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Effects of ZCR-2060 on Allergic Airway Inflammation and Cell Activation in Guinea-pigs

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Abstract—The effects of 2-(2-(4-(diphenylmethyl)-1-piperadinyl) ethoxy) benzoic acid malate (ZCR-2060) on allergic airway inflammation and inflammatory cell activation in guinea-pigs were studied. Allergic airway inflammation was induced by inhalation of antigen into actively-sensitized animals and the increase in inflammatory cells into bronchoalveolar lavage fluid (BALF) was measured. Aeroantigen-induced infiltration of inflammatory cells, especially eosinophils and neutrophils, in BALF gradually increased, and reached a peak at 6 or 9 h after the challenge. ZCR-2060 (1 mg kg^{-1} p.o.) clearly inhibited the increase of eosinophil numbers in BALF. Moreover, the effect of ZCR-2060 on inflammatory cell activation in terms of chemotaxis and superoxide generation in-vitro was studied. ZCR-2060 (10^{-6}–10^{-4} M) inhibited the platelet-activating factor (PAF)-induced chemotaxis of eosinophils and neutrophils, but did not inhibit the leukotriene B_4-induced chemotaxis of eosinophils and the formyl-Met-Leu-Phe-induced chemotaxis of neutrophils. PAF-induced superoxide anion generation by eosinophils, neutrophils and alveolar macrophages was inhibited by ZCR-2060 (10^{-6}–10^{-4} M). However, ZCR-2060 did not affect phorbol myristate acetate-induced superoxide anion generation by eosinophils, neutrophils and alveolar macrophages. These results indicate that ZCR-2060 inhibits allergic airway inflammation, and PAF-induced inflammatory cell activation in guinea-pigs. ZCR-2060 may prove useful for the treatment of allergic airway inflammation or allergic disorders, especially inflammatory cell infiltration and activation.

2-(2-(4-(Diphenylmethyl)-1-piperadinyl) ethoxy) benzoic acid malate (ZCR-2060) is a newly synthesized anti-allergic agent. In previous reports (Omata et al 1994; Yoshida et al 1994), ZCR-2060 has shown a selective histamine H_1-receptor antagonistic action without unwanted CNS side-effects in in-vitro and in-vivo studies. In addition, this compound inhibited immediate allergic reactions including passive cutaneous anaphylaxis in sensitized rats and mice, antigen-induced nasal vascular permeability increase in passively- and actively-sensitized rats, and antigen-induced immediate bronchoconstriction in passively-sensitized guinea-pigs. ZCR-2060 also inhibited antigen-induced immediate- and late-phase reactions which are IgE antibody-mediated biphasic skin reactions and aeroantigen-induced immediate and late bronchoconstriction in actively-sensitized guinea-pigs.

In allergic disorders, allergen provocation induces immediate-phase reaction (IPR) and late-phase reaction (LPR) in skin (Charlesworth et al 1992), nose (Naclerio et al 1985) and airways (Hargreave et al 1974; Cartier et al 1982). Although the pathogenesis of allergic disorders is still obscure, LPR following IPR is thought to be an important phenomenon in the subacute or chronic symptoms of severe allergic disorders (Austen & Orange 1975). Histological and cytological observations have shown that LPR is characterized by infiltration and activation of inflammatory cells, including eosinophils, neutrophils, lymphocytes and other inflammatory cells (Hargreave et al 1974; Charlesworth et al 1992). It is thought that these inflammatory cells, which are activated after allergen provocation, cause inflammation in target organs. Glucocorticosteroids do not prevent IPR, but are effective in the management of LPR in allergic disorders (Pepys & Hutchcroft 1975; Cockcroft & Murdock 1987; Charlesworth et al 1991). It has been shown that, in asthma, allergen provocation induces an influx of neutrophils into the bronchial lumen followed by eosinophils (Metzger et al 1986; Kay 1991). It has been reported that eosinophils infiltrated in the airway lumen may play an important role in inflammatory responses in allergic asthma, caused by the release of inflammatory mediators such as platelet-activating factor (PAF), oxygen radicals and cytotoxic granule proteins to mediate airway injury (Frigas & Gleich 1986; Venge et al 1987; Barnes 1989). Moreover, LPR and airway inflammation accompany non-specific airway hyper-reactivity to spasmogens such as histamine and methacholine (Cartier et al 1982; O'Byrne et al 1987).

