Immunodeficiency and other clinical immunology

In vitro lymphocyte proliferation with milk and a casein–whey protein hydrolyzed formula in children with cow's milk allergy

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Discordant results have been reported about the role of lymphocyte proliferation assays in patients with cow's milk allergy. We studied the peripheral blood mononuclear cell response of 10 children with cow's milk allergy by means of a lymphocyte proliferation test to determine the diagnostic value of this assay, the clinical tolerance of a new therapeutic hydrolyzed formula, and the evolution of lymphocyte proliferation after 3 months of a cow's milk–free diet with the hydrolyzed formula. The lymphocyte proliferation at the time of diagnosis in the patients with cow's milk allergy was not statistically different from the results in the control group. The proliferation test performed after 3 months of diet with the hydrolyzed formula and restriction of cow's milk protein showed that the cellular proliferation remained globally the same compared with the proliferation at the time of diagnosis. The hydrolyzed formula proteins induced a lower cellular proliferation than milk proteins in patients with cow's milk allergy. Our results suggest that the lymphocyte proliferation test cannot be recommended for diagnostic purposes. However, in patients with cow's milk allergy the proliferation test affirmed the absence of immunogenicity of the hydrolyzed formula because it induced no significant T-cell activation. (J ALLERGY CLIN IMMUNOL 1995;96:549-57.)

Key words: *Milk hypersensitivity, infant nutrition, immune tolerance, lymphocyte transformation*

Cow's milk allergy (CMA) is a common allergic disease in infants, and its reported prevalence ranges from 2% to 7.5%.[1-3] However, CMA remains difficult to diagnose because of the absence of one reliable in vitro diagnostic test for all the different clinical presentations. The laboratory workup usually performed includes food-specific serum IgE and IgG determinations, although their sensitivity and specificity are not completely satisfactory.[4] Skin tests and food challenges are considered to be the most accurate procedures.[5] The lymphocyte proliferation test (LPT) was studied for its diagnostic value in 1972 by May and Alberto.[6] These authors concluded that results of this test were positive in control subjects as often as they were in children with allergies. Since then, this laboratory procedure has been applied with a broad range of antigen concentrations. More recently, Taino and Savilahti[7] found that the LPT was valuable in diagnosing CMA, particularly

Abbreviations used

CM:	Cow's milk
CMA:	Cow's milk allergy
CMP:	Cow's milk protein
cpm:	Counts per minute
LPT:	Lymphocyte proliferation test

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when associated with determination of specific IgE. Similarly, Räsänen et al.[4] concluded that the LPT is an accurate test for diagnosis of CMA, particularly in patients with delayed-type symptoms. However, in all these studies lymphocytes from control subjects proliferated in response to cow's milk protein (CMP), although the control subjects clinically tolerated cow's milk (CM).

The treatment of CMA combines a CMP-free diet with therapeutic formulas, which are composed of hydrolyzed casein, whey, or soy proteins and beef collagen. In a preliminary study we found that lymphocyte proliferation in a patient with CMA was markedly diminished after 3 months of CMP-free diet with a new therapeutic casein–whey protein hydrolyzed formula (Damira; Wander, Bern, Switzerland).[8] The mechanism of lymphocyte tolerance to potential food allergens in healthy subjects or in modulating immune response in patients with allergy remains incompletely defined. This study was designed with the following objectives: (1) to determine the diagnostic value of the LPT in a population of infants with CMA, (2) to study the in vitro and clinical tolerance to Damira formula, and (3) to follow T-cell memory during a CM-free diet with the hydrolysate because lymphocyte responsiveness during an antigen avoidance diet is not well defined.

Our results failed to show any diagnostic value for LPT in CMA, because lymphocytes from subjects of both groups (patients with CMA or control subjects) responded similarly to antigen stimulation. In contrast, the proliferation in response to the hydrolyzed formula in patients with CMA was significantly lower than that to milk proteins. Lymphocyte proliferation values remained the same after 3 months of CMP restriction diet.

