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Inhibition of Bacterial Adhesion and Salmonella Infection in BALB/c Mice by Sialyloligosaccharides and Their Derivatives from Chicken Egg Yolk

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The effects of an egg-yolk-derived sialyloligosaccharide (YDS), asialo-YDS, and a sialylglycopeptide of YDS (SGP) on bacterial adhesion to intestinal epithelial cells and on Salmonella infection in BALB/c mice were examined. YDS, its derivative asialo-YDS, and SGP strongly inhibited the binding of *Salmonella enteritidis* but not *E. coli* K-88 to a human epithelial cell line, Caco-2. In a Salmonella infection experiment using BALB/c mice, oral administration of these reagents effectively prevented the bacteria from proliferating in spleen, as well as preventing lethality. An experiment using radioactive SGP orally administered to mice revealed that the compound was absorbed from the intestine into blood and eliminated via urine within 8 h. However, these reagents did not influence the production of TNF-α or NO• in culture macrophages. The results suggest that they inhibit Salmonella infection not by activating macrophages but by inhibiting the entry of bacteria through the gut, suggesting that YDS and its derivatives are useful for preventing Salmonella infection when ingested continuously.

KEYWORDS: Sialyloligosaccharides; sialyologosaccharide-conjugated peptide; bacterial adhesion; Salmonella infection; *Salmonella enteritidis*; *E. coli*; Caco-2 cells

INTRODUCTION

Because the adhesion of bacteria to intestinal epithelial cells is crucial to the initial phase of infection (*1*), blocking the adhesion in the gastric intestine may prevent infections of bacteria such as Salmonella. The adherence of bacteria to cell surfaces is known to be mediated in at least three ways: by lectin−carbohydrate recognition (*2*−*3*), by protein−protein recognition (*4*), and by hydrophobic protein recognition (*5*). On the basis of these mechanisms, several strategies have been proposed to develop lectin-like molecules or dietary compounds containing oligosaccharides or glycoconjugates as binding receptor analogues.

Oligosaccharides in human milk have been shown to prevent bacteria and toxins from attaching to intestinal cells. Galβ1-4GlcNAc β1-3Gal β, a trisaccharide found in human milk, was shown to inhibit the adherence of *Streptococcus pneumoniae* to buccal epithelial cells (*6*). Fucosylated oligosaccharide inhibited the binding of *Campylobacter jejuni*, (*7*), strains of *E. coli* (*8*), and their heat-stable toxin (*9*−*10*) to enterocytes. Blocking of the binding of influenza A virus and S-fimbriated enteropathogenic *E. coli* has been reported to inhibit the binding of their respective target cells by sialylated oligosaccharides in human milk (*11*). On the basis of these findings, the oligosaccharides contained in foods are expected to provide protection against infectious gastric diseases by preventing bacteria and toxins from attaching to target cells.

Koketu et al. (*12*) reported that sialylated oligosaccharides from egg yolk significantly inhibited rotavirus infection both in vitro and in vivo. They pointed out that sialic acid derivatives are effective anti-rotaviral agents, and suggested that the sialic acid moiety of the oligosaccharides plays an important role in the inhibition (*13*). There is also a possibility that sialyloligosaccharides block the binding of typical food-borne bacteria, such as *Salmonella* and *E. coli*, to intestinal epithelial cells.

In this study, we examined the effects of a sialyloligosaccharide and its derivatives on the binding of *Salmonella* and *E. coli* to cells of a human epithelial intestinal cell line, Caco-2, and the protective effect of these compounds on Salmonella infection by oral administration. We also examined the fate of the compound in mice as well as its effects on macrophage activation, because it seemed feasible that the sugar residues on the surface of the bacteria mediate the activation of immune-competent cells such as macrophages.

