

Inhibition of basophil histamine release by gangliosides

Further studies on the significance of cell membrane sialic acid in the histamine release process

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Histamine release from human basophils was inhibited by preincubation of the cells with a glucolipid mixture containing sialic acid-containing gangliosides. This was true for histamine release induced by anti-IgE, Concanavalin A and the calcium ionophore A23187, whereas the release induced by *S. aureus* Wood 46 was not affected. It was demonstrated that the inhibitory capacity of the glucolipid mixture could be attributed to the content of gangliosides, since no inhibition was obtained with cerebroside or with gangliosides from which sialic acid was removed. Preincubation of the cells with the glucolipid mixture increased the sialic acid content of the cells, and this increase was attributed to an insertion of gangliosides into the cell membrane. The inhibition of histamine release was abolished by increasing the calcium concentration, which substantiates our previous findings that cell membrane sialic acid in basophil leukocytes is involved in the regulation of histamine release, possibly by a modulation of the transmembraneous calcium fluxes preceding histamine release.

Key words: basophils; calcium, histamine; gangliosides; sialic acid.

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We have previously shown that cell membrane sialic acid (NANA) modulates basophil histamine release induced by various stimulators (9, 10). Enzymatic removal of membrane NANA increased histamine release and caused a shift to the left of the calcium dependence curve, whereas preincubation of the cells with free NANA caused the opposite reaction, i.e. a diminished histamine release that could be normalized by increasing the extracellular calcium concentration (10). Since the amount of free NANA necessary for inhibition of histamine release was high, we investigated the inhibitory capacity of NANA in exogenously added ganglioside-containing glucolipids. These glucolipids might more readily be inserted into cell membranes due to the lipophilic ceramide end of the gangliosides (2, 17, 23).

MATERIAL AND METHODS

N-acetylneuraminic acid (NANA) type IV, galactocerebrosides types 1 and 2 from bovine brain, and glucocerebrosides from Gaucher's spleen were purchased from Sigma Chem. (St. Louis, USA). A glucolipid mixture (Gmix) from bovine brain was a generous gift from Dr. G. Reuter, University of Kiel, FRG. It was delivered purified (13) and analyzed (14) and contained 20.9% NANA-containing gangliosides, the rest being mainly cerebroside, sulfatides and fatty acids. Only the gangliosides contained NANA (20.4%) and no free NANA was found in the Gmix. The gangliosides consisted of Gm₁ (20.4%), GD₃ (4.1%), GD_{1a} (46.8%), GD_{1b} (17.2%), GT_{1b} (10.9%) and GQ_{1b} (0.5%) (w/w). The Gmix and the cerebroside were dissolved in 0.9% NaCl and sonicated for 10 s on ice

before use. Whole bacteria of the protein A-poor strains of *S. aureus* Wood 46 was prepared as previously described (11). Anti-human IgE (ϵ -chain) 400,000 IU/ml and *V. cholerae* sialidase were obtained from Behringwerke AG (FRG), Concanavalin A (Con A) was from Pharmacia AB (Sweden) and the calcium ionophore A23187 from Calbiochem AG (Switzerland).

Pretreatment of cells

Blood was drawn by venipuncture, anticoagulated by EDTA in a final concentration of 0.01 M and mixed with a double volume of 0.9 % NaCl. Monocytes with approx. 2 % basophils were isolated by the Ficoll-Hypaque gradient method, as previously described (22). To prevent histamine release by activation of complement as a source of error, the cells were washed twice (107 *g* for 10 min) in Tris-AMC (albumin, magnesium, calcium) buffer containing Tris-(hydroxymethyl)-amino methane 25 mM at pH 7.4, NaCl 0.12 M, KCl 5 mM, CaCl₂ 0.6 mM, MgCl₂ 1.1 mM, human serum albumin 0.3 mg/ml, and glucose 1 mg/ml. Thereafter the cells were washed in Tris-AMC, pH 5.6, with CaCl₂ 4 mM, counted (total count of basophils, lymphocytes and monocytes) and finally resuspended in this buffer. One ml cell suspension was incubated for 40 min at 37°C with Gmix in amounts varying from 0 (control) to 50 µg per 10⁷ cells or with a mixture of galactocerebrosides types 1 and 2 and glucocerebrosides, containing 25 or 50 µg of each cerebroside per 10⁷ cells. Thereafter 9 ml ice-cold Tris-AMC, pH 7.4, was added and the cells were washed twice at 150 *g* at 4°C, and resuspended in this buffer. However, different concentrations of calcium were used in the buffer for the calcium experiments (Fig. 3). The cell suspensions were kept at 4°C until the histamine release experiments by different stimulators.

