J. Pharm. Pharmacol. 1993, 45: 286–291
Received May 11, 1992

Effects of NZ-107 on Airway Inflammation and Cell Activation in Guinea-pigs

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Abstract—The effects of NZ-107 on some airway inflammation models and the generation of superoxide anion (O_2^-) were studied in guinea-pigs. Airway inflammation was caused by intra-tracheal injection of murine recombinant interleukin-5 (mrIL-5, 15 μg/animal), inhalation of platelet-activating factor (PAF, 0·003%) and intra-tracheal injection of leukotriene B_4 (LTB_4, 10 μg/animal). NZ-107 (4-bromo-5-(3-ethoxy-4-methoxybenzylamino)-3(2H)-pyridazinone) at a dose of 50 mg kg^{-1}, intraperitoneally reduced mrIL-5- and PAF-induced eosinophilia. This compound at a dose of 25 and 50 mg kg^{-1} also suppressed LTB_4-induced eosinophilia and neutrophilia in bronchoalveolar lavage fluid (BALF). On the other hand, prednisolone at a dose of 20 mg kg^{-1}, i.p., prevented the increased number of macrophages, eosinophils and neutrophils induced by mrIL-5, the increased number of eosinophils induced by PAF and the increased number of eosinophils and neutrophils induced by LTB_4 in BALF. Furthermore, both drugs reduced mrIL-5- or PAF-induced increase in the number of airway epithelial cells in BALF. The generation of O_2^- was measured by the method of cytochrome C reduction. NZ-107 (10–100 μg mL^{-1}) attenuated PAF- and FMLP-induced O_2^- production from macrophages and reduced PAF-induced O_2^- generation by eosinophils but had no effect on that from neutrophils. These results indicate that NZ-107 prevents the increased number of pulmonary eosinophils and airway epithelial cells and the activation of macrophages and eosinophils, suggesting that NZ-107 may be useful as a remedy for airway inflammatory diseases such as bronchial asthma.

(4-Bromo-5-(3-ethoxy-4-methoxybenzylamino)-3(2H)-pyridazinone) (NZ-107) is a newly synthesized pyridazinone derivative. NZ-107 inhibits LTD_4-induced airway smooth muscle contraction in guinea-pigs (Hibi et al 1989). This compound also inhibits the antigen-induced slow reacting substance of anaphylaxis (SRS-A) and histamine release from lung fragments as well as antigen-induced airway smooth muscle contraction in man and guinea-pigs (Yamamoto et al 1991; Nagai et al 1992). Furthermore, NZ-107 inhibits immediate- and late-phase bronchoconstriction and airway hyper-reactivity in-vivo in guinea-pigs when administered orally or intravenously (Iwama et al 1991, 1992b; Suda et al 1992). These observations suggest an application for NZ-107 in bronchial asthma.

In general, many asthmatic patients suffer from an immediate asthmatic response and a late asthmatic response. The latter is accompanied by airway hyper-responsiveness to non-specific stimuli and its magnitude is closely associated with the extent and duration of airway hyper-responsiveness (Hargreave 1989). It has been reported that eosinophil-dominant airway inflammation may play an important role in the onset and development of late asthmatic response and airway hyper-responsiveness (Frigas & Gleich 1986; Barnes 1989; Kay 1991). Eosinophils infiltrated in the airway lumen may release oxygen radicals and granule proteins (e.g. major basic protein, eosinophil peroxidase and eosinophil cationic protein) to damage bronchial epithelium, leading to airway hyper-responsiveness (Frigas & Gleich 1986; Barnes 1989). It has been observed that there are more respiratory epithelial cells as well as eosinophils in the bronchoalveolar lavage fluid (BALF) of guinea-pigs with airways rendered hyper-reactive by antigen challenge than in normal animals (Motojima et al 1989). It has been also reported that clumps of airway epithelial cells, called Creola bodies (Naylor 1985), as well as eosinophils are found in the sputum of asthmatics (Frigas & Gleich 1986; Barnes 1989). Kushima et al (1990) demonstrated that the magnitude of the bronchial epithelial cell desquamation is related to that of the increased bronchial hyper-responsiveness in asthmatics. From these observations, it is clear that the activation of eosinophils followed by the airway epithelium damage play important roles in the pathogenesis of late asthmatic response and airway hyper-responsiveness.

