

Evaluation of the usefulness of lymphocyte proliferation assays in the diagnosis of allergy to cow's milk

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Background: The significance of cell-mediated mechanisms in IgE-mediated milk allergy (IgE-MA) and in milk-induced enterocolitis syndrome (ME) is controversial. Some investigators have claimed that lymphocyte proliferation assays are useful in the diagnosis of food hypersensitivity, despite the great variability in study designs and results reported. This study was undertaken to address many of these variables and to determine whether lymphocyte proliferation assays correlate with clinical diagnoses.

Methods: Lymphocyte proliferative responses to milk antigen were evaluated in two groups of children, 27 with IgE-MA, and nine with ME and in 21 pediatric control subjects. IgE-mediated food allergy was documented by positive double-blind, placebo-controlled food challenges and positive skin prick test results. ME was diagnosed by oral challenge or by a history of repeated episodes of delayed vomiting (>2 hours) after ingestion of milk and by negative skin prick test responses. Peripheral blood mononuclear cells were isolated and cultured. Cultures stimulated with milk (the food antigen of interest), soy antigen (a nonrelevant food antigen), or tetanus antigen (a positive control antigen) and unstimulated controls were performed in quadruplicate. On days 5, 7, and 9, cells were pulsed with tritium-labeled thymidine and incubated for 4 hours. Results were compared as counts per minute (cpm) and as stimulation indices (SIs).

Results: Maximal proliferation was generally seen on day 7. The median cpm (20,941) and the median SI (19.2) in response to milk antigen in the 27 children with IgE-MA were significantly greater than those in the control patients (6969 cpm; SI = 14.2; $p = 0.001$ and $p < 0.05$, respectively). However, the ranges were large and overlapped extensively (IgE-MA, 5616 to 52,053 cpm; controls, 469 to 39,260 cpm). The non-soy-allergic patients with IgE-MA also had a significantly greater response to soy antigen than did the control subjects when cpm were compared ($0.01 < p < 0.05$). There were no differences in background or in response to tetanus antigen. The median response to milk in the patients with ME (11,975 cpm) was significantly greater than that in control subjects (6969 cpm; $0.01 < p < 0.05$), when cpm were compared but not when SIs were com-

pared. There were no significant differences between the patients with IgE-MA and those with ME.

Conclusion: Overall, these results indicate that lymphocyte proliferation assays are neither diagnostic nor predictive of clinical reactivity in individual patients with milk allergy. Lymphocytes of many control patients are highly responsive to milk antigens, and lymphocytes of many patients with milk allergy are not. Statistically significant differences are only evident when the patients are compared as groups. (J Allergy Clin Immunol 1997;99:360-6.)

Key words: Food allergy, lymphocyte proliferation, milk-induced enterocolitis

Hypersensitivity to cow's milk in children has been studied extensively, and IgE-mediated reactions have been well characterized. Four prospective surveys indicate that the prevalence of allergy to cow's milk is approximately 2.5% in the first 3 years of life.¹⁻⁴ In the past several years, there has been a renewed interest in the role of cell-mediated mechanisms in the pathogenesis of hypersensitivity to cow's milk. May and Alberto⁵ reported no differences in lymphocyte proliferative responses to food antigens in atopic children when compared with those in nonatopic children. Endre and Osvath⁶ reported increased lymphoblast transformation in response to milk proteins in a group of 17 children with milk allergy when their responses were compared with those of a group of eight children without milk allergy. Another study evaluated lymphoblast transformation tests in 45 patients with "cow's milk protein intestinal intolerance" and found positive results in only 37.8%.⁷ Van Sickle et al.⁸ found significant differences in cellular responses to milk antigen in patients with milk-induced enterocolitis proven by standardized oral challenge when compared with normal children. More recent studies have also been highly variable in their evaluation of lymphocyte responses to milk antigens.⁹⁻¹³ The disparities in these studies may arise from methodological and analytic differences, including differences in criteria for diagnosis of food allergy, patient populations studied, clinical characteristics of control patients, use of fetal calf serum or human serum in the lymphocyte culture, antigen concentrations, day and duration of tritiated thymidine pulse, reporting results as counts per minute (cpm) or as stimulation indices (SIs), and analyses of data with parametric or nonparametric statistical tests. Despite these many variables, some investigators have proposed that food antigen-stimulated lymphocyte

