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Hajime Otani & Makoto Monnai

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Inhibition of Proliferative Responses of Mouse Spleen Lymphocytes by Bovine Milk κ -Casein Digests

HAJIME OTANI AND MAKOTO MONNAI

Laboratory of Applied Biochemistry of Animal Products, Faculty of Agriculture,
Shinshu University, Minamiminowa-mura 399-45, Japan

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The inhibitory effects of bovine milk κ -casein and its enzymatic digests on the proliferative responses of mouse spleen lymphocytes induced or not induced by mitogens were studied with a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). κ -Casein and its glyco-macropptide (residues 106–169) inhibited the lipopolysaccharide (LPS)-induced proliferative response, but the inhibitory effect was lost significantly after neuraminidase and chymotrypsin digestions. In contrast, trypsin and pronase digestions of κ -casein increased inhibitory effects. The pronase digest inhibited the proliferative responses not only induced by LPS but also in the absence of mitogen or when induced by concanavalin A and phytohemagglutinin. The pronase digest seemed to possess weak cytotoxicity for lymphocytes. The inhibitory peptide was a glycopeptide(s) having specific size, and the inhibitory effects were reduced significantly by neuraminidase and chymotrypsin digestions. Moreover, similar inhibitory effects on proliferation of lymphocytes were observed in pronase-digested bovine milk α_1 -casein and β -casein. These findings suggest that some peptides produced from milk caseins by the action of gastrointestinal proteinases might contribute to down-regulate the immune response of neonates.

Keywords: Bovine κ -casein, lymphocytes, inhibition of proliferation, κ -caseinoglycopeptide, neuraminidase digestion, proteinase digestion

INTRODUCTION

Milk is the normal first food for newborn mammals. Hence, milk is not only an essential nutrient for the developing animal but also a source of biologically active components playing important physiological functions. Perhaps one of the most relevant non-nutritive functions of milk is to provide newborn animals suddenly exposed to a broad spectrum of environmental antigens with a passive host defensive mechanism. It is well known that passively transferred milk proteins such as immunoglobulins, lactoferrin and lysozyme are directly protective against microbes (Lönnerdal, 1985).

On the other hand, milk contains protein components that regulate cells of the immune

system. Lactoferrin has been ascribed to affect the immune system in several ways, inhibiting the activity of natural killer cells (Nishiya & Horwitz, 1982), inhibiting the complement-dependent solubilization of immune precipitate (Kulics & Kijlstra, 1987), inhibiting the lymphocyte proliferation induced by concanavalin A (Con A, Ashorn *et al.*, 1986) and by phytohemagglutinin (PHA; Richie *et al.*, 1987), and suppressing the primary antibody response of B-lymphocytes (Duncan & McArthur, 1981). Human milk secretory component inhibited the production of cytoplasmic immunoglobulin by pokeweed mitogen (PWM)-stimulated peripheral blood lymphocytes (Crago *et al.*, 1981). Kulczycki *et al.* (1987) reported that cow's milk IgG inhibited markedly PWM-induced antibody secretion by human peripheral blood mononuclear cells, while Miura *et al.* (1991) observed that cow's milk IgG₁ had mitogenic activity on mouse splenocytes. Julius *et al.* (1988) isolated an ovine colostrum proline-rich peptide that induced the growth and differentiation of resting B-lymphocytes. It was more recently reported that human milk contained proteins that stimulated and suppressed T-lymphocyte proliferation (Mincheva-Nilsson *et al.*, 1990).

In a previous study (Otani *et al.*, 1992), the authors showed that bovine κ -casein and its glyco-macropptide (GMP, residues 106–169) obtained by rennin (EC 3.4.23.4) digestion inhibited mouse spleen B-lymphocyte proliferation induced by lipopolysaccharide (LPS) stimulation. The present study was undertaken to investigate inhibitory properties of κ -casein on proliferation of mouse spleen lymphocytes.

