Prevention of Intestinal Infection by Glycomacropeptide

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The preventive effects of glycomacropeptide (GMP) against intestinal infection were investigated, and conjugates of GMP with xylooligosaccharide (XOS) and carboxymethyl dextran (CMD) were prepared by the Maillard reaction to enhance the effect of GMP. The binding ability of GMP to intestinal pathogenic bacteria was evaluated by a binding assay with biotinylated bacteria. GMP showed the ability to bind to Salmonella enteritidis and enterohemorrhagic Escherichia coli O157:H7 (EHEC O157). This binding ability was decreased by a sialidase treatment and completely eliminated by periodate oxidation. These results indicate that such carbohydrate moieties as sialic acid in GMP are involved in binding to S. enteritidis and EHEC O157. The preventive effect of GMP on the adhesion of pathogenic bacteria to Caco-2 cells was also investigated. GMP showed an inhibitory effect on the adhesion of EHEC O157 in a dose-dependent manner, although it was not a potent inhibitor of the adhesion of Salmonella infection. However, in the case of Salmonella infection, GMP–XOS and GMP–CMD significantly suppressed IL-8 production which was the index of infection. Our results indicate GMP to be a promising agent for preventing intestinal infection.

Key words: intestinal infection; glycomacropeptide; prevention of infection; Salmonella enteritidis; Escherichia coli O157:H7

Infectious diseases caused by Salmonella enteritidis, enterohemorrhagic Escherichia coli O157:H7 (EHEC O157) and other pathogenic bacteria have been increasing in recent years, and this trend is becoming a major social problem.¹² It is therefore important for effective methods to prevent such infectious diseases to be developed. However, the development of vaccines to such infections has not advanced and involves some problems with safety. Studies in recent years on the mechanisms for infectious diseases have revealed that specific oligosaccharides are involved when infections with bacteria, bacterial toxins and viruses strike the living body. The initial phase of infection is brought about by the adhesion of lectins on the surface of bacteria to specific receptors on intestinal epithelial cells. Mannose and sialic acids have been found to be involved in specific receptors.³⁴ Hence, such infection could be prevented by blocking the adhesion of pathogenic bacteria to the intestinal epithelial cells. Food components which block the adhesion of bacteria are promising as agents for preventing such infection. Studies have so far revealed that several carbohydrate components in food exerted a protective effect against intestinal infection. Sialylated oligosaccharides in bovine and human milk suppressed the adhesion of Helicobacter pylori to gastrointestinal epithelial cells,⁵ while sialylglycopeptides and their derivatives from egg yolk had strong inhibitory activity against the binding of S. enteritidis to Caco-2 cells.⁶ Mannosylated glycopeptides inhibited the binding of enterohemorrhagic Escherichia coli to intestinal cells,⁷ and we have also found that a glycopeptide derived from hen egg ovomucin had the ability to bind EHEC O157.⁸ Our primary objective has been to find novel materials from food that can prevent intestinal infection. In addition, such saccharide molecules as mannose and sialic acid, which are promising for preventing intestinal infection, would be easily adsorbed to the intestines. Our secondary objective has been to enhance the stability of glycopeptides, which have the ability to bind to pathogenic bacteria in a living body, by preparing neoglycoconjugates with non-digestible saccharides.

We investigate in this present study the preventive effects against intestinal infection of the glycomacropeptide (GMP) obtained by the chymocin digestion of...
bovine kappa-casein, GMP is known to have many biological functions such as binding toward cholera and Escherichia coli enterotoxin, suppressing gastric secretions, promoting bifid bacterial growth, modulating immune responses, and inhibiting bacterial and viral adhesion. The amino acid sequence, carbohydrate binding sites and structure of the bound carbohydrate of GMP have been clarified in detail. Since GMP contains sialic acids, we anticipated that GMP would be able to bind to pathogenic bacteria and could be an agent for preventing intestinal infection. We evaluate here the binding ability of GMP to pathogenic bacteria and we attempt to enhance the stability in vivo by conjugating with the non-digestible saccharides, carboxymethyl dextran (CMD) and xylooligosaccharide (XOS). We describe the preventive effect of GMP against intestinal infection and the unique properties of the GMP–CMD and GMP–XOS conjugates.

