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Beta-casomorphin (BCM) and human colonic lamina propria lymphocyte proliferation

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SUMMARY

BCM is a milk-derived peptide with opiate-like properties which is absorbed through the gastrointestinal mucosa. It has been shown to affect gastrointestinal motility, absorption and secretion. Recently, modulation of the immune system by BCM was also reported. In this study we investigated the *in vitro* effect of BCM on the human mucosal immune response as represented by lamina propria lymphocyte (LPL) proliferation. Results show that BCM significantly inhibited concanavalin A (ConA) stimulated LPL DNA synthesis. BCM also inhibited ornithine decarboxylase activity (ODC) in ConA-stimulated LPL. Although BCM also inhibited 12-O-tetradecanoyl phorbol-13-acetate (TPA) stimulated LPL DNA synthesis, the degree of inhibition was much lower than in ConA-stimulated LPL. The anti-proliferative effect of BCM was reversed by the opiate receptor antagonist, neloxone. Our results suggest that BCM may affect the human mucosal immune system, possibly via the opiate receptor.

Keywords beta-casomorphin lamina propria lymphocytes mucosal immunology milk peptides

INTRODUCTION

The human gut immune system is recognized as the first line of defence of the host against foreign peptide antigens (from food proteins) introduced with each meal (Elson *et al.*, 1986). During the digestive process, these peptide antigens are absorbed through the mucosal wall and interact with the mucosal immune system, normally producing immune tolerance.

Mucosal immune processing involves the interaction of ingested peptides with the intestinal epithelium and gut mucosal immune system, particularly the lamina propria lymphocytes (LPL). During this process, the antigenic moiety of the ingested peptide may be changed and introduced to the various mucosal immune components in the lamina propria, i.e. lymphocytes, macrophages, T helper and T suppressor cells (Walker, 1986).

Milk peptides are known to affect the circulatory immune system in human and animal models (Hill *et al.*, 1986) and have been implicated in the immune changes seen in the small and large intestines of patients with 'milk allergy'. Nevertheless, the physiological effect of milk peptides on the normal gut immune system in humans is still unknown.

BCM is an opiate-like peptide originally isolated from human and bovine β-casein following trypsin hydrolysis (Brantl *et al.*, 1979). Several reports have demonstrated the effects of BCM on various organs including the central nervous system (Matthies, Stark & Hartdrodt, 1984), the gastrointestinal tract (Hautefeuille *et al.*, 1986), and the endocrine system (Leaden & Kalra, 1985). Human BCM has also been shown to stimulate phagocytosis by murine peritoneal macrophages and enhance the resistance of adult mice to Klebsiella pneumonia (Parker *et al.*, 1984). These effects were reversed by the specific opiate-receptor antagonist, naloxone. Moreover, specific opiate-like receptors for BCM have been detected in rat brain homogenates (Brantl *et al.*, 1982).

Opiate receptors have been described in various human tissues and systems, including the central nervous system (Koch, Weidermann & Teschemacker, 1985), the gastrointestinal tract (Lord *et al.*, 1977), and the immune system (Mehrishi & Mills, 1983). Furthermore, opiate agonists such as β-endorphin have been shown to affect with human and animal immune functions such as lymphocyte proliferation (Gilman *et al.*, 1982), natural killer (NK) cell activity (Faith *et al.*, 1984) and neutrophil locomotion (van Epps *et al.* 1983).

Our working hypothesis is that, in addition to the various physiological effects of BCM on the gastrointestinal tract, BCM may also affect mucosal immune function during the digestive process. Here we investigated the *in vitro* effect of BCM on human mucosal immune function as represented by LPL DNA synthesis.