MATERIALS AND METHODS

Reagents

LPS of *Salmonella typhimurium* was purchased from Difco Laboratories (Detroit, MI, USA). Con A and PHA-P were obtained from Wako Pure Chemical Industries (Osaka, Japan) and Seikagaku Kogyo (Tokyo, Japan) respectively. Celgrossor-P and Macrophage-SFM media were obtained from Sumitomo Seiyaku (Tokyo, Japan) and Gibco (Grand Island, USA) respectively. Hen ovalbumin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Company (St Louis, MO, USA). Enzymes used were neuraminidase (EC 3.2.1.18, from *Clostridium perfringens*, Sigma Chemical Company), trypsin (EC 3.4.21.4, from bovine pancreas, Boehringer Mannheim GmbH, Mannheim, Germany), chymotrypsin A₄ (EC 3.4.21.1, from bovine pancreas, Boehringer Mannheim GmbH) and pronase E (EC 3.4.24.4, from *Streptomyces griseus*, Merk, Darmstadt, Germany). The other chemicals were of the highest analytical grade commercially available.

Milk Proteins

Bovine α _{s1}-casein, β -casein, κ -casein and β -lactoglobulin were prepared from fresh milk obtained from an individual Holstein cow, as described previously (Otani *et al.*, 1984, 1985, 1986, 1990). Para- κ -casein (residues 1–105) and GMP were purified from κ -casein digested with rennin as reported previously (Otani *et al.*, 1992). α -Lactalbumin and bovine serum albumin (BSA) were purchased from Sigma Chemical Company.

Enzymatic Digestions

Digestion of peptide with neuraminidase was carried out in 0.01 M-phosphate buffer containing 0.15 M-NaCl (pH 7.2, PBS) at 37°C (neuraminidase/substrate, 1/24, w/w). After 1 h, the reaction mixture was diluted 10-fold with PBS and subjected to ultrafiltration on an Amicon Ultrafiltration Cell Model 52 (Amicon Corp., Danvers, USA) with a Diafilter A-15T membrane (cut off m.w.: 15 000, ULVAC, Tokyo, Japan) to remove neuraminidase. The filtrate was recovered as a neuraminidase digest.

Digestions of milk proteins and hen ovalbumin with proteinases were performed in PBS

(proteinase/substrate, 1/100, w/w) at 45°C. After 5 h, proteinases and intact proteins were removed by ultrafiltration as described above, and filtrates were used as proteinase digests.

Lymphocyte Culture

Male C3H/HeN mice, 6 weeks of age, were obtained from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). Mice were sacrificed by vertebral dislocation, and spleens were aseptically removed. Single cell suspensions were prepared by gentle teasing of the tissues in a culture medium (Celgrossor-P). After being washed three times in cold Celgrossor-P, the cells were resuspended at a concentration of 6×10^6 cells ml^{-1} . The viability of the cells determined by trypan blue exclusion was more than 98%.

Cell cultures were set up in quadruplicate on flat-bottomed microtitre plates (Falcon, Cockeysville, USA). Into each well were placed 100 μl of the cell suspension, 10 μl of a test peptide solution dissolved in PBS and 10 μl of a mitogen solution dissolved in PBS. All peptide solutions and mitogen solutions were filter-sterilized through a 0.45 μm pyrogene-free filter. Final concentrations were: lymphocytes, 5×10^6 cell/ml; peptide, 0 to 200 $\mu\text{g ml}^{-1}$; LPS, 50 $\mu\text{g ml}^{-1}$; Con A, 0.5 $\mu\text{g ml}^{-1}$; PHA, 5 $\mu\text{g ml}^{-1}$. The mixtures were incubated in a humidified 5% CO_2 -95% air atmosphere for 48 h. On the final hour, the proliferative response of lymphocytes was estimated by the colourimetric MTT (tetrazolium) assay (Mosmann, 1983). Inhibition of proliferative response in peptide-containing culture was expressed as inhibitory activity (%) calculated by the following formula:

$$\% = \left(1 - \frac{\text{MTT absorbance with test peptide}}{\text{MTT absorbance without the peptide}} \right) \times 100$$

