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Immunoenhancing Effects of Bovine Glycomacropeptide and Its Derivatives on the Proliferative Response and Phagocytic Activities of Human Macrophagelike Cells, U937

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The immunomodulatory effect of GMP and its derivates on the cell proliferative response of human macrophagelike cell, U937, and its effect on phagocytic activities via incorporation of fluorescence beads were studied. GMP was found to be a potent immunoenhancer at low concentrations, significantly enhancing the proliferation and phagocytic activities of U937. The modulatory function could be radically altered by enzymatic treatments. Pepsin digestion significantly enhanced the degree of cell proliferation and phagocytic activities, whereas trypsin had no significant effect. The immunoenhancing effects decreased significantly after sialidase treatment; however, more than 70% of activity was retained after treatment. GMP with different carbohydrate chains was shown to possess different modulatory capabilities. Sialic acid-rich GMP fractions showed an enhanced response. These findings indicate that both the carbohydrate chains compositions, including the terminal sialic acids and the polypeptide portions of GMP, are essential for the stimulatory effects of GMP on cell proliferation and phagocytic activities of U937.

KEYWORDS: Glycomacropeptide; cell proliferation; phagocytic activity; peptic digests; human macrophage; nutraceuticals.

INTRODUCTION

Milk is usually the first food for newborn mammals, as it contains all the essential nutrients required for the development of neonates. All newborn mammals enter the world with a poorly developed immune system. They require a period of fine-tuning of the immune system. Milk is now recognized as containing much more than simple nutrition. It contains an array of bioactivities that not only modulate and enhance gastrointestinal (GI) function but also foster infant growth and health (*1*). Therefore, intense research interest has been focused on identifying biologically active components in milk that have the ability to modulate immune functions.

Otani et al. (*2*) found that bovine κ-casein and its glycomacropeptide (residues 106−169, GMP) released by chymosin hydrolysis during cheese making inhibited the mitogen induced proliferation of mouse spleen lymphocytes and rabbit Peyer's patch cells. GMP suppressed serum IgG antibody production in mice (*3*). GMP was also found to block the action of interleukin-I (IL-1) by binding to IL-1 receptors (*4*). However, the majority of the research interest has focused on the modulatory effect of GMP on antigen-specific immune responses, such as B-lymphocytes, which produce antibodies, and T-lymphocytes, which direct and govern the character of antigen-specific immune reactivity (*5*). To our knowledge, however, there are few reports on GMP that focus on the immunomodulatory effects on components that may be considered as innate immune response, such as macrophages, which are involve in phagocytosis.

Although innate immunity is nonspecific, it acts as a controller and an activator of the antigen-specific response (*6*). Because there is a delay of 4−5 days before the initial antigen-specific immune response takes effect, the immediate innate response has a critical role in controlling infections during this period. Thus, the aim of the present study was to investigate the biological significance of GMP and its derivatives on innate immunity using an in vitro model.

MATERIALS AND METHODS

Preparation of GMP and Its Derivatives. GMP was obtained from Davisco Food Int. Inc. (Eden Prairie, MN). The immunomodulatory effects of enzyme-modified GMP were examined. Pepsin (EC 3.4.23.1, 3460 units/mg, Sigma Chemicals Co, St. Louis, MO) was added to GMP, with enzyme-to-substrate ratio of 1:25, in 0.15 N HCl with 0.33 M NaCl, pH 2.0. Samples were then incubated at 37 °C for 1 h. The reaction was stopped by adding 0.5 M sodium bicarbonate. Trypsin digested samples were prepared by adding trypsin (EC 3.4.21.4, 12 700units/mg, Sigma Chemicals Co.), with enzyme-to-substrate ratio of 1:100, to GMP in 0.1 M Tris-HCl with 0.33 M NaCl, pH 8.0. Solutions were incubated overnight at 37 °C.

