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Carbohydrate Research 342 (2007) 2371–2379
Carbohydrate RESEARCH

Relationship between structure and immunostimulating activity of enzymatically synthesized glycogen

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Received 29 May 2007; received in revised form 27 July 2007; accepted 31 July 2007
Available online 8 August 2007

Abstract—Glycogen acts as energy and carbon reserves in animal cells and in microorganisms. Although anti-tumor activity has recently been reported for shellfish glycogen and enzymatically synthesized glycogen, the activity of glycogen has not yet been fully clarified. We enzymatically prepared various sizes of glycogens with controlled structures to investigate the relationship between the structure and immunostimulating activity of glycogen. The results revealed that glycogens with a weight-average molecular weight (M_w) of more than 10,000K hardly activated RAW264.7, a murine macrophage cell line, whereas glycogens of M_w 5000K and 6500K strongly stimulated RAW264.7 in the presence of interferon-gamma (IFN-γ), leading to augmented production of nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6). Comparing the fine structure of the glycogens, the average-number of chain length, as well as the exterior and the interior chain lengths of the glycogens, had minor correlation between active and less-active glycogen derivatives. The available evidence suggests that the macrophage-stimulating activity of glycogen is strictly related to its molecular weight rather than to any fine structural property.

Keywords: Glycogen; Macrophage; Nitric oxide; TNF-α; Immunostimulating activity; Molecular structure

1. Introduction

Glycogen, a highly α-(1→6)-branched α-(1→4)-glucan, is a polysaccharide with a molecular weight of 10^6–10^9,[1] and is the main carbohydrate-storage component in animals and microorganisms. In mammals, the largest reserves of glycogen are found in the liver and skeletal muscle, whereas a small quantity is also present in other tissues such as brain, thymus, skin, and osteoblast precursor cells.[2] In general, the major functions of glycogen are to supply energy in muscle and to supply glucose to the bloodstream from the liver. Thus, the in vivo functions of glycogen in muscle and liver are clearly understood, and the abnormalities of enzymes involved in the synthesis or degradation of glycogen can cause serious diseases. For example, glycogen-storage diseases are induced by a defect in glycogen synthase, which catalyzes the rate-limiting step for glycogen synthesis.[3] Although very little information is available on the function of glycogen aside from its role as an energy or glucose reservoir, it was reported that glycogens extracted from scallops and oysters had an anti-tumor effect.[4] The authors suggested that this effect may be attributable to the immunomodulating activity of glycogen. However, some preparations of glycogens had no activity, and the authors hypothesized that the anti-tumor activity of glycogen may be closely related to its fine structure, and that the highly stimulating glycogen possessed highly branched structures with shorter exterior chains. Meanwhile, enzymatically synthesized glycogen

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0008-6215/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2007.07.024

(ESG) has been shown to have anti-tumor activity.[5] In this report, ESG facilitated the secretion of nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α) from the murine macrophage cell line J774.1, and prolonged the lifetime of tumor-bearing mice by both local injection and oral administration. Several other α-glucan preparations such as glycogen-like polymer produced by certain types of herb or fungi,[6,7] and α-(1→3)-branched α-(1→6)-glucan have also been reported to have anti-tumor and immunomodulating activities.[8–11] However, the relationship between the structural properties and biological activities of such glucans has not yet been fully clarified.

In this study, we enzymatically prepared various glycogens with controlled structures[12] and verified their immunostimulating activities in RAW264.7, a murine macrophage cell line. We report here the intimate relationship between the structure and immunomodulating activity of glycogen.

2. Results

2.1. Effects of various α-glucans on nitric oxide production

Although a previous report by Ryoyama et al. using the macrophage cell line J774.1 showed that ESG had a greater immunostimulating effect than native glycogens and other α-(1→4)(1→6)-glucans,[5] the relationship between the physicochemical properties and immunostimulating activities of various glycogens was not fully investigated. First, we examined the immunostimulating effect of various α-glucans (Tables 1 and 2) on NO production using another macrophage cell line, RAW264.7. Non-glycogen α-(1→4) or α-(1→4)(1→6)-glucans [highly branched cyclic dextrin (HBCD), β-amylase-treated HBCD (β-HBCD), pullulan, cycloamylose (CA)] hardly stimulated the production of NO (Fig. 1a). This result was consistent with that reported

Table 1. Characteristics of several α-glucans

α-Glucans	M_w[a] (K)	M_n[b] (K)	Endotoxin[c] (ppm)
Highly branched cyclic dextrin (HBCD)	380	140	0.0079
β-Amylase-treated HBCD	220	120	0.0034
Pullulan	220	110	0.0047
Cycloamylose	6.6	5.5	0.0025

[a] M_w: weight-average molecular weight.
[b] M_n: number-average molecular weight.
[c] Endotoxins were assayed with Endospecy ES-24S kit (Seikagaku Co.)[14] and expressed in terms of equivalency to a reference *E. coli* O113:H10 LPS.