Peritoneal Macrophage Culture

Male C3H/HeN mice, 10 weeks of age, received an intraperitoneal injection of 1.5 ml of 2.4% thioglycollate medium (Nissui, Tokyo, Japan). Three days after the injection peritoneal exudate cells were collected by a peritoneal lavage with injection of 5 ml of Hanks' solution (Nacalai Tesque, Kyoto, Japan). The cells were collected by centrifugation, washed three times with cold Hanks' solution, and then resuspended at a concentration of 1.5×10^6 cells/ml in cold Macrophage-SFM supplemented with 10% heat-inactivated fetal calf serum (Irvine Scientific, Santa Ana, CA, USA), 100 U ml^{-1} penicillin (Meiji Seika, Tokyo, Japan) and 100 $\mu\text{g ml}^{-1}$ streptomycin (Meiji Seika). The viability of the cells determined by trypan blue exclusion was more than 95%, and macrophages were more than 90% of the peritoneal cells, as determined by morphology and non-specific esterase staining.

Into each well of a flat-bottomed microtitre plate (Falcon) were placed 100 μl of the cell suspension and 10 μl of a digest solution. The mixtures were incubated in a humidified 5% CO_2 -95% air atmosphere for 72 h, and the number of living macrophages was counted.

Protein and Carbohydrate Determinations

Protein and carbohydrate amounts were determined by the procedures of Lowry *et al.* (1951) and of Dubois *et al.* (1956), respectively.

Statistical Analysis

Results were expressed as the mean inhibitory activity $\pm$ SD. Significance of differences was tested by Student's *t*-test.

RESULTS

Contribution of Carbohydrate Portions of GMP

As shown in Figure 1, GMP inhibited the proliferative response of mouse spleen

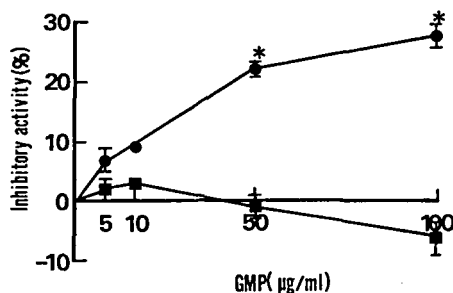


FIG. 1. Inhibitory effects of GMP (●) and its neuraminidase digest (■) on proliferative responses of lymphocytes induced by LPS. Values are means with their SD represented by vertical bars for quadruplicate. * Significant difference from control culture (GMP and its digest free): $P < 0.01$.

lymphocytes induced by LPS in a dose-dependent manner. In contrast, the response to LPS was not significantly influenced by the neuraminidase-digested GMP. The results clearly demonstrated that carbohydrate portions containing sialic acid residues were essential for the inhibition of GMP.

Effects of Proteinase-digested κ -Caseins

Inhibitory effects of κ -casein and its proteinase digests on proliferative response of lymphocytes induced by LPS are shown in Figure 2A. Intact κ -casein inhibited the LPS-induced lymphocyte proliferation in a dose-dependent fashion when up to $100 \mu\text{g ml}^{-1}$ was added, although by adding a higher concentration, no further inhibition was observed. In contrast, the effect of the chymotrypsin digests was far less. Indeed, the LPS-induced lymphocyte proliferation was slightly enhanced in the presence of the chymotrypsin digest. The results indicated that polypeptide portions were responsible for the inhibition of κ -casein.

In contrast, as also shown in Figure 2, the trypsin and pronase digests were much more potent inhibitors than intact κ -casein. The extent of inhibitory activity with the trypsin and pronase digests at a concentration of $200 \mu\text{g ml}^{-1}$ was about 30–40%, whereas that of intact κ -casein at a concentration of $200 \mu\text{g ml}^{-1}$ was about 20%. The inhibitory activity was not considered to be merely a function of increased peptide molecules in the culture medium since the addition of chymotrypsin-digested material in comparable amounts did not result in a similar dose-dependent inhibition. The results suggested that the trypsin and pronase digestions of κ -casein might produce novel peptides that inhibited proliferation of lymphocytes. Therefore, the action of pronase-digested material on proliferative responses of lymphocytes induced and not induced by mitogens was investigated, because the inhibitory activity of the pronase digest on LPS-induced proliferation was higher than that of the trypsin digest.

