

Hemolysis of Human Erythrocytes Is a New Bioactivity of Gangliosides

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Summary

Using sheep erythrocytes and liposomes, an inhibitory effect of gangliosides has been shown on the activation of the alternative pathway of complement. However, in studies using human erythrocytes, we found that gangliosides had hemolytic activity that was possibly mediated through activation of the alternative pathway. Pretreatment of human erythrocytes obtained from healthy volunteers or paroxysmal nocturnal hemoglobinuria (PNH) patients with a ganglioside mixture purified from human erythrocytes enhanced their susceptibility to homologous human complement, and resulted in dose-dependent hemolysis. The enhancement was more marked in PNH erythrocytes than control cells. Protease treatment of the ganglioside mixture did not change its hemolytic activity, but sialidase treatment abolished the activity. Among the major erythrocyte gangliosides, II³NeuAc-LacCer (GM3) was the most potent hemolytic agent. Gangliosides purified from bovine brain were also active, while neither nonsialylated glycosphingolipids, the ceramide moiety, or sialic acid alone were active. Sialic acid residues in the ganglioside molecules were essential to this activity, but the amount of the residue or the source of the gangliosides seemed not to be important. Several treatments inhibiting the alternative but not classical complement pathway markedly reduced the ganglioside hemolytic activity. This novel bioactivity of gangliosides was thus suggested to be mediated partly by activation of the alternative pathway.

Gangliosides are sialylated glycosphingolipids (GSLs)¹ that are functionally located on the outer cell membrane (1). GSLs are well known to participate in various membrane-related biological functions that are associated with cell growth, development, differentiation, and transformation (2, 3). However, the molecular mechanisms of the involvement of GSLs in cellular membrane function are not yet clear. Extensive studies have elucidated new biological functions of GSLs, such as the modulation of cell growth (4, 5), induction of cell differentiation (6), modulation of the immune response (7, 8), and inhibition of viral infection (9), when GSLs are added exogenously to cell culture. In each case, the exogenous GSLs are first inserted into and accumulated in

the lipid layer of the cell membrane (10–14), after which they affect membrane function (3–6, 11, 15). An intensive search for new bioactivities that arise from the interaction of exogenously added GSLs with the functional constituents of cell membranes (4, 11, 16–18) might provide some clues to help our understanding of the critical role of endogenous GSLs in physiological membrane functions. We have recently demonstrated the altered expression of membrane gangliosides in erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH) (19). During our exploration of the physiological significance of ganglioside accumulation in PNH, we also discovered a hemolytic activity of gangliosides that has the potential to be used to enhance our understanding of the physiological role of membrane GSLs.

Materials and Methods

Chemicals. All organic solvents used were of analytical grade. DEAE Sephadex A-25 was obtained from Pharmacia Fine Chem-

¹ Abbreviations used in this paper: DAF, decay-accelerating factor; GSL, glycosphingolipid; PNH, paroxysmal nocturnal hemoglobinuria. GSLs were abbreviated according to the recommendations of the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB) (42), or the nomenclature system of Svennerholm (43).

icals (Uppsala, Sweden) and was converted to its acetate form as described previously (20). Iatrobeads (activated silicic acid, 6RS-8060) were purchased from Iatron Laboratories Inc. (Tokyo, Japan). Precoated high-performance thin-layer plates (Silica Gel 60, 0.25 mm thick) were purchased from E. Merck (Darmstadt, Germany). Sialic acid (*N*-acetylneuraminic acid) was donated by Mect Co. (Tokyo, Japan). Beef brain ceramide was purchased from Serdary Research Laboratories (Ontario, Canada). EDTA and EGTA were purchased from Nacalai Tesque (Kyoto, Japan). The ganglioside mixture used, as well as IV³NeuAc, II³NeuAc-Gg₄Cer (GD1a), were purified from bovine brain in our laboratory by the method described below.

Extraction and Purification of GSLs from Erythrocytes. Gangliosides and neutral GSLs were purified by the methods described previously (19, 21, 22). In brief, erythrocyte membranes were isolated by lysing erythrocytes from healthy volunteers with 0.05% acetic acid and total lipids were extracted from the membranes with organic solvents. After the removal of glyceroglycolipids by mild alkaline hydrolysis, GSLs were then separated into neutral and acidic (ganglioside) fractions by DEAE-Sephadex A-25 column chromatography, and individual GSLs were further purified by Iatrobeads linear-gradient column chromatography (23). Purified glycolipids were then analyzed using TLC and quantitatively determined by densitometric analysis in comparison with standard glycolipids.

