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Preventive Effect of Sialylglycopeptide—Nondigestive Polysaccharide Conjugates on *Salmonella* Infection

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We have previously reported that sialylglycopeptide (SGP) and its derivatives isolated from egg yolk had a preventive effect on *Salmonella* infection in vivo; however, their retention time in the gut was rather short. To improve on this, SGP was conjugated with carboxymethyl cellulose (CMC) or carboxymethyl dextran (CMD). The conjugates inhibited the binding of *Salmonella enteritidis* and *Escherichia coli* to Caco-2 cells. Infection experiments with mice revealed that the SGP−CMD conjugate (SGP−CMD) had a strong protective effect against *Salmonella* infection. A turnover experiment in mice administered with radiolabeled SGP−CMD showed that SGP−CMD was more slowly absorbed into the blood and thus remained longer in the intestinal tract than SGP. SGP−CMD itself did not influence the production of tumor necrosis factor α (TNF-α), interleukin-1 β, or nitrite ion (NO_2^-) by macrophages, although it suppressed that of TNF-α and NO_2^- in zymosan-treated macrophages, suggesting no causative effects of inflammation in SGP−CMD. SGP−CMD is potentially useful as a food ingredient with a preventive effect on *Salmonella* infection.

KEYWORDS: Sialylglycopeptide; carboxymethyl cellulose; carboxymethyl dextran; conjugate; Caco-2 cells; *Salmonella* infection; bacterial adhesion

INTRODUCTION

It has been generally accepted that some oligosaccharides in foods such as milk and eggs have an inhibitory effect on the binding of bacteria or virus to the host cells (*1*−*8*). This property has led to the development of antibacterial and antiviral agents from oligosaccharide derivatives. We previously found that SGP and its sialyl- and asialyloligosaccharide from hen egg yolk not only inhibited the binding of *S. enteritidis* and *E. coli* to human intestinal cells in vitro but also protected mice from Salmonellosis in vivo (*8*). However, these compounds could not be expected to remain in the gut for long because they are rapidly absorbed from the intestine (*8*), affecting their usefulness as protective substances.

In this study, we tried to prepare novel conjugates of SGP in order to improve retention times in the gut and to suppress absorption from the intestines to blood. The protein conjugates seemed to show greater resistance to proteolytic degradation than the unconjugated protein. For instance, proteins conjugated with dextran derivatives were reported to be more resistant to enzymatic degradation than native proteins (*9*, *10*). IgG−poly(ethylene glycol) conjugates were also shown to be more resistant to digestion with pepsin, trypsin, or chymotrypsin than unconjugated IgG (*11*). Hence, it is considered that conjugation improves the effect of SGP by preventing enzymatic digestion in the gut. We chose dextran and cellulose, nondigestive polysaccharides, as modifiers to provide SGP a longer retention time in the gut. Because nondigestive polysaccharides can adsorb harmful materials in the gut and excrete them from the body (*12*, *13*), conjugates of SGP and nondigestive polysaccharides are expected to be excreted through the intestine after binding to pathogenic bacteria. The conjugates might be used to create novel dietary fibers with additional functions such as the prevention of infectious diseases.

In this study, to facilitate the binding of dextran or cellulose to the peptide backbone in SGP using its amino groups (**Figure 1**), CMD and CMC were used. The conjugation of SGP with these polysaccharides was achieved using a water soluble carbodiimide, a cross-linking reagent for amino groups and carboxyl groups. After a covalently bonded SGP−CMD conjugate and SGP−CMC conjugate were prepared, we examined the ability of both conjugates to prevent bacterial infections using in vitro and in vivo assays and the fate of SGP−CMD in