Materials and Methods

Materials. Glycomacropeptide (GMP) was supplied by Meiji Dairies Corporation (Tokyo, Japan). Asialo-GMP was prepared by the sialidase (neuraminidase) treatment. GMP (20 mg) was dissolved in 2 ml of a 0.1 M acetate buffer (pH 5.5). A sialidase solution from Arthrobacter ureafaciens (0.1 U/50 μl) was added to the GMP solution, and the mixture was incubated at 37°C for 24 hr. Desialylation of GMP was confirmed by the periodate-resorcinol method. Periodate oxidation of GMP was performed according to the method of Raqib et al. A GMP solution (5 mg/1 ml of a 0.1 M acetate buffer containing 50 mM sodium periodate (pH 5.5)) was incubated in the dark for 20 min. After dialyzing against distilled water, the solution was lyophilized.

Xylooligosaccharide (XOS) was supplied by Suntory (Osaka, Japan). The total saccharide content was measured by the phenol-sulfuric acid method. The chemical features of GMP were measured with an automatic amino acid analyzer (Hitachi, Tokyo, Japan), after hydrolyzing GMP with 6 M HCl at 110°C for 20 hr in vacuo. The saccharide content of GMP was measured by the phenol-sulfuric acid method. The monosaccharide components of GMP were measured with an ABEE labeling kit plus S (Honen Co., Tokyo, Japan) and a Honenpak C18 column (Honen Co., Tokyo, Japan) according to the manufacturer’s directions.

Preparation of the GMP–CMD conjugate. The GMP–CMD conjugate was prepared by the Maillard reaction. GMP (1.0 g) and CMD (6.3 g) were dissolved in distilled water (100 ml) and lyophilized. The mixture was incubated at 50°C at a relative humidity of 79% for 24 hr. The reaction product was dissolved in water and centrifuged at 25°C and 11,000 rpm for 30 min. The resulting supernatant was dialyzed against distilled water and lyophilized to obtain a GMP–CMD conjugate. Conjugation between GMP and CMD was confirmed by SDS–PAGE. The peptide content and saccharide content of the GMP–CMD conjugate were respectively measured by the absorbance at 230 nm and by the phenol-sulfuric acid method.

Preparation of the GMP–XOS conjugate. The GMP–XOS conjugate was prepared by the Maillard reaction. GMP (500 mg) and XOS (1.6 g) were dissolved in distilled water and lyophilized. The mixture was incubated at 70°C at a relative humidity of 79% for 9 days. The reaction product was dissolved in water and centrifuged at 25°C and 11,000 rpm for 30 min, and the resulting supernatant was recovered. Free CMD in this reaction mixture was removed by salting-out with ammonium sulfate. After dialyzing against distilled water and lyophilizing, the GMP–CMD conjugate was obtained. Conjugation between GMP and CMD was confirmed by SDS–PAGE. The peptide content and saccharide content of the GMP–CMD conjugate were respectively measured by the absorbance at 230 nm and by the phenol-sulfuric acid method.

Bacteria. The bacteria used for the binding assay were EHEC O157 (No. 212, patient), Vibrio parahaemolyticus (VP15, scallop), S. enteritidis (ATCC 13076), Staphylococcus aureus (EKN 5723), Listeria monocytogenes (JCM 2873), Salmonella arizonae (ATCC 13314), Lactobacillus casei (ATCC 393), Legionella pneumophillus (ATCC 33152), Bacillus cereus (ATCC 13061), Morganella morganii (ATCC 25830), Enterococcus faecalis (ATCC 29212), Streptococcus mutans (ATCC 25175), Candida albicans (ATCC 90028), Clostridium
perfringens (ATCC 13124), Serratia marcesens (EKN 420), Yersinia ruckeri (ATCC 29473) and E. coli (ATCC 11775). All bacteria were subjected to a heat treatment and suspended in phosphate-buffered saline (PBS).