Hemolysis Assay. Ham's acidified serum test (24) was modified to assay the hemolytic activity of gangliosides or neutral GSLs. Briefly, erythrocytes were obtained from the peripheral blood of healthy volunteers or PNH patients after consent was given, washed three times with saline, and incubated in saline at 37°C for 5 min with various concentrations of gangliosides or neutral GSLs. These pretreated erythrocytes were washed thoroughly with saline to remove unbound glycolipids and were then adjusted to 3×10^9 /ml in saline. 50 μ l of the pretreated erythrocytes (1.5×10^8 cells) was incubated at 37°C for 60 min with 500 μ l of complement-containing serum obtained just before use from blood type (ABO, Rh)-matched healthy volunteers. Differing from Ham's test, fresh serum was not acidified in our assays. The extent of hemolysis was evaluated by measuring the hemoglobin liberated into the supernatant using a spectrophotometer (DU series 60; Beckman Instruments Inc., Fullerton, CA) at 540 nm. Each assay was performed in duplicate or triplicate.

To examine the involvement of complement in the process of hemolysis, inactivation of serum complement was performed by heating at 56°C for 30 min or chelating with EDTA and EGTA (both 5 mM) (25–27). A combination of both 10 mM EGTA and 5 mM MgCl₂ was used for the selective inhibition of the classical pathway of complement (25–27). The ganglioside fraction was treated with protease from *Staphylococcus aureus* strain V8 (Sigma Chemical Co., St. Louis, MO) at 37°C for 2 h to inactivate possible protein contaminants.

Flow Cytometric Analysis. Erythrocytes pretreated with a ganglioside mixture of erythrocytes were incubated with mouse mAb against II³NeuAc-LacCer (GM3) (Mectolone-015; Mect Co.) at 4°C for 30 min, and subsequently labeled with FITC-conjugated goat anti-mouse Ig (Zymed Laboratories Inc., San Francisco, CA) at 4°C for 30 min. They were then analyzed by laser fluorometry (FACScan®, Becton Dickinson & Co., Mountain View, CA) as described previously (11, 15). The membrane expression of decay-accelerating factor (DAF) by erythrocytes was also fluorocytometrically analyzed as described previously (19).

Results

TLC of Gangliosides Purified from Human Erythrocytes. Fig. 1 shows the results of TLC of gangliosides. As is already well known, the major gangliosides found were GM₃, IV³NeuAc-nLc₄Cer (2-3SPG), IV³NeuAc-Gg₄Cer (GM1), and IV⁶NeuAc-nLc₄Cer (2-6SPG) in erythrocyte membranes (lane 1) (28), and GM₃, GM1, and GD1a in bovine brain (lane 5). GM₃ (lane 2) and 2-3SPG (lane 3) were isolated individually as the major gangliosides found in human erythrocytes.

Hemolysis Assay. Hemolytic activity is often determined by the percent hemolysis calculated from the population of hemolyzed cells. For the sensitive detection of slight hemolysis, we used the change in optical density of the supernatant during incubation. The optical density after the complete hemolysis of 1.5×10^8 erythrocytes with 0.02% acetic acid was ~ 4.0 , and this value corresponded to 100% hemolysis. Using several different concentrations of erythrocytes, the relation between optical density and percent hemolysis was then