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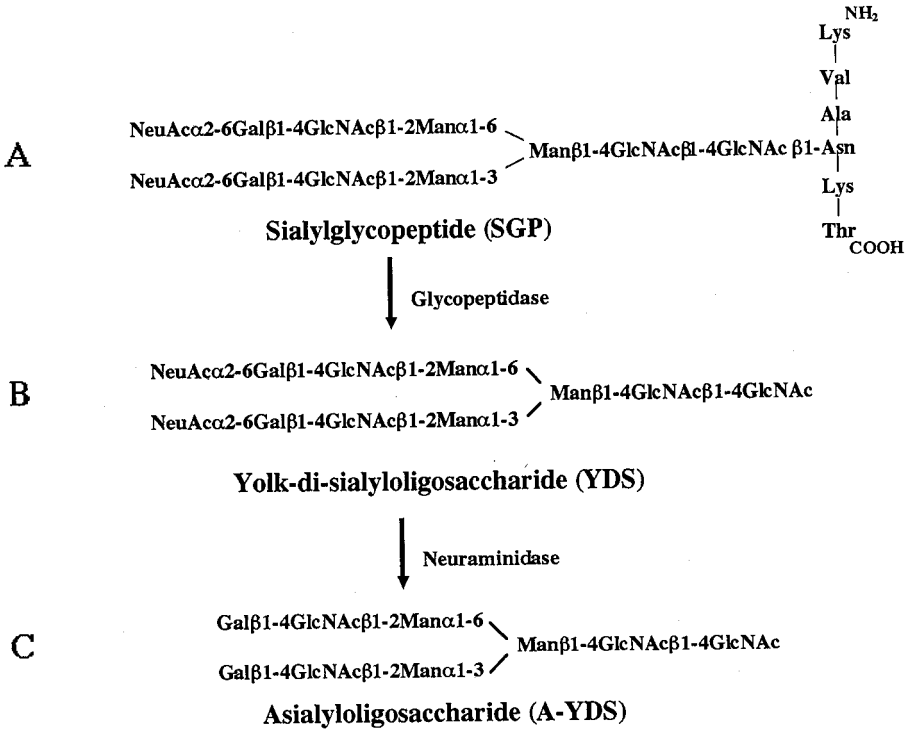

Figure 1. Structure of YDS, asialo YDS, and SGP isolated from egg yolk. A, Sialylglycopeptide (SGP); B, yolk di-sialyloligosaccharide (YDS); C, asialyloligosaccharide (A-YDS).

MATERIALS AND METHODS

Preparation of Oligosaccharides and their Derivatives. Sialylglycopeptide (SGP) and yolk-derived sialyloligosaccharide (YDS) were isolated from chicken egg yolk and purified as described previously (*14*). Asialo YDS (A-YDS) was prepared by sialidase treatment of YDS. In brief, 20 mg of YDS was treated with 400 μL of 0.2 M sodium acetate buffer (pH 5.0) and 0.2 U of *Arthrobactor ureafaciens* neuraminidase (Nakalai Tesque, Kyoto, Japan). The mixture was incubated at 37 °C for 48 h and then diluted with the same volume of distilled water. The enzyme-digested oligosaccharides were applied to a HiLoad Superdex pg column (Amersham-Pharmacia Biotech, Uppsala, Sweden) and monitored by measuring the absorption at 200 nm and refractive index (Shodex RI, Shimadzu, model SE-61, Tokyo, Japan). The released oligosaccharides were pooled, lyophilized, and rechromatographed using a Superdex Peptide HR column (Amersham-Pharmacia Biotech). The asialo YDS (13 mg) was obtained after lyophilization of the corresponding major peak fraction. Oligo mannose-3 (Man 3), oligo mannose-5 (Man 5), and oligo mannose-6 (Man 6) were purchased from Seikagaku Kogyo (Tokyo, Japan).

Bacterial Strains and Culture Conditions. *Salmonella enteritidis* (*S. enteridis*, strain E 930448, phage type 4) was grown overnight in trypticase soy broth (TSB, Becton Dickinson, Mountain View, CA) with shaking at 37 °C. Bacteria were collected by centrifugation at 15000×*g* for 10 min at 4 °C and were washed with ice-cold phosphate-buffered saline (PBS). For binding assays in bacterial infection experiments, appropriate numbers of the washed bacteria were resuspended in tissue-culture media without antibiotics.

