lost in some cases within 1 week off therapy.

Keywords

Food allergy; immunotherapy; milk allergy; basophil; spontaneous histamine release

More than a decade after research into subcutaneous immunotherapy for food allergy was halted because of severe side effects,[1] both oral immunotherapy (OIT) and sublingual immunotherapy (SLIT) show promise as potential therapies for food allergy, but which method is superior is not known. Theoretically, SLIT might be safer than OIT but still efficacious because the mouth has a high density of tolerogenic antigen-presenting cells[2] and because therapeutically important allergen epitopes might be digested by the stomach.[3] Conversely, because OIT doses need not be held in the mouth, much higher doses can be given, which might increase risk but could also be required for maximum efficacy. Both methods have been tried with some success (ie, SLIT for hazelnut,[4] milk,[5,6] peanut,[7] and fruit[8,9] allergies and OIT for peanut,[10-12] egg,[13,14] and milk[6,15-17] allergies), but to our knowledge, they have not been compared head to head. Because studies of immunotherapy are long and potentially risky, it is essential that we identify the best methods to pursue as early as possible.

In addition to which method shows superior clinical outcomes, it is also not known whether these clinical changes represent true long-term tolerance or temporary desensitization. Whether and how fast clinical reactivity is regained after cessation of oral allergen exposure has far-reaching implications for whom and under which conditions this therapy is appropriate.

In this exploratory study we randomized 30 children with persistent IgE-mediated cow's milk (CM) allergy to either SLIT or SLIT followed by OIT to assess (1) the safety and efficacy of these methods, (2) the effect of withdrawal of therapy for 1 and 6 weeks, and (3) mechanistic changes associated with therapy, including changes in CM-specific IgE and IgG_4 antibody levels, end point titration skin test responses, and basophil function and intracellular signaling.

METHODS

Study design

This was an open-label randomized human study of SLIT and OIT for the treatment of milk allergy. The primary end point of the study is the proportion of subjects with a clinical response, which was defined as the ability to tolerate at least 10-fold more milk protein during food challenge compared with baseline, after approximately 15 months of maintenance treatment. Key protocol-defined secondary end points include (1) the proportion of patients who maintain desensitization after 1 and 6 weeks off therapy, (2) differences in clinical response rates between different study arms, (3) the incidence of

serious and common adverse events and comparisons between the groups, and (4) changes in biological markers.

Subject recruitment

Subjects 6 to 21 years of age were recruited from the Johns Hopkins Hospital and Duke University Medical Center pediatric allergy clinics. The study was conducted with investigational new drug approval from the US Food and Drug Administration. The institutional review boards at each institution provided ethics approval. Informed consent was obtained for each subject.

Subject selection

Inclusion criteria included a documented history of CM allergy, a CM-specific IgE level of greater than 0.35 kUa/L, a positive skin prick test response to CM (>3-mm wheal), and a positive double-blind, placebo-controlled food challenge (DBPCFC) result to CM at baseline, which was defined as clear signs or symptoms (eg, rash, upper or lower respiratory tract symptoms, vomiting, and signs or symptoms of hypotension) at a cumulative dose of less than 2.5 g of CM protein. Exclusion criteria were severe persistent asthma, use of greater than 400 μg/d fluticasone or fluticasone equivalent if 12 years old or younger or greater than 600 μg/d if older than 12 years, a history of intubation for asthma, other significant nonallergic medical problems, and a history of a severe episode of anaphylaxis to CM, which was defined as a reaction with hypoxia, incontinence, syncope, or requirement for admission to the intensive care unit.

Study protocol

For an illustration of the study protocol, see Fig 1.

Initial dosing

After eligibility was determined based on DBPCFC and screening results, all subjects began therapy with an initial SLIT dose escalation day (see the Methods section in this article's Online Repository at www.jacionline.org for more details about study visits and the study medication). Subjects started with low-dose SLIT in an attempt to decrease the risk of reactions during dosing. The highest tolerated dose was continued at home daily, with subjects noting the dose, date, time taken, symptoms, and medication use in a daily home diary.

Continued escalation

Subjects returned at 1- to 2-week intervals for dose increases (see Table E1 in this article's Online Repository at www.jacionline.org). After a minimum of 4 weekly SLIT escalations to a dose of 3.7 mg (T2), they were randomized in equal numbers into one of 3 groups: 2 that crossed over to OIT with a target dose of either 2 g (the OITA group: minimum of 19 updosing visits total) or 1 g (the OITB group: minimum of 19 updosing visits) and 1 that continued on SLIT with a goal dose of 7 mg (minimum of 7 updosing visits). Randomization was done with a computerized block-stratified randomization program using a block size of 6. After the target dose was reached (T3), it was continued for 12 more weeks until the next DBPFC (T4). After T4, subjects in the OIT arms had the opportunity to increase their daily maintenance dose to one half the tolerated dose (if greater than their target dose). All subjects continued on a daily maintenance dose for 48 weeks and then underwent an open oral food challenge (OFC; T5) to 8 g of CM protein. If the full challenge was passed without symptoms that required medication, the therapy was stopped and a repeat OFC was done after 1 week off therapy (T6). If the subject passed the 1-week OFC, another OFC was done 5 weeks later (T7), for a total of 6 weeks off therapy.