GMP with different carbohydrate chains were separated using anion exchange high performance liquid chromatography (HPLC) using Waters LC Module I (Millipore Corporation, Bedford, MD). Whole GMP was injected into Mono Q HR 5/5 anionic exchange column (Pharmacia Biotech., Uppsala, Sweden) and eluted with 20 mM Tris-

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HCl buffer at pH 8.0 with linear NaCl gradient from 0 to 1.0 M, at a flow rate of 1 mL/min. Effluents were monitored at 214 nm. Seven peaks were collected. According to the literature, the first peak was asialo-GMP (nonglycosylated), and the following six were sialo-GMPs (glycosylated) (*7*).

Sialidase Treatment and Quantification of Sialic Acids. Sialidase L (V-labs Inc., Covington, LA) was added to GMP in 20 mM sodium acetate, pH 5.5 and incubated at 37 °C overnight. The enzyme-to-substrate ratio was 0.05 U/mg of GMP. The reaction was stopped by adding 0.2 M sodium bicarbonate. Free sialic acids were removed by dialysis with 3.5 kDa membrane. Amount of sialic acid was estimated by colorimetric periodate-resorcinol method described by Jourdian et al. (*8*).

Monocyte Culture and Proliferation Assay. The human macrophage-like cell line U937 was obtained from American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS, from Gibco), 100 U/mL of penicillin and 100 μg/mL of streptomycin (Gibco). A 180-μL aliquot of 1×10^5 cells/mL cell suspension was seeded into 96-well, flat-bottomed tissue culture plates (Costar, Corning, NY). GMP (20 μL) samples were added in triplicate to make the final concentration 10 μg/mL of GMP. All cells were incubated with 0.1% FCS. For negative control, 20 μL of phosphate buffered saline (PBS) was used to replace the volume of GMP sample. As a positive control, cells were incubated in the serum with 10% FCS. The cultures were incubated for 2 days under humidified 5% CO_2 at 37 °C. Almost all U937 cells displayed nonadherent growth. Cell proliferation over the last 3 h of incubation was determined via incorporation of 1 μCi/well of methyl-^{3}H thymidine (Pharmacia Biotech.) into the DNA of the proliferating cells. Cells were harvested onto Whatman GF/C glass microfiber filter (Whatman Int. Ltd., Maidstone, England) using Filtermate Cell Harvester (Perkin-Elmer Packard Canada, Mississauga, Canada). MicroscintO (20 μL) (Perkin-Elmer Packard Canada) was added to each fiber well. The proliferation activity was measured using a TopCount liquid scintillation counter (Perkin-Elmer Packard Canada).

Phagocytic Uptake of Fluorescence Beads by Macrophages. The phagocytic activity of macrophages was determined by the uptake of fluorescence beads under the influence of GMP. An 800-μL aliquot of U937 cell suspension containing 1.0×10^6 cells/mL was seeded in triplicate on cover glasses (13 mm, Sarstedt, Inc., Newton, NC) on 24-well plates. A 200-μL aliquot of 100 μg/mL GMP samples (whole GMP, seven GMP fractions, pepsin-digested and sialidase-digested GMP) were added to the cell culture for 2 days of incubation at 37 °C with 0.1% FCS. As a negative control, PBS was added to replace the sample volume. In positive control, cells were incubated in 10% FCS. At the last 4 h of incubation, 200 μL of fluorescence isothiocyanate (FITC)-labeled beads (1×10^8 beads/mL) (Fluoresbrite Microparticles, 2.0-μm diameter, red, Polysciences Inc., PA) were mixed with the cells. Phagocytic activity was quantified under fluorescence microscope by counting the number of beads per cell and the percentage of macrophages with the beads in a total of 300 macrophages (*9*). Macrophages with strong phagocytic activity was defined as incorporating more than two fluorescence beads per cell.

Statistical Analysis. All results are expressed as the mean ± standard deviation of three independent repeated experiments. Differences are considered to be significant at $p < 0.05$ as determined by Student's *t*-test.