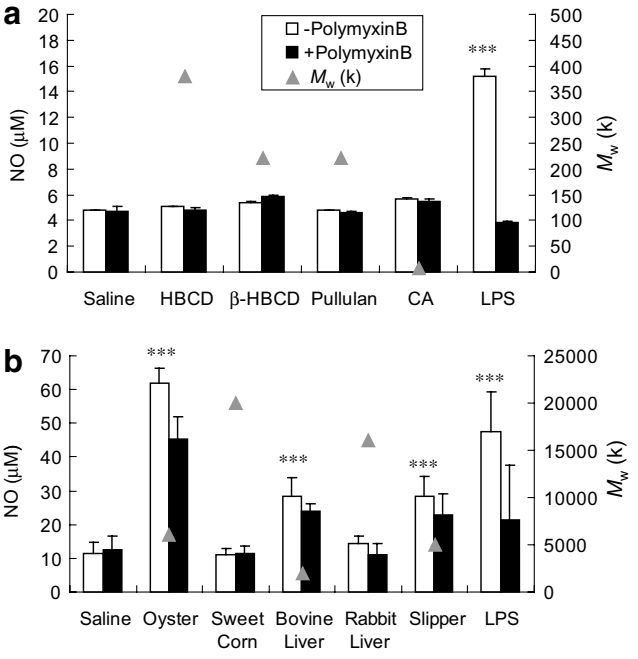

Figure 1. Effects of α-glucans (a) and native glycogens (b) on the production of nitric oxide (NO). RAW264.7 cells (5×10^5) were cultured with each α-glucan (200 μg/mL) or LPS (10 ng/mL) in the presence of IFN-γ (10 ng/mL). After 48 h, the culture fluid was withdrawn, and the amount of NO in the fluid was determined as described in the 'Experimental'. White and black bars indicate the results without and with polymyxin B treatment, respectively. Triangles show M_w of respective glycogens. Values represent mean ± SD. ***: $p < 0.001$ versus control group.

Table 2. Characteristics of several native glycogens

Source of glycogen	M_w[a] (K)	M_n[b] (K)	CL[c]	ECL[d]	ICL[e]	β-Amylolysis (%)	Endotoxin[f] (ppm)
Oyster	6000	4900	9.6	6.3	2.3	44.7	25
Sweet corn	20,000	17,000	14.4	8.7	4.7	46.6	<0.001
Bovine liver	2000	1700	10.4	6.4	3.0	42.1	0.0028
Rabbit liver	16,000	13,000	12.8	7.8	4.0	45.2	0.0028
Slipper limpet	4800	3700	7.6	4.8	1.8	36.8	0.020

[a] M_w: weight-average molecular weight.
[b] M_n: number-average molecular weight.
[c] CL: number-average unit chain length.
[d] ECL: exterior chain length.
[e] ICL: interior chain length.
[f] Endotoxins were assayed with Endospecy ES-24S kit (Seikagaku Co.) and expressed in terms of equivalency to a reference *E. coli* O113:H10 LPS.

by Ryoyama et al.[5] In contrast, native glycogens from oyster, bovine liver and slipper limpet stimulated RAW264.7 and promoted the production of NO (Fig. 1b). On the other hand, glycogens with a relatively high molecular weight from sweet corn and rabbit liver hardly stimulated the cells (Fig. 1b). The activity of oyster glycogen could not be evaluated accurately because of its high endotoxin level (Table 2). These results suggest that glycogens with relatively low molecular weights had macrophage-stimulating activity.