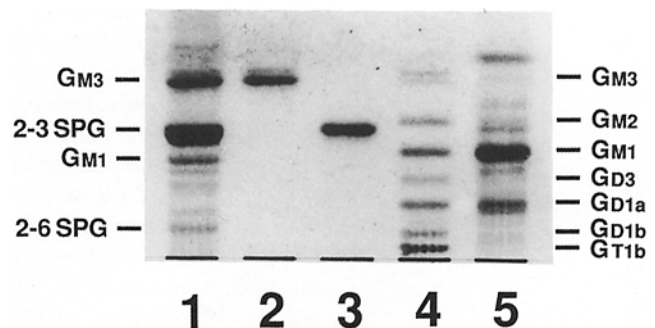


Figure 1. TLC of gangliosides purified from human erythrocytes or bovine brain. Gangliosides were separated with a solvent system of chloroform/methanol/0.5% aqueous CaCl₂ (55:45:10 by vol) and visualized with resorcinol-HCl (21). (Lanes 1–3) Ganglioside mixture, GM₃, and 2-3SPG from human erythrocytes, respectively; (lane 4) GM₃, GM₂, GM₁, GD₃, GD_{1a}, GD_{1b}, and GT_{1b} as marker gangliosides from bovine brain; (lane 5) ganglioside mixture from bovine brain.

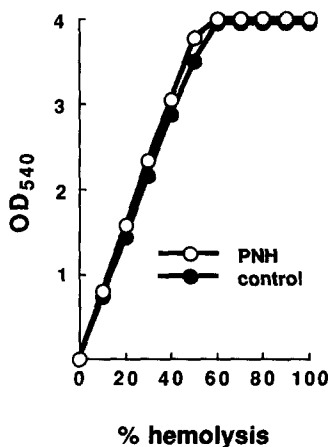


Figure 2. Correlation between optical density (OD₅₄₀) and percent hemolysis in the hemolysis assay. The complete hemolysis of 1.5×10^8 erythrocytes obtained from healthy volunteers (●) or PNH patients (○) corresponds to 100% hemolysis. Using several numbers of erythrocytes, the curve was determined as described in the text.

determined (Fig. 2). Linearity was observed over the range of 0–50% hemolysis, and the standard curve obtained with PNH erythrocytes was almost identical to that for control erythrocytes (Fig. 2). On the basis of this linear relation, the optical density was converted to percent hemolysis in the subsequent hemolysis assays.

Ganglioside-treated erythrocytes obtained from healthy volunteers or PNH patients showed a dose-dependent increase in sensitivity to complement-containing serum, resulting in increased hemolysis (Fig. 3). PNH erythrocytes were originally sensitive to complement (see the percent hemolysis without ganglioside treatment in Fig. 3), and the ganglioside-treated PNH cells (Fig. 3, *a* and *b*) showed even more enhancement of their susceptibility to complement-containing serum when compared with ganglioside-treated control

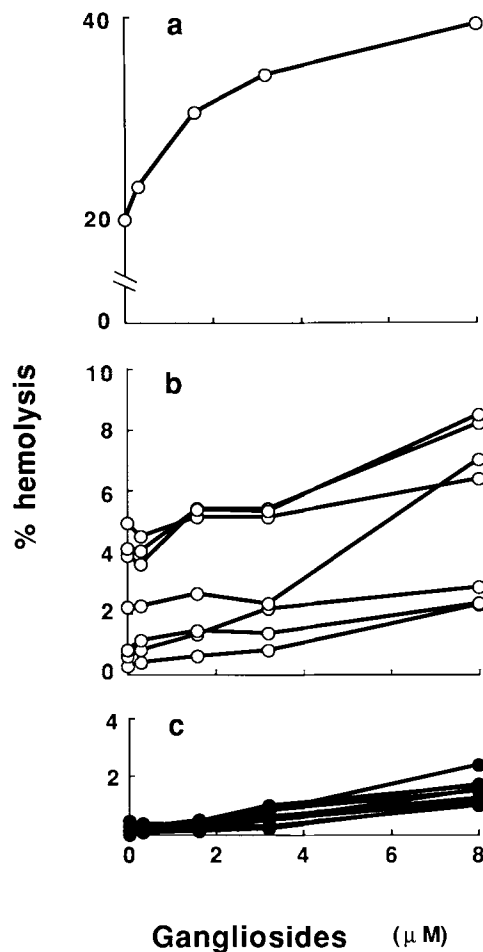


Figure 3. Enhanced hemolysis of ganglioside-treated human erythrocytes. Erythrocytes obtained from nine healthy volunteers (●) and eight PNH patients (○) were incubated with 0–8- μM ($12 \mu\text{g/ml}$) concentrations of a ganglioside mixture isolated from human erythrocytes. (*a*) Erythrocytes with a 98% DAF-negative population from a PNH patient; (*b*) erythrocytes with a 15–70% DAF-negative population from seven PNH patients; (*c*) control erythrocytes from nine healthy volunteers.

