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Stimulatory effect of gangliosides on phagocytosis, phagosome–lysosome fusion, and intracellular signal transduction system by human polymorphonuclear leukocytes

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Gangliosides are known to be differentiation-inducing molecules in mammalian stem cells. We studied the interaction between the molecular structure of glycosphingolipids (GSLs) and their promoting mechanisms of the phagocytic processes in human polymorphonuclear leukocytes (PMN). The effect of various gangliosides from mammalian tissues on adhesion, phagocytosis, phagosome–lysosome (P–L) fusion and superoxide anion production was examined by human PMN using heat-killed cells of *Staphylococcus aureus* coated with GSLs. Gangliosides GM3, GD1a, GD3 and GT1b showed a marked stimulatory effect on the phagocytosis and P–L fusion in a dose-dependent manner, while ganglioside GM1, asialo GM1 and neutral GSLs did not. The relative phagocytic rate of ganglioside GM3-coated *S. aureus* was the highest among the tested GSLs. Both P–L fusion rate and phagocytosis of *S. aureus* were elevated significantly when coated with ganglioside GD1a, GD3 or GT1b, and GT1b gave a five times higher rate than that of the non-coated control. These results suggest that the terminal sialic acid moiety is essential for the enhancement of phagocytosis and that the number of sialic acid molecules in the ganglioside is related to the enhancement of the P–L fusion process. On the other hand, the superoxide anion release from PMN was not affected by ganglioside GM2, GM3, GD1a or GT1b. Furthermore, to clarify the trigger or the signal transduction mechanism of phagocytic processes, we examined the effect of protein kinase inhibitors such as H-7, staurosporine (protein kinase C inhibitor), H-89 (protein kinase A inhibitor), genistein (tyrosine kinase inhibitor), ML-7 (myosin light chain kinase inhibitor), and KN-62 (Ca^{2+}/calmodulin-dependent protein kinase II inhibitor) on ganglioside-induced phagocytosis. H-7, staurosporine and KN-62 inhibited ganglioside-induced phagocytosis in the range of concentration without cell damage, while H-89, genistein and ML-7 did not. Moreover, H-7 and KN-62 inhibited ganglioside-induced P–L fusion. These results suggest that protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II may be involved in the induction of phagocytosis and P–L fusion stimulated by gangliosides.

***Keywords*: gangliosides, phagocytosis, phagosome–lysosome fusion, polymorphonuclear leukocytes, signal transduction system**

Introduction

Glycosphingolipids (GSLs) play an important role in the eucaryotic cell membrane as specific cell surface markers, specific receptors for bacterial toxins, and also as potentiators of differentiation-induction in immature stem cells [1–4]. Experimental evidence suggests that some bacterial lectins play a role as adherence molecules and initiate infection by binding to specific GSLs on host cell surfaces [5, 6].

Gangliosides are sialylated GSLs in the neural membrane of vertebrate tissues. They have been reported to regulate cellular functions, including transmembrane signalling [7–10], cell growth and differentiation [11]. It has also been reported that the acidic GSLs (gangliosides) exhibit a special receptor function for the exogenous bioactive molecule such as bacterial exotoxins, hormones or interferons [12].

However, the precise role of the functional determinants and the specificity of gangliosides in relation to the observed biological effects have not been evaluated. Recently, we reported that acidic GSLs possessing D-glucuronic acid from *Sphingomonas paucimobilis* have a marked stimulatory

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effect on phagocytosis and phagosome-lysosome (P–L) fusion by human polymorphonuclear leukocytes (PMN) [13]. The existence of a free carboxyl group in glucuronic acid moiety was found to be essential for the enhancement of phagocytosis. We have also demonstrated that the ganglioside GM3 and the brain sulfatide (galactosylceramide-3-sulfate) promote phagocytosis and P–L fusion [14]. We have, therefore, attempted to delineate the structural requirements for the action of this ganglioside. In this report, we describe the effect of various gangliosides from bovine brain on the adhesion, phagocytosis, P–L fusion and superoxide anion production by human PMN *in vitro*. Furthermore, since intracellular signalling is important in understanding the mechanisms regulating phagocytosis (endocytosis) and the intracellular killing of pathogens by neutrophils, we have investigated the ganglioside-mediated intracellular signal transduction in human PMN.