Recently, LPR and airway inflammation accompanied by hyper-reactivity have been demonstrated in actively-sensitized guinea-pigs with aeroantigens (Goto et al 1993; Nagai et al 1993). Interestingly PAF, which is one of several important inflammatory mediators, has been shown to produce bronchoconstriction, bronchial hyper-reactivity to a range of spasmogens, and infiltration of inflammatory cells in non-sensitized guinea-pigs (Coyle et al 1988; Takizawa et al 1988; Sanjar et al 1989). The aim of the present study was to assess the effect of ZCR-2060 on antigen-induced infiltration of inflammatory cells in bronchoalveolar lavage fluid (BALF), PAF-, leukotriene B_4 (LTB_4)- and formyl-Met-Leu-Phe (FMLP)-induced chemotaxis of inflammatory cells, and PAF- and phorbol myristate acetate (PMA)-induced superoxide anion production by inflammatory cells in guinea-pigs.

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Materials and Methods

Animals

Male Hartley guinea-pigs, 150–300 g, were purchased from Japan SLC Inc. (Japan).

Drugs

ZCR-2060, ketotifen fumarate (ketotifen) and cetirizine dihydrochloride (cetirizine) were synthesized and terfenadine was extracted from Triludane (Shionogi, Japan) in Central Research Laboratory, Zeria Pharmaceutical Co. Ltd. Prednisolone (Wako Chemicals, Japan), (*RS*)-2-methoxy-3-(octadecyl-carbomoyloxy) propyl- 2-(3-thiazolio) ethyl phosphate (CV-3988, Funakoshi, Japan) and superoxide dismutase (SOD, Sigma, USA) were purchased commercially.

For in-vivo studies, these drugs were suspended in 0·5% methyl cellulose solution. For in-vitro study, these drugs were dissolved in dimethylsulphoxide and diluted in 0·15 M phosphate-buffered 0·9% NaCl (saline) (pH 7·4).

Actively-sensitized guinea-pigs

Actively-sensitized guinea-pigs were prepared with ovalbumin exposure as previously described (Yoshida et al 1994). Briefly, male guinea-pigs were sensitized by exposure for 10 min to aerosolized 1% ovalbumin (Grade V, Sigma) once a day on days 1 to 5 and 8 to 12, repeatedly. Animals were placed in a transparent chamber (20 × 20 × 20 cm) connected to a nebulizer (Devilviss type 5410D). The aerosol was generated using a nebulizer connected to a flow of compressed air (6 L min^{-1}). On days 8 to 12, these animals received intraperitoneal injection of mepyramine (10 mg kg^{-1}, Sigma) 1 h before aeroantigen exposure to prevent hypoxic collapse and death. On day 17, actively-sensitized guinea-pigs were challenged by inhalation of 1% ovalbumin for 10 min as described above. Metyrapone (10 mg kg^{-1}, Aldrich, USA), which inhibits 11β-hydroxylase in glucocorticoid biosynthesis, was injected intravenously 24 and 1 h before aeroantigen challenge, and mepyramine was injected intraperitoneally 1 h before aeroantigen challenge. The cellular infiltrate into the bronchoalveolar lumen associated with aeroantigen challenge was assessed by analysis of BALF.

Preparation of BALF

Non-sensitized and actively-sensitized guinea-pigs were used, and BALF was obtained according to the modified method of Suda et al (1992). After saline- or antigen-exposure, the animals were killed and the lungs were isolated. Bronchoalveolar lavage was performed in the right lung. Briefly, saline (8 mL) was injected into the right lung with a 10-mL syringe through a tube connected to the right main bronchus and then aspirated gently. This procedure was repeated three times. BALF was centrifuged at 400 *g* for 5 min. The pellet obtained was immediately resuspended in 3 mL saline, and total cell number in the BALF was counted in a haemocytometer. Different cell counts were undertaken on centrifuged preparations (Cytospin II; Shandon) stained with Wright–Giemsa, counting 500 cells in each preparation. The cells from the BALF preparation were classified as eosinophils, neutrophils, macrophages and others.

Each drug was administered orally 1 h before the aeroantigen challenge.

Eosinophil preparation

This procedure was performed according to the method of Taylor et al (1991). Briefly, male Hartley guinea-pigs were injected intraperitoneally with 0·5 mL horse serum (J R Scientific) twice a week for two weeks. At 48 or 72 h after the last injection, they were killed and peritoneal exudate cells were collected by lavage with 25 mL Hank's balanced salt solution (HBSS, Gibco, USA) without calcium and magnesium, containing 10 mM HEPES buffer (Gibco) and 0·3% bovine serum albumin (BSA, Sigma). The cells were washed twice with HBSS and suspended in 1·070 g mL^{-1} Percoll (Pharmacia, Sweden). The cell suspensions were layered on the top layer of five layer discontinuous Percoll gradients (1·080, 1·085, 1·090, 1·095 and 1·100 g mL^{-1}) and centrifuged at 1400 rev min^{-1} for 30 min. Eosinophils were obtained from the bottom two bands and washed twice with HBSS. Eosinophils of greater than 93% purity and 95% viability were used for the experiments. Viable cells were assessed by the trypan blue exclusion test.

