

Establishment of T cell lines to bovine β -casein and β -casein-derived epitopes in patients with type 1 diabetes

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Abstract

Enhanced cellular immune response to bovine β -casein has been reported in patients with type 1 diabetes. In this study we aimed to establish β -casein-specific T cell lines from newly diagnosed type 1 diabetic patients and to characterise these cell lines in terms of phenotype and epitope specificity. Furthermore, since sequence homologies exist between β -casein and putative beta-cell autoantigens, reactivity to the latter was also investigated.

T cell lines were generated from the peripheral blood of nine recent onset type 1 diabetic patients with different HLA-DQ and -DR genotypes, after stimulation with antigen pulsed autologous irradiated antigen presenting cells (APCs) and recombinant human interleukin-2 (rhIL-2). T cell line reactivity was evaluated in response to bovine β -casein, to 18 overlapping peptides encompassing the whole sequence of β -casein and to beta-cell antigens, including the human insulinoma cell line, CM, and a peptide from the beta-cell glucose transporter, GLUT-2. T cell lines specific to β -casein could not be isolated from HLA-matched and -unmatched control subjects.

β -Casein T cell lines reacted to different sequences of the protein, however a higher frequency of T cell reactivity was observed towards the C-terminal portion (peptides B05-14, and B05-17 in 5/9 and 4/9 T cell lines respectively). Furthermore, we found that 1 out of 9 β -casein-specific T cell lines reacted also to the homologous peptide from GLUT-2, and that 3 out of 4 of tested cell lines reacted also to extracts of the human insulinoma cell line, CM.

We conclude that T cell lines specific to bovine β -casein can be isolated from the peripheral blood of patients with type 1 diabetes; these cell lines react with multiple and different sequences of the protein particularly towards the C-terminal portion. In addition, reactivity of β -casein T cell lines to human insulinoma extracts and GLUT-2 peptide was detected, suggesting that the potential cross-reactivity with beta-cell antigens deserves further investigation.

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Introduction

Type 1 diabetes develops as a result of beta-cell destruction mediated by an autoimmune process where T cells play the major role (Roep & De Vries 1992, Yoon *et al.* 1998). Environmental factors have been proposed as possible triggers of beta-cell autoimmunity in genetically predisposed individuals (Akerblom & Knip 1998). Particular attention has been focused on cow's milk proteins since substantial evidence has been accumulated on the possible association between cow's milk consumption and an increased risk of developing type 1 diabetes (Virtanen *et al.* 1991, 2000).

Increased cellular and humoral immune responses to different cow's milk proteins including BSA, β -lactoglobulin and β -casein have been reported in patients with type 1 diabetes (Karjalainen *et al.* 1992, Cavallo *et al.* 1996b, Vaarala *et al.* 1996, Ellis *et al.* 1998), but these results have not always been confirmed (Atkinson *et al.* 1992, Roep *et al.* 1999, Saruger *et al.* 1999).

One possible mechanism to explain the relevance of cow's milk in the pathogenesis of type 1 diabetes is that the exposure to cow's milk could prime the immune system to react to beta-cell antigens possessing sequence homologies with cow's milk proteins according to a phenomenon defined as molecular mimicry (Albert & Inman 1999). In

this respect the observation of homologies in the amino acid sequence between several cow's milk proteins and beta-cell-associated antigens is of interest (Karjalainen *et al.* 1992, Becker *et al.* 1995, Cavallo *et al.* 1996b, Vaarala *et al.* 1996). In particular, bovine β -casein contains a sequence of five consecutive amino acids in common with glucose transporter GLUT-2 (Cavallo *et al.* 1996). GLUT-2 has been proposed as one of the autoantigens in type 1 diabetes since antibodies to this molecule were demonstrated in patients with the disease (Inman *et al.* 1993).

We previously reported enhanced cellular immune response to bovine β -casein in patients with type 1 diabetes (Cavallo *et al.* 1996b) and this was confirmed by other authors (Ellis *et al.* 1998). However, a similar increase was noticed also in first degree relatives of type 1 diabetes probands (Ellis *et al.* 1998), pointing out the role of genetic susceptibility in this response.

