

T cell recognition pattern of bovine milk α S1-casein and its peptides

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Abstract

T cell recognition patterns of CAS1.Bovin, its limited hydrolysis, oxidized, reduced/alkylated, cyanogen bromide cleavage fractions and synthetic peptides were examined. Thirteen overlapping peptides covering the intact molecule, with chain lengths varied between 17 and 20 AA, were prepared by f-moc SPPS. In addition, six CNBr-cleavage fragments were obtained and extensively purified using RP/HPLC. Likewise, chemically modified derivatives and limited pepsin hydrolysate, were performed and the specificities were confirmed. Stimulation of PBMC and TCL cultures by the intact CAS1.Bovin molecule, synthetic peptides and modified derivatives were screened by [methyl-³H] thymidine incorporations. PBMC phenotype was performed by flow cytometry and the mean CD4⁺/CD8⁺ ratio of freshly prepared PBMC was compared with the ratio following specific CAS1.Bovin stimulation. CD4⁺ phenotypes (T_H1/, T_H2 and T_H0) were assigned by assay of four marker cytokines IL-4, IL-6, IL-10 and IFN- γ .

Five CNBr fragments and seven of the thirteen tested peptides were recognized by specific TCL. The most reactive epitopes of CAS1.Bovin comprised seven motifs namely: peptides Cas 1–18, Cas 16–35, Cas 67–85, Cas 91–110, Cas 136–155, Cas 152–169 and Cas 166–183. The stimulation range for the seven peptides was 1058–2383 cpm. Stimulation for the CNBr fragments were, respectively, 8670, 5808, 3324, 5465, 2255 and 321 cpm. Cytokine assay showed that CD4⁺ T_H2 phenotype was dominant for half the number of patients, while T_H1 solely or combined T_H0 were represented in the other four cell culture filtrates.

The T cell reactive epitopes described and their antibodies will be useful tools for methods *in progress* for the detection of masked casein epitopes encompassed in processed food.

In conclusion, T cell recognition pattern of CAS1.Bovin was examined using extensively purified synthetic peptides and CNBr fragments. Five large and seven small peptides were clearly recognized. Peptides of chain length less than six AA were left unrecognised. CD4⁺ T_H2 phenotype was the most dominant TCL subpopulations found in atopic patients while CD4⁺ T_H1 was representative in the non-IgE mediated type IV hypersensitivity.

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Keywords: Allergen specific T cell epitopes; Allergen specific TCL; α S1-Casein; CD4⁺ T_H1, T_H0, T_H2 phenotype; CM, CMA, CMP, CNBr-fragments; CAS1.BOVIN; f-moc SPPS; RP/HPLC

1. Introduction

The incidence of allergic diseases has increased during the last two decades and mostly in industrialized countries (Beasley et al., 2000; Kay, 2001a, 2001b). Cow's milk allergy (CMA), most often starts during the first months of life (one-third already during lactation, and mostly within a week after

introduction of Cow's milk (CM) (Burks, 2002; Heine et al., 2002; Host, 2002; Host and Halken, 1990).

Casein hydrolysates in allergen-reduced formulae are widely used in the prevention and treatment of cow's milk allergy in children. Anaphylactic reactions were reported for some hydrolysed formulae and detection of the residual allergenicity of casein epitopes is of clinical relevance (Cantani, 1999; Niggemann et al., 2001). Casein could also be found in unrelated items like meat, fish, cosmetics, drugs or surgical gloves which may endanger CMA patients (Habki et al.,

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2001; Jarmoc and Primack, 1987; Koppelman et al., 1999; Ylitalo et al., 1999). It is therefore of some urgency to establish analytical tools for detecting non-labelled casein or casein hydrolysates in different items.

CAS1.Bovin is a single chain phosphoprotein of 199 residues. It constitutes more than 40% of the major CM casein. CAS1_BOVIN has unique conformation that enables to investigate the reactive determinants without consideration about missing the reactivity (Kumosinski and Brown, 1991).

Of all food allergens, CM proteins (CMP) are of the best-characterized. Casein fraction appears to be an association of four different proteins, α S1-, α S2-, β - and κ -caseins, in approximate proportions of 40:10:40:10 percentages. α S1-casein (CAS1.Bovin), important major allergens of CMP and is widely used in many industrial products in addition to milk hydrolysate formulae and other processed food.

In the present study, we examined the stimulation of peripheral blood mononuclear cells (PBMC) and Allergen specific T lymphocytes cell lines (TCL) generated from CMA patients. For this purpose the intact CAS1_BOVIN, the overlapping peptides, the partial hydrolysate and cyanogen bromide (CNBr)—cleavage fragments and controls were used. Four marker cytokines IL-4, IL-6, IFN- γ and IL-10 released in cell culture filtrates were analysed to examine CD4⁺ subpopulations.

2. Materials and methods

2.1. Preparation of α S1-casein

CAS1.Bovin was provided by the purification of bovine casein, essentially vitamin free, C5679 from Sigma Chemical Co, St. Louis Mercier, USA and bovine α casein (lot No. 125, H09205) with 85% α S-casein, was the initial reagent used through the purification procedures. Purification was performed by gel filtration chromatography (GFC) and reversed phase (RP) high-pressure liquid chromatography (HPLC), 130 A Separation System using BrownleeTM C18, Aquapore RP-300, 7 μ m, 30 mm $\times$ 2.1 mm cartridges, Applied Biosystems, Foster City, CA, USA, were used. Hydrolysis was performed using Sigma pepsin (1% E/S), at room temperature (RT) and time intervals 15, 30, 60 min or 2, 3, 24 h.