Assay for NANA incorporation into cells

Incorporation of NANA-containing gangliosides into the cells was determined after pretreatment of the cells with Gmix as described, except that the total volume was 400 µl. Endogenous NANA in the cells was determined by omitting the addi-

tion of Gmix, and samples of Gmix (50 or 100 µg) served as controls for exogenous gangliosides. After incubation all samples were centrifuged (500 *g* for 6 min at 4°C), and 300 µl of the supernatant was removed and kept for analysis. The pellets were resuspended to 300 µl, and to all samples, including standards containing 0.5–10 µg free NANA, 100 µl 0.125 M H₂SO₄ was added. The samples were hydrolysed at 80°C for 120 min according to Ledéen & Yu (13), and liberated NANA was assayed using Horgan's modification of the Aminoff assay (6).

The incorporation of NANA into the cells was calculated according to the formula: total content of NANA in samples of cells incubated with Gmix minus the sum of endogenous (cellular) and exogenous NANA. Since the gangliosides contained 20.4 % NANA, incorporation of gangliosides can be calculated.

Removal of NANA from the gangliosides in Gmix

In some experiments the cells were pretreated with Gmix where NANA was removed from the gangliosides. Sialidase from *V. cholerae* cleaves off all NANA moieties from the gangliosides, except the so-called internal one in GM₁ (19). All gangliosides present in Gmix are derivatives of GM₁, the differences being in the number of additional NANA molecules, e.g. GD_{1a} and GD_{1b} have one additional, whereas GQ_{1b} has three (24). The action of the sialidase is thus to convert the gangliosides in Gmix to GM₁ (19). 50 µg of Gmix was incubated with 0.1 U sialidase from *V. cholerae* for 60 min at 37°C in Tris-AMC, pH 5.6 with CaCl₂ 4 mM. To prevent an action of sialidase on the cells, the enzymatic activity was blocked by addition of the sialidase inhibitor 2-desoxy-2,3-dehydro-N-acetylneuraminic acid (9, 20), whereafter the cells were added and incubated for 40 min as described. Control experiments were performed with inactive sialidase, where the enzymatic activity was blocked before addition of Gmix. Active sialidase liberated 49 % of the NANA content in Gmix, whereas no liberation was found by inactive sialidase. Liberated NANA was assayed by the Horgan method (6) using free NANA as standards.

Histamine release

90 μ l leukocyte suspension containing 2000–8000 basophils was incubated in plastic tubes at 37°C for 40 min with 10 μ l of the following stimulators: Anti-IgE (400 IU/ml), Con A (300 μ g/ml), A23187 (20 μ M) or *S. aureus* Wood 46 (100 mg wet weight/ml). The reaction was terminated by addition of 3 ml ice-cold Tris-AMC, and histamine release was assayed by estimation of the residual histamine in the cell sediment, as previously described (22). The stimulators were omitted for spontaneous histamine release and for determination of the total histamine content, in the latter case the samples were left at 4°C. The release of histamine was expressed as percentage of the total histamine content of the sample. Since spontaneous histamine release amounted to up to 10%, only histamine release exceeding 10% was considered significant.

Subjects

Eight healthy volunteers served as donors. None had any previous history of atopy and did not receive any medication.

Statistics

Wilcoxon's ranking test for paired data was used.

RESULTS

The capability of the cells to bind gangliosides was examined by incubation of the cells with Gmix, and the increase in NANA content was attributed to incorporation of gangliosides. Table 1 shows that increase in NANA content in cells pretreated with 50 or 100 μ g of Gmix and the calculated amount of gangliosides bound to 10^7 cells. It is seen that the increase in NANA content is similar whether the cells have been incubated with 50 or 100 μ g Gmix, indicating that the cells were saturated with gangliosides already when incubated with 50 μ g Gmix. Therefore, the maximal amount of Gmix used in this study was 50 μ g per 10^7 cells.