For the in vitro assay using a Caco-2 monolayer, S. enteritidis was grown overnight in a tryptose soy broth (TSB, Becton Dickinson) while shaking at 37 °C. The bacteria were collected by centrifugation at 3,500 rpm for 20 min, and washed with saline. Appropriate numbers of bacteria were re-suspended in a tissue culture medium without any antibiotics for the adhesion assay in the bacterial infection experiment.

EHEC O157 was grown overnight at 37 °C in LB broth (Becton Dickinson) for the adhesion assay. Pre-cultured bacteria were 20-fold diluted with fresh Dulbecco’s modified Eagle’s medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) containing 0.45% glycerol, and incubated at 37 °C for 2 hr while shaking at 80 rpm. An appropriate number of bacteria were diluted with DMEM-glycerol for binding assays in the bacterial infection experiments.

Preparation of biotinylated bacteria. The bacterial suspension was adjusted to 1 × 10^10 cells/10 ml (PBS) or the absorbance at 660 nm of the bacterial/PBS suspension (10 ml) was adjusted to 1.0. A 10-µg amount of EZ-Link™ Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, U.S.A.) was added to the suspension (10 ml), which was then incubated for 2 hr at room temperature. After washing three times with PBS, the biotinylated bacteria were resuspended in 10 ml of PBS. The biotinylated bacteria were stored at −80 °C until needed.

Binding assay with biotinylated bacteria. Each sample (0.5 mm; GMP, GMP–XOS, GMP–CMD, XOS and CMD) in PBS was serially diluted two-fold with PBS, and 100-µl portion of the diluted solution was added to each well of a polystyrene microtitration plate (Maxiiser; Nunc, Roskilde, Denmark), before the plate was incubated overnight at 4 °C. After removing the solution, each well was washed three times with 200 µl of PBS. Blocking was performed with 200 µl/well of a blocking reagent for ELISA (Roche Molecular Biochemicals, Basel, Switzerland) at 25 °C for 1 hr, and then the plate was washed three times with PBS. A biotinylated bacterial suspension (100 µl) was added to each well of the plate, and the plate was incubated at 25 °C for 1 hr. After washing three times with PBS, 100 µl of alkaline phosphatase-labeled streptavidin (Zymed) was added to each well, and the plate was again incubated at 25 °C for 1 hr. The plate was then washed three times with PBS, before a 0.1% p-nitrophenyl phosphate disodium/diethanolamine–hydrochloride buffer solution (pH 9.8) was added to each well and the plate incubated at 25 °C for 30 min. After adding a 5 mM sodium hydroxide solution (20 µl) to each well to stop the reaction, the absorbance at 405 nm was measured with an MPR-A4i microplate reader (Tosoh, Tokyo, Japan).

Cell culture. Caco-2 cells were cultured according to the method of Hashimoto and Shimizu,23) DMEM containing 10% fetal calf serum (FCS; Bio Whittaker, Walkersville, MD, U.S.A.), 2 mM L-glutamine (Gibco BRL, Grand Island, NY, U.S.A.) and 100 µg/ml of penicillin/streptomycin (Gibco BRL) were used to culture the Caco-2 cells. The cells were seeded at a density of 1.0 × 10^4/ml/well in 24-well plates (Sumilon Celltigh; Sumitomo Bakelite, Tokyo, Japan) and then cultured at 37 °C in a 5% CO_2 atmosphere for about 10 days. The medium was refreshed every 2 days.