METHODS

Subjects

In this study we enrolled 10 children with CMA (6 boys and 4 girls; age at diagnosis, 3.6 ± 4.6 months [mean ± SEM]) who fulfilled the diagnostic criteria proposed by The European Society for Paediatric Gastroenterology and Nutrition; namely, 2 positive challenge responses to milk with subsequent disappearance of the symptoms after starting an elimination diet.[9] Symptoms had to be gastrointestinal (e.g., stomach pain, vomiting), cutaneous (e.g., eczema, urticaria), or respiratory (chest symptoms, laryngial or nasal symptoms), and symptoms had to be objectively documented by the medical staff. Five patients had IgE-mediated reactions at diagnosis (urticaria and anaphylaxis-related symptoms or atopic dermatitis), and five patients had predominantly gastrointestinal symptoms consisting of enterocolitis with vomiting, diarrhea, and failure to thrive. The control group included six nonatopic children clinically tolerant to CM (1 girl and 5 boys; mean age ± SEM, 23.8 ± 4.6 months) who were undergoing minor surgical procedures and six adults tolerant to CM, three atopic and three nonatopic subjects (3 women and 3 men; mean age, 34 years). Absence of atopy was confirmed by a negative personal and family history in children and adults and by a negative determination of specific IgE to major respiratory allergens by Phadiatop (Pharmacia, Uppsala, Sweden) in adults.

Children were enrolled in the study after informed consent was obtained from their parents. This study was performed in agreement with the requirements of the Institutional Review Board of the University Children's Hospital of Geneva.

Antigens

The therapeutic hydrolyzed formula tested in this study, Damira, is composed of casein and whey proteins hydrolyzed in a gastric phase (with pepsin and HCl) and two pancreatic phases (with trypsin, chymotrypsin, and elastase). Seventy-five percent of the end product has a molecular weight of less than 1 kd and 100% has a molecular weight less than 5 kd. The CM antigen was dehydrated milk powder (Sanolait; Coop, Basel, Switzerland). The proteins used in manufacturing the hydrolysate, either before or after hydrolysis, were used as antigens for the proliferation assay.

LPT

LPTs were performed by harvesting the mononuclear cell fraction isolated from sterile heparinized blood after centrifugation on Ficoll-Paque (Pharmacia). The cells were suspended in Hanks' buffer solution (Biochrom, Berlin, Germany), centrifuged, and washed three times. They were then added to RPMI-1640 culture medium (Biochrom) supplemented with 20% vol/vol pooled human AB serum. One million cells per milliliter were incubated with the antigens at different concentrations in round-bottomed 96-well plates. Seven logarithmic concentrations (from 10^{-8} to 10^{-2} mg/ml) of whole CM, Damira formula, whey protein, hydrolyzed whey protein, casein, and hydrolyzed casein were studied. All test conditions were performed in quadruplicate. After incubation for 7 days, cellular DNA was labeled with methyl-^{3}tritiated thymidine (Amersham, Zurich, Switzerland), collected 4 hours later on a paper filter, and mixed with Kontrogel scintillation liquid (Kontron, Lausanne, Switzerland). Tritiated thymidine incorporation into DNA was used as an index of cell proliferation and measured with a liquid scintillation counter (Beckman Instruments, Fullerton, Calif.). The results were expressed as the mean value in counts per minute (cpm) for each concentration of antigen tested in quadruplicate. Negative controls were performed without antigen. Phytohemagglutinin (Wellcome, Reinach, Switzerland) at 5 mg/ml and tetanus toxoid (Statens Seruminstitut,