2.2. Macrophage-stimulating activities of ESG-A and ESG-B

Native glycogen molecules derived from natural sources bind to many kinds of proteins.[13] Other factors such as contamination by another trace substance may also influence the results. Therefore, we decided to use enzymatically synthesized glycogens to clarify the relationship between the structure and immunostimulating activity of glycogen. We synthesized two glycogens with different molecular weights, ESG-A (24,000K) and ESG-B (5000K) (Table 3). Structural analysis revealed that the ESGs have fine structural properties similar to those of native glycogens (Table 2). Further, the overall molecular shapes of ESGs and native glycogens were spherical and indistinguishable from each other (data not shown). RAW264.7 was cultured with ESG-A and ESG-B at 37 °C for 48 h, and then NO, TNF-α, and interleukin-6 (IL-6) levels in the culture supernatants were determined. As a result, ESG-B-stimulated RAW264.7 released high level of NO, TNF-α, and IL-6, while ESG-A scarcely promoted these productions (Fig. 2). Negligible levels of endotoxin were detected in these samples by the Limulus test (Table 3),[14] and treatment with polymyxin B had no effect on the stimulating activity. These results suggest that the molecular size of glycogen is particularly important in its immunostimulating activity toward macrophages. Moreover, we investigated whether priming of RAW264.7 with interferon-gamma (IFN-γ) was needed for the immunostimulating activities of glycogen. IFN-γ enhances the macrophage proinflammatory cytokine response to both β-(1→3)-glucan and lipopolysaccharide (LPS).[15] As a result, glycogens themselves failed to promote NO, TNF-α, and IL-6 productions by RAW264.7 in the absence of IFN-γ (Fig. 2). Meanwhile, IFN-γ alone hardly promoted the secretion of cytokines. These results show that IFN-γ is essential for macrophages to be responsive to glycogen. IFN-γ may be needed to recognize glycogens on the cell surface of RAW264.7 or to transduce glycogen-stimulated cell-activating signals.

2.3. Relationship between molecular weight and immunostimulating activity of glycogens

Four kinds of glycogens with different sizes were obtained by partially degrading ESG-A and ESG-B with α-amylase. The molecular weights of α1- and α2-ESG-As prepared from ESG-A were 14,000K and 6500K, respectively, while those of α1- and α2-ESG-Bs prepared from ESG-B were 3100K and 1900K, respectively. The immunostimulating activities of these six glycogens were examined with RAW264.7 (Fig. 3). ESG-A (24,000K) and α1-ESG-A (14,000K) scarcely promoted the release of NO into the culture fluid. By contract, α2-ESG-A (6500K) strongly stimulated the NO production. On the other hand, although ESG-B (5000K) intensely enhanced the production of NO by the cells, the activities of α1-ESG-B (3100K) and α2-ESG-B (1900K) were distinctly lower than that of ESG-B. These activities were not decreased by treatment with polymyxin B. Furthermore, tendencies of TNF-α and IL-6 inductions by these glycogens were the same as that of NO induction (Fig. 3b and c). Moreover, peritoneal exudate cells (PECs) from C3H/HeJ mouse in place of RAW264.7 were used in the same cell-stimulatory studies. The immunostimulating activities of six glycogens toward PECs showed similar tendencies as those toward

Table 3. Characteristics of several enzymatically synthesized glycogens

Glycogens	M_w[a] (K)	M_n[b] (K)	CL[c]	ECL[d]	ICL[e]	β-Amylolysis (%)	Endotoxin[f] (ppm)
ESG-A	24,000	20,000	11.6	7.4	3.2	46.1	0.024
α1-ESG-A	14,000	12,000	6.1	3.1	2.0	17.2	0.0057
α2-ESG-A	6500	3600	4.1	2.3	0.8	7.5	0.23
ESG-B	5000	4000	11.6	7.6	3.0	47.9	0.0059
α1-ESG-B	3100	2400	6.1	3.1	2.0	17.9	0.0064
α2-ESG-B	1900	1500	4.3	2.2	1.1	4.2	0.0066

[a] M_w: weight-average molecular weight.
[b] M_n: number-average molecular weight.
[c] CL: number-average unit chain length.
[d] ECL: exterior chain length.
[e] ICL: interior chain length.
[f] Endotoxins were assayed with an Endospecy ES-24S kit (Seikagaku Co.) and are expressed in terms of equivalency to a reference *E. coli* O113:H10 LPS.

