

The threshold for immune cell reactivity to milk antigens decreases in cow's milk allergy with intestinal symptoms

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Background: In cow's milk allergy (CMA) with intestinal symptoms, peripheral blood mononuclear cells (PBMCs) secrete tumor necrosis factor- α (TNF- α), altering intestinal function. However, the type of cow's milk protein (CMP) that triggers symptoms (intact or intestinally processed) is not known, and neither is the minimal amount required.

Methods: PBMCs were isolated from infants with active CMA or cured infants just before a new challenge and stimulated with intact or intestinally processed CMP. Supernatants were tested for cytokine content and for their ability to perturb intestinal barrier capacity, measured in Ussing chambers in HT29-19A intestinal cells.

Results: PBMCs from infants with active CMA secreted more TNF- α , when they were stimulated with intact rather than intestinally processed CMPs, and more TNF- α than PBMCs from cured infants. Accordingly, supernatants from PBMCs stimulated with intact but not intestinally processed CMPs significantly increased intestinal permeability. The CMP concentration required to trigger TNF- α secretion capable of altering intestinal function was very small in infants with active CMA (≈ 2 $\mu\text{g/ml}$), but about 300 times higher in cured infants.

Conclusion: Intact rather than intestinally processed proteins stimulate PBMCs to release TNF- α and alter intestinal barrier capacity. The threshold for PBMC reactivity to milk antigens drops considerably during active CMA with intestinal symptoms. (*J Allergy Clin Immunol* 1996;98:781-9.)

Key words: Food allergy, mononuclear cells, TNF- α , intestinal barrier, Ussing chambers

Cow's milk allergy (CMA), frequently manifested in infancy by skin, respiratory, or gastrointestinal symptoms, is considered to be due to several pathologic mechanisms operating separately or together, depending on the expression of clinical symptoms. In general, immediate reactions are associated with a high level of circulating antigen-specific IgE and with tissue-specific mast

Abbreviations used

CMA:	Cow's milk allergy
CMPs:	Cow's milk proteins
HRP:	Horseradish peroxidase
PBMCs:	Peripheral blood mononuclear cells
PHA:	Phytohemagglutinin
TNF- α :	Tumor necrosis factor- α

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cell degranulation after cross-linking of IgE receptors with the sensitizing antigens. These reactions are most often located in the skin and lead to cutaneous reactions, but intestinal anaphylaxis is also observed, leading to the activation of chloride secretion.¹ In late reactions the mechanism (essentially the enteropathy) remains unclear, although the triggering of a T-cell-mediated event has been suspected.²⁻⁵

For a long time, it has been known that a

malabsorption defect could be induced by β -lactoglobulin and withdrawn with milk protein hydrolysates.⁶ Until now, the principle of elimination and challenge has been the gold standard in the diagnosis of food allergy with predominantly gastrointestinal symptoms.⁷ However, the reintroduction of milk proteins is potentially dangerous in case of anaphylactic shock and is sometimes delayed, mainly in the delayed type of gastrointestinal food allergy. Consequently, there is a great need for predictive tests to overcome these drawbacks.

We recently reported that when peripheral blood mononuclear cells (PBMCs) from infants with CMA displaying intestinal symptoms were stimulated *in vitro* with cow's milk proteins (CMPs), they secreted much greater amounts of tumor necrosis factor- α (TNF- α) than PBMCs from nonallergic or cured infants. This high level of TNF- α secretion directly altered the intestinal epithelial barrier capacity.⁸

Although TNF- α is only one of the mediators involved in the adverse reaction to CMPs, it might be a reliable index of milk sensitization. To explore the clinical value of the TNF- α test, it seemed important to identify the antigen triggering TNF- α release because the CMPs in the intestinal lumen can cross the intestinal epithelium either as intact proteins, which constitute about 10% of the total protein transported,⁹ or in degraded or processed form (i.e., as peptides and probably amino acids). In principle, the peptides could be presented to the lymphocytes by the major histocompatibility complex class II molecules of the enterocytes or other antigen-presenting cells in the lamina propria. It is not known which of the intact or processed proteins directly triggers TNF- α secretion by PBMCs, as it is not known which type of mononuclear cell is involved in the TNF- α secretion induced by milk proteins.

