Immunological effects of dietary peptide derived from soybean protein

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The effect of dietary peptides derived from soybean and casein on the immune responsiveness of Fisher rats has been investigated. The protein efficiency ratio of these diets was similar. The phagocytosis of opsonized sheep red blood cells by alveolar macrophages (AMø) and the mitogenic activity of rats fed both peptides, particularly soybean peptide, were found to be significantly greater than those of rats fed the corresponding proteins of soybean and casein. Furthermore, in the pair-feeding experiment using both peptides, a comparable enhancement of the phagocytic activity of AMø was seen in the rats given the peptides, but little difference in the natural killer cell activity was noted among rats fed each diet. It was suggested that immune activating factors would be presented in the soybean peptide.

Keywords: peptide from soybean; phagocytosis of alveolar macrophages; natural killer cell activity; mitogenic activity

Introduction

There are many studies on the effects of protein levels in diet on the humoral and cellular immune response,[1-4] but few reports dealing with the type and quality of dietary protein in the immune system.[5] In the present study, the possibility of immunological activity of peptides was investigated. Jollès et al.[6] observed the immunostimulating properties of peptides that are characterized by a hexapeptide and tripeptide.[7-9] We previously reported the presence of immunostimulating properties in soybean protein, which is one of the main protein sources of the Japanese, and suggested that the protein would contain peptides having a potential effect on the immunological responses.[10,11]

The present paper describes the result of a comparative study on the effect of peptide and protein from soybean on the immune responses of rats. In addition, an attempt was made to evaluate the difference in the immune response of rats fed peptide derived from casein.

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Methods and materials

Animals and diets

Specific pathogen-free, male Fisher strain rats (Japan SLC, Inc., Shizuoka, Japan) (4-weeks old) were used and divided into four dietary groups. In the first experiment, they were fed isoenergetic diets (*Table 1*) containing 15% peptide and protein from soybean for 2 and 4 weeks. The rats of the casein peptide- and casein-fed groups were given equivalent amounts of the diets in reference to the control groups for 2 and 4 weeks. In the second pair-feeding experiment, the food intake of each of the respective groups was adjusted to that of the soybean peptide group. All rats were given water ad libitum and housed individually in an air-conditioned room at 22 ± 2° C. Body weight and food intake were measured daily.

Preparation of peptides derived from soybean and casein

After the homogenates were adjusted to a 2% level, hydrolysis was carried out at 37° C for 20 hr with stirring. The hydrolysate was immediately transmitted through an ultrafiltration membrane (Amicon, Lexington, MA USA; Membrane YM 10, fraction molecular weight : 10000) and the transmitted solution was applied to a strong acidic cation exchange resin, Dowex 50 W (H^+ form, 50–100 mesh, 4.5 × 20 cm). This column was sufficiently washed with deionized water prior to elution treatment using 2N NH_4OH, and the eluate was concentrated under reduced pressure. The concentrated solution was applied to a Sephadex G-25 column (medium, 2.5 × 150 cm) preliminarily buffererd with deionized water at a flow rate of

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30 mL/hr. Each fractionating amount was 8.6 mL. The peptide fraction collected by repeating the gel filtration column chromatography was freeze dried to prepare a peptide powder (molecular weight 300–2000).

Amino acid analyses of the proteins and peptides were carried out for the hydrolysates with 6N HCl containing 0.1% phenol or 4N methansulfonic acid containing 0.2% tryptamine at 110° C for 20 hr using an amino acid analyzer PICO-TAG (Waters Ltd., Milford, MA USA).

Table 1 Composition of experimental diets (%)

Ingredients	Concentration (%)
Protein and peptide	15
Cornstarch	62
Soybean oil	8
Cellulose	10
Vitamin mixture*	1
Salt mixture*	4

*Prepared by Oriental Yeast Co. LTD.

Spleen, thymus, and preparation of splenocytes

The spleen and thymus were removed and weighed. The splenocytes were aseptically excised, weighed, and minced with scissors. The splenocytes were dissociated using a stainless-steel screen and adjusted to 1×10^6 cells per 1 mL of RPMI 1640 medium.