In the present study, we aimed to elucidate thoroughly the characteristics of the immune response to bovine β -casein by establishing specific T cell lines to this protein. Furthermore, stimulation of β -casein-specific T cell lines with beta-cell antigens including the GLUT-2 peptide and the human insulinoma cell line, CM, was investigated. The human insulinoma cell line, CM, has previously been described for functional characteristics, islet antigen expression and beta-cell genes patterns (Cavallo *et al.* 1996a, Baroni *et al.* 1999). In the present study, to evaluate T cell reactivity to beta-cell antigens, we used two different extracts obtained from CM cells cultured at high and low glucose levels since the expression of relevant beta-cell-associated antigens is known to be regulated by glucose concentration.

Materials and Methods

Patients

Mononuclear cells from venous blood were collected from 9 patients (6 males and 3 females, mean age 14 years \pm 8.8 S.D.) with recent onset type 1 diabetes (<1 month duration) who showed positive T cell reactivity to bovine β -casein (stimulation index (S.I.) >3) in primary proliferation assays, and from 7 control subjects including 2 non HLA-matched and 2 HLA-matched unrelated controls and 3 first degree relatives matched for HLA.

The project was approved by the Ethical Committee of the University of Rome 'La Sapienza' and informed consent was obtained by participating patients or their parents.

HLA genotyping

The HLA-DQB1 and -DRB1 typing of type 1 diabetic patients was performed by PCR- and sequences

specific oligonucleotide probed (SSO)-reverse line blot as described elsewhere (Erlich *et al.* 1991).

Antigens

Purified bovine milk proteins (β -casein, α -casein, β -lactoglobulin, BSA) and ovoalbumin (OVA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). These proteins were reconstituted in distilled water at a final concentration of 1 mg/ml.

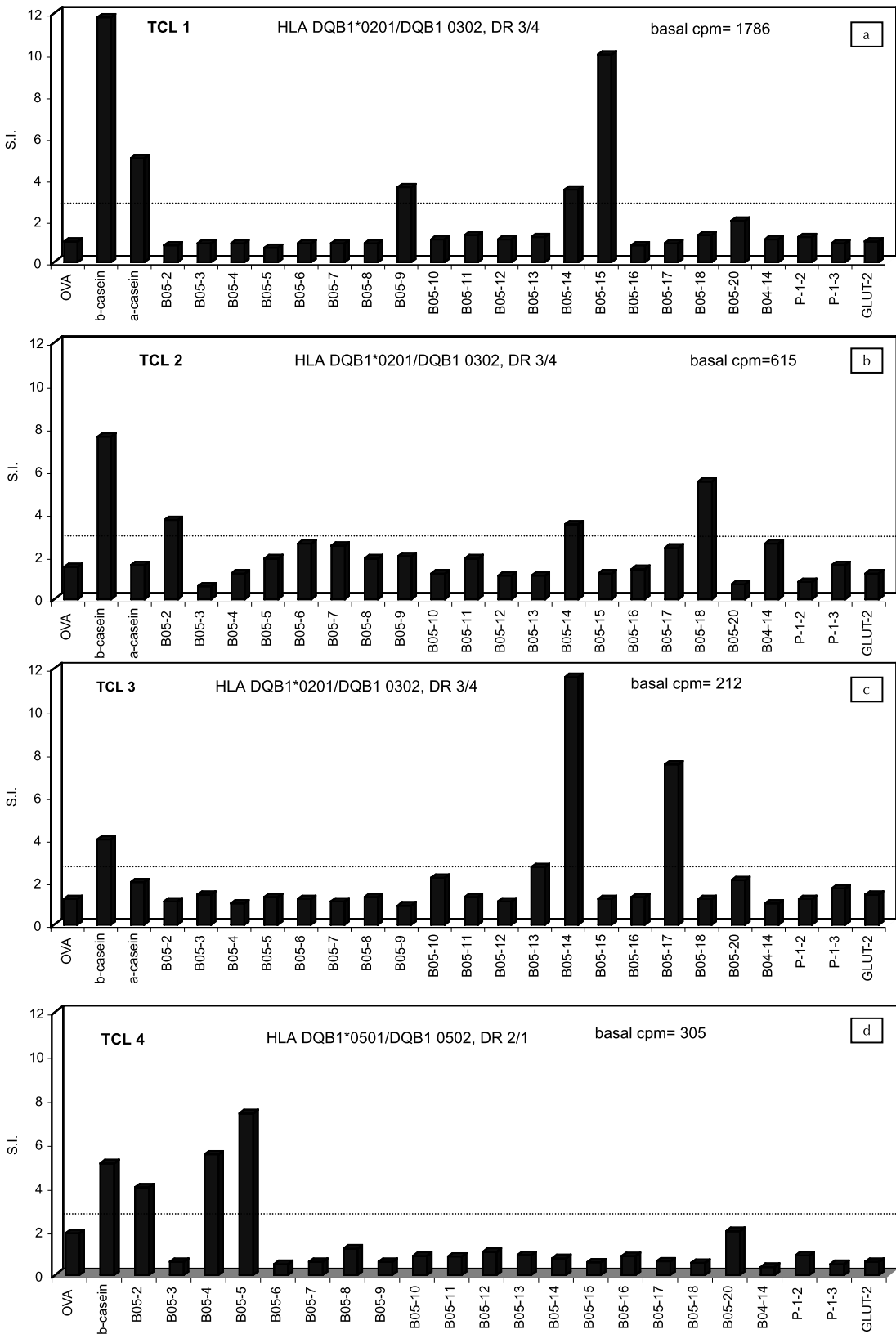
All peptides used to stimulate β -casein T cell lines were obtained by a robot system for multiple peptide synthesis (SYRO, MultiSyntech, Bochum, Switzerland) and kindly provided by Prof. G Jung, University of Tübingen, Germany. They all exhibited a purity of at least 95%. They included a set of 18 consecutive peptides (20 mers) encompassing the sequence of bovine β -casein with an overlap of 10 amino acids (peptides from B05-2 to B05-20), a peptide from the GLUT-2 glucose transporter (17 mer) and a peptide from β -casein (B04-14, 20 mer) sharing 5 consecutive amino acids in the central region with the GLUT-2 peptide, and 2 peptides of random sequence (20 mer) used as negative controls (P-1-2 and P-1-3).