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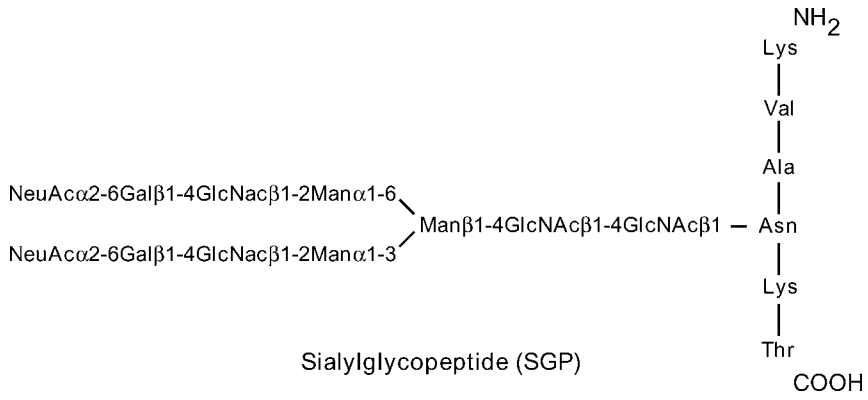

Figure 1. Structure of SGP isolated from hen egg yolk.

mice. We also examined the effect of SGP−CMD on macrophage activation because it seemed feasible that carbohydrate polymers might modulate macrophage functions. Our results demonstrated the possibility of using a functional conjugate such as SGP−CMD as a food ingredient with a protective effect on *Salmonella* infection.

MATERIALS AND METHODS

Materials. Dextran (500 kDa), cellulose (100 kDa), and LPS (*E. coli* O55:B5) were purchased from Sigma Chemical Co. (St. Louis, MO). EDC was obtained from Dojindo (Kumamoto, Japan). TSA was purchased from Becton Dickinson and Company (Sparks, MD). Griess−Romijin reagent was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Carboxymethylation of Dextran and Cellulose. Dextran and cellulose were carboxymethylated with monochloroacetic acid under alkaline conditions according to the method of Hattori et al. (*14*). In brief, 2 g of dextran or 1.5 g of cellulose dissolved in 9.4 mL of a 15% monochloroacetic acid solution containing 1.4 g of sodium hydroxide (pH 11.5) was incubated at 40 °C for 48 h. After the solution was cooled, the reaction solution was neutralized to pH 6.5 with acetic acid to stop the reaction. After dialysis against distilled water and lyophilization, CMD and CMC were obtained.

Preparation of the Conjugates. SGP was isolated from chicken egg yolk and purified as described previously (*8*). The SGP−CMD and SGP−CMC conjugates were prepared using the method of Hattori et al. with some modifications (*14*). In the case of the SGP−CMC conjugate, SGP (25 mg) or CMC (180 mg) was dissolved in 14 mL of distilled water and adjusted to pH 4.75 with 1 M HCl. An EDC solution (41.3 mg/mL) was added dropwise to the mixture over 30 min while keeping the pH at 4.75 with 1 M HCl before incubation for 3 h at room temperature. The reaction was terminated by adding 2 M acetate buffer (pH 5.5). After dialysis against distilled water and lyophilization, SGP−CMC was obtained. SGP−CMD was prepared in the same manner except that 450 mg of CMD was used as a starting material.

Assay of Sialic Acid. The amount of SGP introduced into each polysaccharide was determined from the amount of sialic acid in the conjugates based on there being two sialic acid residues per SGP molecule (**Figure 1**). The assay was performed according to the method of Jourdian et al. (*15*). Briefly, 0.1 mL of 0.04 M sodium periodate and each conjugate solution (0.5 mL) were mixed and incubated on ice for 20 min. In addition, 1.25 mL of 0.6% resorcinol solution (0.6 g of resorcinol in 16.8% HCl containing 25 μmol of copper sulfate) was added to the mixture before incubation on ice for 5 min and at 100 °C for 15 min and cooling under running water. After the addition of 2-methyl-2-propanol (1.25 mL), the solution was incubated at 37 °C for 3 min to stabilize its color development. The solution was cooled to room temperature, and then, the absorbance at 630 nm was measured with a spectrophotometer (Shimadzu, Kyoto, Japan).

