

Cord blood mononuclear cells and milk-specific T-cell clones are tools to evaluate the residual immunogenicity of hydrolyzed milk formulas

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Background: Hydrolyzed milk formulas (HFs) are given to infants allergic to cow's milk proteins and, for preventive reasons, to atopy-prone newborns for which breast feeding is not feasible. The ultimate properties of HFs are not only a reduced allergenicity but also decreased immunogenic capacity combined with good taste and caloric value. No information is available concerning the capacity of HFs to induce immune responses.

Objective: We sought to determine the residual immunogenic capacity of partially (pHF) and extensively hydrolyzed milk formula (eHF), and we studied the cellular reactivity of cord blood-derived ($n = 71$) mononuclear cells induced by 10 different HFs.

Methods: To test the effect of HF on T-helper cells, β -casein-specific T-cell clones (TCCs, $n = 21$) from individuals allergic to milk were established, and T-cell proliferation and cytokine profiles (interferon- γ and IL-4) were determined on stimulation with HF.

Results: We found significantly reduced proliferative responses of eHF compared with milk proteins. Whey-based pHF displayed the same proliferative capacity as unmodified milk proteins. As expected, extensively processed whey products displayed lower cellular responses compared with partially hydrolyzed products (pHF whey vs eHF whey, $p < 0.0001$). No difference in cellular response was found between casein-based pHF and casein-based eHF. β -Casein-specific TCCs ($n = 21$) proliferated in response to casein-derived hydrolysates (14% with casein/whey-based pHF, 4% with casein-based pHF, and 0% with casein-based eHF). Whey-based pHF was also found to induce proliferation in β -casein-specific TCCs, indicating the presence or the generation of peptides displaying cross-reactivity with these whey-derived hydrolysates. TCCs stimulated with whey- or casein-based pHF or eHF produced the same amount of cytokines (IL-4, interferon- γ) as the same clones stimulated with unmodified products.

Conclusion: Our data indicate that whey- and casein-derived eHFs display highly reduced immunogenic properties at the T-cell level. In contrast, pHFs display residual immunogenic

properties detectable at the T-cell level, reflecting a potential for the induction of pathogenetically important T-cell responses. (*J Allergy Clin Immunol* 1998;101:514-20.)

Key words: Cord blood cells, hydrolyzed milk formulas, proliferative response, milk proteins, T-cell clones, cytokines

The concept of reduced allergenicity and immunogenicity in cow milk-based formula can be viewed from two perspectives. The first involves a reduction in allergenicity (the capacity to elicit an IgE-mediated effector function) sufficient to cause no clinically significant symptoms in infants already allergic to cow's milk protein. This task is demanding because only a minute residual molecule is required for recognition of the antigen by the previously sensitized individual.¹ A second perspective relates to allergy prophylaxis in atopy-prone infants. Although the molecular requirements in this instance are less well understood, prophylaxis implicates a reduction in immunogenicity (the capacity to induce an immune response) sufficient to delay or prevent sensitization that may result in clinical symptoms of allergy.

To study the residual immunogenicity and allergenicity of milk formula, a combination of physicochemical and immunologic testing is required. The following approaches have been used in this context: extent of protein hydrolysis,^{2,3} sodium dodecylsulfate-polyacrylamide gel electrophoresis,^{4,5} skin prick tests in patients with allergic symptoms,^{6,7} leukocyte histamine release tests,^{7,8} RASTs, and ELISA with ELISA inhibition.^{4,8}

Recently, a crucial role for T cells in the induction and maintenance of Type I allergy has been demonstrated.⁹ Therefore in vitro tests investigating T-cell recognition should represent a part of the analysis process of hydrolyzed milk formula (HF). Some investigators used cellular proliferation in response to milk proteins and milk protein hydrolysates in patients with milk allergy or intolerance to define their beneficial effect on the immune system.^{10,11} Because the initiation of immune responses to some antigens is likely to be triggered early in the neonatal period,^{12,13} knowledge of the residual immunogenicity of hydrolyzed milk protein formula appears essential. Although the use of milk protein-based hydrolyzed formula is recommended in addition to breast feeding for babies at risk, it is not known whether these HFs are recognized by T cells. In a recent

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Supported by the Fonds zur Förderung der Österreichischen Forschung (FWF) project #S06701-Med and #S06704-Med and by BMWF GZ 49.847/5.