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MATERIALS AND METHODS

Materials

Tissue culture media and supplies were obtained from GIBCO (Grand Island, NY). Collagenase, bovine BCM-7 (Tyr–Pro–Phe–Pro–Gly–Pro–Ile), dithiothreitol (DTT), EDTA, TPA, naloxone, trypan blue, ethidium bromide, acridine orange, and other chemicals were purchased from Sigma Chemical Co. (St Louis, MO). Concanavalin A (ConA) was purchased from ICN Immuno Biologicals (Lisle, IL). Radioactive materials, ^{3}H-thymidine (83 mCi/mmol) and L-^{14}C-ornithine (50 mCi/mmol) were purchased from NEN DuPont (Boston, MA). Fluorescein-conjugated goat anti-mouse IgG, CD3, CD19, CD4, and CD8 were purchased from Becton Dickinson (Mountain View, CA).

Human colon specimens

Twenty human colon and three ileal specimens were obtained from adult patients (aged 41–85 years) who underwent colon resection. None of the patients had any known immune disorders or milk allergy. Macroscopically normal colon and ileal specimens (at least 5 cm away from any diseased area) were transferred in ice-cold RPMI 1640 medium to the laboratory within 30 min of surgical resection. Only specimens that were subsequently confirmed to be histologically normal were included for this study. In addition, flow cytometry characterization of LPL cell components was done on LPL obtained from each colon or ileal specimen to assure normal mucosal immune cell population. The study was approved by the Human Investigation Committee of Wayne State University.

Isolation of LPL

Isolation of LPL was done according to the method of Bull & Bookman (1977), as described (Elitsur, Bull & Luk, 1990). Briefly, mucosa was separated from the muscle layer and was minced into small pieces (0·5 × 0·5 cm). The fragments were treated in calcium- and magnesium-free HBSS (CMF-HBSS) containing 0·1 M DTT, followed by EDTA (0·75 mM) and collagenase (0·1 mg/100 ml) digestion overnight. The resulting cell suspension was centrifuged over Ficol–Hypaque gradient, washed and then resuspended in standard culture media (RPMI 1640, 25 mM HEPES buffer, 2 mM L-glutamine, 10% human AB serum, penicillin 100 U/ml, amphotericin-B 2·5 μg/ml, and streptomycin 100 μg/ml) for thymidine incorporation studies. Cell viability was determined by trypan blue exclusion or ethidium bromide and acridine orange staining. Viability was found to be always above 90%.

To confirm normal mucosal immune cell components, B and T cell distribution was assessed by cell surface antigen labelling and quantified using flow cytometry. The mean (±s.e.m.) percentage of T cells (CD3) and T cell subpopulation ratio (CD4/CD8) were 66·9 ± 3·2 and 2·6 ± 0·4, respectively. There was no difference between LPL subpopulations obtained from colon or ileal specimens. The distribution was found to be within the published normal range and similar to our previous results (Elitsur *et al.*, 1990).

Thymidine incorporation assay

Aliquots of 0·2 ml of ConA (5 μg/ml) or TPA (50 ng/ml) stimulated LPL (10^{6} cells/ml) were incubated in standard culture media in triplicate in 96-well microtitre plates (37°C, 5% CO_2), with different concentrations of BCM (10–250 μg/ml) for 5 days (previous studies have indicated that thymidine incorporation into LPL peaked after 5 days of incubation). BCM is a water-soluble peptide and was dissolved in RPMI 1640. BCM (10 μl/well) was added to the tested wells for the final concentration of 10–250 μg/ml. Control cultures were only added 10 μl/well of RPMI 1640 as a vehicle. Six hours before cell harvesting, cultures were pulsed with ^{3}H-thymidine (0·5 μCi/well) and radioactivity was quantified by liquid scintillation in a Packard 1500 beta counter. As previously described (Elitsur *et al.*, 1990), the results were expressed as fraction of control (FC) according to the formula

$$FC = \frac{\text{Experimental} - \text{Background release}}{\text{Control} - \text{Background release}}$$

and presented as the mean ± s.e.m.

In addition, a time-course study was done to characterize further the effect of BCM on LPL proliferation. In this experiment ConA-stimulated LPL were incubated in standard culture media in multi-well plates (0·2 ml/well, 10^{6} cells/ml) with BCM (50 μg/ml, 10 μg/well). Control cultures did not contain BCM. Cultures were harvested after 1, 3, 4, and 5 days of incubation and radioactivity was quantified as described above.

