Action Sites of Antiallergic Drugs on Human Neutrophils

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Abstract—Sites of the inhibitory action of antiallergic drugs (azelastine, oxatomide, tranilast, repirinast and amlexanox) on human neutrophils were investigated by measuring leukotriene B₄ formation, arachidonic acid release and superoxide generation. Results obtained in this study were as follows: (i) Formations of leukotriene B₄ by neutrophils activated with a calcium ionophore (A23187) were effectively inhibited by all types of antiallergic drugs examined here, although the required concentrations were within a range of 20–200 μM. (ii) Releases of arachidonic acid from activated cells were diminished by azelastine and oxatomide that were classified as basic antiallergic drugs. On the contrary, acidic antiallergic agents including repirinast, amlexanox and tranilast enhanced the arachidonic acid liberation. (iii) Generations of superoxide from neutrophils activated with either phorbol 12-myristate 13-acetate or n-formyl-methionyl-leucyl-phenylalanine were effectively diminished only by the basic antiallergic drugs.

In the process of allergic reactions, a variety of substances are released as "chemical mediators" and these include histamine, serotonin, bradykinin and leukotrienes (LTs) (1). Leukotrienes including LTB₄, LTC₄ and LTD₄ and LTE₄ can be produced in mast cells, basophils as well as neutrophils, and they are released from human or guinea pig lungs upon immunological challenge (2). Since they possess a potent vasoconstricting activity and cause contraction of bronchial air way (3, 4), LTs may play an important role in immediate hypersensitivity responses as mediators of allergic bronchoconstriction and vascular permeability (5). LTB₄ which can be produced by human neutrophils is a potent chemotactic and aggregating agent for many types of cells, and it may have an important role in inflammation and tissue damage (6, 7).

It is widely known that all of the prostaglandins as well as LTs can be produced from arachidonic acid, which could be released from membrane phospholipids by phospholipase A₂ (8–10). For LTs formation, arachidonic acid is converted to 5-hydroxyeicosatetraenoic acid by enzyme lipooxygenase and then further enzymatically transformed to LTs B₄, C₄, D₄ and E₄ (11). Therefore, there are at least two stage where drugs can influence the formation of LTs: (i) the liberation of arachidonic acid from phospholipids and (ii) the catabolic steps which convert the arachidonic acid to LTs. Thus, the development of inhibitors of LT release in vivo became of therapeutic relevance, and such drugs are now classified as "antiallergic agents" (12). The first representative of this category of antiallergic agent, disodium chromoglycate, was reported in 1967 (13), and many drugs such as ketotifen and azelastine are now being used clinically for treatments of various allergic diseases including bronchial asthma.

Although their chemical structures are quite different as seen in Fig. 1, the agents may be classified into two groups: (i) the basic antiallergic drugs (azelastine and oxatomide) containing amines in their structure and (ii) the acidic antiallergic drugs (repirinast, amlexanox and tranilast) which have carbonic acid in their molecules. Furthermore, the antiallergic properties are different among the various types of agents in which the basic drugs are known to act not only as an inhibitor of LT release but also has antihistaminic activity (14). On the other hand, the acidic
agents were developed on the basis of observations that DSCG (disodium cromoglycate), bicalein and caffeic acid are inhibitors of chemical mediator release (12, 15).

We have previously shown that superoxide generation, arachidonic acid release as well as changes of membrane potential were suppressed by one of the basic antiallergic agents, azelastine (16–18). In this study, we attempted to compare the inhibitory effects of antiallergic drugs on the release of arachidonic acid, leukotriene B₄ and superoxide from activated human neutrophils to clarify the action sites of the various basic and acidic antiallergic drugs.

Materials and Methods

Preparation of neutrophils: Human neutrophils were separated from the blood of healthy volunteers. Preparations containing 90–95% polymorphonuclear leukocytes were obtained by the dextran sedimentation of the whole blood followed by Ficoll-Hypaque gradient centrifugation (19). Contaminating erythrocytes were removed by hypotonic lysis. After restoring the osmolarity, the cells were washed twice with Gey’s balanced salt solution containing 0.1% BSA and 10 mM HEPES, pH 7.4 (modified Gey’s solution) and resuspended in an appropriate buffer.

Measurement of leukotriene production: Neutrophils were resuspended (10⁶ cells/ml) in Dulbecco’s phosphate buffered saline, pH 7.4. The cell suspension was preincubated with or without drugs at 37°C for 5 min. The reaction was started by the addition of 2 μM A23187 and carried out for a further 2 min. At the end of the incubation period, the same volume of ice cold buffer was added. After centrifugation (1500×g, 10 min), the supernatant was used for the assay of leukotriene B₄. Leukotriene B₄ was measured by using a leukotriene B₄ radioimmunoassay kit (Amerham). The value of A23187-induced leu-
kotriene B₄ production from human neutrophils was 20.5±2.6 nmoles/10⁶ cells in 2 min.