Mechanistic correlates

At T1, T3 to T5, and T7, blood was collected for measurement of CM-specific IgE and IgG_4 antibody levels, basophil histamine release (HR), constitutive CD63 expression, CD203c expression, and intracellular spleen tyrosine kinase (Syk) levels. At T2, basophil studies were done, and plasma was analyzed for serologic measures in some participants. End point skin prick testing to CM was done at T1, T3 to T5, and T7. Details are shown in the Methods section in this article's Online Repository.

Statistical analysis

Differences between SLIT versus OIT and OITA versus OITB in fold-increase food challenge threshold were evaluated by using Mann-Whitney *U* tests. Binary outcomes were evaluated by using χ^2 tests. Incidence rate ratios (IRRs) were generated by using negative binomial analysis with generalized estimating equations to compare the rates of adverse effects across different types of doses. Laboratory outcomes were evaluated by using linear regression models with generalized estimating equations to account for repeated measures over time. Because of the small sample size, we combined the OIT groups for these analyses. All groups were combined at T2 because treatment was identical at this point.

Subjects in the OIT groups were divided into those who passed the final food challenge (tolerant) and those who failed one of the challenges at T5 to T7 or withdrew (not tolerant). Differences in laboratory markers between these groups at T1 and T5 were evaluated by using Mann-Whitney *U* tests. When appropriate, variables were log-transformed for analysis. Analyses of HR to peanut were restricted to those subjects who had a peanut-specific IgE level of greater than 0.35 kUa/L. All analyses were performed with STATA/SEv11 software (StataCorp, College Station, Tex).

RESULTS

Study participants

Thirty-three participants underwent screening DBPCFCs. Two subjects failed screening because they tolerated at least 2.5 g at the initial DBPCFC. One subject declined to continue in the study after the food challenge. Thirty subjects (24 at Johns Hopkins and 6 at Duke) were randomized (Table I). Twenty-eight subjects completed the study and underwent OFC after 60 weeks of maintenance. Two subjects withdrew during therapy because of adverse events. One, in the OITB group, withdrew during dose escalation at a dose of 490 mg because of persistent eczema exacerbation. The other, in the OITA group, withdrew during maintenance because of concerns for therapy-induced eosinophilic esophagitis. Endoscopy and biopsy results were consistent with reflux esophagitis, which persisted even after avoidance of milk; this was thought to be unrelated to study treatment.

The time to complete dose escalation ranged from 8 to 14 weeks in the SLIT/SLIT group (median, 10 weeks) and 20 to 41 weeks in the SLIT/OIT group (median, 28 weeks), with differences related both to participants' scheduling constraints, the need to delay or repeat escalation because of symptoms, and manufacturing delays.

Challenge threshold after therapy

The food challenge threshold increased in all subjects who completed the full maintenance period (Fig 2). After 12 weeks of maintenance (T4), the median fold increase was 7-fold for the SLIT group, 64-fold for the OITB group, and 79-fold for the OITA group. After the full 60 weeks of maintenance (T5), the median increase was 40-fold for subjects in the SLIT/SLIT group, 159-fold for subjects in the SLIT/OITB group, and 54-fold for subjects in the SLIT/OITA group. The challenge threshold increased by at least 10 times baseline at T5 for

60% of those randomized to SLIT and for 90% in each of the OIT groups ($P = .053$, SLIT vs OIT).

Desensitization versus tolerance

For more information on desensitization versus tolerance, see Table II. At T5, 1 of 10 subjects in the SLIT/SLIT group, 6 of 10 subjects in the SLIT/OITB group, and 8 of 10 subjects in the SLIT/OITA group were not reactive to the entire 8-g challenge and were taken off all therapy ($P = .002$, SLIT vs OIT; $P = .33$, SLIT/OITA vs SLIT/OITB). After 1 week off therapy (T6), 2 subjects, both in the SLIT/OITB group, reacted on food challenge (1 at 4140 mg and 1 at 8140 mg). Both required epinephrine for abdominal pain/vomiting and urticaria/flushing. After 6 weeks off therapy (T7), an additional subject in the SLIT/OITB group and 3 subjects in the SLIT/OITA group failed the challenge, 1 at 2540 mg, 1 at 6140 mg, and 2 at 8140 mg. Symptoms included hives (1 subject), rhinorrhea (3 subjects), abdominal pain (3 subjects), and wheeze/dyspnea (1 subject); 1 subject was given epinephrine. This left 1 of 10 subjects in the SLIT/SLIT group, 3 of 10 subjects in the SLIT/OITB group, and 5 of 10 subjects in the OITA group who were considered tolerant ($P = .09$, SLIT vs OIT; $P = .36$, SLIT/OITA vs SLIT/OITB).