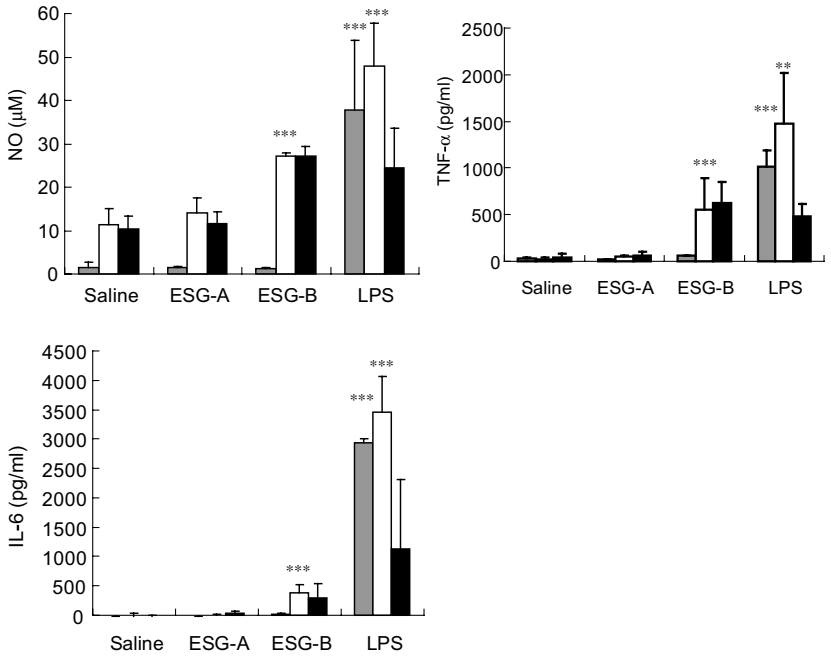

Figure 2. Effects of ESG-A and ESG-B on the production of NO, TNF-α, and IL-6. RAW264.7 cells (5×10^5) were cultured with each ESG (200 μg/mL) or LPS (10 ng/mL) in the presence or absence of IFN-γ (10 ng/mL). After 48 h, the culture fluid was withdrawn, and the amounts of NO, TNF-α, and IL-6 in the fluid were determined as described in the 'Section 4.4'. Gray bars show the results without IFN-γ priming. White and black bars indicate the results without and with polymyxin B treatment in the presence of IFN-γ, respectively. Values represent mean ± SD. **: $p < 0.01$, ***: $p < 0.001$ versus control group.

RAW264.7 (Fig. 4). The molecular weight of glycogen stimulated both cells strongly was 5000–6500K. C3H/HeJ mouse is a Toll-like receptor 4 mutant mouse and is only slightly sensitive against LPS. It was verified that the macrophage-stimulating activities of six glycogens were independent of contamination by LPS. These results suggest that the immunostimulating activity of glycogen is closely related to its molecular size and is optimum at around 5000–6500K.

2.4. Effect of the fine structure of glycogens

Glycogen is a spherical molecule, and α-amylase trims its outer chains without changing the overall shape of the molecule. To investigate whether or not the immunostimulating activity of glycogen is only related to its molecular size, we evaluated fine structures by determining number-average unit chain lengths (CLs), exterior chain lengths (ECLs), and interior chain lengths (ICLs) of six glycogens (Table 3). The parameters of ESG-A and ESG-B were equivalent to those of native glycogens, but partially degraded products (α1- and α2-ESG-As, and α1- and α2-ESG-Bs) had significantly decreased CLs, ECLs, and ICLs, which suggest that these products have highly branched structures, especially in their molecular surfaces. On the other hand, Figures 3 and 4 show that α2-ESG-A and ESG-B had higher stimulating activities toward macrophages than ESG-A, α1-ESG-A, α1-ESG-B, or α2-ESG-B. Taken together, these results suggest that there are minor correlations between these chain lengths and the immunostimulating activity of glycogen, and that the activity of glycogen is related to its molecular weight rather than its fine structural properties.

2.5. The binding activities of ESG-A and ESG-B to RAW264.7 cells

To clarify the glycogen-recognition mechanism of macrophages, we synthesized biotinylated ESG-A and ESG-B and investigated the binding activities of these biotinylated glycogens to RAW264.7 in the presence or absence of IFN-γ. Labeling did not alter the stimulating properties of ESGs when they were tested for the NO-production assay. Interestingly, RAW264.7 cells were equivalently bound to not only highly active ESG-B, but also to inactive ESG-A (Fig. 5), and this binding was independent of treatment with IFN-γ (data not shown).

3. Discussion

In this study, we prepared enzymatically synthesized glycogens of various molecular weights and investigated in detail the relationship between the structure and