erythrocytes from healthy volunteers (Fig. 3 *c*). The ganglioside-induced enhancement of susceptibility of control erythrocytes to complement was weak but reproducible. Gangliosides at $<2 \mu\text{M}$ ($3 \mu\text{g/ml}$) could also clearly enhance hemolysis in some cases of PNH. Pretreatment with $8 \mu\text{M}$ ($12 \mu\text{g/ml}$) ganglioside mixture for 5 min induced, respectively, 20%, 2–5%, and 1–2% enhancement of the hemolysis of PNH erythrocytes that were 98% DAF-negative from a PNH patient with coexisting C9 deficiency (Fig. 3 *a*) (29), PNH erythrocytes that were 15–70% DAF negative (Fig. 3 *b*), and control erythrocytes from healthy volunteers (Fig. 3 *c*). Fig. 4 shows the dependency of ganglioside-induced hemolysis on the duration of pretreatment of erythrocytes with $8 \mu\text{M}$ ganglioside mixture. The hemolytic effect reached its maximum level within 20 min in control erythrocytes (Fig. 4 *b*) and increased with time in PNH erythrocytes (Fig. 4 *a*).

Fig. 5 shows the hemolytic activity of the individual gangliosides. GM3 purified from human erythrocytes possessed a potent activity, whereas 2-3SPG from human erythrocytes or GD1a from bovine brain were not biologically active. The ganglioside mixture obtained from bovine brain also showed a marked hemolytic activity at similar doses to those of erythrocyte gangliosides (data not shown).

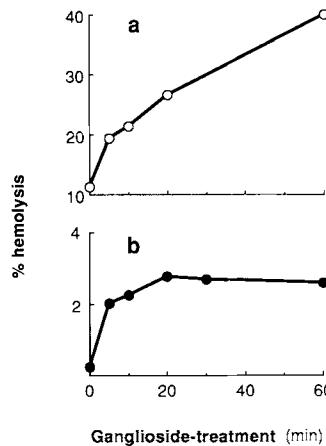


Figure 4. Dependency of hemolysis on the duration of ganglioside treatment. Erythrocytes from a healthy volunteer (*b*) or a PNH patient (*a*) were treated with $8 \mu\text{M}$ ($12 \mu\text{g/ml}$) ganglioside mixture for 0–60 min at 37°C , as described in the text.

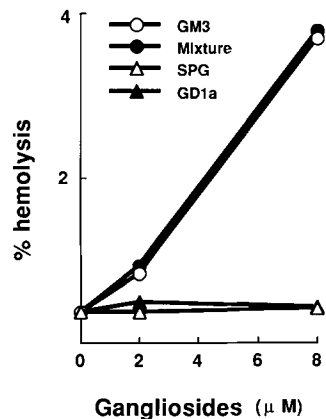


Figure 5. Hemolytic activity of individual gangliosides. Erythrocytes with a 5% DAF-negative population were incubated for 5 min at 37°C with GM3 (○) and 2-3SPG (△) purified from human erythrocytes, GD1a purified from bovine brain (▲), or the ganglioside mixture obtained from human erythrocytes (●). All gangliosides were used at $8 \mu\text{M}$.

Neutral GSLs, ceramide, and sialic acid alone did not have any hemolytic activity at 8 μM (data not shown). Neuraminidase treatment of gangliosides inhibited their hemolytic effect. Heating and protease treatment of the ganglioside mixture to inactivate possible protein contaminants did not change its hemolytic activity (data not shown). To investigate the involvement of complement in ganglioside-induced hemolysis, several types of pretreatment of complement-containing serum were performed (Fig. 6). Heating at 56°C for 30 min (Fig. 6, column 2) and the use of both 5 mM EDTA and 5 mM EGTA (Fig. 6, column 3) reduced, respectively, the hemolysis to 50% and 45% of the control level achieved with untreated serum (Fig. 6, column 1). Serum obtained from a patient with C9 deficiency (Fig. 6, column 5) (29) also reduced the hemolysis to 44% of the control level. However, addition of both 10 mM EGTA and 5 mM MgCl_2 (which inhibits the classical but not alternative pathway of complement) did not reduce hemolysis (data not shown).

