

Influence of Neuraminidase and N-Acetylneuraminic Acid on Basophil Histamine Release *in Vitro*

C. JENSEN, U. HENRIKSEN, B. T. DAHL, P. STAHL SKOV and S. NORN

Department of Pharmacology, University of Copenhagen, Copenhagen, Denmark

Since N-acetylneuraminic acid (NANA) in cell membrane glucocalyx mediates or modulates a variety of actions, such as mediator release, we examined a possible modulating role of this amino sugar in histamine release from human basophil leukocytes. Removal of NANA from the cell membrane by the enzyme neuraminidase caused a dose-dependent histamine release. Removal of smaller amounts of NANA enhanced histamine release induced by anti-IgE, Concanavalin A and the calcium ionophore A23187, and reduced the interval between addition of antigen and initiation of histamine release. Pretreatment with free NANA had the opposite effects, i.e. a diminished and delayed maximal histamine release. The hypothesis that NANA in the cell membrane modulates the cellular response to stimulation was further substantiated by demonstrating that the altered response was reflected by a change in the sensitivity of the cell to extracellular calcium. NANA in the cell membrane glucocalyx thus seems to modulate the basophil response to stimulation by modulating transmembraneous calcium transport.

Key words: basophil leukocytes; histamine release; neuraminidase; N-acetylneuraminic acid; modulation; potentiation; calcium dependence.

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The presence of a sialic acid N-acetylneuraminic acid (NANA) in cell membrane glucocalyx mediates or modulates a variety of actions, such as mediator release or smooth muscle contraction (2, 20, 21). We therefore investigated a possible modulating role of NANA in the cell membrane in histamine release from human basophil leukocytes by enzymatic cleavage of NANA from the membrane and by addition of NANA to the cells.

MATERIAL AND METHODS

Twelve healthy individuals aged between 18 and 39 years served as donors. None had any previous history of atopy and they received no medication.

N-acetylneuraminic acid, type IV, N-acetylneuraminlactose grade II, phospholipase C (EC

3.1.4.3) type IX, and trypsin (EC 3.4.21.4) type III were purchased from Sigma Chem. (St. Louis, USA). Neuraminidase (EC 3.2.1.18) from *Cl. Perfringens* was from Boehringer (Mannheim, FRG), Concanavalin A (Con A) from Pharmacia AB (Sweden), rabbit anti-human IgE (ϵ -chain) 400,000 IU/ml from Behringwerke AG (FRG), and calcium ionophore A23187 from Calbiochem AG (Switzerland).

Preparation of cell suspension

Blood was drawn by venipuncture, anticoagulated with EDTA in a final concentration of 0.01 M and mixed with double volume of 0.9 % NaCl. Monocytes with approx. 2 % basophils were isolated by the Ficoll-Hypaque gradient method, as described earlier (22). To prevent histamine

release by activation of plasma 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)-aminomethane 25 mM at pH 7.4, NaCl 0.12 M, KCl 5 mM, CaCl_2 0.6 mM, MgCl_2 1.1 mM, human serum albumin 0.3 mg/ml, and glucose 1 mg/ml (22). The cells were finally resuspended in this buffer.

Pretreatment of cells

The cell suspension was divided into three parts of 1.0 ml each and incubated for 40 min at 37°C at pH 7.4 with neuraminidase (25 $\mu\text{U}/10^6$ cells), NANA 8 mg/ml and without these agents (control). Thereafter 9 ml of ice-cold Tris-AMC was added and the cells were washed twice at 150 g at 4°C and kept at 4°C.

For the calcium experiments, the Tris-AMC was replaced by Tris-A containing 1 mM EDTA for the first wash and Tris-AM for the second. These cells were resuspended in Tris-AMC with calcium in final concentrations varying between 0.018 and 18.0 mM.

Histamine release

Ninety μl leukocyte suspension containing 2000–8000 basophils was incubated in plastic tubes at 37°C with 10 μl of the following agents: neuraminidase, anti-IgE, Con A, and calcium ionophore A23187. The incubation period was 40 min, unless otherwise stated. The reaction was terminated by addition of 3 ml ice-cold Tris-A containing 1 mM EDTA, 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 a percentage of the total histamine content of the sample. Since spontaneous histamine release amounted to up to 10 %, only histamine release > 10 % was considered significant.

Pretreatment with neuraminidase to remove NANA from the cell surface was performed at

pH 7.4 instead of pH 5.0 where the enzyme activity is maximal (4). To ensure that the activity was adequate, it was determined at pH 7.4 and compared with that of pH 5.0. Four μg of N-acetylneuraminlactose was dissolved in 400 μl phosphate-buffered saline at pH 5.0 and 7.4. To these tubes enzyme was added in the range of 0.5 to 50 mU. The samples were incubated at 37°C for 60 min and the hydrolysis of N-acetylneuraminlactose to NANA was assayed using the method described by Horgan (8). NANA served as standard, and the enzyme was omitted for blanks.