CM insulinoma cells were cultured in RPMI medium containing high glucose (11 mM). At confluence the cells were split and cultured in low glucose medium (0.8 mM) for 20 weeks and then re-stimulated with 11 mM glucose for 48 h. Crude extracts from both CM cell sets were obtained following homogenisation in buffer containing a proteinase inhibitor cocktail as described elsewhere (Monetini *et al.* 1999). The protein content was determined by BCA reagent (Pierce, Rockford, USA). The homogenate from the human fibroblasts MRC-5 (Jacobs *et al.* 1970) was obtained using the same procedure.

Generation of T cell lines

Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque density gradient centrifugation at 800 *g* for approximately 30 min at room temperature. Cells at interface were washed twice and resuspended in RPMI 1640 complete medium supplemented with 10% of autologous serum, 10 mg/ml streptomycin (Sigma Chemical Co.), 104 U/ml penicillin (Sigma Chemical Co.) and 200 mM 1% glutamine (Sigma Chemical Co.). Cells (2×10^6) were placed in flat-bottomed 24-well plates (Nunc, Roskilde, Denmark) in a final volume of 1 ml and incubated in the presence of β -casein (20 μ g/ml) for 4 days with 5% CO₂ at 37 °C. After this period, recombinant human (rh) interleukin (IL)-2 (Sigma Chemical Co.) at a final concentration of 10 IU/ml was

Figure 1 Proliferative response to antigens and peptides by T cell lines (TCL) 1–4. Each graph represents one single experiment performed on a T cell line obtained from one diabetic patient. Each column in the graphs represents the proliferative response of TCL to one antigen/peptide expressed as stimulation index (S.I.). The broken line indicates the cut-off value for positivity (S.I. = 3.0).