RESULTS AND DISCUSSION

Dose Response of GMP on Cell Proliferation. To find the optimal concentration of GMP for cell proliferation of U937, 20 μL of GMP with different concentrations was added to the cell culture to make final concentrations of 1, 5 10, 50, 100 and 250 μg/mL of GMP. The dose responses of GMP on cellular proliferation of U937 are shown in **Figure 1**. GMP significantly enhanced the proliferation activity of U937 at the dose of 1 μg/mL to a maximum of 10 μg/mL. However, its enhancing activity decreased rapidly when the dose reached 100 μg/mL. It was suggested that GMP could act as a potent immunoenhancer at low concentration. For consistent results, 10 μg/mL GMP was used for the rest of the experiments.

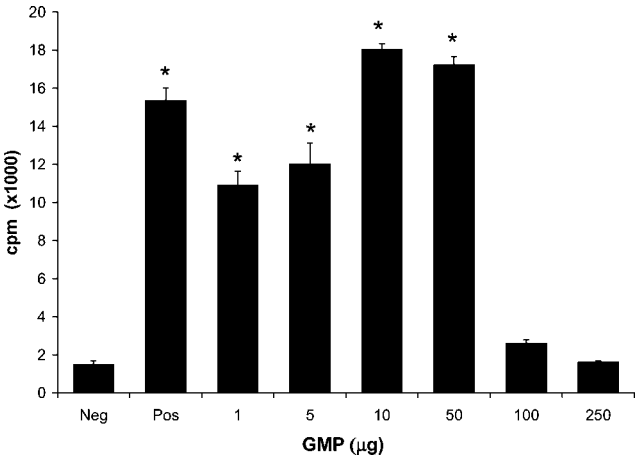

Figure 1. Dose responses of GMP on the proliferation of human monocytic cells, U937. All cells were incubated with 0.1% FCS. The 0.1% FCS acted as a negative control (Neg), while 10% FCS acted as a positive control (Pos) without GMP. Results were expressed as mean ± SD of three independent experiments. *Significantly different from negative control, $p < 0.05$.

Our findings were contradictory to the findings of Otani et al. (2), who demonstrated that GMP acted as an immunosuppresser. GMP inhibited lipopolysaccharide (LPS)- and PHA-induced proliferation of mouse splenic cells and rabbit Peyer's patch cells when tested with 50 μg/mL and 100 μg/mL GMP. Yet, in our experiment, GMP significantly enhanced the proliferation of U937 without mitogen stimulus at a concentration of 50 μg/mL of GMP. At 100 μg/mL of GMP, the enhanced proliferation response reduced significantly. Moreover, the proliferation response reduced significantly at 250 μg/mL of GMP compared to the one with 100 μg/mL GMP. However, they did not exhibit an inhibitory effect on U937 proliferation compared to the negative control. This difference might be due to the dosage and cell species differences. The experimental design also caused different interpretation of the results. In contrast to the immunosuppressive qualities of GMP, there are some evidences that certain subfractions of GMP can promote lymphocyte proliferation (*2*). A sub-fraction from GMP isolated by size-exclusion chromatography was found to promote proliferation of murine spleen cells, in the absence of extraneous mitogens (*10*).

The wide and cryptic range of in vitro immunomodulatory effects of a single milk peptide has been demonstrated. It has been suggested that GMP might have a bimodal effect; at high concentration, GMP could act as a potent immuno-suppresser, whereas at low concentration, GMP could be a potent immunoenhancer.

Effects of Peptic and Tryptic Digestion of GMP on Cell Proliferation of U937. To carry out immunoenhancing properties, GMP must pass through the intestinal epithelium to enter into circulation. However, most of the proteins are susceptible to the degradative effects of gastric processing and the extensive hydrolysis that takes place upon exposure to enzymes in the digestive tract. In particular, food proteins are broken down by proteolytic enzymes such as pepsin in the stomach and trypsin in the intestine into smaller fragments or even amino acids. The immunomodulatory effects of pepsin- and trypsin-digested GMP were examined.