Flow Cytometric Analysis of Ganglioside-treated Erythrocytes. After ganglioside treatment of erythrocytes, we performed flow cytometric analysis with anti-glycolipid (GM_3) antibody, which showed that the ganglioside-treated erythrocytes were stained more intensely than the untreated erythrocytes (data not shown).

Discussion

We detected and investigated a new biological activity of gangliosides. A ganglioside mixture that was isolated from human erythrocytes induced hemolysis in a dose-dependent manner when added to human erythrocytes. PNH erythrocytes are susceptible to complement (30, 31) due to the lack of complement regulatory proteins (32–34), and they showed more enhancement of their susceptibility to complement-containing serum than the control erythrocytes. The discrepancy in the time course of ganglioside-mediated hemolysis between the control and PNH erythrocytes may suggest

that the maximum level of ganglioside hemolytic activity depends on the size of the population of labile erythrocytes susceptible to complement attack. Several methods of treatment of serum to inactivate complement significantly reduced the extent of ganglioside-induced hemolysis. Moreover, C9-deficient serum also reduced the hemolysis. Inhibition of the classical but not the alternative pathway of complement did not decrease the hemolysis. Antibody-dependent cytolysis is known to be associated with the classical pathway of complement (35). However, we could not detect serum antibodies directed against either gangliosides or erythrocytes using TLC immunostaining (19) or laser fluorocytometry (data not shown). These results thus suggest that ganglioside-induced hemolysis occurs at least partly through activation of the alternative pathway of complement, but is not antibody dependent.

The following findings also suggested the involvement of additional mechanisms in the hemolytic process. Even in the absence of complement-containing serum, gangliosides still induced hemolysis dose dependently but weakly. Inhibition of the activation of complement with chelating agents or heating was remarkably effective in reducing hemolysis, but did not completely eliminate it. Also, gangliosides induced the hemolysis of even control erythrocytes, which express sufficient complement-regulatory membrane proteins to prevent hemolytic attack by complement. Finally, even in the presence of C9 deficiency serum, gangliosides still induced hemolysis. These results all imply the involvement of other mechanisms in ganglioside-induced hemolysis in addition to complement activation.

We investigated the possible presence of contaminants with a hemolytic activity, especially proteins or neutral glycolipids, in ganglioside mixture. Pretreatment of the mixture using proteases or heat did not produce any change in the hemolytic activity. Organic solvents like chloroform and methanol, which denature proteins, were actually used in purification of the gangliosides, so it is quite unlikely that any proteins with hemolytic activity survived in the ganglioside mixture. Neutral GSLs isolated from human erythrocytes did not have any hemolytic activity, even at higher doses of up to 200 μM . Alkaline hydrolysis used in the purification of gangliosides could eliminate lyso-phospholipids that are known to possess hemolytic activity. Thus, our results suggest that only the gangliosides in the mixture possessed any hemolytic activity. The structural difference between gangliosides and neutral GSLs is solely the presence of sialic acid residues in the former, suggesting that these residues are more critical than the other carbohydrate or ceramide moieties of the gangliosides with respect to the hemolytic activity. To confirm this new biological activity and examine the structure-activity relationships of gangliosides, we isolated the major gangliosides GM_3 and 2-3SPG, which comprise >80% of the gangliosides in human erythrocytes (28). GM_3 showed a potent hemolytic activity, while 2-3SPG (the most abundant ganglioside in erythrocytes) did not show any hemolytic activity. A ganglioside mixture isolated from bovine brain also possessed remarkable hemolytic activity. Bovine brain $\text{GD}1a$ did