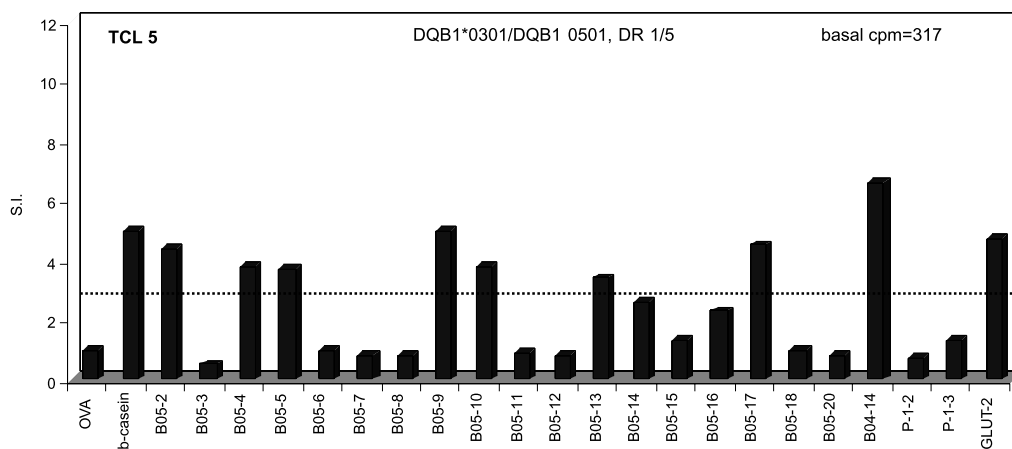


Figure 2 Proliferative response to antigens and peptides by TCL 5. This cell line reacts to the GLUT-2 peptide and the β -casein peptide (B04-14). These peptides share a sequence homology of five consecutive amino acids.

added to the wells and the plates were cultured for a further 4–5 days. Cells were stimulated to antigen-driven proliferation by adding irradiated autologous PBMC as antigen presenting cells (APCs). Three days later, T cell lines were fed with rhIL-2 and split when necessary using RPMI 1640 complete medium containing 10% autologous serum.

Antigen specificity, T cell epitope mapping and cross-reactivity study

T cell lines were assessed for specific reactivity to β -casein after 8–10 days from the resting period with rhIL-2. T cells (10×10^3) were cultured in round bottomed 96-well plates with 30×10^3 irradiated autologous PBMC as APCs in the presence of β -casein (20 $\mu\text{g}/\text{ml}$). Other cow's milk proteins including β -lactoglobulin, α -casein and BSA were used at the same concentration. Irrelevant antigens included tetanus toxoid (10 $\mu\text{g}/\text{ml}$), OVA (20 $\mu\text{g}/\text{ml}$) and phytohaemagglutinin (10 $\mu\text{g}/\text{ml}$) (Sigma Chemical Co.).

After 72 h, T cells were pulsed with [^3H]thymidine (Amersham, Life Science) (0.5 $\mu\text{Ci}/\text{well}$) and the proliferation was measured following thymidine uptake by liquid scintillation counting and expressed as S.I. (Cavallo *et al.* 1996b).

To map the specificity of T cell responses to β -casein, T cell lines were tested against a panel of 18 overlapping β -casein synthetic peptides. T cell lines were also tested against the GLUT-2-derived peptide containing the homology region (amino acids 415 to 419) with β -casein. The above sequence is identical to the sequence in

position 63–67 of β -casein corresponding to one of the regions of variation between bovine and human β -casein. The final concentration of peptides was 40 $\mu\text{g}/\text{ml}$.

The extracts from CM cells and human fibroblasts MRC-5 used as control were added at a concentration of 10 $\mu\text{g}/\text{ml}$.