2.2. Oxidation, cyanogen bromide (CNBr)-cleavage, reduction and alkylation

Oxidation was performed as described previously (Bruserud et al., 1992; Elsayed and Stavseng, 1994). Briefly, CAS1.Bovin (5 mg/mL) was treated with 70% formic acid to give 1% (w/v) 10 mL solution, for 15 min. H₂O₂ (70 mL) was then added, and the solution was lyophilized and stored until used. CNBr-cleavage was done by the addition of CNBr in acetonitrile, 50–100-fold molar excess over methionine residues (200 μ g/100 μ g protein) in a small volume of 70%

formic acid. The sample was kept in dark under N₂, at RT for 24 h. The sample was then 10-folds diluted and lyophilized.

Reduction and alkylation was performed on CAS1_Bovin (74.3 mg), 6 M guanidine-HCl, Fluka, 50940 Buchs, Switzerland, add 1.680 mL, 500 mM Tris-HCl (Sigma T-3253), 10 mM EDTA pH 8.50, Sigma EDSS), and carefully shaken. DL-dithiothreitol, Cleland's reagent (DDT) (4.6 mg, DDT 99% Sigma D-0632), were added in molar excess. The mole ratio DDT/casein = 100. The addition was done under N₂ for 2 min in dark before further incubation (2 h at 37 °C). Freshly prepared iodoacetic acid (Sigma I-6250) in 100 μ L 0.5 N NaOH was added, in molar excess with respect to DDT (mol ratio iodoacetic acid/DDT = 1.1). This was followed by shaking and incubation for 30 min at RT, in the dark. Finally, 1% of 2-mercaptoethanol (Merck 805740, Darmstadt, Germany) was added slightly in excess of iodoacetic acid. The mixture was shaken then dialyzed (12 KD cut off), against 50 mM NH₄HCO₃ (BDH 10302) overnight at 4°. The mixture was then lyophilized and stored at –20 °C until used.

2.3. Hydrolysis

CAS1.Bovin was hydrolysed using pepsin E/S ratio 1/100 at pH 4.0. The hydrolysis proceeded at intervals of 1, 2, 3, and 24 h at RT.

2.4. Reversed phase High performance liquid chromatography (RP/HPLC)

RP/HPLC was performed as described in previous publications (Elsayed and Stavseng, 1994; Eriksson et al., 1998).

2.5. Solid phase peptide synthesis (SPPS)

Thirteen overlapping peptides covering the whole CAS1.Bovin molecule were synthesized using f-moc SPPS and a semiautomatic outfit (BiolyxTM 4175, LKB, Sweden). Eleven peptides overlapped at five amino acid (AA) windows while two peptides 1–18 and 16–35 with only two AA overlap). The peptides were purified and their N-terminal AA sequences were ascertained (Applied Biosystem, Foster City, CA) (Elsayed and Vik, 1993). The primary structure of the intact CAS1_Bovin and the locations of the SPPS peptides are shown in Fig. 1.

2.6. Cell donors

Eight CMA patients (seven females and one male, age range 14–64 years), referred to the routine laboratory diagnosis at the Department of Medical Biochemistry, University Hospital Bergen and two healthy volunteers. Patients and control individuals donated the blood samples, after informed consents. Some of the patients have high titre of specific IgE to CM and other allergens. The two healthy control volunteers (age 25 and 66 years) have low values of s-total and specific IgE.