As shown in Figure 2B, the pronase-digested κ -casein inhibited those proliferative responses of lymphocytes not only induced by LPS but also induced by PHA and Con A. Moreover, the proliferation in the absence of mitogen was also inhibited markedly by the addition of pronase digest. The results confirmed that the pronase digestion of κ -casein produced novel peptide(s) which inhibited the proliferation of lymphocytes, because intact κ -casein hardly affected proliferation of mouse lymphocytes in the absence of mitogen or induced by Con A (Otani *et al.*, 1992).

Effects of Various Milk Proteins and Hen Ovalbumin Digested with Pronase

To examine whether the potent inhibitory effect was unique for the κ -casein digest, effects of various milk proteins and hen ovalbumin digested with pronase on proliferation of

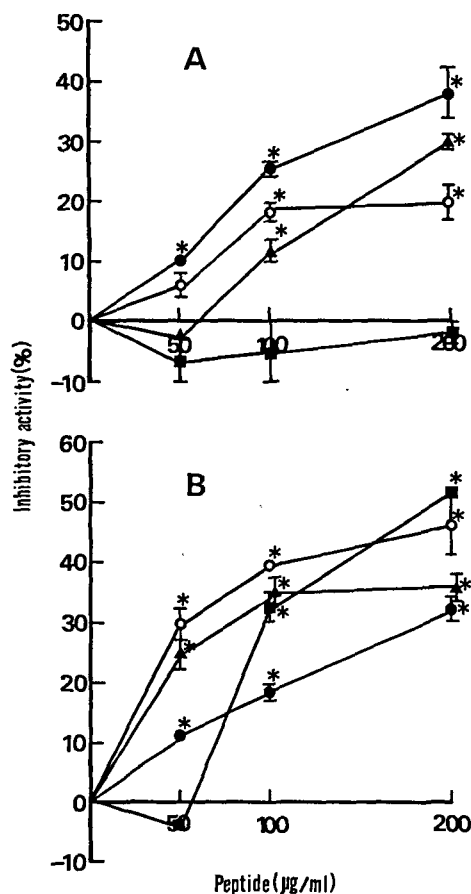


FIG. 2. Inhibitory effects of κ -casein and its proteinase digests on proliferative responses of lymphocytes. (A) Inhibitory effects of various proteinase digests on proliferative responses induced by LPS. ○: intact κ -casein, ▲: trypsin digest, ■: chymotrypsin digest, ●: pronase digest. (B) Inhibitory effects of pronase digest on proliferative responses induced by various mitogens. ○: in the absence of mitogen, ●: LPS stimulation, ▲: PHA stimulation, ■: Con A stimulation. Values are means with their SD represented by vertical bars for quadruplicate. * Significant difference from control culture (κ -casein and its digest free): $P < 0.01$.

lymphocytes, both in the absence of mitogen or induced by LPS, PHA and Con A, were investigated. The results are shown in Figure 3. Bovine milk α_1 -casein and β -casein digests demonstrated significant inhibition of lymphocyte proliferation in the absence of mitogen and induced by PHA and Con A, although neither of the casein digests inhibited the proliferative response induced by LPS. α -lactalbumin, BSA and ovalbumin digests, on the other hand, had little influence on the proliferation regardless of mitogens. Moreover, β -lactoglobulin digest showed inhibition of the proliferative response induced only by PHA. From this study, it was demonstrated that the production of inhibitory peptides by the action of pronase was common to casein components.