In the present study the effects of NZ-107 on some airway inflammation models and superoxide anion production by inflammatory leucocytes have been studied.

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Materials and Methods

Animals

Male Hartley guinea-pigs, 300–500 g (Nihon SLC, Japan), were used.

Materials

Murine recombinant interleukin-5 (mrIL-5) was kindly provided by Dr S. Takatsu (the University of Kumamoto, (Tokyo Engineering). NZ-107 was donated by Nissan Chemical Ind. Ltd, Tokyo, Japan. Platelet-activating factor (PAF), phorbol myristate acetate (PMA), Formyl-Met-Leu-Phe (FMLP), cytochalasin B, cytochrome C (Type III), polymyxin B sulphate and superoxide dismutase (SOD) (Sigma Chemical Co., Japan). Leukotriene B_4 (LTB_4) was donated by Dr F. Sato, St Louis, USA), prednisolone acetate (Takeda Chemical Ind. Ltd, Osaka, Japan), Ficoll 400 (Pharmacia Fine Chemicals, A.B. Uppsala, Sweden), Con-

ray (Daiichi Pharmaceutical Co. Ltd, Tokyo, Japan), May-Gruenwald solution, Giemsa solution (Merk Co. Inc., NJ, USA), Turk solution (Wako Pure Chemicals, Japan), bovine serum albumin (BSA) (fraction V, Miles, IL, USA) were purchased from each company.

mrIL-5 and BSA were dissolved in 0·9% NaCl solution (saline). PAF was dissolved in saline containing 0·1% BSA. LTB_4 in methanol was dried under N_2 gas and was dissolved in 100% ethanol (EtOH) diluted with saline (finally 0·6% EtOH). FMLP was dissolved in 100% EtOH, and PMA and cytochalasin B in 100% dimethylsulphoxide (DMSO). Cytochrome C and SOD were dissolved in Hanks balanced salt solution (HBSS, pH = 7·3). NZ-107 and prednisolone acetate were suspended in saline containing 0·5% carboxy methyl cellulose sodium for in-vivo study, and NZ-107 was dissolved in 100% DMSO for in-vitro study.

Treatment with PAF, mrIL-5 and LTB_4 in-vivo

Guinea-pigs were exposed to PAF aerosol as follows. Animals were positioned inside a plastic body apparatus which was partitioned into 10 equal sections with head and body compartments separated by a neck restrainer. The snout of each animal was placed in a nose-space connected to an ultrasonic nebulizer Tur-3200 (Nihon Koden, Japan). A 0·003% PAF aerosol (spray volume 3 mL min^{-1}, mass particle diameter distribution 2·0–6·0 μm) was generated for 15 min using the ultrasonic nebulizer Tur-3200. The lungs were lavaged 24 h after the inhalation of PAF.

Each of mrIL-5 and LTB_4 was injected intra-tracheally according to the method previously described (Iwama et al 1992a). In brief, trachea was surgically exposed, and 0·25 mL of sterile LTB_4 or mrIL-5 solution was injected into the trachea using a 26-G syringe under sodium pentobarbitone anaesthesia. The wound was closed with sterile stitches. The lungs were lavaged 4 h (LTB_4) or 24 h (mrIL-5) after the exposure. NZ-107 was administered intraperitoneally 30 min, and prednisolone was given intraperitoneally 3 h, before the treatment with mrIL-5, PAF or LTB_4.