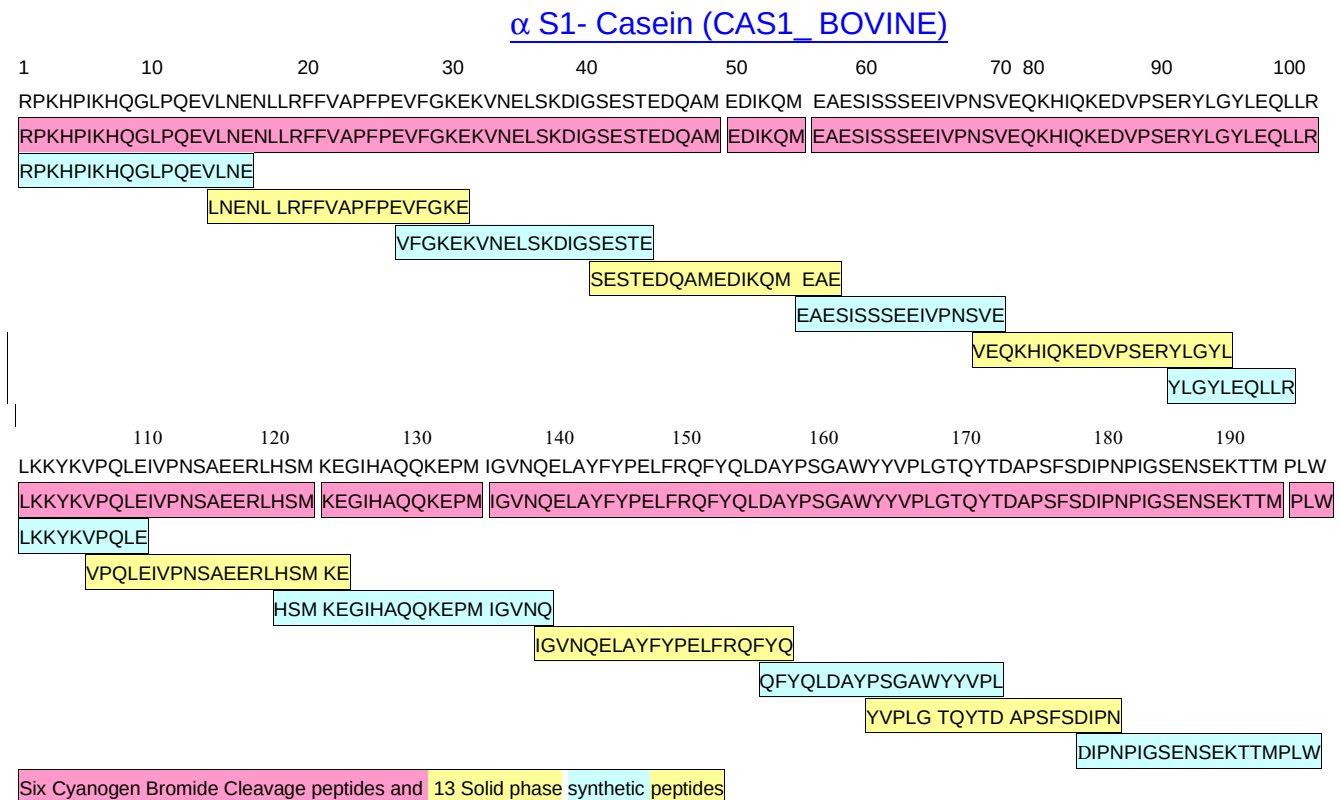


Fig. 1. The primary structure of wild CAS1_BOVIN showing the locations of the CNBr fragments in red boxes and SPPS in yellow and blue boxes. The location of the peptides was ascertained by N-terminal AA sequence analyses.

2.7. PBMC proliferation assay

PBMC were collected from the interface after Ficoll Paque (Research grade, code No. 17-0840-02, Pharmacia Biotech, Sweden) centrifugation, washing and resuspension in complete RPMI 1640/AB+ medium (Holen and Elsayed, 1996). PBMC (12×10^6 per well) were cultured in 24-well cell culture clusters (Costar, Cambridge) with or without 100 allergen (0.1, 1 and 10 mg/mL), final culture volume 2 mL. Five days after initiating the cultures, rIL-2 (20 U/mL, Boehringer Mannheim, Germany) was added to each well. On day 7, 200 μ L in triplicate from each well were cultured in 96-well cell culture clusters (Costar) and pulsed overnight by adding 20 μ L 0.05 mCi/mL [methyl- ^{3} H] thymidine (Amersham, Denmark). The results were

expressed either in cpm or as stimulation index (SI)

$$= \frac{\text{cpm of the culture}}{\text{cpm of the control back ground}} \times 100$$

Cells from wells expanded by allergen-induced proliferation were isolated by Ficoll Paque centrifugation. Isolated T cell blasts were recultured in 24-well clusters and restimulated by phytohaemagglutinin (PHA, 0.8 μ g/mL), rIL-2 (20 U/mL) and autologous irradiated (30 Gy) PBMC (1×10^5 per well). For maintaining the TCL cultures the procedure was repeated each 14th day.

2.8. Allergen specific TCL proliferation assay

Specific TCL proliferation responses were tested 14 days after the last addition of PHA and rIL-2. In 24-well plates, enriched T cells ($(1-2) \times 10^6$ cells/well) added irradiated (30 Gy) autologous PBMC (10^5 cells/well) and specific allergen (final concentration = 0.5 mg/mL) were cultured for 4 days. From the 24-well plates, 200 μ L of each culture were seeded in 96-well plates and added 20 μ L, 0.05 mCi/mL [methyl- ^{3} H] thymidine. The radioactive uptake was measured the next day and cpm was given as means of triplicate cultures. The 24-well cultures were harvested on day 5; pelleted cells were resuspended in cRPMI-1640 and phenotype markers were stated by flow cytometry. The culture filtrates were stored at -20° for cytokine assay.

2.9. Phenotype determination

Freshly prepared PBMC (day 1) and established TCL following specific allergen challenge (harvested on day 5) were analysed for the expression of dual $CD4^+$ / $CD8^+$ and $CD2^+$ / $CD19^+$ using Coulter Epics XL-MCL Flow Cytometry System II TM soft ware, fully automates set-up supplied by Coulter Corporation, Luton, UK. Monoclonal antibody T4RD/T8-FITC (Coulter 6603802) and T11RD1/B4-FITC (Coulter 6603801), respectively, were used for the analysis.

Table 1

CNBr-cleavage fragments were numerated from I to VII after their elution by GFC

CNBr fragment no., no. of AA: M_r	Amino acid sequence
I Cas 55–123, 63 AA:8440.59	EAESISSEEIVPNSVEQKHQKEDVPSERYLGYLEQLRLKKYKVPQLE IVPNSAEERL HSM
II Cas 125–194, 61 AA:8093.96	IGVQNQELAYFYPELFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSEN SEKTTM
III Cas 1–54, 54 AA:7130.73	RPKHPIKHQGLPQEVLENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAM
IV Cas 123–134, 12 AA:1593.69	KEGIHAQ QKEPM
V Cas 55–60, 6 AA:852.97	EDIKQM
VII Cas 195–197, 3 AA:450.54	PLW

Fragments I–V and VII are in descending molecular weights. AA sequence analysis ascertained their locations (Fig. 1).

2.10. Cytokine analysis

Estimations of IL-4, IL-6, IFN- γ , and IL-10 in culture filtrates of specific allergen stimulated TCL were performed according to manufacturer's instructions, using commercial enzyme linked immunosorbent assay kits (PeliKine CompactTM human ELISA kits, CLB, 1006 AD Amsterdam, Netherlands). The manufacturer gave the specificity and the sensitivity of the kits. The lower detection limits for the different cytokines in pg/mL were as follows: 0.2 for IL-4; 0.2 for IL-6; 0.8 for IFN- γ and 1.0 for IL-10. In order to calculate the results, absorbencies were read at λ = 450 nm, by a spectrophotometer (Lab systems Multiscan Bichromatic Type 348) and Genesis II (WindowsTM Based Micro plate software) was used for calculating the cytokine concentrations.

3. Results

3.1. Preparation and purification

Purification of casein, mostly removed proteins with M_r values <25 and >45 kD. Void volume peak from GFC was purified by RP/HPLC. Peaks with retention times R_t 25.37 and 25.51 represented the highly purified CAS1_BOVIN. The partially purified intact molecule showed two bands by SDS/PAGE, also when stained by blotting showing that both have affinities to specific IgE from CMA patients. The major and intensive band was further run on RP/HPLC and finally one symmetrical peak for each band was found. Those peaks were collected, lyophilized and their amino terminal sequence AA analyses (R P K H) were identical. This ascertained the homology and homogeneity of the fractions.