Cell Culture. Caco-2 cells (ATCC, HTB37) were maintained according to Dharmsathaphorn (*15*) and Hashimoto and Shimizu (*16*). These cells were seeded at 1×10^5 cells/mL of DMEM containing 10% heat-inactivated fetal bovine serum, nonessential amino acids, and antibiotics in 24-well plates (Iwaki Glass, Tokyo, Japan), and were cultured for about 2 wk before use until the cells differentiated to a epithelial cell-like monolayer. Three hours before inoculation of bacteria, the growth medium was removed from each well and replaced with a fresh medium devoid of antibiotics. Various concentrations of oligosaccharide, its derivatives, and oligomannose were dissolved in the antibiotic-free culture medium and incubated with 1×10^7 cfu/mL of *S. enteridis* at 4 °C for 30 min. A 1-mL aliquot of each mixture containing *S. enteridis* with or without test compounds was applied to the apical side of Caco-2 cells. Cells were incubated at 4 °C for 1 h and washed 3 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 trypticase soy agar (TSA). The activity to inhibit Salmonella from binding to Caco-2 cells was estimated as follows:

$$\text{inhibitory activity (\%)} = \frac{\text{cfu of bound bacteria with oligosaccharide}}{\text{cfu of bound bacteria without oligosaccharide}} \times 100$$

Bacterial Infection. Female BALB/c mice (6 wk old, Nihon SLC, Shizuoka, Japan) were given water containing 0.1% oligosaccharide, its derivatives, or nothing for 2 days before infection. Infection was carried out orally with 5×10^6 cfu/head of *S. enteridis*, and the spleen was removed at 0, 3, 6, 9, or 12 days after infection. During the experiments, mice were continuously given water with or without test reagents. The isolated spleen was homogenized in sterile ice-cold PBS, and the diluted homogenates were plated on TSA. The colonies were counted after an overnight culture to estimate the viable Salmonella number in spleen. As a negative control, mice were given water with no additives followed by a sham administration of saline, and were not infected with Salmonella.

Preparation of Radioactive SGP. About 10 mg of SGP was dissolved in 0.1 M sodium borate buffer, pH 8.0 (buffer A), at 28.5 mg/mL, chilled on ice, and mixed with 10 μL of 250 μCi [^{125}I] Bolton−Hunter reagent (NEX120, NEN Life Science Products, Inc., Boston, MA). The reaction was performed on ice for 20 min, and quenched with 0.5 mL of 0.2 M glycine in buffer A. Finally, the reaction mixture was applied to a PD-10 column (Amersham-Pharmacia Biotech U.K. Ltd., Buckinghamshire, U.K.), which had been equilibrated with saline (Otsuka Pharmaceutical Co., Tokushima, Japan). [^{125}I] Radioactivity in aliquot of each fraction was measured in a gamma counter (Aloka, Tokyo, Japan), and the peak fractions were pooled and stored at 4 °C until use.

Distribution of [^{125}I] SGP in Mice. BALB/c mice, about 25-g body weight, were administered 0.2 mL of 1 mg/mL [^{125}I] SGP (specific radioactivity, 83.9 cpm/mg) orally. Mice were divided into 7 groups, and fed a standard diet (Oriental Co., Tokyo) and water ad lib. At times