Measurement of arachidonic acid release: Arachidonic acid release from human neutrophils was measured by a previously described method (20). Neutrophils were resuspended in modified Gey's solution at the concentration of 1×10⁷ cells/ml. The cell suspension was incubated with [³H]-arachidonic acid (2.96 TBq/mmoles, NEN) in order to incorporate the radiolabelled arachidonic acid into neutrophils at the final concentration of 9.25 KBq/ml at 37 °C for 10 min. The incorporation was terminated by the addition of 5 volumes of ice cold buffer and centrifuged (400×g, 2 min). The pellet was washed twice with modified Gey's solution and resuspended in the same buffer to give a final concentration of 1×10⁷ cells/ml (58026±12514 dpm/5×10⁶ cells). The labeled cells (5×10⁶ cells/0.5 ml) were preincubated with or without drugs at 37 °C for 5 min. The reaction was started by the addition of 2 µM of A23187 and then carried out for a further 10 min before termination by the addition of 1.75 ml of methanol : chloroform (2:1, vol./vol.). Extraction and separation of lipids were carried out according to the method described by Bligh and Dyer (21). The chloroform extract was evaporated under a stream of nitrogen, and the residue was spotted on a TLC plate (silica gel, Merk) and developed in petroleum ether : diethyl ether : acetic acid (80:20:1, vol./vol.). Spots detected by iodine vapor were scraped into a counting vial, and the radioactivity was measured by a liquid scintillation counter after the addition of 10 ml of Scintisol (Wako Pure Chemical Indust.) to each vial. The value of A23187-stimulated release of arachidonic acid from neutrophils was 8245±1880 dpm. In the absence of stimuli, the control value was 2061±343 dpm.

Results

To examine the inhibitory effects of currently developed antiallergic drugs on neutrophils, we employed azelastine, oxatomide, tranilast, repirinast and amlexanox which are classified as "inhibitors of chemical mediator release". Human neutrophils were isolated and activated by A23187 and PMA or FMLP for the assays of the arachidonic acid release or LTB₄ formation and superoxide generation, respectively. The effects of various antiallergic drugs on arachidonic acid release and LTB₄ production are summarized in Table 1 where the inhibitory effects are expressed as a percent of remaining activity from a control activity obtained from the stimulated neutrophils in the absence of drugs. A shown in the Methods section, 100% radioactivities of the arachidonic acid released was 6184±1717 dpm/5×10⁶ cells. In the presence of drugs, the cells were preincubated for 5 min before the addition of the metabolic stimuli (A23187) and incubated for a further 10 and 2 min for the arachidonic acid release and LTB₄, respectively.

As seen in Table 1 and Fig. 2, the formation of LTB₄ by neutrophils was inhibited in a dose-dependent manner by all the antiallergic drugs examined here, although the IC50s of the drugs varied in a concentration range of 20–200 µM. The mean values were obtained from 3 to 4 healthy individuals, but some of the results (over 200 µM of the drug concentrations) were the data from just one experiment. The approximate potency of the inhibition in terms of the IC50 values was:

Measurement of superoxide anion production: Superoxide anion production was measured as described previously (20) using 0.3 µg phorbol 12-myristate 13-acetate (PMA), 20 µM ferricytochrome c and 10⁶ cells with or without various drugs. The values of PMA and n-formyl-methionyl-leucyl-phenylalanine (FMLP) (100 nM)-induced superoxide anion generation were 6.63±1.12 nmoles/min/10⁶ cells and 5.317±0.638 nmoles/min/10⁶ cells, respectively.

Chemicals: Azelastine, clemastine, oxatomide, repirinast, amlexanox and tranilast were obtained from Eisai Co. (Tokyo, Japan). PMA and cytochrome c were purchased from Sigma Chemical Co., and A23187 was obtained from Calbiochem-Behring Co. Clemastine, oxatomide and tranilast were dissolved in ethanol, and repirinast and amlexanox were dissolved in dimethyl sulfoxide. The final concentration of ethanol and dimethyl sulfoxide were under 0.5% and did not affect the neutrophil functions.
Table 1. Leukotriene production and arachidonic acid release from neutrophils stimulated by A23187