The effect of enzyme-digested GMP on the proliferation activities of U937 is shown in **Figure 2**. Trypsin-digested GMP

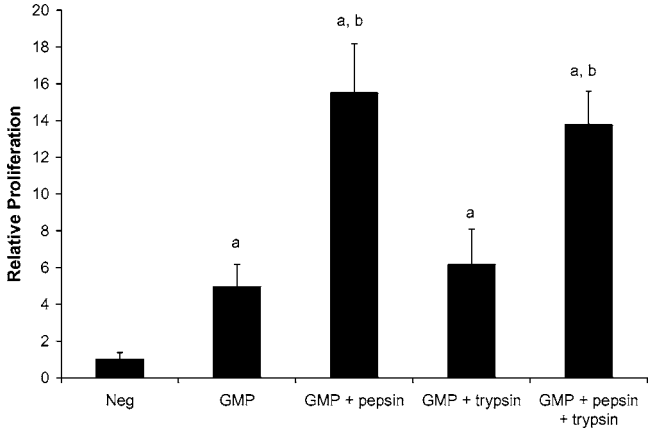

Figure 2. Immunomodulatory effect of pepsin- and trypsin-digested GMP on the cell proliferation of U937. 0.1% FCS acted as a negative control (Neg) without GMP. All measurements were expressed as means ± SD of three independent experiments and expressed relative to the negative control. a, Significantly different from negative control, $p < 0.05$. b, Significantly different from 10 μg/mL GMP, $p < 0.05$.

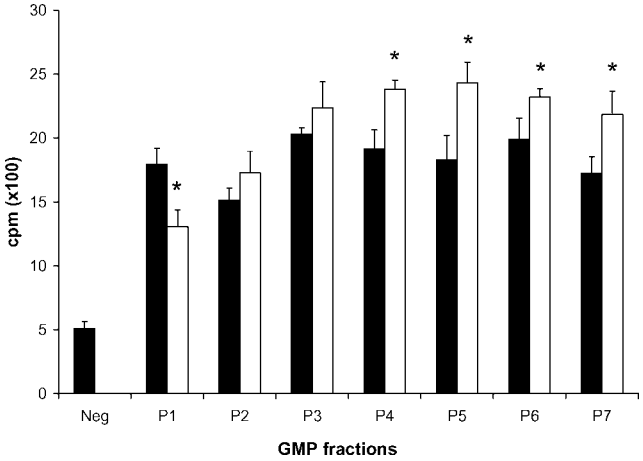

Figure 3. Effect of GMP with heterogeneous carbohydrate chains on cell proliferation of U937. The 0.1% FCS acted as a negative control (Neg) without GMP. All measurements were expressed as means ± SD of three independent experiments. (■) Non-digested GMP fractions; (□) Pepsin-digested GMP fractions. All were significantly different from negative control, $p < 0.05$. Significantly different from non-digested GMP, $p < 0.05$.

did not significantly affect the proliferation response of macrophages. However, pepsin digestion had an opposite effect; the digested fragments enhanced cell proliferation about three times more than the nondigested GMP. Similar results were observed with the GMP digested in both pepsin + trypsin. This results clearly demonstrated that the enhanced immunostimulatory effect of GMP was due to pepsin-digested fragments. It has been reported that a marked variation in the modulatory potential of GMP, following its in vitro digestion with different commercial enzymes (*2*).

GMP has three lysine residues at positions 111, 112, and 116, where trypsin can cleave (*11*). In contrast, pepsin digestion was more nonspecific. GMP has several pepsin cleavage sites. When matching the amino acid sequence of GMP with the pepsin cleavage sites, pepsin formed not only smaller peptides but also tri-peptides, di-peptides, and even amino acid residues. Some of which had sugar(s) attached. Because pepsin-digested GMP showed an enhancement in its immunostimulatory activities, these activities might be due to the action of these small peptides, possibly those fragments with sugar chains. This work clearly showed that the modulatory function of GMP could be radically altered by proteinase treatment. The molecular size of the peptide might be responsible for the immunoenhancing function of GMP. The isolation and identification of the active GMP derived peptides and their sequences including sugar content are under in progress in our laboratory. This may partially explain the often radical differences observed between the results of in vitro or in vivo studies of immune modulation (*1*).