Materials and methods

Reagents and bacterial strain

Gangliosides (GM1, GM2, GM3, GD1a, GD3, GT1b and asialo GM1) isolated from bovine brain were purchased from Sigma Co. (St Louis, MO). Stock solutions of gangliosides were prepared by dissolving the gangliosides in a mixture of chloroform:methanol (1:1 v/v). Glucosylceramide and galactosylceramide from bovine sources were purchased from Funakoshi Co. (Tokyo, Japan). Lactosylceramide from bovine sources was purchased from Sigma Co., and dissolved in a mixture of chloroform:methanol (1:1 v/v). Sialic acid was purchased from Sigma Co., and dissolved in a 0.1% gelatin-Hanks buffer (Nissui Pharm. Co. Ltd, Tokyo, Japan).

Staphylococcus aureus 209P was grown in a nutrient agar at 37 °C for 24 h and killed by autoclaving.

Preparation of PMN

Human PMN were isolated from the heparinized venous blood of healthy donors with Mono-poly resolving medium (Flow Lab. Inc., Irvine, Australia) by centrifugation at $400 \times \mathbf{g}$ for 30 min. The PMN were washed three times with RPMI 1640 medium (Nikken Bio Medical Lab. Inc., Kyoto, Japan) with 100 U ml^{-1} of penicillin G and 100 μg ml^{-1} of streptomycin and resuspended at a concentration of 5×10^6 cells ml^{-1} in RPMI 1640 medium supplemented with 10% of heat-inactivated fetal bovine serum (GIBCO, New York). The PMN (1×10^6 cells) were incubated on a cover slip (Thermanox, Lux Miles Lab. Inc., Naperville) for 90 min at 37 °C, followed by washing three times with RPMI 1640 medium to remove the non-adherent cells.

Estimation of adhesion rate of ganglioside-coated *S. aureus* to PMN

Ganglioside in methanol (0.2–5.0 μg ml^{-1}) was mixed with the heat-killed cell suspension of *S. aureus* 209P (1×10^8 cells ml^{-1}) and the solvent was evaporated off by rotary evaporation. The ganglioside-coated *S. aureus* was then suspended in 0.1% gelatin-Hanks buffer by mixing vigorously. The estimation of adhesion was initiated by the addition of 200 μl of ganglioside GM3, GD3 or GT1b (0.2–5.0 μg ml^{-1})-coated killed *S. aureus* suspension to the PMN on cover slips. After incubation for 90 min at 4 °C, the cover slips were washed three times with RPMI 1640 medium, and the PMN fixed with methanol, followed by staining with methylene blue. The ganglioside coated *S. aureus* that had adhered to the PMN was observed under a microscope (Nikon OPTIPHOT). One hundred and twenty microscopic fields were examined at random, and the number of bacteria per cell was counted.

Estimation of phagocytic rate of ganglioside-coated *S. aureus* by PMN

Generally, assay of phagocytosis has been measured with ganglioside-coated polystyrene latex or yeast particles [3, 15]. However, as shown in the previous paper [13, 16], the method using bacteria coated with GSLs is better for observing the interaction between particles and receptors on the surface of PMN. In brief, 200 μl of the ganglioside-coated bacterial suspension (5×10^7 killed *S. aureus* cells ml^{-1}) was added to the PMN culture (5×10^6 cells ml^{-1}) on a cover slip, then incubated for 60 min at 37 °C. The phagocytic rate of ganglioside-coated bacteria by PMN was estimated according to the method described in the section of adhesion. Furthermore, the phagocytosis by PMN of bacteria coated with neutral GSLs (glucosylceramide, galactosylceramide, lactosylceramide or asialo GM1) was also estimated.

Estimation of phagosome-lysosome (P–L) fusion rate of ganglioside-coated *S. aureus* by PMN

Estimation of the rate of P–L fusion by PMN was performed according to the previous method [5, 13, 16]. Cover slips with PMN attached were prelabelled with 5 μg ml^{-1} of acridine orange in PBS for 15 min at 37 °C and washed three times with antibiotic-free RPMI 1640 medium. P–L fusion was initiated by the addition of killed *S. aureus* cells coated with ganglioside (PMN to bacteria ratio of 1:10) in a total volume of 200 μl of RPMI 1640 medium supplemented with 10% fetal bovine serum. After incubation for 60 min, the cover slips were washed three times with RPMI 1640 medium and allowed to dry in the air. P–L fusion was estimated by counting acridine-stained bacteria under a fluorescent microscope (BH-2, Olympus, Tokyo) with emission wavelength of 520 nm. PMN were then fixed with methanol and stained with methylene blue to estimate the phagocytosis. One hundred microscopic fields were examined at random and both the number of cells per field and the number of bacteria per cell were calculated.