Received for publication June 26, 1997; revised Nov. 24, 1997; accepted for publication Nov. 25, 1997.

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0091-6749/98 \$5.00 + 0 1/1/87935

Abbreviations used

CBMC:	Cord blood mononuclear cell
eHF:	Extensively hydrolyzed milk formula
HF:	Hydrolyzed milk formula
IFN:	Interferon
PBMC:	Peripheral blood mononuclear cell
pHF:	Partially hydrolyzed milk formula
TCC:	T-cell clone

article we have shown that cord blood mononuclear cells (CBMCs) from almost every term newborn proliferate in response to some milk proteins,¹⁴ indicating prenatal contact with milk proteins.

In this study we focused on the identification of the “residual immunogenicity” of HF. The capacity of a variety of partially hydrolyzed milk formulas (pHFs) and extensively hydrolyzed milk formulas (eHFs) to induce proliferation in cord blood cells from term newborns and to stimulate allergen-specific T-cell clones (TCCs) was assessed.

METHODS

Cord blood cells

Human umbilical cord blood from term infants ($n = 71$) was drawn and placed on heparin before expulsion of the placenta, and CBMCs were prepared as described.¹⁵ The protocol has been approved by the local ethical committee of the University of Vienna.

Cord blood and maternal serum IgE concentrations were measured with a commercially available ELISA (Kallestad, Sanofi Diagnostics Pasteur; sensitivity 0.5 IU/ml). For exclusion of possible maternal blood contamination, cord blood serum was analyzed for total IgA and IgM.

Three adults with cow's milk allergy, as determined by positive skin prick test responses to cow's milk proteins, detection of cow's milk protein-specific IgE, and clinical reaction with predominantly dermatologic upper respiratory and gastrointestinal symptoms, were screened for cellular responsiveness to milk proteins. Thereafter, β -casein-specific T-cell lines and TCCs were established according to described protocols.¹⁶

Milk formula and antigens

Samples used in this study included five eHFs, three pHFs, two adapted milk formulas, and two extensive peptide components for manufacturing products with the following characteristics: Aptamil 1 (Milupa; an adapted cow's milk formula); Beba HA (Nestlé; a partial whey hydrolysate); Nidina HA (Nestlé; a partial whey hydrolysate); Alfaré (Nestlé; an extensive whey protein hydrolysate); Prophylac (ALK-Epipharma; an extensive whey hydrolysate); Hipp HA (Hipp; an extensive whey hydrolysate); Peptamine Junior (Nestlé; an extensive whey-based hydrolysate for supplementation of mother's milk in premature newborns); extensive whey hydrolysate (MOH, Milupa; an extensive whey-based peptide component for HA formula); Aptamil hypoallergen (Milupa; an extensive whey/casein hydrolysate); Damira (Sandoz-Wander; an extensive whey/casein hydrolysate); partial casein hydrolysate (CHA, Milupa; a partial casein-based peptide component for HA formula); and Nutramigen (Mead Johnson; an extensive casein hydrolysate).

α -Lactalbumin, bovine serum albumin, α -casein, β -casein, and κ -casein were purchased from Sigma (Deisenhofen, Germany). β -Lactoglobulin (a kind gift from Jean-Michel Wal [France]), used for T-cell cloning experiments, was purified by high-performance liquid chromatography and affinity chromatography.

Cell preparation and T-cell proliferation

CBMCs were separated from heparinized blood by density gradient centrifugation and cultured at a concentration of 10^5 cells/200 ml in Ultra Culture Medium (BioWhittaker, Inc., Walkersville, Md.) supplemented with 1% human serum, 2 mmol/L glutamine, and 50 mg/ml gentamicin. The cells were incubated at 37° C in a humidified atmosphere with 5% CO₂ for 7 days in the presence or absence of various pHFs and eHFs (50 μ g/ml).^{14, 16} Stimulation with purified milk proteins (β -lactoglobulin, α -lactalbumin, α -casein, β -casein, and κ -casein) were used as comparative controls, recombinant human IL-2 (20 IU/ml; Boehringer Mannheim, Mannheim, Germany) served as a positive control,¹⁴ and medium alone served as a negative control. After 6 days of culture, tritiated thymidine (Amersham; 0.5 μ Ci/well) was added for 16 hours, and the incorporated radioactivity was measured by scintillation counting. Proliferation was expressed as stimulation index (SI) of a geometric mean of triplicates (ratio between cpm obtained in cultures with CBMCs plus antigen and cpm obtained in cultures without antigen).^{14, 16}