Inhibition of Bacterial Binding to Caco-2 Cells by the SGP−Polysaccharide Conjugates. The bacterial strains used in this experiment were *S. enteritidis* (strain E 930448, phage type 4) and *E. coli* (K-88, isolated from swine intestine). Caco-2 cells (ATCC, HTB37) were maintained according to the methods of Dharmsathaphorn (*16*) and Hashimoto and Shimizu (*17*). The precultures of bacteria and the binding assay were performed as described previously (*8*). In brief, 3 h before the inoculation of bacteria, the Dulbecco's modified Eagle medium containing 10% fetal calf serum was removed from wells and replaced with the fresh medium devoid of antibiotics. Various concentrations of each conjugate, CMC, CMD, and SGP were dissolved in the antibiotic-free culture medium and incubated with 1×10^7 cfu/mL of *S. enteritidis* or *E. coli* at 4 °C for 30 min. Each mixture (1 mL) containing *S. enteritidis* or *E. coli* with or without test compounds was applied to the apical side of Caco-2 cells. The cells were incubated at 4 °C for 1 h and then washed three times with PBS to remove the unbound bacteria from the cell surface. After treatment of the cells with PBS containing 0.1% Triton X-100, the cell extracts were diluted with PBS and plated on TSA. The activity of a sample to inhibit bacterial binding to Caco-2 cells was estimated as follows: adhesion (%) = (cfu of bound bacteria with a test compound/cfu of bound bacteria without a test compound) × 100.

Bacterial Infection. Female BALB/c mice (6 weeks old, Japan SLC, Inc., Shizuoka, Japan) were given water containing 0.1% SGP−CMC, SGP−CMD, CMC, and SGP ad libitum from 2 days before inoculation. The same water with or without test compounds was continuously given to mice throughout this experiment. The mice were divided into eight groups, each of which consisted of 10−11 animals. Oral inoculation was performed with 2.2×10^8 cfu/head of *S. enteritidis*, and mortality was observed for 13 days after infection. As a positive control, the mice administered water with no additives were infected with *S. enteritidis*, whereas the mice given water containing each additive without *S. enteritidis* infection were used as a negative control.

Distribution of [^{125}I]SGP−CMD Conjugate in Mice. [^{125}I]SGP was prepared according to a method described previously (*8*). With this [^{125}I]SGP, a [^{125}I]SGP−CMD conjugate was prepared in the same manner as SGP−CMD. The amount of SGP introduced into [^{125}I]SGP−CMD was determined from the level of [^{125}I]SGP radioactivity, as 11.25 residues of SGP per mol.

BALB/c mice about 25 g in body weight were orally administered 0.2 mL of 0.04% [^{125}I]SGP (specific radioactivity, 9.32×10^6 cpm/mg) or [^{125}I]SGP−CMD (specific radioactivity, 2.16×10^5 cpm/mg). The mice were divided into five groups (four heads/group), kept individually in a metabolic cage, and fed a standard diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum. At 0, 6, 24, 48, and 72 h after administration, blood was taken by heart puncture, and urine and feces were collected. Plasma was obtained from the blood after centrifugation at 3000*g* for 5 min at 4 °C. The radioactivity in each sample was measured with a γ-counter as described previously (*8*). The distribution of [^{125}I]SGP or [^{125}I]SGP−CMD in each sample was expressed as a ratio of the radioactivity in 1 mL of plasma, 1 mL of urine, or 100 mg of feces to the overall radioactivity calculated from the dose of [^{125}I]SGP and [^{125}I]SGP−CMD administered.