The fusion index and the phagocytic index were defined as the percentage of the positive fusion multiplied by the mean number of fused phagosomes per cell and the percentage of the positive phagocytosis multiplied by the mean number of phagocytosed *S. aureus* per cell, respectively. In addition, the fusion index of asialo GM1 was estimated as a control.

Determination of superoxide anion production by PMN

Superoxide anion production was determined by the reduction of cytochrome c according to a previous method [13, 16]. The standard reaction mixture contained 80 μM cytochrome c, 1×10^6 PMN per ml, and 5×10^7 cells of *S. aureus* coated with ganglioside per ml in a final volume of 1.0 ml of HEPES-saline buffer. After incubation for 60 min at 37 °C, the suspension was centrifuged at $5000 \times \mathbf{g}$ for 1 min. The absorbance at 550 nm of each supernatant solution was measured with a spectrophotometer (Hitachi type 100-20). The reduced amount of cytochrome c was measured by the absorbance at 550 nm and was expressed in nanomoles using a molar extinction coefficient $E_{550} = 2.1 \times 10^4\ M^{-1}\,cm^{-1}$.

Estimation of effect of staurosporine, H-7, H-89, genistein, ML-7 or KN-62 on phagocytic rate by PMN of ganglioside-coated *S. aureus*

We used 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) as a specific inhibitor of protein kinase C with a K_i value of 6 μM [17], staurosporine as a protein kinase C inhibitor with a K_i value of 1–3 nM [18], *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide (H-89) as a protein kinase A inhibitor with K_i value of 0.05 μM [19], genistein as a tyrosine kinase inhibitor with a K_i value of < 40 μM [20], 1-(5-Iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride (ML-7) as a myosin light chain kinase inhibitor with a K_i value of 0.3 μM [21] or 1-[*N*,*O*-bis(1,5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) as a Ca^{2+}/calmodulin-dependent protein kinase II inhibitor with a K_i value of 0.9 μM [22]. Staurosporine or genistein was purchased from Sigma Co.. Stock solutions were prepared by dissolving them in methanol or ethanol. H-7 was purchased from Seikagaku Kogyo Co. (Tokyo, Japan), and dissolved in distilled water. H-89 was purchased from Funakoshi Co., and dissolved in a mixture of ethanol:water (1:1 v/v). ML-7 or KN-62 was purchased from Seikagaku Kogyo Co.. Stock solutions were prepared by dissolving them in methanol or dimethylsulfoxide. To study the signal transduction of phagocytosis of ganglioside-coated *S. aureus* by PMN, we examined the role of protein kinase C, protein kinase A, tyrosine kinase, myosin light chain kinase or Ca^{2+}/calmodulin-dependent protein kinase II. The heat killed *S. aureus* coated with ganglioside GM3 (5 μg ml^{-1}) was incubated with PMN in the presence of staurosporine (40–500nM), H-7 (16–240 μM), H-89 (0.4–10 μM), genistein (0.8–20 μM), ML-7 (5–15 μM) or KN-62 (10–30 μM) for 60 min at 37 °C, respectively.

Results

Adhesion of ganglioside-coated *S. aureus* to PMN

The time-course study showed that the adhesion of ganglioside (GM3, GD3 or GT1b)-coated *S. aureus* to PMN increased linearly during incubation for 60 min at 4 °C and reached a plateau at 90 min (data not shown). The adhesion rate of ganglioside (GM3, GD3 or GT1b)-coated *S. aureus* was only slightly enhanced at a concentration of 5 μg ml^{-1} (Table 1) but these changes were not significantly different from the control. Also, the aggregation of ganglioside GT1b-coated staphylococci was slightly enhanced under the microscope (data not shown).

Effect of ganglioside-coated *S. aureus* on phagocytosis by PMN

Since only slight stimulatory effects of gangliosides GM3, GD3 or GT1b were observed on the adhesion of bacteria to PMN, we tested the effect of ganglioside coating of staphylococci on their phagocytosis by PMN. The time-course study of phagocytosis of the killed *S. aureus* coated with ganglioside GM3 by PMN is shown in Figure 1. The rate of phagocytosis increased linearly during an incubation time of 60 min at 37 °C and reached a plateau at 75 min. The phagocytic rate was markedly enhanced in a dose-dependent manner.

