Oral Ingestion of Phosphorylated Dextrin Stimulates Antibody Responses in Mice

Hajime OTANI, Tatsunori MORIMOTO and Takeshi KAWAHARA
Graduate School of Agriculture, Shinshu University, Minaminokawa-mura, Nagano 399–4598, Japan
(Received December 14, 2006)

Summary Corn dextrin with an average degradation degree of 16.7, or molecular mass of 2,700, was covalently introduced with 4.5 mol of phosphoric acid per mole by dry-heating with sodium phosphate. The effect on immunoglobulin production of the phosphorylated dextrin in mice that orally ingested lipopolysaccharide (LPS) from Salmonella typhimurium was investigated. No significant difference in body weight gain was observed between mice fed a phosphorylated dextrin-containing diet and those fed a control (dextrin-containing) diet. Fecal and intestinal anti-LPS immunoglobulin (Ig) A, intestinal and serum anti-LPS IgG, and fecal and intestinal total IgA levels were significantly higher in the mice given the phosphorylated dextrin. In contrast, serum and intestinal levels of IgM specific to the LPS were similar between the two groups. Moreover, spleen cells from mice fed the phosphorylated dextrin-added diet had significantly higher levels of anti-LPS IgG and IgA than those from mice on the control diet. These results suggest that dietary phosphorylated dextrin protects against local and systemic invasions of pathogenic microorganisms in mice.

Key Words phosphorylated dextrin, mucosal IgA stimulator, anti-lipopolysaccharide, oral immunoadjuvant, serum IgG

The mucosal immune system is recognized to play a crucial role in the protection of a host against invasion by microbes and dietary antigens. Immunoglobulin (Ig) A is the predominant antibody in the mucosal immune system (1). However, at birth and for much of infancy in mammals, much of the defense system involving the immune system is not equivalent to that of older animals or adults. Hence, it is beneficial for newborn animals to be given mucosal immuno-enhancing factors.

Recently, Otani et al. (2–4) demonstrated that dietary bovine casein phosphopeptide preparations named CPP-I and CPP-III, mainly consisting of αs2-casein (1–32) and β-casein (1–28), stimulated intestinal production of IgA specific to peritoneally or orally ingested proteins and orally ingested lipopolysaccharide (LPS) from Salmonella typhimurium in mice and piglets. Kitamura et al. (5) observed that the mean fecal IgA level of 7 volunteers who ingested a cake containing casein phosphopeptides for 30 d was increased markedly compared with that of the same subjects immediately before the ingestion, and the mean IgA level noticeably decreased when the volunteers ingested a placebo cake after ingesting the cake containing casein phosphopeptides for 30 d. These results suggest that the oral ingestion of bovine casein phosphopeptides stimulates intestinal IgA production in mammals. Bernard et al. (6), however, proposed that the phosphoserine-rich region of calcium-sensitive casein might be recognized as an epitope in some milk allergy patients because IgE antibodies in sera from some patients with clinical symptoms of cow milk allergy bound strongly to bovine β-casein (1–25) but hardly at all to dephosphorylated β-casein (1–25). Thus, the potential allergenicity of casein phosphopeptides should be taken into account when considering their biological effect and possible use in functional foods.

In an accompanying paper (7), the authors have found that the phosphorylated dextrin (PD) prepared by dry-heating of corn dextrin (average molecular mass of 2,700) at 140˚C for 24 h with sodium phosphate stimulated IgA production in mouse spleen cell cultures. The dextrin seemed to display little allergenicity because of its small molecular mass. Steward (8) described that dextrin of less than 50,000 was far less immunogenic. In general, however, orally ingested dextrin is easily hydrolyzed into glucoses by gastrointestinal enzymes. Hence, it is necessary to learn whether the orally ingested PD stimulates intestinal IgA production in animals when considering its biological effect and possible use in functional foods.

Thus, the present study was carried out to investigate adjuvant effects of PD on the production of immunoglobulins in mice that had orally ingested LPS from Salmonella typhimurium.