Preparation and purification of alveolar macrophages (AMø)

The renal arteries were cut under anesthesia by i.p. injection of sodium pentbarbital to exsanguinate the rats. After the chest cavity was opened, a salivary gland and connective tissue were removed to expose the trachea. The trachea was cannulated with a cut tube from a Butterfly-21 infusion set. The lungs were washed with 5 mL of physiological saline at 37° C. The procedure was repeated several times until 50 mL of lavaged fluid was obtained per rat. The lavaged fluid was centrifuged at 1800 rpm for 10 min. The total number of the AMø cells collected was assessed by nonspecific esterase staining. The AMø cells were layered on the wells of a Multiwell plate (Falcon Plastic, Oxnard, CA USA) containing 1 mL of RPMI 1640 medium with 5% fetal bovine serum. After 60 min, nonadherent cells

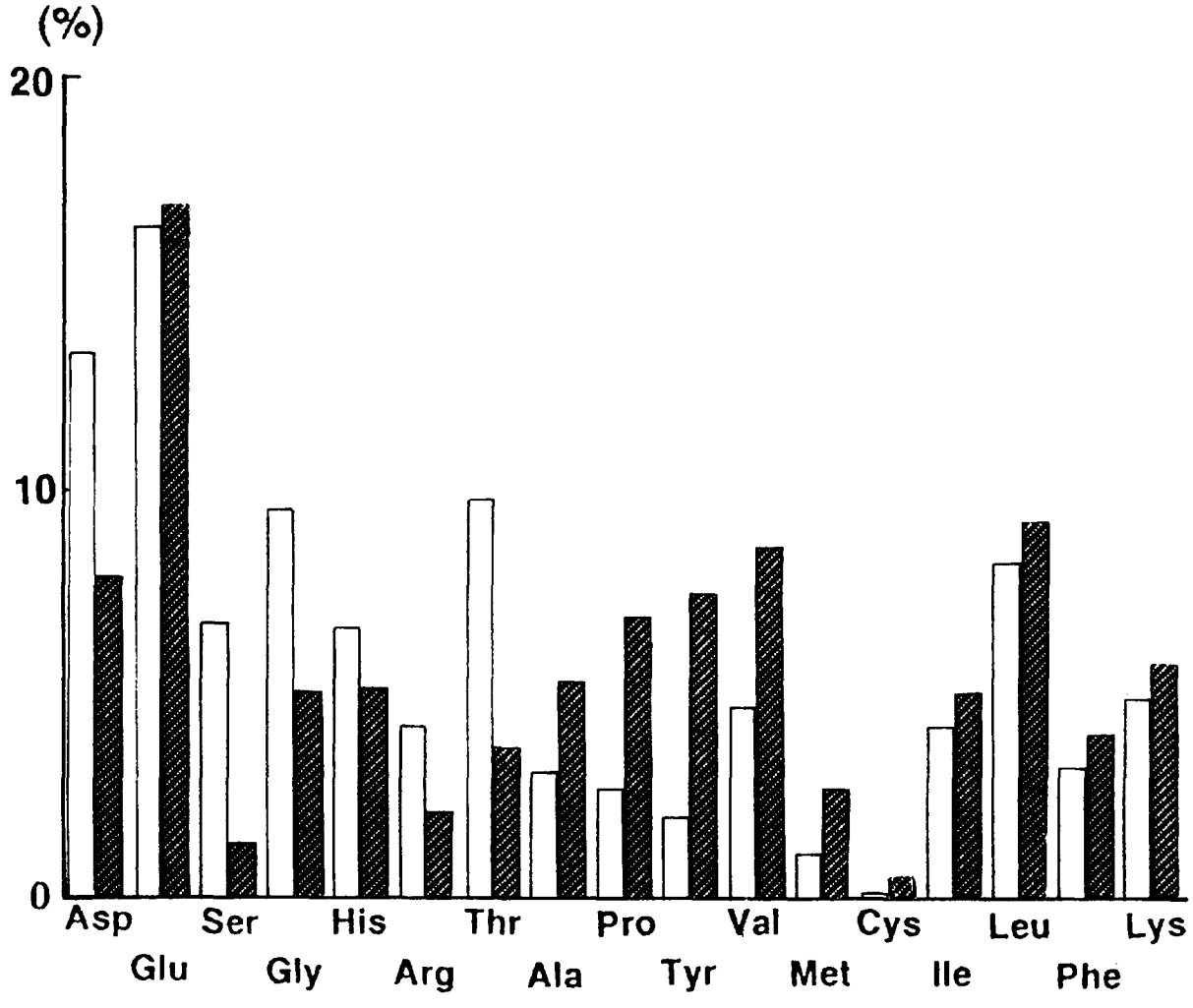

Figure 1 Ratios of individual amino acid in the dietary peptiides derived from soybean and casein. □ soybean peptide, ▨ casein peptide.