The aims of this study were to determine: (1) whether intact or intestinally processed milk proteins induce TNF- α secretion that alters intestinal function, (2) the minimal amount of milk proteins triggering a potentially harmful TNF- α secretion, and (3) the type of mononuclear cells responsible for this secretion.

METHODS

Patients

Forty-seven infants were included in the study. Their mean age was 21.8 ± 1.8 months. All were initially diagnosed as being allergic to CMPs according to the criteria of the European Society for Gastroenterology and Nutrition⁷ (i.e., mainly by using the principle of

elimination and challenge and favorable response to the elimination diet). CMA was diagnosed by cow's milk elimination, followed by a single open challenge with an unprocessed infant formula. The challenge was started by placing a drop of cow's milk on the lips; thereafter, milk was given in increasing doses, up to 100 ml, at 1-hour intervals. When this first challenge was followed by clinical symptoms and infants had an appropriate clinical history and biologic evidence, they were diagnosed as having CMA. They were maintained on a cow's milk-free diet for at least 3 months before another challenge was attempted. A blood sample of 2 to 3 ml was collected just before that challenge, and the infants were then divided into two groups: the active CMA group, comprising those whose second challenge result was positive, and the cured of CMA group, comprising those whose second challenge result was negative. Informed consent for inclusion in the study was obtained from all parents or guardians. The active CMA group comprised 24 infants with intestinal symptoms and 11 with skin symptoms. Of the 24 with intestinal symptoms (mean age, 22.4 ± 2.4 months), only 27% had specific CMP IgE antibodies. They were first seen with at least one of the following symptoms: vomiting, diarrhea, failure to thrive, and anemia. In this group the results of the dual lactulose-mannitol test¹⁰ correlated with the effects of cow's milk ingestion. Among the 11 infants with active CMA manifested by skin lesions (mean age, 18.2 ± 4.6 months), 83% had high specific IgE. The second group of infants, who had recovered from CMA, comprised 12 subjects (mean age, 23.7 ± 3.0 months) who were initially diagnosed as being allergic to CMPs but whose second challenge result was negative. All initially had intestinal symptoms, only two had high total IgE, and none demonstrated high specific IgE.

Because of the small size of blood samples available, the whole set of experiments described below was not performed for each infant. In particular, results concerning the effect of reducing the amount of milk proteins on TNF- α release were confined to a separate set of 31 infants. Infants with active CMA and skin symptoms were tested exclusively in this dose-response study. All experimental procedures were performed in a blinded manner with respect to the clinical outcome.

Isolation and stimulation of PBMCs

Immediately after blood sampling, mononuclear cells were isolated from heparinized blood by using a Ficoll gradient (MSL; Eurobio, Les Ulis, France), washed three times with buffered saline solution, and resuspended in RPMI-1640 culture medium (Eurobio) containing 10% heat-inactivated human blood group AB serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 4 mmol/L glutamine. The cell concentration was adjusted to 10^6 /ml. In some experiments cells were allowed to adhere overnight at 37°C, nonadherent cells were removed, and cell concentrations were adjusted to 10^6 cells/ml before stimulation.

All the cells (i.e., adherent plus nonadherent), or in some cases nonadherent cells alone or adherent cells alone, were stimulated for 6 days with a mixture of intact bovine milk proteins comprising equal amounts of β -lactoglobulin, α -lactalbumin, and whole casein (Sigma, St. Quentin, France) at final concentrations ranging from 600 to 0.6 $\mu\text{g/ml}$. The highest CMP concentration used comprised 200 $\mu\text{g/ml}$ β -lactoglobulin, α -lactalbumin, and casein (total, 600 $\mu\text{g/ml}$); and serial quarter dilutions allowed us to test concentrations of 150, 37.5, 9.3, 2.3, and 0.6 $\mu\text{g/ml}$. Intestinally processed CMPs, prepared as described below, were also used, as well as the nonmilk protein ovalbumin (200 $\mu\text{g/ml}$). Parallel control cultures were run without stimulation. Supernatants obtained from control PBMCs or from PBMCs stimulated with either intact CMPs or intestinally processed CMPs were each collected in aliquots and frozen at -80°C until use.