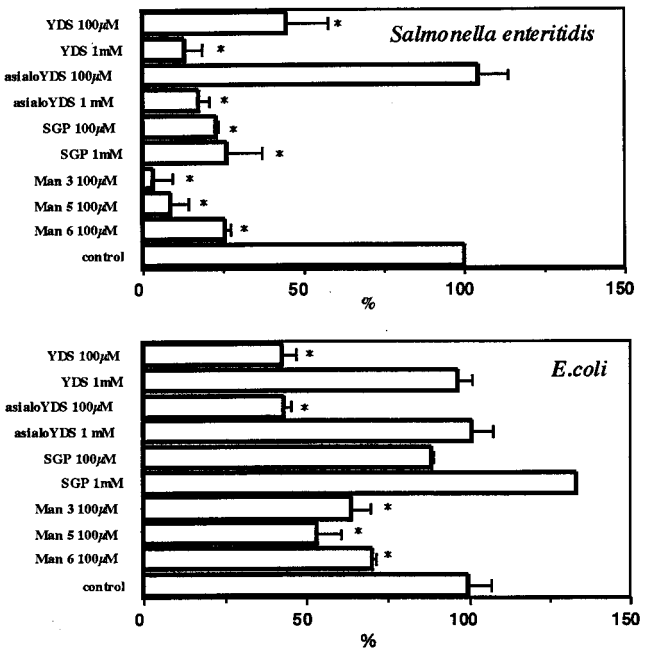

Figure 2. Inhibitory effect of YDS, asialo YDS, and SGP on the binding of *S. enteridis* to Caco-2 cells. *S. enteridis* and *E. coli* K-88 were preincubated with various concentrations of YDS and its derivatives for 30 min and then added to tissue culture wells containing differentiated monolayers of Caco-2 cells. After incubation with bacteria at 4 °C for 1 hr, the cells were washed and then treated with 0.1% Triton X-100 in PBS. The cell extracts were diluted and spread on LB agar plates. The results were expressed as the relative bacteria number, compared with the nontreated control as 100%. The results are the means ± SE for three independent experiments. Asterik (*) indicates significantly different from control ($p < 0.05$).

0, 2, 4, 6, 8, 12, and 24 h after administration, mice in each group were sacrificed, and blood, urine, whole intestine, spleen, brain, and kidney were collected. Total radioactivity was measured in a gamma counter as described above. Plasma was obtained by centrifugation at 3000*g* for 5 min at 4 °C, and the radioactivity in a 0.05-mL volume was measured. The radioactivity in urine was measured with 0.1 mL of the sample.

Effect of YDS and its Analogues on Macrophage Activation in Vitro. To examine the direct effect of YDS and its analogues on the activation of macrophages, we first checked the contamination by endotoxin in each reagent. The levels were as follows: YDS, 0.140; asialoYDS, 0.183; and SGP, 0.245 EU/mL/100 nmol. A murine macrophage-like cell line, J774.1/JA-4, maintained and cultured as described previously (*17*), was used for the experiments. For testing macrophage activation by assaying TNF-α and nitric oxide production, 2×10^5 cells/0.5 mL Ham's F12 medium + 10% FBS (GIBCO, Grand Island, NY)/well were seeded onto a 24-well flat-bottomed plate (Costar 3524, Cambridge, MA) and incubated at 37 °C overnight in a CO_2 incubator (5% CO_2/95% humidified air). The medium was replaced with 0.5 mL of fresh medium, then YDS and its analogues were added at 100 μM in the presence or absence of 100 ng/mL LPS (*E. coli* O55: B5, Sigma) and/or 10 U/mL IFN-γ (kindly provided by Toray Co., Tokyo). The cells were incubated at 37 °C for a further 20 h, and the culture supernatants were collected into microfuge tubes and centrifuged at 10000*g* for 1 min at 4 °C. The supernatants (0.25 mL) were recovered from the top and used for TNF-α ELISA (Genzyme, Cambridge, MA) and to estimate the nitrite concentration with Griess−Romijin reagent (Wako Pure Chemicals, Tokyo), as described previously (*18*−*19*).

RESULTS AND DISCUSSION

Sialyloligosaccharide isolated from egg yolk (YDS) is a biantenally branched oligosaccharide containing sialic acid (**Figure 1B**), which was prepared from sialylated glycopeptide (SGP, **Figure 1A**) by enzymatic digestion. Further, treatment of YDS with neuramidase generates an asialoligosaccharide, bi-Galβ1-4GlcNAc β1-2Manα1 (asialo-YDS, **Figure 1C**)).

The inhibitory effects of YDS, asialo-YDS, and SGP on the binding of *S. enteritidis* or *E coli*. K-88 to Caco-2 cells are shown in **Figure 2**. Because oligomannose has been reported to be a typical inhibitor of the binding of *Salmonella typhimurium* (*20*) and *E. coli* pilli (*21*) to epithelial cells, tri-mannose, penta-mannose, and hexa-mannose were used as positive controls. In the binding assay of *S. enteritidis*, YDS and its derivatives prevented *S. enteritidis* from attaching to Caco-2 cells as effectively as the control oligomannoside. This inhibition was concentration-dependent, with an approximately 80% reduction at 1 mM. However, the binding of *E. coli* K-88 to Caco-2 cells was not inhibited by these oligosaccharides stoichiometrically. A low dose of YDS and asialo-YDS (100 μM) had a stronger inhibitory effect than a high dose (1 mM), whereas SGP did not inhibit the binding significantly at 100 mM but enhanced it at 1 mM. The extent of the inhibition with YDS and asialo YDS was around 50% at the concentration of

