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Suplatast tosilate, a new type of antiallergic agent, prevents the expression of airway hyperresponsiveness in guinea pigs

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Abstract

Suplatast tosilate (suplatast) is an antiallergic agent capable of down-regulating the functions of $CD4^+$ T cells. We now investigated the effects of suplatast on the antigen-induced airway hyperresponsiveness and the underlying allergic inflammatory response in sensitized guinea pigs. Animals that had been immunized twice by ovalbumin inhalation on day 0 and day 7 developed an increased airway responsiveness against inhaled acetylcholine 24 h after the ovalbumin challenge on day 14. Suplatast (10 and 100 mg/kg per day) and ketotifen (10 mg/kg per day) given orally from day 0 to day 14 effectively inhibited the expression of airway hyperresponsiveness. They also inhibited the infiltration of eosinophils and macrophages into broncho-bronchiolar walls and lumen. Interestingly, suplatast, but not ketotifen, inhibited the infiltration of lymphocytes including $CD4^+$ T cells. Collectively, these results strongly suggest that suplatast prevents the expression of airway hyperresponsiveness due to the ability to suppress the infiltration of inflammatory cells into lung tissues.

Keywords: Suplatast tosilate; Airway inflammation; Airway hyperresponsiveness; Eosinophil; Lymphocyte; T cell

1. Introduction

An allergic reaction is characterized by a series of events that are initiated by immunoglobulin (Ig) E-mediated activation of mast cells/basophils to release chemical mediators. The IgE-mediated early response is followed by an influx of inflammatory cells, including eosinophils and mononuclear cells, into the site of the response. Activated inflammatory cells have been shown to release a wide range of active mediators that evoke a delayed and sustained local inflammation (Capron, 1992). Allergic inflammation is of great clinical importance in that it accounts for the morbidity and severity of chronic allergic diseases including bronchial asthma, allergic rhinitis, and atopic dermatitis (Robinson et al., 1993; Charlesworth et al., 1993). Particularly in asthma, chronic airway inflammation renders asthmatic patients more sensitive to specific as well as non-specific stimulation (Adelroth et al., 1986; Robinson et al., 1993). Recently, it has been reported that cyclosporin A effectively suppresses the allergic inflammatory response in both human and animal models (Fukuda et al., 1991; Elwood et al., 1992), supporting the recent understanding of the pathogenesis of allergic diseases in that type-2 $CD4^+$ T (Th2) cells producing interleukin-4 and interleukin-5 play a causative role in allergic inflammation. Suplatast tosilate (suplatast) is a new type of antiallergic agent, and has been shown to suppress: (1) IgE synthesis in both mice and humans without having a direct effect on B cells (Matsuura et al., 1992; Yanagihara et al., 1993), (2) the synthesis of interleukin-4 and interleukin-5 in both human and murine Th2 (like) cells (Yamaya et al., 1995; Yanagihara et al., 1993), (3) the expression of local eosinophilia that is regulated by Th2 cells (Yamaya et al., 1995), and (4) the induction of mast cells from mouse spleen cells (Konno et al., 1994). A more important finding, suplatast was proved to be effective in the treatment of

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patients with atopic diseases. In the present study, we further investigated the effects of suplatast on the expression of airway hyperresponsiveness in sensitized guinea pigs. The results obtained revealed that suplatast suppresses antigen-induced airway hyperresponsiveness, at least in part, by inhibiting the accumulation of inflammatory cells in lung tissues.

2. Materials and methods

2.1. Animals

Male Hartley guinea pigs weighing 300–450 g were purchased from Charles River Japan (Kanagawa, Japan). They were maintained at a temperature of 22 ± 2°C and humidity of 55 ± 15% until used in the experiments.

2.2. Drugs

Suplatast tosilate (suplatast, (±)-[2-[4-(3-ethoxy-2-hydroxypropoxy) phenylcarbamoyl] ethyl]dimethylsulfonium *p*-toluenesulfonate) was synthesized by Taiho Pharmaceutical (Tokyo, Japan). Ketotifen was purchased from Sigma (St. Louis, MO, USA). Suplatast and ketotifen were dissolved in distilled water for oral administration.