MATERIALS AND METHODS

This experiment was conducted under the guidelines for the Regulation of Animal Experimentation of the Faculty of Agriculture and the Graduate School of Agriculture in Shinshu University, and according to Law No. 105 and Notification No. 6 of the Japanese Government.

Materials. Corn dextrin with an average degradation degree of 16.7 (molecular mass of 2,700) was obtained from Sanwa Cornstarch (Nara, Japan). Ovalbumin (Grade II, OVA) and bovine serum albumin (Fraction V, BSA) were obtained from Sigma Chemical
Company (St. Louis, MO, USA). Goat anti-mouse IgA (Fc), horseradish peroxidase-bound sheep anti-mouse IgA (Fc) and horseradish peroxidase-bound sheep anti-mouse IgG were purchased from Bethyl Laboratories (Montgomery, TX, USA). Horseradish peroxidase-conjugated sheep anti-mouse IgM was obtained from Tago (Burlingame, CA, USA). Lipopolysaccharide (LPS) of Salmonella typhimurium was obtained from Difco Laboratories (Detroit, MI, USA). Defined fetal bovine serum (BFS) and RPMI 1640 were from Perbio Science Company (Logan, UT, USA) and Mediatech (Herndon, VA, USA), respectively.

Preparation of PD. PD was prepared principally according to the procedure of Nakano et al. (9). The procedure is outlined in the accompanying paper (7).

Mice and feeding procedure. Male C3H/HeN mice were obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan) at 3 wk of age. Three-week-old mice were placed on test regimens and fed a commercial mouse pellet feed (MF, Oriental Yeast, Tokyo, Japan) according to the procedure of Nakano et al. (9).

PD-added diet. Feces were collected on the 0, 7th, 14th, and 21st, 28th and 35th days after the start of feeding with the control or PD-added diet. Feces were separately ground for 20 min at 2˚C with sea sand (1 g) in 0.01 M sodium phosphate buffer, pH 7.2, containing 0.15 M sodium chloride (PBS, 2.5 mL). The ground material was centrifuged at 1,200 g for 30 min at 4˚C, and the supernatant was collected as fecal extract for immunoglobulin assay.

Table 1. Compositions of diets used in the present work.

| Component                  | Dextrin-added diet (Control diet) | Phosphorylated dextrin-added diet (PD-added diet) |
|----------------------------|----------------------------------|--------------------------------------------------|
| Phosphorylated dextrin (PD)| 0.3 (%): P | 0.3 (%) P |
| Dextrin                    | 0.3 (%)              | — (%)   |
| Ovalbumin                  | 25.0 (%)              | 25.0 (%) |
| Protein-free diet          | 74.7 (%)              | 74.7 (%) |
| (PM15765)                  |                      |        |
| Total                      | 100.0 (%)             | 100.0 (%)|

Preparation of serum sample, fecal extract and intestinal extract for immunoglobulin assay. Blood samples and intestinal tracts (duodenum to rectum) consisting of tissues and contents were carefully collected on the 35th day after the start of feeding with the control or PD-added diet. Feces were collected on the 0, 7th, 14th, 21st, 28th and 35th days after the start of feeding with the diets. The feces (125 mg) and intestinal tract (1 g) were separately ground for 20 min at 2˚C with sea sand (1 g) in 0.01 M sodium phosphate buffer, pH 7.2, containing 0.15 M sodium chloride (PBS, 2.5 mL). The ground material was centrifuged at 1,200 g for 30 min at 4˚C, and the supernatant was collected as fecal or intestinal extracts.

Cell culture. The spleen cell culture was prepared from mice fed the control or PD-added diet for 35 d. Briefly, mice were sacrificed by cervical dislocation and their spleens were removed aseptically. Single-cell suspensions were prepared by gentle manipulation of the tissues in a culture medium (RPMI 1640 medium containing 10% (v/v) BFS, 100 units penicillin/mL and 100 μg streptomycin/mL). The cells were washed 3 times in the culture medium and resuspended at a concentration of 6×10^6 viable cells/mL. Cell cultures were set up in quadruplicate on flat-bottomed microtiter plates (Falcon Labware, Oxnard, CA, USA). Into each well were placed 100 μL of cell suspension and 10 μL of PBS. The final concentration of spleen cells was 5×10^6 viable cells/mL. The mixtures were cultured at 37˚C in a humidified 5% (v/v) CO₂–95% (v/v) air atmosphere for 72 h.