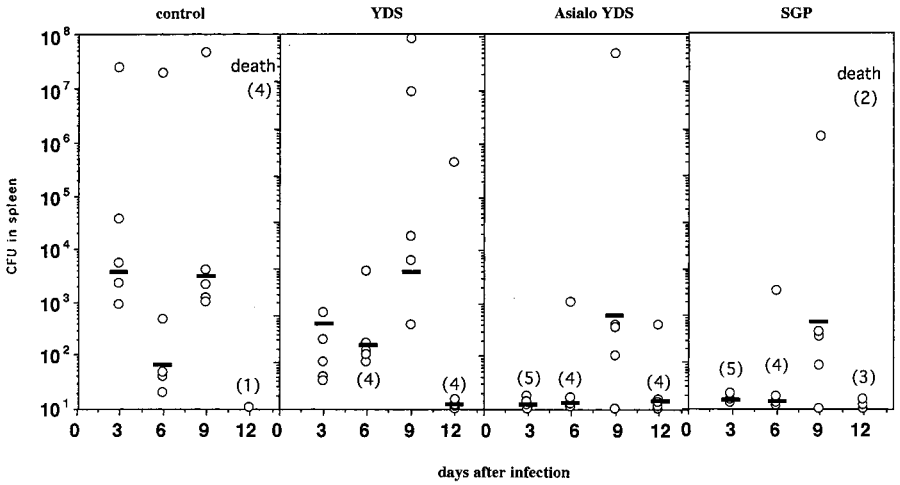

Figure 3. Effects of YDS, asialo YDS, and SGP on bacterial numbers in spleen after *S. enteridis* infection. Mice were given water containing 1 μM YDS, asialo YDS, or SGP, and then infected with 5×10^6 cfu of *S. enteridis*. After infection, the spleen was removed at day 3, 6, 9, or 12. This was followed by homogenization, dilution, and plating on agar. Bars express the median of bacterial number of spleen in each group ($N = 5$). At day 12, the median was not calculated in the group mice died (control and SGP).