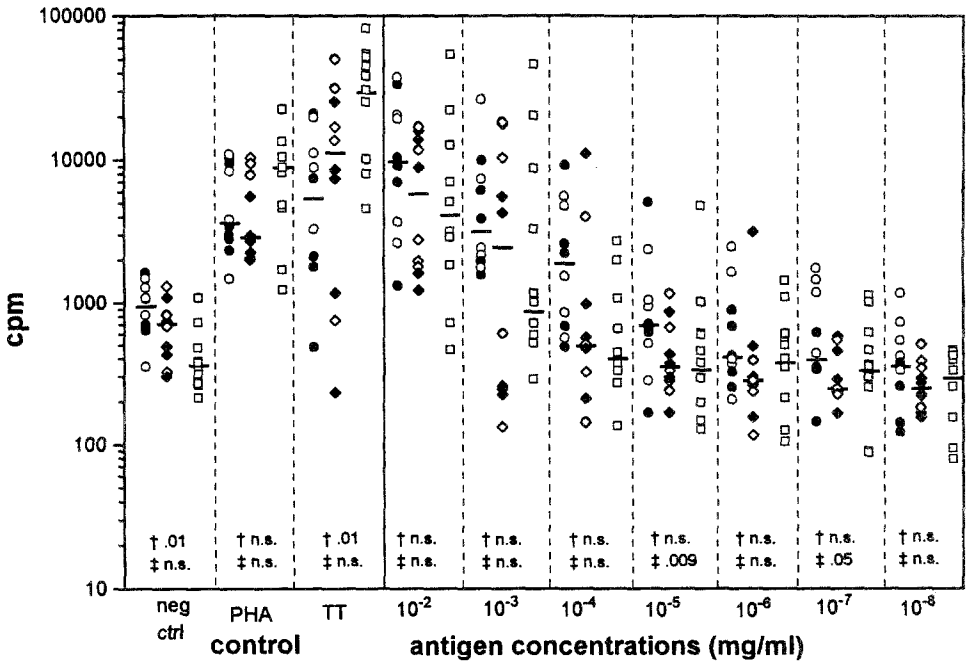

FIG. 1. LPT with milk; results are expressed in cpm. *Circles* represent test 1 (baseline), and *diamonds* represent test 2 (at 3-month follow-up) for patients with CMA. *Open symbols* indicate patients with IgE-mediated symptoms, and *solid symbols,* patients with non-IgE-mediated symptoms. *Squares* represent the control group. The median for each group is shown by a bar. Counts per minutes are plotted on the y axis on a log scale. Controls and different concentrations of antigen are plotted on the x axis. Statistical analysis: †*p* values when results of test 1 are compared in patients with CMA and the control group; ‡*p* values when results of tests 1 and 2 are compared in patients with CMA. There is no statistical difference between patients with IgE-mediated symptoms and those with non-IgE-mediated symptoms. *PHA,* Phytohemagglutinin; *TT,* tetanus toxoid, *n.s.,* not significant.

Copenhagen, Denmark) at 50 mg/ml were used as positive controls.

Statistics

Statistical analysis was done by Wilcoxon signed-rank test with a two-tailed alpha of 0.05 as the level of significance for the paired data; the Mann-Whitney test was used for between-group comparison.

RESULTS

The clinical tolerance of Damira formula was excellent during the study period. The boys had an average weight gain of 2140 gm (125% of predicted value) and an increase from the 25th to the 25th-50th percentile during the study period. The girls' weight increased by an average of 1900 gm (90% of predicted value) and remained above the 50th percentile (from 75th to 50th-75th percentile).

Figs. 1 to 3 show cpm values for lymphocyte proliferation with whole CMP in control subjects, as well as in patients with CMA at the time of diagnosis (test 1) and after 3 months of CM restriction diet with Damira formula (test 2). Results of LPTs for whole milk are plotted in Fig. 1. Controls with mitogens or without antigen showed cpm values between 100 and slightly over 1000 for basal values without antigen and cpm values up to 100,000 with mitogens, indicating normal lymphocyte function. In patients with CMA, cpm in test 2 were slightly lower than in test 1 at all concentrations; however, this difference was not confirmed by statistical analysis. Cell proliferation at the high antigen concentration of 10^{-2} mg/ml was close to the proliferation values induced by mitogens. Proliferation with antigen concentrations below 10^{-5} mg/ml was similar to values for proliferation without antigen. Concentrations with definite proliferation in response to antigens in this test are between 10^{-3} and 10^{-5} mg/ml. The median cpm value was slightly lower in control subjects than in patients with CMA, as well as in test 1 compared with test 2 at the same antigen concentrations. However, because of a large cpm variation between patients, this difference was not statistically