Symptoms with dosing

For more information on symptoms with dosing, see Table III. There were symptoms with 1,802 (29%) of 6,246 SLIT doses and 2,402 (23%) of 10,645 OIT doses. Taking into account intrapersonal correlation, there were no significant differences in the rate of total, oral, or skin symptoms between SLIT and OIT ($P = .73$, .70, and .50, respectively). However, compared with SLIT dosing, during OIT, there were significantly more multisystem (IRR, 11.5; $P < .001$), upper respiratory tract (IRR, 4.7; $P = .004$), gastrointestinal (IRR, 3.3; $P = .01$), and lower respiratory tract (IRR, 8.9; $P < .001$) symptoms, as well as more need for β-agonists (IRR, 8.6; $P < .001$) and antihistamines (IRR, 8.2; $P < .001$). There were no significant differences in the rates of any type of reactions or medication use between the OITA and OITB doses (data not shown). Epinephrine was given twice after subjects receiving SLITaspirated the dose and 4 times during OIT dosing, twice in conjunction with possible accidental ingestions at home during updosing, once during maintenance, and once during office updosing.

Serologic and skin prick test measures

For more information on serologic and skin prick test measures, see Fig 3 and Table E2 in this article's Online Repository at www.jacionline.org. CM-specific IgE levels increased at T2 ($P = .04$) and then significantly decreased by T5 in the combined OIT group ($P < .001$) but not in the SLIT group ($P = .09$ and $P = .03$, SLIT vs OIT). CM-specific IgG_4 levels increased from T1 to T3 in all groups ($P < .001$) and remained high. By T3, the increase was greater in the subjects receiving OIT than in the subjects receiving SLIT ($P = .001$, SLIT vs OIT) but was not different by T4 ($P = .52$ at T4 and $P = .45$ at T5). Skin prick test reactivity to CM decreased in all groups by T3 ($P < .05$ for both OIT and SLIT), without significant differences between the groups ($P = .15$, SLIT vs OIT) and remained less than baseline values in all groups ($P < .001$).

Basophil activity

For more information on basophil activity, see Fig 4. At baseline, basophils from 18 of 24 subjects exhibited high spontaneous histamine release (SHR), which was defined as greater than 10% HR in the absence of stimulation (median, 28% [range, 11% to 79%] of total histamine content). SHR did not change withtreatment in the SLIT/SLIT group at any time but significantly decreased in the combined SLIT/OIT group by T3 ($P < .007$) and remained

lower at T4, T5, and T7 compared with baseline values ($P = .02$, $P = .03$, and $P = .02$, respectively). HR to CM, peanut, or N-formylmethionine did not change significantly at any of the time points tested ($P > .1$ for all time points for each variable), and HR to anti-IgE actually increased in the SLIT/OIT group by T5 compared with baseline values ($P = .04$ at T5 and $P = .005$ at T7). No change in basophil Syk expression was observed at any point.

Constitutive expression of CD203c increased transiently at T2 in all groups ($P < .01$) and then decreased compared with baseline values at T5 in both the SLIT/SLIT and SLIT/OIT groups ($P = .004$ for both) and remained low ($P = .001$ at T7 for the OIT group). Constitutive CD63 expression decreased at T5 only in the SLIT/SLIT group ($P = .01$), although, statistically, there was no difference between the groups in changes in either CD63 or CD203c expression ($P > .15$ for all interaction terms).

DISCUSSION

In this study we explored several vital questions about food allergy immunotherapy. The first, whether and how long the clinical effects of immunotherapy last when exposure ceases, informs the risk-benefit analysis for these treatments and offers important lessons about how immunotherapy might need to be conducted to be safe. The second, comparing the safety and efficacy of SLIT and OIT methods, underscores the differences and limits of currently available forms of therapy. Finally, our laboratory studies shed light on the mechanism of immunotherapy.

Until now, there have been sparse data about whether mucosally delivered immunotherapy for food allergy leads to a permanent state of tolerance or whether the effects represent temporary desensitization. Two small studies of egg immunotherapy offered different answers to this question in studies of very different lengths. Two of 4 subjects regained reactivity 3 months off therapy in one study[13] compared with no regaining of reactivity after 1 month in the other study.[14] In our current study we found that 6 of the 15 subjects who passed a full milk challenge after 60 weeks of maintenance lost desensitization within 6 weeks. It is reassuring that even after avoidance, the lowest reaction threshold was equivalent to about 2.5 oz of milk, which is well above most accidental exposures, and most did not react until a full cup of milk. In contrast, at the start of therapy, the median food challenge threshold was less than 1 teaspoon. However, it is clear that for most subjects treated with the doses and times in this study, the posttreatment clinical state is far from nonallergic. It is also important to note that 13 other subjects were still reactive after therapy and were therefore not eligible to participate in this test of tolerance. In all likelihood, they would have been much more prone to losing the desensitization that they had achieved with the withdrawal of therapy. Whether a desensitized but not tolerant outcome justifies the risk and effort of immunotherapy needs to be debated.

Two of the subjects regained reactivity within just 1 week, shedding further light on this quandary. The 1-week time point was chosen because we would expect that most patients would avoid an oral allergen for at least 1 week at some point whether because of illness, travel, or aversion to the food. If reactivity can return within this short period, as we demonstrate here, patients will need to maintain strict adherence to inclusionary diets for an indefinite period of time. Doing this might be less difficult for milk and egg for which we expect, but do not conclusively know, that cooked forms will maintain desensitization. For peanut, however, indefinite daily consumption might be impossible. In a study of children with naturally resolved peanut allergy, Fleischer et al[18] found that only 7% ate peanut daily and only 31% ate it at least once per week, likely because of persistence of their learned aversion to peanut. We continue to see this problem in patients with resolved peanut allergy, even when these diets are explicitly recommended. Therefore even after apparently

successful immunotherapy, patients might be at high risk for allergic reactions if they do not consume the allergen regularly but feel falsely protected from accidental exposures.