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Abbreviations used

cpm:	Counts per minute
DBPCFC:	Double-blind, placebo-controlled food challenge
IgE-MA:	IgE-mediated cow's milk allergy
ME:	Milk-induced enterocolitis syndrome
PBMCs:	Peripheral blood mononuclear cells
SI:	Stimulation index

proliferation assays should be used as a diagnostic or predictive test for food allergy.¹⁰⁻¹²

This study was designed to evaluate the clinical utility of *in vitro* lymphocyte proliferation assays in both IgE-mediated milk allergy (IgE-MA) and in milk-induced enterocolitis syndrome (ME). Many of the variables found in previous studies were controlled by using strict diagnostic criteria such as the double-blind, placebo-controlled oral food challenge (DBPCFC) for evaluation of our patient groups, by using autologous sera in the lymphocyte culture, by reporting and comparing our results in both cpm and SIs, and by analyzing statistical significance with nonparametric tests because the data are not normally distributed.

METHODS

Subjects

Patients were selected from the Pediatric Allergy Clinic and from children referred to us for evaluation of food allergy. The first patient group consisted of 27 children with IgE-MA confirmed by positive skin prick test responses, and inpatient DBPCFCs were performed as previously described.¹⁴ These children had immediate symptoms of allergy after milk ingestion, such as morbilliform rash, urticaria, pruritus, angioedema, vomiting, laryngeal edema, or wheezing. The median age of the children was 4 years. The second patient group consisted of nine children with ME diagnosed by the oral challenge procedure described by Powell¹⁵ or by a history of repeated episodes of isolated delayed vomiting more than 2 hours after the ingestion of milk protein. Patients with ME are not typically atopic and have no detectable IgE antibodies to milk; that is, they have negative skin test and RAST responses. Symptoms generally consist of vomiting, diarrhea, and colic; however, a small but significant percentage will experience hypotension and will require intravenous fluid resuscitation. They will display increased numbers of polymorphonuclear neutrophils on their differential white blood cell counts 4 to 6 hours after milk ingestion. Symptoms resolve completely within 48 to 72 hours of eliminating milk protein from the diet. Their symptoms recur once the milk-based formula is resumed. Up to 60% of these patients will also have similar reactions to soy antigen, if they begin receiving a soy-based formula at an early age.¹⁶ This association is less common in IgE-MA, in which concurrent allergy to soy antigens is seen in approximately 5% to 15% of children allergic to cow's milk (unpublished data). All the patients with ME had negative skin prick test responses to milk; the median age of this group was 1.3 years.

The control group comprised 21 children (median age, 5 years) who had no evidence of milk allergy. Sixteen of these children, although not allergic to milk, had other documented food allergies.

Lymphocyte culture

Before oral challenges, each patient had heparinized venous blood drawn in a sterile manner. All patients with milk sensitivity were on strict milk elimination diets before challenge. Peripheral blood mononuclear cells (PBMCs) were purified by centrifugation on a Ficoll-Hypaque gradient, washed twice, and resuspended in RPMI-1640 (GIBCO Laboratories, Grand Island, NY) containing L-glutamine (2 mmol/L), penicillin (100 U/ml), streptomycin (100 µg/ml), and 5% autologous serum. Preliminary studies indicated that optimal proliferative responses were obtained with autologous sera compared with pooled human AB sera or fetal calf serum. Cells were plated at 2×10^5 cells/200 µl/well in round-bottom microtiter plates.

