Inhibitory Effect of N-(3,4-Dimethoxycinnamoyl)anthranilic Acid on Release of SRS from Alveolar Macrophages in Vitro

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Abstract—The effects of N-(3,4-dimethoxycinnamoyl)anthranilic acid (N-5') on the release of the slow-reacting substance (SRS) by zymosan- or Ca ionophore-stimulated rat and human alveolar macrophages (AM) were examined in vitro. Disodium cromoglycate (DSCG) was used as a control. N-5' at concentrations of 10^{-4} - 10^{-3} M significantly inhibited the release of SRS from both rat and human AM stimulated by zymosan. N-5' had almost the same inhibitory effect when added to the AM culture system at any time from 180 min before to 30 min after the addition of zymosan. N-5' (10^{-4} - 10^{-3} M) also significantly inhibited the release of SRS by Ca ionophore-stimulated rat AM. N-5' (10^{-6} - 10^{-3} M) had no significant effect on phagocytosis of yeast particles by rat AM. DSCG (10^{-6} - 10^{-3} M) did not inhibit the release of SRS from the zymosan-stimulated rat AM. N-5' was concluded to have a relatively specific inhibitory effect on the non-immunological release of SRS from stimulated AM. It is postulated that N-5' inhibits the process of release of SRS from AM by acting after the initial stage.

Previously, we found that human and rat alveolar macrophages (AM) release slow-reacting substances (SRS) in vitro when they are stimulated by zymosan (Zy) and that these substances are leukotriene C_{4} and D_{4} (1).

N-5' was reported to inhibit the release of histamine from sensitized rat mast cells (2-4), and release of SRS-A from sensitized guinea pig lung tissue (5) in vitro. Moreover, N-5' was found to be effective for preventing experimental asthma in rats (6) and atopic bronchial asthma in humans (7). Therefore N-5' is thought to be effective for treatment of various kinds of allergic diseases because it inhibits the release of chemical mediators such as histamine and SRS-A from mast cells caused by the antigen-antibody reaction.

In the present study, we examined the effect of N-5' on the release of SRS from rat and human AM stimulated by Zy and Ca ionophore in vitro.

Materials and Methods

1) Alveolar macrophages (AM) of rat and humans
   a) Rat AM: Male Wistar strain rats weighing about 150 g were used. The rats were killed by bleeding from the abdominal aorta under pentobarbital anesthesia, and the lung from the trachea down was removed en bloc with other thoracic organs. A plastic cannula was inserted into the trachea and tied tightly with thread. A syringe was inserted into the cannula and bronchoalveolar lavage was carried out 5 times with 6-7 ml volumes of saline, with gentle massage of the lung tissue. The lavage fluids were pooled, and centrifuged at 250xg at 4°C for 5 min. The precipitated cells were washed twice with RPMI-1640 medium (Nissui Seiyaku Co), containing 100 unit/ml of penicillin G, 100 μg/ml of streptomycin and 25 μM/ml of HEPES, and suspended in the same medium. A part of the suspension was used for cell
analysis after staining the cells with May-Giemsa stain. Cell viability was determined by the trypan blue dye exclusion method. Preparations that showed over 85% cell viability and contained over 85% AM were used.

b) Human AM: Humam AM were obtained from five normal male volunteers by bronchoalveolar lavage (BAL). Informed consent was obtained from all volunteers. BAL was carried out in S4 or S5 of the right middle lobe as described previously (8). Each region was lavaged 3 times with saline (50 ml) using a bronchofiberscope (Olympus BF, 1T), and the lavage fluid was processed described above to obtain AM.

2) Preparation of stimulants and test drugs

Stimulating substances and test drugs were added as solutions or suspensions in RPMI-1640 medium to AM culture systems in a final volume of 1 ml. Zymosan (Zy, Sigma) and Ca ionophore A23187 (Eli Lilly) were used as stimulants. Zy was suspended in RPMI-1640 medium at a concentration of 20 mg/ml, sterilized, diluted with the same medium and added to the AM culture system at a final concentration of 200 μg/ml. Ca ionophore A23187 was dissolved in ethanol at a concentration of 10 mg/ml, diluted with RMPI-1640 medium and added to the AM culture system at a final concentration of 500 ng/ml. N-5' (Kissei Pharmaceutical Co.) and disodium cromoglycate (DSCG, from Fujisawa Pharmaceutical Co.) were used as test drugs. Just before use, N-5' was dissolved into 1% NaHCO₃ at a concentration of 10⁻⁶ M with heating, diluted with RPMI-1640 medium and added to the AM culture system at a final concentration of 500 ng/ml.

3) Culture of AM

AM were suspended at a concentration of 1×10⁶ cell/ml in RPMI-1640 medium and 1 ml portions of the suspension was transferred to plastic tissue culture plates (Falcon 3047) and incubated at 37°C in an incubator under 5% CO₂ in air for 1 hr. Then the culture medium was discarded and the adhering AM monolayer was washed with the same medium.