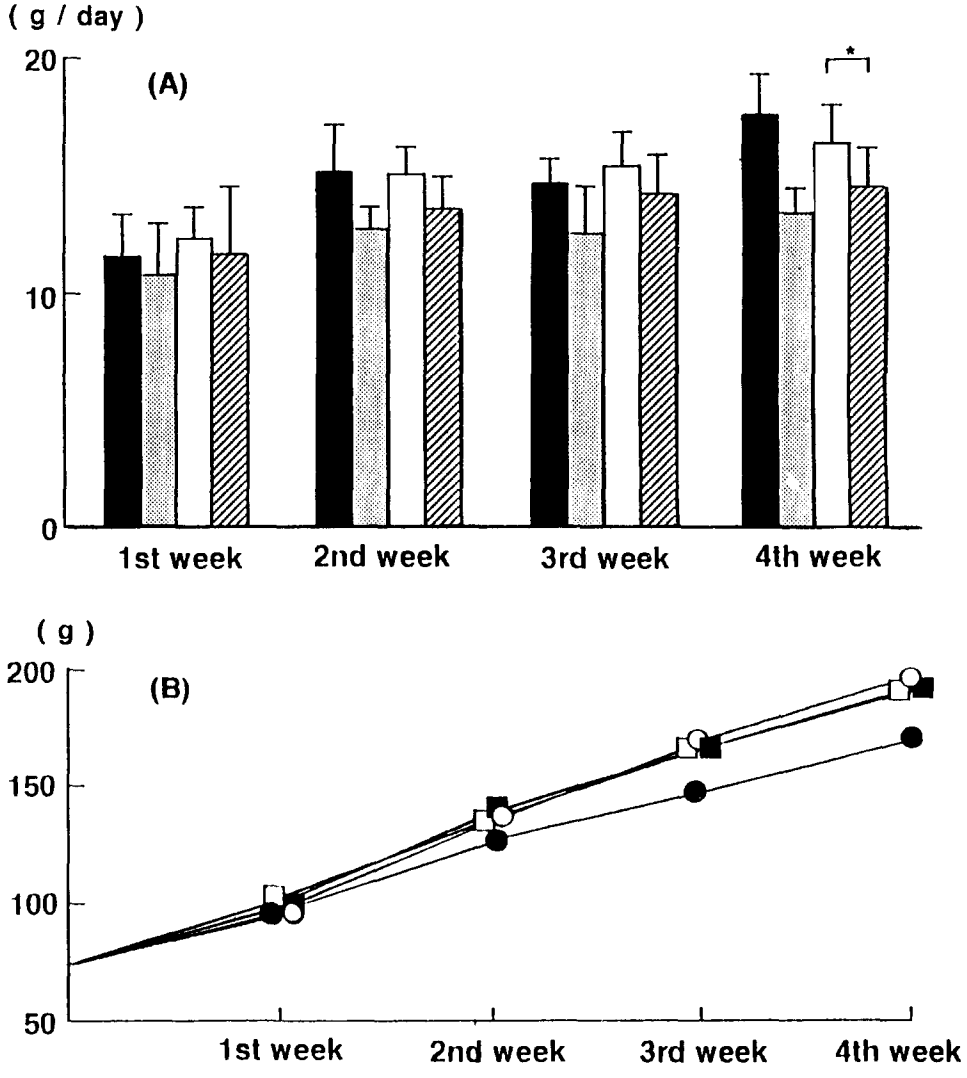

Figure 2 Changes of food intakes (A) and body weights (B) in rats fed each diet. Each value presents the mean ± SD from 6 rats. *Significantly different between protein and peptide dietary groups ($P < 0.05$). ■-○-soybean, ▨-●-soybean peptide □-□-casein, ▨-■-casein peptide.

Table 2 Protein efficiency ratio

Groups	Protein efficiency ratio (PER)
Soybean protein	1.91 ± 0.04
Soybean peptide	1.79 ± 0.05
Casein protein	1.83 ± 0.04
Casein peptide	2.00 ± 0.07

PER is expressed as the body weight gain per 1 g of protein or peptide. Values are means ± SEM for 5 rats.

were removed by washing the plates with the medium. AMø cells were then used for each assay.

Phagocytosis of AMø

AMø cells (2×10^5 cells/well) were incubated with opsonized sheep red blood cell (RBC) labeled with 200 μCi Na_2CrO_4 (Japan Atomic Energy Research Institute, Tokyo Japan) for 2 hr at 37° C.[12] Nonphagocytosed sheep RBC were hemolysed by distilled water and washed by adding 0.1N NaOH, and the radioactivity of the lysate was determined using a gamma counter.