Since ganglioside GM3 stimulated phagocytosis by PMN, the phagocytic rate of *S. aureus* coated with other gangliosides by PMN was compared, and the relationship between the structure of the ganglioside and its ability to regulate phagocytosis by PMN was defined (Table 1). Killed *S. aureus* cells coated with various gangliosides (GM1, GM2, GM3, GD1a, GD3, GT1b or asialo GM1) were incubated with PMN for 60 min at 37 °C. Ganglioside GM3- or GT1b-coated *S. aureus* cells were clearly observed in the cytoplasm of PMN (data not shown). The rate of phagocytosis by PMN of ganglioside (GM2, GM3, GD1a, GD3 or GT1b)-coated *S. aureus* increased in a dose-dependent manner, especially in ganglioside GM3 and GD3, while that of ganglioside GM1 did not (Figure 2). Furthermore, neutral GSLs such as glucosylceramide, galactosylceramide, lactosylceramide or asialo GM1 had no effect on the phagocytic rate of killed staphylococci by PMN (Figure 3). The phagocytic rates of GM3- or GD3-coated *S. aureus* by PMN were about four times higher at their maximum than that of the control (lactosylceramide). The stimulation of phagocytosis appeared to be greatest at a concentration of 5.0 μg ml^{-1} ganglioside (Figure 2, Table 1).

Table 1. The adhesion, phagocytosis and P–L fusion of human PMN with ganglioside-coated staphylococci.

Gangliosides	*Structure*	*Relative value (%)*[a]		
		Adhesion	*Phagocytosis*	*P–L fusion*
Non-coated	—	100	100	100
GM3	Gal-Glc-Cer | NeuAc	165 ± 5	460 ± 66*	150 ± 35
GM2	GalNAc-Gal-Glc-Cer | NeuAc	NT[b]	150 ± 22	158 ± 52
GM1	Gal-GalNAc-Gal-Glc-Cer | NeuAc	NT	100 ± 4	NT[b]
GD1a	Gal-GalNAc-Gal-Glc-Cer | | NeuAc NeuAc	NT	220 ± 22*	242 ± 15*
GD3	Gal-Glc-Cer | NeuAc | NeuAc	182 ± 7	383 ± 1*	221 ± 63*
GT1b	Gal-GalNAc-Gal-Glc-Cer | | NeuAc NeuAc | NeuAc	130 ± 1	235 ± 10*	500 ± 31**
Asialo GM1	Gal-GalNac-Gal-Glc-Cer	NT	100 ± 10	NT

[a] Relative values are expressed as percentage for non-coated control.
[b] NT not tested.
Gal, galactose; Glc, glucose, GalNAc, *N*-acetylgalactosamine; NeuAc, *N*-acetylneuraminic acid; Cer, ceramide.
Heat-killed *S. aureus* cells were coated with 5 μg ml^{-1} of each ganglioside.
*$p < 0.05$, **$p < 0.01$ versus each control. All other differences $p > 0.05$

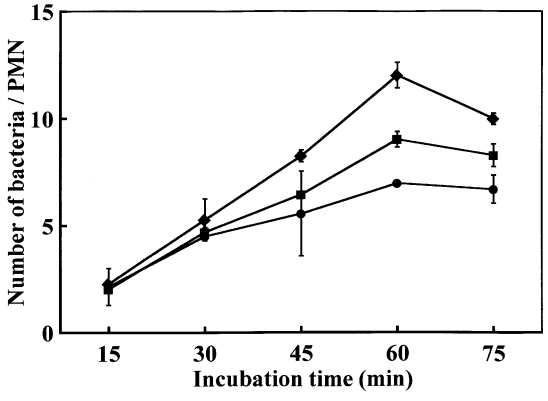

Figure 1. The time course for the phagocytosis of killed *S. aureus* coated with ganglioside GM3 by PMN. Ganglioside GM3-coated *S. aureus* (5×10^7 ml^{-1}) was incubated with human PMN (5×10^6 ml^{-1}) at 37 °C on a cover slip. PMN on the cover slip were fixed with methanol, followed by staining with methylene blue. The number of ganglioside-coated *S. aureus* phagocytosed by PMN was counted under a microscope. The values represent mean ± SD. (●) 0 μg ml^{-1}; (■) 1 μg ml^{-1}; (◆) 5 μg ml^{-1}.