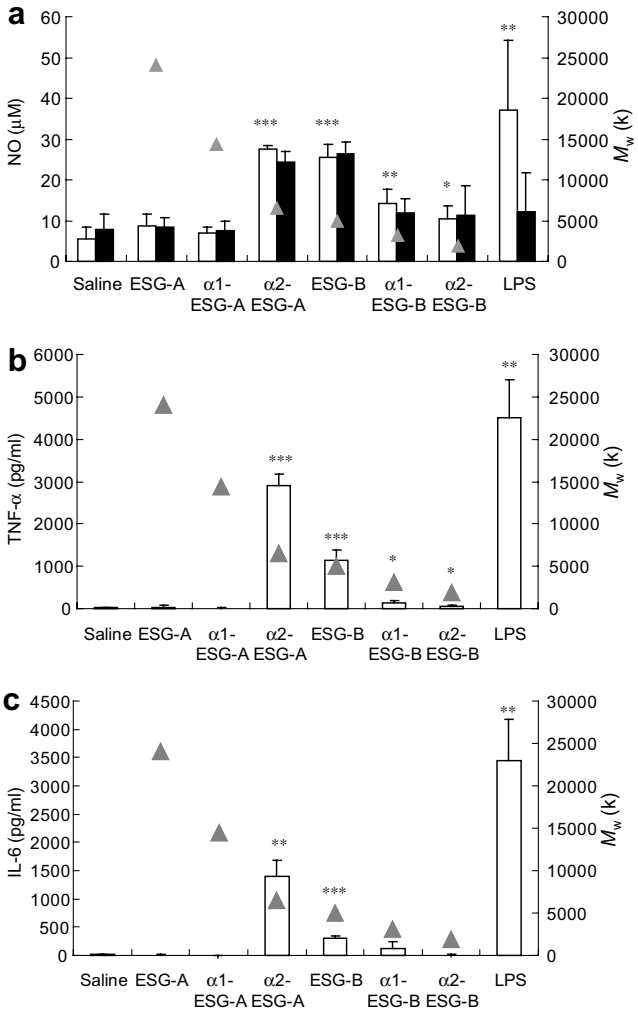

Figure 3. Relationship between molecular weight and immunostimulating activity of glycogens. RAW264.7 cells (5×10^5) were cultured with each ESG (200 μg/mL) or LPS (10 ng/mL) in the presence of IFN-γ (10 ng/mL). After 48 h, the culture fluid was withdrawn, and the amounts of NO (a), TNF-α (b), and IL-6 (c) in the fluid were determined as described in the 'Experimental'. White bars show the results without polymyxin B treatment, and black bars indicate the results with polymyxin B treatment. Triangles represent M_w of respective glycogens. Values represent mean ± SD. *: $p < 0.05$, ***: $p < 0.01$, ***: $p < 0.001$ versus control group.

immunostimulating activity of glycogens. These results clarified that the molecular weight of glycogen is particularly important in elevating the NO, TNF-α, and IL-6 productions of macrophages, and glycogens with molecular weights of about 5000–6500K, but not above 10,000K, are appropriate for stimulating macrophages (Fig. 3). Moreover, NO and cytokine augmentations were dependent on the presence of IFN-γ. These results suggest that extracellular glycogen may act as an immunostimulator.

β-(1→3)-Glucans from fungi and yeasts have been reported to have anti-tumor activities by stimulating host immunocytes such as macrophages, natural killer

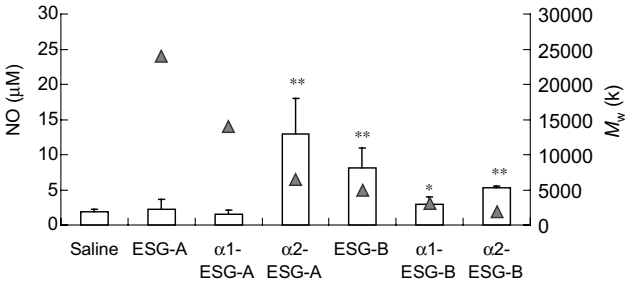

Figure 4. Relationship between molecular weight and immunostimulating activities of glycogens toward peritoneal exudate cells (PECs) from C3H/HeJ mice. PECs (5×10^5) from C3H/HeJ mice were cultured with each ESG (500 μg/mL) in the presence of IFN-γ (10 ng/mL). After 48 h, the culture fluid was withdrawn, and the amount of NO in the fluid was determined as described in the 'Experimental'. Triangles show M_w of respective glycogens. Values represent mean ± SD. *: $p < 0.05$, **: $p < 0.01$ versus control group.

cells, and cytotoxic T lymphocytes.[16,17] These anti-tumor effects of β-glucans depend on their molecular weight, degree of branching, and conformation (single- and triple-helix).[18–20] For instance, Adachi et al. determined the anti-tumor activities of β-glucans with molecular weights of 800K, 250K, 210K and 6.4K prepared from *Grifola frondosa*.[18] β-Glucan with a molecular weight of 800K potently activated biological activities such as anti-tumor, alternative complement pathway, and lysosomal enzyme production in macrophage among the β-glucans tested. These reports indicate that anti-tumor glucan is not always a multiple enhancer of host defense mechanisms and that a large molecular weight is required to augment multiple immunological activities. In another report, the anti-tumor activities of lentinan (LNT) with various molecular weights were determined.[21] LNT obtained from *Lentinus edodes* has been shown to have strong anti-tumor activity against sarcoma 180 implanted subcutaneously in mice. Since LNT with a molecular weight of 1.5×10^6 showed much higher anti-tumor activity than any other LNTs, there was a significant correlation between anti-tumor activity and the molecular weight of LNT. These results and our present results indicate that the molecular weights of polysaccharides can be important factors in their biological activities.