Natural killer cell activity of splenocytes

Splenocytes from each group (1×10^6 cells/mL) were incubated in a CO_2 incubator with ^{51}Cr-tumor cells (YAC-1) for 4 hr at 37° C.[13] The supernatant was collected and the radioactivity was measured by a gamma counter using Equation 1.

$$\% \text{ lysis} = \frac{\text{experimental } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}{\text{maximum } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}} \times 100 \quad (1)$$

Spontaneous ^{51}Cr release was determined from target cell cultures incubated with the medium alone. The maximum ^{51}Cr release was determined by dissolving all tumor cells with 0.1 mL of 1N NaOH.

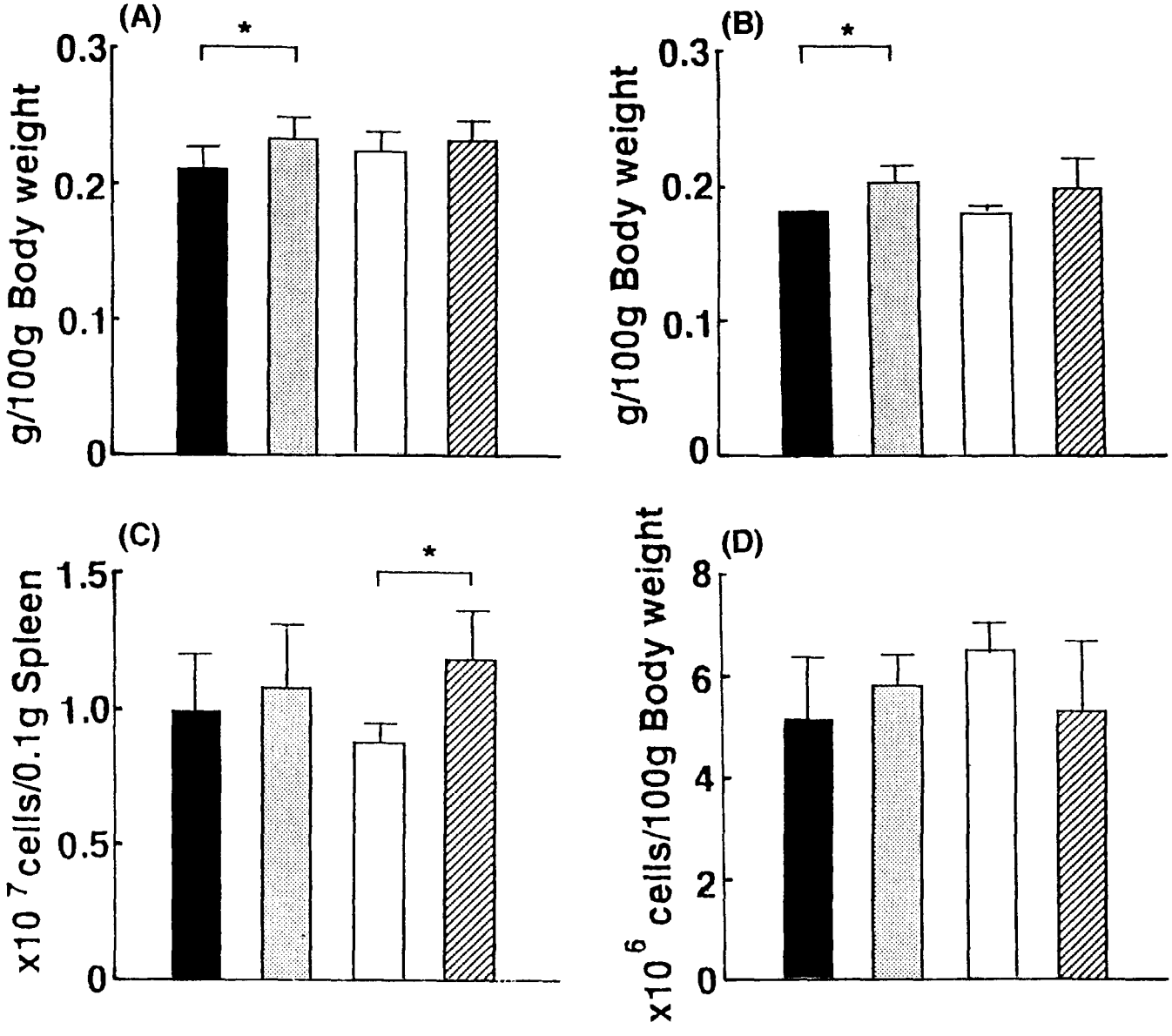

Figure 3 Weights of spleen (A) and thymus (B), and numbers of splenocytes (C) and alveolar macrophages (D) in rats fed each diet for 4 weeks. Each value presents the mean ± SD from 6 rats. *Significantly different between protein and peptide dietary groups ($P < 0.05$). ■ soybean, ▨ soybean peptide, □ casein, ▨ casein peptide.