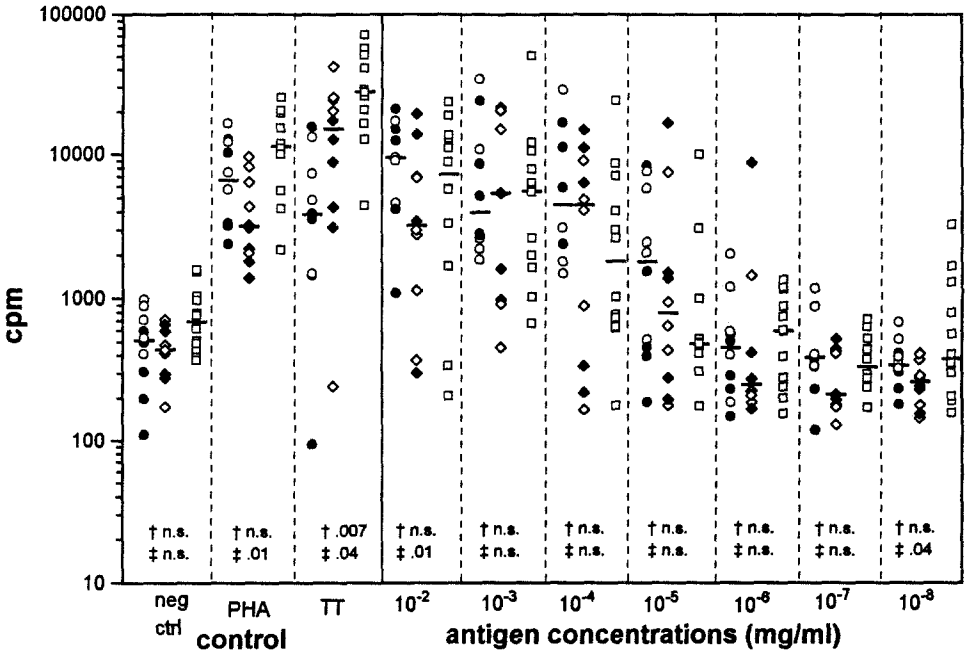

FIG. 2. LPT with casein; results are expressed in cpm. *Circles* represent test 1 (baseline), and *diamonds* represent test 2 (at 3-month follow-up) for patients with CMA. *Open symbols* indicate patients with IgE-mediated symptoms, and *solid symbols,* patients with non-IgE-mediated symptoms. *Squares* represent the control group. The median for each group is shown by a bar. Counts per minute are plotted on the y axis on a log scale. Controls and different antigen concentrations are plotted on the x axis. Statistical analysis: †*p* values when results of test 1 are compared in patients with CMA and the control group; ‡*p* values when results of tests 1 and 2 are compared in patients with CMA. There is no statistical difference between patients with IgE-mediated symptoms and those with non-IgE-mediated symptoms. *PHA,* Phytohemagglutinin; *TT,* tetanus toxoid, *n.s.,* not significant.

significant. Lymphocyte proliferation values for casein are represented in Fig. 2. As seen with whole milk, casein induced a strong lymphocyte proliferation at the higher antigen concentrations (10^{-2} to 10^{-4} mg/ml). Values were similar for all three tests, since the medians were within the same range. At the lower concentrations (10^{-6} to 10^{-8} mg/ml), cpm were close to values in blank controls. There was a statistical difference between test 1 and test 2 at 10^{-2} mg/ml, but these proliferation numbers were close to those obtained with mitogens. Fig. 3 shows data for whey protein. Similar results for whole milk and casein could be observed. The highest antigen concentrations resulted in cpm close to those with phytohemagglutinin or tetanus toxoid; whereas cpm with low antigen concentrations were comparable to those without antigen. A statistically significant difference could only be observed at 10^{-3} mg/ml. There was no statistically significant difference for lymphocyte proliferation values between the groups of patients with enterocolitis and those with IgE-mediated symptoms for all three milk proteins tested.

Figs. 4 to 6 compare cpm at diagnosis for hydrolyzed and nonhydrolyzed milk proteins in the group of patients with CMA. Proliferation values for milk were up to 5 times higher than those for Damira formula at concentrations from 10^{-2} to 10^{-5} mg/ml, as shown in Fig. 4. There was a statistically significant difference between milk and Damira formula at these concentrations. At low concentrations, cpm were close to blank values for both antigens, and there was no statistical difference. Fig. 5 represents cpm values for casein and hydrolyzed casein. Proliferation at the higher antigen concentrations was lower for hydrolyzed casein than for nonhydrolyzed casein. The difference was statistically significant for concentrations from 10^{-2} to 10^{-5} mg/ml. Low concentrations (10^{-6} mg/ml and lower) of both casein and hydrolyzed casein induced no cell proliferation. Cell proliferation with whey protein and hydrolyzed whey protein is plotted in Fig. 6. Again, cpm for high concentrations of antigen (10^{-2} to 10^{-4} mg/ml) were similar to mitogen proliferation values, but cpm were lower for hydrolyzed proteins at all concentrations. From 10^{-2} to 10^{-5} mg/ml, the