Effects of Carbohydrate Chains on Cell Proliferation of U937. Besides enzymatic influence, heterogeneous carbohydrate chains in GMP were shown to possess different modulatory capabilities. Seven subfractions were separated from GMP isolated by Mono Q anion exchange chromatography (*12*, *13*). According to Kawakami et al. (*12*), the first peak was identified as sugar-free (asialo-GMP), followed by six heterogeneous glycosylated GMPs (sialo-GMP). The cell proliferation response of U937 under the stimulus of GMP with different sugar chains is shown in **Figure 3**. Asialo-GMP (peak 1) showed a significantly higher proliferation response in its nondigested state. In contrast, sialo-GMPs, particularly sialic acid rich fractions (fractions 4−7) showed a significant enhancement on macrophage proliferation after peptic digestion. These results were similar to the findings of Otani et al. (*14*). They demonstrated that different sized subfractions of GMP possessed different modulatory capabilities, with low carbohydrate-containing fractions showing no suppressive activity, whereas other with higher sugar content were potent suppressants against T-lymphocytes (*14*). This result suggested that both the peptide and the carbohydrate moiety played vital roles in the immunoenhancing effect of GMP. The peptide fragment size, the attachment position, and the stereo configuration of the sugar chains might contribute to the stimulatory effect of the U937 proliferation response.

Table 1. Sialic Acid Content in Each GMP Fraction Before and After Sialidase Digestion

	concentration of sialic acid (μg/mL)		
sample[a]	before digestion	after digestion	% of digestion
com GMP	0.091	0.000	100
P1	0.000	0.000	
P2	0.000	0.000	
P3	0.079	0.000	100
P4	0.041	0.000	100
P5	0.114	0.031	67.5
P6	0.141	0.039	72.3
P7	0.183	0.041	77.6

[a] Com GMP = commercial GMP; P1–P7 represent the seven heterogeneous peaks of GMP purified by Mono Q HR 5/5 anionic chromatography.

Effects of Sialic Acids on the Immunomodulatory Function of GMP. Sialic acid, a class of important ketoses that contain nine carbon atoms, is a prominent terminal sugar unit of GMP. The unique structural features of this molecule, which includes a negative charge owing to a carboxyl group, enable it to play an important role in cellular functions, such as cell-to-cell repulsion, recognition, transportation of positively charged compounds, and tumor cell metastasis (*15*). The sialic acid in the terminal sugar unit of GMP might be involved in the recognition of sialic acid receptors on the macrophage surface and in turn release signals for proliferation. Many researchers claim that sialic acid plays an important role in GMP functions (*14*, *16*, *17*). Thus, the biological importance of sialic acids of GMP on its immunoenhancing function was investigated.

The amount of sialic acids in GMP before and after sialidase treatment is shown in **Table 1**. The terminal sialic acids of GMP

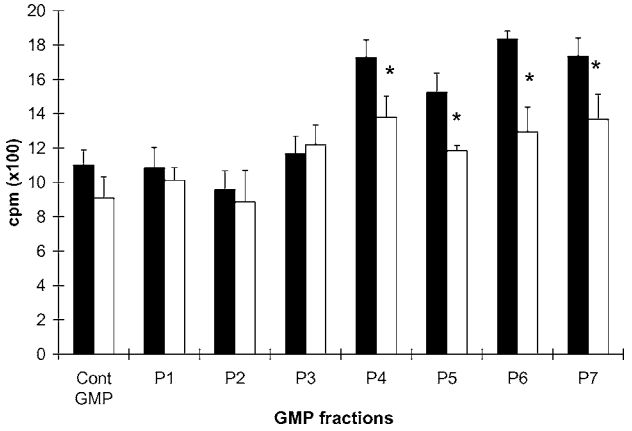

Figure 4. Effect of sialic acids on cell proliferation of U937. All measurements were expressed as means ± SD of three independent experiments. (■) Non-digested GMP fractions; (□) Sialidase-digested GMP fractions. *Significantly different from non-digested GMP, $p < 0.05$.

