

# Stimulation of human peripheral blood lymphocytes by bioactive peptides derived from bovine milk proteins\*\*

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**Abstract** The *in vitro* modulation of the proliferation of human peripheral blood lymphocytes by different synthetic peptides derived from milk proteins was investigated. Therefore, proliferation changes were followed up after incorporation of BrdU into the DNA, and the influence on protein biosynthesis was measured using the [<sup>3</sup>H]leucine incorporation test. Tyr-Gly and Tyr-Gly-Gly significantly enhanced (maximal 90 and 35%, respectively) the proliferation of PBL. For  $\beta$ -casomorphin-7 and  $\beta$ -casokinin-10, lymphocyte proliferation was suppressed at lower concentrations, but stimulated at higher concentrations ( $\geq 10^{-7}$  mol/l). Protein synthesis was stimulated (maxima at 25%) only with Tyr-Gly and Tyr-Gly-Gly. The findings point to a need for further studies on the possible function of peptides derived from milk proteins as orally bioavailable immunopotentiatory compounds.

**Key words:** Bioactive peptide; Immunoepitope; Milk protein; Human peripheral blood lymphocyte

## 1. Introduction

A wide range of biologically active peptides which can be liberated from milk proteins during enzymatic *in vitro* or *in vivo* digestion have been identified (for review see [1]). Casein phosphopeptides and casomorphins have already found interesting applications, both as pharmaceutical preparations and as dietary supplements. Casein derived immunoepitopes including immunoepitopes from  $\alpha_{s1}$ -casein (residues 194–199) and  $\beta$ -casein (residues 63–68 and 191–193) have been shown to stimulate phagocytosis of sheep red blood cells by murine peritoneal macrophages, and to exert a protective effect against *Klebsiella pneumoniae* infection in mice after intravenous treatment [2,3]. There are various hypotheses about the physiological action of such peptides. They might stimulate the proliferation and maturation of T-cells and natural killer cells for the defence of the newborn against different bacteria, particularly enteric bacteria.

This paper reports on the immunoreactivity of human peripheral blood lymphocytes stimulated or suppressed by various synthetic peptides corresponding to bioactive sequences of bovine milk proteins.

## 2. Materials and methods

### 2.1. Materials

RPMI-1640, Ficoll-Hypaque, macromolecular dextran, Con A, penicillin/streptomycin solution, Tyr-Gly (YG), Tyr-Gly-Gly (YGG),  $\beta$ -casomorphin-7 (YPFPGPI), all cell culture dishes and multiwell plates were from Sigma (Deisenhofen, Germany). BrdU-proliferation assay kit was obtained from Boehringer (Mannheim, Germany). L-[4,5-<sup>3</sup>H]leucine (128 Ci/mmol) was purchased from Amersham Life Science (Braunschweig, Germany). All other substrates and chemicals were obtained from the specific suppliers as indicated.

### 2.2. Peptide synthesis

$\beta$ -Casokinin-10 (YQQPVLGPVR) was synthesized by the Fmoc-polyamide solid-phase method [4] using a resin derivatised with Fmoc-Arg(Mtr) (Novabiochem, Cambridge) and a continuous flow peptide synthesizer as described in reference; the overall yield of the HPLC-purified peptide was 53.2%.

### 2.3. Isolation of human peripheral blood lymphocytes (PBL)

PBL in the heparin-anticoagulated blood of normal subjects were enriched according to the method of Bøyum [5]. Briefly, buffy coat was diluted 2-fold with PBS (pH 7.2) and PBL together with monocytes were recovered by centrifugation on Ficoll-Hypaque cushions. Cells were washed twice with 0.9% NaCl and subsequently diluted in RPMI-1640 medium containing 10% heat-inactivated calf serum (Sigma, Deisenhofen, Germany) and 2  $\mu$ g/ml Con A in an atmosphere containing 5% CO<sub>2</sub>. After 24 h of differentiation, PBL were separated from adherent monocytes and diluted in fresh medium for further assays.

### 2.4. Assessment of PBL proliferation

To quantify PBL proliferation the colorimetric Cell Proliferation ELISA (Boehringer Mannheim, Germany) was used according to manufacturer's instructions. Cells ( $2 \times 10^5/200 \mu$ l) cultured in a 96-well microtiter plate in the presence of various peptides were pulsed with 5-bromo-2'-deoxyuridine (BrdU) for 12 h. Incorporation of BrdU into the DNA was detected by the anti-BrdU antibody peroxidase conjugate (POD). The amount of POD retained in the immunocomplex was quantified by a substrate reaction using TMB. Results were read out on an ELISA spectrophotometer at 450 nm (reference wavelength: 620 nm).