These experiments revealed that the activity of the enzyme at pH 7.4 was adequate, i.e. approx. 66 % of the activity at pH 5.0. A possible contamination of the neuraminidase preparation by NAN-aldolase would interfere with the determination of the neuraminidase activity. Such a contamination could, however, be excluded, since no difference was obtained in NANA standards incubated with or without 0.1 U of neuraminidase at 37°C for 60 min.

Enzyme impurity assays

The commercially available neuraminidase preparation may contain proteases or phospholipase C which might disturb the cell function and thereby the histamine release. The actual activities of these enzymes were therefore assayed using methods including casein and phosphatidyl choline as substrates (12, 24). Trypsin and pure phospholipase C were used as standards. The neuraminidase preparation contained neither detectable protease nor phospholipase C activity (< 1 μU) when assayed in amounts up to 6 mU, which is more than 25 times greater than the maximal amount used in this study.

Statistics

Wilcoxon's ranking test for paired data was used.

RESULTS

The influence of pretreatment of basophils with neuraminidase or NANA on histamine release

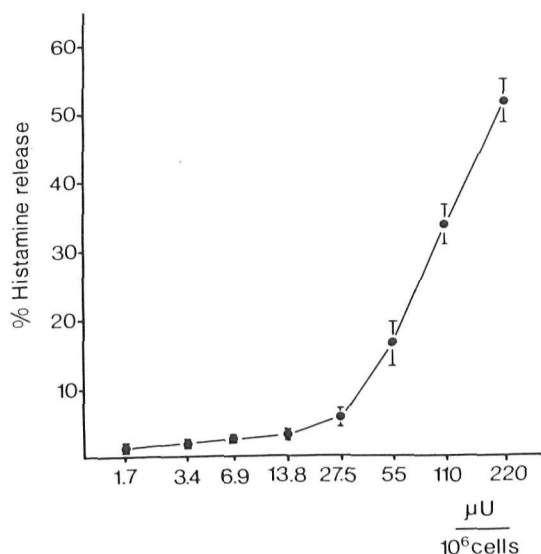


Fig. 1. Basophil histamine release induced by incubation of the cells with neuraminidase. Mean of 12 experiments $\pm$ SEM is given.

from normal individuals was examined *in vitro*. A histamine releasing capability of the neuraminidase was investigated in the range between 1.7 and 220 $\mu\text{U}/10^6$ cells. In all 12 persons tested, no histamine release was found in the range between 1.7 and 27.5 $\mu\text{U}/10^6$ cells, whereas the enzyme caused dose-dependent histamine release above this range (Fig. 1). Incubation of the cells with NANA in concentrations up to 10 mg/ml did not cause histamine release in any of the persons investigated (data not shown).

The effect of pretreatment of the cells with amounts of the enzyme not causing histamine release (25 $\mu\text{U}/10^6$ cells) and with free NANA (8 mg/ml) was investigated. After this pretreatment the cells were washed twice and challenged with either anti-IgE, Con A, or calcium ionophore A23187. These results are presented in Fig. 2. The figure shows that pretreatment with neuraminidase both enhanced the maximal response and caused a shift to the left of the dose-response curve for all the three stimulators. Pretreatment with NANA, in contrast, resulted in both a diminished maximal response and a shift to the right of the dose-response curve.

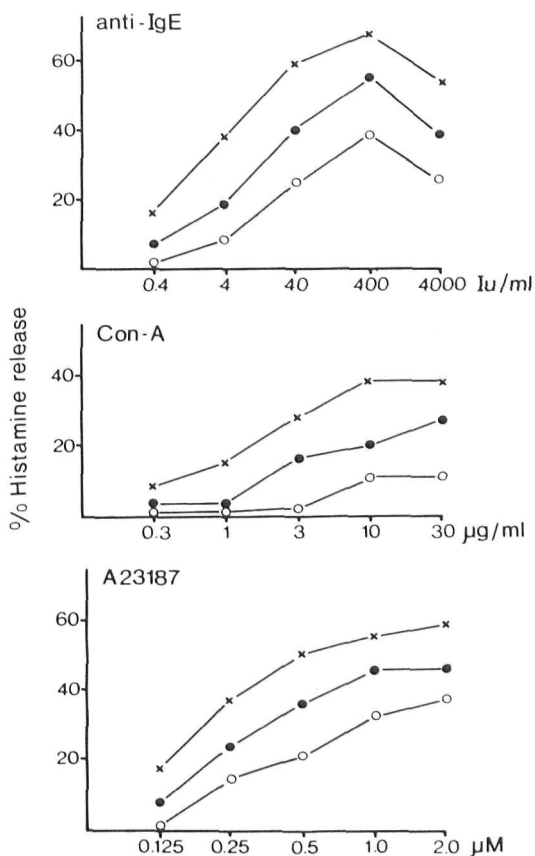


Fig. 2. Effect of pretreatment of the cells with neuraminidase or NANA on histamine release induced by anti-IgE, Con A or A23187. A representative example of 8 experiments is given. Histamine release was significantly enhanced and reduced, respectively, in cells treated with neuraminidase and NANA as compared with controls ($P < 0.01$). ●: control; ×: neuraminidase-treated; ○: NANA-treated.

