Immunomodulatory Effects of Phosphorylated Dextrin in Mouse Spleen Cell Cultures

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Summary  Corn dextrin preparations with average degradation degrees of 16.7 and 167, or average molecular masses of 2,700 and 27,000, were covalently introduced with 4.5 and 11.2 to 35.4 mol of phosphoric acid, respectively, per mole by dry-heating at 110 to 140˚C for 24 h with sodium phosphate, and their effects on cell proliferation and production of immunoglobulin A (IgA) were investigated in C3H/HeN mouse spleen cell cultures. All the phosphorylated dextrin had mitogenic effects and stimulated proliferative responses induced by lipopolysaccharide (LPS) and concanavalin A (ConA). The phosphorylated dextrin with an average degradation degree of 16.7 was divided into 3 fractions, which had 3.6, 4.0 and 6.5 mol of phosphoric acid per mole when the molecular mass of each dextrin was postulated to be 2,700. LPS-induced proliferation was greatest with the preparation having 3.6 mol of phosphoric acid per mole of dextrin, whereas ConA-induced proliferation was strongest with 6.5 mol of phosphoric acid. On the other hand, the divided dextrin enhanced production of IgA by approximately 1.2-, 2.0- and 2.2-fold in accordance with the increasing amount of phosphoric acid. These results indicate that phosphorylated dextrin stimulates humoral immune responses in mouse spleen cell cultures.

Key Words  phosphorylated dextrin, immunomodulator, mouse spleen cell culture, mitogen, IgA

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 A (IgA) is the predominant antibody in this system and the intestinal tract is an efficient site to induce mucosal production of IgA (1). The mucosal administration of an antigen, however, generally induces little immune response and requires the use of a specific mucosal adjuvant in order to enhance the response. Some bacterial enterotoxins such as a heat-labile enterotoxin produced by Escherichia coli and a cholera toxin produced by Vibrio cholerae are powerful mucosal adjuvants (2). However, these toxins are for pharmaceutical or experimental use, not for dietary use.

Otani and Watanabe (3) demonstrated that a casein phosphopeptide, bovine β-casein (1–28), significantly stimulated production of IgA by enhancing the formation of interleukin (IL)-5 and IL-6 in mouse spleen cell cultures. In addition, Otani et al. (4, 5) demonstrated that mice fed casein phosphopeptide-containing diets exhibited a sustained enhancement of intestinal IgA production in responses to peritoneally or orally ingested proteins and lipopolysaccharide from Salmonella typhimurium. Kitamura et al. (6) reported that the dietary ingestion of casein phosphopeptides by pregnant sows raised levels of colostral IgA and IgG during the pregnancy. Moreover, Otani et al. (7) indicated that the stimulatory effect of casein phosphopeptides on IgA production was attributable to the sequence SerP-X-SerP. In view of these studies, it might be expected that phosphopeptides containing SerP-X-SerP would be used as an oral adjuvant to stimulate production of IgA against pathogenic bacteria in human intestinal tracts.

Casein phosphopeptides have been known to solubilize calcium phosphate, and to enhance calcium absorption in the mammalian ileum. The calcium-solubilizing ability of phosphopeptides is attributable to phosphate groups (8). Recently, Nakano et al. (9) demonstrated that phosphorylated starch and dextrin had calcium phosphate-solubilizing ability. This finding suggests that certain kinds of phosphorylated starch and dextrin may have immunomodulatory properties such as regulatory effects on the proliferation of immunocompetent cells and stimulatory effects on the production of IgA.

Thus, this study was carried out to investigate whether phosphorylated dextrin influenced proliferative responses and production of IgA in mouse spleen cell cultures.

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 Govern-
mend.

Materials. Corn dextrin preparations (average degradation degrees of 16.7 and 167; average molecular masses of 2,700 and 27,000, respectively) were obtained from Sanwa Cornstarch (Nara, Japan). Lipopolysaccharide (LPS) of *Salmonella typhimurium* and concanavalin A (ConA) were purchased from Difco Laboratories (Detroit, MI, USA) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Nissui Pharmaceutical (Tokyo, Japan) and Funakoshi (Tokyo, Japan), respectively. Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), Biomedicals (Montreal, Canada) and O. E. M. Concept (Toms River, NJ, USA). Male C3H/HeN mice, 6 wk of age, were obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan). The spleen cell culture was prepared as described previously (7). 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 5% (v/v) FBS, 100 units penicillin/mL and 100 μg streptomycin/mL). The cells were washed 3 times in the RPMI 1640 medium and resuspended at a concentration of 6×10⁶ 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. 10 μL of test dextrin solution dissolved in 0.01 M sodium phosphate buffer, pH 7.2, containing 0.15 M sodium chloride (PBS), and 10 μL of mitogen solution dissolved in PBS. Final concentrations were: spleen cells, 5×10⁶ viable cells/mL; test dextrin, 0 to 100 μg/mL; LPS, 0 or 50 μg/mL; Con A, 0 or 2 μg/mL. The mixtures were cultured at 37°C in a humidified 5% (v/v) CO₂–95% (v/v) air atmosphere for 72 h for the estimation of proliferative responses or for 24 to 120 h for the estimation of IgA levels. The proliferative response was determined by MTT assay (10). The IgA level was assayed by a sandwich enzyme-linked immunosorbent assay (ELISA).