Establishment of β -casein-specific TCCs and autologous Epstein-Barr virus-transformed B-cell lines as antigen-presenting cells

Allergen-specific TCCs and Epstein-Barr virus-transformed B cells were obtained by techniques described.^{16, 17} Briefly, peripheral blood mononuclear cells (PBMCs) (10^6 /ml) were stimulated with optimal β -casein concentrations (5 to 100 μ g/ml, determined by titration in a proliferation assay with PBMCs) in Ultra Culture Medium supplemented with 1% human serum, 2 mmol/L glutamine, and 2×10^{-5} mol/L 2-mercaptoethanol in 24-well, flat-bottomed culture plates (Costar, Cambridge, Mass.) for 5 days. Subsequently, human rIL-2 (10 IU/ml) was added, and the culture continued for an additional 7 days. Viable T-cell blasts were then separated by Ficoll/Hypaque (Pharmacia, Uppsala, Sweden), and allergen specificity was assessed by a tritiated thymidine incorporation assay. Thereafter, T-cell blasts were seeded in limiting dilution (0.3 cells/well) in 96-well, round-bottom plates (Nunc, Nunc) in the presence of 10^5 irradiated (5000 rad) allogeneic PBMCs used as feeder cells, 1% phytohemagglutinin (vol/vol) (Gibco) and rIL-2 (4 IU/well) in the above-described medium. Growing microcultures were then expanded at weekly intervals with fresh feeder cells and rIL-2.

Proliferation assays

The specificity of the established TCCs was assessed as previously described.¹⁶ Briefly, 2×10^4 T-cell blasts were incubated in triplicate cultures in 96-well plates in the presence of 10^5 autologous EBV-B cells and antigen (milk proteins and HFs). Microtiter plates were then incubated for 48 hours at 37° C in a 5% CO₂ humidified atmosphere. After pulsing for 16 hours with 0.5 μ Ci/well of tritiated thymidine, cells were harvested, and the incorporated radioactivity was measured by liquid scintillation counting. Only TCCs inducing an SI greater than 5 were considered positive.