Neutrophil preparation

Neutrophils were obtained according to the method of Suzuki & Furuta (1988). Briefly, male Hartley guinea-pigs received an intraperitoneal injection of 40 mL 1% glycogen (type II from Oyster, Sigma). After 16–24 h, peritoneal exudate cells were obtained as above and washed twice with HBSS containing 0·3% BSA and 10 mM HEPES. Neutrophils were enriched by centrifugation on a discontinuous Percoll gradient as described above. Neutrophils in two bands at density 1·085 and 1·095 g mL^{-1} were collected and washed twice with HBSS. Neutrophils of greater than 95% purity and 95% viability were used for experiments.

Alveolar macrophage preparation

BALF was obtained from normal male guinea-pigs. Alveolar macrophages were separated by the method of Bachelet et al (1986). Briefly, BALF was centrifuged at 400 *g* for 5 min and washed with RPMI 1640 (Gibco). The cells were suspended with RPMI 1640 which contained 10% foetal calf serum (Gibco) and adhered to a 90 mm Petri-dish (Iwaki, Japan) for 2 h at 37°C in a 5% CO_2 incubator. Nonadhering cells were removed and adhered cells were washed with HBSS without phenol red. The mean values of purity and viability of alveolar macrophages were 87·3 and >95%, respectively.

Chemotaxis assay

Chemotaxis assay was performed by a modified Boyden chamber method (Richards & McCullough 1984), using a 96-well microchemotaxis chamber (Neuroprobe). Briefly, purified eosinophils (5×10^5 cells mL^{-1}) and neutrophils ($2{\cdot}5 \times 10^5$ cells mL^{-1}) were suspended in complete HBSS containing 2% BSA and 10 mM HEPES buffer. The cells were preincubated for 30 min at 37°C with each drug before the chemotaxis assay was carried out. PAF (10^{-7} M, Avanti Polar Ltd, USA), LTB_4 (10^{-8} M, Cascade Biochem, UK) or FMLP (10^{-8} M, Sigma) in 30-μL suspension was placed in the lower compartment of the chamber, and 100 μL cell suspensions with drugs were added to the upper compartments. The

Table 1. Incidence of inflammatory cells in BALF from non-sensitized and actively-sensitized guinea-pigs.

Inhalation	n	Number of cells in BALF ($\times 10^5$ cells)			
		Total cells	Eosinophils	Neutrophils	Macrophages
Non-sensitized animals					
Non	15	32·4 ± 2·6	1·9 ± 0·6	0·4 ± 0·2	23·3 ± 2·9
Saline	14	27·7 ± 2·4	2·6 ± 0·7	0·2 ± 0·1	23·2 ± 2·2
Ovalbumin	6	90·8 ± 21·3**	8·5 ± 1·6**	24·8 ± 10·7**	50·9 ± 10·1*
Actively-sensitized animals					
Non	7	49·3 ± 14·6	6·0 ± 3·3	0·4 ± 0·2	40·6 ± 10·9
Saline	6	39·0 ± 5·2	7·0 ± 3·4	0·7 ± 0·3	29·5 ± 2·5
Ovalbumin	8	110·3 ± 4·7**	42·5 ± 3·5*‡	14·0 ± 1·7**	49·8 ± 1·9

Each value indicates the mean ± s.e. * $P < 0.05$, ** $P < 0.01$ compared with saline. ‡ $P < 0.01$ compared with non-sensitized animals.

two compartments were separated by a polycarbonated framed filter (pore size; 8 μm for eosinophils and 3 μm for neutrophils, Neuroprobe). The chamber was incubated for 2 h at 37°C in a 5% CO_2 incubator. Thereafter, the filters were removed, fixed and stained with Diff-Quik (International Reagents Corp., Japan). The cells that had migrated to the lower side of the filter, were counted by light microscopy.

Superoxide anion generation

Superoxide anion was measured by 2-methyl-6-phenyl-3,7-dihydroimidazo (1,2-a)pyrazin-3-one (CLA, Tokyo Kasei, Japan)-dependent luminescense on PMA (Sigma)- and PAF-induced superoxide anion generation by guinea-pig eosinophils, neutrophils and alveolar macrophages, according to the method of Nakano (1990). Briefly, separated eosinophils, neutrophils and alveolar macrophages were suspended in HBSS without phenol red. The cell suspensions (10^5 cells mL^{-1}) were preincubated for 5 min at 37°C with each drug and 0·5 μM CLA, PAF (10^{-7} M) and PMA (10 ng mL^{-1}) were added; incubation at 37°C followed. CLA-dependent chemiluminescence was detected with a luminometer analyser (Packard Picolite, Packard) and was expressed as the peak production of superoxide anion after the reactions.