The influence of this incorporation of NANA-containing gangliosides on histamine release from human basophil leukocyte was examined *in vitro*. Fig. 1 shows the effect of pretreatment of the cells with Gmix in amounts varying from 0.8 to 50 μ g per 10^7 cells on subsequent histamine release induced by various stimulators. This pretreatment did not cause any release of histamine by itself. After pretreatment of the cells with Gmix, the cells were washed twice in order to remove unbound substances and challenged with anti-IgE, Con A, A23187, or *S. aureus* Wood 46. Gmix caused a dose-dependent inhibition of the histamine release, and maximal inhibition was obtained at 25 to 50 μ g of Gmix. However, no inhibition was seen in connection with *S. aureus* Wood 46. As might be expected from the studies on incorporation of NANA into the cells, pretreatment of the cells with 100 μ g Gmix per 10^7 cells revealed no further inhibition (data not shown).

From Fig. 1 also the lack of inhibitory capacity of a mixture of cerebroside is seen. The cerebroside are precursors of gangliosides but do not contain NANA. Even when added in amount of 150 μ g cerebroside per 10^7 cells, these substances did not inhibit histamine release induced by any of the stimulators.

If NANA in the gangliosides are responsible for inhibition of histamine release, a reduction of the NANA content in the gangliosides should diminish or abolish the inhibitory effect of Gmix. Fig. 2 shows that this is the case. As previously, Gmix was found to reduce histamine release caused by anti-IgE and A23187, but not by *S. aureus* Wood 46. However, the inhibitory capacity of Gmix was completely abolished when the NANA content in the gangliosides was reduced to 51% by treatment with sialidase. The abolishment was caused by removal of NANA from the gangliosides and not by the enzyme acting on the cells, since the activity of the enzyme was terminated before addition of the cells by addition of the sialidase inhibitor 2-desoxy-2,3-dehydro-N-acetylneuraminic acid. Furthermore, no abolishment was obtained when Gmix was treated with sialidase in combination with the sialidase inhibitor (inactive sialidase) (Fig. 2).

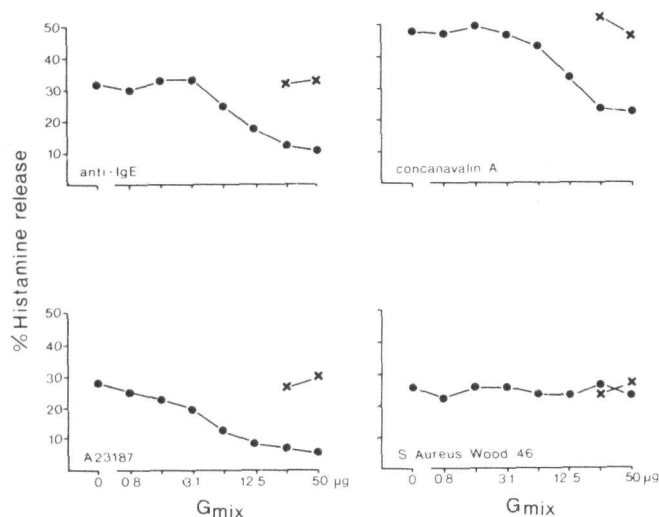


Fig. 1. Inhibition of histamine release by Gmix. Cells (10^7) were preincubated with Gmix in amounts ranging from 0 (control) to 50 μ g, washed twice and challenged with stimulator (●). Note that a mixture of 3 cerebrosides consisting of 50 μ g of each (x) caused no inhibition of histamine release. A representative example of 4 experiments.