Preparation of intestinally processed CMPs

Monolayers of HT29-19A intestinal cells, grown on micropore filters as described below, were used to process the mixture of intact CMPs comprising 200 $\mu\text{g/ml}$ each of β -lactoglobulin, α -lactalbumin, and casein. This mixture was placed for 48 hours in the apical (mucosal) compartment (1.5 ml) of Transwell filters (Costar, Brumath, France) on which HT29-19A cells were cultured (exposed surface, 4.7 cm^2). Proteins were allowed to be transported and processed during transport through the intestinal cell layer, and the basal (serosal) compartments (2.5 ml) were collected after 48 hours. This basal culture medium containing the intestinally processed CMPs was assayed for immunoreactive β -lactoglobulin by using an ELISA as previously described,¹¹ which produced a 50 $\mu\text{g/ml}$ concentration for immunoreactive β -lactoglobulin. The proteins other than this immunoreactive β -lactoglobulin were considered to be partially or totally processed, and no attempt was made to characterize them further in these experiments. The intestinally processed CMPs in the basal compartment were used to stimulate PBMCs without further dilution.

Cytokine release in PBMC culture supernatants

Supernatants from PBMCs cultured under different experimental conditions were tested for the release of IL-4, interferon- γ , IL-6, and TNF- α . We used commercially available ELISA kits (Tebu, Le Perray en Yvelines, France). In the last set of experiments, in which increasing amounts of intact CMPs were used to stimulate PBMCs from infants with active CMA manifested by intestinal or skin symptoms, only TNF- α was tested.

The intestinal HT29-19A cell line as a model of human intestinal epithelium

The human colon carcinoma cell line HT29-19A, cloned from HT29 by Augeron and Laboisse¹² and

cultured on permeable supports, was used as a model to assess intestinal barrier capacity after cell treatment with PBMC supernatants, as previously described.⁸ Between passages 9 and 35, intestinal cells were cultured at 37°C in Dulbecco's modified Eagle medium (Eurobio) containing 10% heat-inactivated fetal calf serum, 4 mmol/L glutamine, and 50 $\mu\text{g/ml}$ gentamycin, in a humidified atmosphere of 5% CO_2 /95% air. The medium was changed daily, and cells were passaged every week and seeded at 10^6 cells per 25 cm^2 . For experimental purposes, cells were seeded at a density of 0.8×10^6 cells/ cm^2 on polycarbonate Transwell filters with a pore diameter of 0.4 μm and a surface area of 1.13 or 4.7 cm^2 . Cells formed a monolayer that reached stable electrical resistance of about $150 \Omega \cdot \text{cm}^2$ after 3 weeks and were used from that time.

Measurement of intestinal barrier capacity after treatment with PBMC supernatants

CMPs, intestinally processed CMPs, or control supernatants from infants with active (intestinal symptoms) CMA or cured infants were applied for 48 hours to the basal compartment of the Transwell-grown HT29-19A intestinal cells at a final concentration of 10%. The inserts were then removed from the culture plates, and filters were gently cut out and mounted in Ussing chambers with an exposed area of 0.15 cm^2 . Monolayers were exposed on each side to 1.5 ml of oxygenated, thermostated Ringer's solution as previously described.¹³ Cell monolayers were short-circuited with an automatic voltage clamp (WPI, Aston, England) after appropriate correction for fluid and system resistance. Electrical parameters including potential difference, short-circuit current, and ionic conductance were recorded at regular intervals during the 130-minute experiments. Ionic conductance was considered as an index of paracellular epithelial permeability. After equilibration, permeability markers were added to the apical compartment. Horseradish peroxidase (HRP) was added at a final concentration of 0.4 mg/ml (10 $\mu\text{mol/L}$) with a tracer dose of tritiated HRP (37 KBq/ml), labeled, and stored as previously described.¹⁴ Mannitol (5 mmol/L) was introduced on both sides of the intestinal layer, and carbon 14-labeled mannitol (12.2 KBq/ml) was introduced on the apical side only. Finally, sodium 22 was added apically to obtain a specific activity of 14.8 KBq/ml. The three markers were applied simultaneously in the apical compartment, and their basal appearance was monitored by sampling 800 μl of the basal compartment at 20-minute intervals for 130 minutes. Radioactivities (^3H , ^{14}C , and ^{22}Na) were measured on 500 μl samples by liquid scintillation photometry with three appropriate channels and triple label correction. HRP was immediately assayed on 200 μl aliquots by using its enzymatic activity,¹⁵ and intact HRP fluxes were calculated. Tritiated HRP-equivalent fluxes were calculated and constituted the total amount of HRP transported in either intact or degraded form. Degraded HRP fluxes were