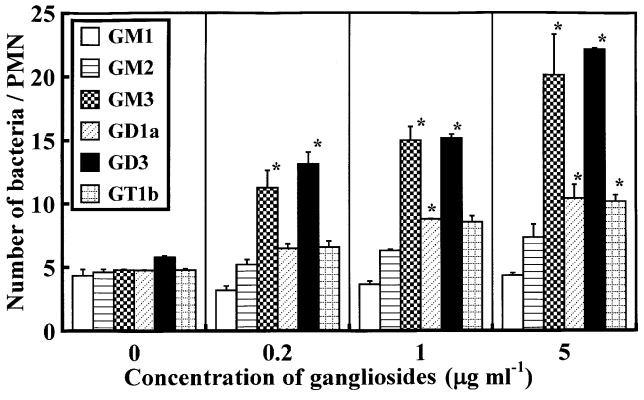

Figure 2. Comparison of phagocytosis promoting activity of ganglioside (GM1, GM2, GM3, GD1a, GD3 and GT1b). Heat-killed *S. aureus* were coated with GM1, GM2, GM3, GD1a, GD3 or GT1b of indicated concentrations and then phagocytosis rate was estimated as described in the Materials and methods. The values represent mean ± SD. *$p < 0.05$ versus each control.

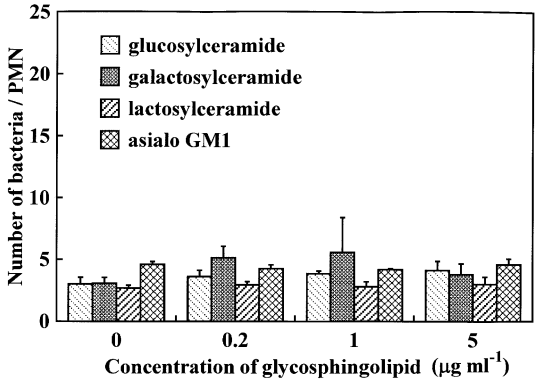

Figure 3. Comparison of phagocytosis promoting activity of neutral GSLs. Heat-killed *S. aureus* were coated with glucosylceramide, galactosylceramide, lactosylceramide or asialo GM1 of the indicated concentrations and then the phagocytic rate was estimated as described in Materials and methods. The values represent mean ± SD.

Effect of ganglioside-coated *S. aureus* on phagosome-lysosome fusion by PMN

Since P–L fusion and a respiratory burst are important mechanisms for PMN intracellular killing, we tested the P–L fusion of PMN, prelabelled with acridine orange, by observing the presence of stained *S. aureus* under a fluorescent microscope. Ganglioside-coated *S. aureus* were incubated at 37 °C for 60 min. The fusion index was defined as the percentage of positive fusions multiplied by the mean number of fused phagosomes per cell [7]. The effect of ganglioside (GM3, GD1a, GD3, GT1b or asialo GM1)-coating on P–L fusion was also examined with a concentration of 0.2–5.0 μg ml^{-1} (Figure 4). The fusion index of *S. aureus* coated with gangliosides GD1a, GD3 and GT1b was increased significantly, while that of ganglioside asialo GM1 was not (Figure 4). Ganglioside GT1b-coated staphylococci showed the highest stimulatory effect – about five times higher than that of the control (Figure 4, Table 1).

Effect of ganglioside on superoxide anion production by PMN

One of the bacteriacidal mechanisms of PMN is the production of active oxygen species such as superoxide anion or hydroxyl radicals. We therefore examined the effect of ganglioside (GM2, GM3, GD1a or GT1b)-coating of staphylococci on superoxide anion release from PMN. Superoxide anion release from PMN was not enhanced by coating with gangliosides up to a concentration of 5 μg ml^{-1} (Figure 5).