Statistics

Results are expressed as mean ± s.e. Statistical analysis was performed using an unpaired Student's or Welch's t-test after the F test and paired t-test. $P < 0.05$ and $P < 0.01$ were considered to indicate a significant difference. IC50 values were calculated from linear regression analysis.

Results

The cell population in BALF from actively-sensitized guinea-pigs

The number of infiltrated cells in BALF at 24 h after exposure to antigen is indicated in Table 1. Saline exposure did not affect the population of infiltrated cells in non-sensitized or sensitized animals. In non-sensitized animals,

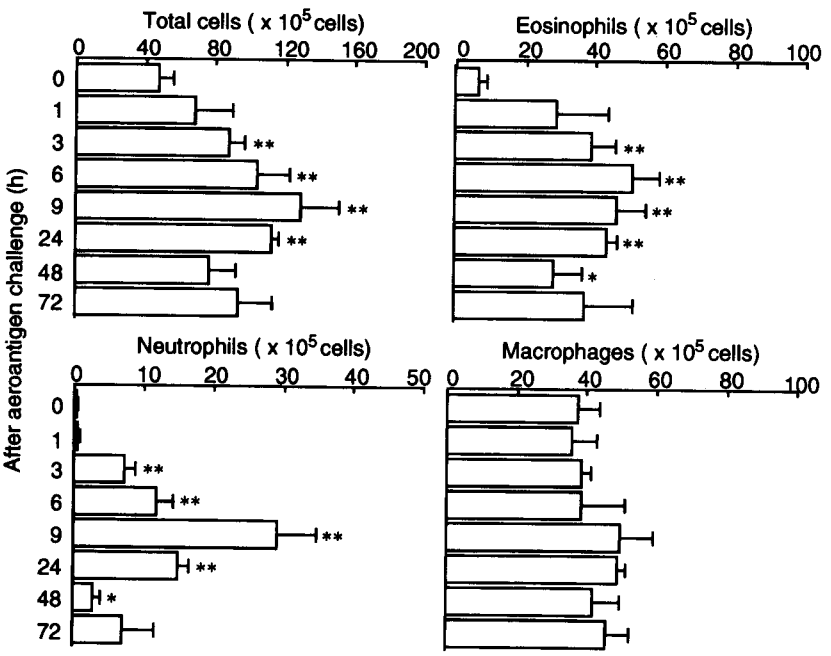

FIG. 1. Time course for the infiltration of inflammatory cells in BALF from actively-sensitized guinea-pigs after the aeroantigen challenge. Each column indicates the mean ± s.e. of 5–14 animals. * $P < 0.05$, ** $P < 0.01$ compared with time zero.

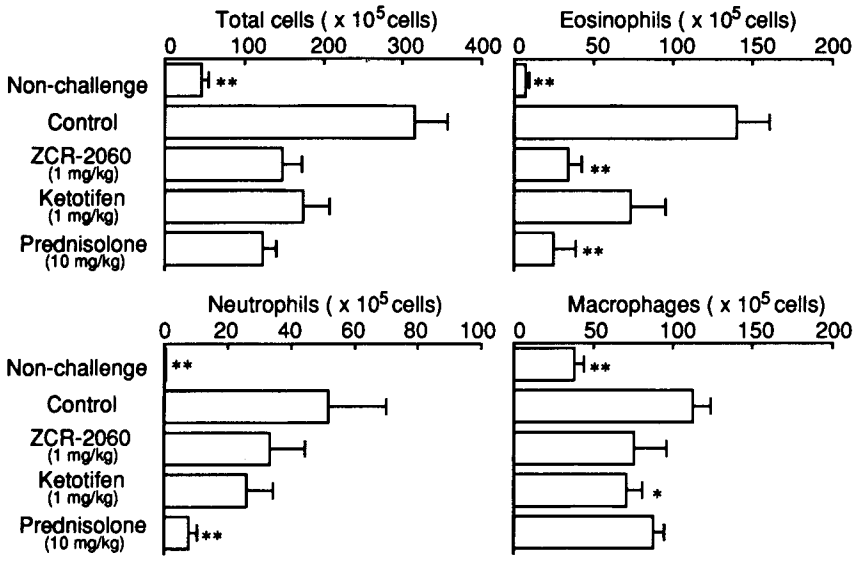

FIG. 2. Effects of ZCR-2060, ketotifen and prednisolone on aeroantigen-induced increase of inflammatory cell infiltration in BALF from actively-sensitized guinea-pigs at 6 h after the aeroantigen challenge. Each drug was given orally 1 h before the aeroantigen challenge. Each column indicates the mean ± s.e. of 4–6 animals. * $P < 0.05$, ** $P < 0.01$ compared with control.