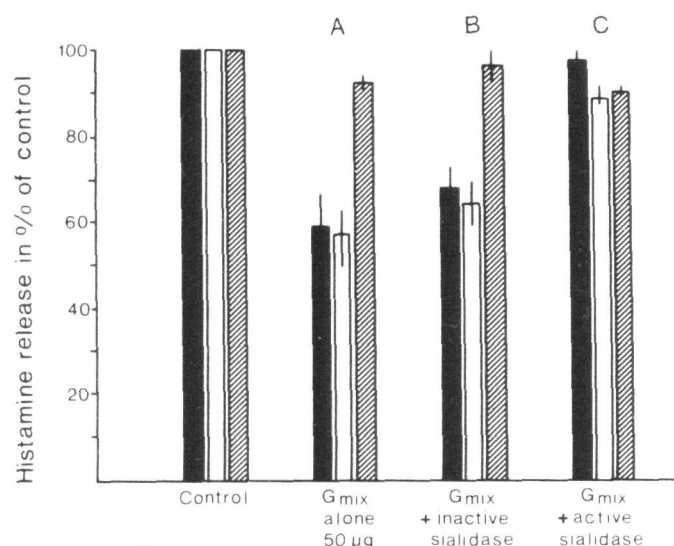


Fig. 2. Responsibility of NANA for the inhibitory effect of Gmix on histamine release. The cells were pretreated with Gmix (A), NANA-deprived Gmix (B) and by Gmix combined with sialidase and sialidase inhibitor (C). Histamine release caused by anti-IgE (■), A23187 (□), and *S. aureus* Wood 46 (▨). Mean of 3 experiments $\pm$ SEM.

Since NANA in the cell membrane in other cell systems is involved in the regulation of transmembraneous calcium fluxes (3, 7), we examined whether the inhibition of histamine release caused by the NANA-containing gangliosides in Gmix could be counteracted by increasing the extracellular calcium concentration. After pretreatment with Gmix and

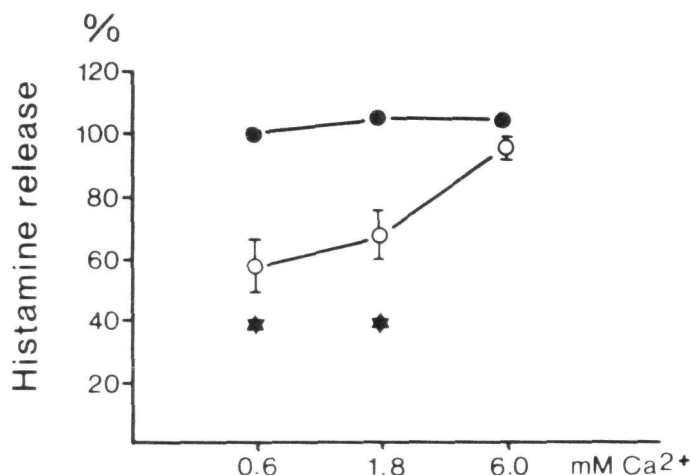


Fig. 3. Counteracting effects of calcium inhibition of histamine release by Gmix. Cells pretreated with Gmix (○—○) and without Gmix (●—●). Histamine release induced by anti-IgE. Mean of 5 experiments $\pm$ SEM. * $P < 0.01$.

repeated washings the cells were resuspended in buffer containing either 0.6, 1.8, or 6.0 mM CaCl_2 and stimulated with anti-IgE. Fig. 3 shows that the inhibitory effect of Gmix on histamine release was clearly seen at concentrations of 0.6 and 1.8 mM CaCl_2 , but it was counteracted by increasing the calcium concentration to 6 mM.

DISCUSSION

We have previously shown that the content of sialic acid (NANA) in the membrane of human basophil leukocytes modulates the cellular response, since enzymatic removal of this membrane constituent by the enzyme sialidase increased the histamine release induced by various stimulators and lowered the extracellular calcium concentration necessary for histamine release (9, 10). In contrast, preincubation of the basophils with free NANA inhibited histamine release and increased the calcium concentration necessary for the release (10). Since the concentration of free NANA needed for induction of this inhibition was high, 8 mg/ml, we examined NANA-containing substances which might more readily be inserted into the cell membrane, and gangliosides were chosen.

Gangliosides contain NANA and the lipophilic ceramide portion of gangliosides makes it pos-

sible to insert these substances into cell membranes, and the hydrophilic NANA moieties then represent the exterior part of the molecules (2, 24). These substances are present in the highest amounts in the grey matter of the brain, but have also been found in extraneural cells such as erythrocytes, lymphocytes and platelets (19). The significance of gangliosides in extraneural tissue is not clarified, but *in vitro* they are potent immunomodulatory agents capable of inhibiting e.g. mitogen activation of lymphocytes or the stimulatory action of interleukin 2 and γ -interferon (4, 18, 23).