2.3. Immunization and antigen challenge

Guinea pigs were immunized and challenged by exposure to aerosolized ovalbumin (Seikagaku, Tokyo, Japan) according to the method of Arimura et al. (1994). Briefly, animals were made to inhale aerosolized 1% ovalbumin in saline for 10 min on days 0 and 7. The aerosol was generated by an ultrasonic nebulizer (NE-U12, Omron, Tokyo, Japan) having an airflow of 17 l/min. Seven days after the second immunization (day 14), the guinea pigs were challenged with aerosolized 1% ovalbumin for 10 min. The animals were injected intraperitoneally with 10 mg/kg of pyrilamine maleate (Sigma) 30 min prior to antigen challenge.

2.4. Measurement of antibodies

The anti-ovalbumin IgG and IgE titer was measured by homologous passive cutaneous anaphylaxis. Briefly, 0.1 ml of a twofold dilution of antiserum was injected intradermally, and passive cutaneous anaphylaxis was elicited 4 h and 8 days after skin sensitization for the determination of IgG and IgE antibodies, respectively, by the intravenous injection of 1 ml physiological saline containing 1.0 mg ovalbumin and 2.0 mg Evans blue. The IgG and IgE titer was expressed as the maximum dilution displaying a positive blueing spot (5 mm diameter).

2.5. Measurement of airway responsiveness to acetylcholine

Airway responsiveness was determined by measuring respiratory resistance in response to acetylcholine (Sigma) 4 h before and 24 h after an antigen challenge. Respiratory resistance was measured automatically using an Animalasto (TMC-2100, Chest-MI, Japan), according to the method of Iijima et al. (1987). In brief, the animals were placed inside a body plethysmograph, and 30-Hz sine wave oscillation was applied to the body surface. The flow rate through the mask and box pressure were measured by a differential pressure transducer and respiratory resistance was calculated by analog computer based on the 30-Hz components of the mask flow and box pressure. The guinea pigs were forced to inhale an aerosol of physiological saline for 60 s to determine the baseline value for respiratory resistance. Subsequently, they were made to inhale acetylcholine for 60 s per concentration (0.078–5.0 mg/ml) with the concentration doubled at 2-min intervals. Respiratory resistance was monitored for 60 s at each concentration of acetylcholine, and the peak respiratory resistance was plotted against the acetylcholine concentration. The concentration of acetylcholine that produced a twofold increase in respiratory resistance as compared with the baseline respiratory resistance was determined from the dose-response curve for acetylcholine and was expressed as PC200. ΔlogPC200 was calculated from the following formula: log ((PC200 of acetylcholine before the antigen challenge)/(PC200 of acetylcholine after the antigen challenge)).

2.6. Assessment of cellular infiltration

2.6.1. Airway lumen

The animals were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg) 24 h after antigen challenge. The trachea was cannulated with a polyethylene tube through which the airways were lavaged 3 times with 10 ml of physiologic saline. The bronchoalveolar lavage fluid was centrifuged at 134 × *g* for 5 min, and the cells were resuspended in 5 ml of physiologic saline. Total cell counts were determined with a hemocytometer. Cytospin preparations were stained with Wright-Giemsa, and more than 300 cells were counted to determine the cell differential.

2.6.2. Lung tissue

The lungs were removed from anesthetized guinea pigs 24 h after the antigenic challenge, were fixed in phosphate-buffered 10% formalin solution and embedded in paraffin. Sections of 3-μm thickness were stained with Luna. For each section, three bronchioles and their bronchiolar branches were selected randomly, and eosinophils, stained red, that were located in a cuff 50 μm outside the

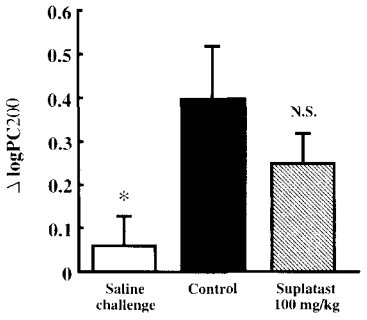


Fig. 1. Effects of single treatment with suplatast on the airway hyperresponsiveness in guinea pigs. Groups of 7–8 animals were immunized twice by ovalbumin inhalation on day 0 and day 7. They were then challenged with saline or inhaled ovalbumin. Suplatast or ketotifen was given orally 1 h prior to ovalbumin challenge on day 14. Respiratory resistance to increasing concentrations of inhaled acetylcholine was measured 4 h before and 24 h after ovalbumin challenge, and PC200 was calculated based on the dose-response curve for acetylcholine. The changes in PC200 following antigen challenge were expressed as ΔlogPC200. Each column and vertical bar represents the mean ± S.E. * $P < 0.05$, as compared with the control.