ovalbumin exposure clearly induced an increase in total cells, eosinophils, neutrophils and macrophages in BALF when compared with saline- or with non-exposure experiments. In actively-sensitized animals, aeroantigen challenge caused a significant increase in the number of total cells, eosinophils and neutrophils in BALF when compared with saline- and non-exposure. The time-course for the infiltration of inflammatory cells in BALF was assessed after the aeroantigen challenge in actively-sensitized animals. As shown in Fig. 1, total cell, eosinophil and neutrophil infiltration in BALF gradually and significantly increased after the aeroantigen challenge. The largest increase in the number of total cells, eosinophils and neutrophils were observed at 9, 6 and 9 h after the aeroantigen challenge, respectively. A significant increase of inflammatory cell infiltration in BALF was still seen 24 and 48 h after the aeroantigen challenge; in contrast, the number of macrophages in BALF did not change at any time.

The effect of ZCR-2060 (1 mg kg^{-1}, p.o.) was compared with that of ketotifen (1 mg kg^{-1}, p.o.) and prednisolone

Table 2. Effects of ZCR-2060, ketotifen, terfenadine, cetirizine and CV-3988 on PAF- and LTB_4-induced chemotaxis of guinea-pig eosinophils.

Drugs	Concn (M)	PAF-induced		LTB_4-induced	
		Cells[a]	Inhibition (%)	Cells[a]	Inhibition (%)
Control	—	97 ± 27	—	197 ± 63	—
ZCR-2060	10^{-7}	89 ± 19	8·2	—	—
	10^{-6}	60 ± 16*	38·1	197 ± 63	0
	10^{-5}	47 ± 11	51·5	192 ± 64	2·5
	10^{-4}	27 ± 12*	72·2	182 ± 54	7·6
Ketotifen	10^{-7}	84 ± 22	13·4	—	—
	10^{-6}	85 ± 25	12·4	184 ± 53	6·6
	10^{-5}	50 ± 13*	48·5	202 ± 68	−2·5
	10^{-4}	38 ± 12*	60.8	212 ± 68	−7·6
Terfenadine	10^{-7}	82 ± 22	15·5	—	—
	10^{-6}	71 ± 24	26·8	210 ± 73	−6·6
	10^{-5}	76 ± 26	21·6	215 ± 82	−9·1
Cetirizine	10^{-7}	61 ± 13	37·1	—	—
	10^{-6}	55 ± 23*	49·3	207 ± 76	−5·1
	10^{-5}	49 ± 16*	49·5	195 ± 71	1·0
	10^{-4}	54 ± 19	44·3	192 ± 64	2·5
CV-3988	10^{-7}	82 ± 23	15·5	—	—
	10^{-6}	35 ± 5	63·9	188 ± 64	4·6
	10^{-5}	24 ± 11*	75·3	170 ± 65	13·7

Each value indicates the mean ± s.e. of four experiments for PAF-induced chemotaxis and three experiments for LTB_4-induced chemotaxis. [a] Number of cells in the observed light-microscopy field. * $P < 0.05$ compared with control.

Table 3. Effects of ZCR-2060, ketotifen, terfenadine, cetirizine and CV-3988 on PAF- and FMLP-induced chemotaxis of guinea-pig neutrophils.

Drugs	Concn (M)	PAF-induced		FMLP-induced	
		Cells[a]	Inhibition (%)	Cells[a]	Inhibition (%)
Control		259 ± 58	—	127 ± 11	—
ZCR-2060	10^{-7}	288 ± 104	−11·2		
	10^{-6}	216 ± 58	16·6	117 ± 10	7·9
	10^{-5}	171 ± 57**	34·0	117 ± 10	7·9
	10^{-4}	157 ± 50**	39·4	118 ± 25	7·1
Ketotifen	10^{-7}	240 ± 56	7·3	—	—
	10^{-6}	205 ± 48*	20·8	118 ± 12	7·1
	10^{-5}	176 ± 43*	32·0	127 ± 15	0
	10^{-4}	145 ± 50*	44·0	138 ± 23	−8·7
Terfenadine	10^{-7}	208 ± 34	19·7	—	—
	10^{-6}	225 ± 58	13·1	144 ± 33	−13·4
	10^{-5}	282 ± 116	−8·9	152 ± 48	−19·7
Cetirizine	10^{-7}	259 ± 73	0	—	—
	10^{-6}	211 ± 74	18·5	144 ± 44	−13·4
	10^{-5}	201 ± 65	22·4	124 ± 21	2·4
	10^{-4}	170 ± 72*	34·4	136 ± 30	−7·1
CV-3988	10^{-7}	224 ± 69	13·5	—	—
	10^{-6}	171 ± 48**	34·0	118 ± 20	7·1
	10^{-5}	156 ± 48**	39·8	119 ± 14	6·3