Bronchoalveolar lavage fluid (BALF) study

Guinea-pigs were killed by intraperitoneal injection of sodium pentobarbitone (150 mg kg^{-1}). The trachea was cannulated and the airway lumen was washed twice with 5 mL saline containing 0·1% BSA warmed at 37°C. BALF from each animal was pooled in a plastic tube cooled in ice and then centrifuged (150 *g*) at 4°C for 10 min. The cell pellets were resuspended in physiological saline (2 mL). The total leucocyte number was counted after Turk staining and the differentiated cell type count was made on a smear prepared with a cytocentrifuge and a May-Gruenwald and Giemsa dye staining under a microscope (× 500). Results are expressed as the total number of cells recovered in the BALF.

Preparation of leucocytes for in-vitro study

Alveolar macrophages. Guinea-pig alveolar macrophages were isolated from BALF. Briefly, after the injection of sodium pentobarbitone (150 mg kg^{-1}, i.p.), the trachea was cannulated and the airway lumen was washed with three portions (10 mL) of saline containing 0·1% BSA warmed at 37°C. Lavage fluid from each animal was centrifuged (150 *g* at 4°C for 10 min). The cell pellets were gently washed with Ca^{2+}, Mg^{2+}-free HBSS (pH = 7·3) and suspended at 2×10^6 cells mL^{-1} in HBSS (pH = 7·3) containing 0·1% BSA. These cells contained more than 90% alveolar macrophages as identified by May-Gruenwald and Giemsa staining.

Peritoneal eosinophils. The eosinophils of guinea-pigs were obtained according to the method described by Yamashita et al (1985). Briefly, 1 mL sterile polymyxin B solution (4 mg mL^{-1}) was intraperitoneally injected into guinea-pigs once a week. More than ten weeks later, peritoneal exudate cells were harvested and washed with 20 mL saline containing 0·1% BSA three times and then centrifuged (150 *g*, 10 min, 4°C). The cell pellets were suspended in 0·2% NaCl solution at room temperature (21°C) for 30 s to lyse contaminating red blood cells. The osmotic pressure of the cell suspension was adjusted to physiological conditions by adding an equal volume of 1·6% NaCl, and washed with saline. The washed cells were suspended in sterile saline (10^7 cells mL^{-1}), and then placed on Ficoll-Conray solution (d = 1·078 g mL^{-1}) for enriching the eosinophils by centrifugation (100 *g*, 25 min, 15°C). The sedimented cells were gently washed with Ca^{2+},

Table 1. Effects of PAF, mrIL-5 and LTB_4 on the cell number in guinea-pig BALF.

		Leucocytes (× 10^6)				
	Total cells	Macrophages	Eosinophils	Neutrophils	Lymphocytes	Epithelial cells (× 10^5)
mrIL-5						
Saline	6·00 ± 1·61	4·68 ± 1·04	0·50 ± 0·24	0·50 ± 0·17	0·32 ± 0·17	0·73 ± 0·23
mrIL-5	18·96 ± 4·38*	9·23 ± 1·16*	2·25 ± 0·54*	6·63 ± 2·59*	0·78 ± 0·30	4·73 ± 1·11*
PAF						
0·1% BSA	4·69 ± 0·61	3·34 ± 0·72	0·16 ± 0·10	0·13 ± 0·05	0·15 ± 0·04	0·50 ± 0·41
PAF	5·29 ± 0·58	4·06 ± 0·32	0·83 ± 0·30*	0·15 ± 0·05	0·19 ± 0·04	1·97 ± 0·49*
LTB_4						
0·6% EtOH	7·35 ± 0·62	6·79 ± 0·67	0·24 ± 0·12	0·10 ± 0·23	0·22 ± 0·06	0·72 ± 0·41
LTB_4	10·04 ± 1·59	7·23 ± 1·14	1·11 ± 0·28*	1·24 ± 0·27*	0·46 ± 0·14	0·54 ± 0·21