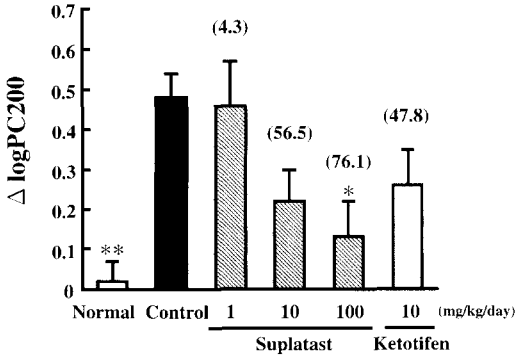


Fig. 2. Effects of chronic treatment with suplatast or ketotifen on the airway hyperresponsiveness in guinea pigs. Groups of 7–8 animals were immunized and challenged as described in the legend to Fig. 1. Agents were given orally from day 0 to day 14. Each column and vertical bar represents the mean ± S.E. for respiratory resistance. Values in parentheses indicate % inhibition. * $P < 0.05$, ** $P < 0.01$, as compared with the control.

bronchial smooth muscle, muscle layer and basement membrane were counted with a video micrometer VM-30 (Olympus, Tokyo, Japan) in a blind fashion. Further, the lungs were fixed in a phosphate-buffered 4% paraformaldehyde solution and frozen in O.C.T. compound (Miles, Elkhart, USA). Cryostat sections of 6-μm thickness were treated with normal rabbit serum and incubated with mouse anti-guinea pig $CD4^+$ T cell monoclonal antibody (CT7, Serotec, Oxford, UK) (Steerenberg et al., 1991), followed by biotinylated rabbit anti-mouse IgG antibody. The sections were then incubated with streptavidin conjugated with alkaline phosphatase, followed by reaction with fast red substrate in veronyl acetate buffer (Histofine SAB-AP

Table 1
Effects of suplatast and ketotifen on the production of ovalbumin-specific IgG antibody in guinea pigs

Drugs	Dose (mg/kg per day)	IgG titer
Control		9.1 ± 0.3
Suplatast	10	9.1 ± 0.4
	100	8.8 ± 0.1
Ketotifen	10	9.2 ± 0.2

Guinea pigs were immunized on day 0 and day 7 by ovalbumin inhalation. The ovalbumin-specific IgG and IgE titer in serum obtained 7 days after the second inhalation was measured by homologous passive cutaneous anaphylaxis and the results were expressed as two to the Nth power. Note that the anti-ovalbumin IgE titer in control animals was less than 2^1. Each value represents the mean ± S.E. for 8 animals.