Effect of staurosporine, H-7, H-89, genistein, ML-7 or KN-62 on ganglioside-induced phagocytosis or P–L fusion by PMN

It is of interest to know how a ganglioside can stimulate or enhance the intracellular killing system of PMN. To clarify

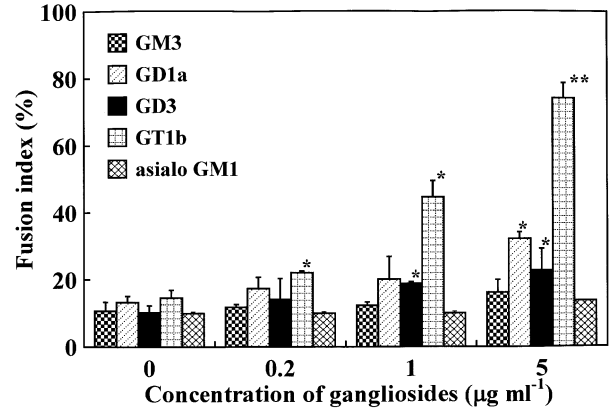

Figure 4. Effect of coating of *S. aureus* with ganglioside (GM3, GD1a, GD3, GT1b or asialo GM1) on phagosome–lysosome (P–L) fusion by PMN. The P–L fusion was examined in the concentration of 0.2–5.0 μg ml^{-1}. The fusion index was defined as the percentage of positive fusion multiplied by the mean number of fused phagosomes per cell. The values represent mean ± SD. *$p < 0.05$ versus each control. **$p < 0.01$ versus each control.

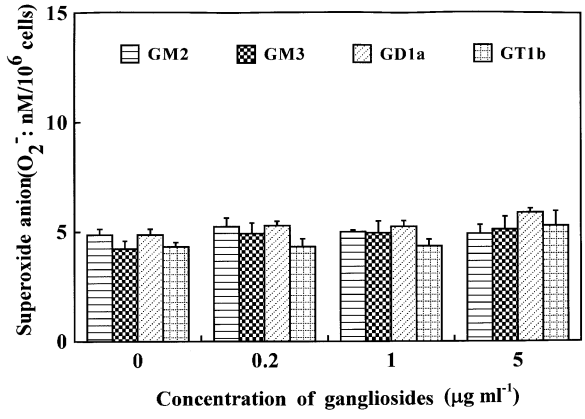

Figure 5. Effect of coating of *S. aureus* with ganglioside (GM2, GM3, GD1a or GT1b) on superoxide anion (O_2^-) release from PMN. Results are mean ± SD of three different experiments.

the signal transduction mechanism, we examined the effect of protein kinase inhibitors. The PMN phagocytosis of ganglioside GM3 (5 μg ml^{-1})-coated *S. aureus* was inhibited in a dose-dependent manner with staurosporine, H-7 or KN-62, within concentrations that did not damage the PMN (Figure 6), but was not inhibited with H-89, genistein or ML-7 (data not shown). It is suggested that protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II are required for enhancing the phagocytosis induced by ganglioside GM3. Furthermore, we tested the effect of H-7 or KN-62 on ganglioside GM3- or GT1b-induced P–L fusion. The P–L fusion of *S. aureus* coated with ganglioside GT1b was inhibited with H-7 and KN-62 (Figure 7). It is suggested that protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II are also directly or indirectly involved in stimulating the ganglioside-induced P–L fusion by PMN.

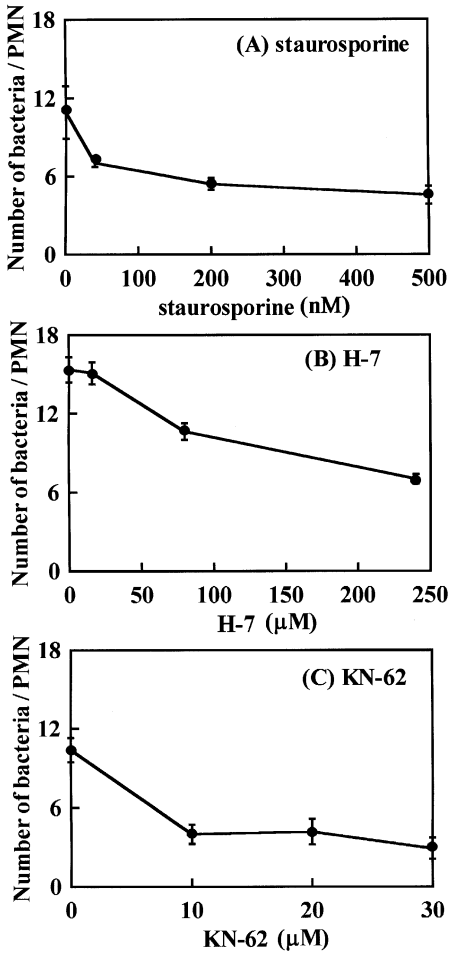

Figure 6. Effect of protein kinase inhibitor (staurosporine, H-7 or KN-62) on ganglioside GM3-induced phagocytosis by PMN. Staurosporine of H-7; a protein kinase C inhibitor. KN-62; a Ca^{2+}/calmodulin-dependent protein kinase II inhibitor. The values represent mean ± SD.