RPMI-1640 medium and stimulants were added to the AM monolayer, and the cultures were incubated at 37°C in a CO₂-incubator for 3 hr. Unless otherwise stated, Zy at a final concentration of 200 ng/ml was used as a stimulant.

For examination of the effects of the test drugs, the AM were preincubated with the test drugs for 30 min and then the stimulant was added. After culture, the supernatants from 3 chambers were collected and centrifuged at 250×g at 4°C for 15 min. The activity of the resultant supernatant on the contraction of smooth muscle was assayed.

4) Assay of activity for inducing smooth muscle contraction

Activity was assayed by the method of Magnus as described previously (1). A piece of guinea pig ileum was immersed in 10 ml of Tyrode's solution. The contraction of guinea pig ileum induced by 0.01–1 ng/ml LTD was determined. Then the effect of 1 ml of the AM culture supernatant on muscle contraction was assayed in the presence of 5×10⁻⁷ M atropine sulfate and 1×10⁻⁷ M mepyramine to exclude the influences of acetylcholine and histamine.

N-5' or DSCG added to the AM culture medium was diluted 10-fold. In a preliminary experiment, N-5' at a concentration below 1×10⁻⁴ M was shown to have no significant effect on the contraction of the guinea pig ileum induced by 10⁻³ g/ml Ba²⁺. N-5' at a concentration of 3×10⁻³ M showed a slight inhibitory effect (20–39%), as reported previously by Nakazawa et al. (9). N-5' at a concentration above 3×10⁻⁴ M inhibits SRS-induced contraction of the guinea pig ileum and trachea and that below 1×10⁻⁴ M does not (4–5). Thus the effect of N-5' on the release of SRS from AM in vitro was examined at a concentration below 1×10⁻⁴ M to exclude the direct inhibitory action of N-5' on the contraction of smooth muscle and the antagonistic action of N-5' for smooth muscle-contracting action of SRS.

The effect of the AM culture supernatant was expressed as a percentage of the contraction induced by 0.1 ng/ml LTD. The activity in the presence of a test drug was expressed as the percentage of that in its absence.

5) Phagocytic activity of AM on yeast
The phagocytic activity of AM was determined as described previously (10) using yeast particles as foreign bodies. The AM were incubated with yeast particles for 1 hr and more than 200 AM were examined to determine the percentage of AM that had phagocytized at least one yeast particle/cell. This percentage is shown as an "index". AM that phagocytized one, two and three or more particles were scored as one, two and three units, respectively, and the total score for 100 AM was recorded as the "score".

6) Statistical analysis

Data are presented as the mean±1 S.D. from 5 samples. Significance of difference was determined using Student’s t-test for unpaired variables.

Results

1) Effect of N-5' on in vitro release of SRS by Zy-stimulated rat AM

a) Effect of concentration of N-5': Figure 1 shows the amounts of SRS released from Zy-stimulated rat AM in the presence of various concentrations (10⁻⁶–10⁻³ M) of N-5’. The agent showed significant inhibition at a concentration of 10⁻⁴ M (P<0.005) but not at 10⁻⁶–10⁻⁵ M. N-5’ at 10⁻³ M caused about 80% inhibition in this experiment. The inhibitory effect of N-5’ varied to some extent in different experiments, and 10⁻³M N-5’ was distinctly inhibitory in some experiments.

b) Effect of preincubation time: Preincubation of AM with 10⁻³ M N-5’ for 180, 60, 30 or 15 min before the addition of Zy did not influence the inhibitory effect of N-5’. Therefore, in the following experiments, a preincubation time of 30 min was used, unless otherwise stated.

Next the effect of the time of addition of N-5’ after Zy on its inhibitory effect was examined. As shown in Fig. 2, N-5’ had almost the same inhibitory effect when added at 0, 10, 20 or 30 min after Zy as when added at 30 min before Zy, but it had no significant inhibitory effect when added 60 min after Zy.

2) Dose-dependence of the effect of N-5’ on the release of SRS from Zy-stimulated human AM in vitro

As shown in Fig. 3, the inhibitory effect of N-5’ on the release of SRS was detectable at the concentration of 10⁻⁴ M (P<0.005) and was dose-dependent.

3) Dose-dependence of N-5’ on the release of SRS from Ca ionophore-stimulated rat AM in vitro

As shown in Fig. 4, N-5’ also inhibited the release of SRS from Ca ionophore-stimulated rat AM, and the activity was also dose-dependent at concentrations above 10⁻⁴ M.

4) Effect of N-5’ on phagocytosis of yeast by rat AM

The effect of N-5’ on phagocytosis of yeast by rat AM was examined in the presence and absence of 5% rat serum. In the absence of N-5’, the mean phagocytic index in 5 experiments was 87.2±3.2 in the absence of serum and 94.7±2.0 in the presence of serum. The score was 223±18 in the absence and 261±20 in the presence of serum. N-5’ at concentrations between 10⁻⁶ and 10⁻³ M had no significant effect on phagocytic activity, expressed as either the...
Fig. 2. Effect of time of addition of N-5' on in vitro release of SRS by Zy-stimulated rat AM. 10^{-3} M N-5' was added to the AM culture system at the indicated times from 30 min before to 60 min after the addition of Zy, and the AM were cultured for 3 hr after the addition of zymosan. Points and vertical lines are means±S.D. from 5 samples.