Hydrolysis of CAS1_BOVIN for 1 h allowed two peptide-populations as detected by RP/HPLC. Using the same RP column, no significant HPLC-signals for the 2 h hydrolysis or longer periods were demonstrated.

Reduced alkylated and oxidised products of casein were similarly run on RP/HPLC. The chemical modifications did not alter the R_t but resulted in broader denatured protein peaks (R_t 25.92 for the reduced; R_t 25.3 for the oxidised; R_t 25.72 for the native).

CNBr cleavage showed six peaks on RP/HPLC were demonstrated. Preparative collection of the fragments,

lyophilization and N-terminal sequence analysis ascertained their location as shown in Table 1.

The primary structures of CAS1_BOVIN and the synthetic peptides and cleavage fragments were ascertained by their NH₂-AA sequence analysis and were accordingly located as in Fig. 1.

3.2. Peptide synthesis

SPPS of 13 overlapping peptides (with three to five AAs windows) covering the whole CAS1_Bovine molecule were synthesized, cleaved from the resin and purified by GFC and RP/HPLC. All the synthetic peptides reached high degree of homogeneity as judged by the symmetrical peaks obtained. Their first three to four NH₂-terminal AA sequence was done to ascertain their locations.

The primary structures of CAS1_Bovine and the synthetic peptides and cleavage fragments were shown in Fig. 1.

3.3. Primary PBMC proliferation assay

The intact CAS1_BOVIN showed high PBMC stimulation; the highest [methyl-³H] thymidine incorporation was found at relatively high antigen concentrations. All the eight patients whose serum showed high CM serum specific IgE showed high ³H-thymidine incorporation. Similar dose response relationships were found for all patients primary stimulations and one representative histogram for patient AL is showed in Fig. 2.

3.4. Specific TCL proliferation

Allergen specific TCL proliferation was shown in Fig. 3; generated from one representative patient (L I) and illustrated high proliferations for CAS1_BOVIN (casein native) 50,365 cpm, reduced-alkylated 29,514 cpm, CNBr-fragments 5452 cpm, pepsin hydrolysate 34,771 cpm. As positive control NANTM Nestle that contains 40% casein (18,085 cpm) was used. For negative control a non-related birch pollen major allergen (BV neg contr) and hen's egg ovomucoid (OM) showed 742 and 1826 cpm, respectively. High background cpm for negative control ovomucoid was due to patients multiple hypersensitivities to hen's egg white proteins. Three peptides were selected as positive controls: P8 (Cas 106–125) showed 951 cpm; peptide P10 (Cas 136–155)

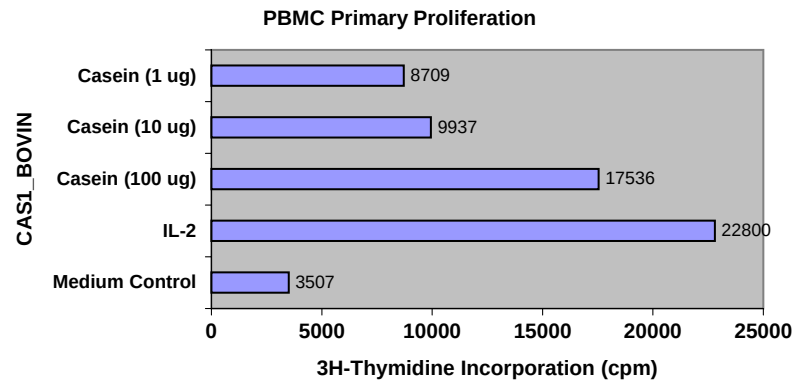


Fig. 2. Allergen specific primary proliferation, PBMC from one CMA patient (AL). The antigens used were in the following order: α S1-casein—1 ug/well, 10 ug/well, 100 ug/well, IL-2 (specific mitogen) and medium control without addition of antigen. A dose/stimulation response relationship was found for all the patients examined.