Mitogenesis of splenocytes

Splenic T- and B-lymphocyte responses to mitogens such as phytohemagglutinin (PHA), concanavalin A (Con A), and lipopolysaccharide (LPS) from *Escherichia coli* were determined.[14] Single cell suspensions of 1×10^6 cells/mL were prepared in RPMI 1640 medium supplemented with 25 mmol/L 4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 100 μ/mL penicillin, 100 μg/mL streptomycin, and 50 μmol/mL 2-mercaptoethanol. Splenocytes with or without mitogens were plated in 96-well microtiter plates, incubated in 5% CO_2 at 37° C for 72 hr, and pulsed with 1.0 μCi[^{3}H]thymidine (specific activity 25 Ci/mmol, New England Nuclear, Boston, MA USA). After 24 hr they were harvested. The radioactivity was measured by a liquid scintillation counter, and data are presented as counts per min (cpm). Some of the splenocytes were immediately pulsed with [^{3}H] thymidine after isolation in single cells.

Statistical analysis

All data were subjected to analysis of variance, and Student's *t* test was used to determine significant differences between treatment means.

Results

Preparation of peptides derived from soybean and casein

In this experiment, the soybean and casein proteins prepared by enzymolysis using pepsin and the hydrolysate were further purified by an ultrafiltration membrane, ion exchange resin column, and gel filtration column chromatography. Peptide fractions obtained by these procedures had a peak in a fractional molecular