were released by the sialidase treatment, and most of the sialic acids were removed. However, the sialidase used was only specific for alpha (2−3)-linked sialic acid in GMP (terminal sialic acids), not the alpha(2−6) linkages (branched chain sialic acids). Because fractions 5−7 contained more sialic acids, some of which may be in alpha (2−6) linkages (*18*), it can be explained why some of the GMP fractions still contained sialic acids.

The effects of sialic acid on immunomodulatory properties of GMP are illustrated in **Figure 4**. The response of cell proliferation decreased with sialidase treatment. This effect was more significant in fractions with higher sialic acid content (fractions 5−7). However, its decrease was not drastic, as expected. More than 70% of activity on cell proliferation was retained after enzyme treatment. The P3 and P4 sialidase-digested GMP fractions, from which 100% of sialic acid was removed, displayed a higher proliferative response than P1 and P2 GMP fractions, which do not contain sialic acid. In addition, P5 sialidase-digested GMP fraction, which contains 1 (mol/mol) sialic acid exhibited a lower proliferative activity than P4 enzyme treated GMP fraction. These results suggested that the immunoenhancing property of GMP was partially sialic acid dependent, but not it's critical component of GMP cell proliferative activity. The terminal sialic acids (or in this case, the alpha (2−3) linked sialic acid) could be responsible for triggering macrophage proliferation response; however, other carbohydrate components such as galactose and *N*-acetylgalactosamine and their structure may contribute to GMP cell proliferative activity as well as peptide molecular size. This should be further considered for future work.

Interestingly, asialo-GMP also showed a decreased response in cell proliferation. One possible explanation might be that sialidase was not completely removed from the GMP sample and partially digested the sialic acid on the cell surface to hinder cell proliferation.

The role of sialic acid in GMP on biological activities has been well recognized. However, the heterogeneous modulatory capabilities of GMP sub-fractions and the enhancement of immunostimulatory effect of pepsin-digested GMP was not dependent only on the terminal sialic acids. One might notice that the stimulatory effects on U937 proliferation were not reduced by a large significant amount after sialidase treatment. Perhaps the stimulatory effect of the peptide backbone slightly offset the effect of sialic acids. Therefore, the sialic acid and the peptide fragments may interact with some specific receptors on the cell surface to stimulate the proliferation response of U937.

Table 2. Distribution of Fluorescence Beads as a Percentage in a Total of 300 U937 Cells Under the Influence of GMP and Its Derivatives[a]