However, it might be that with longer treatment we will find longer-term resolution. For venom and aeroallergen immunotherapy, at least 3 years of treatment is usually required to have long-lasting effects,[19,20] and OIT might have similar require-ments. In a study of peanut OIT, IgE levels continued to decrease at 30 months of treatment.[10] Although cumbersome, we will need longer-term studies to evaluate this hypothesis.

The second novel feature of this study was the direct comparison of SLIT and OIT. Here we sought to minimize the rate of adverse reactions by initiating therapy with SLIT and then to determine whether subsequent treatment with SLIT or OIT might be most promising for future use. At the doses we were able to use, given the limitations of SLIT dosing imposed by both volume and concentration, SLIT followed by OIT was much more effective at desensitization than SLIT alone but was accompanied by an increased risk of systemic side effects. Only 1 of 10 subjects treated with SLIT was able to consume a full serving of milk without symptoms after therapy compared with 14 of 20 subjects treated with SLIT/OIT. However, multisystem reactions were more than 11 times more likely with OIT. The difference in efficacy and safety between the groups is likely related to the dose of allergen given; the cumulative dose that the subjects in the SLIT group received was at least 140-fold lower than the minimum cumulative OIT dose.

Higher SLIT doses might have improved efficacy[4,5] but will require more concentrated forms of therapy. We administered the highest volume we thought practical, 700 μL 3 times, but only achieved 7 mg of CM protein daily. Even this small volume was too much for some subjects: 2 subjects aspirated the liquid and required epinephrine for lower respiratory tract symptoms, a complication of SLIT with liquid extracts, which should be noted. Potentially, multiple doses per day might be more effective.[21] However, given that exercise after dosing is a concern,[15,22] this option might not be feasible for children.

We also examined 2 OIT maintenance doses and did not find significant differences in either the overall rate of reaction or efficacy between these regimens. However, we lacked the power to detect relatively small differences between these regimens, and there was a trend toward improved efficacy with the higher OIT doses. Previous studies have used a wide range of OIT doses[5,6,15,16,23] and protocols, making historical comparisons difficult. Given the suggestion of increased efficacy, it seems reasonable to aim for higher doses with OIT.

The mechanisms of action of specific mucosal immunotherapy remain unclear. Similar to what has been found with SLIT using inhalant allergens,[19,20] we found that CM-specific IgE antibody levels increased early in dose escalation. In the subjects receiving OIT, CM-specific IgE levels subsequently decreased compared with baseline values but remained high throughout the study. Thus deletion of CM-specific plasma cells and decreases in IgE levels are unlikely to be the principal mechanisms of clinical improvement over the interval of this study. The decrease in CM-specific IgE levels was associated with a decrease in SHR, but the clinical significance of this is not clear. IgG_4 antibody has been shown to suppress IgE-mediated mast cell and basophil degranulation by competing with IgE for binding to allergen and by causing coligation of FcεRI and FcγRIIB.[24] CM-specific IgG_4 antibody levels did increase, but these changes did not correlate with clinical outcome (see Results and Fig E1 in this article's Online Repository). However, we had limited power to examine predictors of tolerance and other clinical outcomes.

Many studies have focused on the basophil as a key cell type underlying clinical desensitization. Decreased expression of basophil activation markers, specifically CD63, which is often used as a surrogate for HR,[25,26] after allergen stimulation has been observed

in children undergoing immunotherapy for food allergy.[10] Despite the overall favorable clinical outcomes in this study, we did not observe any significant decrease in allergenor anti-IgE–induced basophil HR at any point, suggesting that immunotherapy did not induce a state of intrinsic basophil suppression. This is consistent with the observed lack of change in expression of Syk, a tyrosine kinase that functions as a key determinant of HR.[27] Prior reports of reduced allergen-induced CD63 expression with immunotherapy used whole-blood assays,[10,28] in contrast to our washed suspensions. We hypothesize these washes might have disrupted binding of IgG or another serum inhibitory factor, which could account for the discrepant results. Interestingly, constitutive expression of CD63 and CD203c did decrease significantly with therapy, potentially suggesting a state of reduced extrinsic basophil activation *in vivo*.

CD203c expression on basophils increased very early in therapy. High constitutive CD203c expression has been associated with clinical inflammation, such as that found with asthma exacerbations.[29] We found that subjects who had a greater increase in CD63 and CD203c expression early in therapy had statistically significantly poorer outcomes later in the study, suggesting a potential early marker of clinical response. However, no marker at the time before therapy was withdrawn distinguished those who were tolerant from those who were not. For more details, see Fig E1 and the Results section in this article's Online Repository.

There are several limitations to this study. In the absence of a placebo, we cannot know how many subjects would have spontaneously improved. However, over the time frame of this study, we would not expect resolution of CM allergy in subjects of this age who reacted at the required baseline challenge dose. A previous placebo-controlled study of CM immunotherapy in subjects drawn from our same clinical population did not find any improvement from baseline.[15] This study was also not designed to fully assess whether starting with SLIT increased safety on transition to OIT. Future studies should compare various initial dose escalation schemes for immunotherapy and different durations of therapy. An additional potential limitation is that we do not know whether subjects who tolerated a full challenge to 8 oz of milk would react at higher doses. Also, we cannot account for day-to-day variation in food challenge threshold.