Evaluation of the effect of GMP on the adhesion of EHEC O157 to Caco-2 cells. EHEC O157 was pre-cultured in LB broth (Becton, Dickinson and Company) for 18 hr. The resulting EHEC O157 suspension was 20-fold diluted with DMEM without antibiotics and incubated at 37 °C in a 5% CO_2 atmosphere for 6 hr. The GMP sample and EHEC O157 suspension were mixed. A 1-ml amount of the mixture containing 1.0 × 10^5 cells/ml was added to the Caco-2 cells in each well and incubation was continued at 37 °C in a 5% CO_2 atmosphere for 30 min. The culture medium was removed from the wells, and the wells were washed 5 times with HBSS (Invitrogen, Carlsbad, CA, U.S.A.). The bacteria adhering to the Caco-2 monolayer were released by vigorous agitation with 1 ml of 0.1% Triton X-100 in PBS. The resulting bacterial suspension was diluted and plated on tryptose soy agar (TSA; Becton Dickinson). After incubating overnight at 37 °C, the number of colonies was counted.

Evaluation of the effect of GMP on the adhesion of S. enteritidis to Caco-2 cells. A 40-µl suspension of S. enteritidis (5 × 10^8 cells/ml) was mixed with 3,960 µl of a sample solution (GMP, GMP–XOS, GMP–CMD, XOS, or CMD; 0.001–0.5 mM). The mixture (1 ml) was added to the Caco-2 monolayer in a 24-well plate, and the plate was incubated at 37 °C for 1 hr. Each well was then washed three times with PBS to remove any unbound bacteria. The bacteria adhering to the Caco-2 monolayer were released by vigorously agitation with 200 µl of 0.1% Triton X-100 in PBS. The resulting bacterial suspension was diluted and plated on tryptose soy agar (TSA; Becton Dickinson). After incubating overnight at 37 °C, the number of colonies was counted.

Detection of IL-8. Caco-2 cells cultured for about 10 days in a 48-well plate (Sumilon Celltigh; Sumitomo Bakelite, Tokyo, Japan) were infected with S. enteritidis (5 × 10^7 cfu/ml) for 90 min from the apical side. After removing the apical and basal media, the cultured cells were further incubated for 5.5 hr in the presence of 100 µg/ml of gentamycin. The culture supernatant was collected 0, 1, 2, 3, 5 and 7 hr after infection. The IL-8
concentration was determined with a human IL-8 ELISA kit (Pharmingen, San Diego, CA, U.S.A.) according to the manufacturer’s instructions.

Result and Discussion

Characteristics of the GMP and GMP–saccharide conjugates

The chemical features of GMP used in this study were investigated by an amino acid analysis, SEC and SDS–PAGE. As shown in Table 1, the amino acid composition of the GMP sample used in this study was similar to the theoretical value for the amino acid composition of kappa-casein f106–169. We considered that this GMP sample did not contain other proteins or peptides and was of sufficient quality for the present study.

As shown in Fig. 1a, a single peak was apparent for GMP in the SEC analysis. The molecular weight of the GMP sample was calculated to be 45 kDa. The theoretical value for the molecular weight of GMP is 7.5 kDa, so we consider that GMP existed as an aggregated form in the phosphate buffer. It has been reported that GMP aggregated in a solution, and that this aggregation was influenced by the pH value of the solution.24) As shown in Fig. 2, the SDS–PAGE pattern portrayed some bands for GMP indicating that interaction among the GMP molecules was so strong that there would not be any dissociation to a monomer in the presence of SDS. This observation is consistent with the results of Nakano and Ozimek.25) The carbohydrate composition of GMP is shown in Table 2. GMP contained about 8.1% of saccharide, more than half of this being sialic acid.

The degree of polymerization of XOS was 3.7 residues from the results of the Somogyi–Nelson and phenol-sulfuric acid methods.1 H-NMR revealed that CMD had 23 carboxymethyl groups per molecule. The average molecular weights of XOS and CMD were 555 and 12,000, respectively.

The GMP–XOS and GMP–CMD conjugates were subjected to SDS–PAGE and SEC. As shown in Figs. 1b and 1c, it was clarified that GMP–XOS and GMP–CMD each had a higher molecular weight than GMP. The composition of the conjugate determined by the absorbance at 230 nm and by the phenol-sulfuric acid method indicated that the respective molar ratios of GMP to XOS and CMD in the conjugate were 5:8 and 10:1.