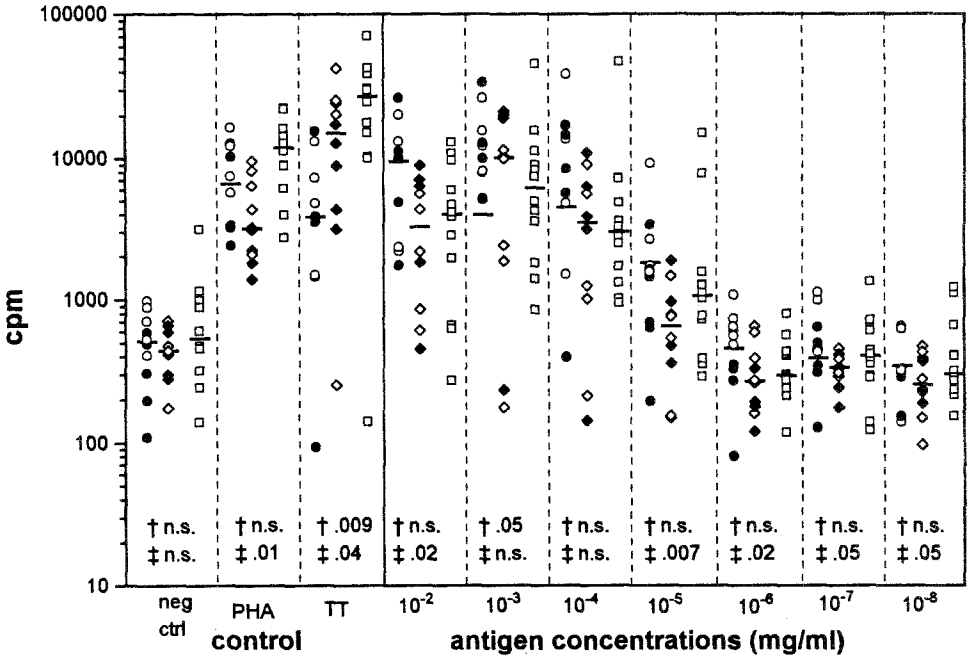

FIG. 3. LPT with whey protein; results are expressed in cpm. *Circles* represent test 1 (at diagnosis), and *diamonds* represent test 2 (at 3-month follow-up) for patients with CMA. *Open symbols* indicate patients with IgE-mediated symptoms, and *solid symbols,* patients with non-IgE-mediated symptoms. *Squares* represent the control group. The median for each group is shown by a bar. Counts per minute are plotted on the y axis on a log scale. Controls and different antigen concentrations are plotted on the x axis. Statistical analysis: †*p* values when results of test 1 are compared in patients with CMA and the control group; ‡*p* values when results of tests 1 and 2 are compared in patients with CMA. There is no statistical difference between patients with IgE-mediated symptoms and those with non-IgE-mediated symptoms. *PHA,* Phytohemagglutinin; *TT,* tetanus toxoid, *n.s.,* not significant.

difference between hydrolyzed and nonhydrolyzed whey proteins was statistically significant.

DISCUSSION

This study shows that peripheral blood mononuclear cells from patients with CMA proliferate in response to CMP but that the results of lymphocyte proliferation assays are not statistically different when compared with those of healthy control subjects who are tolerant to CM. After 3 months of a diet of hydrolyzed formula and restriction of CM protein, there was no change in cellular proliferation for all tested milk proteins. Furthermore, there was no T-cell proliferation with the therapeutic formula tested in this study.

Several authors have studied the diagnostic value of LPTs in CMA. May and Alberto[6] in 1972 and Scheinmann et al.[10] 4 years later did not find any significant difference of cell proliferation between allergic and nonallergic subjects. Several more recent studies technically improved the LPT and expanded it to include the most relevant milk proteins. In a study by Minor et al.[11] one of five patients with CMA and seven of nine control subjects had positive LPT results for CM, α-lactalbumin, β-lactoglobulin, and casein. In contrast, other investigators found good sensitivity, specificity, or both for the LPT. Van-Sickle et al.[12] reported that peripheral blood lymphocytes from children with enterocolitis symptoms were more responsive than those from control subjects, suggesting that lymphocytes were probably involved in the pathogenesis of the symptoms. In the report by Räsänen et al.[4] seven of 11 patients with "immediate reactions" and nine of 11 patients with "delayed symptoms" to milk had positive LPT results, whereas lymphocytes from only one of six milk-tolerant control subjects proliferated in response to CM and casein. Similarly, two other recent studies reported good sensitivity when β-lactoglobulin and casein were tested in patients with CMA.[7, 13] In a study on lymphocyte proliferation in pediatric patients with atopic dermatitis and egg or milk allergy, Kondo et al.[14] found a good correlation between lymphocyte proliferation and clinical sensitivity in 23 patients. However, it