Stock solutions of food antigens were prepared in RPMI-1640. The final concentrations in culture were as follows: milk antigens were a mixture of casein at 1000 µg/ml and whey at 100 µg/ml or a mixture of casein at 500 µg/ml and whey at 50 µg/ml; soy protein concentrations were 250 µg/ml and 125 µg/ml. Milk was chosen as the food antigen of interest, and soy antigen was chosen as a nonrelevant food antigen. The food antigen concentrations were chosen for optimal proliferation as described by Van Sickle et al.⁸ Similarly, a recent study, in which 7 logarithmic dilutions of milk proteins (10^{-2} to 10^{-8} mg/ml) were used, demonstrated optimal proliferation with the highest concentrations of milk protein.¹³ Casein and whey proteins were kindly provided by Ross Laboratories (Columbus, Ohio). Soy antigen was prepared by dissolving soy flour (Arrowhead Mills, Hereford, Texas) in phosphate-buffered saline and solubilizing the soy protein. All food antigens were sterile-filtered (Millipore, Bedford, Mass.) and frozen at -20°C until use.

Tetanus toxoid (lot no. 40106-515; Massachusetts Department of Public Health) was used as a positive control antigen to which all immunized children should have a cellular response. The final concentration per well for tetanus antigen was 0.2 Lf/ml.

Culture with each antigen concentration was performed in quadruplicate. Negative control cultures (no antigen added) were prepared simultaneously. Cultures were incubated at 37°C in a 5% CO_2 environment. Cells were pulsed for 4 hours with 1 µCi of tritium-labeled thymidine per well on days 5, 7, and 9. Cells were harvested on glass fiber filter paper by using a cell harvester (Cambridge Technologies, Inc., Cambridge, Mass.). Incorporated cellular radioactivity was measured with a liquid scintillation counter (Beckman Instruments, Inc., Irvine, Calif.) with toluene and 2,5 diphenyloxazole.

Statistical analysis

Mean cpm were calculated for each antigen concentration and for background control wells. Results were expressed in cpm and as the SI, which is the mean cpm for antigen-stimulated cells divided by the mean cpm for unstimulated cells. The cpm or SI reported is the maximal value for each antigen on day 5, 7, or 9. In most patients, maximal proliferation occurred on day 7 with the higher antigen concentration.

Comparative analyses of the results were done by using the Wilcoxon rank-sum test because the data were not normally distributed.

RESULTS

The median proliferative response, in cpm, to milk antigen in the 27 children with IgE-MA was significantly greater than that in the control patients ($p = 0.001$) (Fig. 1). Although the difference between medians in these two groups was significant, the ranges were quite large

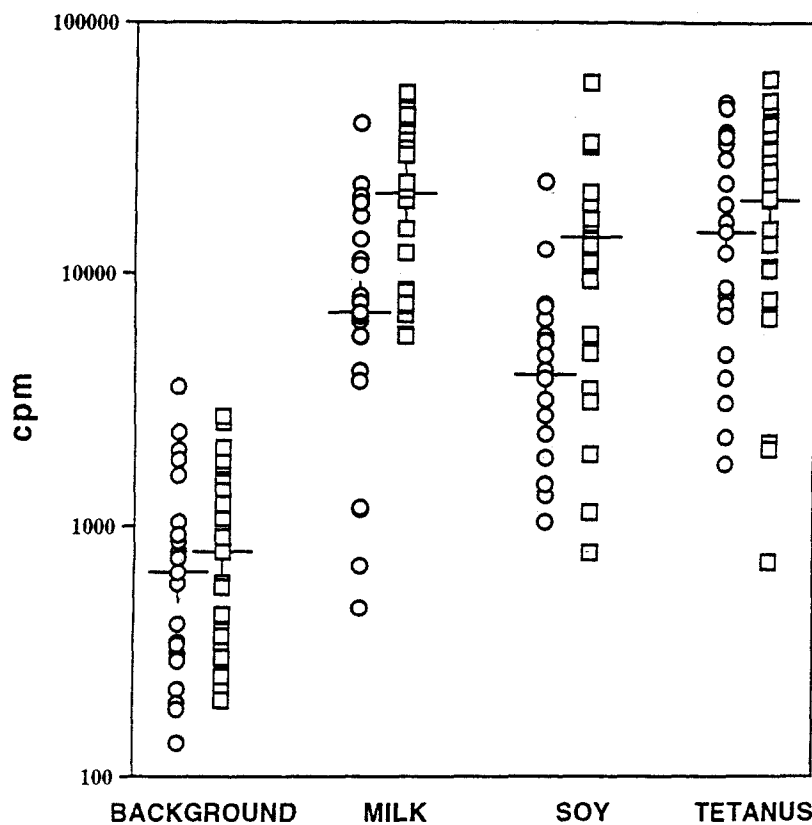


FIG. 1. Comparison (in cpm) of lymphocyte proliferation in response to antigen stimulation in control subjects (○) and in patients with IgE-MA (□).