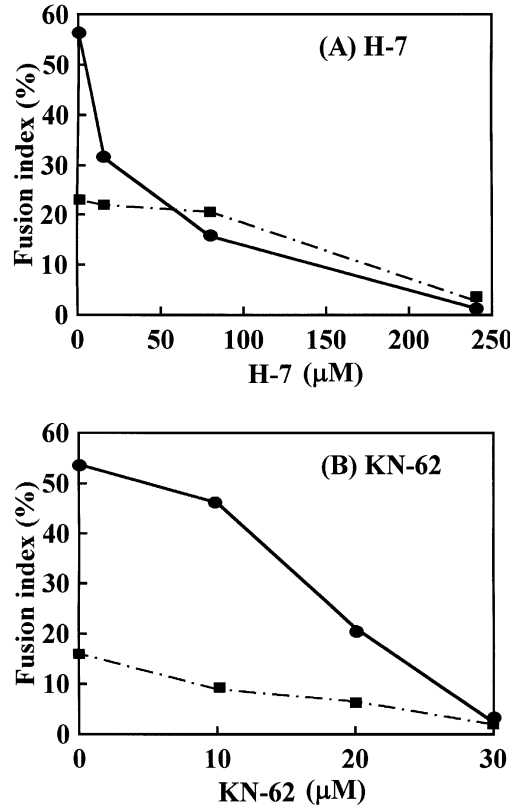

Figure 7. Effect of H-7 (protein kinase C inhibitor) or KN-62 (Ca^{2+}/calmodulin-dependent protein kinase II inhibitor) on ganglioside GM3- or GT1b-induced phagosome-lysosome fusion by PMN. (●) GT1b 5 μg ml^{-1}; (■) GM3 5 μg ml^{-1}.

Discussion

In this report, we attempted to elucidate the relationship between the molecular structure of ganglioside and the effect on phagocytic activity of PMN. Previously, we have reported that the acidic GSLs from a gram-negative aerobic bacterium '*Sphingomonas*' have a marked stimulative activity on phagocytosis [13] and that the sulfatide (galactosylceramide-3-sulfate) from bovine brain is also significant in promoting phagocytosis by PMN [14]. Attachment to host cells in mucous membranes is the initial event of the infectious process and the molecules promoting this step are one important virulence factor. This may be followed by penetration of the host mucous membrane and interaction with phagocytic cells. Such a function is required for viral infection, bacterial toxin penetration, and infection of some intracellular pathogens. In such processes, membrane lipid-associated oligosaccharides are of particular interest [5]. To reveal the roles of various gangliosides, we therefore examined adhesion, phagocytosis, P–L fusion, superoxide anion production, and intracellular signal transduction of PMN with ganglioside-coated staphylococci.

Coating *S. aureus* with ganglioside (GM3, GD3 or GT1b) had a slight stimulative effect on adhesion to PMN (Table 1). Furthermore, we examined the effect of various gangliosides (GM1, GM2, GM3, GD1a, GD3 or GT1b) on phagocytosis by PMN. Gangliosides GM3, GD1a, GD3 and GT1b significantly accelerated the phagocytosis in a dose-dependent manner, while GM1 and neutral GSLs did not (Figures 2 and 3). P–L fusion of ganglioside (GD1a, GD3 or GT1b)-coated *S. aureus* to PMN was also stimulated in a dose-dependent manner, while that of ganglioside asialo GM1 was not (Figure 4). Relative phagocytic rates of ganglioside (GM3, GD1a, GD3 or GT1b)-coated *S. aureus* were from 2.2 to 4.6 times higher than that of the non-coated control, and ganglioside GM3 showed the greatest stimulatory effect (Figure 2, Table 1). The fusion index of *S. aureus* coated with gangliosides (GD1a, GD3 or GT1b) increased markedly as did phagocytosis, and with ganglioside GT1b it was five times higher than that of the non-coated control (Figure 4, Table 1). As shown in Table 1, gangliosides