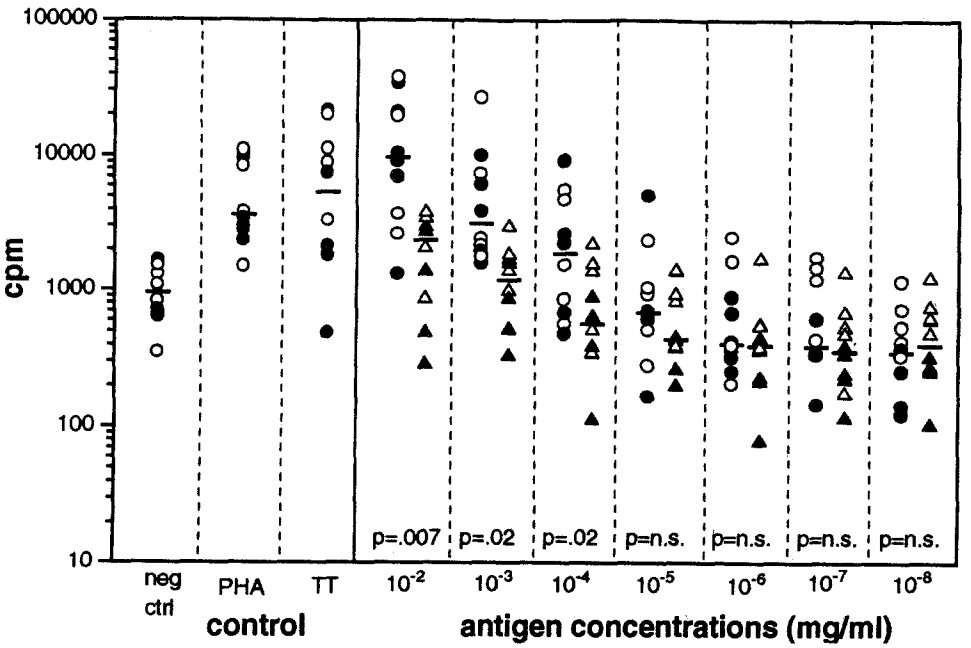

FIG. 4. LPT with milk and Damira formula in patients with CMA; results are expressed in cpm. *Circles* represent milk, and *triangles* represent Damira formula. *Open symbols* indicate patients with IgE-mediated symptoms, and *solid symbols,* patients with non-IgE-mediated symptoms. The median for each group is shown by a bar. Counts per minute are plotted on the y axis on a log scale. Controls and different antigen concentrations are plotted on the x axis. Statistical analysis was performed to compare both proteins at the same concentration. *PHA,* Phytohemagglutinin; *TT,* tetanus toxoid, *n.s.,* not significant.

must be noted that the LPT methods were not identical in all the above cited reports. Sometimes, not all relevant antigens were tested, and varying antigen concentrations might, in part, explain these divergent results.

We applied the same method for LPTs as that of most authors (quadruplicate test conditions, antigen incubation time of 7 days).[4, 7, 13] We chose to test the antigens at concentrations ranging from 10^{-8} to 10^{-2} mg/ml in all patients. In our study all children with CMA had significant cell proliferation for at least one of the milk proteins. As compared with the control subjects, proliferation was higher in the patients with CMA, although not statistically significant. Comparable to other studies[6, 11] a majority of milk-tolerant children in our study (5 of 6) had a positive cellular response to antigens to which they were otherwise clinically tolerant. Globally, a large variability in proliferation values was observed and could explain the absence of statistical significance. These results, similar to those of more recent reports, show no diagnostic value for LPT in CMA.