and overlapping. There was no significant difference between the patients with IgE-MA and the control subjects in background proliferation or in cellular response to tetanus antigen. When these two groups were compared for their response to soy, eight of the children with milk allergy and three of the control subjects were not used in this comparison because they had documented soy antigen allergy; therefore, soy antigen was not a nonrelevant food antigen in these 11 patients. Interestingly, the 19 children with milk allergy who were not allergic to soy antigen had a significantly greater response to soy antigen (median, 13,058 cpm) than did the 18 control patients who were not allergic to either soy or milk antigens (median, 4,427 cpm; $0.01 < p < 0.05$). As seen in Fig. 1, there was also a large overlap between the two groups in their cellular response to soy antigen.

When the patients with IgE-MA were compared with control patients by using SIs instead of cpm (Fig. 2), the statistical significance was less striking. The median SI in response to milk antigen in the group with milk allergy was 19.2, compared with 14.2 in the control subjects ($p < 0.05$). Again, the overlap of responses was quite large. When the SIs of these two groups were compared for their response to soy antigen (excluding the patients with

soy allergy in both groups, as before), there was no significant difference in median SI values ($p > 0.10$). There was no significant difference between any of the groups when their responses to tetanus were compared by using SIs or cpm.

The cellular proliferation in response to milk was compared in the second patient group, the nine children with ME, and the 21 control patients (Fig. 3). The median cpm in the patient group was 11,975 cpm, a value that was significantly higher than the median value found in the control subjects (6,969 cpm; $0.01 < p < 0.05$). Background proliferation and response to tetanus were not significantly different between the two groups. Proliferation in response to soy as a nonrelevant antigen was not used in this patient group because the majority of the patients with ME had either documented reactions to soy antigen or had never ingested soy antigen before their enrollment in the study. When the proliferative response to milk antigen was compared in the patients with ME and the control subjects by using SIs instead of cpm (Fig. 4), the difference was not statistically significant ($p < 0.2$).

When the lymphocyte responses of patients with ME were compared with those of the patients with IgE-MA, there was no significant difference in their response to

milk antigen ($p = 0.812$) or tetanus toxoid or in their background proliferation. There was also no significant difference when SIs were used instead of cpm.

Because most patients and control subjects demonstrated significant proliferative responses to milk antigens, one might question whether milk proteins have mitogenic properties. Although the optimal response to milk proteins is at 7 days, suggesting an antigenic response, we stimulated six egg antigen-specific T-cell lines with milk antigens to rule out any mitogenic response to these proteins. Antigen-specific T-cell lines were used to rule out a mitogenic response to milk antigens because even cord blood lymphocytes have been shown to respond to food antigens, as a result of in utero exposure and sensitization.¹⁷ The egg antigen-specific T-cell lines demonstrated no proliferation above background to milk antigens but had significant proliferation to egg proteins (median counts: background, 4970 cpm; milk antigen, 4473 cpm [SI, 0.9]; and egg antigen, 11,928 cpm [SI, 2.4]), suggesting no mitogenic response to the milk proteins.

DISCUSSION

We have evaluated and compared the lymphocyte proliferative responses to milk antigen in 27 children with IgE-MA, nine children with ME, and 21 children without milk allergy. When compared as groups with the control group, both the patients with IgE-MA and the patients with ME showed significantly increased proliferative responses to milk antigen. However, the ranges of all three groups' responses were quite large and overlapped extensively when either cpm or SIs were compared. Many control patients demonstrated in vitro proliferative responses to milk that were higher than those of some patients with milk allergy. Interestingly, patients with IgE-MA had higher proliferative responses to soy antigen than did the control patients. Because none of the patients in this particular comparison were allergic to soy antigen, it would suggest a lack of specificity in food antigen-stimulated lymphocyte responsiveness or that patients with food allergy have a greater cellular reactivity to a variety of food antigens. There were no differences among the groups in overall lymphocyte responsiveness to a common recall antigen, as reflected by the proliferation in tetanus antigen-stimulated cultures or by activation status, as seen in the unstimulated cultures.