Effects of Pronase Digests on Viabilities of Lymphocytes and Macrophages

Lymphocytes and macrophages were incubated for 72 h in the presence of pronase-digested κ -casein and BSA, and the numbers of living macrophages remaining in the wells were

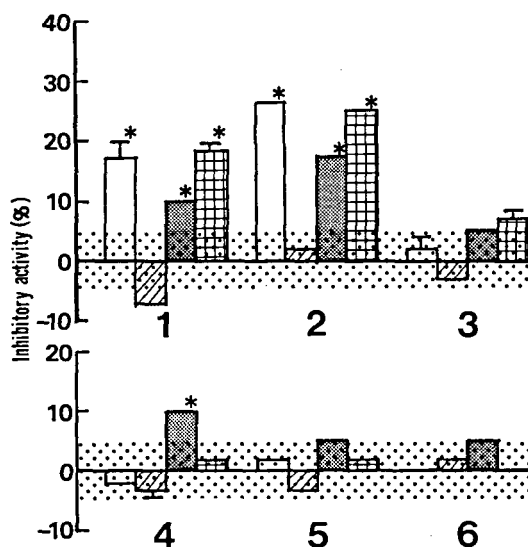


FIG. 3. Inhibitory effects of various milk proteins and ovalbumin digested with pronase on proliferative responses of lymphocytes. Digested peptide concentration is $50 \mu\text{g ml}^{-1}$. 1: α S₁-casein, 2: β -casein, 3: α -lactalbumin, 4: β -lactoglobulin, 5: BSA, 6: ovalbumin. □: in the absence of mitogen, ▨: LPS stimulation, ▤: PHA stimulation, ▤: Con A stimulation. Values are means with their SD represented by vertical bars for quadruplicate. Inhibitory activity represented by shadowed area is not statistically significant. * Significant difference from control culture (digest free): $P < 0.01$.

counted. As shown in Figure 4, the number of lymphocytes was decreased by the addition of κ -casein digest whereas the BSA digest had no effect on viability of lymphocytes even at a concentration of $100 \mu\text{g ml}^{-1}$. On the other hand, the κ -casein digest was found to have no effect on viability of macrophages. The results suggested that the inhibitory effect of the κ -casein digest might be due to weak cytotoxicity specific for lymphocytes.

Characterization of Inhibitory Peptides in κ -Casein Digest

To examine whether the inhibitory peptides were derived from GMP (residues 106–169) or para- κ -casein (residues 1–105) the effects of pronase-digested GMP and para- κ -casein were investigated. As shown in Figure 5, the addition of $50 \mu\text{g ml}^{-1}$ of GMP digest gave significant inhibition whereas the inhibitory activity of para- κ -casein digest was slight but non-significant. The results provided evidence that the inhibitory peptide can be attributed to within the GMP region.

The pronase-digested GMP was chromatographed on a Sephadex G-50 column. The chromatographed peptides were pooled in two consecutive fractions named A and B as shown in Figure 6. Each fraction was concentrated by ultrafiltration using a Diafilter G-05T membrane (cut off m.w.: 5000, ULVAC), and the inhibitory effect was determined (Figure 6). On the basis of carbohydrate concentration, fraction A was more than three times active as an inhibitor than fraction B. Fraction A was richer in carbohydrates than fraction B. The results suggested that the inhibitory component in the pronase digest was a glycopeptide(s) having a specific size.

Figure 7 shows the inhibitory effects of intact fraction A and sialidase- and chymotrypsin-digested fraction A on proliferation of lymphocytes in the absence of mitogen or