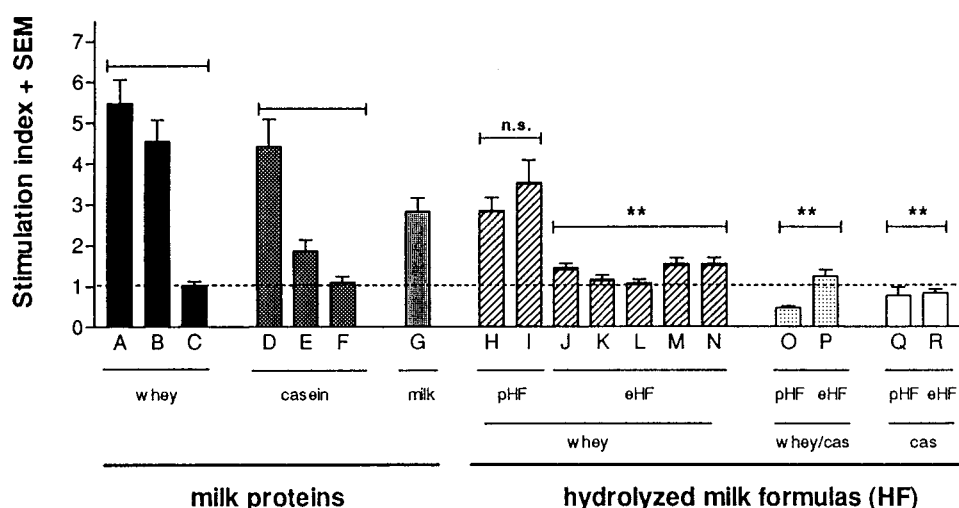


FIG. 1. Cellular proliferative response of CBMCs to HFs. CBMCs from 71 term newborns were incubated with 50 μ g/ml HF, and mean of proliferation index (+SEM) was determined after 6 days of culture in triplicates. Milk proteins and HFs: whey proteins (A: α -lactalbumin, B: β -lactoglobulin, and C: bovine serum albumin), casein (D: α -casein, E: β -casein, and F: κ -casein), adapted cow's milk (G: Aptamil 1), partial whey-based HFs (H: Beba HA and I: Nidina HA), extensive whey-based HFs (J: Alfaré, K: Prophylac, L: Hipp HA, M: Peptamine Junior, and N: MOH), partial whey/casein-based HF (O: Aptamil hypoallergen), extensive whey/casein-based HF (P: Damira), partial casein-based peptide component (Q: CAH), and extensive casein-based HF (R: Nutramigen). Background level is defined at stimulation index of 1. Generalized linear model was used for comparison of groups of antigens. Probability values are for comparison between groups ($p < 0.0001$).

Phenotype analysis of TCCs

Fluorescein isothiocyanate-labeled monoclonal antibody anti-Leu-4/CD3, anti-Leu-3a/CD4, anti-Leu-2a/CD8, anti-T-cell antigen receptor TCR-1a/b WT31, and anti-TCR-g/d I (Becton Dickinson, Mountain View, Calif.) were used to analyze the phenotype of the TCCs. TCCs and irradiated feeder cells alone were harvested and washed, and 10^5 cells were incubated for 30 minutes on ice with the appropriate dilution of monoclonal antibodies or isotype-matched control antibodies. After washing three times with phosphate-buffered saline (containing 0.3% bovine serum albumin and 0.3% NAN3), the cells were stained with propidium iodide to allow exclusion of dead cells and analyzed on a FACScan (Becton Dickinson).

Lymphokine measurements

Interferon- γ (IFN- γ) secretion by CBMCs was determined with a commercial ELISA kit (Medgenix, Fleurus, Belgium) with a sensitivity threshold of 0.03 IU/ml (corresponding to 1.5 pg/ml). IL-4 levels were measured with a sandwich ELISA technique (Quantikine; R&D Systems, Minneapolis, Minn.) with a sensitivity threshold of 20 pg/ml. Recombinant and natural cytokine standards were used as references.

For TCC cytokine analysis, 10^5 clone cells/200 ml were washed and incubated with 10^5 irradiated autologous antigen-presenting cells (EBV-transformed B cells) in the presence of β -casein (50 μ g/ml). Supernatants were collected after 48 hours of incubation.

Statistical analysis

Pairwise comparison of the 18 variables defining the antigens was performed in 153 individual tests with the paired t test. For differences with a sample size of less than 30, the nonparametric Wilcoxon signed-rank test was applied. To control for the multiple level α , the sequential Bonferroni-Holm correction was applied.

Comparing groups of variables, a further generalization of the generalized linear model was used. For computational reasons, only equal variance for all of the subjects could be specified. The multiple level of significance was again controlled for the Bonferroni-Holm procedure.

Statistical analysis was carried out through the SAS package, version 6.11.

RESULTS

Cord blood cells proliferate in response to milk protein hydrolysates

To test whether HFs exhibit residual proliferative responses in CBMCs, CBMCs were incubated with various pHFs and eHFs. Fig. 1 illustrates that pHFs and eHFs elicit proliferative responses in mononuclear cells. Whey-based pHFs (H and I) displayed similar proliferative capacity as unmodified milk (G) or milk proteins (A and B). As expected, whey-based eHFs induced lower cellular responses compared with pHFs (pHF vs eHF; $p < 0.0001$). Moreover, the SI of less than 1 in both casein-based pHFs and casein-based eHFs reflected no lymphocyte-stimulatory capacity. Stimulation of CBMCs with whey-based eHFs (J, K, L, M, and N) or a whey/casein-based eHF (P) induced significantly lower proliferation compared with unmodified casein proteins and unmodified whey proteins, respectively ($p < 0.0001$, Fig. 1). Compared with the background level SI of 1, residual stimulatory capacity was found in whey-based eHFs (J, M, and N) and a whey/casein-based eHF (P) but not in a whey/casein-based pHF (O), a casein-based pHF (Q), or a casein-based eHF (R).