In summary, in this study we demonstrated that although most patients with even severe milk allergy can be desensitized to milk with OITand SLIT, tolerance is elusive for most after 60 weeks of maintenance. In some subjects desensitization can be lost within 1 week of allergen avoidance, and we lack reliable surrogate markers to predict those who will lose desensitization. Moreover, although most of the adverse events experienced by study participants were mild, there were occasional multisystem reactions, even at doses that had been tolerated for some time without reaction, as has been previously described.[12,15,30,31] Taken to-gether, these findings raise fundamental questions about whether and how immunotherapy should be conducted and emphasize that more work needs to be done before these therapies are ready for the general allergy clinic.

METHODS

Details of study visits and procedures

Screening—The screening visit at T1 included a detailed history, pulmonary function testing, end point skin prick testing, phlebotomy, and physical examination, followed by a DBPCFC to CM.

Food challenges—Food challenges were prepared by the study nutritionist using either CM powder or placebo powder (Prophree; Abbott Nutrition, Columbus, Ohio). Placebo or CM powder was mixed with the subject's choice of vehicle (rice milk with chocolate or

strawberry syrup, applesauce, or orange juice) in random order. All vehicles were chosen to mask the content of the challenge. Challenges were performed in the research unit by a study physician who was blind to the content of the challenge. The challenge doses were 0.1, 1, 10, 40, 100, 400, 800, and 1200 mg, for a cumulative dose of 2551.1 mg of CM protein for the first challenge, and 40, 100, 400, 800, 1200, 1600, 2000, and 2000 mg, for a cumulative dose of 8140 mg for subsequent challenges. Doses were given every 15 minutes. Challenges were stopped when clear symptoms of an allergic reaction developed, including rash, upper or lower respiratory tract symptoms, abdominal pain and vomiting, or signs or symptoms of hypotension.

Study medication—Sublingual dosing was with glycerinated CM allergenic extract provided by Greer Laboratories in prefilled vials that dispensed a set amount of liquid per squirt. Subjects were instructed to hold the set number of squirts (up to 5 squirts at a time ×3) under the tongue for 2 minutes without swallowing. On the initial escalation day, the first dose was 1.67×10^{-6} mg, with a goal of 0.067 mg. Doses were given at 30-minute intervals. Subsequent doses are detailed in Table E1.

Oral dosing was with nonfat CM powder provided by Greer Laboratories that was distributed in individual-dose vials. The powder was mixed with a food vehicle of the subject's choice at the time of dosing. See Table E1 for the dosing schedule.

Subjects were instructed to take the daily dose at the same time each day and to abstain from vigorous exercise for at least 2 hours after taking a dose. A study physician was available 24 hours a day by telephone. Each subject maintained an emergency kit at all times with self-injectable epinephrine. Participants who experienced more than a mild reaction at home decreased their dose to the previously tolerated dose and re-escalated under observation.

Instructions after treatment—After the study, subjects who reacted to more than 4 g of milk protein at the final challenge on treatment were prescribed an individually determined minimum and maximum dose of dietary milk to be maintained in their diet. Subjects who were fully desensitized but subsequently reacted to less than 4 g of milk protein after avoidance were given the opportunity to re-escalate with milk powder.

Over the course of the study, as it became apparent that clinical outcomes were better with OIT than with SLIT, the protocol was changed with US Food and Drug Administration and institutional review board approval to allow subjects in the SLIT/SLIT arm who reacted to less than 4 g of milk protein at the final challenge to re-escalate with OIT. The results of re-escalation are not presented here.

Laboratory measures

Measurement of milk IgE and IgG levels—Blood for serologic measurements was collected from all subjects at T1, T3 to T5, and T7. Plasma collected at T2 was also analyzed in 21 subjects at Johns Hopkins. CM-specific IgE and IgG_4 levels were measured with the ImmunoCAP-System FEIA (Phadia Diagnostics, Uppsala, Sweden). The sum of α-lactalbumin, β-lactoglobulin, and casein-specific IgG_4 antibody levels were used as a surrogate for IgG_4 anti-CM antibody to avoid the problem of interference with the IgG antibodies specific for bovine albumin found in most sera.[E1] Because of concerns about interference with IgG_4 anti-CM, CM-specific IgE was additionally run by using ImmunoCap at a dilution of 1:20. For samples with a coefficient of variation of greater than 20%, the diluted sample value was used. The lower limit of detection used in this study was 0.1 kUa/L for IgE assays and 0.1 mg/mL for IgG_4 assays.

End point titration skin prick testing—End point titration skin prick testing was performed with the Greer Pick by using a commercial 1:20 wt/vol CM extract (Greer Laboratories) diluted with albumin saline containing phenol to dilutions of 1:1, 1:10, 1:100, 1:1,000, and 1:10,000. The response variable was the average of all 5 dilution wheal sizes.