mrIL-5 (15 μg), LTB_4 (10 μg) and their vehicles (saline for mrIL-5 and 0·6% EtOH for LTB_4) were injected into the guinea-pig trachea, and PAF aerosol (0·003%) and its vehicle (0·1% BSA = saline containing 0·1% BSA) aerosol were inhaled by guinea-pigs. The BALF was obtained 24 h after the treatment with PAF and mrIL-5 and 4 h after the treatment with LTB_4. Results are expressed as the means ± s.e.m. of 6–11 experiments. *$P < 0·05$, significantly different from the corresponding vehicle response (saline for mrIL-5, 0·1% BSA for PAF and 0·6% EtOH for LTB_4) using Dunnett's multiple range test. There was no significant change between each vehicle (saline, 0·1% BSA and 0·6% EtOH)-treatment group and the corresponding normal group.

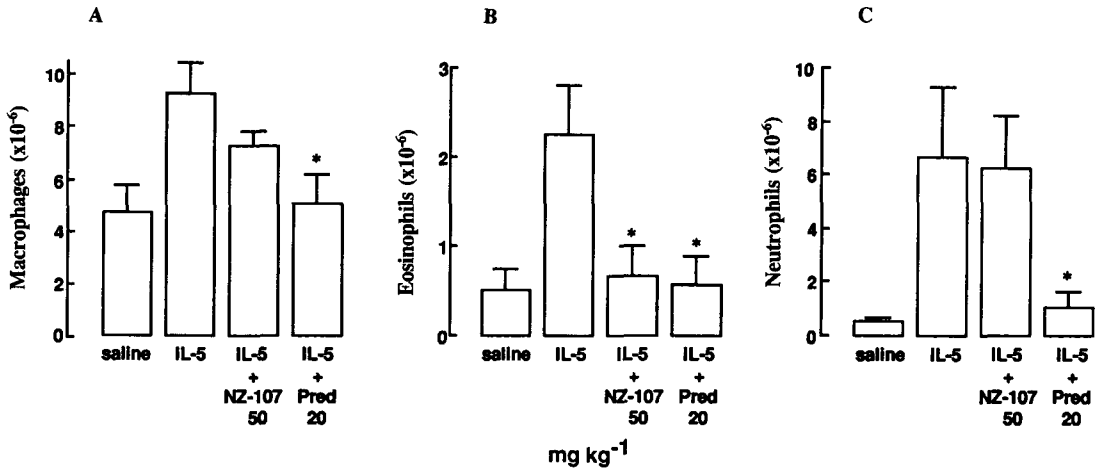

FIG. 1. Effects of NZ-107 and prednisolone on mrIL-5-induced increase in the number of macrophages (A), eosinophils (B) and neutrophils (C) in guinea-pig BALF. NZ-107 and prednisolone were administered intraperitoneally. BALF was obtained 24 h after the intra-tracheal injection of mrIL-5 (15 μg/animal) and saline (mrIL-5 vehicle). Pred = prednisolone, IL-5 = mrIL-5. Results are expressed as the means ± s.e.m. of 6 experiments. $*P < 0.05$, significantly different from mrIL-5 (no drug) response using Dunnett's multiple range test.

Mg^{2+}-free HBSS (pH = 7·3) containing 0·1% BSA and were finally suspended at 10^6 cells mL^{-1} in HBBS containing 0·1% BSA. These cells contained more than 90% eosinophils as identified by May-Gruenwald and Giemsa staining.

Peritoneal neutrophils. Guinea-pig neutrophils were prepared in accordance with the method described by Shimazaki et al (1990). Briefly, 16–18 h after the injection of sterile 2% sodium caseinate suspension (25 mL/animal, i.p.), the peritoneal exudate cells were collected and washed by centrifugation (150 *g*, 10 min, 4°C). After the removal of contaminating red blood cells by the method described above, the collected cells were resuspended at 3×10^6 cells mL^{-1} in HBSS. These cells contained more than 90% neutrophils as identified by May-Gruenwald and Giemsa staining.