Effect of the SGP−Polysaccharide Conjugates on Macrophage Activation in Vitro. To examine the direct effect of the conjugates on the activation of macrophages, we used a murine macrophage-like cell line (J774.1/JA-4) as described previously (*18*). To test for macrophage activation, levels of TNF-α, IL-1β, and nitric oxide production were

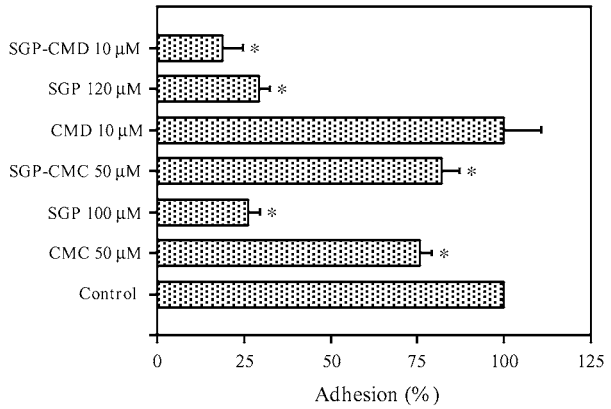

Figure 2. Inhibitory effect of SGP–CMC, SGP–CMD, CMC, CMD, and SGP on the binding of *S. enteritidis* to Caco-2 cells. *S. enteritidis* was preincubated with various concentrations of these compounds for 30 min and then added to tissue culture wells containing differentiated monolayers of Caco-2 cells. After they were incubated at 4 °C for 1 h, the cells were washed and then treated with 0.1% Triton X-100 in PBS. The cell extracts were diluted and spread on TSA plates. The results were expressed as the relative bacterial number, as compared with the untreated control at 100%. The results are the means ± SEM for three independent experiments; *, significantly different from the control ($p < 0.05$).

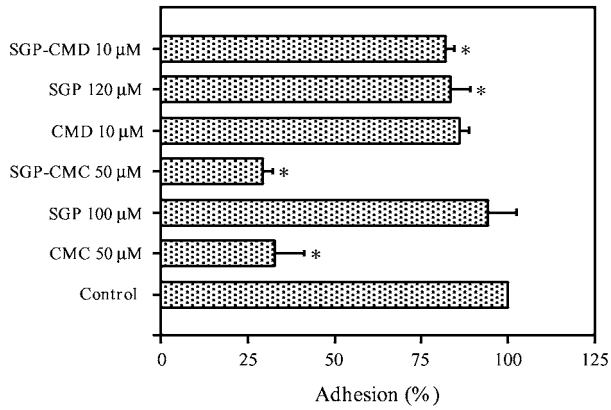

Figure 3. Inhibitory effect of SGP–CMC, SGP–CMD, CMC, CMD, and SGP on the binding of *E. coli* K-88 to Caco-2 cells. *E. coli* K-88 was preincubated with various concentrations of these compounds for 30 min and then added to tissue culture wells containing differentiated monolayers of Caco-2 cells. After they were incubated at 4 °C for 1 h, the cells were washed and then treated with 0.1% Triton X-100 in PBS. The cell extracts were diluted and spread on TSA plates. The results were expressed as the relative bacterial number, as compared with the untreated control at 100%. The results are the means ± SEM for three independent experiments; *, significantly different from the control ($p < 0.05$).

measured. In brief, 2×10^5 cells/0.5 mL of Ham's F12 medium containing 10% fetal bovine serum (GIBCO, Grand Island, NY) were seeded into each well of a 24 well flat-bottomed plate (Costar 3524, Cambridge, MA), and the plate was incubated at 37 °C overnight in a CO_2 incubator (5% CO_2/95% humidified air). The medium in the wells was replaced with 0.5 mL of fresh medium, and then, the conjugates were added at 0.1% in the presence or absence of 0.5 mg/mL of zymosan. The plate was incubated at 37 °C for a further 20 h, and then, the culture supernatants were collected into microcentrifuge tubes. After centrifugation at 10000*g* for 1 min at 4 °C, the supernatants (0.25 mL) were recovered from the top of the tubes and used for TNF-α ELISA (Genzyme, Cambridge, MA), for IL-1β ELISA (R&D, Minneapolis, MN), and for estimation of the nitrite concentration with Griess−Romijin reagent as described previously (*19*, *20*).

Statistical Analyses. Data were presented as the mean ± SEM. Student's *t*-test was performed on various parameters obtained from each subject.