Basophil HR—Peripheral blood was collected from subjects at Johns Hopkins (24 total, 8 in each randomization group) in EDTA at T1 to T5 and T7. Blood was subjected to double Percoll (Pharmacia Biotech, Piscataway, NJ) density centrifugation, as previously described.[E2] The lower fraction of cells using this protocol consists of basophil-enriched mononuclear cells (BECs) that were washed once in piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES)/albumin/glucose and then again in column buffer (PIPES containing 1% BSA and 2 mmol/L EDTA). Basophils were counted after staining with Alcian blue. BECs (20,000 basophils per condition) were then cultured in a final volume of 50 μL in conditioned Iscove modified Dulbecco medium (Invitrogen Life Technologies, Carlsbad, Calif) supplemented with 5% FCS (Invitrogen Life Technologies), 1× nonessential amino acids (Invitrogen Life Technologies), and 10 μg/mL gentamicin (Invitrogen Life Technologies), pH 7.2 to 7.4. Cells were stimulated with crude CM extract (10 μg/mL; Greer Laboratories), crude peanut extract (10 μg/mL, Greer Laboratories), polyclonal goat anti-human IgE antibody (100 ng/mL, generated in house), or N-formylmethionine (10^{-6} mol/L; Sigma Chemical Co, St Louis, Mo). After a 45-minute incubation at 37°C, 1 mL of PIPES/albumin/glucose was added, and HR was measured in the cell-free culture supernatants by using automated fluorometry.[E3] Percentage release under each condition was calculated relative to total histamine content, which was determined by treating an identical number of BECs with perchloric acid (1.6% final). SHR was subtracted from values reporting HR in response to CM, peanut, anti-IgE, and N-formylmethionine. Results from 1 subject, a nonresponder whose basophils did not produce histamine after stimulation with anti-IgE, were excluded from our analysis.

Flow cytometry—At each time point, an aliquot of BECs was fixed in 4% buffered paraformaldehyde (Sigma Chemical Co) and stored in 10% dimethyl sulfoxide/PBS (Sigma Chemical Co) at –80°C. BECs from all time points were then analyzed simultaneously for constitutive expression of CD63 and CD203c by using flow cytometry. Cells were first washed in PBS and then blocked with human IgG (1 mg/mL, Sigma Chemical Co). The following antibodies were used: CD63-allophycocyanin (AbD Serotec, Raleigh, NC), CD203c-allophycocyanin (Miltenyi Biotech, Bergisch Gladbach, Germany), CD123-phycoerytrhin (BD PharMingen, San Jose, Calif), and BDCA2-fluorescin isothiocyanate (Miltenyi Biotech). Stained cells were analyzed with a FACSCalibur (BD PharMingen). Basophils were identified by gating on cells that were $CD123^+$ and $BDCA2^-$, and mean fluorescence intensity (MFI) for CD63 and CD203c was determined by using CellQuest software (BD PharMingen). Measurement of Syk protein expression by means of intracellular flow cytometry was done with the anti-Syk antibody 4D10 (Santa Cruz Biotechnology) on freshly prepared BECs, as previously described.[E4,E5] Instrument variability was corrected for by using CaliBRITE allophycocyanin calibration beads (BD PharMingen). MFI values are reported after subtraction of MFI from appropriate isotype controls.

RESULTS

Laboratory correlates of outcome

For more information on laboratory correlates of outcome, see Fig E1. To determine whether any laboratory markers correlated with outcome within the OIT-treated subjects, we separated them into 2 groups: tolerant (defined as passing the final challenge after 6 weeks

of avoidance) and not tolerant. At baseline, CM-specific IgE levels were significantly lower in those who became tolerant, although there was substantial overlap between the groups (median CM-specific IgE level of 20.7 kUa/L [range, 1.19-88.3 kUa/L] in those who became tolerant compared with 74.5 kUa/L [range, 10.0-226 kUa/L] in those who did not become tolerant, $P = .03$). No other biomarker at baseline predicted outcome, including CM-specific IgG_4 levels ($P = .94$), CM end point skin prick test titration ($P = .37$), basophil CD63 expression ($P = .13$), CD203c expression ($P = .87$), SHR ($P = .26$), and milk-induced HR ($P = .95$). Similarly, except for CM-specific IgE levels, which showed a trend toward being lower in those who became tolerant at T5 ($P = .08$), no other marker at T5 discriminated between those who were tolerant and not tolerant, including CM-specific IgG_4 levels ($P = .13$), CM end point skin prick test titration ($P = .33$), basophil CD63 expression ($P = .38$), CD203c expression ($P = .66$), SHR ($P = .77$), or CM-induced release ($P = .19$; see Fig E1).

There were some differences in changes in basophil markers with therapy between the groups. Subjects who did not become tolerant had early increased basophil activation that was not seen in subjects who became tolerant with therapy. Both constitutive CD63 and C203c expression increased during SLIT build-up in those who did not become tolerant ($P < .001$ and 5 .001, respectively, T2 vs T1); CD203c expression did not change in those who became tolerant ($P = .34$), and CD63 expression actually decreased by T2 in those who became tolerant ($P = .01$). This difference between the groups at T2 was statistically significant ($P < .001$ and $P = .001$ for the respective interaction terms). There were no differences in changes in CM-specific IgE level, CM-specific IgG_4 levels, or skin prick test responses between the groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

BEC	Basophil-enriched mononuclear cell
DBPCFC	Double-blind, placebo-controlled food challenge
CM	Cow’s milk
HR	Histamine release
IRR	Incidence rate ratio

MFI	Mean fluorescence intensity
OFC	Oral food challenge
OIT	Oral immunotherapy
PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)
SHR	Spontaneous histamine release
SLIT	Sublingual immunotherapy
Syk	Spleen tyrosine kinase

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Clinical implications

Both SLIT and OIT showed efficacy for the treatment of CM allergy, with increased efficacy but systemic reactions seen with OIT. Clinical desensitization was lost in as little as 1 week.