ODC assay

ODC was determined by the micro-method of Beaven *et al.* (1978) as previously described (Elitsur *et al.*, 1990). Briefly, the cell pellet was homogenized in a buffer containing 50 mM Tris (pH 7·5), 0·25 M sucrose, 0·1 mM EDTA, 0·4 mM pyridoxal 5′ phosphate, and 1 mM DTT. Homogenates were centrifuged at 100 000 *g* for 60 min and the supernatants were assayed for ODC activity. The incubation mixture contained 50 mM HEPES, 1 mM EDTA, 0·25 mM pyridoxal 5′ phosphate, 1 mM DTT, 130 μM ornithine, and 0·144 μM ^{14}C-ornithine. Protein concentration in the assay mixture was determined using Bradford's protein dye method.

Naloxone and the effect of BCM on ConA-stimulated LPL

Naloxone is a known opiate receptor antagonist which inhibits opiate receptor-mediated effects. To determine whether the inhibitory effect of BCM was mediated via opioid receptors, ConA-stimulated LPL were incubated with BCM (50 μg/ml) in the presence of naloxone (10^{-9}–10^{-6} M). Control cultures were ConA-stimulated LPL with BCM alone or without BCM or naloxone. Cultures were then incubated at 37°C in 5% CO_2 for 5 days. Six hours before cell harvest, ^{3}H-thymidine (0·5 μCi/well) was added, and radioactivity was quantified as described above.

Statistical analysis

Since ratio variables based on actual measurements were used in our study, parametric statistical analysis, i.e. an unpaired Student's *t*-test, was used for comparison between the test group and the control. $P < 0·05$ was considered significant.

RESULTS

The time-course study showed that BCM inhibited thymidine incorporation into ConA-stimulated LPL over time, with maximal inhibition occurring at 5 days (Fig. 1).

BCM did not alter the rate of DNA synthesis in unstimulated LPL. BCM (10–250 μg/ml) significantly suppressed DNA synthesis in Con A-stimulated colonic or ileal LPL after 5 days

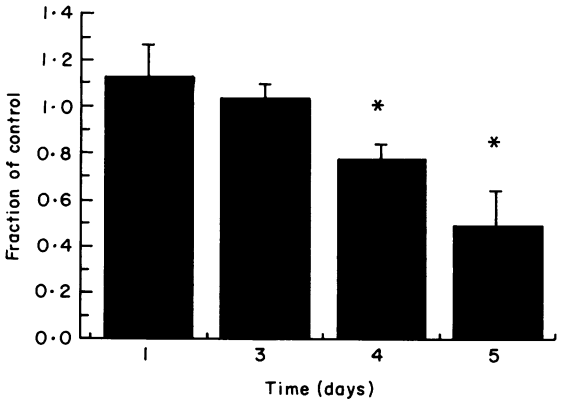

Fig. 1. Time-course of the effect of BCM (50 μg/ml) on thymidine incorporation into ConA-stimulated LPL. Results are expressed in mean ct/min + s.e.m. of one representative experiment (out of two), each done in triplicate. Untreated control was denoted arbitrarily as 1·0 for fraction of control calculations. $*P < 0.05$ compared with control.

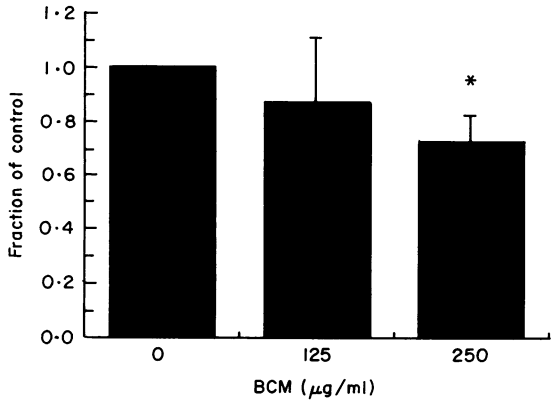

Fig. 4. Effect of BCM on thymidine incorporation into phorbol ester (TPA) stimulated LPL. LPL were incubated with TPA (50 ng/ml) and BCM (125, 250 μg/ml) for 5 days. Control cultures did not contain BCM. The results shown are the mean + s.e.m. of three different experiments, each done in triplicate determinations. Average ct/min for unstimulated (background) and of TPA-stimulated (control) LPL were 1391 and 12 523, respectively. $*P < 0.05$ compared with control.

