Effects of Azelastine on Neutrophil Chemotaxis, Phagocytosis and Oxygen Radical Generation

Hirohiko Akamatsu, Yoshiki Miyachi¹, Yasuo Asada and Yukie Niwa²

Department of Dermatology, Kansai Medical University, Osaka 570, Japan
¹Department of Dermatology, Tenri Hospital, Nara 632, Japan
²Niwa Institute for Immunology, Kochi 787-03, Japan

Received May 14, 1991 Accepted September 21, 1991

ABSTRACT—The effects of azelastine, an orally active anti-allergic drug, on several inflammatory parameters of human neutrophils, including human neutrophil chemotaxis, phagocytosis and generation of reactive oxygen species (ROS), was examined. ROS generated in a cell-free, xanthine-xanthine oxidase system was also assessed. The species investigated were superoxide radical anion (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radical (OH·). Azelastine significantly inhibited human neutrophil phagocytosis and the generation of O₂⁻, H₂O₂, OH· by human neutrophils. However, the drug did not markedly affect human neutrophil chemotaxis or the ROS levels generated in the xanthine-xanthine oxidase system. The present study indicates that azelastine may exert an anti-inflammatory action by inhibiting human neutrophil phagocytosis as well as oxygen radical generation at the sites of inflammation.

Immunoglobulin E (IgE)-mediated allergic diseases such as bronchial asthma, allergic rhinitis and urticaria can be thought of as inflammatory events occurring as the result of mast cell activation. There is increasing evidence that inflammatory cells such as neutrophils play an important role in these reactions (1, 2). It has been reported that high molecular weight neutrophil chemotactic factors (3–6), leukotriene B₄ (7, 8) and platelet-activating factor (9) are produced by mast cells in IgE-mediated allergic reactions, resulting in the accumulation of neutrophils at the site of the allergic inflammatory reaction. The attracted neutrophils are thought to produce and release various mediators and enzymes as well as reactive oxygen species (ROS) which are some of the most toxic mediators, with the resultant development of inflammation.

Azelastine [4-(p-chlorobenzyl)-2-(hexahydro-1-methyl-1H-azepine-4-yl)-1-(2H)-phthalazinone hydrochloride] is an orally active anti-allergic drug. Regarding its mechanisms of action, the drug has been demonstrated to inhibit not only histamine release but also leukotriene release, and it also antagonizes histamine as well as leukotrienes (10–12). It has been recently reported that azelastine decrease O₂⁻ generation by neutrophils (13, 14). However, the effects on neutrophil chemotaxis, phagocytosis and each kind of ROS, especially OH· which is the most toxic oxidant, generated by both neutrophils and the xanthine-xanthine oxidase system, have not yet been examined.

In the present study, we investigated the effects of azelastine on the inflammatory parameters of human neutrophils such as neutrophil chemotaxis, phagocytosis and ROS generation; ROS generation in a cell-free system was also assessed.
MATERIALS AND METHODS

Chemicals
Azelastine (Eisai Co., Tokyo, Japan) was added to the following neutrophil function assay systems at concentrations of 0.05, 0.5, or 5 μg/ml.

Neutrophil preparation
Neutrophils were isolated from the heparinized venous blood of seven healthy volunteers by a modification of a previously described method (15). After centrifugation of the blood over a Ficoll-Hypaque gradient, the plasma-containing upper layer, mononuclear cell layer, and remaining cell pellet were respectively separated. The plasma was freed from platelets by centrifugation. The cell pellet, containing leukocytes and erythrocytes, was washed with saline solution and resuspended in plasma containing dextran 170 (molecular weight, 170,000) at a final concentration of 1%. The neutrophils were recovered after sedimentation at unit gravity, and a few contaminating erythrocytes were lysed by treatment of the preparation with 0.876% NH₄Cl. By this procedure, the viability of the harvested neutrophils was always greater than 99% as assessed by the trypan blue exclusion test; and [¹⁴C]inulin uptake, which measures phagocytic activity, was greater than 900 dpm [¹⁴C]inulin uptake/mg protein. Neutrophils showed less than 600 dpm [¹⁴C]inulin uptake/mg protein, their function was considered to have been impaired, and the results were discarded.