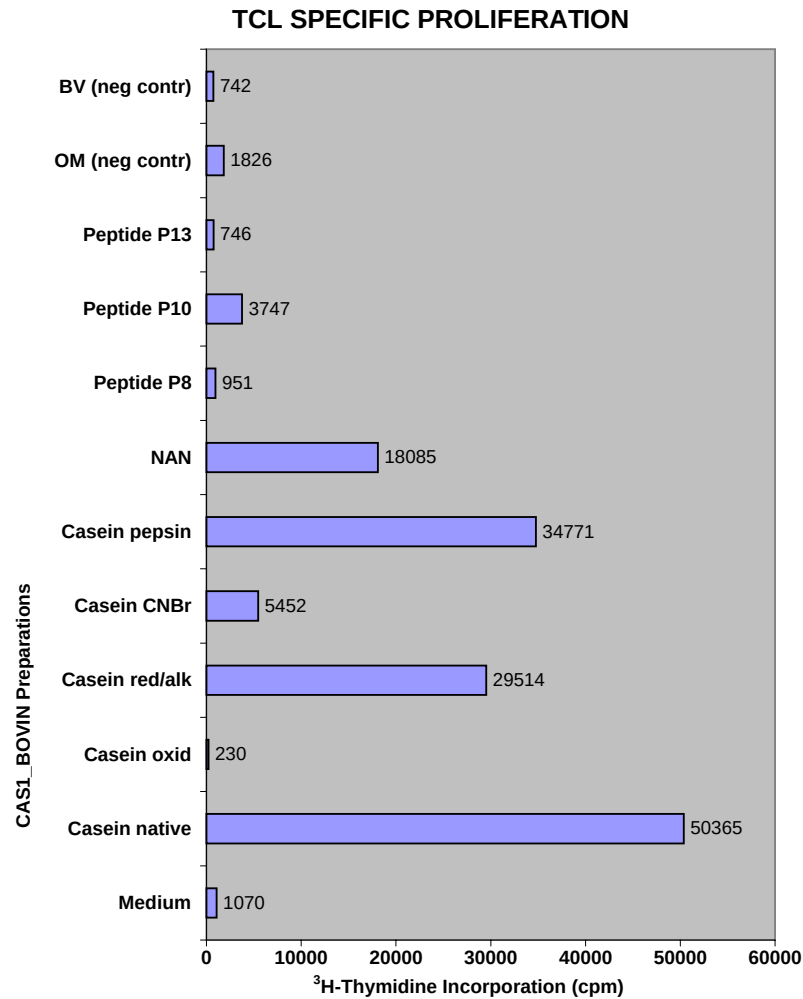


Fig. 3. Specific proliferation using a TCL generated from one CMA patient (Li), illustrated significantly high allergen specific TCL proliferation for CAS1_Bovine (casein native) 50,365 cpm, reduced/alkylated 29,514 cpm, CNBr fractions 5452 cpm, and pepsin limited hydrolysate 34,771 cpm. As a positive control NANTM Nestle (40% casein) showed 18,085 cpm. Negative non-related allergen from birch pollen (BV neg contr) showed low cpm while hen's egg ovomucoid (OM) was relatively high (patient is allergic to hens egg white protein). Three control peptides from other laboratories P 13, P 10 and P 8. Only P 10 showed moderate stimulations.

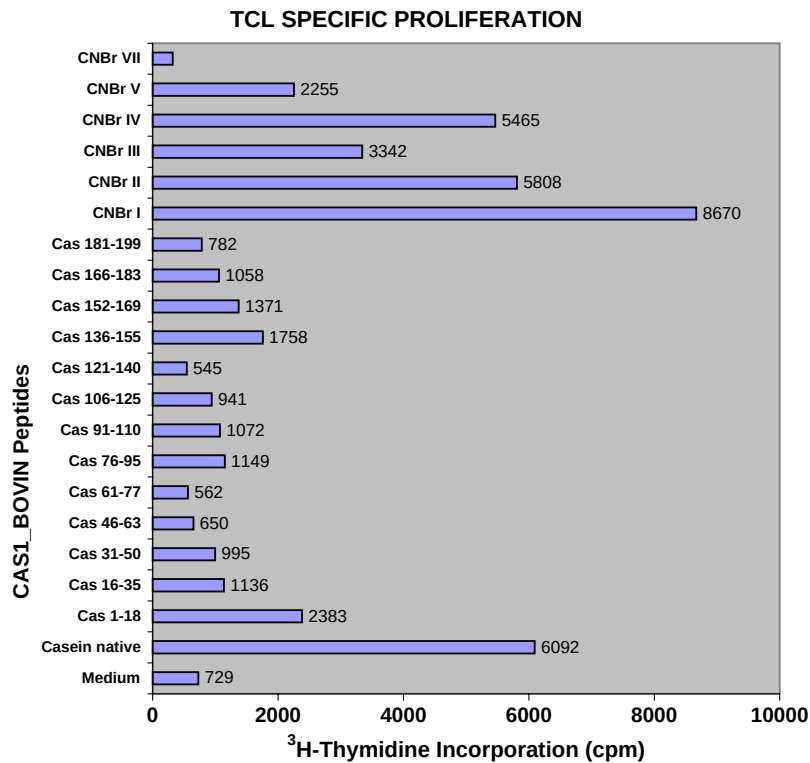


Fig. 4. TCL specific proliferation for CMA patient (AL). High specific stimulation was obtained for CAS1.BOVINB (casein native) 6092 with very high stimulation index 835.7. Similarly, significant proliferation was found for peptide Cas 1–18 and Cas 136–155. The proliferation induced by the other 11 peptides was relatively low. Five of the seven CNBr fragment isolated I–IV. V showed high stimulation (8670, 5808, 3342, 465, 2255, and 2255 cpm, respectively). CNBr VI was designated CNBr VII due to its elution sequence in RP HPLC.

gave 3747 cpm; and peptide P13 (Cas 181–199) showed 746 cpm. Those peptides were previously shown to be highly potent T cell epitopes (Nakajimaadachi et al., 1998). Compared with the extensively purified peptides and derivatives in this study, only one peptide (P10) showed good specificity.

Seven peptides of the 13 in question could specifically stimulate TCL. Particularly higher proliferations were found for peptides Cas 1–18 and Cas 136–155. CNBr-cleavage fragments I, II, III, IV and V (2255–8670 cpm) as compared to a negative control (729 cpm). Seven peptides with low/moderate stimulation stimulations Cas 16–35, Cas 31–50, Cas 76–95, Cas 91–110, Cas 106–125, Cas 152–169 and Cas 166–183 (range 995–1371 cpm). Peptides with no stimulations Cas 46–63, Cas 61–77, Cas 121–140, and Cas 181–199 (Fig. 4).

3.5. PBMC and TCL phenotype

CD4⁺/CD8⁺ ratio for PBMC (non-stimulated, allergic and healthy) was 1.24 (reference range 1–1.6) ($n = 8$) while for the TCL (stimulated, allergic) was 1.98 (reference range 0.3–3.5) ($n = 8$)

3.6. Cytokine assay

Four cytokines IL-4, IL-6, IFN- γ and L-10 secretions were assayed in culture filtrates of both the primary and

allergen specific TCL for patients (MBS, SS, AL, TMW, SM, RKSG, CAM, and CAR) and controls. CAS1.BOVIN, oxidised, reduced alkylated, CNBr, partial hydrolysate and NANTM were used for stimulation. All the control and primary cultures showed low levels of IL-4. CAS1.BOVIN specific patient AL was higher than all the other patients. Another patient (RKSG) showed high secretions of IL-4 in all cultures. Patient AL was highly atopic with multiple-allergen sensitivity including CMP and OM (hen's egg white ovomucoid). While RKSG has type IV mechanisms for several food allergens and high values of IL-4 secretion in all TCL cultures (Fig. 5).