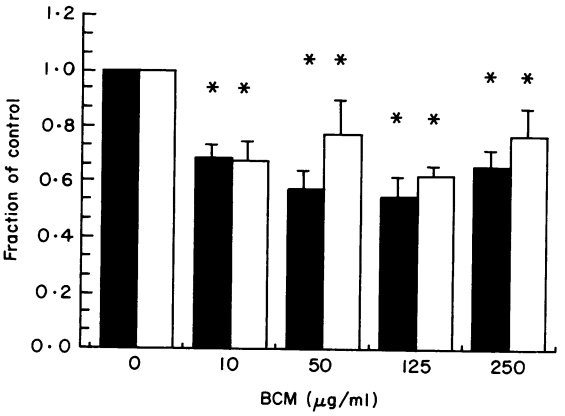

Fig. 2. The effect of varying concentrations of BCM on thymidine incorporation into ConA-stimulated LPL. Average ct/min for unstimulated (background) and of ConA (5 μg/ml) stimulated (control) LPL were 443 and 34 082, respectively. (See Fig. 1 legend for control data). ■, Colon; □, ileum. $*P < 0.05$ compared with control.

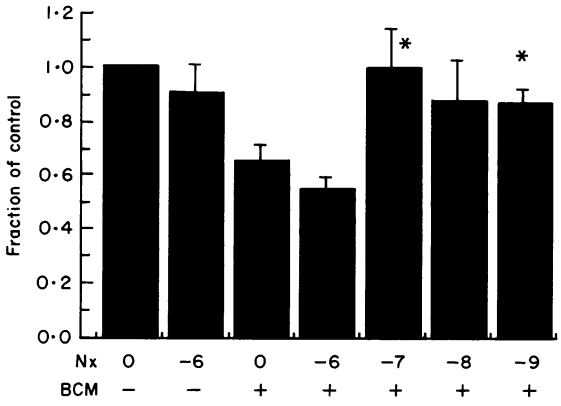

Fig. 5. The effect of BCM (50 μg/ml) and varying concentrations of naloxone (Nx) on thymidine incorporation into ConA-stimulated LPL. ConA (5 μg/ml) stimulated LPL were incubated with BCM (50 μg/ml) in the absence or presence of varying concentrations of naloxone (10^{-9}–10^{-6} M). Results are the mean + s.e.m. of four to six different experiments, each done in triplicate determinations. Average ct/min for unstimulated (background) and of ConA-stimulated (control) LPL were 189 and 35 337, respectively. $*P < 0.05$ compared with BCM treatment alone.

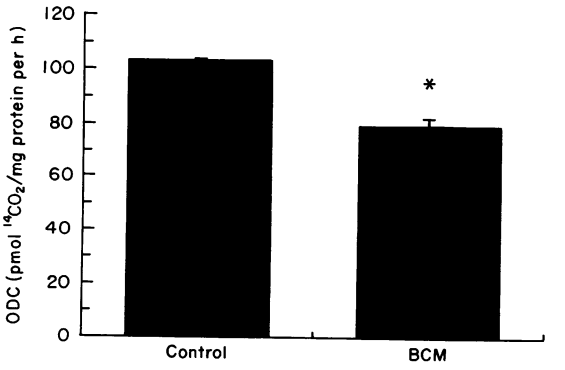

Fig. 3. Effect of BCM (50 μg/ml) on ornithine decarboxylase (ODC) activity of ConA-stimulated LPL. Results are the mean + s.e.m. of three different experiments, each done in duplicate determinations. $*P < 0.05$ compared with control.

of incubation (Fig. 2). The data represent the results of nine (colon) and three (ileum) different experiments, each done in triplicate determinations.