To define differences among the groups of pHFs and

TABLE I. Comparison between individual antigen stimulation variables of cord blood cells of unselected newborns

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
A	—	NS	*	NS	*	*	*	*	NS	*	*	*	*	*	*	*	*	*
B		—	*	NS	*	*	*	†	NS	*	*	*	*	*	*	*	*	*
C			—	*	†	NS	*	*	*	*	‡	NS	*	*	*	NS	NS	NS
D				—	*	*	‡	‡	NS	*	*	*	*	*	*	*	*	*
E					—	‡	‡	‡	*	NS	‡	†	NS	NS	*	NS	†	†
F						—	*	*	*	†	NS	NS	†	‡	*	*	NS	NS
G							—	NS	NS	*	*	*	*	*	*	*	*	*
H								—	*	*	*	*	*	*	*	*	*	*
I									—	*	*	*	*	*	*	*	*	*
J										—	†	†	NS	NS	*	NS	†	*
K											—	NS	†	‡	*	NS	‡	†
L												—	*	*	*	NS	NS	‡
M													—	NS	*	NS	*	*
N														—	*	NS	*	*
O															—	*	NS	*
P																—	*	NS
Q																	—	NS
R																		—

Individual antigen stimulations were compared with each other with the Wilcoxon signed-rank test when $n < 30$ (I and P) or with the paired t test when $n = 71$ (all other antigens).

Milk proteins and HFs: whey proteins (A, B, and C), casein (D, E, and F), adapted cow's milk (G), partial whey-based HFs (H and I), extensive whey-based HFs (J, K, L, M, and N), partial whey/casein-based HF (O), extensive whey/casein-based HF (P), partial casein-based peptide component (Q), and extensive casein-based HF (R); NS, Not significant.

* $p < 0.001$

† $p < 0.01$

‡ $p < 0.05$

eHFs, we compared different products of each group with one another. The whey-based pHFs (H and I) did not differ from one another. In contrast, the whey-based eHFs (J, M, and N) were each significantly different from the whey-based eHFs (K and L) ($p < 0.01$, Table I). No difference in cellular response was found between a casein-based pHF (Q) and a casein-based eHF (R).

HF-induced proliferation of TCCs

The results (percent reactive TCCs) of 21 β -casein-specific TCCs stimulated with milk proteins, whey, and casein hydrolysates is shown in Fig. 2. Interestingly, most of the clones reacting with β -casein (SI > 5) reacted also with κ -casein (15 of 21, 71%), possibly because of cross-reactivity or cross-contamination. Moreover, most β -casein-specific clones also proliferated in response to adapted milk (G, 13 of 21, 61%). Because TCCs were β -casein specific, they showed no proliferative response to unmodified whey proteins (A, B, and C). Whey-based pHFs were found to induce proliferation in β -casein-specific TCCs (H, 12 of 21, 57%; I, 14 of 21, 66%), indicating the presence or generation of peptides displaying cross-reactivity with these whey-based HFs. Furthermore, TCCs reacted occasionally with whey- and whey/casein-based eHFs (Fig. 2). None of the clones proliferated in response to the whey-based eHFs (M, and N) or the casein-based eHF (R).

We tested whey-based pHFs (H, and I) and a whey-based eHF (I) on the proliferative response of TCCs specific for an unrelated inhalant allergen (birch pollen

major allergen Bet v 1). None of the Bet v 1-specific TCCs proliferated in response to the particular whey-based HFs (data not shown), reflecting the specificity of the assay.

HF-induced cytokine production in TCCs

Twenty-one β -casein-specific TCCs had the T-helper lymphocyte phenotype (CD4+/CD8- and TCRa/b+). The majority of the clones were T_{H0} -like ($n = 12$), producing low or equal amounts of IFN- γ and IL-4; six were T_{H1} -like, producing more IFN- γ than IL-4; and three were T_{H2} -like, producing more IL-4 than IFN- γ .

Fig. 3 shows supernatants from three representative TCCs (bC15, bC21, and bC71) in response to HF stimulation. We demonstrate that both proliferation and cytokine measurement are important because some clones, such as bC71, displayed relatively low proliferative response but produced high amounts of cytokines, whereas in other cases, such as bC21, high proliferation was combined with lower cytokine production in the supernatants, perhaps because of autocrine consumption.