RESULTS AND DISCUSSION

Our previous study demonstrated that SGP, its sialyloligosaccharide, and its asialooligosaccharide derived from hen egg yolk inhibited the binding of bacteria to intestinal cells and had the ability to prevent Salmonellosis. However, these compounds did not last long in the gut due to rapid absorption from the gastrointestine (*8*), which might reduce their effectiveness. To overcome this problem, we prepared novel glycoconjugates of SGP by conjugation with carboxymethylated nondigestive polysaccharides (CMD and CMC). Among the derivatives of egg yolk, SGP is the only compound with amino groups, which react with the carboxyl groups in CMD and CMC through an EDC reaction, within the molecule (**Figure 1**). Confirmation of the conjugation was obtained by the assay of sialic acid and by the phenol−sulfuric acid method. The molar ratio of SGP to each polysaccharide in SGP−CMC and SGP−CMD was about 2:1 and 12:1, respectively. In view of these values and the molecular masses of the polysaccharides, it is considered that the binding efficiencies of CMC and CMD for SGP are similar. The molecular masses of SGP−CMC and SGP−CMD were more than 100 kDa and more than 500 kDa, respectively.

Figure 2 shows the ability of the conjugates to inhibit the binding of *S. enteritidis* to Caco-2 cells. SGP−CMD had a significant inhibitory effect at a concentration of 10 μM. Also, 120 μM SGP, which is nearly equal to the SGP concentration in 10 μM SGP−CMD, inhibited the binding but less effectively than SGP−CMD, whereas 10 μM CMD, which corresponds to the CMD concentration in SGP−CMD, did not affect the binding at all. This result showed that SGP−CMD retained the inhibitory activity of SGP against the adhesion of *S. enteritidis* to human intestinal cells. The inhibitory effect of SGP−CMD would reflect that of SGP. On the other hand, SGP−CMC at a concentration of 50 μM showed slight inhibition, which was also observed at the same concentration of CMC. The inhibitory effect of SGP−CMC was weaker than that of SGP alone, suggesting that the conjugation of SGP with CMC results in an attenuation of the biological effect of SGP. The difference in inhibitory effect between the two conjugates would result from a difference of structure.

As for the binding of *E. coli* to Caco-2 cells (**Figure 3**), SGP−CMD and SGP showed weak inhibition. In contrast, SGP−CMC strongly inhibited the binding of *E. coli* to the intestinal cells, but the inhibition seemed not to be due to the effect of SGP because CMC caused similar inhibition of the binding. The effect of SGP−CMC would result from the inhibitory activity of CMC. Interestingly, chelating agents including CMC are inhibitors of several bacterial adhesins and CMC inhibited strongly the adhesion of *E. coli* to *Saccharomyces cerevisiae* (*21*). A similar effect may have been brought about in our experiment. From the assay of the conjugates using Caco-2 cells, it was demonstrated that SGP−CMC and SGP−CMD have the ability to inhibit the adhesion of *S. enteritidis* and *E. coli* to human intestinal epithelial cells.

To confirm the inhibitory effect of these conjugates in vivo, infection experiments were performed with mice. The mice were administered water containing SGP, each polysaccharide, and the conjugates starting from 2 days before *Salmonella* inoculation to the end of the experiment. Observation of the mice was performed for 13 days after the administration. **Figure 4** shows the effect of the conjugates on the mortality of mice after *Salmonella* infection. Although the negative control group showed no deaths during this experiment (data not shown), all groups infected with *S. enteritidis* began to lose members on