Children with CMA are free of symptoms when fed with "high-degree" hydrolyzed formulas (also called therapeutic formulas or hydrolysates). These formulas are part of their diet for long periods. Careful clinical and immunologic evaluation is required, because anaphylactic events have been reported during use of hydrolyzed formulas in patients with CMA.[15, 16]

Cordle et al.[17] applied an immunoassay model in mice to test hydrolyzed and native proteins and were able to demonstrate lower immunogenicity of the hydrolysate. Immunogenicity of several hydrolyzed formulas versus CM was similarly tested by Strobel and Fairclough.[18] They immunized mice with whole protein formulas, constituents of the hydrolyzed formulas, and the final hydrolysates. Antibody response and cellular immunity were lower with hydrolyzed than with nonhydrolyzed proteins. After animal studies, human tolerance studies are crucial to assess absence of immunogenicity of therapeutic formulas. For this purpose, Sampson et al.[19] first studied the immunochemical properties of a hydrolyzed formula by immunoassay, then challenged children with CMA with the hydrolysate. In their conclusions they insisted on the necessity of doing carefully designed clinical tolerance studies after immunologic in vitro testing of hydrolysates.

To study the cellular tolerance to Damira for-

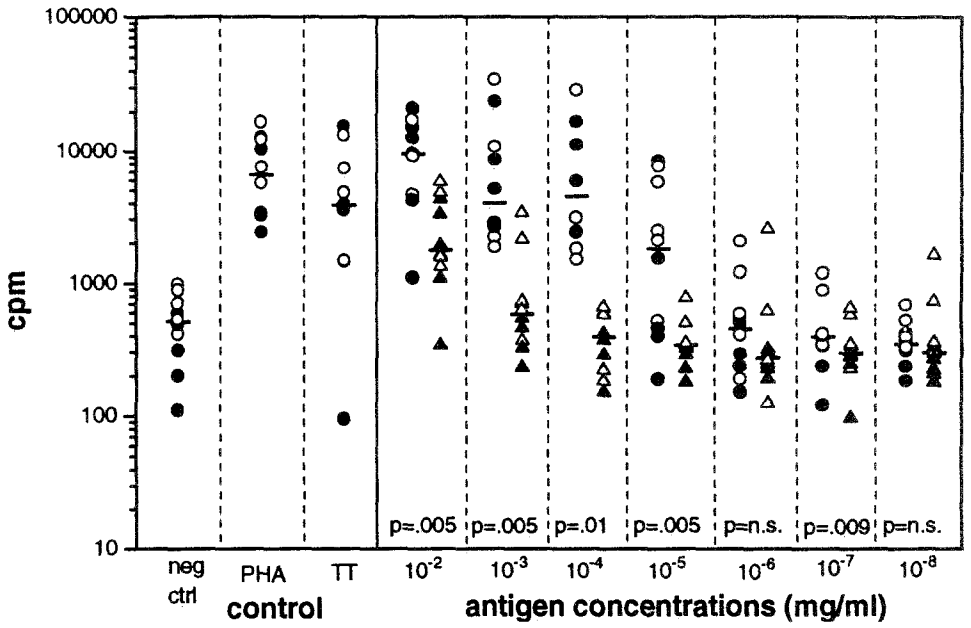

FIG. 5. LPT with casein and hydrolyzed casein in patients with CMA; results are expressed in cpm. *Circles* represent casein, and *triangles* represent hydrolyzed casein. *Open symbols* indicate patients with IgE-mediated symptoms, and *solid symbols,* patients with non-IgE-mediated symptoms. The median for each group is shown by a bar. Counts per minute are plotted on the y axis on a log scale. Controls and different antigen concentrations are plotted on the x axis. Statistical analysis was performed to compare both antigens at the same concentration. *PHA,* Phytohemagglutinin; *TT,* tetanus toxoid, *n.s.,* not significant.