Table 1. Amino Acid Composition of GMP

<table>
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<tr>
<th>Amino acid</th>
<th>Experimental value (residues)</th>
<th>Theoretical value (residues)</th>
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<tr>
<td>Asp</td>
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<tr>
<td>Thr</td>
<td>11.5</td>
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</tr>
<tr>
<td>Ser</td>
<td>5.9</td>
<td>6</td>
</tr>
<tr>
<td>Glu</td>
<td>12.4</td>
<td>8</td>
</tr>
<tr>
<td>Gly</td>
<td>1.4</td>
<td>1</td>
</tr>
<tr>
<td>Ala</td>
<td>5.8</td>
<td>5</td>
</tr>
<tr>
<td>Val</td>
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</tr>
<tr>
<td>Ile</td>
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<td>7</td>
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<tr>
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</tr>
<tr>
<td>Phe</td>
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<td>0</td>
</tr>
<tr>
<td>Lys</td>
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<td>3</td>
</tr>
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<td>His</td>
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</tr>
<tr>
<td>Arg</td>
<td>0.3</td>
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Table 2. Carbohydrate Composition of GMP

<table>
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<tr>
<th>Saccharide</th>
<th>Composition (%)</th>
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<tr>
<td>Galactose (Gal)</td>
<td>17.1</td>
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<tr>
<td>Mannose (Man)</td>
<td>0.8</td>
</tr>
<tr>
<td>Sialic acid (Sia)</td>
<td>53.5</td>
</tr>
<tr>
<td>N-acetylglucosamine (GlcNAc)</td>
<td>6.9</td>
</tr>
<tr>
<td>Fucose (Fuc)</td>
<td>0.7</td>
</tr>
<tr>
<td>N-acetylgalactosamine (GalNAc)</td>
<td>21.0</td>
</tr>
</tbody>
</table>

Fig. 1. Size-Exclusion Chromatographic Pattern for GMP.
Conditions: column, TSK-gel G3000SWXL (7.8 ID × 300 mm; Tosoh, Tokyo, Japan); flow rate, 1.0 ml/min; eluent, 0.067 M phosphate buffer containing 0.3 M NaCl (pH 7.0); detection, absorbance at 230 nm.

Fig. 2. SDS–PAGE Pattern for GMP.
SDS–PAGE was performed according to the method of Laemmli22) with 15% polyacrylamide gel. After electrophoresis, the protein bands were stained with Coomassie brilliant blue. 1, marker; 2, GMP.
Preventive effect of GMP and its conjugates on intestinal infection

The binding ability of GMP to pathogenic bacteria was evaluated by binding assay with biotinylated bacteria (EHEC O157, S. enteritidis, Vibrio parahaemolyticus, Staphylococcus aureus, and Listeria monocytogenes) as probes (Fig. 3). Among these bacteria, GMP could bind EHEC O157 and S. enteritidis. The binding ability of GMP to EHEC O157 was completely lost and this ability to S. enteritidis was lowered after desialylation. In addition, periodate oxidation deprived GMP of its binding ability to both EHEC O157 and S. enteritidis. In both cases, the carbohydrate structure is considered to have been crucial for the binding ability of GMP. In particular, silaic acid was considered to have been important for the binding of GMP to EHEC O157.

The effect of GMP on EHEC O157 infection was evaluated by an adhesion assay with Caco-2 cells. As shown in Fig. 4, GMP showed an inhibitory effect against the adhesion of EHEC O157 to Caco-2 cells in a dose-dependent manner. Significant inhibition was observed in the case of a high dose of GMP (0.53 mM). These results are consistent with the results of the binding assay with biotinylated bacteria. We consider the binding assay with biotinylated bacteria to be a useful method to search for substances that inhibit the adhesion of pathogenic bacteria to the intestines and that GMP is promising as an effective agent to prevent EHEC O157 and S. enteritidis infection.

When we consider the application of GMP for preventing intestinal infection, the following problems may exist: i) the effect on the population of lactic acid bacteria and other microflora bacteria and ii) low effectiveness as a result of absorption from intestines owing to low molecular weight of GMP.