Neutrophil chemotaxis assay
Neutrophil chemotactic activities were measured using an agarose plate (18). Agarose plates were prepared by mixing 2.5 ml of 2.4% agarose solution with 2.5 ml of 2 × normal RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. Three wells, with a diameter of 3 mm, were cut on a straight line, at 8-mm intervals. Ten microliters of a neutrophil suspension in RPMI 1640 medium containing various concentrations of azelastine and 1 × 10⁸ cells/ml was added to the center well. To the outer well, 10 μl of 10⁻⁷ M or 10⁻⁶ M N-formyl-methionyl-leucyl-phenylalanine (fMLP) was added as a chemoattractant. To the inner well, 10 μl of the RPMI 1640 medium was added as a control. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO₂ in air for two hours. The distances traveled by the ten fastest-moving neutrophils toward the outer well (true chemotaxis) and the inner well (random migration) was measured with a microscope. In the test of agarose plate neutrophil chemotaxis, 10⁻⁷ M or 10⁻⁶ M platelet activating factor (PAF) was also used as a chemoattractant.

Neutrophil phagocytosis assay
Emulsions of paraffin oil containing oil red O were prepared as previously described (19), except that the lipopolysaccharide solution (endotoxin) was replaced with normal human serum. The emulsion was incubated with an equal volume of normal human serum at 37°C for 30 min for opsonization. Neutrophils (2 × 10⁷ cells/0.9 ml KRP) which had been preincubated for 5 min with each concentration of azelastine were added with 0.1 ml of the opsonized emulsion, the mixture was incubated for 5 min at 37°C, and then 9 ml ice-cold KRP was added to the solution to stop the reaction. The cells were washed three times
with ice-cold KRP to remove the paraffin oil droplets that had not been ingested. Paraffin oil containing oil red O was extracted from the cells by the method of Bligh and Dyer (20), using chloroform and methanol (v/v, 1:2), and the optical density of the chloroform layer was determined at a wavelength of 525 nm.

The mean optical density of oil red O extracted from $2 \times 10^7$ neutrophils incubated with opsonized paraffin oil droplets was 0.0161 ± 0.001 (average ± S.D. of five experiments); and examination under a microscope revealed that a majority of the neutrophils were heavily loaded with oil droplets. On the other hand, when non-opsonized paraffin oil droplets were incubated with neutrophils, the optical density was less than 0.005, and under the microscope, only a few neutrophils were loaded. These findings confirmed that most of the extracted oil red O represented droplets ingested by the neutrophils.

**Neutrophil ROS generation assays**

In studies of $O_2^-$ formation, $1 \times 10^6$ neutrophils were preincubated at 37°C for 10 min with 1 mg/ml opsonized zymosan (Sigma) and various concentrations of azelastine, and then 0.1 mM ferriytochrome c (type II, Sigma) was added. The neutrophils were incubated for another 30 min. Immediately after sedimentation of the neutrophils and opsonized zymosan by centrifugation, 0.1 ml of the supernatant was assayed for reduced cytochrome c by measuring absorbance at 550 nm (21, 22) in 2 ml of 100 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA (pH 7.8). The results were converted to nmoles of reduced cytochrome c, using $A_{550} = 2.1 \times 10^4/M/cm$ (23). SOD (Sigma) was also added to the neutrophil medium at a concentration of 400 units/ml to inhibit the reduction of cytochrome c by $O_2^-$. 