### 2.5. Assessment of protein biosynthesis

In order to demonstrate the influence of bioactive peptides on the protein biosynthesis of PBL the [<sup>3</sup>H]leucine incorporation assay was used [6]. Therefore,  $2 \times 10^5$  PBL in 200  $\mu$ l of leucine-free RPMI-1640 were added to each of 96 wells of microtiter plates with an increasing amount of the respective peptide or Con A as positive control. After 18 h, 1  $\mu$ Ci of [<sup>3</sup>H]leucine was added to each well and incubation was continued for 6 h at 37°C. The incubations were stopped by removing the cells onto Whatman 3MM glass fiber plates. The incorporation of [<sup>3</sup>H]leucine into acid-precipitable material was measured by liquid scintillation counting.

## 3. Results and discussion

The effects of Tyr-Gly, Tyr-Gly-Gly,  $\beta$ -casomorphin-7 as well as  $\beta$ -casokinin-10 on the proliferation of human peripheral blood lymphocytes activated with ConA were examined

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**Abbreviations:** PBL, peripheral blood lymphocytes; BrdU, 5-bromo-2'-deoxyuridine; ConA, concanavalin A; TMB, tetramethylbenzidine; PBS, phosphate-buffered saline.

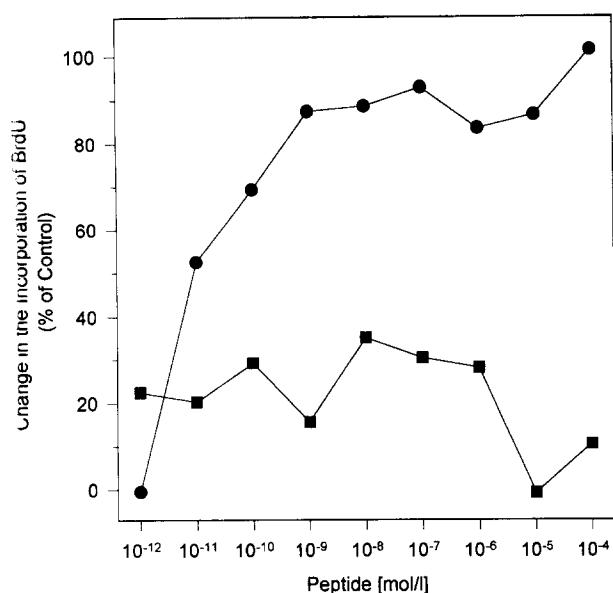


Fig. 1. Effect of Tyr-Gly (●) and Tyr-Gly-Gly (■) on incorporation of BrdU by human lymphocytes activated with ConA. Each data point represents the mean percentage change; the results have been reproduced five times with an S.E.M.  $\leq 20\%$ . Cells cultured in medium only gave an absorbance of  $(A_{450} - A_{620}) = 0.484 \pm 0.026$ .

in vitro by separately quantifying the incorporation of BrdU after 72 h and  $[^3\text{H}]$ leucine after 18 h to assess alterations in DNA synthesis and protein synthesis, respectively. Tyr-Gly and Tyr-Gly-Gly significantly enhanced the incorporation of BrdU by PBL at respective concentrations of  $10^{-11}$  to  $10^{-4}$  mol/l (Fig. 1). Maximal stimulation was achieved with  $10^{-4}$  mol/l Tyr-Gly and  $10^{-8}$  mol/l Tyr-Gly-Gly, respectively. It should be stressed that Tyr-Gly already revealed 93% of maximal stimulation at  $10^{-9}$  mol/l, and Tyr-Gly-Gly showed 74% of maximal stimulation at  $10^{-12}$  mol/l. In contrast,  $\beta$ -caso-

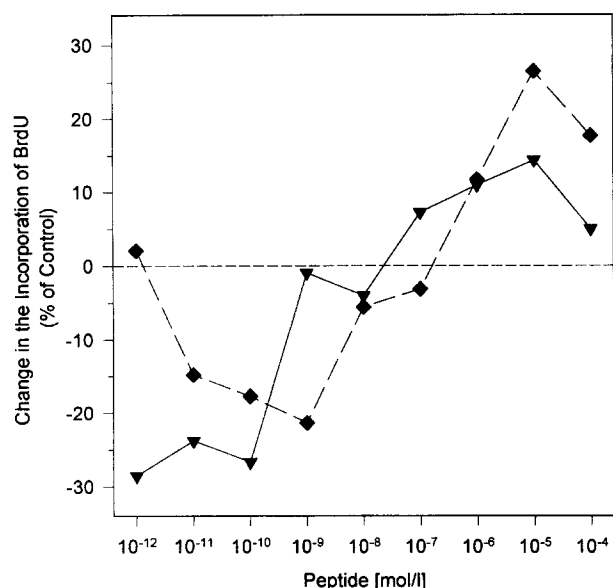


Fig. 2. Effect of  $\beta$ -casokinin-10 (▼) and  $\beta$ -casomorphin-7 (◆) on incorporation of BrdU by human lymphocytes activated with ConA. Each data point represents the mean percentage change; the results have been reproduced five times with an S.E.M.  $\leq 17\%$  and  $\leq 10\%$ , respectively.