IL-6 analysis; except for the oxidised CAS1.BOVIN derivative, all the TCL culture filtrates showed very high values of IL-6 (>150 pg/mL). Four patients (MBS, SS, AL, TMW) spontaneously secreted IL-6 even in the control cultures with no exposure to the allergen.

IFN- γ secretion was extensive for three patients (SS, RKSG, CAR) for the primary CAS1.BOVIN. For the CAS1.BOVIN, reduced/alkylated and CNBr-cleavage particularly two patients RKSG and CAR showed high IFN- γ (>100 pg/L). Only RKSG showed extremely high IFN- γ in all cultures tested even in specific control where no allergen was included (Fig. 6).

IL-10 secretions were high for all patients (>4 pg/mL) in all the CAS1.BOVIN primary cultures. For the allergen specific cultures most of the patients showed low values (Fig. 7).

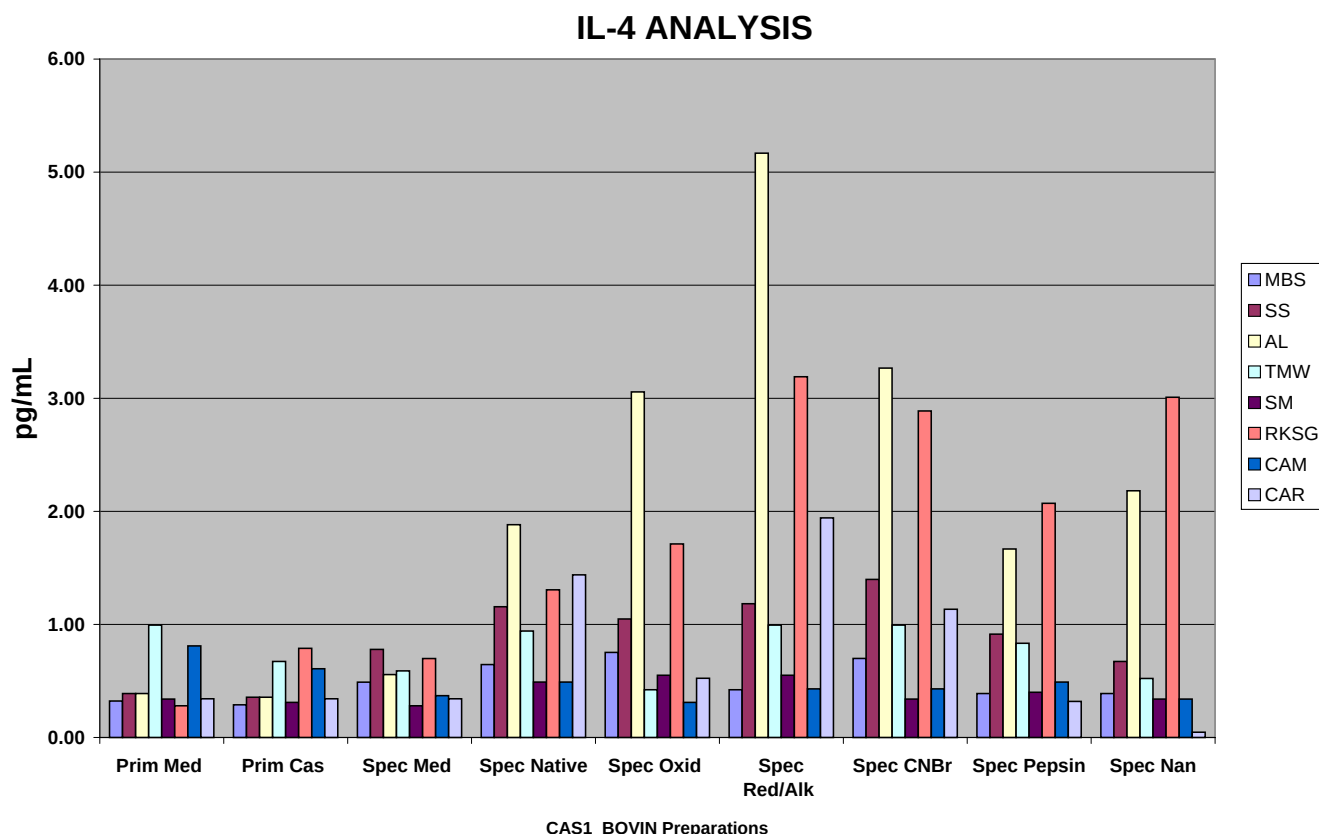


Fig. 5. IL-4 assay of cell culture filtrates from eight patients. One patient showed relatively high IL-4 secretion in the allergen specific TCL. Increased IL-4 indicated a T_H2 polarization for this patient (typical IgE mediated allergic patient).

4. Discussion

We have examined the recognition of CAS1_BOVIN, its purified synthetic peptides and derivatives in primary by specific TCL cultures using highly purified preparations. To ascertain the locations and homogeneity of all the synthetic peptides and the CNBr fragments, their N-terminal AA sequence (three to five residues) was performed and confirmed their locations (Fig. 1).

PBMC proliferations showed dose/response relationships in primary cultures; the higher the concentration of CAS1_BOVIN the higher values of ^3H -thymidine incorporation (Fig. 2).