$H_2O_2$-generation was measured by quantifying the decrease in fluorescence intensity of scopoletin (Sigma) due to its peroxidase-mediated oxidation by $H_2O_2$ (23). After incubation of $2.5 \times 10^6$ neutrophils for 10 min at room temperature in KRP containing 5 mM glucose and 0.1 mg/ml gelatin in the presence of each concentration of azelastine and 1 mg/ml opsonized zymosan, 0.1 ml of 50 mM scopoletin in KRP and 0.05 ml of 1 mg/ml horseradish peroxidase (type II; Sigma) in phosphate-buffered saline (PBS) were added. The rate of decrease in fluorescence intensity of the scopoletin was quantified using a fluorescence spectrophotometer (Hitachi Co. Ltd., Tokyo, Japan). To calculate the $H_2O_2$ concentration, we assumed that 1 mole of $H_2O_2$ was oxidized 1 mole of scopoletin (23). Incubation of the supernatants with an excess of 400 units/ml catalase (Sigma) inhibited fluorescence reduction.

$OH^-$ was quantitated by the amount of ethylene formed from $\alpha$-keto-methylbutyric acid (KMB) (Sigma) plus $OH^-$ generated by neutrophils (24). Neutrophils ($2 \times 10^6$) in 2 ml KRP containing glucose were preincubated with 1 mM KMB and each concentration of azelastine in a stopped tube and gently mixed in a 37°C shaker bath for 5 min. Opsonized zymosan was then added, and the cells were incubated for 10 min. Thereafter, aliquots of gas in the tube were sampled using a gas-tight syringe, and the ethylene content was determined by gas chromatography (Hitachi). The total amount of ethylene formed during 10, 20 and 30 min served as the $OH^-$ value.

**ROS generation assay in the xanthine-xanthine oxidase system**

All ROS were also measured in the xanthine-xanthine oxidase system. Instead of adding neutrophils and opsonized zymosan, 0.1 mM hypoxanthine, 1.25 mM EDTA and 16.5 $\mu$M ferriytochrome c were mixed in a total volume of 2 ml (125 mM phosphate buffer). After the addition of the various concentrations of azelastine, approximately 0.006 U/ml of dialyzed xanthine oxidase was added to generate ROS (25).

Triplicate assays were performed in each experiment; the results are expressed as the mean ± S.D. of replicate assays. Statistical significance was ascertained by Student's $t$-test.
RESULTS

Azelastine had no significant effect on neutrophil chemotaxis or random neutrophil locomotion (both \( P > 0.05 \)) (Table 1).

Neutrophil phagocytosis was significantly reduced in the presence of azelastine (27.3\% inhibition by 5.0 \( \mu \text{g/ml} \), \( P < 0.05 \)) (Fig. 1).

The levels of \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \) and \( \text{OH}^- \) generated by neutrophils were markedly decreased in a dose-dependent fashion by azelastine (\( \text{O}_2^- \): 9.1\% inhibition by 0.5 \( \mu \text{g/ml} \), \( P < 0.05 \); 29.2\% inhibition by 5 \( \mu \text{g/ml} \), \( P < 0.01 \), \( \text{H}_2\text{O}_2 \): 11.3\% inhibition by 0.5 \( \mu \text{g/ml} \), \( P < 0.05 \); 28.3\% inhibition by 5 \( \mu \text{g/ml} \), \( P < 0.01 \), \( \text{OH}^- \): 13.9\% inhibition by 0.05 \( \mu \text{g/ml} \), 23.9\% inhibition by 0.5 \( \mu \text{g/ml} \); and 35.9\% inhibition by 5.0 \( \mu \text{g/ml} \), \( P < 0.01 \)) (Fig. 2). On the other hand, none of the ROS generated in a cell-free, xanthine-xanthine oxidase system were affected in the presence of azelastine (Table 2).

Neutrophil chemotaxis induced by \( 10^{-6} \)M or \( 10^{-7} \)M PAF in the presence of azelastine was similar to that caused by fMLP; neutrophil chemotaxis by \( 10^{-6} \)M or \( 10^{-7} \)M PAF was not affected by the addition of azelastine (data not shown).