	distribution of cells (%)		
sample[b]	0[c]	1[c]	>2[c]
negative control	55.51 ± 4.77	24.4 ± 4.43	20.09 ± 1.87
positive control	29.37 ± 2.64	24.35 ± 4.62	46.28 ± 5.23
Com GMP	61.89 ± 5.17	14.78 ± 3.5	23.33 ± 5.21
+P	53.17 ± 5.51	14.00 ± 2.31	32.83 ± 3.09[d]
+S	61.78 ± 5.19	17.00 ± 2.52	21.22 ± 2.67
P1	52.00 ± 3.78	17.78 ± 2.87	30.22 ± 4.84
+P	52.00 ± 4.17	12.67 ± 2.21	35.33 ± 5.04
+S	49.33 ± 4.63	23.45 ± 4.74	27.22 ± 2.01
P2	44.78 ± 4.88	23.89 ± 2.01	31.33 ± 3.61
+P	40.44 ± 2.53	20.44 ± 1.83	39.11 ± 3.24[d]
+S	67.00 ± 4.36	14.00 ± 2.89	19.00 ± 3.51[d]
P3	36.78 ± 2.33	17.00 ± 1.67	46.22 ± 4.91
+P	28.11 ± 4.86	14.55 ± 4.88	54.00 ± 3.21[d]
+S	52.89 ± 5.97	16.44 ± 3.83	30.67 ± 3.56[d]
P4	41.44 ± 6.56	17.33 ± 4.67	41.22 ± 3.53
+P	28.34 ± 2.01	13.11 ± 3.02	58.55 ± 2.91[d]
+S	53.33 ± 5.48	21.11 ± 3.19	25.56 ± 3.75[d]
P5	28.67 ± 2.77	15.50 ± 3.17	55.83 ± 4.86
+P	20.77 ± 4.50	16.67 ± 3.18	62.56 ± 6.35
+S	59.89 ± 8.10	18.55 ± 5.58	21.56 ± 2.62[d]
P6	35.11 ± 6.38	12.89 ± 3.36	52.00 ± 6.64
+P	19.89 ± 4.22	20.33 ± 3.58	59.78 ± 4.34
+S	53.67 ± 6.06	16.10 ± 3.34	30.33 ± 3.34[d]
P7	35.55 ± 3.20	17.45 ± 1.84	47.00 ± 6.64
+P	35.78 ± 3.25	11.11 ± 1.07	53.11 ± 2.34
+S	52.11 ± 4.55	21.55 ± 3.67	26.34 ± 4.51[d]

[a] Phagocytic activity is quantified by counting the number phagocytic activity is defined as incorporating more measurement is the mean ± SD of three independent cells per trial. [b] Number of fluorescence beads incorporated per. [c] +P, pepsin digested GMP; +S, sialidase digested GMP. [d] Significantly different from non-digested GMP, $p < 0.05$.

Effects of GMP and its Derivatives on Fluorescence Bead Uptake by U937. Macrophages participate in many aspects of the innate immunity. The most common function of macrophages is to engulf invading organisms by the process of phagocytosis. The effects of GMP and its derivatives on phagocytic activities of U937 are shown in **Table 2**. The results were similar to the effects on cell proliferation responses. In general, GMP not only stimulated the growth of macrophages (increased the density of cells observed under microscope) but also enhanced its phagocytic activities. GMP fractions with different heterogeneous sugar chains exhibited heterogeneous phagocytic activities. Those which contained more sialic acids (fractions 5−7) enhanced phagocytosis with more than 50% of the cells having strong phagocytic activities. Pepsin-digested GMP further enhanced phagocytosis, whereas sialidase digestion had the opposite effect; the digested fragments suppressed the phagocytic uptake of nonspecific target (fluorescent beads). Again, the results clearly demonstrated that the size of GMP and sugar chains, particularly the terminal sialic acids, were important key factors affecting GMP function.

In conclusion, the present study demonstrated that GMP could be a potential immunoenhancer at low concentration in vitro. This immunoenhancing property could be a potential altered by enzymatic modifications. Moreover, GMP with different

sugar chains was shown to have heterogeneous modulatory capabilities. GMP fractions with more sialic acids showed a higher enhancing activity; however, sialidase treatment of GMP did not cause drastic decrease of its cell proliferative activity, resulting in more than 70% activity retained. All results indicated that the peptide molecular size and carbohydrate chains, including the terminal sialic acids, played an important role in the immunostimulatory function of GMP.

However, macrophages participate in many aspects of the innate immune response and most of the current GMP research on immune functions are in vitro experiments, and definitive evidence is lacking that in vitro modulatory effects can be replicated in vivo (*19*). There is a clear scope for future progress with in vivo experimentation, to establish whether these effects are maintained when GMP are included in the diet. This scope is under in progress in our laboratory including human subject study.

ABBREVIATIONS USED

GMP, glycomacropeptide; HPLC, high performance liquid chromatography; FCS, fetal calf serum; PBS, phosphate buffer saline

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