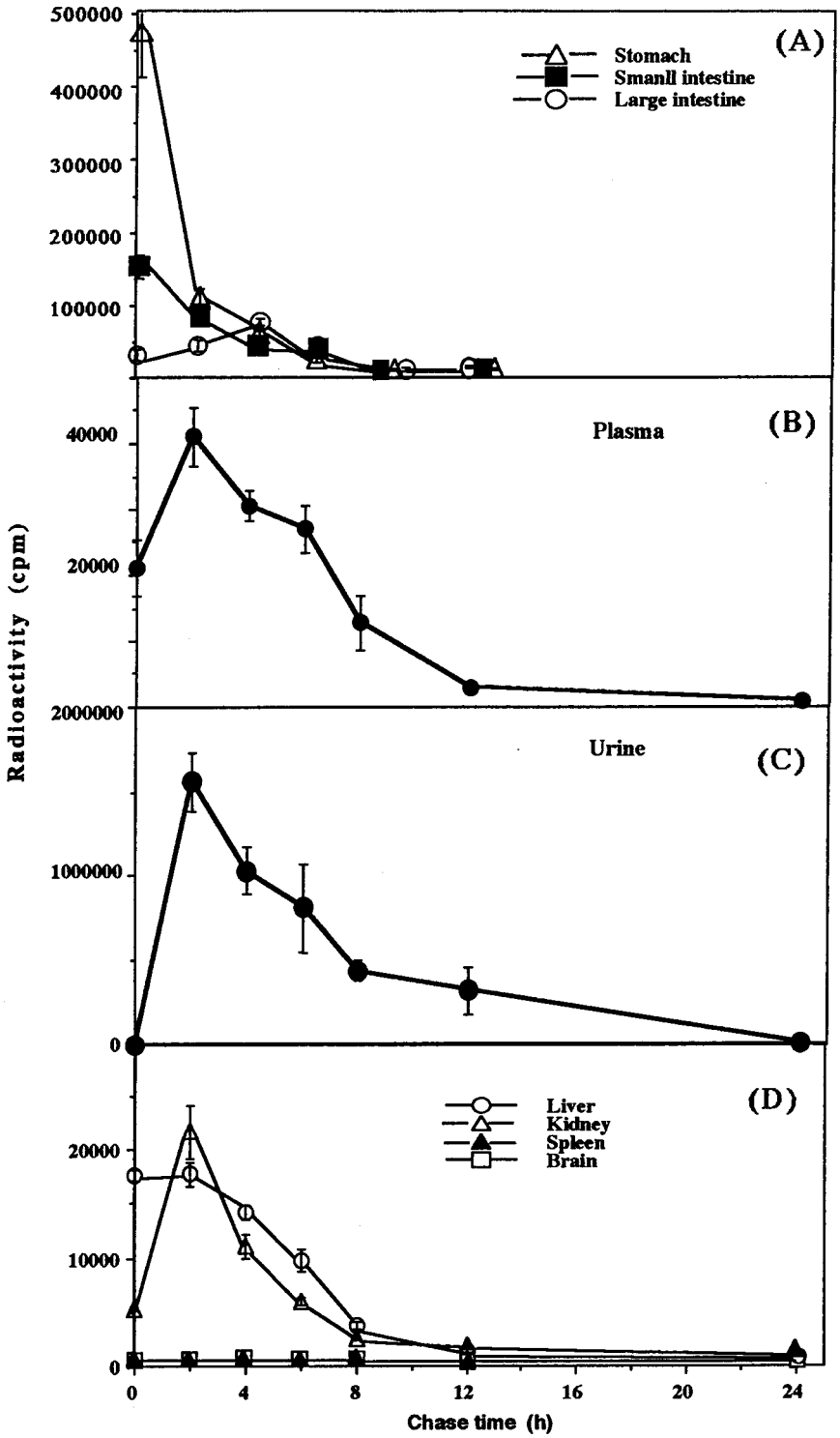

Figure 4. Fate of SGP after oral administration. Mice were given ^{125}I-SGP orally and then blood, urine, intestines, kidney, spleen, liver, and brain were collected at 2, 4, 6, 8, 12, and 24 hrs after administration. Their radioactivity was measured with a γ-counter. (A) Time course of radioactivity in the gastrointestinal tract: △, stomach; ■, small intestine; ○, large intestine; (B) time course of radioactivity in plasma; (C) time course of radioactivity in urine; (D) time course of radioactivity in other organs: ○, liver; △, kidney; ▲, spleen; and □, brain.

100 μM, similar to those of Man 3, Man 5, and Man 6, which were used as positive controls.

These results show that YDS and its derivatives prevent *S. enteritidis* from attaching to Caco-2 cells, and suggest that the inhibitory effects of these oligosaccharides are selective. Sialic acid residues (NeuAc-R2) have been reported to be possible binding sites of *E. coli* fimbrie (*22*). However, the sialic acid contained in YDS does not seem to play a crucial role in the binding of *S. enteritidis* and *E. coli* K-88 to Caco-2 cells, because YDS and asialo-YDS showed similar inhibitory effects on the bacterial attachment. Instead, it seems feasible that a biantenary oligosaccharide structure is involved in the recognition of Caco-2 by *S. enteritidis*. Besides, SGP was less effective than YDS in stopping the binding of *S. enteritidis* even at 1 mM. Although SGP has the same glyco-structure as YDS, its peptide may regulate the competition between the oligosaccha-