On the other hand, a glycogen-like polymer prepared from the medicinal plant *Tinospora cordifolia* was recently reported to exhibit immunostimulating activity.[6,11] This glucan activated lymphocytes such as natural killer (NK) cells, T cells, and B cells, and elicited the synthesis of several Th1 cytokines, IL-1β, IL-6, IFN-γ, and TNF-α. Further, the tumor-killing activity of NK cells was significantly augmented by glucan stimulation. The molecular weight of this glucan was estimated to be >550K by gel-filtration chromatography using dextran as a marker. However, in this method, the molecular weight could be underestimated because of the differ-

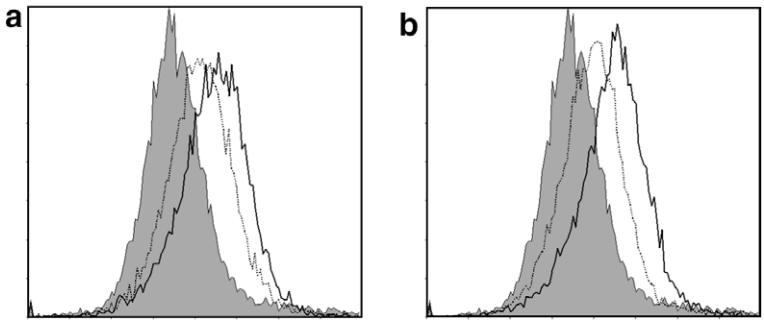

Figure 5. ESG-A (a) and ESG-B (b) bind equivalently to RAW264.7 cells. To determine the binding activities of ESG-A and ESG-B to RAW264.7 cells, biotinylated ESG-A and biotinylated ESG-B were synthesized by periodate oxidation.[26] The cells were incubated with biotinylated ESG-A or biotinylated ESG-B in the presence of IFN-γ for 30 min. After washing, the amounts of ESG-A-biotin and ESG-B-biotin bound to RAW264.7 were determined by staining with Streptavidin-Alexa 488 conjugate (Invitrogen, USA) and using a flow cytometer (FC 500 MPL; Beckman–Coulter, Co.). The shaded, dotted-line, and solid-line histograms in each graph show the results with 0, 100, and 1000 μg of ESGs, respectively.

ences in the molecular shape of dextran and this glucan, and the precise molecular weight of this glucan may be much higher. In another study, a glycogen-like polymer on the cell surface of a fungus, *Pseudallescheria boydii*, stimulated macrophages and increased their phagocytotic activity.[7] Further, this glucan induced cytokine secretion by cells of the innate immune system in a mechanism that involved Toll-like receptor 2 (TLR2), CD14 and MyD88. It would be very interesting to compare these glucans with our glycogens.

IFN-γ is well-known as a Th1-oriented immunostimulative cytokine especially on macrophage-mediated host defense systems. IFN-γ primes macrophages to up regulate inducible NO synthase (iNOS) and increases the background level of NO production as shown in Figure 3. The further NO production was accelerated by triggering with other inflammatory cytokines or Toll-like receptor agonists, such as IL-6, LPS, or CpG oligonucleotides.[22,23] The iNOS metabolizes L-arginine to produce NO, and NAD(P)H-oscillation is concomitantly required. The triggering molecules like CpG oligonucleotides stimulate the NAD(P)H oscillation in high frequency and result in higher NO production.[22] In the present study, we have shown that glycogen possessing a molecular weight around 5000K significantly triggered the higher NO production in the presence of IFN-γ. However, the difference between the glycogen stimulation and Toll-like receptor signaling is the requirement of IFN-γ for the NO, TNF-α, and IL-6 productions. Briefly, LPS, a TLR4 agonist, could induce a significant level of NO, TNF-α, and IL-6 productions without IFN-γ priming, whereas ESG-B needed the IFN-γ priming for the significant increment of these productions (Fig. 2). Similar results were observed in IL-6-triggered NO production, whereby the NO production and concomitant high frequent NAD(P)H oscillation only occurred after IFN-γ priming.[23] These findings suggest that cell signaling pathways activated with glycogen may be distinct from those with Toll-like receptor 4 or 9 agonists, including LPS or CpG.