Fig. 3. Concentration-dependence of inhibition by N-5' of in vitro release of SRS by Zy-stimulated human AM. Conditions were as for Fig. 1 except that human AM were used.

Fig. 4. Concentration-dependence of inhibition by N-5' of in vitro release of SRS by Ca ionophore-stimulated rat AM. Conditions were as for Fig. 1 except that AM were stimulated with Ca ionophore (500 ng/ml).

index or the score, in either the presence or absence of serum.

5) Effect of DSCG on release of SRS from Zy-stimulated rat AM in vitro

DSCG at concentrations of 10^{-6}–10^{-3} M did not inhibit the release of SRS, and in fact, it rather enhanced its release at concen-
Fig. 5. Effect of DSCG on in vitro release of SRS by Zy-stimulated rat AM. Rat AM were preincubated with 10^{-6} to 10^{-3} M DSCG and then cultured for 3 hr. Subsequent procedures were as in Fig. 1.

In this study, we examined the effects of N-5' and DSCG on the release of SRS from Zy and Ca ionophore-stimulated AM in vitro.

As described in the Materials and Methods, we examined the effect of N-5' on the release of SRS from AM in vitro at a concentration below 10^{-3} M to exclude its direct inhibitory action on the contraction of smooth muscle and its antagonistic action for smooth muscle-contracting action of SRS when the amount of SRS released was measured.

We have found that the release of SRS from both rat and human AM stimulated in vitro by Zy was inhibited by N-5' at concentrations of 10^{-4}--10^{-3} M. Previous studies showed that the in vitro release of SRS by AM occurred only when the AM were stimulated with substances such as Zy and that the amount of SRS released into the culture medium reached a plateau about 3 hr after the addition of the stimulants. We found that N-5' had almost the same inhibitory effect when added to the AM culture system at any time from 180 min before to 30 min after the addition of Zy. These results indicate that AM do not release SRS within 30 min after the addition of Zy and that the release is inhibited by N-5' when the agent is added to the AM culture system before this time. Thus, these results also indicate that N-5' inhibits the release of SRS from AM by acting on a stage later than the initial stage in the process of SRS release.

N-5' is thought to inhibit the degranulation of mast cells, which occurs when a causative antigen binds to IgE antibody located on the surface membrane of the cells (2-5, 11). However, AM have been shown to be morphologically and functionally different from mast cells (12), and the release of SRS by AM is an IgE-independent, non-immunological process. Therefore, the mechanism of release of SRS by AM is presumably different from that of release of SRS following degranulation of mast cells, particularly in its initial stage. The initial stage in mast cell activation (degranulation) by immunological stimuli is the perturbation of its cell membrane caused by cross-linkage of the IgE receptors (13). It is unknown whether the mechanism of release of SRS by Zy-stimulated AM is the same as that by Ca ionophore-stimulated AM, but we found that the same concentrations of N-5' inhibited SRS release from both Zy-stimulated AM and Ca ionophore-stimulated AM. Therefore, this also indicates that N-5' inhibits release of SRS by AM by acting on a stage later than the initial one.

Phagocytosis is a primary function of AM. N-5' did not inhibit phagocytosis of yeast particles by AM at concentrations of 10^{-6}--10^{-3} M, suggesting that its inhibitory effect on release of SRS was not due to inhibition of overall functions of AM, but was rather specific.

DSCG at concentrations of 10^{-6}--10^{-3} M was reported to inhibit in vitro release of chemical mediators from guinea pig mast cells (14). In the present work, DSCG at concentrations of 10^{-8}--10^{-3} M did not inhibit release of SRS by Zy-stimulated AM, and in fact, it rather enhanced SRS release. These results suggest that the pharmacological action of N-5' is different from that of DSCG.

AM are thought to be involved in the pathophysiology of various kinds of immune and inflammatory diseases of the lower respiratory tract and bronchial asthma.
because: 1) they play a central role in the immune and inflammatory process in the lower respiratory tract (15), 2) they release various kinds of mediators such as SRS and neutrophil chemotactic factors (16) in response to stimuli, 3) they release a neutrophil chemotactic factor (17) and SRS (18) by an IgE-mediated mechanism.

The results of Figs. 1 and 3 suggest that the inhibitory effect of N-5' on release of SRS by AM in vitro is almost the same in rats and humans. It has been shown that serum N-5' level rises up to $10^{-4}$ M in patients with bronchial asthma after its oral administration of 5 mg/kg (19). These findings suggest that its clinical effect on bronchial asthma is partly due to its inhibitory effect on the release of chemical mediators from AM.

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