Table 1. Effect of azelastine on neutrophil chemotaxis

| Azelastine (\( \mu \text{g/ml} \)) | True chemotaxis (fMLP) | Random chemotaxis |
|---------------------------------|-------------------------|-----------------|
|                                 | \( 10^{-6} \)M | \( 10^{-7} \)M |                 |
| 0                               | 20.0 ± 0.0 | 19.0 ± 1.00 | 8.1 ± 0.32 |
| 0.05                            | 19.0 ± 1.00 | 18.6 ± 1.53 | 8.3 ± 0.29 |
| 0.5                             | 19.3 ± 1.15 | 17.3 ± 2.31 | 7.9 ± 0.34 |
| 5.0                             | 19.3 ± 0.58 | 19.0 ± 1.00 | 7.8 ± 0.28 |

The experiment was performed as described in Materials and Methods. The distance traveled by the ten fastest-moving neutrophils toward the outer well was measured with a microprojector and expressed as mm.
Fig. 2. Effect of azelastine on ROS generation by neutrophils. Closed circle (●) denotes \( \text{O}_2^- \); open circle (○), \( \text{H}_2\text{O}_2 \); and cross symbol (×), \( \text{OH}^- \) levels. *: \( P < 0.05 \) vs. control, **: \( P < 0.01 \).

Table 2. Effect of azelastine on ROS levels generated in the xanthine–xanthine oxidase system

| Azelastine (\( \mu \text{g/ml} \)) | Reactive oxygen species (ROS) |
|---------------------------------|--------------------------------|
| 0                               | 1.25 ± 0.10 \( \text{O}_2^- \) (nmol/min) | 776.3 ± 22.6 \( \text{H}_2\text{O}_2 \) (pmol/min) | 48.3 ± 2.50 \( \text{OH}^- \) (pmol/min) |
| 0.05                            | 1.27 ± 0.11 \( \text{O}_2^- \) (nmol/min) | 795.7 ± 28.0 \( \text{H}_2\text{O}_2 \) (pmol/min) | 57.3 ± 3.23 \( \text{OH}^- \) (pmol/min) |
| 0.5                             | 1.25 ± 0.12 \( \text{O}_2^- \) (nmol/min) | 771.7 ± 30.3 \( \text{H}_2\text{O}_2 \) (pmol/min) | 55.5 ± 4.22 \( \text{OH}^- \) (pmol/min) |
| 5.0                             | 1.23 ± 0.09 \( \text{O}_2^- \) (nmol/min) | 754.3 ± 42.6 \( \text{H}_2\text{O}_2 \) (pmol/min) | 48.7 ± 3.11 \( \text{OH}^- \) (pmol/min) |

The experiment was performed as described in Materials and Methods. All ROS were measured in the cell-free, xanthine-xanthine oxidase system.
DISCUSSION

The present study demonstrates that azelastine significantly decreased neutrophil phagocytosis and one of the most potent inflammatory mediators, ROS, which are generated by neutrophils, while the drug did not markedly affect neutrophil chemotaxis or ROS levels generated in a cell-free, xanthine-xanthine oxidase system. Taniguchi et al. (13, 14) have previously reported that azelastine inhibits the amount of O$_2^-$ generated by neutrophils, and this was confirmed by the results of the present investigation. In addition, our results show that the drug reduced the amount of H$_2$O$_2$ and OH· generation by neutrophils, respectively. The results obtained in the cell-free system seem to indicate that azelastine does not scavenge generated ROS.

The activation of neutrophils is thought to be induced by an increase of intracellular calcium concentration due to either extracellular entry or mobilization from calcium storage with subsequent activation of the activity of methyltransferase and phospholipase A$_2$ which leads to the induction of the arachidonic acid cascade and the production of various inflammatory mediators (26–29). Recently, it was reported that arachidonic acid may be essential for the activation of NADPH oxidase in the plasma membrane, which mediates the production of most ROS by neutrophils (30, 31).