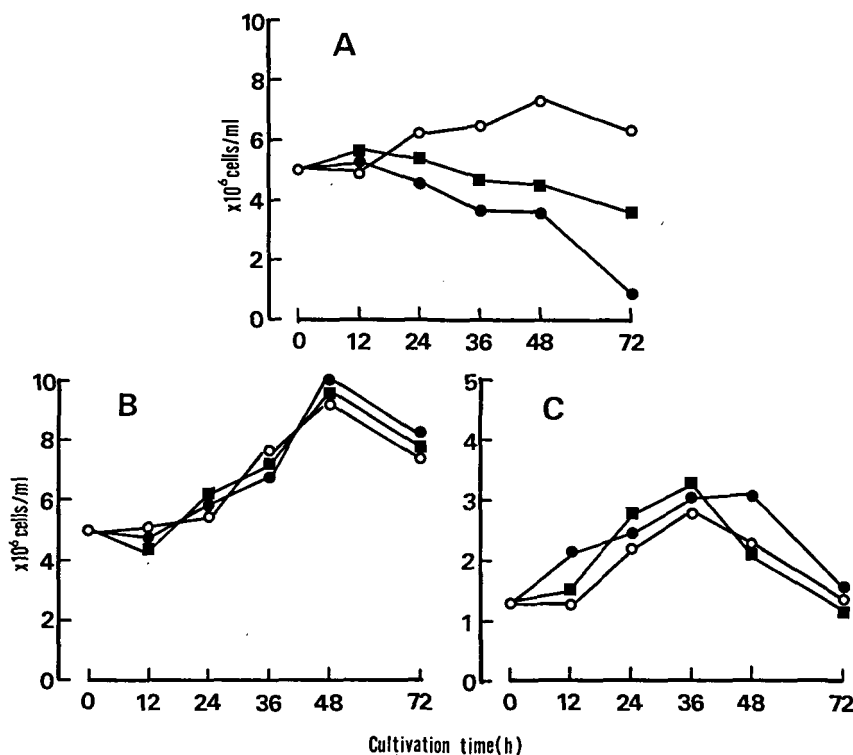


FIG. 4. Effects of κ -casein and BSA digested with pronase on viabilities of lymphocytes and macrophages. A: κ -casein digest to lymphocytes, B: BSA digest to lymphocytes, C: κ -casein digest to macrophages. Digested peptide concentrations are $\circ$: none, $\blacksquare$: $50 \mu\text{g ml}^{-1}$ and $\bullet$: $100 \mu\text{g ml}^{-1}$.

induced by LPS and Con A. The extent of inhibitory activity with intact fraction A was about 20–30% at a concentration of $10 \mu\text{g ml}^{-1}$, while neuraminidase- and chymotrypsin-digested material significantly lowered the extent of inhibition. The reduced inhibitory activities of the digested fraction A indicated that both carbohydrate and polypeptide portions of GMP were responsible for the inhibition of lymphocyte proliferation.

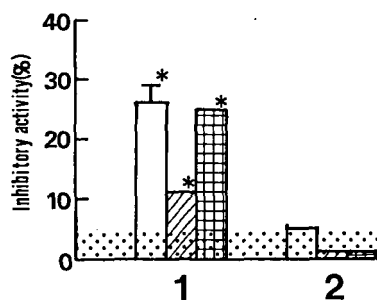


FIG. 5. Inhibitory effects of GMP and para- κ -casein digested with pronase on proliferation of lymphocytes. 1: GMP digest, 2: para- κ -casein digest. $\square$: in the absence of mitogen, ▨ : LPS stimulation, ▩ : Con A stimulation. Values are means with their SD represented by vertical bars for quadruplicate. Inhibitory activity represented by shadowed area is not statistically significant. * Significant difference from control culture (digest free): $P < 0.01$.

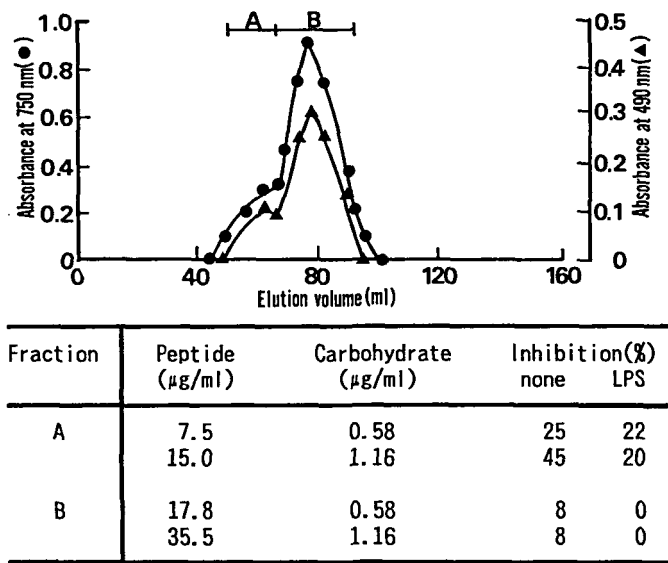


FIG. 6. Sephadex G-50 column chromatography of GMP digested with pronase and inhibitory effects of fractionated peptides.