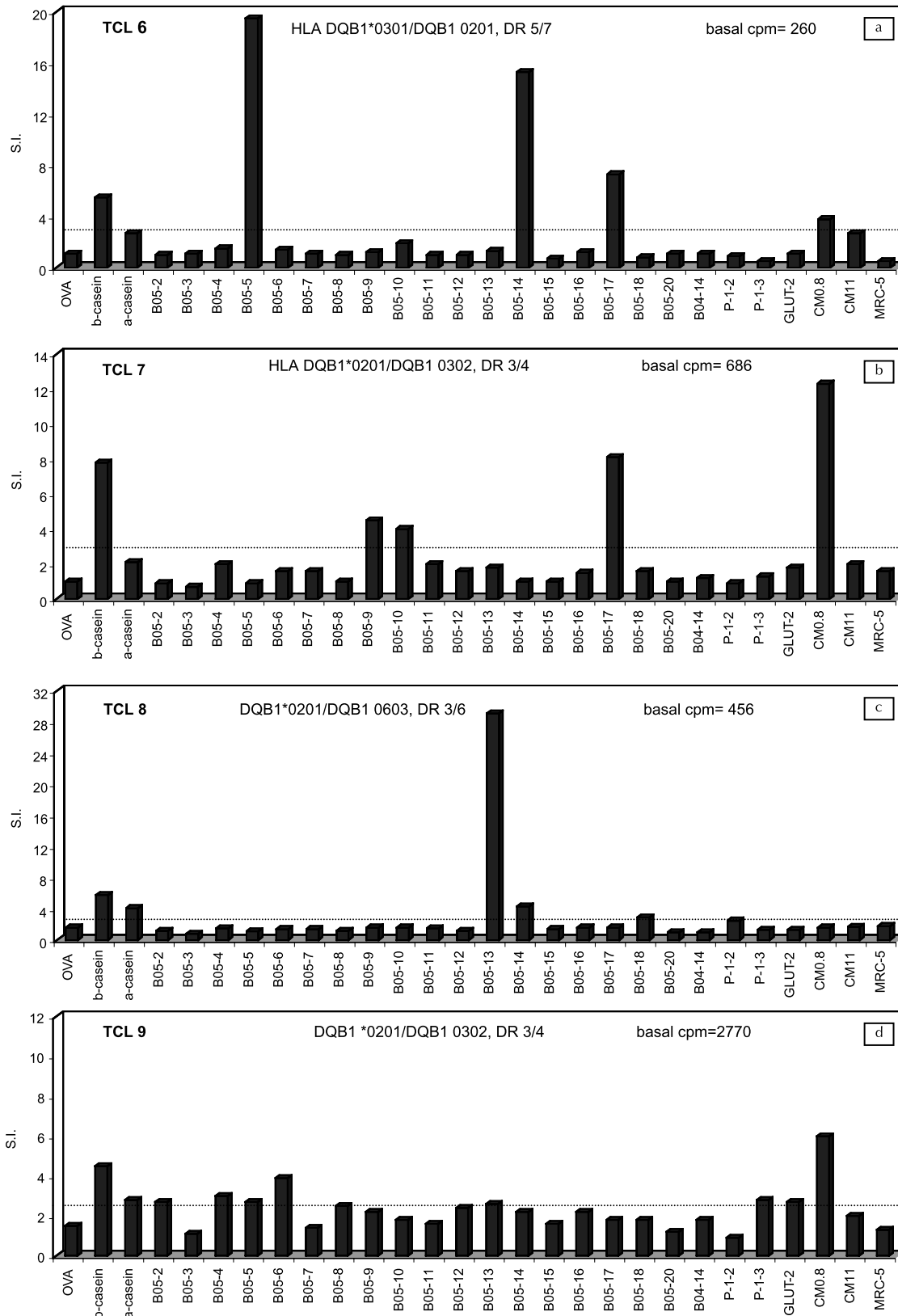
To determine the HLA restriction of T cell lines, APCs were incubated with monoclonal antibody (moAb) DK22 specific for HLA-DR (kindly donated by Dr Beales, St Bartholomew's Hospital, UK) at a final concentration of 5 $\mu\text{g}/\text{ml}$ for 30 min at 37 $^\circ\text{C}$ before the proliferation assay. The moAb anti class I (W6/32) was used as a control.

Characterization of T cell subsets and cytokine measurement

β -Casein-specific T cell lines were phenotypically characterised for surface molecular markers using phycoerythrin and fluorescein isothiocyanate (FITC)-conjugated antibodies to CD4 and CD8 (Becton Dickinson, Baltimore, USA) antigens and analysed by FACScan analysis.

T cell lines were assessed for cytokine production (IL-4 and interferon- γ (IFN- γ)) by using ELISA assays routinely carried out at the National Institute for Biological Standard and Controls (Potters Bar, UK). Briefly, the plates were coated with antibodies (100 $\mu\text{l}/\text{well}$) overnight at 4 $^\circ\text{C}$. The plates were washed twice with PBS+0.05% Tween 20 (PBS/Tween) and non-specific binding was blocked by adding PBS+0.05% BSA (PBS/BSA) for 30 min at room temperature (RT). Appropriate dilutions of the standard and the samples were added to the plates and left for 3 h at RT. After washing with PBS/BSA, the biotinylated detecting antibody was added for 2 h at RT. The plates

Figure 3 Proliferative response to antigens and peptides by TCL 6–9. These cell lines were stimulated also with crude extracts from CM cells cultured at high (11 mM; CM11) and low (0.8 mM; CM0-8) glucose levels. A positive T cell response was observed towards homogenates from CM cells grown at 0.8 mM glucose. MRC-5 represents a control cell extract (fibroblasts).