To evaluate the first possibility, the binding ability of GMP to 12 strains of bacteria, including Lactobacillus, was investigated (Fig. 5). GMP showed strong binding
Fig. 4. Effect of GMP on the Adhesion of EHEC O157 to Caco-2 Cells.

The GMP sample and EHEC O157 suspension were mixed. A 1-ml of the mixture containing $1.0 \times 10^5$ cells/ml was added to the Caco-2 cells in each well, and incubation was continued at 37°C in a 5% CO$_2$ atmosphere for 30 min. The culture medium was removed from the wells, and the wells were washed 5 times with HBSS. The bacteria adhering to the Caco-2 monolayer were released by vigorous agitation with 1 ml of 0.1% Triton X-100 in PBS. The resulting bacterial suspension was diluted and plated on TSA. After incubating overnight at 37°C, the number of colonies was counted.

Fig. 5. Binding Ability of GMP and Asialo-GMP to Various Bacteria.

The binding assay was performed as described in the Materials and Methods section. The bacteria used were Salmonella arizonae (a), Legionaires pneumophilus (b), Lactobacillus casei (c), Bacillus cereus (d), Morganella morganii (e), Enterococcus faecalis (f), Streptococcus mutans (g), Candida albicans (h), Clostridium perfringens (i), Serratia marcesens (j), Yersinia ruckeri (k) and E. coli (l). ●, GMP; ○, asialo-GMP.
ability to M. morganii and E. coli, which are pathogenic microfloral bacteria, but not to L. casei. In respect of this binding to E. coli, GMP and asialo-GMP showed similar binding ability, suggesting that the peptide moiety of GMP was responsible for the binding. We can therefore consider that the presence of GMP did not disrupt the homeostasis of intestinal microfloral bacteria.

To cope with the second possibility, we prepared conjugates of GMP and non-digestible saccharides. The effect of GMP and its conjugates on the adhesion of S. enteritidis to Caco-2 cells is shown in Fig. 6. The adhesion assay of S. enteritidis showed that GMP and GMP–CMD at a concentration of 0.5 mM slightly reduced the adhering bacteria (to about 70%) compared with the control case, although this difference was not significant. GMP–XOS also showed no significant reduction (to about 90%). GMP and its conjugates were not potent inhibitors of the adhesion of S. enteritidis to Caco-2 cells. However, the GMP–XOS and GMP–CMD conjugates were both effective for suppressing IL-8 production (Fig. 7) in the IL-8 production assay which is an index of S. enteritidis invasion into Caco-2 cells, although the mechanism remains unclear. These results suggest that these conjugates would inhibit the invasion of S. enteritidis into intestinal cells, even at a low concentration.

**Conclusion**

We evaluated in this study the binding ability of GMP to several pathogenic bacteria by applying a binding assay with biotinylated bacteria. This binding ability was specific for certain bacteria, the binding ability of GMP to EHEC O157 being particularly strong in the present study. In addition, GMP showed binding ability to S. enteritidis, M. morganii, and E. coli. Since the binding ability of GMP to EHEC O157 was almost eliminated by desialylation and peroxidation, the carbohydrate chains, especially sialic acid, are considered to have been important for this ability. The binding ability to S. enteritidis was eliminated by peroxidation, whereas half of this binding ability remained after the sialidase treatment. These results suggest that carbohydrate structures other than sialic acid were involved in the binding, and that the binding to S. enteritidis was not as specific as that to EHEC O157. We established an *in vitro* adhesion assay using Caco-2 cells. GMP showed an inhibitory effect on the adhesion of EHEC O157 in this assay. Although GMP was not a potent inhibitor of S. enteritidis adhesion, its conjugates (GMP–XOS and GMP–CMD) were effective for suppressing IL-8 production by infected Caco-2 cells. GMP is considered to be a promising agent for preventing intestinal infection.
We intend to clarify the mechanisms for the preventive effect of GMP against intestinal infection and to develop methods to enhance this effect.

Acknowledgments

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