**Preparation of phosphorylated dextrin.** Phosphorylated dextrin was prepared principally as described previously (9). In brief, the dextrin was suspended at a 2% (w/v) concentration in 0.1 M phosphate buffer (pH 5.5), incubated at 80°C for 10 min and lyophilized. The lyophilized dextrin was heated at 110°C, 120°C, 130°C or 140°C for 24 h (in the case of the high molecular mass dextrin) or at 140°C for 24 h (in the case of the low molecular mass dextrin) in a drying-oven (model DX-58, Yamato Scientific, Tokyo, Japan). The heated dextrin was dissolved at a 2% (w/v) concentration in distilled water, poured into Seamless Celulose Tubing (Sanko Jynyaku, Tokyo, Japan) and eluted with PBS at a flow rate of 25 mL/h. The effluent was monitored for neutral sugar using the phenol–sulfuric acid method. The organic phosphorus of the lyophilized dextrin was determined according to procedures described previously (9). The lyophilized dextrin prepared from the high molecular mass dextrin heated at 110°C, 120°C, 130°C and 140°C contained 11.2, 17.4, 26.3 and 35.4 mol of organic phosphoric acid per mole, respectively, when the molecular mass of the dextrin was postulated to be the original one (27,000), and the low molecular weight preparation had 4.5 mol of organic phosphoric acid per mole when the molecular mass was postulated to be 2,700.

**Spleen cell cultures.** Male C3H/HeN mice, 6 wk of age, were obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan). The spleen cell culture was prepared according to procedures described previously (7). 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 5% (v/v) FBS, 100 units penicillin/mL and 100 μg streptomycin/mL). The cells were washed 3 times in the RPMI 1640 medium and resuspended at a concentration of 6×10⁶ 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. 10 μL of test dextrin solution dissolved in 0.01 M sodium phosphate buffer, pH 7.2, containing 0.15 M sodium chloride (PBS), and 10 μL of mitogen solution dissolved in PBS. Final concentrations were: spleen cells, 5×10⁶ viable cells/mL; test dextrin, 0 to 100 μg/mL; LPS, 0 or 50 μg/mL; Con A, 0 or 2 μg/mL. The mixtures were cultured at 37°C in a humidified 5% (v/v) CO₂–95% (v/v) air atmosphere for 72 h for the estimation of proliferative responses or for 24 to 120 h for the estimation of IgA levels. The proliferative response was determined by MTT assay (10). The IgA level was assayed by a sandwich enzyme-linked immunosorbent assay (ELISA).

**Gel filtration.** One hundred milligrams of phosphorylated dextrin derived from the low molecular preparation was dissolved in 4 mL of PBS and centrifuged at 1,200 × g for 15 min. The supernatant containing 45 mg of dextrin (3.5 mL) was applied to a Bio-Gel P-6 column and eluted with PBS at a flow rate of 25 mL/h. The effluent was monitored for neutral sugar using the phenol–sulfuric acid reaction.

**Statistical analysis.** Results were expressed as the mean ± SD for the MTT value (absorbance at 570 nm) or IgA level (absorbance at 490 nm). Significant differences between the culture without dextrin and the culture with dextrin were tested with Student’s t-test.

**RESULTS**

Mouse spleen cells were cultured with the high molecular mass dextrin (untreated) or the phosphory-
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The phosphorylated dextrin derived from it at a concentration of 0–100 μg/mL for 72 h in the absence and presence of LPS or Con A. The mitogenic effect and effect on proliferative responses of the phosphorylated dextrin were investigated. As shown in Fig. 1, mitogenic activity was significantly displayed in all cultures with the phosphorylated dextrin, and the strength of the activity seemed to increase with the strength of phosphoric acid when the mole number was below 26.3. As shown in Fig. 2, all the phosphorylated dextrin significantly stimulated proliferative responses of mouse spleen cells induced by LPS and Con A. The stimulatory activity of the dextrin seemed to be greater in the Con A-treated culture than LPS-treated culture.