Our results are in agreement with those of an early study by May and Alberto,⁵ who found significant lymphocyte stimulation in response to food proteins in children with or without allergy. Scheinman et al.⁷ reported positive lymphoblast transformation to α -lactalbumin or β -lactoglobulin in 17 of 38 patients with "cow's milk protein intestinal intolerance." They found only four patients of 43 to be positive in their nonatopic control group. A more recent study claimed that the lymphocyte stimulation test was specific, despite results that showed a large overlap in lymphocyte proliferation in response to β -lactoglobulin in both patient and con-

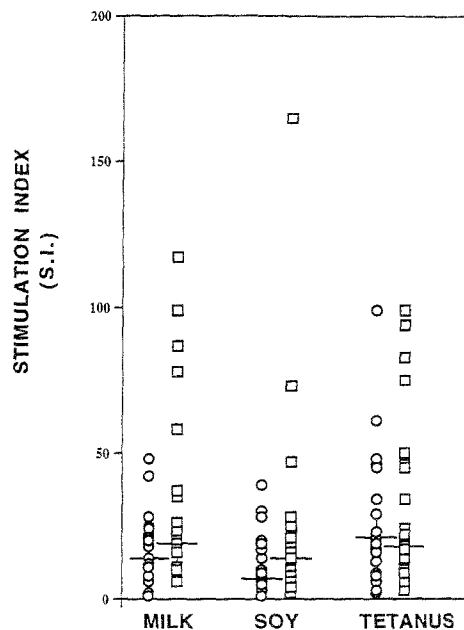


FIG. 2. Comparison (in SIs) of control subjects (○) and patients with IgE-MA (□).

trol groups.¹⁰ In that study, open oral challenges were used to confirm milk allergy in the patient group; some patients reportedly reacted up to 24 hours after ingestion of milk.

In a recent study by Suomalainen et al.,¹⁸ lymphocyte responses to cow's milk proteins were reported to decrease if blood from patients with milk allergy was drawn after a positive milk challenge. (In our study, all blood samples were drawn before food challenges.) In their study, milk challenges were performed openly over a 48-hour period; whole blood was used in lymphocyte cultures, and iodine 125-labeled 5-iodo-2' deoxyuridine was added 16 to 18 hours before cell harvest on day 7. Despite these methodological differences, results of this study were similar to those reported here, showing large overlaps in lymphocyte proliferative responses to milk in both patients and control subjects. Suomalainen et al.¹⁸ also observed a difference in statistical significance when results were analyzed by using cpm rather than SIs.

In another study, Tainio and Savilahti⁹ reported a sensitivity of 88% and a specificity of 67% when a combination of lymphocyte proliferative responses to β -lactoglobulin and specific IgE antibodies to cow milk was measured. Patients were diagnosed with milk allergy on the basis of an open oral milk challenge protocol in which the ingested quantity of milk protein was increased every 4 hours, up to 72 hours, until a reaction was observed. In this study, patients with milk allergy were diagnosed on the basis of DBPCFCs, as described previously.¹⁴ Another difference included performing the thymidine pulses on the third day of the culture. Although frequently optimal for mitogen-stimulated lymphocytes, 3 days of culture is generally too short a