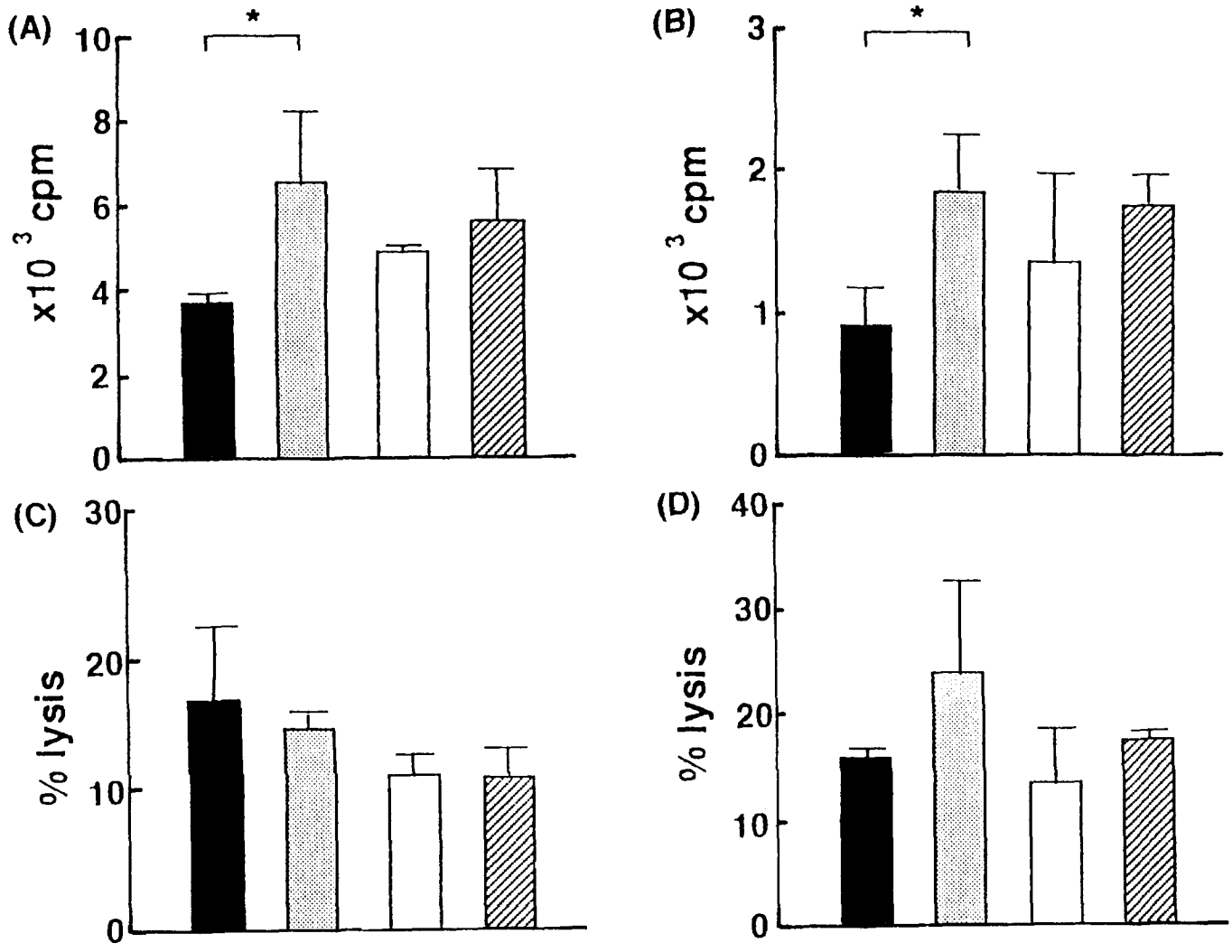

Figure 4 Phagocytosis of alveolar macrophages from rats fed each diet for 2 weeks (A) and 4 weeks (B), and NK activity of splenocytes from rats fed each diet for 2 weeks (C) and 4 weeks (D). Each value presents the mean ± SD from 6 rats. *Significantly different between protein and peptide dietary groups. ($P < 0.05$). ■ soybean, ▨ soybean peptide, □ casein, ▧ casein peptide.

weight range of 300–2000 and a molecular weight range of 200–5000. As shown in *Figure 1*, the ratios of the individual amino acids of the soybean and casein peptides were generally similar except for higher levels of Asp, Gly, Arg, and Thr in soybean peptide than in those of casein peptide, and lower levels of Ala, Pro, Thr, Vat, and Met in soybean peptide than in casein peptide.

Food intake and body weight

Figure 2 shows the changes in food intake and body weight of rats fed diets containing soybean peptide, casein peptide, and corresponding proteins of soybean and casein. The food intake was slightly reduced in both peptide diet groups as compared with both protein groups from the second week of the experiment. With respect to the changes in body weight, there was no significant difference between the experimental groups up to the second week, and a favorable weight gain was shown. However, in the period from the second week to the fourth week, growth delay was shown in the soybean peptide group. It was suspected that delayed growth in the soybean peptide group was due to the reduction in food intake. As shown in *Table 2*, there was no difference in the protein efficiency ratio (body weight gain per 1 g protein or peptide) among the respective groups fed each diet for 4 weeks.

Weights of the spleen and thymus, and number of splenocytes and AMø

The weights of the spleen and thymus per 100 g body weight of rats fed each diet for 4 weeks is shown in *Figure 3*. The soybean peptide group showed a significant increase in the weight of both organs. The number of splenocytes in the casein peptide group increased significantly, but no significant difference in the number of AMø was observed among the respective groups.