The phosphorylated dextrin derived from the low molecular preparation was fractionated by gel filtration on a Bio-Gel P-6 column. As shown in Fig. 3, the eluted dextrin was conveniently divided into 3 fractions named A, B and C. A, B and C were calculated to contain 3.6, 4.0 and 6.5 mol of phosphoric acid per mole when the molecular mass of A, B and C was postulated to be the original one (2,700). Mouse spleen cells were cultured with the unfractionated phosphorylated dextrin, A, B or C at a concentration of 0–5 μg/mL for 72 h in the absence and presence of LPS or Con A, and effects of the dextrin on proliferative responses were investigated. As shown in Fig. 4, all the dextrin significantly displayed a mitogenic effect on the resting cells (LPS- and Con A-free) and stimulated proliferative responses of the cells induced by both mitogens. The stimulatory effect on LPS-induced proliferation was greatest in the presence of A, whereas that on Con A-induced proliferation was strongest in the presence of C.

Mouse spleen cells were cultured with the low molecular mass dextrin (untreated), A, B or C at a concentration of 5 μg/mL for 0–120 h, and the effect of the dextrin on production of IgA was investigated. As shown in Fig. 5, all the fractionated dextrin significantly stimulated the production of IgA, although the untreated...
The spleen is a major secondary lymphoid organ along with the lymph node, and contains a variety of immunocompetent cells such as B cells, T cells, accessory cells, etc. Contact with a specific antigen or mitogen causes B cells and T cells to proliferate rapidly and differentiate into plasma cells or immunoregulatory cells. In the present study, all the phosphorylated dextrin induced the proliferation of resting mouse spleen cells, or displayed mitogenic effects on spleen cells. It seemed unlikely that the phosphorylated dextrin was contaminated with LPS from Gram-negative bacteria, which has strong mitogenic activity, since the mitogenic activity of the phosphorylated dextrin was not inhibited by addition of polymyxin B (results not shown), which inactivates LPS ($^{11}$).

The influx of calcium into B and T cells plays an important part in the triggering of the cell stimulation and the calcium ionophore A 23187 is mitogenic towards the cells because it enhances the intake of calcium ($^{12}$). Casein phosphopeptide forms a complex with calcium, solubilizes the calcium, and enhances its uptake via phosphate groups ($^{13}$). Hata et al. ($^{14}$) found that a commercially available phosphopeptide preparation, CPP-III, had a mitogenic effect on mouse spleen cells while the removal of phosphate groups from CPP-III with acid phosphatase reduced significantly the mitogenic effect, and concluded that the mitogenic activity of CPP-III was attributable to the calcium-solubilizing ability of CPP-III via phosphate groups.

Recently, Nakano et al. ($^{9}$) demonstrated that phosphorylated dextrin formed a complex with calcium and solubilized the calcium via phosphate groups. In the present study, the authors indicated that the strength of the mitogenic activity was related to the concentration of phosphoric acid in dextrin (Fig. 1). Thus, the mitogenic activity of the phosphorylated dextrin may be at least partly attributable to the calcium-solubilizing ability of the dextrin.

**DISCUSSION**

The spleen is a major secondary lymphoid organ along with the lymph node, and contains a variety of immunocompetent cells such as B cells, T cells, accessory cells, etc. Contact with a specific antigen or mitogen causes B cells and T cells to proliferate rapidly and...
tively (15). All the phosphorylated dextrin enhanced the proliferative responses of the spleen cells induced by LPS and ConA (Figs. 2 and 4). These findings suggest that the phosphorylated dextrin stimulates the proliferative responses of both B and T cells. However, the stimulatory effect on LPS-induction was greatest with the dextrin having 3.6 mol of phosphoric acid, whereas that on ConA-induction was greatest with the dextrin having 6.5 mol of phosphoric acid (Fig. 4). The reason for this difference is unclear in the present study. Further study is in progress in order to clarify this point.

Phosphorylated dextrin stimulated IgA production in the spleen cell cultures (Fig. 5). The stimulatory activity was dependent on the amount of phosphorous introduced into the dextrin, with the dextrin having the highest phosphorous level displaying the strongest stimulatory activity. These observations suggest that the phosphorylated dextrin stimulates not only proliferative responses but also the differentiation of B and/or T cells.

In recent years, many immunomodulatory substances have been characterized. Sato et al. (16) reported that the introduction of phosphoric acid into dextran from Leuconostoc mesenteroides augmented B cell-specific mitogenic activity and antigen-presenting activity of dendritic cells. The molecular mass of the dextran used by Sato et al. (16) was more than 10,500. As described above, the authors found that the dextrin with a lower molecular mass, i.e., 2,700, possessed immunomodulatory activities, in particular, stimulating not only the proliferation of B and T cells but also the production of IgA. Phosphorylated dextrin with a low molecular mass is widely used in foods to improve the uptake of calcium. In order to use phosphorylated dextrin of low molecular mass as an immunomodulatory agent in food, further characterization of dextrin is needed.

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