In vitro experiments have demonstrated that azelastine is a potent inhibitor of the arachidonic acid release and O$_2^-$ generation by fMLP-stimulated neutrophils, but weak inhibitors of phospholipase A$_2$ (13). Furthermore, O$_2^-$ generation by phorbol 12-myristate 13-acetate (PMA)-stimulated neutrophils is also reduced in the presence of the drug (14). These reports seem to indicate that azelastine may inhibit the process of phospholipase A$_2$ activation and/or the mobilization of calcium from the intracellular storage sites.

The present study demonstrates that azelastine is not an ROS scavenger, but rather appears to decrease neutrophil-derived ROS generation by acting on neutrophil metabolism. This may occur through or correlate with inhibitory effects on the process of phospholipase A$_2$ activation with resultant failure in induction of the arachidonic acid cascade. Our study provides further background for azelastine therapy in inflammatory allergic diseases.

REFERENCES

1 Simehowitz, L., and Spilberg, I.: Evidence for the role of superoxide radicals in neutrophil-mediated cytotoxicity. Immunology 37, 301–309 (1979)
2 Sacks, T., Moldow, C.F., Craddock, P.R., Bowers, T.K. and Jacob, H.S.: Oxygen radicals mediate endothelial cell damage by complement-stimulated granulocytes. J. Clin. Invest. 61, 1161–1167 (1978)
3 Wasserman, S.I., Soter, N.A., Center, D.M. and Austen, K.F.: Cold urticaria; recognition and characterization of a neutrophil chemotactic factor which appears in serum during experimental cold challenge. J. Clin. Invest. 60, 189–196 (1977)
4 Atkins, P.G., Norman, M., Weiner, H. and Zweiman, B.: Release of neutrophil chemotactic activity during immediate hypersensitivity reactions in humans. Ann. Intern. Med. 86, 415–418 (1977)
5 Soter, N.A., Wasserman, S.I., Austen, K.F. and McFadden, E.R., Jr.: Release of mast-cell mediators and alterations in lung function in patient with cholinergic urticaria. N. Engl. J. Med. 302, 604–608 (1980)
6 Soter, N.A., Wasserman, S.I., Pathak, M.A., Parrish, J.A. and Austen, K.F.: Solar urticaria; release of mast cell mediators into the circulation after experimental challenge. J. Invest. Dermatol. 72, 282 (1979)
7 Salari, H., Baegeat, P., Broquet, P. and Siros, P.: Studies on the biosynthesis of leukotrienes in guinea pig lung in vitro by use of high-performance liquid chromatography. J. Allergy Clin. Immunol. 77, 720–727 (1986)
8 Atkins, P.C., Valenzano, M., Goetzl, E.J., Ratnoff, W.D., Graziano, F.M. and Zweiman, B.: Identification of leukotriene B$_4$ as the neutrophil chemotactic factor released by antigen challenge from passively sensitized guinea pig lungs. J. Allergy Clin. Immunol. 83, 136–143 (1989)
9 Grandel, K.E., Farr, R.S., Wanderer, A.A., Eisenstadt, T.C. and Wasserman, S.I.: Association of platelet-activating factor with primary acquired cold urticaria. N. Engl. J. Med. 313, 405–409 (1985)
10 Field, D.A.S., Pillar, J., Diamantis, W., Perbach,
J.L., Jr., Sofia, R.D. and Chand, N.: Inhibition of azelastine of nonallergic histamine release from rat peritoneal mast cells. J. Allergy Clin. Immunol. 73, 400–403 (1984)

11 Chand, N., Pillar, J., Diamantis, W. and Sofia, R.D.: Inhibition of IgE-mediated allergic histamine release from rat peritoneal mast cells by azelastine and selected antiallergy drugs. Agents Actions 16, 318–322 (1985)

12 Katayama, T., Tsunoda, H., Sakuma, Y., Kai, H., Tanaka, I. and Katayama, K.: Effect of azelastine on the release and action of leukotriene C4 and D4. Int. Arch. Allergy Appl. Immunol. 83, 284–289 (1987)