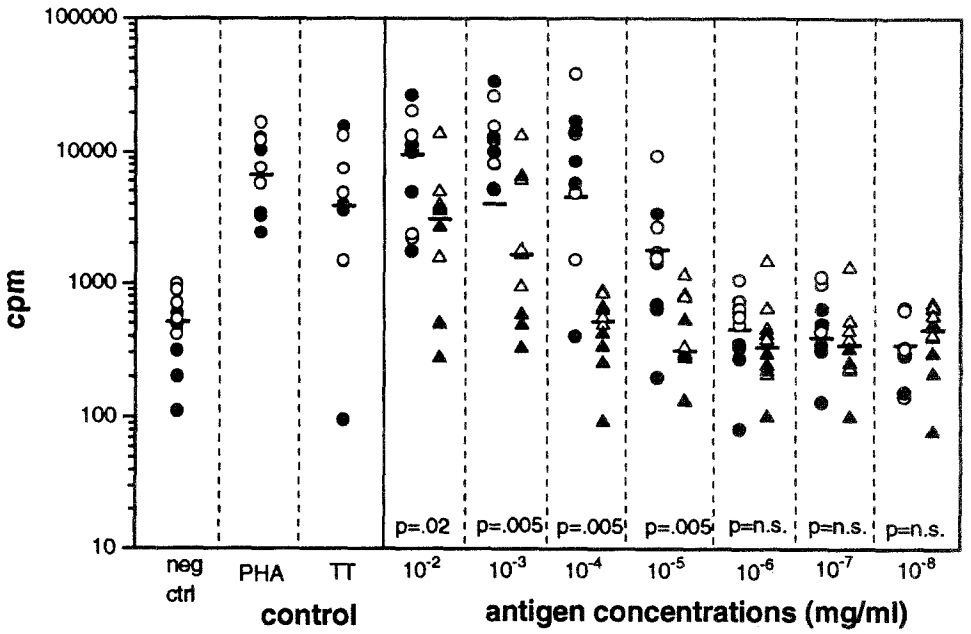

FIG. 6. LPT with whey protein and hydrolyzed whey protein in patients with CMA; results are expressed in cpm. *Circles* represent whey protein, and *triangles* represent hydrolyzed whey protein. *Open symbols* indicate patients with IgE-mediated symptoms, and *solid symbols,* patients with non-IgE-mediated symptoms. The median for each group is shown by a bar. Counts per minute are plotted on the y axis on a log scale. Controls and antigen concentrations are plotted on the x axis. Statistical analysis was performed to compare both proteins at the same concentration. *PHA,* Phytohemagglutinin; *TT,* tetanus toxoid, *n.s.,* not significant.

mula, we performed LPTs with the hydrolyzed formula in native and hydrolyzed forms on peripheral blood lymphocytes of patients with CMA. Cellular proliferation in response to hydrolyzed components of Damira formula (casein and whey protein) was close to basal values without antigen. These results were statistically lower than proliferation values with milk antigens for all concentrations when proliferation was observed. These findings, combined with a symptom-free diet with Damira formula for all patients, support the immunologic hypoallergenicity of the therapeutic hydrolysate and demonstrate the absence of any immunologically recognizable milk protein. This therapeutic hydrolysate is therefore suitable for patients with CMA. It is, however, uncertain whether the peptides in the hydrolyzed formula act as in vitro tolerogens and in what way, if any, they influence the natural course of CMA.

It is a common observation that food allergy is a disease of young children, which in most cases is outgrown.[20] It is then obvious that a reliable test to predict loss of sensitivity is needed. Children who acquire tolerance can continue to have positive skin test results for years, and therefore skin test results are not predictive of outgrowing the food sensitivity.[20, 21] Mainly T lymphocytes are involved in oral tolerance to potential antigens, and failure of tolerance mechanisms (probably clonal deletion or clonal anergy) leads to food hypersensitivity.[22] However, it still remains unclear which immunologic mechanisms lead to the disappearance of food sensitivity and to what extent T lymphocytes are implicated. Evolution of cellular sensitivity by LPT was investigated in children with atopic dermatitis and egg or milk allergy.[14, 23] All of the 27 patients with egg allergy and 16 patients with milk sensitivity reportedly had a significant decrease in lymphocyte responsiveness after a finite period of an elimination diet. The authors conclude that lymphocyte proliferative response is partially suppressed during the elimination diet. However, the second test was performed at different times, ranging from 3 to 18 months after diagnosis, which makes an interpretation of these results more difficult. In our patients the results of the second LPT, after 3 months of an elimination diet, showed a slight, but not statistically significant, diminution of cellular proliferation with CMP when compared with results of the first test.

In conclusion, we studied the accuracy of the LPT in the diagnosis of CMA and the cellular tolerance to a therapeutic formula. The results in patients with CMA showed a significant cellular proliferation in response to CM antigens. However, when compared with control subjects, patients with CMA do not have a statistically different cellular response to milk antigens. Furthermore, there was no difference in the proliferative response to CMP in either group of patients (enterocolitis or IgE-mediated symptoms). The LPT can therefore not be recommended as a diagnostic test for CMA. The hydrolyzed formula and its components induced no significant cellular proliferation in patients with CMA. These results affirm its immunologic hypoallergenicity, which is confirmed by a good clinical tolerance. The second LPT performed in patients with CMA after 3 months of diet with the hydrolyzed formula and restriction of CM showed no statistically significant difference.

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