DISCUSSION

This study reports the investigation of residual immunogenicity of pHFs and eHFs by cellular techniques. As a read-out system, cord blood bulk cultures derived from term newborns and milk protein-specific TCCs derived from adults allergic to milk were used. The present data show significantly reduced immunogenic properties of most eHFs. However, residual immunogenic properties

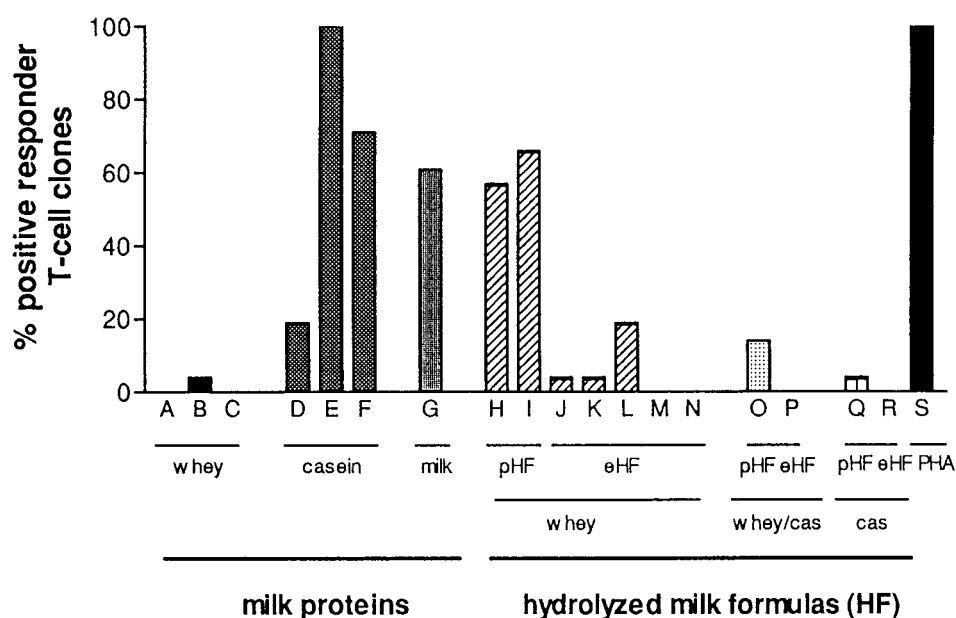


FIG. 2. β -Casein-specific TCCs proliferate in response to pHF and eHF. Twenty-one β -casein-specific TCCs were stimulated with milk proteins and hydrolyzed milk formula. Percent proliferating TCCs is expressed. Milk proteins and HFs: whey proteins (A, B, and C), casein (D, E, and F), adapted cow's milk (G), partial whey-based HFs (H and I), extensive whey-based HFs (J, K, L, M, and N), partial whey/casein-based HF (O), extensive whey/casein-based HF (P), partial casein-based peptide component (Q), and extensive casein-based HF (R). S represents positive control on stimulation of TCCs with mitogen phytohemagglutinin at 10 mg/ml.

of pHFs, and occasionally of eHFs, could be demonstrated.

We tested 71 unselected cord blood samples from term newborns. Analyzing the data, we found a comparable proliferative response to a particular HF in all 71 CBMC samples (Fig. 1), indicated by low SEM values. We therefore concluded that a limited number of cord blood samples might be representative for the expected response to a tested HF. Neither the parents' histories with respect to allergic disease nor other factors seem to influence the cellular response to HF in cord blood cultures.¹⁴

Characterization of HF has been attempted before with different methods.^{2,3,5-8} All these systems provided evidence for the presence of residual allergenicity of the tested HFs. The reports of occasional anaphylactic reactions after ingestion of pHFs and eHFs in patients allergic to milk in which anaphylactic reactions occurred after ingestion of unmodified milk products^{18,19} provides clinical evidence for the residual allergenicity of these products. Recent studies with lymphocyte transformation tests focused on patients sensitized to milk proteins or on healthy volunteers. These results indicated reduced proliferative responses to milk proteins in vitro after avoidance of milk products or substitution with HF. In previous studies we and others observed that CBMCs derived from term newborns showed considerable proliferative responses on stimulation with various milk proteins, indicating that prenatal allergen contact occurs.^{14,21} We therefore chose cord blood cells