Specific proliferation using TCL generated from patient Li, illustrated high proliferation for CAS1_BOVIN, reduced-alkylated, CNBr-cleavage, limited hydrolysate. A positive control (NAN Nestle™, 40% casein) was used and showed large stimulation as compared to control. Oxidation of the allergen inhibited T cell stimulation. Three peptides, suggested by other laboratories to be T cell epitopes, were used in this study as control: peptides P8 (106–125); P10 (136–155); and P13 (181–199) (Nakajimaadachi et al., 1998). Only peptide P10 showed stimulation larger than control (Fig. 3). The lack of activity of P13 and P8 could be due to: (a) heterogeneity of the

preparations; (b) truncated peptides sequences; (c) diversity of TCL response.

At least 7 of the examined 13 f-moc peptides could stimulate casein specific TCL. Relatively high affinity was found for peptide Cas 1–18 and Cas 136–155 (SI = 3.26 and 2.41). Fragments of CNBr I (63 AA), II (61 AA), III (54 AA), IV (12 AA) and V (6 AA) showed high proliferation as compared to control (SI 11.89, 7.96, 4.58, 6.26 and 3.09, respectively). Peptides Cas 31–50, Cas 46–63, Cas 61–77, Cas 106–125, Cas 121–140, and Cas 181–199 showed no activity, SI was similar to background. Higher stimulation was found for CNBr-1 than the intact molecule. This peptide may be located in hydrophobic parts of the molecule where they were not accessible in the native conformation and became bio available after cleavage (Fig. 4).

All the CNBr-cleavage peptides, except CNBr VII (Cas 195–197) were recognized by specific TCL. Higher stimulation was found for CNBr-I than for the intact molecule. Cas 55–60 (6 AA) CNBr V showed moderately high cpm than expected, the reason was probably contamination by other longer peptides. The nature of a peptide sequence and its length are important for MHC binding. Peptides bound by MHC class II molecules are longer than CNBr V and VII (~13–15 AA) (Walden, 1996).

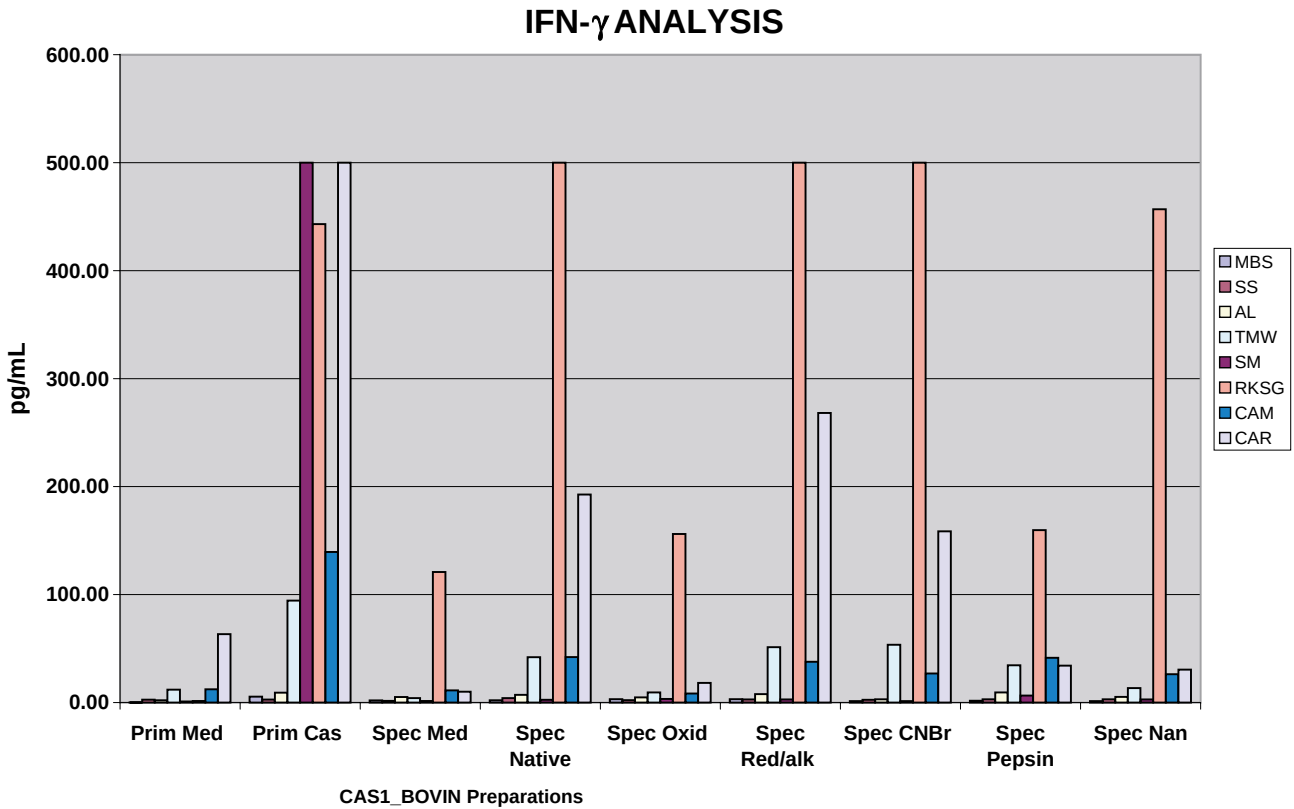


Fig. 6. Assay of IFN- γ for eight CMA patients in box. Primary cell cultures gave high values of IFN- γ for four patients (as compared to primary control culture). Specific proliferation cultures from three patients with chronic type IV hypersensitivity (MBS, SS and RKSG) showed high values and T_H1 phenotype profile. Particularly one patients (RKSG) secreted high values of IFN γ even by the partial hydrolysis preparations (spec pepsin and NANTM).