These three preparations always contained greater than 95% viable cells as demonstrated by staining with trypan blue.

Measurement of superoxide anion (O_2^-) production

Yamashita's method was used (Yamashita et al 1985). Cells in 1 mL HBSS containing 0·1% BSA were preincubated with cytochrome C (1·24 mg mL^{-1}) and cytochalasin B (5 μg mL^{-1}). After 10 min, cells were stimulated using PAF (10^{-6} M), PMA (2×10^{-7} M) or FMLP (1×10^{-6} M) for 10 min. NZ-107 (10–100 μg mL^{-1}) and SOD (50 μg mL^{-1}) were added 5 min before stimulation by these agonists. The reaction was terminated by transfer to an ice-bath and was followed by centrifugation at 1500 *g* for 10 min at 4°C. An aliquot of the supernatant was then measured spectrophotometrically at 550 nm. The amount of reduced cytochrome C was calculated from the molar extinction coefficient of 21·1 mM^{-1} cm^{-1} (Yamashita et al 1985). Results are expressed as the number of nmol of the SOD-inhibitable reduction of cytochrome C.

Statistical analysis

All data were represented as mean ± s.e.m. and analysed using Dunnett's multiple range test and paired *t*-test.

Results

mrIL-5, PAF- and LTB_4-induced airway inflammation

Intra-tracheal injection of mrIL-5 (15 μg/animal) induced an increase in the number of macrophages, eosinophils and neutrophils in BALF 24 h after the injection (Table 1). NZ-107 (50 mg kg^{-1}, i.p.) selectively reduced mrIL-5-induced accumulation of eosinophils, but not macrophages and neutrophils (Fig. 1). On the other hand, prednisolone (20 mg

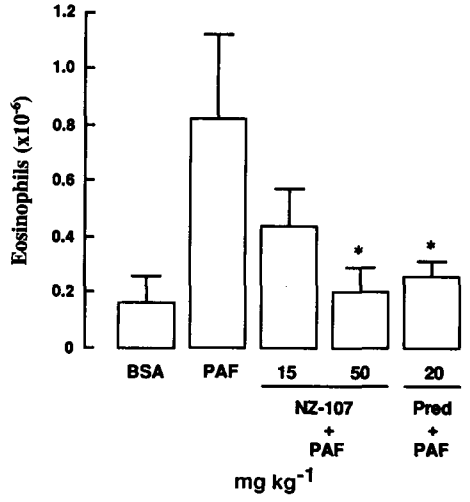

FIG. 2. Effects of NZ-107 and prednisolone on PAF-induced eosinophilia in guinea-pig BALF. NZ-107 and prednisolone were administered intraperitoneally. BALF was obtained 24 h after the inhalation of PAF (0·003%) and BSA (0·1%, PAF vehicle). Pred = prednisolone. Results are expressed as the mean ± s.e.m. of 6–11 experiments. $*P < 0.05$, significantly different from PAF (no drug) response using Dunnett's multiple range test.