Immunoglobulin assay. An enzyme-linked immunosorbent assay (ELISA) was used to determine levels of IgG, IgM or IgA specific to LPS from Salmonella typhimurium. A sandwich ELISA was carried out for the determination of total IgA levels.

Immunostimulatory Effect of Phosphorylated Dextrin

Fig. 1. Changes of body weight in mice during the experimental period. ○, Control diet; ●, PD-added diet. Each point represents an average for 5 mice.
bated for 1.5 h at 25˚C and washed with PBS-Tween. Peroxidase substrate (100 µL, *ortho*-phenylenediamine dihydrochloride in 0.1 M citrate-0.2 M sodium phosphate buffer containing 0.03% (v/v) hydrogen peroxide, pH 5.0, 4 mg/mL) was added to each well. After 20 min at 25˚C, the reaction was stopped by the addition of 25 µL of 2 M H₂SO₄. The plates were read at 490 nm on a Bio-Rad model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The immunoglobulin level was calculated with the following formula: Immunoglobulin level = absorbance/dilution-fold of the test sample.

**Statistical analysis.** Results were expressed as means±SE for body weight (g) and the immunoglobulin level (A490 nm). Significant differences between the control diet and PD-containing diet groups were tested with Student’s *t*-test.

**RESULTS**

Figure 1 shows the body weight of mice fed the control diet or PD-added diet during the test period. Mice given PD gained body weight at levels quite similar to those on the control diet.

Figures 2 and 3 show the levels of fecal total or LPS-specific IgA, and the levels of intestinal LPS-specific IgG, IgM or IgA and total IgA, respectively, in mice fed the control or the PD-containing diet for 35 d. The mice given PD had higher fecal and intestinal total and LPS-specific IgA levels than those on the control diet. Similarly, the intestinal LPS-specific IgG level was higher in the mice given PD. The level of intestinal LPS-specific IgM, by contrast, was similar to that in mice on the control diet. Figure 4 shows serum LPS-specific IgG, IgA and IgM levels. Mice given the PD-added diet had significantly higher IgG levels than those given the control diet, although there was no significant difference in IgA or IgM levels between the two groups.

Spleen cells were collected from the mice and cultured for 72 h to determine LPS-specific IgG, IgM, or IgA levels in culture supernatants. As shown in Fig. 5, the spleen cells from mice fed the PD-added diet exhibited a significantly larger increase in LPS-specific IgG and IgA production than the control.

**DISCUSSION**

The primary function of dietary proteins is to supply
the body with indispensable amino acids and organic nitrogen. In addition, however, the peptides originating from dietary proteins should be considered potential modulators of various regulatory processes in the body. The authors demonstrated that mice fed a mixture of a commercially available casein phosphopeptide preparation and OVA showed substantially higher serum, fecal, and intestinal levels of total IgA and IgA specific to an orally ingested LPS from Salmonella typhimurium than those fed OVA alone (4). Moreover, the stimulatory effect of casein phosphopeptide on IgA production was found to be attributable to phosphorys groups in the sequence SerP-X-SerP (10). In view of these findings, the authors have investigated whether dietary glucose polymers containing phosphorys groups, or PD, stimulated responses to LPS from Salmonella typhimurium in mice.