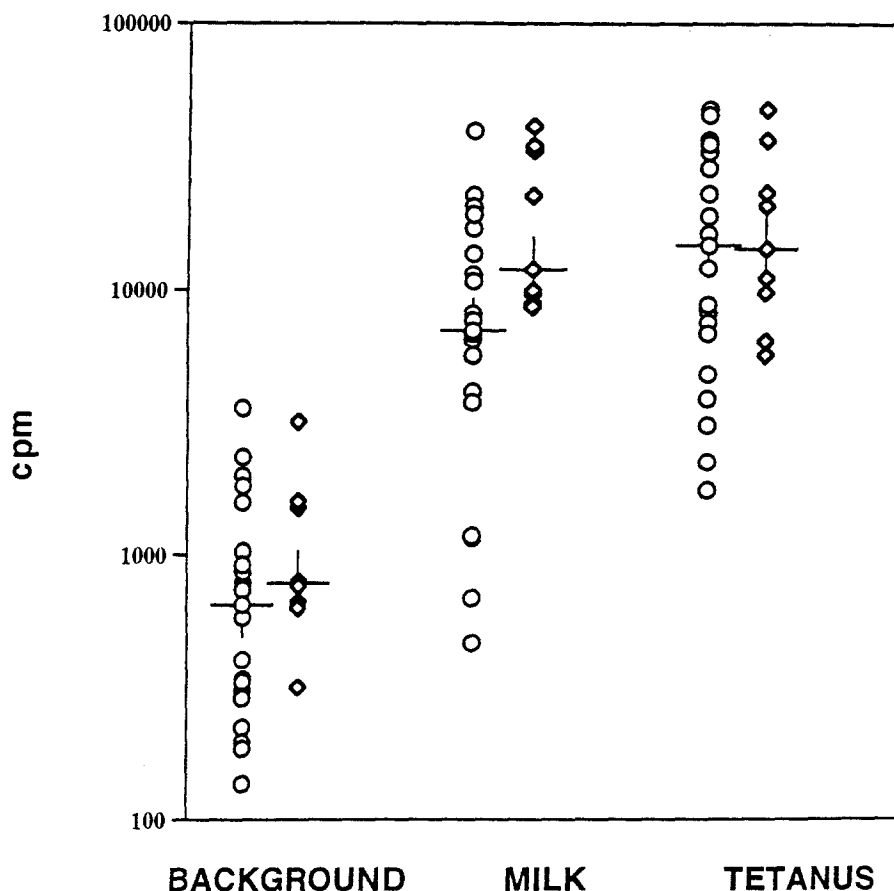


FIG. 3. Comparison (in cpm) of control subjects (○) and patients with ME (◇).

time in which to achieve peak proliferation caused by food protein stimulation, which requires antigen presentation. In this study peak proliferation generally occurred on day 7, which is in agreement with results of previous studies.^{5, 8, 10, 18}

Findings in this study differ from those of an earlier study of patients with atopic dermatitis by Kondo et al.¹¹ On the basis of food challenges, patients were divided into those with immediate symptoms and those with late (>2 hours) symptoms of allergy to food. In contrast, our patients with IgE-MA, 21 of whom had atopic dermatitis (AD), all had positive DBPCFCs, with symptoms manifesting within 2 hours of ingestion of milk, consistent with previous studies^{14, 19, 20} that used the challenge protocol initially described by May.²¹ Another methodological difference in the study by Kondo et al.¹¹ was the use of bovine serum albumin as the stimulating milk protein, whereas casein and whey were used in this study. Kondo et al.¹¹ reported peak proliferation on days 3 through 5, in contrast to day 7 in our study. Their mean cpm and SI values were quite low in their group with milk allergy (<2000 cpm; SI, <4, respectively) with wide overlapping ranges. Our median cpm and SI values were much greater (>2000 cpm; SI, >19), although also with wide overlapping ranges. Although the study by Kondo

et al.¹¹ states that the results of patient and control groups do not reach statistically significant differences, they still suggest that "the combination of RAST and the detection of proliferative responses of PBMCs to each food antigen is very useful in the diagnosis of hypersensitivity in children with AD who are sensitive to food allergens."¹¹ The results of our study and those of others do not support the contention that lymphocyte proliferation assays are useful in the diagnosis of food allergy. However, in the rare cases in which lymphocyte proliferation to milk proteins *in vitro* is absent, it is very unlikely that a patient would experience an allergic response because some lymphocyte activation is necessary for both hypersensitivity and normal immune responses to food antigens.