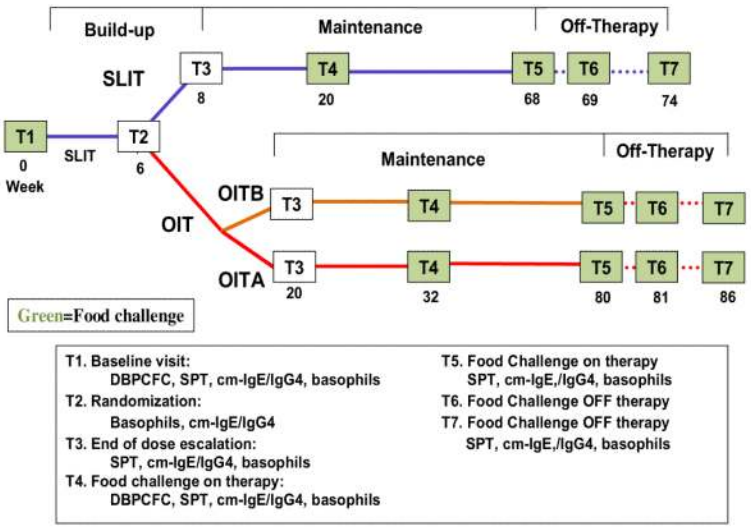

FIG 1.

Study timeline with key features highlighted. Subjects were randomized at T2 to either SLIT (goal dose, 7 mg), OITB (goal dose, 1000 mg), or OITA (goal dose, 2000 mg). CM-specific IgE, CM-specific IgG_4, and CM-titrated skin prick test (*SPT*) results were obtained in all subjects at T1, T3 to T5, and T7. Basophil studies were done at T1, T2 to T5, and T7.

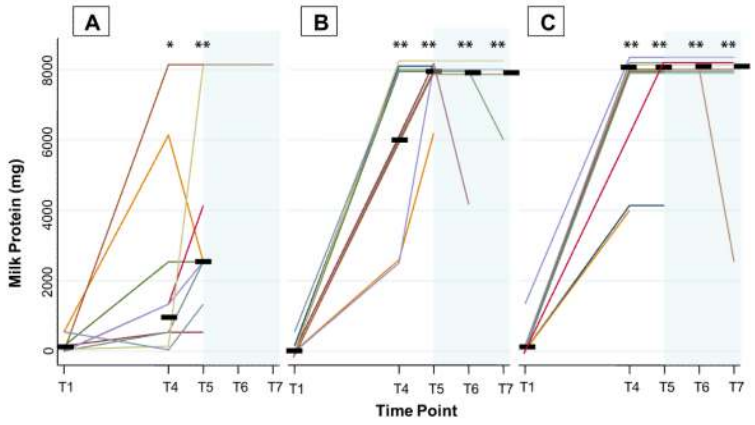

FIG 2.

Food challenge outcome. The CM protein threshold is shown for baseline (T1), after 12 weeks of maintenance (T4), after 60 weeks of maintenance (T5), and 1 week (T6) and 6 weeks (T7) off therapy. **A**, SLIT/SLIT group. **B**, SLIT/OITB group. **C**, SLIT/OITC groups. *Bars* represent medians. **P* < .05 and ***P* < .01 compared with T1.

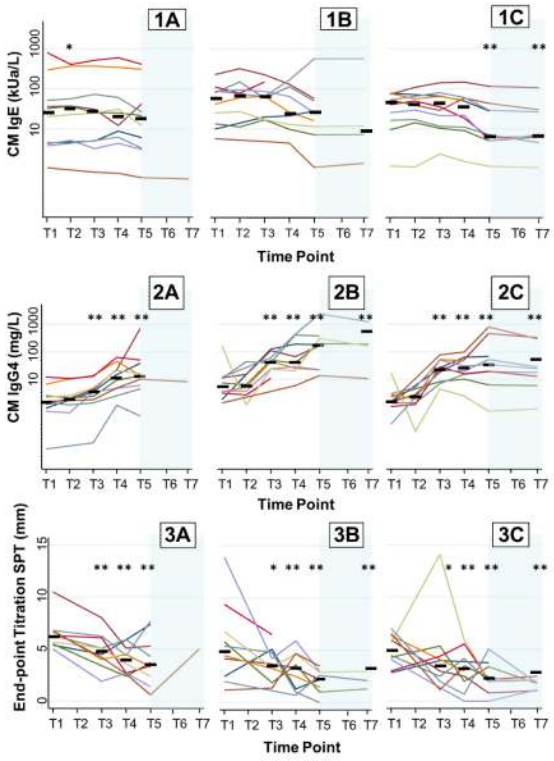

FIG 3.