TABLE I. Effect of various stimuli on the release of cytokines by PBMCs isolated from infants with active CMA or PBMCs from cured infants

	Active CMA				Recovered from CMA			
	TNF- α	IFN- γ	IL-6	IL-4	TNF- α	IFN- γ	IL-6	IL-4
Nonstimulated controls	145 $\pm$ 41 (25)	12 $\pm$ 12 (5)	17 $\pm$ 10 (5)	Not detectable	26 $\pm$ 11 (13)	Not detectable	Not detectable	Not detectable
PHA (10 μ g/ml)	229 $\pm$ 50 (4)	671 $\pm$ 67 (4)	187 $\pm$ 51 (4)	103 $\pm$ 2 (3)	146 $\pm$ 24 (5)	801 $\pm$ 101 (5)	182 $\pm$ 23 (5)	134 $\pm$ 18 (5)
CMPs	1332 $\pm$ 158* $\dagger$ (16)	149 $\pm$ 43* (7)	627 $\pm$ 128* (7)	Not detectable	385 $\pm$ 89 $\ddagger$ (13)	170 $\pm$ 15* (6)	606 $\pm$ 60* (6)	32 $\pm$ 20* (6)
Intestinally processed CMPs	310 $\pm$ 69 (7)	118 $\pm$ 33 (7)	569 $\pm$ 123 (7)	Not detectable	328 $\pm$ 74 (7)	179 $\pm$ 10 (7)	679 $\pm$ 124 (7)	42 $\pm$ 20 (7)
Ovalbumin	177 $\pm$ 56 (10)	ND	ND	ND	77 $\pm$ 37 (8)	ND	ND	ND

CMPs comprised a mixture of β -lactoglobulin, α -lactalbumin, and casein at a concentration of 200 μ g/ml each. Ovalbumin was used at the same concentration. Number of infants is given in parentheses. Cytokine concentrations are expressed in picograms per milliliter.

IFN, Interferon; ND, not done.

* Significantly different from PHA ($p < 0.002$).

$\dagger$ Significantly different from the corresponding intestinally processed CMP ($p < 0.001$).

$\ddagger$ Significantly different from active CMA value ($p < 0.0001$).

then calculated as total minus intact fluxes. HRP fluxes were expressed in nanograms per hour times square centimeters (ng/hr $\cdot$ cm²), and mannitol and sodium fluxes were calculated according to their specific activity and expressed in micromoles per hour times square centimeters (μ mol/hr $\cdot$ cm²) and micro-equivalents per hour times square centimeters (μ Eq/hr $\cdot$ cm²), respectively. Values for all fluxes are given at steady-state (i.e., as mean values of fluxes measured during the second hour of experimentation).

Statistical analysis

Data were analyzed by using the SAS software package¹⁶ (SAS Institute, Cary, N.C.). Analysis of variance was performed by the general linear model procedure, and Student's t test was used to compare means of the different groups. Results are expressed as means $\pm$ SEM.