Patients with ME in this study had PBMC proliferation in response to milk antigen similar to that reported by Van Sickle et al.⁸ However, our control group had a wider range of proliferative responses to food antigens and a higher median proliferation than those seen in the study by Van Sickle et al.,⁸ in which the control group's proliferative responses did not overlap with the responses of the patient group. This difference might be explained by the young age of the patients in their control group (mean age, 4.7 months). Also, none of the

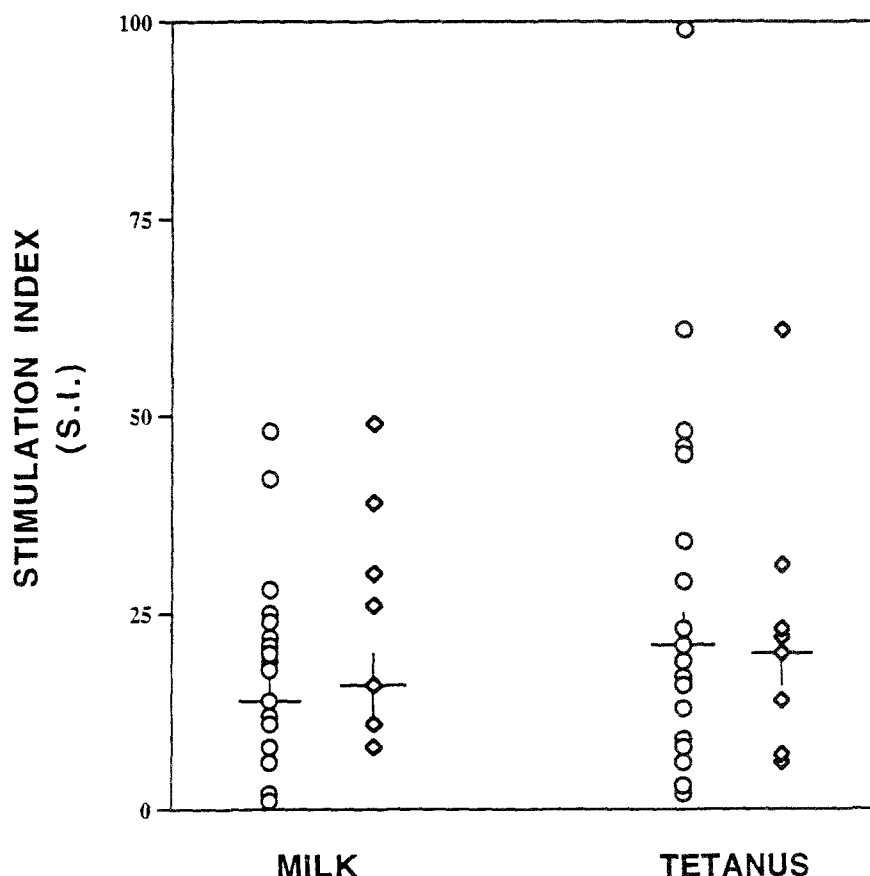


FIG. 4. Comparison (in SIs) of control subjects (O) and patients with ME (◇).

patients in their control group were known to be atopic. Although not statistically significant, patients with IgE-MA in our study tended to have higher proliferative responses to milk than did the patients with ME; the latter group is affected by a non-IgE-mediated disorder in which cell-mediated hypersensitivity is believed to play a prominent role. Although the higher proliferative responses overall in both patient groups might implicate a pathogenic role for sensitized lymphocytes in milk allergy and in ME, the wide range of responses in both patients and control subjects would suggest that proliferation alone does not sufficiently reflect the immunologic mechanisms involved in the clinical manifestations of these conditions.

In conclusion, the lymphocyte proliferation assay does not appear to be useful as a diagnostic test in food hypersensitivity. Significant lymphocyte proliferation occurs in patients with IgE-MA, in patients with ME, and in control subjects. Future investigation into the profile of cytokines elaborated by lymphocytes proliferating in response to food antigen stimulation may provide insight into the role of cell-mediated immunity in the pathogenesis of food hypersensitivity.

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