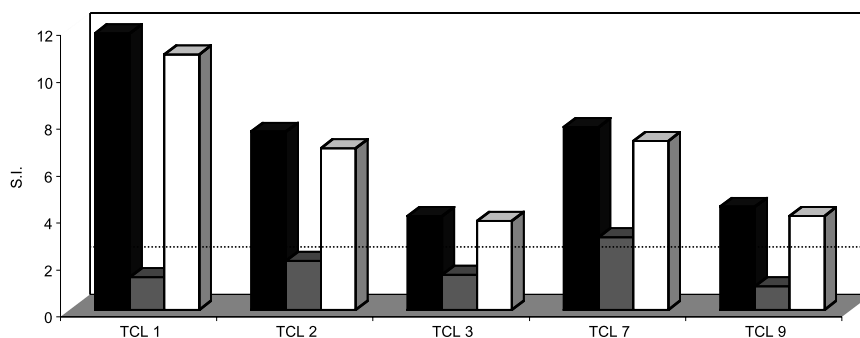


Figure 4 Inhibition of proliferative response to β -casein in 5 T cell lines after pre-incubation with anti HLA-DR moAb (DK22). MoAb anti class I (W6/32) was used as a control. Solid bars, β -casein; shaded bars, anti DR (DK22); open bars, anti class I (W6/32).

were washed again with PBS/Tween and streptavidin-horseradish peroxidase (HRP) conjugate antibody was added for 1 h at RT. After the final wash with PBS/Tween, TMB substrate was added for 10–15 min. The reaction was stopped by adding 50 μ l/well 2 M H_2SO_4 . The plates were read at 450 nm.

Results

Antigen specificity, T cell epitope mapping and cross-reactivity study

Nine β -casein-specific T cell lines were isolated from nine type 1 diabetic patients within 1 month of diagnosis. The proliferative response to β -casein, β -casein peptides and the other panel of antigens is shown in Figs 1, 2 and 3. In these figures each graph represents one single experiment performed on a T cell line obtained from one diabetic patient. Each column in the graphs shown in Figs 1–3 represents the proliferative response of T cell lines to the various antigens and peptides, expressed as stimulation index (S.I.). S.I. is calculated by dividing the mean c.p.m. value of triplicates in the presence of antigen by the mean c.p.m. with medium alone. Positive responses were considered with S.I. above 3.

Different peptides of β -casein were involved in T cell reactivity, however T cell proliferation was preferentially directed towards the C-terminal portion of the protein. Five out of nine T cell lines reacted to residues 130–150 (B05–14 peptide) and four out of nine recognised residues 160–180 (B05–17 peptide). All T cell lines were also tested for reactivity to both the GLUT-2 peptide and the homology β -casein peptide B04–14. These two peptides share a homology sequence of 5 consecutive amino acids in the central region. Only one T cell line reacted to the GLUT-2 peptide and to the β -casein homology peptide B04–14 (Fig. 2).

In two cases, T cell lines specific to β -casein reacted also to α -casein indicating that the two proteins may share T cell epitopes (Fig. 1a, 3c).

Crude extracts from CM cells cultured at different glucose concentrations (11 and 0.8 mM) were used to stimulate β -casein T cell lines. In only four cases were we able to study the reactivity of β -casein T cell lines against CM cells. Three out of four T cell lines proliferated in the presence of CM antigen extracts, particularly towards CM cells cultured at 0.8 mM glucose (Fig. 3). The response to CM cells cultured at 0.8 mM was higher compared with extracts of cells cultured at 11 mM glucose. This may be explained with the hypothesis that long term exposure of cells to 11 mM glucose induces a downregulation of antigen expression whereas, in cells grown with low glucose, the antigen expression might be restored.

The recognition of β -casein was not associated with particular HLA-DR or -DQ alleles. In addition, we observed that different peptides were presented from the same HLA haplotype.

Proliferative responses to β -casein were abrogated after pre-incubation with anti HLA-DR (DK22) but not with anti HLA class I (W6/32) (Fig. 4).

β -Casein-specific T cell lines could not be isolated from HLA-matched (including first degree relatives) and non HLA-matched control subjects using the same procedure.

Characterisation of T cell subsets and cytokine measurement

By FACScan analysis the vast majority of T cells were composed of CD4+ T cells.

T cell line supernatants were collected and examined for the presence of cytokine including IFN- γ and IL-4. T cell lines produced IFN- γ whereas IL-4 was not detected (Table 1), indicating a predominance of Th1 lymphocytes. The assay sensitivities for IL-4 and IFN- γ were 50 pg and 30 pg respectively.