RESULTS

Cytokine content of PBMC culture supernatants

Table I shows the cytokines released by PBMCs from the infants with active CMA manifested by intestinal symptoms or PBMCs from infants cured of CMA, cultured in the presence of phytohemagglutinin (PHA), CMPs, intestinally processed CMPs, or ovalbumin. As previously reported,⁸ but even more strikingly, TNF- α release was greater in PBMCs from infants with active CMA than in those from cured infants. Furthermore, intestinally processed CMPs did not induce such high secre-

tion of TNF- α . Ovalbumin, a nonmilk-related protein, did not stimulate TNF- α release above control values, suggesting that the reaction was specific to CMPs.

In general, CMPs were more potent in stimulating the release of IL-6 and TNF- α than PHA, which rather triggered interferon- γ and IL-4 release. This suggests that CMPs are more likely to stimulate monokine secretion, whereas PHA stimulates lymphokine secretion.

IL-6 secretion also increased in PBMCs from infants with active CMA, but this high secretion was also found in cells from cured infants, suggesting that this cytokine was not responsible for the intestinal disease.⁸ Note that IL-4 secretion was not stimulated by CMPs in the infants with CMA manifested by intestinal symptoms.

Effect of PBMC supernatants on intestinal function

As Fig. 1 shows, HT29-19A cell monolayer permeability was modified after 48 hours of treatment with PBMC culture supernatants from the infants with active CMA manifested by intestinal symptoms. Ionic conductance, G , an index of paracellular epithelial permeability, rose significantly when PBMCs were stimulated with intact CMPs, but this increase was no longer significant when they were stimulated with intestinally processed CMPs. The same pattern was observed for the

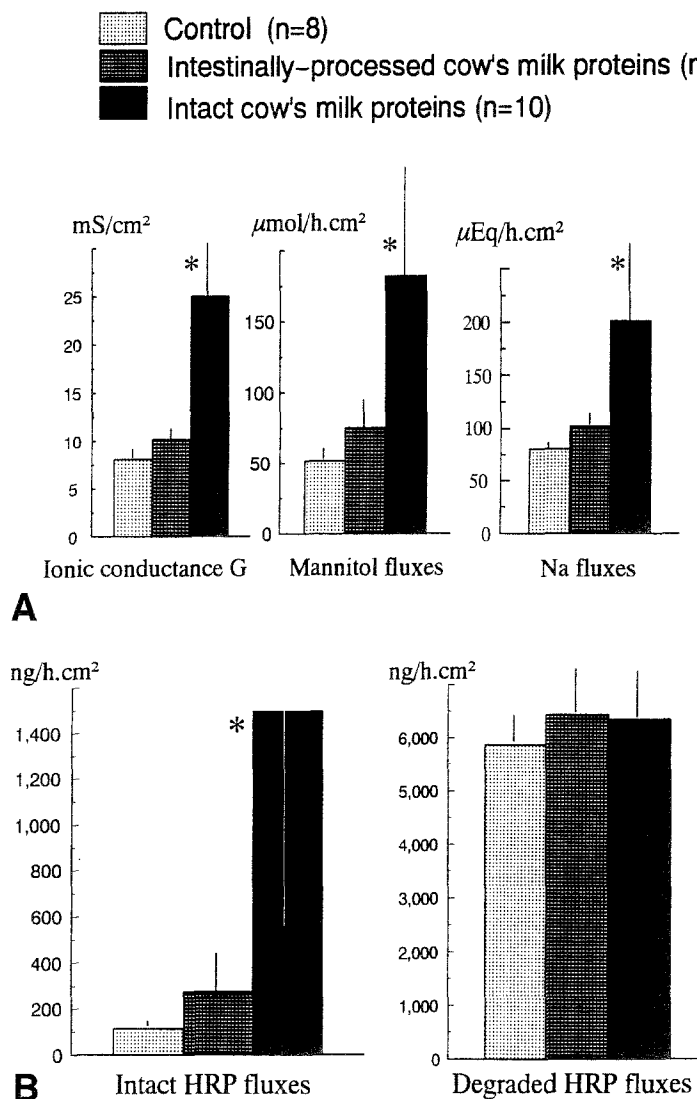


FIG. 1. Effect of supernatants of PBMCs from infants with active CMA, unstimulated PBMCs (control), or stimulated PBMCs with intact or intestinally processed CMPs on intestinal permeability to ions and small markers (**A**) and HRP (**B**) in intact and degraded form, measured on intestinal HT29-19A cells. *Significantly different from control and intestinally processed CMP ($p < 0.03$). n , Number of infants tested; mS/cm^2 , $\mu mol/hr \cdot cm^2$, $\mu Eq/hr \cdot cm^2$, $ng/hr \cdot cm^2$.