The effect of BCM on ODC activity

We have previously shown that ConA-stimulated ODC activity peaked after about 24 h of incubation (Elitsur *et al.*, 1990). Consequently, ConA-stimulated LPL were incubated in the absence or presence of BCM (50 μg/ml) for 24 h. Cells were pelleted by centrifugation and ODC activity was determined in LPL. BCM (50 μg/ml) significantly suppressed ODC activity in ConA-stimulated LPL (Fig. 3).

The effect of BCM on TPA-stimulated LPL

We previously reported that the optimal concentration of TPA for maximal stimulation of LPL DNA synthesis was 50 ng/ml (Elitsur *et al.*, 1990). BCM (125 and 250 μg/ml) suppressed thymidine incorporation in TPA-stimulated LPL (Fig. 4). The degree of suppression was less than that observed with ConA-stimulated LPL, but reached statistical significance at 250 μg/ml of BCM.

The effect of naloxone on LPL thymidine incorporation

There was no effect of naloxone alone (10^{-6} M) on ConA-stimulated LPL DNA synthesis. BCM at 500 μg/ml suppressed DNA synthesis to 62% of control. Naloxone reversed the anti-proliferative effect of BCM on ConA-stimulated LPL DNA synthesis. Naloxone at concentrations of 10^{-9}–10^{-7} M returned LPL DNA synthesis to 81–99% of control (Fig. 5). Data represent the results of four to six different experiments, each done in triplicate determinations.

DISCUSSION

Normal immune processing of foreign peptides is one of the major functions of the mucosal immune system, providing immune protection and possibly preventing the development of autoimmune diseases. Most previous studies have investigated the effect of food peptides on the circulatory immune system (Hamosh, Hong & Hamosh, 1989). We are not aware of any study that investigated the *in vitro* effect of food peptides on the human mucosal immune cell population of the intestinal wall.

The aetiologic agent of milk allergy has not been ascertained. Potential candidates include the milk peptides, one of which is BCM, which is provided from β-casein in milk (Hamosh *et al.*, 1989). Following the absorption of BCM, interaction with the mucosal immune system occurs, particularly with the mucosal LPL components. In this *in vitro* study, we report that BCM significantly inhibited DNA synthesis in ConA-stimulated human colonic and ileal lamina propria lymphocytes. This effect was reversed by the specific opiate receptor antagonist, naloxone.

Whether the *in vitro* anti-proliferative effect of BCM on LPL proliferation is physiologically relevant is an important question. Previous studies have shown that β-casein is the major component of human milk casein, and its concentration in human milk averaged 4·67 g/l (Hamosh *et al.*, 1989). To be biologically active *in vitro*, BCM needs to be released from its precursor, β-casein, in the gut lumen. Indeed, several studies have shown that β-casein and BCM are found in the human intestinal lumen. BCM has been found in the intestinal aspirates of human volunteers who had ingested bovine milk (Svedberg *et al.*, 1985). In the same study, bovine milk was subjected to an *in vitro* enzymatic digestion process to simulate 'gastric' and 'intestinal' digestive conditions. BCM was found in the 'intestinal phase' but not in the 'gastric phase'. Moreover, it was also found that BCM could be produced as a result of the proteolytic activity of bacteria (Hamosh *et al.*, 1989). Overall, the current data strongly suggest that BCM is present in the intestinal lumen and thus may affect gastrointestinal function locally.

Although the serum immunoreactive BCM concentration in newborn calves after milk ingestion has been reported to be 1 nM (Umbach *et al.*, 1985), the concentrations of immunoreactive BCM in human intestinal contents was much higher and ranged between 10 and 45 μM (Svedberg *et al.*, 1985). We found a significant *in vitro* suppressive effect of BCM on LPL proliferation at BCM concentrations as low as 10 μM. To our knowledge, no data are available on the concentration of BCM in the intestinal mucosa.