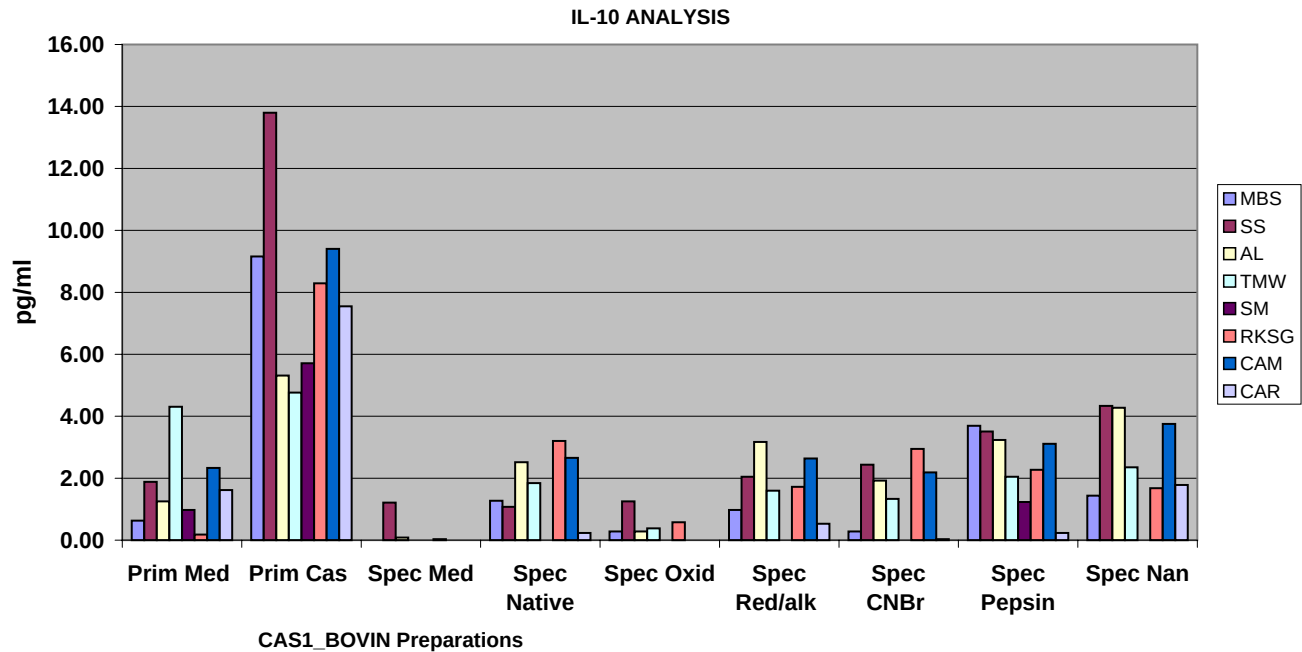


Fig. 7. IL-10 analysis showed high levels for the primary cultures of all patients. Patients with extensive reactions have low levels of IL-10 in the allergen specific cultures.

T lymphocytes have large impact on allergen recognition and induction of allergic diathesis. Helper CD4⁺ T cells differentiate into T_H1 or T_H2 cells, which have been characterized by cytokine secretion patterns that activate either cellular or humoral immunity (Chinen and Shearer, 2003). The particular profile of cytokine production could provide important information regarding the nature of the immune response (T_H1 versus T_H2 response) (Mosmann and Coffman, 1987; Romagnani, 2000). T_H2 cytokines, such as interleukin IL-4 account for all the allergic manifestations. IL-4 is the main cytokine that trigger the isotype switching to IgE isotype. IL-4 stimulates HLA II and accessory molecules and lead naïve T_H0 towards T_H2 phenotype. IL-4 is important in maintaining the allergic immune response by preventing apoptosis of TCL (Vella et al., 1997). In accordance with the T_H1/T_H2 hypothesis cytokine assay showed that cultures, which gave high IL-4, gave low IFN- γ . Modulating the immune response to increase the production of IFN- γ and to down regulate the production of IL-4 are main issue in regulating allergic response and developing new therapies for the disease (Bhatia et al., 2002).

TCL from an atopic patient AL with multiple CM allergies (α -lactalbumin, β -lactoglobulin, casein in addition to OM) high serum total and specific IgE showed high levels of IL-4 and low levels for all the cultures of IFN- γ clearly suggesting a major CD4⁺ T_H2 profile. IL-4 assay for culture filtrates of TCL suggest a T_H2 patterns for three patients (SS, AL, TMW) Fig. 5. The results also suggested that two patients (RKSG and MBS) have CD4⁺ Th0, secreting both T_H1 and T_H2 cytokines. Those patients suffer non-IgE mediated type IV mechanism. allergy resulting the cytokine profiles found.

All cultures showed very high levels of IL-6, an anti- and pro-inflammatory cytokine (>150 pg/mL) for all the eight patients confirming the inflammatory condition. An important biological function for IL-6 is its ability to stimulate the final stages of B lymphocyte maturation and hepatocytes synthesis of acute phase proteins. Overproduction of IL-6 in animal model of bronchial inflammation lessens bronchial hyper reactivity (DiCosmo et al., 1994).

IL-10 is a regulatory cytokine and product of both T_H1 and T_H2. In the presence of IL-10 not only TCL not activated but rendered tolerant and responsible for inhibition of T_H1 and T_H2 cytokine production (Borish et al., 1996; Prete et al., 1993). ELISA results showed that this cytokine was relatively high for all the primary proliferation of PBMC for the intact CAS1_BOVIN but not for the modified protein or peptides. IL-10 down regulates IL-4-induced IgE production and is secreted by both T_H1 and T_H2. An enhanced secretion of IL-10 indicated some inflammatory down-regulation of IL-4 (Mendel and Shevach, 2002; Punnonen et al., 1993).

Casein may be found in many items of no relation to CM; a new process for restructured meat and fish has been introduced to the market recently. Its main compound is casein, and it may therefore endanger CMA patients (Habki et al., 2001; Jarmoc and Primack, 1987; Koppelman et al., 1999; Ylitalo et al., 1999). Our present study allowed the establishment of screening of T cells epitopes found in processed food

items that eventually can cause an allergic injuries to highly CMA children.

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