small molecules (mannitol and sodium) and the macromolecular tracer HRP. The transcellular transport measured by degraded HRP fluxes was not modified. In infants cured of CMA, there were no permeability changes with intact or intestinally processed supernatants compared with control supernatants (not shown).

Effect of increasing amounts of CMPs on TNF- α release by PBMCs from infants with active CMA and infants cured of CMA

Because TNF- α release was highly activated after treatment of PBMCs with intact CMPs (Table I), this cytokine was tested in a dose-response

assay. The test was conducted in a separate group of 31 infants who had active CMA ($n = 26$) with either intestinal symptoms ($n = 18$) or skin symptoms ($n = 8$) and in cured infants ($n = 5$).

Fig. 2 shows the release of TNF- α by PBMCs, which were isolated from these infants and stimulated with increasing amounts of CMPs. For CMP concentrations of 0.6 to 600 $\mu g/ml$, TNF- α release by PBMCs from infants with active CMA manifested by intestinal symptoms was greater than that by PBMCs from cured infants. The CMP concentration allowing the release of an amount of TNF- α previously shown to alter epithelial barrier function (i.e., 500 pg/ml ⁸) was very low in the

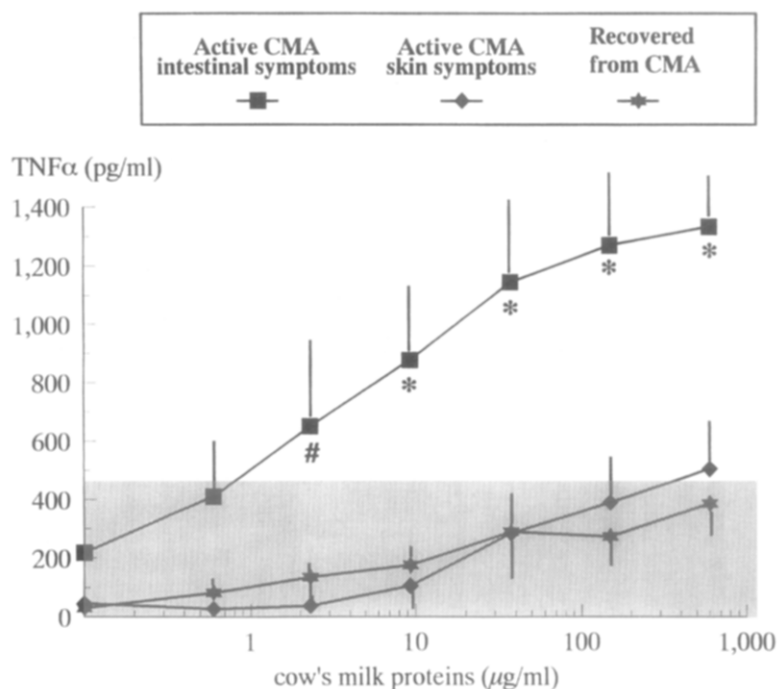


FIG. 2. TNF- α release from PBMCs (10^6 /ml) stimulated with increasing amounts of CMPs. Shaded area, Zone in which the TNF- α concentration did not induce intestinal dysfunction.⁸ In infants with active CMA manifested by intestinal symptoms ($n = 18$), CMP doses as low as 2 μ g/ml induced permeability disorders, whereas infants who had recovered from CMA ($n = 5$) secreted less TNF- α , even at the very high CMP concentration of 600 μ g/ml. In contrast, in infants with active CMA manifested by skin symptoms ($n = 11$), TNF- α secretion remained low.

infants with active CMA (2 μ g/ml), but it was about 300 times higher in cured infants (600 μ g/ml). Unlike PBMCs from infants with active CMA manifested by intestinal symptoms, PBMCs from infants with active CMA manifested by skin symptoms did not release more TNF- α than PBMCs from cured infants.