to test proliferative responses on stimulation with HF. In our opinion these cells are representative of the immunologic situations in which HFs are actually applied. Interestingly, some partially hydrolyzed products (Beba HA, Nidina HA) showed no differences in proliferative response compared with unmodified milk formula (Aptamil 1), suggesting that the T-cell relevant immunogenic fragments in HF were unchanged. T cells are known to recognize linear peptides with a length of 12 to 20 amino acids presented by major histocompatibility complex class II molecules on the surface of antigen-presenting cells.^{22,23} Considering the amino acid length requirements of hydrolyzed milk formulas (<5000 d, corresponding to approximately 40 amino acids in length), one would expect to have the immunogenic structures conserved in the HF, but to our surprise T-cell stimulatory capacity of almost all eHF was lost.

Considering the application of HF in atopy-prone newborns, it might be advisable to use those HFs with the lowest residual potency of stimulation of cord blood cells (i.e., the most eHFs). Although these T-cell responses are crucial in starting an immune response, it is not clear whether the proliferation response of cord blood cells reflects activation and differentiation processes of allergen-specific T cells. These would subsequently supply B cells with IL-4, which is a prerequisite for an immunoglobulin germ line switch to IgE production.²⁴ Furthermore, it is uncertain whether the residual immunogenic capacity of pHF is harmful (by initiating a