Takaya et al. reported that glycogen preparations with high anti-tumor activities possess highly branched structures with short unit chains (DP <5).[4] To investigate whether highly branched external structures of glycogen were involved in the immunomodulatory activities toward RAW264.7, we determined the CLs, ECL, and ICLs of the six above-mentioned glycogens (Table 3). The ECLs of α2-ESG-A and ESG-B, with high macrophage-activating activities were 2.3 and 7.6, respectively. Meanwhile, the ECLs of α1- and α2-ESG-Bs with low activities were 3.1 and 2.2, respectively, and those of ESG-A and α1-ESG-A with no activity were 7.4 and 3.1, respectively. These results suggest that the ECLs of glycogen are not important for the stimulation toward RAW264.7. In addition, β-amylolysis of these glycogens demonstrated that there is a minor correlation between their exterior structures and macrophage-stimulating activities. Takaya et al. did not report the molecular weight of scallop glycogens in detail.[4] Furthermore, they noted that the preparation method significantly influenced the immunostimulating activity: a mild method using protease treatment yielded highly active glycogen, whereas extraction using acid, alkali, or organic solvent resulted in inactive glycogen. Since the molecular weights of native glycogens are highly influenced by these extractions,[24] we speculate that the anti-tumor activities of scallop glycogens may be related to not only their fine structures as they discussed, but also to their molecular weights.

In general, mannan and β-glucans are recognized by a mannose receptor and by Dectin-1, respectively.[25,26] Such C-type lectins play important roles in the innate immune response and recognize sugar ligands through the carbohydrate recognition domain (CRD) in a Ca^{2+}-dependent manner.[27–29] We speculate that glycogen is also recognized by particular C-type lectin(s) on the cell surface that transmit signals intracellularly to

enhance the release of NO, TNF-α, and IL-6. In this paper, as it was revealed that the molecular weight of glycogen was important in macrophage stimulation, we thought that the molecular weight might be closely related to glycogen recognition by macrophages. However, in the experiment using biotinylated ESGs, it was indicated that both ESG-A and ESG-B were equivalently bound to RAW264.7 (Fig. 5). Moreover, the binding activities of glycogens to RAW264.7 were independent on IFN-γ, which was essential for the macrophage-stimulating activity (data not shown). These results suggest that glycogen-recognition receptor(s) constitutively exist on the macrophage cell surface and bind to glycogen in a molecular size-independent manner. We clarified that the binding of glycogen to RAW264.7 was not sufficient for the immunostimulating activities, and both IFN-γ stimulation and 5000–6500K-glycogen stimulation were essential. We surmise that IFN-γ promotes the incorporation of 5000–6500K glycogens into RAW264.7 and activates the immunoactivating signal transduction pathways in this cell. The detailed mechanism of the macrophage-stimulating activity of glycogen is now under investigation.

4. Experimental

4.1. Chemicals

Lipopolysaccharide (LPS) (*E. coli* 055:B5), polymyxin B, RPMI1640, Hank's balanced salt solution, bovine liver glycogen, rabbit liver glycogen, and slipper limpet glycogen were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Pullulan, oyster glycogen, and phytoglycogen were acquired from Hayashibara Co. (Okayama, Japan), Wako Pure Chemicals (Osaka, Japan), and Q.P. Corporation (Tokyo, Japan), respectively. Highly branched cyclic dextrin (HBCD), β-amylase-treated HBCD (β-HBCD), and cycloamylose (CA) were prepared as described previously.[5,30,31]

4.2. Cell lines and animals

RAW264.7, a murine macrophage cell line, was obtained from RIKEN Cell Bank (Tsukuba, Japan). C3H/HeJ mice between 6 and 8 weeks of age were purchased from Japan SLC, Shizuoka.

4.3. Preparation of ESGs

ESG-A was synthesized from sucrose and maltotetraose using sucrose phosphorylase (SP, EC 2.4.1.7), α-glucan phosphorylase (GP, EC 2.4.1.1) and branching enzyme (BE, EC 2.4.1.18) as reported previously.[5] In brief, sucrose was phosphorolyzed to glucose 1-phosphate and fructose by SP in the presence of inorganic phosphate, and the glucosyl moiety of glucose 1-phosphate was transferred to the non-reducing end of maltotetraose by GP to liberate inorganic phosphate and elongate linear α-(1→4)-glucans. Finally, BE was introduced into the α-(1→6)-glucosidic linkages in the elongated chains. α1-ESG-A and α2-ESG-A are partially degraded ESG-A, prepared by slight and extensive actions of α-amylase, respectively. Meanwhile, ESG-B was synthesized from dextrin (partially hydrolyzed starch with a trade name Pine-Dex #100, Matsutani Kagaku Co., Hyogo, Japan) using isoamylase (EC 3.2.1.68), amylomaltase (EC 2.4.1.25), and BE as follows. The α-(1→6)-glucosidic linkages of the dextrin was completely digested by using *Pseudomonas amyloderamosa* isoamylase to produce short-chain amylose. After heating for inactivating isoamylase, recombinant amylomaltase from *Thermus aquaticus*[31] and BE from *Aquifex aeolicus*[32] were added to the reaction mixture. Since amylomaltase was able to elongate the length of short chain amylose with which BE was found difficult to react, this enzyme was added for raising the efficiency of the BE reaction. Finally, ESG-B was constructed from elongated amyloses by the BE reaction. α1-ESG-B and α2-ESG-B are partially degraded ESG-B prepared using α-amylase. The molecular weights of all α-glucans were determined using high-performance size-exclusion chromatography with a multi-angle laser light scattering photometer and a differential refractive index detector (HPSEC-MALLS-RI) as described previously.[32]