In the present study a glucolipid mixture (Gmix) containing 20.9 % gangliosides was used. The gangliosides had one (GM₁) to four (GQ₁b) sialic acid moieties, and it was demonstrated that gangliosides could be inserted into the cell membrane (Table 1). The exact mode of insertion is unknown. The maximal ganglioside binding capacity amounts to an insertion of 0.50 μ g NANA per 10⁷ cells

The effect of inserting Gmix into the cell membrane on basophil histamine release was investigated using the following stimulators: Anti-IgE acting by cross-linking adjacent IgE molecules in the basophil membrane (8), the plant lectin Concanavalin A (Con A) acting on carbohydrates in the cell membrane (21), the calcium ionophore A23187 acting by raising the intracellular calcium concentration (15), and bacterium *S. aureus* Wood 46 (protein A-poor), which we have previously shown to release histamine by an interaction between carbohydrates in the bacterial cell wall and lectin situated in basophil cell mem-

brane (11). Pretreatment of the cells with Gmix inhibited the histamine release induced by anti-IgE, Con A or A23187, but the response to Wood 46 was not inhibited (Fig. 1). Maximal inhibition was obtained with 25 to 50 μ g of Gmix corresponding well to the maximal binding capacity of the cells (Table 1). The reason for the lack of effect of Gmix in connection with Wood 46 remains obscure, but bacterial histamine release has also in other ways shown different release mechanism (5, unpublished results).

The content of NANA in Gmix, i.e. the NANA-containing gangliosides, was found to play a crucial role in the inhibitory capacity of Gmix, whereas the cerebroside were without effect. Thus, removal of NANA for Gmix by sialidase completely abolished the inhibitory effect and hereby all gangliosides were converted to the monosialoganglioside GM₁. This ganglioside seems therefore to be without effect on histamine release.

Gangliosides in membranes bind calcium ions, and exogenously added ganglioside has been shown to inhibit Con A and calcium ionophore-induced mitogen stimulation of lymphocytes by an inhibition of the transmembraneous influx of calcium that is essential for mitogenesis (1, 12). Similar to these findings on lymphocytes we found that the insertion of Gmix into the membrane was reflected in the sensitivity of the cells to extracellular calcium, since the inhibition produced by Gmix at normal calcium concentrations was reverted by increasing the calcium concentration to 6.0 mM. An impaired calcium permeability would thus provide a possible explanation to the mechanism by which Gmix inhibits histamine release. Although it seems reasonable to attribute the inhibitory capacity of Gmix to content of gangliosides, it cannot from these experiments be deduced which of the gangliosides are the most potent inhibitors. Since the gangliosides lost their inhibitory capacity when converted to the monosialoganglioside GM₁, it might be speculated that the inhibitory capacity of gangliosides is correlated to their content of NANA. If so, a tetrasialoganglioside, e.g. GQ₁b, might be the most potent and monosialogangliosides, as for instance GM₁, the least potent.

These results with NANA-containing gangliosides correspond well with our previous find-

Table 1
Binding of gangliosides to cells

Cells preincubated with Gmix	Incorporation of NANA (μ g)	Calculated amount of gangliosides (μ g) bound to 10 ⁷ cells
50 μ g	0.46 $\pm$ 0.06*	2.25
100 μ g	0.51 $\pm$ 0.06*	2.49

Cells (10⁷) were incubated with Gmix for 40 min, and the incorporation of NANA was determined in the cell pellets. The amounts of NANA-containing gangliosides were calculated. Mean of 4 experiments $\pm$ SEM. **P* < 0.01 compared with controls not incubated with Gmix.

ings that the content of NANA in the basophil membrane is of significance in the cellular response and that a possible role of NANA in the membrane is to regulate transmembraneous calcium fluxes (9, 10, 12). The biological significance *in vivo* of extraneural gangliosides is unknown (16, 19) as is the presence of endogenous gangliosides in the basophil membrane. But since exogenously added gangliosides *in vitro* are highly potent immunomodulatory agents (12, 16, 19, 23) these substances might also play a modulatory role in immune responses *in vivo*.

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