<table>
<thead>
<tr>
<th>Drugs</th>
<th>% of control</th>
<th>Leukotriene B₄ production</th>
<th>Arachidonic acid release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Basic antiallergics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azelastine</td>
<td>20 μM</td>
<td>86±13 (3)***</td>
<td>60±30 (4)*</td>
</tr>
<tr>
<td></td>
<td>50 μM</td>
<td>57±16 (3)***</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 μM</td>
<td>10±4 (3)***</td>
<td>13±11 (4)***</td>
</tr>
<tr>
<td>Oxatomide</td>
<td>20 μM</td>
<td>92±7 (3)***</td>
<td>130±25 (3)</td>
</tr>
<tr>
<td></td>
<td>50 μM</td>
<td>2±3 (3)***</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 μM</td>
<td>1±2 (3)***</td>
<td>29±30 (3)***</td>
</tr>
<tr>
<td>Acidic antiallergics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tranilast</td>
<td>20 μM</td>
<td>98±23 (3)</td>
<td>85±25 (3)</td>
</tr>
<tr>
<td></td>
<td>100 μM</td>
<td>88±19 (3)</td>
<td>122±11 (4)**</td>
</tr>
<tr>
<td></td>
<td>200 μM</td>
<td>33 (1)</td>
<td>111 (1)</td>
</tr>
<tr>
<td>Amlexanox</td>
<td>20 μM</td>
<td>100±12 (3)</td>
<td>160±56 (4)</td>
</tr>
<tr>
<td></td>
<td>50 μM</td>
<td>104±9 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 μM</td>
<td>63±28 (4)</td>
<td>154±36 (4)*</td>
</tr>
<tr>
<td></td>
<td>200 μM</td>
<td>3 (1)</td>
<td>174 (1)</td>
</tr>
<tr>
<td>Repirinast</td>
<td>20 μM</td>
<td>86±24 (4)</td>
<td>159±51 (3)</td>
</tr>
<tr>
<td></td>
<td>50 μM</td>
<td>48±13 (3)***</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 μM</td>
<td>12±18 (3)***</td>
<td>158±21 (4)***</td>
</tr>
</tbody>
</table>

Control values (100%) of leukotriene B₄ production and arachidonic acid release was 20.5±2.6 nmoles/10⁶ cells in 2 min and 6184±1717 dpm/5×10⁶ cells in 10 min, respectively. Each value represents the mean±S.D. of 3 or 4 independent experiments. Significantly different from the control: ***P<0.01, **P<0.02, *P<0.05.

Oxatomide (IC50<50 μM)<azelastine and repirinast<amlexanox<tranilast (IC50>100 μM). On the other hand, the releases of arachidonic acid as a precursor of LTB₄ were effectively diminished only by the basic antiallergic drugs, such as azelastine and oxatomide, but the acidic agents including tranilast, amlexanox and repirinast up to 200 μM failed to inhibit the liberation of fatty acid. In fact, the acidic antiallergic drugs, especially amlexanox and repirinast rather enhanced the arachidonic acid release even at lower concentrations (20 μM). Since a similar activation phenomena was observed in the superoxide generation from neutrophils, we will discuss this further in a later section.

These data clearly indicate that the basic drugs inhibited both arachidonic acid release as well as LTB₄ formation, whereas the acidic agents inhibited only the LTB₄ formation. Furthermore, the effects of antiallergic drugs on superoxide generation from neutrophils stimulated by PMA were compared with that on the LTB₄ formation by A23187. As shown in Fig. 2, the results were similar to those seen in Table 1. The inhibitions of superoxide generation were achieved by the basic agents which suppressed the arachidonic acid release. The most effective inhibitor for superoxide generation was azelastion which almost completely suppressed the respiratory burst at 20 μM. These inhibitory effects of cellular responses were not due to the cellular damage, since the trypan blue exclusion tests indicated that at least over 80% of the neutrophils were negative under any conditions employed.

As seen in Table 1 and Fig. 2, there were fair correlations between the potency of inhibition of arachidonic acid release and superoxide generation by the basic drugs. That is, the basic agents were effective inhibitors in terms of three kinds of metabolic bursts of neutrophils including superoxide generation,
arachidonic acid release and formation of LTs. Especially, azelastine almost completely inhibited the superoxide generation even at lower concentration (20 μM). On the contrary, all of the acidic drugs up to 100 μM failed to inhibit the PMA stimulated superoxide generation. A slight activation of the superoxide generation was observed with the PMA activated neutrophils in the presence of acidic drugs. A striking enhancement of the superoxide generation was found neutrophils were activated by chemotactic factor (FMLP) with repirinast as demonstrated in Fig. 3. Over 200% of the superoxide production was observed when FMLP was challenged to the incubation medium in the presence of 100 μM of repirinast. The enhancement of the respiratory burst by repirinast was seen even at lower concentration (20 μM), but it had only a slight effect on PMA stimulated superoxide generation. Although the degrees were not identical, activations were commonly observed in arachidonic acid release as well as superoxide generation by acidic antiallergic drugs. In contrast, effective inhibition of the superoxide generation by azelastine was observed regardless of the type of metabolic stimuli employed.