Mechanistic changes. Individual line plots showing changes with treatment for the 3 randomization groups (**A**, SLIT/SLIT group; **B**, SLIT/OITB group; and **C**, SLIT/OITA group) in CM-specific IgE level (*1*), CM-specific IgG_4 levels (*2*), and end point titration skin prick test responses. Shown is the average wheal size over 5 CM dilution. *Bars* represent medians. The shaded blue area represents the period of withdrawal of therapy. $*P < .05$ and $**P < .01$ compared with T1.

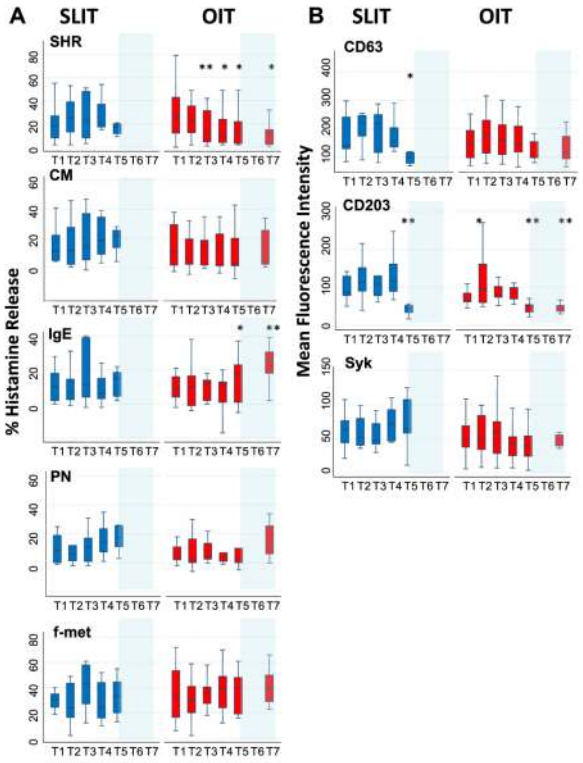

FIG 4.

Basophil activity. A, HR in media alone (SHR) or after stimulation with CM extract, goat anti-human IgE antibody (IgE), peanut extract (*PN*), or N-formylmethionine (*f-met*) at T1 to T5 and T7. **B**, Constitutive surface expression of CD63 and CD203c and intracellular Syk by basophils at T1 to T5 and T7. A *box-and-whisker plot* is shown; outliers are not shown. $*P < .05$ and $**P < .01$ compared with T1.

TABLE I

Baseline characteristics

Characteristic	SLIT group	OITB group	OITA group
No.	10	10	10
Sex			
Male	4 (40%)	7 (70%)	7 (70%)
Age (y), median (range)	8 (6-11)	9 (6-15)	8 (6-16)
CM-specific IgE (kUa/L), median (range)	23 (1.1-572)	47 (3.9-226)	39.85 (1.2-77.2)
CM SPT wheal (mm), median (range)	11 (7-33)	10 (6-22)	9 (6-18)
History of anaphylaxis to CM	8 (80%)	9 (90%)	6 (60%)
Food challenge threshold (mg), median (range)	101 (1-551)	31.1 (1.1-551.1)	81.1 (11.1-1351)
Other allergic diseases			
Other food allergies	9 (90%)	8 (80%)	6 (60%)
No. (range)	4 (0-7)	2 (0-6)	4 (0-7)
Asthma	8 (80%)	9 (90%)	9 (90%)
Atopic dermatitis	10 (100%)	6 (60%)	6 (60%)

No significant ($P < .05$) differences are shown between any groups.

SPT, Skin prick test.

TABLE II

Clinical outcomes

Group	SLIT/SLIT	SLIT/OITB	SLIT/OITA
Withdrew	0	1	1
Failed full desensitization challenge (T5)	9	3	1
Failed challenge 1 wk off therapy (T6)	0	2	0
Failed challenge 6 wk off therapy (T7)	0	1	3
Considered tolerant	1	3	5
Total no.	10	10	10

TABLE III

Symptoms with dosing

	Symptoms (% doses)							Medications used (% doses)	
Type of dose (no. of doses)	**Total**	**Oral**	**GI**	**Skin**	**Upper**	**Lower**	**Multisystem**	**Antihistamine**	**β-Agonist**
SLIT escalation (2021)	30.28%	26.82%	2.97%	2.23%	0.59%	0.45%	0.10%	1.43%	0.35%
SLIT maintenance (4205)	28.25%	27.99%	0.38%	0.10%	0.07%	0.02%	0.02%	0.52%	0.05%
Initial OIT escalation (1842)	36.21%	29.64%	7.17%	1.79%	1.41%	2.17%	0.71%	11.13%	1.52%
OITB escalation and maintenance (1230)	30.41%	24.07%	7.97%	0.73%	1.30%	2.28%	0.08%	3.50%	2.03%
OITA escalation and maintenance (1396)	26.72%	21.99%	3.15%	1.72%	2.79%	1.00%	0.43%	11.03%	1.50%
Post dose-adjustment maintenance (6029)	16.42%	12.71%	2.82%	1.68%	0.53%	1.01%	0.68%	4.01%	0.81%

Epinephrine was used for 2 SLIT doses and 4 OIT doses.

GI, Gastrointestinal; *Lower*, lower respiratory tract; *Upper*, upper respiratory tract.