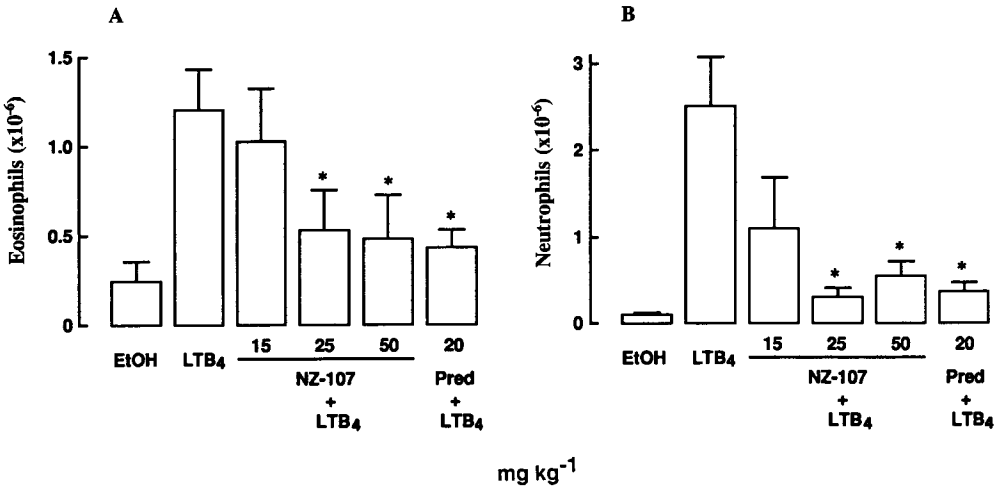

FIG. 3. Effects of NZ-107 and prednisolone on LTB_4-induced bronchial eosinophilia (A) and neutrophilia (B) in guinea-pig BALF. NZ-107 and prednisolone were administered intraperitoneally. BALF was recovered 4 h after the intra-tracheal injection of LTB_4 (10 μg/animal) and EtOH (0·6%, LTB_4 vehicle). Pred = prednisolone. Results are expressed as the mean ± s.e.m. of 6–10 experiments. $*P < 0.05$, significantly different from LTB_4 (no drug) response using Dunnett's multiple range test.

kg^{-1}, i.p.) nonselectively suppressed the accumulation of macrophages, neutrophils and eosinophils induced by mrIL-5 (Fig. 1). PAF aerosol (0·003%) caused an increase in the number of eosinophils in BALF 24 h after the inhalation (Table 1). NZ-107 (50 mg kg^{-1}, i.p.) and prednisolone (20 mg kg^{-1}, i.p.) suppressed PAF aerosol-induced eosinophilia in the BALF (Fig. 2). The intra-tracheal injection of LTB_4 (10 μg/animal) induced an increase in the number of eosinophils and neutrophils in BALF 4 h after the treatment (Table 1).

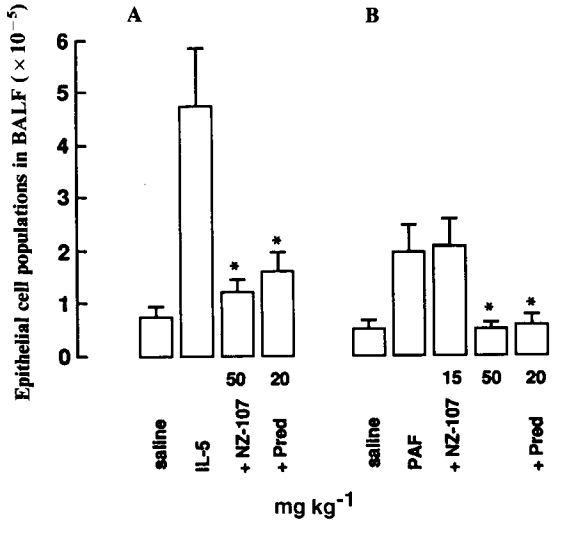

FIG. 4. Effects of NZ-107 and prednisolone on mrIL-5- and PAF-induced increase in the number of pulmonary epithelial cells in guinea-pigs. NZ-107 and prednisolone were administered intraperitoneally. BALF was obtained 24 h after A, the intra-tracheal injection of mrIL-5 (15 μg/animal) and saline (mrIL-5 vehicle) and B, the inhalation of PAF (0·003%) and BSA (0·1%, PAF vehicle). Pred = prednisolone, IL-5 = mrIL-5. Results are expressed as the mean ± s.e.m. of 6–11 experiments. $*P < 0.05$, significantly different from corresponding IL-5 (no drug) or PAF (no drug) response using Dunnett's multiple range test.