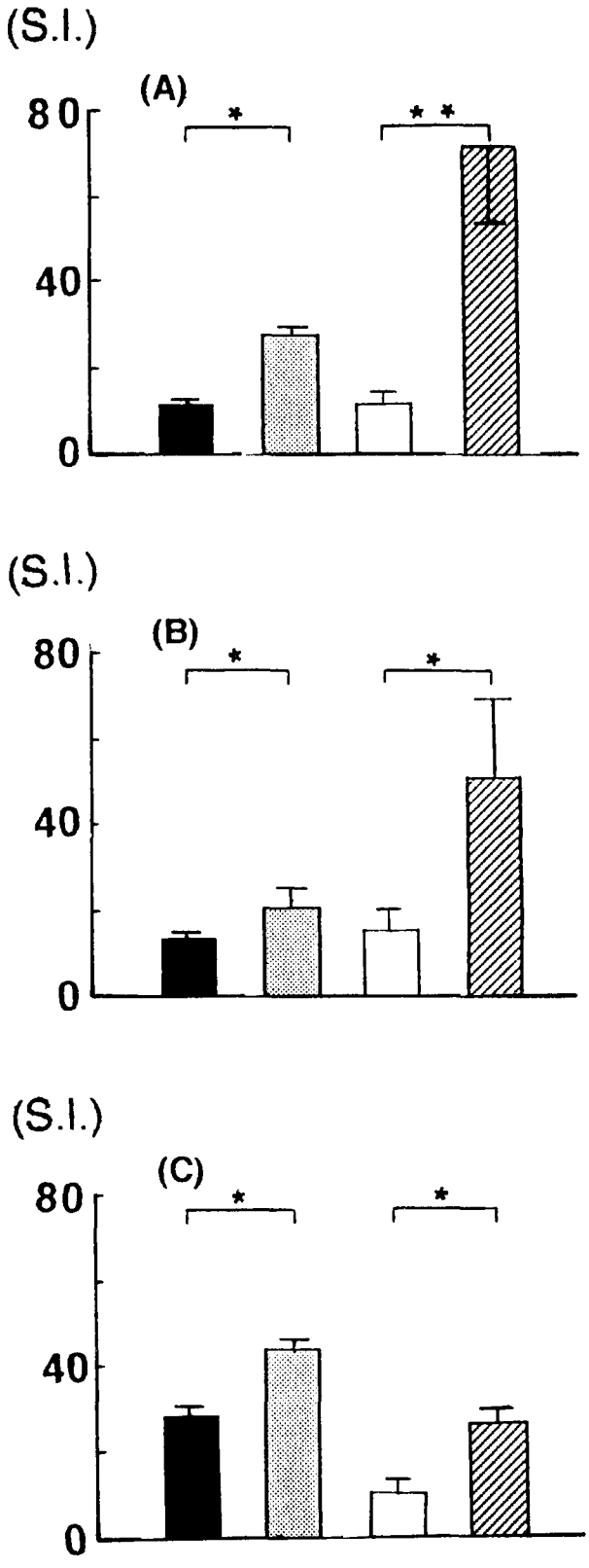

Figure 5 Effect of PHA, Con A, and LPS on mitogenesis of splenocytes in rats fed each diet. Each value presents the mean ± SD from 6 rats. *,**Significantly different between protein and peptide dietary groups (*$P < 0.05$, **$P < 0.01$). ■ soybean, ▨ soybean peptide, □ casein, ▨ casein peptide.

Phagocytic activity of AMø and NK activity of splenocytes

The phagocytic activity of AMø after 2 and 4 weeks was significantly higher in the soybean peptide group as compared with the soybean protein group as shown in *Figure 4* A and B.

The NK activity of splenocytes increased in both peptide groups after 2 weeks compared with the corresponding protein groups (*Figure 4* C), but no significant difference was seen in the respective group after 4 weeks (*Figure 4* D).

Mitogenic activity of splenocytes

The lymphocyte transformation assay was performed with isolated rat splenocytes from the protein and peptide groups. As shown in *Figure 5*, there were significant differences in the mitogenic responses stimulated by PHA, Con A, and LPS between the protein groups and peptide groups.

Phagocytic activity and NK activity of pair-fed rats

In rats of the 4-week pair-feeding experiments, as shown in *Figure 6*, phagocytosis of AMø and mitogenesis of splenocytes were increased in both peptide groups compared with those of the corresponding protein groups. However, the NK activity of splenocytes was not different in rats fed both peptide groups (data not shown).

Discussion

To evaluate the effect of peptides derived from soybean and casein on immune responsiveness, we have chosen to compare their corresponding proteins. The difference noted was higher weights of the spleen and thymus per body weight in rats fed soybean peptide. Phagocytosis of AMø and mitogenesis of splenocytes were higher in the soybean peptide group, as well as the casein peptide group, as compared to the corresponding protein diet groups.

We cannot readily explain why the peptide from soybean actually had a beneficial effect on cellular immunity. Although any difference between these peptides could theoretically be interpreted as being crucial to the immunological effect, a decrease in food intake of the soybean peptide group first attracted our attention because previous experiments showed that a moderate restriction of food or protein intake enhanced the immune responses.[12,15] In this study, no difference in protein efficiency ratio was observed among the experimental groups. In further experiments involving pair-feeding for 4 weeks, the peptide also had a similar stimulatory effect on the phagocytic activity of AMø and the mitogenic activity of splenocytes for defense of the host (data not shown). The factor responsible for the immune effect of soybean peptide does not appear to be related to its nutritional deficiency by a decrease in food intake. The reason for the difference in immune responses seems likely to lie either in qualitative or quantitative differences in the peptide constitution of