13 Taniguchi, K., Urakami, M. and Takanaka, K.: Effects of various drugs on superoxide generation, arachidonic acid release and phospholipase A2 in polymorphonuclear leukocytes. Japan. J. Pharmacol. 46, 275–284 (1988)

14 Taniguchi, K., Masuda, Y. and Takanaka, K.: Action sites of antiallergy drugs on human neutrophils. Japan. J. Pharmacol. 52, 101–108 (1990)

15 Niwa, Y., Sakane, T., Shingu, M., Yanagida, I., Komura, J. and Miyachi, Y.: Neutrophil-generated active oxygen in linear IgA bullous dermatosis. Arch. Dermatol. 121, 73–78 (1985)

16 Skosey, J.L., Damgaard, E., Chow, D.C. and Sorensen, L.B.: Modification of zymosan-induced release of lysosomal enzymes from polymorphonuclear leukocytes by cytochalasin B. J. Cell. Biol. 62, 625–634 (1974)

17 Johnson, A.R. and Erdos, G.: Metabolism of vasoactive peptides by human endothelial cells in culture. J. Clin. Invest. 59, 684–695 (1977)

18 Nelson, R.D., Ouic, P.G. and Simmons, R.L.: Chemotaxis under agarose. A new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. J. Immunol. 115, 1650–1656 (1977)

19 Stossel, T.P.: Evaluation of opsonic and leukocyte function with a spectrophotometric test in patients with infection and with phagocytic disorders. Blood 42, 121–130 (1973)

20 Bligh, E.G. and Dyer, W.J.: A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917 (1959)

21 Johnston, R.B., Jr. and Lehmeyer, J.E.: Elaboration of toxic oxygen by-products by neutrophils in a model of immune complex disease. J. Clin. Invest. 57, 836–841 (1976)

22 Massey, V.: The microestimation of succinate and the extinction coefficient of cytochrome c. Biochim. Biophys. Acta 34, 255–256 (1959)

23 Root, R.K. and Metcalf, J.A.: H2O2 release from human granulocytes during phagocytosis. J. Clin. Invest. 60, 1266–1279 (1972)

24 Klebanoff, S.J. and Rosen, H.: Ethylene formation by polymorphonuclear leukocytes. J. Exp. Med. 148, 490–505 (1978)

25 Niwa, Y., Kasama, T., Miyachi, Y. and Kanoh, T.: Neutrophil chemotaxis, phagocytosis and parameters of reactive oxygen species in human aging: cross-sectional and longitudinal studies. Life Sci. 44, 1655–1664 (1989)

26 Hirata, F. and Axelrod, J.: Phospholipid methylation and biological signal transmission. Science 209, 1082–1090 (1980)

27 Ishizaka, T., Hirata, T. and Ishizaka, K.: Stimulation of phospholipid methylation, Ca2+ influx, and histamine release by bridging of IgG receptors on rat cells. Proc. Natl. Acad. Sci. U.S.A. 77, 1903–1906 (1980)

28 Bareis, D.L., Hirata, F., Schiffmann, E. and Axelrod, J.: Phospholipid metabolism, calcium influx and receptor mediated induction of chemotaxis in rabbit neutrophils. J. Cell. Biol. 93, 690–697 (1982)

29 Nishizuka, Y.: Studies and perspectives of protein kinase C. Science 233, 305–312 (1986)

30 McPhail, L.C., Shirley, P.S., Clayton, C.C. and Snyderman, R.: Activation of the respiratory burst enzyme from human neutrophils in a cell-free system. J. Clin. Invest. 75, 1735–1739 (1985)

31 Curnutte, J.T.: Activation of human neutrophils nicotine adenine dinucleotide phosphate, reduced (triphosphopyridine nucleotide, reduced) oxidase by arachidonic acid in a cell-free system. J. Clin. Invest. 75, 1740–1743 (1985)