NZ-107 at doses of 25 and 50 mg kg^{-1} (i.p.) prevented eosinophilia and neutrophilia induced by LTB_4 (10 μg/animal), but NZ-107 at a dose of 15 mg kg^{-1} had no effect on either (Fig. 3). Prednisolone (20 mg kg^{-1}, i.p.) also reduced both bronchial eosinophilia and neutrophilia by LTB_4 (Fig. 3). Twenty four hours after the intra-tracheal injection of mrIL-5 (15 μg/animal) and the inhalation of PAF (0·003%), the number of epithelial cells increased in BALF, but the intra-tracheal injection of LTB_4 (10 μg/animal) caused no significant increase in the number of airway epithelial cells (Table 1). Both NZ-107 (50 mg kg^{-1}) and prednisolone (20 mg kg^{-1}) reduced the increased number of epithelial cells in BALF caused by mrIL-5 and PAF (Fig. 4).

Effect of NZ-107 on superoxide anion production

NZ-107 (100 μg mL^{-1}) inhibited PAF- and FMLP-induced O_2^- production from alveolar macrophages; however, NZ-107 had no effect on PMA-induced O_2^- generation (Fig. 5A). NZ-107 (30–100 μg mL^{-1}) was also effective on PAF (not PMA)-induced O_2^- production by eosinophils (Fig. 5B). In contrast, NZ-107 was ineffective against O_2^- generation from neutrophils caused by either PAF or PMA (Fig. 5C).

Discussion

Airway inflammation is an important pathogenic symptom in asthmatics. An increased number of eosinophils, in particular, have been shown to be present in the airway of patients with late asthmatic response and airway hyperresponsiveness (Kay 1991). In addition to an important role of this inflammatory leucocyte, much attention has been paid to the role of airway epithelial cells in these pathogeneses, since eosinophils infiltrated into the airway lumen are activated and release oxygen radicals and granule proteins, resulting in the damage of bronchial epithelium (Frigas & Gleich 1986; Barnes 1989). This activation of the eosinophil-

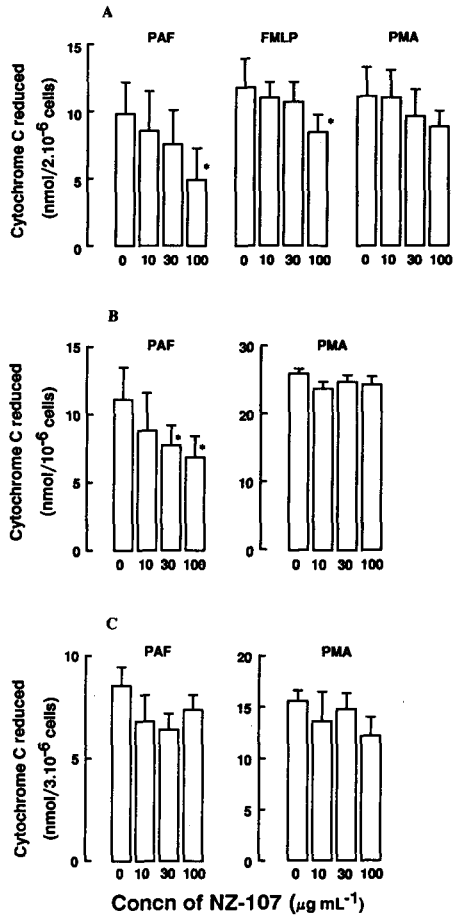

FIG. 5. Effects of NZ-107 on PAF-, FMLP- and PMA-induced generation of superoxide anion from macrophages (A), eosinophils (B) and neutrophils (C) from guinea-pigs. Results are expressed as the mean ± s.e.m. of 4 experiments. *$P < 0.05$, significantly different from the zero-concentration response using paired t-test.

epithelium pathway, therefore, may be closely related to the onset of airway hyper-responsiveness. In the present study, we have focused on the roles of eosinophils and epithelial cells and have evaluated the effect of NZ-107 on them.