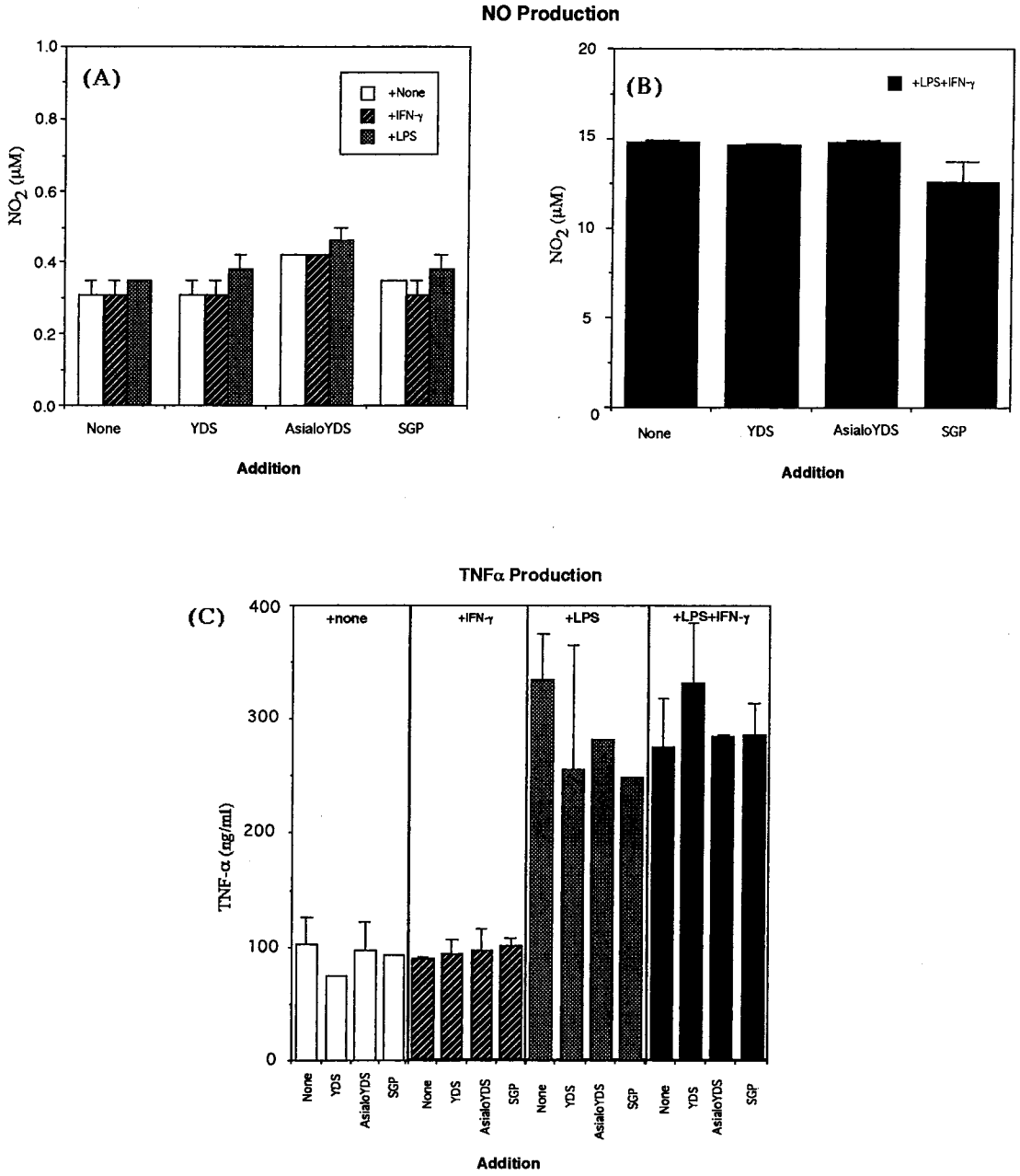

Figure 5. Effects of YDS, asialo YDS, and SGP on macrophage activation. J774.1 macrophage-like cell line was seeded at 2×10^5 cells/0.5 mL/well and was incubated with 100 μM YDS, asialo YDS, or SGP in the presence or absence of 100 ng/mL LPS and/or 10 U/mL IFN-γ at 37 °C for 20 h, as described in the text. The culture supernatants of these macrophages were collected and analyzed for nitrite (A, B) or TNF-α (C). The results are the means ± SE for three independent experiments.

ride and the binding site of the bacteria. The difference in binding abilities to bacteria between YDS and SGP may be due to the changing of electrostatic charges or conformation of the whole molecule.