4.4. Determination of NO levels

NO production by RAW264.7, as evaluated by the NO concentration, was determined with the Griess reagent.[33] Briefly, RAW264.7 cells [5×10^5 cells in 1 mL per well] were cultured in a 24-well flat-bottomed plate (Sumilon Bakelite Co., Tokyo). Specimens or polymyxin B-treated specimens (200 μg/mL) were added to this culture with or without IFN-γ. Polymyxin B-treated specimens were prepared by incubating 200 μg of the specimen with 10 μg of polymyxin B at room temperature (rt) for 30 min. After 48 h, 50 μL of culture fluid from each well was recovered and mixed with 50 μL of Griess reagent. The mixtures were incubated for 10 min at rt in the dark, and the absorbance at 550 nm minus that at 630 nm for each well was determined with a CORONA MTP-450. NO concentrations were detected using sodium nitrite as a standard.

4.5. Quantification of cytokines

The inflammatory cytokines TNF-α and IL-6 were quantified by ELISA as follows.[34]

4.5.1. TNF-α. Immune plates (Nunc) were coated with capture antibody for rat anti-mouse/rat TNF monoclo-

nal antibody (mAb) (PharMingen, CA, USA) in 0.2 M sodium phosphate buffer (pH 6.0) by incubation at 4 °C overnight. The plates were washed with PBS containing 0.05% Tween-20 (PBST) and blocked with PBST containing 1% bovine serum albumin (BPBST) at rt for 2 h. After being washed, they were incubated with recombinant mouse TNF-α (PharMingen) or samples at rt for 3 h. They were then washed and treated with biotinylated rat anti-mouse TNF mAb (PharMingen) and incubated at rt for 1 h. The plates were then incubated with peroxidase-conjugated streptavidine (Zymed Lab., Inc.) at rt for 30 min, and developed with a TMB substrate system (KPL, Inc., MD, USA). The development of color was stopped with 1 N phosphoric acid, and the optical density at 450 nm was determined.

4.5.2. IL-6. Immune plates (Sumitomo Bakelite Co., Tokyo) were coated with capture antibody for rat anti-mouse IL-6 mAb (PharMingen, CA, USA) in 0.1 M bicarbonate buffer (pH 9.5) by incubation at 4 °C overnight. The plates were washed with PBST and blocked with BPBST at 37 °C for 40 min. After being washed they were incubated with recombinant mouse IL-6 (PharMingen) or samples at 37 °C for 1 h. They were washed again, treated with biotinylated rat anti-mouse IL-6 mAb (PharMingen) and incubated at 37 °C for 40 min. The plates were then incubated with peroxidase-conjugated streptavidine at 37 °C for 40 min, and developed with a TMB substrate system. The development of color was stopped with 1 N phosphoric acid, and the optical density at 450 nm was determined.

4.6. Analysis of the fine structures of glycogens

The fine structural properties, CLs, ECLs, ICLs, and β-amylolysis of the glycogens were determined as described by Hizukuri and co-workers.[35–37]

4.7. Determination of endotoxin

Endotoxin contents of glucan samples were determined by the Limulus test using a kit (Endospecy ES-24S Kit; Seikagaku Corp., Tokyo, Japan).[14]

4.8. Binding ability of ESGs to RAW264.7 cells

IFN-γ-primed RAW264.7 cells (2.5×10^5 cells) were incubated for 16 h at 37 °C. Thereafter, the cells were treated with biotin-labeled ESGs and incubated for 30 min at 37 °C. The cells were further treated with Streptavidin-Alexa 488 conjugate, and the cell-associated fluorescence intensity was measured by using a flow cytometer (FC 500 MPL, Beckman–Coulter Co.).

Acknowledgments

This work was supported in part by a grant from the 'Program for new technology development to activate agriculture, forestry, fisheries, and food industry by cooperating industry, academia, and the government' of the Ministry of Agriculture, Forestry, and Fisheries, Japan.

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