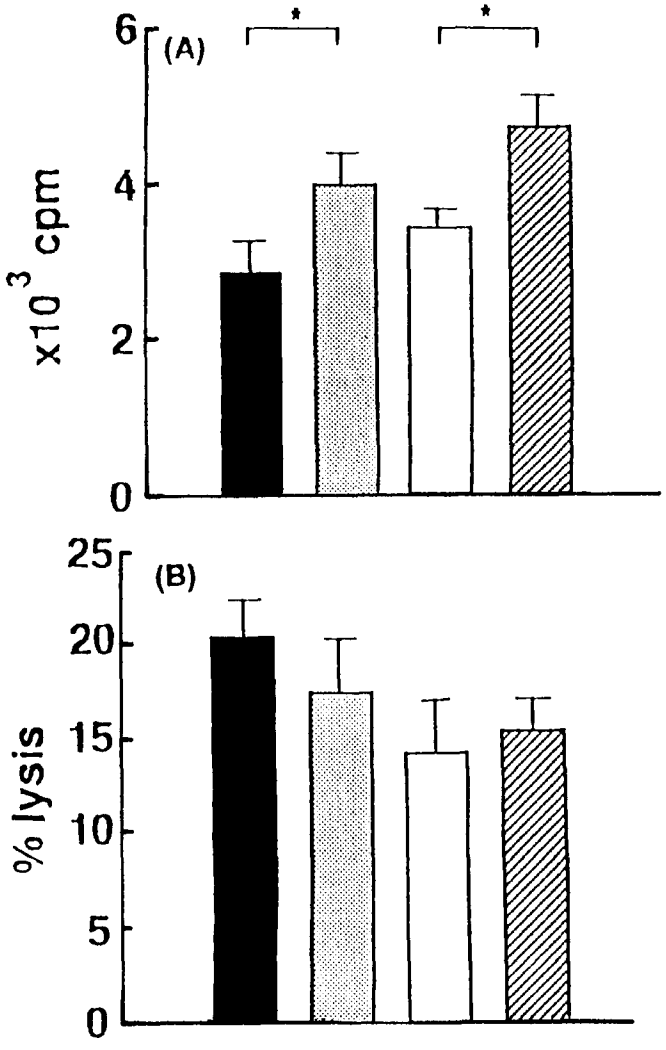

Figure 6 Phagocytosis of alveolar macrophages (A) and NK activity of splenocytes (B) from pair-fed rats for 4 weeks. Each value presents the mean ± SD from 6 rats. *Significantly different between protein and peptide dietary groups ($P < 0.05$). ■ soybean, ▨ soybean peptide, □ casein, ▨ casein peptide.

the protein hydrolysates or in the basic amino acid composition of the peptides. Bounous et al.[5] suggested that dietary protein types may influence systemic immunity by changing the relative composition of the intestinal microflora in view of the immunological interactions between the host and its intestinal microbes. They investigated the effect on the response capacity of splenocytes to mitogens in mice fed lactalbumin, casein, soybean, and wheat, and indicated that all of these functions were significantly accelerated in the lactalbumin group as compared with the other three groups.

In the present experiment, the fresh soybean was prepared by enzymolysis using pepsin, and the hydrolysate was further purified by an ultrafiltration membrane, an exchange resin column, and gel filtration column chromatography. Peptide fractions obtained by these procedures had a peak in a fractional molecular weight range of 300–2000 and a molecular weight range of 200–5000. Peptide fractions from casein were also obtained by the same preparation. There was no great difference between the molecular weight distribution of the peptides or between the amino acid composition in both peptides. It is considered that the difference between the amino acid sequences of the constitutional peptides exerted an effect on the host immunological responses. Jollès et al.[6] speculated on the possibility of enzymatic release of immunomodulating peptides from milk casein during the digestive process, and recently Miglior-Samour et al.[16] have obtained hexapeptide (Val-Glu-Pro-Ile-Pro-Tyr) and tripeptide (Gly-Leu-Phe) as immune activating peptides from the same casein subjected to trypsin treatment. It was known that these peptides activate in vitro phagocytosis of macrophages and increase the production of antibodies, and tripeptide enhances the survival rates of mice infected with *Klebsiella pneumoniae*. Other active peptides, which are implicated in the stimulation of the immune system, α-casein–derived exorphins,[17] and angiotensin I-converting enzyme inhibitors have been purified by different investigators.[18] Iwamoto et al.[19] also described that fish protein hydrolysates stimulate mitogenesis of peripheral lymphocytes by Con A. Therefore, dietary peptides should be taken into account in connection with the effect on the immune function.

It is considered that soybean peptides are of the approximate molecular weight range of saponins[20] and the protease inhibitor[21] as an anti-promoter for cancer. From the present results, the immune activating factors would probably be present in soybean peptides, and favorable peptide composition requires clarification through further studies.

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