In the present study, the mice fed the diet containing PD showed substantially higher serum, fecal and intestinal levels of IgA specific to the orally ingested LPS from Salmonella typhimurium than those fed the control diet. Serum and intestinal LPS-specific IgG levels were also higher although serum and intestinal IgM levels were not significantly different from those in mice given the control diet. It is well established that mice fed diets containing insufficient amounts of protein exhibit a loss of body weight (11). In the present study, however, the mice fed on the control diet gained weight with no significant difference from those fed the PD-containing diet. These findings indicate that the differences in total IgA and LPS-specific IgA or IgG levels between the control diet group and the PD-added diet group are not attributable to a non-specific effect of the PD-added diet on immune responses. Thus, it is concluded that the dietary PD has a significant enhancing effect on both the total level of IgA and the level of IgA or IgG specific to the orally ingested bacterial LPS, or food-borne antigen in mice.

The gastrointestinal tract is the most common site of entry for infectious agents at mucosal surfaces. Secretory IgA is the main immunoglobulin (1). Mucosal IgA prevents the adhesion of bacteria or viruses, and reduces the absorption of food antigens in the intestine.

Hence, various attempts have been made to improve the mucosal immune system. The present study indicates that orally ingested PD enhances the mucosal response to bacterial LPS ingested orally. The immune cells at the mucosal level are associated with gut-associated lymphoid tissue (GALT). After antigen-stimulation in GALT, specific IgA-producing precursor cells should increase in number in distant mucosa-associated lymphoid tissues (1). Thus, dietary PD, or phosphorylated dextrin, may increase intestinal antigen-specific IgA levels in local immune systems such as respiratory tracts, mammary glands, etc., and may be of practical use as an immunostimulator to enhance mucosal immunity via IgA and host defense.

Acknowledgments
This study was partly supported by a Grant-in-Aid from the Japan Society for the Promotion of Science to H. O. (No. 16380180).

REFERENCES
1) Mestecky Y, McGhee JR. 1992. Prospect for human mucosal vaccines. Adv Exp Med Biol 327: 13–23.
2) Otani H, Kihara Y, Park MK. 2000. The immunoenhancing property of a dietary casein phosphopeptide preparation in mice. Food Agric Immunol 12: 165–173.
3) Otani H, Kitamura H, Park MK, Kihara Y, Oshida T, Kusuhara S, Sawada K. 2000. Enhancement of intestinal IgA levels in piglets by oral administration of a commercially available casein phosphopeptide preparation. Milchwissenschaft 55: 429–432.
4) Otani H, Nakano K, Kawahara T. 2003. Stimulatory effect of a dietary casein phosphopeptide preparation on the mucosal IgA response of mice orally ingested lipopolysaccharide from Salmonella typhimurium. Biosci Biotechnol Biochem 67: 729–735.
5) Kitamura H, Oshida T, Otani H, Wakaduki S, Kusuhara S. 2002. Milk immunoglobulin levels in sows given a diet containing a commercially available casein phosphopeptide preparation, CPP-I, during pregnancy. Milchwissenschaft 57: 486–489.
6) Bernard H, Meisel H, Creminon C, Wal J. 2000. Post-translation phosphorylation affects the IgE binding capacity of caseins. FEBS Lett 467: 239–244.
7) Otani H, Sakakibara I, Aoki T. 2007. Immunomodulatory effects of phosphorylated dextrin in mouse spleen cell cultures. J Nutr Sci Vitaminol 53: 349–353.
8) Steward MW. 1976. Outline Studies in Biology Immunochernistry, p 12. John Wiley & Sons Inc, New York.
9) Nakano T, Salvador AS, Tamochi J, Sugimoto Y, Ibrahim HR, Toba Y, Aoe S, Kawakami H, Aoki T. 2003. Phosphorylation of starch and dextrin by dry-heating in the presence of phosphate, and their calcium phosphate-solubilizing ability. Nahrung 47: 274–278.
10) Otani H, Watanabe T, Tashiro Y. 2001. Effects of bovine β-casein (1–28) and its chemically synthesized partial fragments on proliferative responses and immunoglobulin production in mouse spleen cell cultures. Biosci Biotechnol Biochem 65: 2489–2495.
11) Schaedler RW, Dubos RJ. 1959. Effect of dietary proteins and amino acids on the susceptibility of mice to bacterial infections. J Exp Med 110: 921–934.