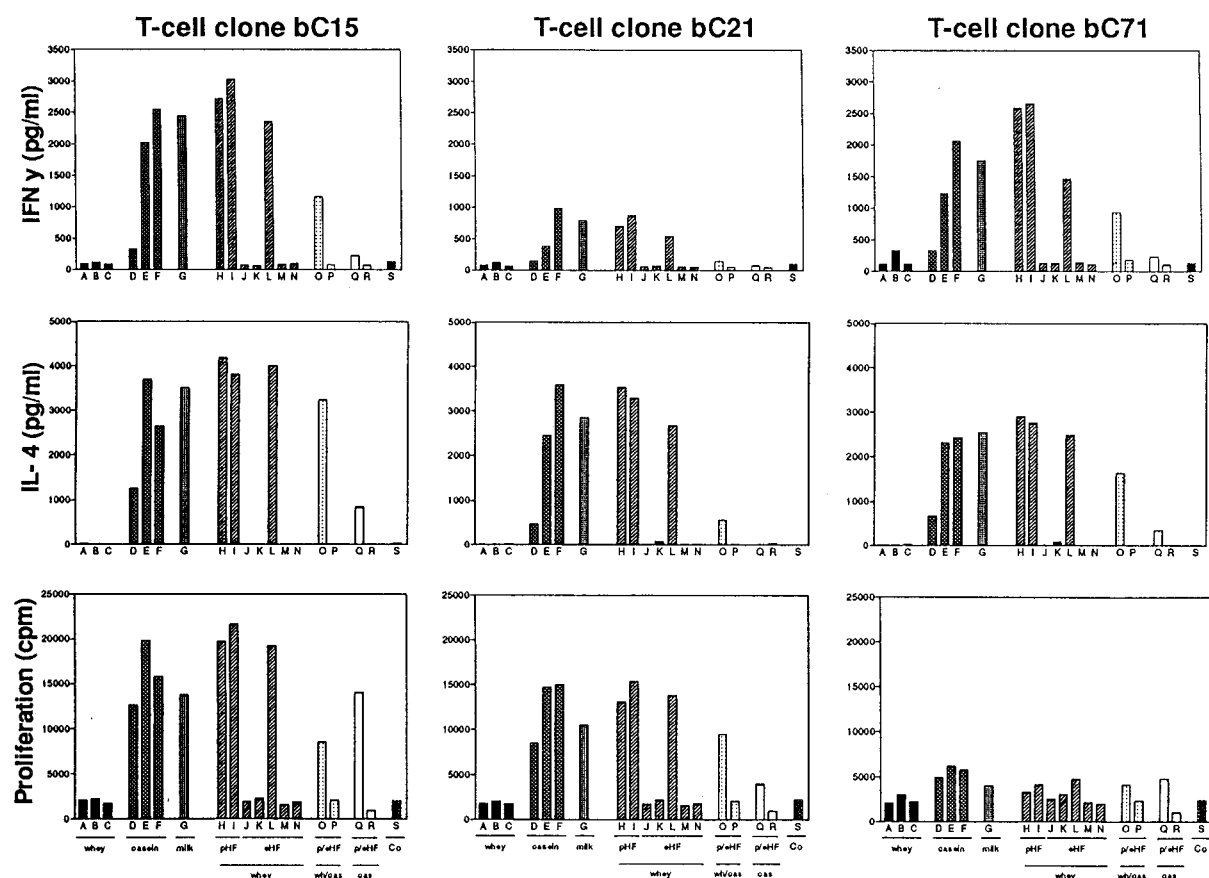


FIG. 3. β -Casein-specific TCCs proliferate and produce cytokines in response to pHFs and eHFs. TCCs (10^5) were incubated with antigen (50 μ g/ml) for 48 hours, and proliferation was assessed by tritiated thymidine incorporation for 16 hours. Soluble cytokines (IFN- γ and IL-4) were measured in supernatants after 48 hours by specific ELISA. Results from three representative TCCs (bC15, bC21, and bC71) are shown. All figures representing proliferation, IFN- γ production, and IL-4 production, respectively, are shown in same scale. Proliferation is expressed as counts per minute (cpm). Cytokines are expressed in picograms per milliliter. Milk proteins and HFs: whey proteins (A, B, and C), casein (D, E, and F), adapted cow's milk (G), partial whey-based HFs (H and I), extensive whey-based HFs (J, K, L, M, and N), partial whey/casein-based HF (O), extensive whey/casein-based HF (P), partial casein-based peptide component (Q), and extensive casein-based HF (R).

pathogenetic immune response) or beneficial (by inducing oral tolerance mechanisms).

Therefore we investigated the proliferative and cytokine response of β -casein-specific TCCs established from adults allergic to milk. TCCs are commonly used to identify immunodominant T-cell epitopes of proteins,^{16,25} to investigate their pathogenetic importance (i.e., their cytokine production profile),²⁶ and to establish models of anergy or tolerance induction.²⁷ We used TCCs because stimulation of CBMCs with the appropriate antigen did not promote measurable cytokine levels of IL-4 by ELISA.^{15,28} In general, cytokine production levels correlated closely with clonal proliferation in response to antigen challenge. For the TCCs bC21 and bC15, the data obtained demonstrate that the proliferative response to antigen (unmodified protein or HF) parallels cytokine production (IL-4 and IFN- γ) induced by most antigens. However, it is certainly necessary to measure cytokines and proliferation because strong pro-

liferation with low cytokine production can be observed (bC15 and bC21), as well as low proliferation and high cytokine responses (bC71).

Polyclonal T-cell responses might give better representation of the diversity of T-cell responses to allergens than responses measured with TCCs because a bias towards certain TCCs might be selected during the *in vitro* cloning procedure.⁹ Therefore at present it appears suitable to use both cell devices (cord blood cells for polyclonal analysis and TCCs for monoclonal analysis) to investigate HF. Intracellular cytokine determination and the polymerase chain reaction technique might overcome some limitations of the analysis of cord blood cell cultures.

An unexpected finding in the evaluation of the T-cell responses to HF was the strong stimulatory capacity of whey-based pHF (H, and I) and one whey-based eHF (L) on TCCs specific for β -casein (Fig. 2, H, I, L). A mitogenic effect could have been one reason for this

observation, but this was unlikely because TCCs specific for an unrelated allergen (birch pollen major allergen Bet v 1) were not stimulated by whey-based pHFs (*H*, and *I*) and one whey-based eHF (*L*) (data not shown). Another explanation may be the presence or generation of casein immunogenic peptides in the particular whey-derived hydrolysates. Further investigations will clarify this point.

An important aspect of this study is the effect of HF on the T-cell level. In our opinion it is not sufficient to study the IgE-binding capacity of HF *in vitro*,⁷ to measure skin prick tests,^{6, 7, 19} to define the molecular weight of the HF, or even to administer the HF *in vivo*.^{18, 19} None of these approaches can obviously give evidence whether the HF under investigation is capable of initiating an immune response. CBMCs may therefore provide a good tool to answer this question. In summary, our study provides evidence that, for the currently used HF, an improved characterization of the residual immunogenicity is necessary and feasible.

We thank Dr. Wal (France) for providing purified β -lactoglobulin.

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