Each value indicates the mean ± s.e. of four experiments for PAF-induced chemotaxis and three experiments for FMLP-induced chemotaxis. [a] Number of cells in the observed light-microscopy field. * $P < 0.05$, ** $P < 0.01$ compared with control.

Table 4. Effects of ZCR-2060, ketotifen, terfenadine, cetirizine, CV-3988 and superoxide dismutase (SOD) on PAF-induced superoxide anion generation by guinea-pig eosinophils, neutrophils and alveolar macrophages.

Drugs	Concn (M)	% of control		
		Eosinophils	Neutrophils	Macrophages
ZCR-2060	10^{-7}	119·0 ± 25·5	98·0 ± 13·1	—
	10^{-6}	62·1 ± 7·7*	62·6 ± 4·0*	90·6 ± 7·9
	10^{-5}	47·3 ± 10·7*	60·2 ± 15·3	77·8 ± 15·5
	10^{-4}	40·2 ± 17·5*	30·7 ± 9·3*	78·4 ± 5·5*
Ketotifen	10^{-5}	62·1 ± 12·4	61·9 ± 17·3	105·5 ± 19·1
	10^{-4}	51·2 ± 19·8	37·7 ± 13·5*	56·1 ± 9·5**
Terfenadine	10^{-6}	55·9 ± 13·1	76·7 ± 7·9	81·4 ± 14·6
	10^{-5}	30·5 ± 6·0*	50·0 ± 6·9	56·7 ± 5·0
Cetirizine	10^{-5}	42·9 ± 10·1*	64·3 ± 14·6	99·4 ± 18·2
	10^{-4}	38·9 ± 8·8*	54·3 ± 13·7*	87·2 ± 20·0
CV-3988	10^{-6}	36·1 ± 14·0*	43·2 ± 4·8*	37·2 ± 6·4*
SOD	0·1 μg mL^{-1}	30·0 ± 3·5**	14·5 ± 5·7*	14·4 ± 2·9*

The chemiluminescent intensity of control in eosinophils, neutrophils and alveolar macrophages were 92·1 ± 26·8, 63·9 ± 20·5 and 58·4 ± 26·8 mV, respectively. Each value indicates the mean ± s.e. of three or four experiments. * $P < 0·05$, ** $P < 0·01$ compared with control.

(10 mg kg^{-1}, p.o.) on the aeroantigen-induced increase in inflammatory cell infiltration in BALF at 6 h after the aeroantigen challenge in actively-sensitized animals. As illustrated in Fig. 2, ZCR-2060 significantly inhibited the aeroantigen-induced increase in the number of eosinophils in BALF. Prednisolone strongly inhibited the aeroantigen-induced increase of eosinophils and neutrophils. Ketotifen slightly inhibited the aeroantigen-induced increase of the number of macrophages.

Effects on the PAF- and LTB_4-induced chemotaxis of eosinophils

As indicated in Table 2, eosinophils exhibited a marked migration in response to PAF and LTB_4. ZCR-2060, ketotifen and CV-3988 also inhibited the PAF-induced chemotaxis of eosinophils. The IC50 values of ZCR-2060, ketotifen and CV-3988 were 6·2, 15·0 and 1·1 μM, respectively. Cetirizine at concentrations of 10^{-6} and 10^{-5} M significantly inhibited the PAF-induced chemotaxis of eosinophils, but dose-dependency was not observed. Furthermore, these drugs did not inhibit the LTB_4-induced chemotaxis of eosinophils. In contrast, terfenadine at a concentration of 10^{-7}–10^{-5} M did not affect the PAF- and LTB_4-induced chemotaxis of eosinophils.

Effects on the PAF- and FMLP-induced chemotaxis of neutrophils

The chemotaxis of neutrophils induced by PAF and FMLP is shown in Table 3. ZCR-2060, ketotifen, cetirizine and CV-3988 also slightly but clearly inhibited the PAF-induced chemotaxis of neutrophils in a concentration-dependent fashion. However, the inhibitory effects of these drugs was not observed in the FMLP-induced chemotaxis of neutrophils. On the other hand, terfenadine at a concentration of

Table 5. Effects of ZCR-2060, ketotifen, terfenadine, cetirizine, CV-3988 and superoxide dismutase (SOD) on PMA-induced superoxide anion generation by guinea-pig eosinophils, neutrophils and alveolar macrophages.