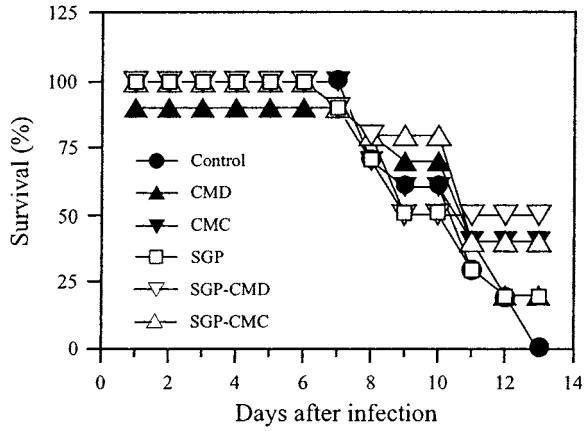

Figure 4. Effects of SGP–CMC, SGP–CMD, CMC, CMD, and SGP on the mortality of mice after *S. enteritidis* infection. Mice ($N = 10$–11) were continuously given water containing 0.1% of each compound from 2 days before *S. enteritidis* infection (2.2×10^8 cfu) to the end of the experiment. After infection, the mice were observed until day 13. The positive control group was given PBS and infected with the same dose as the other group. The negative control group was given each test compound without infection.

day 8. In the positive control group, all mice had died 13 days after infection. However, some of the mice in each group administered these compounds survived 13 days. In particular, in the SGP−CMD-treated group, 50% of mice survived under the conditions. In contrast, the survival rate for the group treated with SGP or CMD was 20%. Hence, it is conceivable that the reduced mortality from SGP−CMD is due to the effect of the conjugation of SGP and CMD. On the other hand, the survival rate of the SGP−CMC-treated group was 40%, which was equal to that of the CMC-treated group. Hence, this protective effect of SGP−CMC does not seem to exclude the possibility of a nonspecific effect of CMC. Although the mechanism of the effect of CMC is largely unknown at present, the chelating properties of CMC (*21*) may be involved. Taken together, SGP−CMD seems to be the best of these compounds for preventing *Salmonella* infection in vivo.

To evaluate the half-life of SGP−CMD in vivo, the fate of the conjugate in mice was examined with [^{125}I]SGP−CMD (**Figure 5**). As a reference, [^{125}I]SGP was also administered and treated similarly throughout this experiment. The level of [^{125}I]SGP−CMD in plasma was less than 0.1% of the total radioactivity administered until 72 h after ingestion (**Figure 5a**). A peak of SGP appeared at 6 h after ingestion in this study (**Figure 5a**), although it might not be the maximum because the level in plasma was highest at 2 h after administration in our previous study (*8*). Nevertheless, SGP was detected in plasma at higher levels than SGP−CMD throughout this experiment. In urine (**Figure 5b**), the amount of [^{125}I]SGP−CMD was about 8% of the total administered, whereas that of [^{125}I]SGP was approximately 36% at 6 h after the administration. In feces (**Figure 5c**), about 22% of [^{125}I]SGP−CMD was detected at 6 h postadministration and then the amount decreased gradually. On the other hand, the amount of [^{125}I]SGP remained below 1% until 72 h. Consequently, SGP−CMD was mostly detected in feces, whereas SGP existed mainly in urine. These results suggest that SGP−CMD possesses different properties from SGP, being poorly absorbed into the blood and mostly remaining in the gastrointestinal tract until excreted. Therefore, the properties of SGP−CMD make it suitable as a food ingredient for the prevention of intestinal infectious diseases.