Discussion

In this study we generated nine β -casein T cell lines from nine type 1 diabetic patients with different HLA

Table 1 Cytokine production by β -casein specific T cell lines (TCL). Supernatants were collected after 3 days stimulation of T cells with irradiated APC in the presence of antigen. The results shown are corrected for cytokine levels detected after stimulation without antigen

	IFN- γ (ng/ml)	IL-4 (pg/ml)
TCL 1	2.4	nd
TCL 2	2.03	nd
TCL 3	4.75	nd
TCL 4	0.84	nd
TCL 5	5.17	nd
TCL 6	1.6	nd
TCL 7	5.3	nd

nd, not detectable. IL-4 measurement was always below the detection limit of the assay 50 pg/ml.

haplotypes, who reacted to β -casein in primary proliferation assays. These cell lines were tested for epitope specificity and for reactivity with beta-cell molecules in order to determine whether potential cross-reactivity with pancreatic beta-cells could be envisaged.

By using a panel of 18 overlapping β -casein peptides we found that T cell reactivity was spread along the whole molecule although the C-terminal portion of the protein was more frequently involved in T cell recognition. We also found that 2 out of 7 β -casein-specific T cell lines responded also to α -casein indicating that sequence homologies present between α - and β -casein may be cross recognised. β -Casein T cell lines were not stimulated to proliferate in the presence of other cow's milk proteins including β -lactoglobulin and BSA or diabetes-associated antigens like glutamic acid decarboxylase (GAD) or insulin (data not shown). β -Casein-specific T cell lines could not be isolated from control subjects. As controls, we tested both HLA-matched (including first degree relatives) and non HLA-matched subjects.

One of the mechanisms by which cow's milk proteins may trigger an autoimmune response to beta-cells is molecular mimicry. This phenomenon has been described to be relevant in the pathogenesis of different autoimmune diseases such as experimental autoimmune encephalomyelitis where an autoimmune response to myelin basic protein (MBP) may be evoked by virus infection bearing antigen similarities to host MBP (Wucherpfennig & Strominger 1995).

Homologies between beta-cell antigens and cow's milk protein have been described (Karjalainen *et al.* 1992, Becker *et al.* 1995, Cavallo *et al.* 1996b, Vaarala *et al.* 1996). We have also found that the sequence in position 63–67 of β -casein corresponding to one of the regions of variation between bovine and human β -casein is identical to residues 415–419 of the beta-cell-specific glucose transporter GLUT-2 (Cavallo *et al.* 1996b).

In our study, only one β -casein T cell line out of nine reacted with both the GLUT-2 peptide and the homologous peptide on β -casein (B04-14) in the context of HLA DQB1*0301/DQB10501-DR 1/5, and it is possible that T cell recognition of both peptides was restricted by that particular HLA.

We also found that 3 out of 4 β -casein-specific T cell lines reacted also with the human insulinoma cell line, CM, used as a source of beta-cell antigens. β -Casein T cell lines showed higher proliferative response to the homogenate obtained from CM cells cultured with 0.8 mM glucose and then stimulated with 11 mM glucose for 48 h compared with the extract from CM cells cultured long term with 11 mM glucose. Since the capacity of insulin secretion by CM cells is reduced by high glucose concentration, it may be possible that the expression of other relevant beta-cell antigens is also affected by this culture condition. By culturing CM cells at low glucose concentrations the capacity to produce insulin is retained, as recently demonstrated (Baroni *et al.* 1999), and it is likely that the expression of other beta-cell antigens, potentially cross-reactive with β -casein, may be restored. This hypothesis would explain the difference between the S.I. of β -casein-specific T cell lines in response to the two different CM homogenates.

It has been speculated that the existence of sequence homologies between beta-cell components and cow's milk proteins may be responsible for the activation of autoreactive T cell clones in type 1 diabetes. The results presented in this study show that β -casein-specific T cell lines do indeed have the potential to undergo activation in response to beta-cell antigens. In addition we showed that β -casein T cell lines are predominantly composed of CD4+, Th1 lymphocytes which are the T cell subset believed to be involved in the autoimmune destruction of beta-cells.

In conclusion, we have provided evidence for the first time that β -casein T cell lines can be isolated from patients with type 1 diabetes and that different epitopes are recognised within this protein. Of particular interest is the reactivity of β -casein-specific T cell lines with beta-cell antigens. These results may have important implications since cow's milk has been epidemiologically associated with the development of type 1 diabetes. Therefore, the hypothesis that environmental factors, such as cow's milk proteins, may be capable of activating 'ignorant' autoreactive T cell clones by means of antigens that mimic the host proteins certainly deserves further consideration.

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