Discussion

Although mast cells and basophils are known to play central roles in the immunological responses of human allergic diseases, the current therapy for allergies emphasizes, the action of compounds of LT release (22, 23), and one of the major suppliers of the arachidonic acid metabolites is neutrophils. Therefore, it is worth-while to examine the effects of various antiallergic drugs on the metabolic responses of human neutrophils.

In previous studies, we have demonstrated that metabolic bursts of rabbit polymorphonuclear leukocytes such as superoxide generation and arachidonic acid releases were effectively diminished by the newly developed antiallergic drug, azelastine (17, 20). In this
study, attempts were made to examine five representative new drugs including oxatomide, azelastine, tranilast, repirinast and amplexanox on superoxide generation, arachidonic acid release and LTB₄ formation from the stimulated human neutrophils. The results obtained here suggested that the drugs mentioned above can be classified into two groups according to how they inhibit the metabolic activations as well as the chemical structures of the agents. That is, the amine-containing antiallergic drugs inhibit three kinds of metabolic bursts including superoxide, arachidonic acid and LTB₄ formations, but the carbonic acid containing agents inhibited only the production of LTB₄. The initiation of arachidonic acid cascade in neutrophils starts with an increase of intracellular calcium concentration due to either extracellular entry or mobilization from calcium storage (24). Under our experimental conditions, a calcium ionophore increases intracellular calcium concentration (25), so that phospholipase A₂ or glyceride lipase liberates fatty acids from phospholipids and then lipoxigenase systems convert arachidonic acid to LTs (26, 27).

The present results suggest that the liberation of arachidonic acid from phospholipids is inhibited by the basic antiallergic agents, and catabolic steps which convert arachidonic acid to LTs are inhibited by the acidic antiallergic drugs. The superoxide generation was also effectively diminished only by the basic drugs indicating that there was a close relationship between the inhibitory effects on the phospholipase A₂ activation and the plasma membrane located NADPH oxidase. In other words, it is likely that the basic antiallergic drugs may inhibit plasma membrane located enzymes such as phospholipase A₂ and NADPH oxidase. In this respect, there is a possibility that the inhibition of these enzymes may be due to changes in the local charge of membranous proteins. This assumption can be supported with the results demonstrated by Miyahara et al. which suggest that a charged moiety of the neutrophils play a key role in metabolic stimulation (28, 29). They showed that the inhibition and activation of super-
oxide generation by leukocytes manipulated by anionic and cationic agents. Therefore, the initiation of the respiratory burst, which is generally accepted as linked to protein kinase C activation (30), might be related to the presence of a net charge on the plasma membrane.

As shown in Table 1 and Fig. 3, the acidic antiallergic drugs activate the arachidonic acid release as well as superoxide generation, although the degrees of the effects are quite different. The most striking activation of superoxide generation is seen with the combination of FMLP and repirinast. Presumably, this could be largely due to the anionic property of repirinast, which may mobilize the signal transport proteins as well as membranous enzymes. However, PMA can penetrate into the cytosol where regulatory proteins are phosphorylated. So that, the enhancement by repirinast with PMA are less than the activation seen by FMLP. (Further details are under investigation in our laboratory.) The anionic property of the acidic agents may, therefore, enhance the activation process or mobilization of the plasma membrane located enzymes such as NADPH oxidase and phospholipase A2. On the other hand, the inhibition of arachidonic acid release and superoxide generation by the basic drugs could be due to the cationic property of the antiallergic agents.

Although the roles of fatty acid liberated in the activation process of superoxide generation have been a matter of debate, recent investigation show that there is a possibility that the arachidonic acid is essential for the activation of NADPH oxidase in the plasma membrane (31, 32). These observations indicate that the arachidonic acid liberated by phospholipase A2 is playing a key role in the metabolic burst of PMNs. The inhibition of the lipase activity by agents may result in the inhibition of superoxide generation. In any case, further investigations on the initiation process of the metabolic activation are necessary to discuss the relationship between superoxide generation and arachidonic acid release from PMNs.

To clarify the precise mechanisms involved, further studies are currently being performed using the HL60 cell line in our laboratories and the relevance of the mechanisms under in vivo conditions should be examined for therapeutic uses.

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