Because YDS and its derivatives were found to have anti-adhesive effects on *S. enteritidis* in in vitro assays, we examined the protective effects of these reagents in mice. To maximize the inhibitory effects on the binding of *S. enteritidis* to mouse intestine, we administered the reagents prior to infection, expecting a rapid delivery of the agents to the intestinal tract. In this experiment, the mice were given water containing YDS or its derivatives from 2 days before the infection. After the infection, the bacterial number in spleen, recognized to be an indicator of systemic infection of Salmonella, was counted (**Figure 3**). The numbers at 0, 3, 6, 9, and 12 days after infection showed that in control mice the level of bacterial infection was more than 10^3 cfu/spleen at day 3, while in YDS-, asialo-YDS- or SGP-treated mice, it was suppressed to less than 10^3 cfu/spleen or to below the detection level (less than 20 cfu/spleen). These results show that YDS and its derivatives inhibit *S. enteritidis* from moving from the gut lumen to the spleen. Although the bacterial numbers in spleen increased with time in the mice treated with reagents, the extent of the increase was significantly less than that in the control mice (**Figure 3**). By day 12, 4 out of 5 mice in the control group had died (mortality 80%), whereas all of the mice given YDS or asialo-YDS were still alive. However, 2 out of 5 mice given SGP died on day 12. These results suggest that YDS and its derivatives provide protection against lethal infection of *S. enteritidis* in vivo. If the agent is to be administered orally, it is important to know

its fate in the gastrointestinal tract. Ideally, it would remain in the tract for some time, providing complete inhibitory activity. For the experiments on the fate of these reagents, SGP was used because its structure lends itself to radio labeling with [^{125}I] Bolton−Hunter reagent. BALB/c mice were administered with ^{125}I −SGP orally and the distribution of the radioactivity to tissues was traced. As shown in **Figure 4A**, ^{125}I−SGP disappeared from the stomach within 2 h, moved to the small intestine, and then appeared in the large intestine where a peak of the radioactivity was observed at 2 h after ingestion. The fate of ^{125}I−SGP in plasma and urine showed the similar patterns with a peak at 2 h after ingestion followed by a gradual decrease (**Figure 4B,C**). Radioactivity was also found in the liver and kidney, but little radioactivity was detected in brain or spleen (**Figure 4D**). The percentage of ^{125}I−SGP recovered in blood within 8 h was estimated at about 0.2%. More than 90% of the^{125}I−SGP was found in feces. These results show that SGP is absorbed by the intestine within 4 h of ingestion and subsequently eliminated via blood and urine. These results also indicate that the reagent would disappear from the gastrointestinal tract within a short period, suggesting the necessity for modifications.

Oligosaccharides on bacterial cells are known to act as ligands for immune-competent cells such as macrophages (*23*). For example, the binding of bacteria to macrophages stimulates the production of cytokines in these cells, leading to activation of the macrophages and subsequent regulation of the immune system. To assess the biological effects of YDS and its derivatives on the activation of macrophages either directly or via LPS and/or IFN-γ, the compounds were added to cultures of a mouse macrophage-like cell line, J774.1/JA-4, and TNF-α and nitrite production were examined (**Figure 5**). None of the reagents alone induced the production of nitrite (**Figure 5A**) or TNF-α (**Figure 5C**) by macrophages. Nor did they stimulate or suppress production of nitrite (**Figure 5A,B**) or TNF-α (**Figure 5C**) in the presence of LPS and/or IFN-γ, although LPS (but not IFN-γ) induced production of TNF-α (**Figure 5C**), and both LPS and IFN-γ were necessary to induce significant production of nitric oxide (**Figure 5B**) in this system. These results suggest that YDS and its derivatives per se have little effect on macrophage activation.

Taken together, these findings suggest that YDS, asialo-YDS, and SGP have anti-bacterial properties and provide protection against gastric diseases such as Salmonella infection by preventing bacteria from binding to the intestine rather than by activating macrophages. These reagents were found to be rapidly absorbed from the gastrointestinal tract. If this turnover can be slowed, these oligosaccharides and their derivatives have the potential to be used as functional food ingredients to prevent Salmonella infection.

ABBREVIATIONS USED

YDS, egg-yolk-derived sialyloligosaccharide; SGP, sialylglycopeptide of YDS; LPS, lipopolisaccharide; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha; *S. enteritidis*, *Salmonella enteritidis*; E. coli, *Escherichia coli*; PBS, phosphate-buffered saline.

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