consist of two structural components, a lipid moiety (ceramide) inserted into the lipid bilayer of the plasma membrane, and a carbohydrate portion extending outward into the extracellular milieu. Therefore, gangliosides are amphipathic molecules. Ganglioside GD1a, GD3 or GT1b have relatively more sialyl residues than GM2 or GM3, especially ganglioside GT1b which is the most hydrophilic and anionic (Table 1). The structural difference of gangliosides seemed to play a critical role in the modulating activity of phagocytic processes. Saito *et al.* reported that the biological activities of gangliosides could be demonstrated in relation to their chemical structure, especially the sugar moiety [12]. Van Oss *et al.* have reported that the more hydrophobic bacteria can be readily phagocytosed, while the more hydrophilic bacteria resisted this process [23]. The stimulatory effect of gangliosides may, however, be affected not only by the size of the hydrophilic carbohydrate chain, but also by the positional distribution of the anionic charge (for instance the terminal sialic acid).

In a previous report, we have described how negative charge on D-glucuronic acid in glycosphingolipids plays an important role in promoting phagocytosis by PMN [13, 16]. Recently, we found that a sulfatide (galactosylceramide-3-sulfate) showed a marked stimulatory effect on the phagocytic processes of PMN and the existence of a negative charge on the carbohydrate moiety may be essential for the induction of differentiation of phagocytic cells [14]. In the case of gangliosides, the phagocytosis of serum-opsonized *S. aureus* was not promoted in GM3-pretreated PMN (data not shown), however, as demonstrated in this report, the existence of sialic acid may be essential for the enhancement of both phagocytosis and P–L fusion. We therefore tested whether GM3-mediated phagocytosis was affected by the presence of free sialic acid, but the addition of sialic acid did not affect ganglioside-induced phagocytosis (data not shown). This might be caused by the difference between free sialic acid and sialic acid as a component of the ganglioside structure. Recently, Brandly *et al.* reported that selectin is a carbohydrate-binding protein requiring a carboxyl group in sialic acid [24], and Yuen *et al.* showed that E-selectin can bind to sulfated Lewis^a or sialyl-Lewis^x structure [25]. Further, Ladish *et al.* reported that a potent immunosuppressing activity is usually associated with the gangliosides containing a terminal sialic acid in the human T cell proliferative responses [7, 8]. In the current study, it was shown that a terminal sialic acid was required for the enhancement of phagocytosis and the number of sialic acids affected the P–L fusion. To date, the mechanism by which such a structure is required for the induction of phagocytosis is unknown. Both phagocytosis and P–L fusion are common events in actin-dependent cell functions. It is possible that a particular carbohydrate structure transmits a signal via an unknown regulator molecule. It is also assumed that phagocytosis and P–L fusion proceed by different mechanisms or different intracellular signal transduction systems.

We, therefore, also examined the effect of protein kinase or phosphatase inhibitors (staurosporine, H-7, H-89, genistein, ML-7 or KN-62) on the ganglioside-induced phagocytosis in order to study the ganglioside-mediated intracellular signal transduction. We used a much higher concentration of inhibitors than suggested by the K_i value for protein kinase. Zheng *et al.* used the solution at a concentration of 50 μM H-7 or 120 nM staurosporine with opsonized *S. aureus* for incubation with human monocytes to look at the role of protein kinase C isozymes [26]. An incubation time of 60 min was necessary to observe the inhibitory effect of H-7, because the effect was insufficient at 45 min (data not shown). The ganglioside-induced phagocytosis was inhibited by staurosporine, H-7 or KN-62 (Figure 6), but not by H-89, genistein or ML-7 in a concentration which did not cause cell damage (data not shown). Furthermore, both H-7 and KN-62 also inhibited ganglioside GT1b-induced P–L fusion (Figure 7). It has been suggested that the stimulatory effect of ganglioside on phagocytosis and P–L fusion might be induced via signal transduction in which protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II are involved. Generally, protein kinase C is involved in the oxidant-mediated amplification of bacterial ingestion [27, 28], but gangliosides did not enhance the release of superoxide anion up to 5 μg ml^{-1} (Figure 5). Recently, it has been reported that there may be functional co-operation between small GTP-binding proteins such as Rho, Rab and Rac to regulate sequential reactions – Rho can regulate cell motility, Rab can regulate exocytosis and endocytosis, and Rac can regulate superoxide anion production [29]. These results suggest that the signalling pathway for the stimulatory effect of ganglioside on phagocytosis and P–L fusion may be different from that of superoxide anion production. Further, the mechanism for activation of phagocytosis via cellular transduction in human phagocytic cells may be of particular interest and this should be studied in the future.

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