Fig. 3 shows the time course of histamine release induced by anti-IgE (400 IU/ml). Pretreatment with neuraminidase reduced the interval between addition of stimulator and initiation of histamine release (the lag phase), whereas pretreatment with NANA increased the lag phase. It should be noted that the concentration of anti-IgE giving maximal histamine release was used in these experiments.

Since NANA in the cell membrane is known to be a major calcium-binding site in other cell systems (13) we examined the influence of removal from and addition to the cells of NANA on histamine release induced by anti-

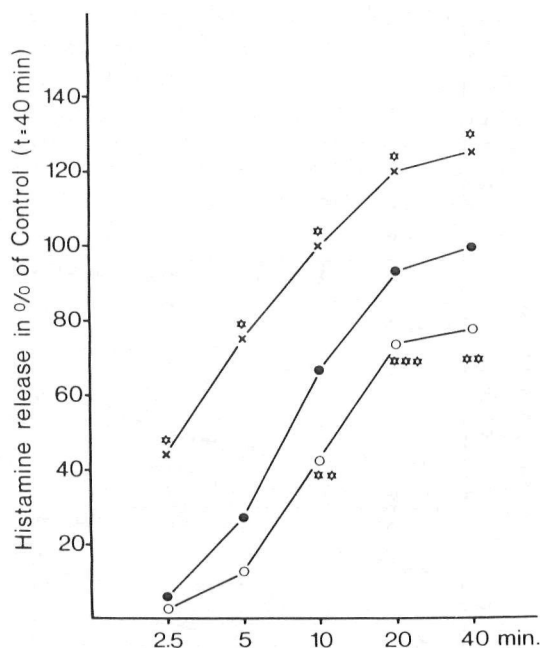


Fig. 3. Effect of pretreatment with neuraminidase or NANA on the time course of histamine release by anti-IgE. Histamine release is expressed as a percentage of control at 40 min. Mean of 6 experiments. *, ** and *** = $P < 0.01$, 0.02, and 0.05, respectively, compared with controls. ●: control; ×: neuraminidase-treated; ○: NANA-treated.

IgE as a function of increasing concentrations of extracellular calcium. After pretreatment with enzyme or free NANA, both free and membrane-associated calcium was removed by washing with buffer containing 1 mM of EDTA. These experiments showed that pretreatment with neuraminidase markedly increased the sensitivity of the cells to extracellular calcium (Fig. 4). In concentrations between 0.018 and 0.06 mM Ca^{++} , where the control cells were unable to respond to stimulation, the enzyme-treated cells released histamine, thus showing a shift to the left in the calcium dependence curve. Again, with pretreatment with NANA the opposite picture was seen, i.e. higher concentrations of calcium were required for histamine release than was the case with control cells. Indeed, at a calcium concentration of 18 mM, NANA-treated cells showed the same histamine release as seen with the control cells at 0.6 mM of calcium. The inhibitory effect of

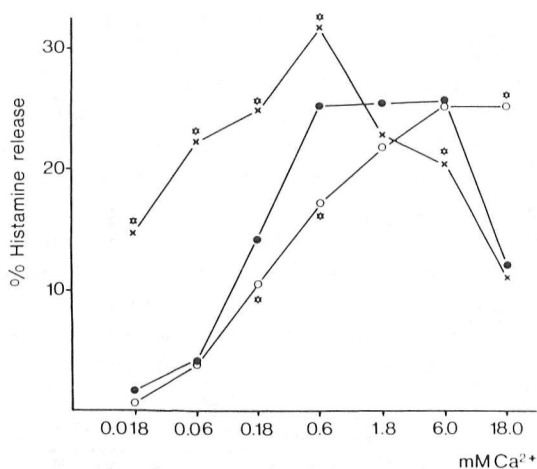


Fig. 4. Effect of pretreatment with neuraminidase or NANA on histamine release as a function of the calcium concentration. The cells were challenged with anti-IgE 40 IU/ml. Mean of 6 experiments. * = $P < 0.01$. ●: control; ×: neuraminidase-treated; ○: NANA-treated.

addition of exogenous NANA could thus be overcome by increasing the extracellular calcium concentration.