Drugs	Concn (M)	% of control		
		Eosinophils	Neutrophils	Macrophages
ZCR-2060	10^{-6}	106·7 ± 11·6	114·0 ± 3·1	105·6 ± 9·1
	10^{-5}	108·5 ± 5·9	110·5 ± 4·8	97·7 ± 5·8
	10^{-4}	100·6 ± 8·9	131·7 ± 3·8	99·2 ± 7·0
Ketotifen	10^{-5}	92·4 ± 11·0	102·4 ± 5·2	105·9 ± 10·0
	10^{-4}	81·6 ± 12·3	93·2 ± 6·5	85·8 ± 5·1
Terfenadine	10^{-6}	88·1 ± 13·6	96·8 ± 7·9	86·6 ± 8·9
	10^{-4}	87·7 ± 8·7	75·2 ± 10·1	84·0 ± 14·2
Cetirizine	10^{-5}	82·0 ± 15·8	98·5 ± 11·2	79·1 ± 4·6
	10^{-4}	77·6 ± 17·8	87·0 ± 11·4	84·0 ± 9·2
CV-3988	10^{-6}	107·0 ± 3·6	74·4 ± 10·0	59·5 ± 13·0
SOD	0·1 μg mL^{-1}	38·9 ± 4·3*	24·4 ± 3·9**	32·9 ± 8·1

The chemiluminescent intensity of control in eosinophils, neutrophils and alveolar macrophages were 275·5 ± 34·0, 196·0 ± 7·3 and 173·5 ± 25·2 mV, respectively. Each value indicates the mean ± s.e. of three or four experiments. * $P < 0·05$, ** $P < 0·01$ compared with control.

10^{-7}–10^{-5} M did not affect the PAF- and FMLP-induced chemotaxis of neutrophils.

Effects on PAF- and PMA-induced superoxide anion generation

The efficacy of ZCR-2060, reference drugs and superoxide dismutase (SOD) was studied on PAF- and PMA-induced superoxide anion generation by eosinophils, neutrophils and alveolar macrophages. As shown in Table 4, ZCR-2060 and cetirizine significantly inhibited PAF-induced superoxide anion generation by eosinophils and neutrophils, although both drugs slightly inhibited PAF-induced superoxide anion generation by alveolar macrophages. Ketotifen, terfenadine and CV-3988 also inhibited PAF-induced superoxide anion generation by each type of cell. As shown in Table 5, PMA-induced superoxide anion generation in all cells was not inhibited by ZCR-2060. Other antihistamines also did not affect PMA-induced superoxide anion generation. In contrast, SOD at a concentration of 0·1 $\mu g\,mL^{-1}$ strongly inhibited PAF- and PMA-induced superoxide anion generation.

Discussion

In the present study, we have examined the effects of ZCR-2060 on the aeroantigen-induced activation of and increase in inflammatory cells in the BALF of actively-sensitized guinea-pigs. The function of the inflammatory cells was examined by means of PAF-, LTB_4- or FMLP-induced chemotaxis of eosinophils and neutrophils, and superoxide anion generation by eosinophils, neutrophils and alveolar macrophages.