ODC is a rate-limiting enzyme for polyamine biosynthesis, which has been found to be crucial for proliferation of human PBL (Bowlin, McKown & Sunkara, 1987). We recently found that ODC is also essential for LPL proliferation (Strom, Elitsur & Luk, 1989). In this study, we found that BCM suppressed ODC activity in ConA-stimulated LPL after 24 h of incubation. These results suggest that BCM may act at a signal transduction pathway proximal to ODC induction.

TPA is a known activator of protein kinase C (PKC) and ultimately leads to DNA synthesis and cell proliferation. TPA alone was shown to activate human peripheral blood lymphocytes (PBL) (Abb, Bayliss & Deinhardt, 1979) and LPL (Elitsur *et al.*, 1990). It has also been shown to be one of the most potent inducers of ODC in lymphocytes (Mustelin, Poso & Andersson, 1986). We found that BCM suppressed DNA synthesis in TPA-stimulated LPL. However, the degree of inhibition of DNA synthesis was less than that observed with ConA-stimulated LPL. Our TPA results combined with our ODC results suggest that BCM acts at or pior to PKC activation.

BCM and the β-endorphins are opioid peptides which may exert their effects via opiate receptors. Both peptides bind to opiate receptors and their effects are antagonized by the specific opiate receptor antagonist naloxone. Thus, BCM might be considered as a β-endorphin-like peptide. In fact, some investigators have described BCM as an 'exorphin' (Hamosh *et al.*, 1989). Since little data are available on the mechanism of action of BCM on the immune system (Parker *et al.*, 1984), our results might be more easily interpreted in the context of the available data on β-endorphins.

Previous reports have suggested that β-endorphins have different mechanisms for different immune functions. The effect of β-endorphin on cytolytic activity of human PBL was thought to be an opiate receptor-mediated phenomenon (Mandler *et al.*, 1986), while lymphocyte proliferation in human and animal models was not (Gilman *et al.*, 1982). Brown & van Epps (1985) have suggested that this effect is dependent on arachidonic acid metabolism. Furthermore, it has been shown that the opioids may act on human leucocytes by binding to two different receptors, namely the opiate receptor and the specific non-opiate receptor (Hazum, Chang & Cuatrecasas, 1979; Lopker *et al.*, 1980), while naloxone binds selectively to the opiate receptor. Moreover, lymphocyte proliferation is an end result of a complex immune cascade, involving the participation of macrophages, different T cell populations, and various lymphokines. Indeed, β-endorphin was previously shown to affect several of these participants, i.e. NK cells (Faith *et al.*, 1984), T cell cytotoxicity and lymphokine production (Mandler *et al.*, 1986). It should also be noted that, although opiate receptors have been found on human lymphocytes, to our knowledge no such receptors have yet been found on human LPL. Although it was previously suggested that β-endorphin may inhibit adenyl cyclase (Koski & Klee, 1981), stimulate GTP hydrolysis (Roder & Klein, 1979), or modify cell permeability to ions (Faith *et al.*, 1984), the intracellular mechanism by which BCM exerts its effect is also not defined. Similar to a previous report (Hautefeuille *et al.*, 1986), we found that naloxone reversed the

antiproliferative effect of BCM on ConA-stimulated LPL, suggesting that in our system, BCM's effect may be mediated via the opiate receptor. Because of cell heterogeneity in our experimental model, the cellular target for BCM cannot be elucidated from our study.

We have demonstrated that the human milk-derived peptide BCM inhibits ODC activity and suppresses DNA synthesis in ConA-stimulated human LPL. We found that the specific opiate receptor antagonist, naloxone, reversed this effect. We postulated that the inhibitory effect of BCM may involve an intracellular metabolic step at or prior to the activation of PKC. We conclude that BCM, a food-derived peptide, modulates the human mucosal immune system and thus may have an important function during the digestive process.

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