It was demonstrated that the change in the cell response induced by neuraminidase was actually caused by the enzyme itself and not by contaminating impurities such as NAN-aldolase, proteases, and phospholipase C, since such a contamination was not found (data not shown). Furthermore, the enhancement of maximal response and the shift to the left of the dose-response curve by pretreatment of the cells with neuraminidase (Fig. 2) were competitively inhibited by addition of substrate (N-acetylneuraminlactose) to the cells together with the enzyme (data not shown).

DISCUSSION

NANA (N-acetylneuraminic acid) is present as the ultimate carbohydrate moiety in cell membrane glycoproteins and -lipids (21). This weak acid, which at physiological pH preferentially binds calcium ions (11), is in part responsible for cell surface negative charge, and is in many cell systems involved in cell-to-cell interaction and inflammatory cell responses (5, 7, 17, 21). Removal of NANA by the enzyme neuro-

minidase has been widely used for studying the significance of NANA in the cell membrane. The removal of NANA has been shown to enhance cellular responses, such as lymphocyte proliferation, neutrophil cytotoxicity and smooth muscle contraction (18, 25). Bach & Brashler (1) have shown that pretreatment of rat mast cells with neuraminidase enhanced antigen-induced histamine release when the enzyme was used in minute amounts, whereas pretreatment of the cells with greater amounts of the enzyme caused histamine release by itself.

Similar to the effect on rat mast cells, we found that treatment of the basophils with neuraminidase in greater amounts caused the cells to release histamine in a dose-dependent manner. The amount of enzyme causing significant histamine release was the same in all persons tested. The release did not require the presence of extracellular calcium (data not shown) and might thus be due to an unspecific damage to the cell, possibly induced by changes in the surface charge and/or in conformation of cell membrane constituents (6, 21).

The effect of pretreating the cell with neuraminidase in small amounts, insufficient to induce histamine release themselves, was examined on the cellular response to different stimulators, using the following three stimulators: anti-IgE acting by cross-linking adjacent IgE molecules on the cell surface (9), the plant lectin Con A shown to interact with carbohydrates on the cell membrane regardless of the presence of IgE molecules (23), and the calcium ionophore A23187 which acts as a calcium carrier bypassing the calcium channels (15). The effect of pretreatment with neuraminidase on histamine release induced by these three stimulators proved to be identical, i.e. an increased maximal release and a shift to the left of the dose-response curve.

Our results are in accordance with those obtained by Baxter & Adamik (2) using isolated rat mast cells. However, to differentiate between an effect on the cell or on the stimulator, the enzyme was in our experiments washed away before addition of the stimulator. The experiments demonstrate an effect of the cell.

Removal of NANA from the cell membrane thus seems to cause a generalized increase in the cellular response.

The lag phase in histamine release depends on the concentration of stimulator, i.e. increasing concentrations causing decreasing lag phase (16). In our studies of the time course of histamine release we used the concentration of stimulator resulting in maximal histamine release, and compared the time course with that obtained in cells pretreated with neuraminidase. This partial removal of membrane NANA resulted in a reduced lag phase as well as in an increased maximal histamine release, compared with control cells. The findings suggest a regulatory role of NANA in the basophil cell membrane, since a partial removal renders the cell more alert. Therefore, it could be expected that an increase in membrane NANA content would cause the opposite effect, and since NANA can be covalently bound to glucoproteins in the absence of enzyme (19), a possible effect of the addition of NANA to the cells was studied. These experiments supported our hypothesis, since pretreatment with NANA caused the opposite effect of pretreatment with neuraminidase, i.e. a diminished and delayed histamine release.

Our findings may be due to changes in cell surface charge resulting in an altered binding of the stimulators to the cell surface, since NANA is a charged molecule involved in cell surface charge (5, 13, 21). This was suggested by Coleman (3) in rat experiments using charged molecules as liberators of 5-hydroxytryptamine from mast cells, and might thus provide a possible explanation of our findings. However, it could also be speculated whether NANA in the membrane regulates the events occurring between stimulation of the cell membrane and histamine release. NANA together with membrane phospholipids provides a low affinity, high capacity reservoir for calcium (10, 13). It might therefore be probable that NANA in the cell membrane is involved in the transmembraneous calcium fluxes preceding histamine release (14). An increased exchangeability for calcium induced by partial removal of membrane NANA has been shown in cultured heart cells and in

guinea pig taenia coli (6, 10, 13). We therefore examined whether pretreatment with neuraminidase or NANA would change the requirement for extracellular calcium in the cellular response, and we found that removal of membrane NANA diminished and addition of membrane NANA increased the concentrations of calcium necessary for histamine release. Alterations in transmembraneous calcium fluxes might thus be a possible explanation of our findings.

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Address:

Carsten Jensen

Department of Pharmacology

University of Copenhagen

Juliane Maries Vej 20

DK-2100 Copenhagen Ø

Denmark

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