DISCUSSION

For a long time, milk caseins have been considered principally as a source of amino acids for young animals. More recently, however, caseins have been considered not only to have a nutritional role but also a physiological importance, since various peptides from milk caseins have been demonstrated to have biological significance (Fiat & Jollès, 1989). GMP is a heterogeneous group of glycopeptides released from κ -casein by the action of rennin and pepsin. A part of the heterogeneity of GMP relates to the degree of sialylation (Eigel *et al.*, 1984). Some biological functions of GMP have been reported; specifically, a growth-promoting effect on bifidobacteria (Azuma *et al.*, 1984), suppression of gastric secretion (Stan *et al.*, 1983), inhibition of adhesion of oral actinomyces to the cell

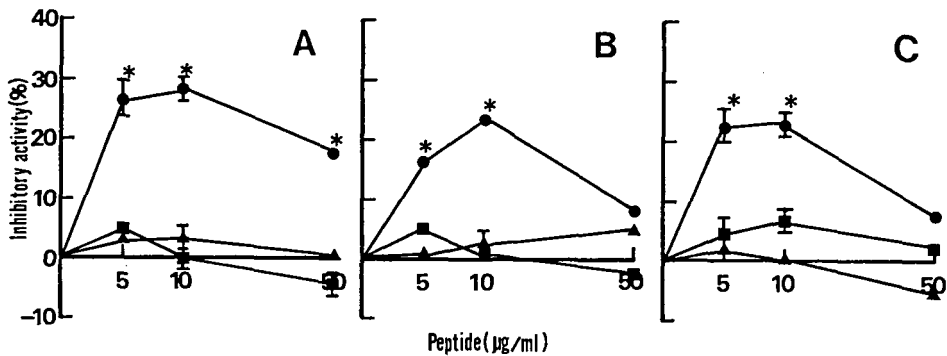


FIG. 7. Inhibitory effects of fraction A digested with neuraminidase and chymotrypsin on proliferation of lymphocytes. A: in the absence of mitogen, B: LPS stimulation, C: Con A stimulation. ●: intact fraction A, ▲: neuraminidase digest, ■: chymotrypsin digest. Values are means with their SD represented by vertical bars for quadruplicate. * Significant difference from control culture (fraction A and its digest free): $P < 0.01$.

membrane (Neeser *et al.*, 1988), inhibition of cholera toxin binding to its receptor (Kawasaki *et al.*, 1992) and inhibition of interferon- β production of human diploid fibroblasts (Yamada *et al.*, 1991). Azuma *et al.* (1984) and Kawasaki *et al.* (1992) reported that the promoting and inhibiting activities of GMP related closely to both carbohydrate and polypeptide portions, while Nesser *et al.* (1988) suggested that the inhibiting activity was due to polypeptide portions. The present authors have found previously (Otani *et al.*, 1992) that GMP inhibited the proliferation and blastogenesis of mouse spleen lymphocytes induced by LPS. The data obtained in the present study provide evidence that both carbohydrate, particularly sialic acid residues, and polypeptide portions are essential for inhibition of the proliferative response of mouse lymphocytes.

In contrast, the digestion of bovine κ -casein with trypsin and pronase produced peptides that had higher inhibitory activity than intact bovine κ -casein on the proliferative response of mouse spleen lymphocytes induced by LPS. Since the inhibitory activity of the pronase digest was higher than that of the trypsin digest, the authors studied in detail the inhibitory properties of the pronase digest on proliferation of mouse spleen lymphocytes. The pronase digest inhibited proliferation of lymphocytes not only induced by LPS but also in the absence of mitogen or induced by PHA and Con A. The inhibitory peptide was a glycopeptide(s) having specific size. The inhibitory activities of peptides were decreased significantly by neuraminidase and chymotrypsin digestions, as observed in the case of intact κ -casein and GMP, suggesting that both carbohydrate and polypeptide portions contribute to the inhibition. Moreover, inhibitory effects on proliferation of lymphocytes in the absence of mitogen or induced by PHA and Con A were found in pronase-digested bovine α s1-casein and β -casein, but not observed in pronase-digested bovine α -lactalbumin, BSA and hen ovalbumin. These results indicated that the formation of inhibitory peptides by pronase digestion is common to bovine casein components.