morphin-7 and  $\beta$ -casokinin-10 suppressed the incorporation of BrdU at lower concentrations, but revealed stimulation at higher concentrations (Fig. 2). Protein synthesis was enhanced with Tyr-Gly and Tyr-Gly-Gly (Fig. 3); however, no marked effect was found with  $\beta$ -casomorphin-7 and  $\beta$ -casokinin-10 (data not shown).

The peptides Tyr-Gly and Tyr-Gly-Gly are partial sequences in the primary structure of bovine  $\kappa$ -casein and  $\alpha$ -lactalbumin, respectively. Recently, these peptides were used for immunotherapy of human immunodeficiency virus infection: In a large multicenter trial, a dialysed leukocyte extract from normal donors in which Tyr-Gly-Gly and Tyr-Gly are thought to be the nonspecific active components was applied to inhibit the development of infections in patients with pre-AIDS. Encouraging results after a bi-weekly treatment of 93 patients with an AIDS-related complex showed a significant reduction either to progress to a clinically relevant endpoint or to AIDS [7].

$\beta$ -Casomorphin-7 derived from  $\beta$ -casein [8] was used as a possible immunostimulating substrate since it is a ligand of  $\mu$ -type opioid receptors that have also been found on the surface of human T-lymphocytes [9,10]. Furthermore, casomorphins may function as both opioid agonists and inhibitors of angiotensin-converting-enzyme (ACE) [11].  $\beta$ -Casokinin-10 is a non-opioid peptide fragment of casein that inhibits ACE [4]. A common structural feature of several ACE-inhibitory peptides and some immunomodulatory peptides is the presence of arginine as the C-terminal residue [12–15]. It is assumed that the positively charged arginine residue contributes substantially to the bioactivity of these peptides.

Milk proteins are precursors of different bioactive peptides that are inactive within the sequence of the precursor protein and can be released during gastrointestinal passage [1]. This also applies to the immunomodulating peptides investigated in the present study. Such peptides may interact with the gut-associated lymphoid tissue (GALT). Moreover, di- and tripeptides like Tyr-Gly and Tyr-Gly-Gly can, in principle, pass

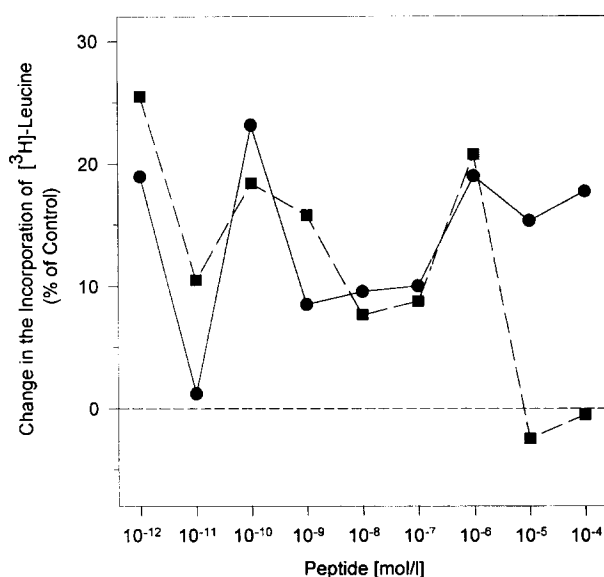


Fig. 3. Effect of Tyr-Gly (●) and Tyr-Gly-Gly (■) on uptake of  $[^3\text{H}]$ leucine by human lymphocytes activated with ConA. Each data point represents the mean percentage change; the results have been reproduced five times with an S.E.M.  $\leq 9\%$ . Cells cultured in medium only gave 22070  $\pm$  960 cpm.

across the intestine in quantitatively significant amounts to reach peripheral lymphocytes. Taken together, the effects of these peptides on PBL lend support to the claim that several as yet unknown immunomodulatory sequences are located within bovine milk proteins. These peptides may function as orally bioavailable compounds. Thus, the results justify further experiments, e.g. using cells from Peyer's patches, to elucidate the putative physiological significance as well as the structure-activity relationship of immunopotentiatory peptides derived from milk proteins.

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