Cytokine, PAF and LTB_4 are postulated to play important roles in airway inflammation, especially eosinophilia, followed by late asthmatic response and airway hyper-responsiveness (Barnes 1989; Kay 1991). It has been reported that IL-2 (Renzi et al 1991), GM-CSF and TNFα (Kings et al 1990) induce pulmonary eosinophilia in rat and guinea-pigs. We also have reported that IL-5, a potent eosinophil activator, caused airway eosinophilia in guinea-pigs (Iwama et al 1992a). Anti-mouse IL-5 monoclonal antibody inhibits antigen-induced bronchial eosinophilia in guinea-pigs (Chand et al 1992). mRNA for IL-5 is strongly expressed in mucosal bronchial biopsies from asthma and IL-5 mRNA-positive asthmatics tend to develop more severe symptoms than do negative asthmatics (Hamid et al 1991). Coyle et al (1988) and Richards et al (1989) reported the efficacies of PAF- and LTB_4-antagonists on antigen-induced accumulation of eosinophils in the airway lumen. In addition to antigen-induced airway inflammation, endotoxin-induced bronchial eosinophilia and neutrophilia are reduced by a PAF antagonist in guinea-pigs (Rylander et al 1988). Moreover, the inhalation of PAF produces the selective accumulation of eosinophils (Coyle et al 1988) and the intratracheal instillation of LTB_4 induced those of eosinophils and neutrophils (this study) in the airways of guinea-pigs. These observations suggest an important role for IL-5, PAF and LTB_4 in airway inflammation. Our results demonstrate that NZ-107 prevented both IL-5- and PAF-induced eosinophilia and LTB_4-induced eosinophilia and neutrophilia in guinea-pig airways, while a steroid, prednisolone, nonselectively suppressed the increased numbers of inflammatory leucocytes caused by IL-5 and LTB_4. In this work, mrIL-5 and PAF, but not LTB_4, increased the number of epithelial cells in BALF. Both NZ-107 and prednisolone reduced the accumulation of pulmonary epithelial cells. These results suggests that IL-5 and PAF may increase the eosinophil number and activate them in BALF, resulting in the damage to bronchial epithelium. Accordingly, an explanation for the protective effect of NZ-107 on epithelium damage may involve suppression of the accumulation of inflammatory cells, especially the eosinophil, in the airway. As there is no effect of LTB_4 on bronchial epithelium, LTB_4 may probably have a weaker stimulating effect on eosinophils and neutrophils when compared with IL-5 and PAF. This seems to be the reason why LTB_4 did not show an increase of epithelial cells in BALF.

Oxygen radicals may be released by various inflammatory leucocytes accumulated into the airways resulting in bronchial epithelium damage, and may contribute to the bronchial hyper-responsiveness in asthma (Barnes 1989). In the present study, we have investigated the effects of NZ-107 on the generation of superoxide anion produced by macrophages, eosinophils and neutrophils. NZ-107 reduced PAF- or FMLP-induced O_2^- production from macrophages and eosinophils, whereas it had no effect on PMA-stimulated O_2^- production, suggesting that NZ-107 may act on a part of the outer side of the cell membrane to prevent the cell activation in addition to the inhibitory effect of NZ-107 on airway eosinophilia, since PAF and FMLP may attack their receptors on the surface or on the outer side of the cell membranes, while PMA may directly act on protein kinase C on the internal side of the cell membrane. On the other hand, NZ-107 had no effect on the generation of O_2^- from neutrophils.

In conclusion, we have examined the effects of NZ-107 on mrIL-5-, PAF- and LTB_4-induced airway inflammation and superoxide anion production from inflammatory leucocytes. NZ-107 prevented airway inflammation, especially eosinophilia and the desquamation of epithelial cells, and PAF- and FMLP-induced O_2^- production from macrophages and eosinophils. These results suggest that NZ-107 may be useful for the treatment of the late asthmatic response and airway hyper-responsiveness in which PAF, IL-5 and LTB_4 may play important roles.

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