At present, the authors have not identified specific inhibitory peptides from the casein digests, and do not have any data on the mechanism of action of inhibition of the digests at the cellular level. The first and the most studied biologically active peptides from milk caseins are opioid peptides (Fiat & Jollès, 1989). It was reported that opioid peptides influenced a variety of immune responses, including mitogen stimulation (McCain *et al.*, 1982), immunoglobulin production (Johnson *et al.*, 1982) and cytotoxic activity (Mathews *et al.*, 1983). Migliore-Samour and Jollès (1988) suggested that opioid casein peptides such as β -casomorphins and exorphins with a great affinity for μ -opiate receptor had endorphin-like activity on neonate immune cells, particularly with respect to the development of T-lymphocyte functions and cellular immunity. Thus, the inhibition of lymphocyte proliferation by the casein digests found in the present study might be caused by such peptides having opioid activity. On the other hand, the inhibition of proliferation in the absence of mitogen and induced by three chemically different mitogens (LPS, PHA and Con A) rules out the direct binding of mitogen by casein digests. Yamada *et al.* (1991) reported that GMP inhibited interferon- β production of human diploid fibroblasts. Moreover, it is well known that various lymphokines regulate the proliferative response of lymphocytes (Hamblin, 1988). Possibly, the casein digests might affect the production of lymphokines regulating the proliferation of lymphocytes.

Hypersensitivity reactions to food proteins are rare, because a state of immunological tolerance (named oral tolerance) develops (Mowat, 1987). Several immunoregulatory mechanisms are implicated in the induction of the oral tolerance. Most workers agree that the oral tolerance is achieved principally by T-lymphocyte unresponsiveness (Strobel & Ferguson, 1984; Mowat, 1987). Caseins are major proteins in bovine and human milk (Mepharm, 1976). The caseins are easily degraded by gastrointestinal proteinases. However, in the case of neonates, both gastric secretion and pepsin activity are weak (Agunod *et al.*, 1969). Hence, GMP is almost the only single peptide to be released from caseins in the stomach of young animals. The major portion of the caseins ingested by neonates leaves the stomach after minimal protein digestion (Berfenstam *et al.*, 1955). Digestion of milk caseins

occurs mainly in the proximal small intestine through the action of various proteases such as trypsin, chymotrypsin and carboxypeptidase. Intact κ -casein and GMP inhibited proliferation of mouse spleen lymphocytes induced by LPS, as reported previously (Otani *et al.*, 1992). The data obtained in the present study indicated that the trypsin and pronase digestions of κ -casein produced peptides that inhibited proliferation of lymphocytes in the absence of mitogen or induced by LPS, PHA and Con A, although the inhibitory peptide(s) was inactivated by chymotrypsin and neuraminidase digestions. Moreover, pronase digestions of bovine α_s1 and β -casein also produced peptides that inhibited proliferation of lymphocytes in the absence of mitogen or induced by PHA and Con A. LPS activates B-lymphocytes directly. Both PHA and Con A activate T-lymphocytes specifically, but their functions are different in several aspects such as target cell population and adherent cell requirement, etc. As to target cell population, it is generally accepted that helper T-lymphocytes are preferentially stimulated by PHA and suppressors by Con A (Nakamura *et al.*, 1986). These facts indicate that the pronase digestions of bovine α_s1 -casein and β -casein produce peptides which suppress T-lymphocyte proliferation while that of κ -casein forms peptides which inhibit both T- and B-lymphocyte proliferation. Thus, it is suggested that some peptides produced from caseins by the action of gastrointestinal proteinases might contribute to down-regulate the immune system, or oral tolerance of neonates. Further investigations are necessary using caseins, gastrointestinal proteinases and lymphocytes from the same animal origin. Further studies are in progress to purify the inhibitory peptides to homogeneity and to elucidate the inhibitory mechanism.

ACKNOWLEDGEMENTS

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