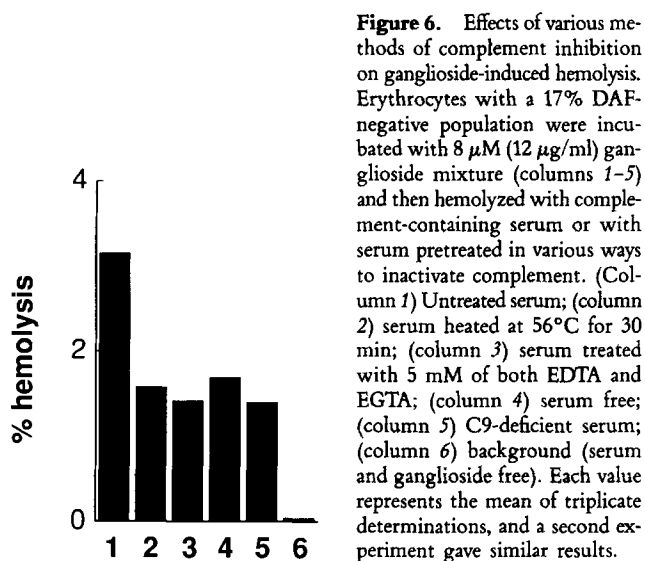


Figure 6. Effects of various methods of complement inhibition on ganglioside-induced hemolysis. Erythrocytes with a 17% DAF-negative population were incubated with 8 μM (12 $\mu\text{g}/\text{ml}$) ganglioside mixture (columns 1–5) and then hemolyzed with complement-containing serum or with serum pretreated in various ways to inactivate complement. (Column 1) Untreated serum; (column 2) serum heated at 56°C for 30 min; (column 3) serum treated with 5 mM of both EDTA and EGTA; (column 4) serum free; (column 5) C9-deficient serum; (column 6) background (serum and ganglioside free). Each value represents the mean of triplicate determinations, and a second experiment gave similar results.

not show any hemolytic activity. Thus, the two ganglioside mixtures and GM₃ itself from human erythrocytes were indicated to possess a potent hemolytic activity, and the sialic acid content of the molecules seemed not to be critical to the production of hemolysis. These results suggest that neither ganglioside-induced hemolysis was due to artificial damage using high levels of lipids, nor that the hemolysis required sialic acid simply for the solubility of the lipids.

Regarding the mode of action of the exogenously added gangliosides, fluorocytometric analysis suggested that they were functionally incorporated into the erythrocyte membrane. The incorporation of gangliosides into cell membranes has been reported to be essential in their biological activities at varying doses (10–14). Thus, the next problem is to clarify how the incorporated gangliosides induced hemolysis. We have suggested the possible involvement of the alternative pathway in the production of hemolysis. There have already been reports that gangliosides regulate the activation of the alternative pathway (36–38), but they were actually reported to inhibit the activation of the alternative pathway, although our findings suggested a quite different conclusion. This discrepancy may be partly explained by differences in the experimental conditions. First, the molecular species and doses of the gangliosides used were different. Next, the previous studies evaluated the effects of gangliosides by measuring C3 conversion on the surface of ganglioside-containing liposomes or animal erythrocytes like sheep erythrocytes. In contrast, we assessed the hemolysis of ganglioside-treated human erythrocytes. Thus, the possibility of conformational differences in the membrane localization of gangliosides when they are incorporated into liposomes or intact human blood cells can be suggested (39–41). In particular, intact cells and above

all human erythrocytes possess both well-known and perhaps unknown complement-regulatory membrane proteins in contrast to liposomes. It seems likely that the presence of such complement regulatory proteins has various effects on the activation of complement. The different results in various studies may thus reflect the presence of several sorts of complement-regulatory membrane proteins, the functions of which could be modulated by gangliosides. In fact, the selective and functional interaction of gangliosides (especially GM₃) with membrane proteins has been reported previously (4, 16, 41). Accordingly, we propose that the exogenously added gangliosides were first inserted into the erythrocyte membrane where they interacted with certain membrane constituents that might restrain complement activation, abolished this restriction, and produced hemolysis after activation of the alternative pathway. Interestingly, ganglioside-treated white blood cells also became susceptible to complement-containing serum (data not shown). Further studies are still required to clarify the molecular mechanisms of ganglioside-induced activation of the alternative pathway to produce hemolysis.

This is apparently the first report of a hemolytic effect of gangliosides on human erythrocytes. The use of intact PNH erythrocytes with increased susceptibility to complement and the establishment of a sensitive method for the quantitation of hemolytic activity using spectrophotometry enabled us to reproducibly detect this novel hemolytic activity of gangliosides. We have previously reported the increased expression of gangliosides in the membranes of PNH erythrocytes (19). These findings, taken together, suggest that the increased expression of membrane gangliosides in PNH erythrocytes may lead to abnormal hemolysis.

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