We investigated the aeroantigen-induced increase of inflammatory cell infiltration in the BALF of actively-sensitized guinea-pigs and compared it with that of non-sensitized guinea-pigs. In non-sensitized guinea-pigs, aeroantigen single exposure induced the increase of leucocyte infiltration in BALF. These findings indicate that ovalbumin, a heterologous protein, mediated non-immunological airway inflammation in non-sensitized guinea-pigs. The magnitude of aeroantigen-induced infiltration of inflammatory cells is weak in saline-treated animals, compared with the actively-sensitized animals. These results suggest that the antigen-induced infiltration of inflammatory cells in actively-sensitized guinea-pigs might consist of two components, stimulation by the antigen itself and an allergic mechanism. The time-course of the aeroantigen-induced increase in inflammatory cell infiltration in the BALF was therefore also examined. Total leucocytes, eosinophils and neutrophils in BALF increased gradually and reached a peak at 9, 6 and 9 h after the aeroantigen challenge, respectively. Our model shows different kinetics of eosinophil and neutrophil infiltration from previous reports. Hutson et al (1988) reported that eosinophil in BALF was not significantly elevated above baseline until 17 h after the antigen challenge, and neutrophil numbers increased rapidly, reaching a peak at 17 h. Terashi et al (1988) reported that eosinophil and neutrophil reached maximum at 6 and 3 h after the antigen challenge, respectively. These differences would be based on a different sensitizing schedule. In the present model, a significant increase of eosinophil was observed. ZCR-2060 inhibited the aeroantigen-induced increase of eosinophil infiltration in BALF to a greater degree than it inhibited other leucocyte infiltration. Ketotifen slightly inhibited the increase of leucocyte infiltration in BALF. Prednisolone strongly inhibited the increase of infiltration by both eosinophils and neutrophils in BALF. In previous reports (Omata et al 1994; Yoshida et al 1994), we have pointed out a different efficacy of ZCR-2060 from other agents. Whereas ZCR-2060 inhibited some experimental immediate allergic models at almost the same potency as that of ketotifen, ZCR-2060 strongly inhibited aeroantigen-induced biphasic airway obstruction in actively-sensitized guinea-pigs, unlike ketotifen. Simultaneously, prednisolone inhibited late-phase response, but not immediate-phase response. Considering the present results combined with these previous results, ZCR-2060 would appear to have more beneficial efficacy for bronchial asthma than either ketotifen or prednisolone. We reported previously (Goto et al 1993; Nagai et al 1993) that ketotifen, when administered repeatedly and at high doses, inhibited the antigen-induced airway hyper-reactivity and the increase in inflammatory cell infiltration in the BALF of actively-sensitized guinea-pigs. It seems likely, therefore, that repeated administration of high doses of ketotifen would also inhibit airway inflammation in this model. The inhibitory mechanism of glucocorticosteroids on airway inflammation is still not clearly understood. It is believed, however, that the inhibitory action of these compounds on the inflammatory cell infiltration would make them highly effective in the treatment of severe allergic disorders. Indeed, it has been demonstrated that glucocorticosteroids inhibit inflammatory cell infiltration into the target organs of allergic disorders and decrease the allergic symptoms (Charlesworth et al 1991). These findings suggest that inflammatory cell infiltration in target organs plays an important role in asthma and allergic disorders.

We compared the effects of ZCR-2060 on inflammatory cell activation with those of newer antihistamines. Among many allergic mediators, PAF is one of the most important mediators in terms of airway inflammation and airway hyper-reactivity. PAF induces the airway hyper-reactivity and the increase of eosinophil and neutrophil infiltration into the BALF of non-sensitized guinea-pigs (Coyle et al 1988; Takizawa et al 1988; Sanjar et al 1989). Cetirizine, one of the newer histamine H_1-receptor antagonists, has been reported to be effective against the increase of tissue eosinophil infiltration induced by antigen and PAF (Fadel et al 1987; Charlesworth et al 1992). Further, it has been reported that cetirizine (De Vos et al 1989) and ketotifen (Nabe et al 1991) prevent the human eosinophil chemotaxis elicited by PAF in-vitro. Fadel et al (1987) suggested that the inhibitory effect of cetirizine on eosinophil chemotaxis was not dependent on histamine H_1-receptor antagonistic action. In the present study, ZCR-2060 as well as ketotifen and cetirizine selectively inhibited PAF-induced chemotaxis of guinea-pig eosinophils and neutrophils, but did not inhibit the LTB_4-induced eosinophil chemotaxis and FMLP-induced neutrophil chemotaxis. In contrast, terfenadine did not affect the chemotaxis of guinea-pig eosinophils or neutrophils. Moreover, ZCR-2060 and other antihistamines have been shown to inhibit PAF-induced superoxide anion generation by eosinophils, neutrophils and alveolar

macrophages in guinea-pigs, but did not inhibit PMA-induced superoxide anion generation by each type of inflammatory cell. In addition, CV-3988, a specific PAF antagonist (Takizawa et al 1988), selectively inhibited PAF-induced chemotaxis and superoxide anion generation. In our preliminary studies, ZCR-2060 did not have PAF-receptor antagonistic action (data not shown). The above findings, however, suggest that ZCR-2060 selectively inhibits PAF-induced inflammatory cell migration and activation in-vitro.

In conclusion, ZCR-2060 inhibited certain features of aeroantigen-induced airway inflammation, especially infiltration of eosinophils, in actively-sensitized guinea-pigs, PAF-induced eosinophil and neutrophil chemotaxis, and PAF-induced superoxide anion generation by eosinophils, neutrophils and alveolar macrophages in guinea-pigs. These results suggest that ZCR-2060 may be useful for the treatment of allergic disorders in which inflammatory cell infiltration and activation play important roles.

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