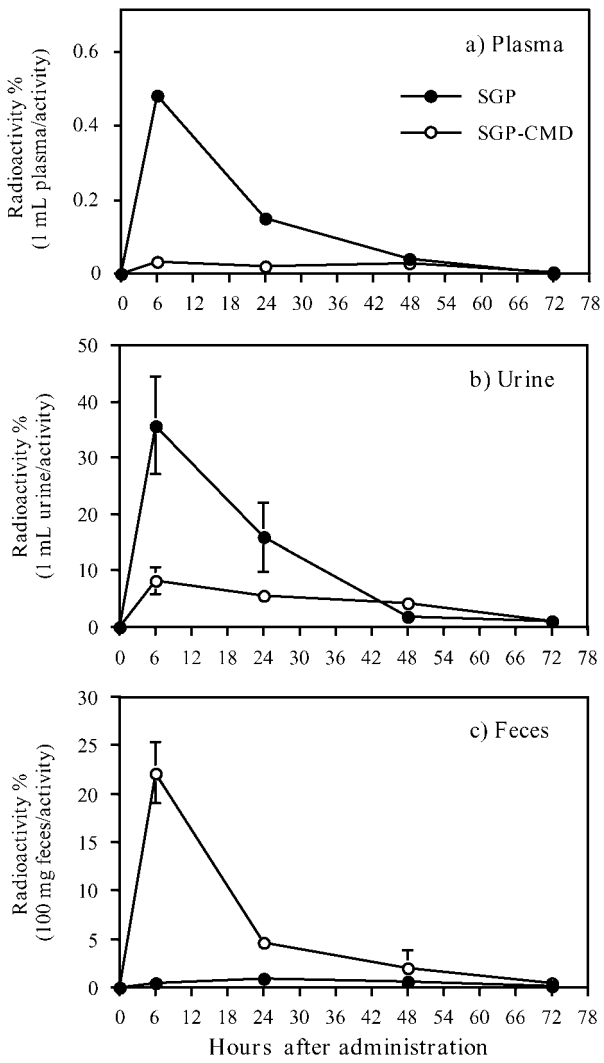

Figure 5. Fate of SGP and the SGP–CMD conjugate after oral administration in mice. Mice ($N = 4$) were orally given [^{125}I]SGP or [^{125}I]SGP–CMD, and then, blood, urine, and feces were collected at 6, 12, 24, 48, and 72 h after administration. Radioactivity was measured with a γ-counter. Time course of radioactivity in plasma (**a**), urine (**b**), and feces (**c**). The amount of [^{125}I]SGP and [^{125}I]SGP–CMD in these fractions was evaluated as the amount of radioactivity recovered in 1 mL of plasma, 1 mL of urine, or 100 mg of feces relative to the overall radioactivity calculated from the dose of [^{125}I]SGP and [^{125}I]SGP–CMD administered.

Furthermore, the influence of the conjugates on the immunosystem was examined using a mouse macrophage cell line. None of the reagents themselves significantly induced TNF-α, IL-1β, or NO_2^- production by the macrophages (**Figure 6**). However, in zymosan-treated macrophages, CMD, SGP−CMD, CMC, and SGP−CMC partly inhibited TNF-α production (**Figure 6a**), and CMD and SGP−CMD strongly inhibited NO_2^- production (**Figure 6c**). In contrast, SGP−CMC promoted IL-1β production slightly (**Figure 6b**), while none of the others did. These results suggest that SGP−CMD does not induce inflammation itself but has antiinflammatory effects to reduce TNF-α and NO_2^- production by zymosan-treated macrophages. It seems feasible that these antiinflammatory effects are attributable to CMD, the polymer support of SGP−CMD. It is not known why IL-1β production from zymosan-treated macrophages was not inhibited by CMD, SGP−CMD, CMC, or SGP−CMC as TNF-α production was, but it may be because