Although more infants should be examined before a diagnostic or predictive test based on TNF- α release by isolated PBMCs can be considered, we analyzed the results of such a test in terms of sensitivity and specificity. The best discrimination between infants with active (intestinal symptoms) CMA and cured infants was obtained at a cutoff value of 200 pg/ml and a CMP concentration of 10 μ g/ml. In these conditions the predictive power of the test was 90%, its specificity was 80%, and its sensitivity was 94%.

Comparison of TNF- α release by whole PBMCs and nonadherent PBMCs from infants with CMA

In eight infants, we conducted parallel tests of TNF- α secretion from whole PBMCs and from

nonadherent PBMCs. As Table II indicates, nonadherent cells alone (i.e., T and B lymphocytes) did not secrete TNF- α . In addition, in two infants, we tested TNF- α secretion after direct stimulation of isolated adherent cells with the highest concentration of CMPs, and none was observed (not shown). This suggests that both nonadherent cells (lymphocytes) and adherent cells (monocytes/macrophages) are essential for TNF- α secretion.

DISCUSSION

The results of this study confirm that TNF- α secretion by mononuclear cells greatly increases during active CMA with intestinal symptoms but not during CMA with skin symptoms. They further indicate that in such infants with CMA, the threshold of immune reactivity to milk antigens drops considerably and might be used as a test for prediction of the outcome at a new challenge.

It has long been a puzzling observation that most infants with intestinal symptoms caused by CMA (also called cow's milk sensitive enteropathy) have a predominantly non-IgE-dependent disease for which the effectors remained uncharacterized. Re-

TABLE II. TNF- α secretion by whole PBMCs (lymphocytes + monocytes) or nonadherent PBMCs (lymphocytes) from infants with CMA

	CMP concentration ($\mu\text{g/ml}$)						
	0	0.6	2.3	9.3	37.5	150	600
Whole PBMCs	66 $\pm$ 38	101 $\pm$ 35	223 $\pm$ 97	448 $\pm$ 252	497 $\pm$ 261	627 $\pm$ 247	778 $\pm$ 223
Nonadherent PBMCs	20 $\pm$ 9	17 $\pm$ 7	18 $\pm$ 8	19 $\pm$ 7	21 $\pm$ 8	33 $\pm$ 7	85 $\pm$ 39
<i>p</i> Value	NS	NS	NS	0.03	0.027	0.0065	0.001

PBMCs were collected from eight infants and stimulated with increasing amounts of CMPs. Cells were allowed to adhere for 24 hours before nonadherent cells were collected and stimulated with CMPs. Results are given for eight infants (two with active CMA and intestinal symptoms, three with active CMA and skin symptoms, and three who had recovered).

NS, Not significant.

cent results from our laboratory⁸ showed that the release of the proinflammatory cytokine TNF- α was greatly enhanced by PBMCs from infants with active CMA and intestinal symptoms. In this study we compared the capacity of intact versus intestinally processed milk proteins to induce such TNF- α release. We also attempted to determine the minimal amount of milk proteins able to stimulate such high TNF- α secretion and the type of immune cells involved in TNF- α release.