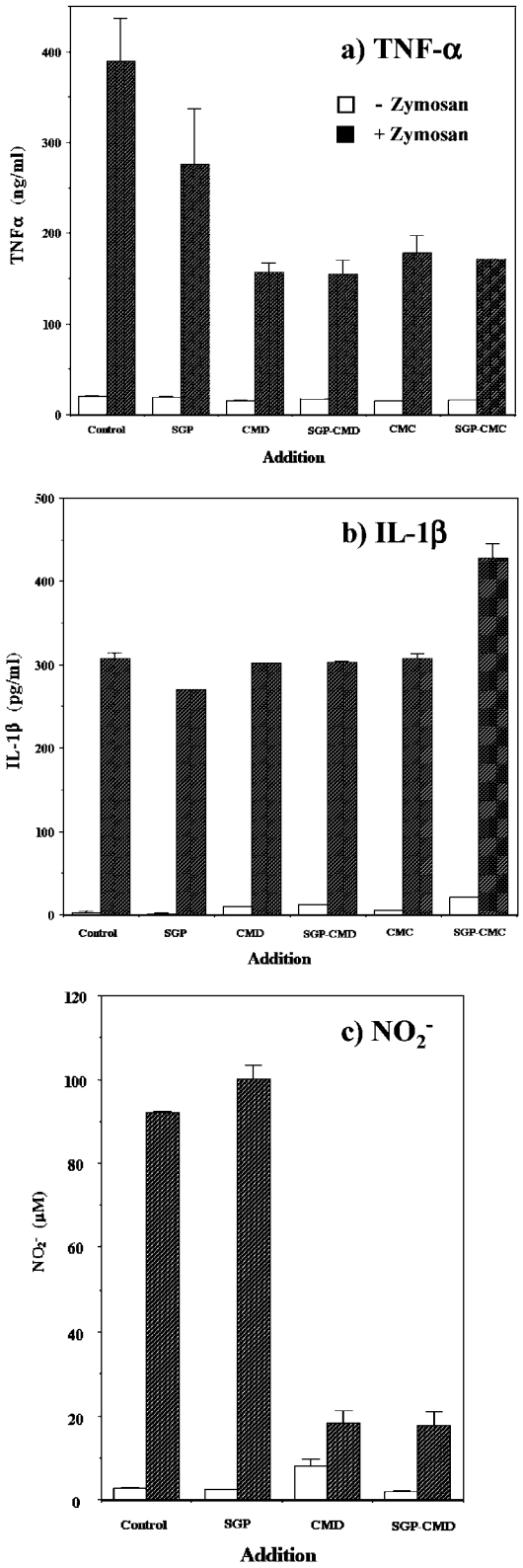

Figure 6. Effects of the SGP–CMD and SGP–CMC conjugates, CMC, CMD, and SGP on macrophage activation. J774.1/JA-4 macrophages were precultured overnight and treated with SGP–CMD, SGP–CMC, CMC, CMD, or SGP with or without 0.5 mg/mL of zymosan at 37 °C for 20 h. The culture supernatants were collected and analyzed for TNF-α (**a**), IL-1β (**b**), and NO_2^- (**c**) as described in the text. In panel **c**, SGP–CMC and CMC interfered with the Griess reagents resulting in turbidity of the reaction mixture. Results are the means ± SEM for three independent experiments.

the activation of macrophages and the production of cytokines by macrophages do not always share common pathways of induction or regulation (*22*, *23*).

Taken together, although SGP−nondigestive polysaccharide conjugates had inhibitory activity against the adhesion of pathogenic bacteria to human intestinal cells, SGP−CMD was superior to SGP−CMC in terms of the preventive effect on *S. enteritidis* infection in vivo. In addition, it was found that SGP−CMD could remain longer in the gut than SGP and a large portion of SGP−CMD would be excreted in feces. It is expected that such a novel conjugate would be excreted through the intestine after binding to pathogenic bacteria. SGP−CMD is potentially useful as a food ingredient with a preventive effect on *Salmonella* infection. In this study, the conjugates of SGP and the polysaccharides were prepared using an EDC reaction. However, for applications in food, a safer method such as an enzymatic reaction needs to be introduced into the process of preparing the conjugates. On the basis of the idea of creating conjugates of oligosaccharides with other functions and nondigestive polysaccharides, novel multifunctional glycoconjugates will be developed soon.

ABBREVIATIONS USED

CMC, carboxymethyl cellulose; CMD, carboxymethyl dextran; *E. coli*, *Escherichia coli*; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; ELISA, enzyme-linked immunosorbent assay; IL-1β, interleukin-1 β; NO_2^-, nitrite ion; PBS, phosphate-buffered saline; *S. enteritidis*, *Salmonella enteritidis*; SGP, sialylglycopeptide; TNF-α, tumor necrosis factor α; TSA, trypticase soy agar.

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