Luminal processing of food proteins makes them lose most of their antigenic properties, thus rendering them inactive in relation to the immune system. However, small amounts of intact food proteins do reach the proximal intestine,¹⁷ undergo transcytosis, and are processed through the enterocytes.⁹ Recent reports have presented evidence that in addition to their absorptive capacity, enterocytes are able to express the class II major histocompatibility complex^{18, 19} and to act as occasional antigen-presenting cells in relation to the underlying intraepithelial lymphocytes^{20, 21} or even the lamina propria lymphocytes.²² Therefore it is possible that peptides formed during transcytosis in the enterocytes and expressed at the basal membrane in conjunction with class II molecules, or released in the lamina propria, stimulate underlying immune cells. In this study we submitted intact milk antigens (i.e., β -lactoglobulin, α -lactalbumin, and casein) to enterocytic processing with intestinal HT29-19A cells grown in vitro on permeable filters. Both this cell line and the Caco2 cell line were previously used to study the transcytosis of intact macromolecules, mainly HRP²³ or lactoferrin.²⁴ As in the entire intestine,⁹ the two macromolecular transport pathways comprised one minor direct pathway responsible for 10% of the transport, along which the transported protein remains intact, and a major degradative pathway, which is responsible for the remaining 90% of

transport and involves intracellular processing. Therefore the basal compartment of the filters on which HT29-19A cells were grown was used as the fraction rich in intestinally processed proteins. Although the exact content of such a preparation was virtually unknown, particularly the nature of the milk protein peptides generated and the possible release of cytokines by HT29-19A cells, it constituted a first approach to the determination of these peptides' capacity to stimulate immune cells. Thus when such intestinally processed CMPs were used, TNF- α secretion by PBMCs was significantly lower than that obtained with intact CMPs, and the intestinal permeability of filter-grown HT29-19A intestinal cells was not altered.

This raised the question of what minimal amount of intact protein is able to trigger TNF- α release by mononuclear cells from infants with active CMA. Although it is remarkable that tiny amounts of milk antigens in allergic infants are sometimes enough to stimulate symptoms, the actual threshold of immune reactivity to milk antigens has never been assessed. In this study our TNF- α release test showed that in infants with active CMA, amounts as small as 2 $\mu\text{g/ml}$ triggered a TNF- α release large enough to induce an in vitro increase in intestinal permeability. In cured infants even 300 times more CMP (600 $\mu\text{g/ml}$) did not trigger release of such large amounts TNF- α . Therefore it may be postulated that intestinal symptoms in infants with active CMA are due to the lowering of the threshold of milk antigens able to activate the submucosal immune system, with subsequent release of harmful mediators such as TNF- α . The lowering of the reactivity threshold might be due to an increase in the number of circulating and/or local CMP-specific mononuclear cells, which would increase the probability of such cell interaction with small amounts of antigen. Accordingly, if few mononuclear cells have the

potential to be activated, more proteins will be needed to trigger their activation.

Finally, we attempted to characterize the type of mononuclear cells (i.e., monocytes/macrophages or lymphocytes) responsible for TNF- α secretion. Several of our findings argue in favor of the involvement of monocytes/macrophages: (1) the profile of the cytokines released is more likely to be of the monokine than the lymphokine type because TNF- α and IL-6, rather than IL-4 and Interferon- γ , were secreted, (2) nonadherent PBMCs secreted less TNF- α than whole PBMCs, and (3) TNF- α secretion was even more pronounced after 24 hours than after 6 days (not shown), suggesting the involvement of macrophages. However, CMPs did not seem to directly stimulate monocytes/macrophages, suggesting TNF- α secretion by an indirect action, probably through activated lymphocytes.

In this study we found that in infants with active CMA manifested by skin symptoms, TNF- α was not one of the main cytokines released by mononuclear cells. This strengthens the hypothesis that different mechanisms operate in CMA. Children with intestinal symptoms have a low incidence of IgE-mediated disease, and in this study TNF- α seemed to be involved in the pathogenesis. In contrast, most infants with skin symptoms are atopic, and it is possible that other mediators are involved in this pathogenesis.

In conclusion, the results of this study suggest that TNF- α release by PBMCs is a good marker of immune reactivity to milk antigens in children with CMA and intestinal symptoms (cow's milk sensitive enteropathy). Such immune reactivity might be used as a factor to distinguish infants with active CMA from cured infants, because infants with active allergy have a threshold of reactivity 300 times lower than cured infants. We are presently starting a larger study with whole blood instead of isolated PBMCs to further assess the feasibility and effectiveness of the TNF- α test.

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