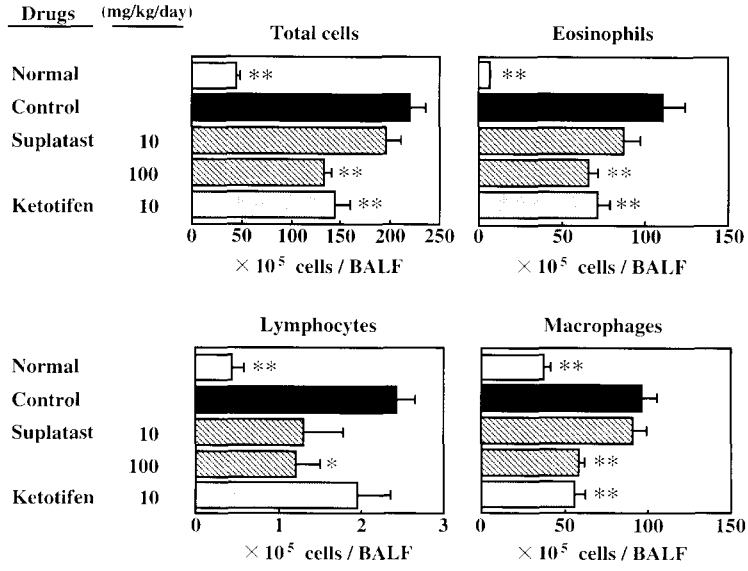


Fig. 3. Effects of suplatast and ketotifen on cellular infiltration to the airway lumen. Groups of 8 guinea pigs were immunized and challenged as described in the legend to Fig. 1. Cell differential counts in bronchoalveolar lavage fluid collected 24 h after antigen challenge were determined. Each column and horizontal bar represents the mean ± S.E. * $P < 0.05$, ** $P < 0.01$, as compared with the control.

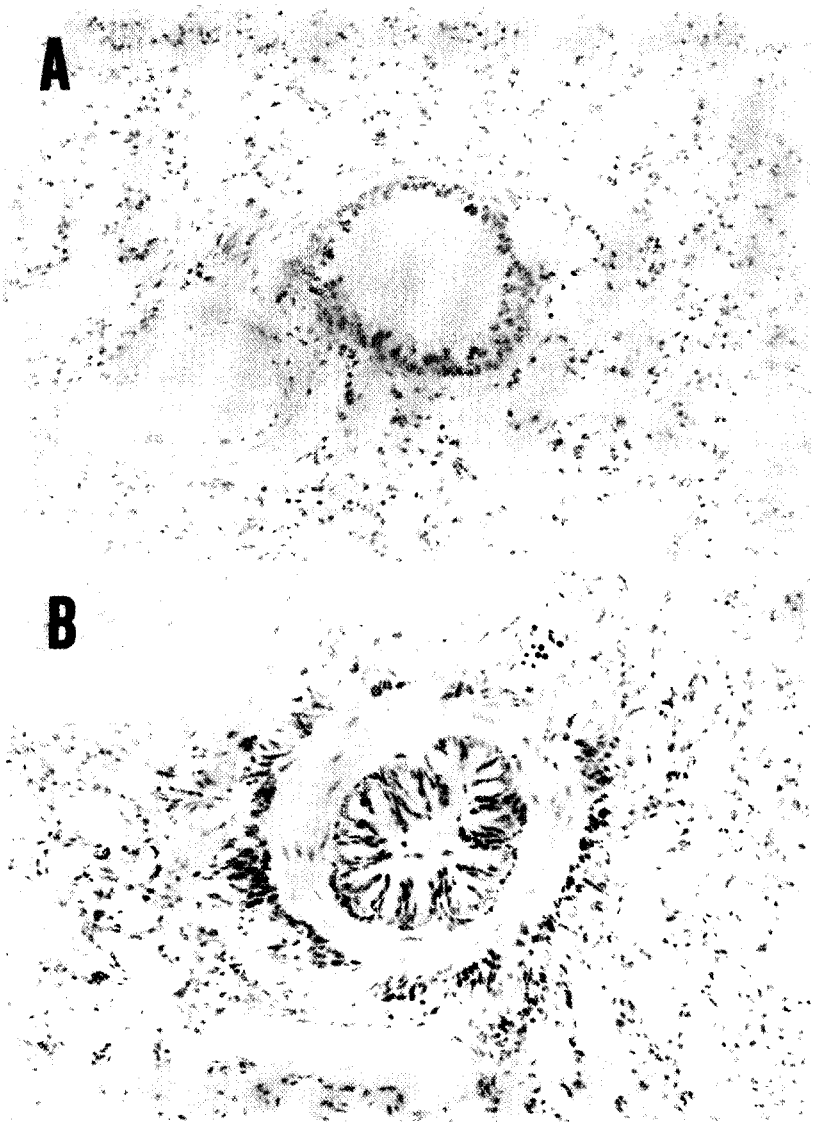


Fig. 4. Antigen-induced accumulation of eosinophils in lung tissue. The lungs were removed from normal or immunized animals 24 h after ovalbumin challenge. (A) Normal group; (B) immunized group (Luna stain, ×200).

kit, Nichirei, Tokyo, Japan). The immunostained cells were counted as described for the eosinophils.

2.7. Data analysis

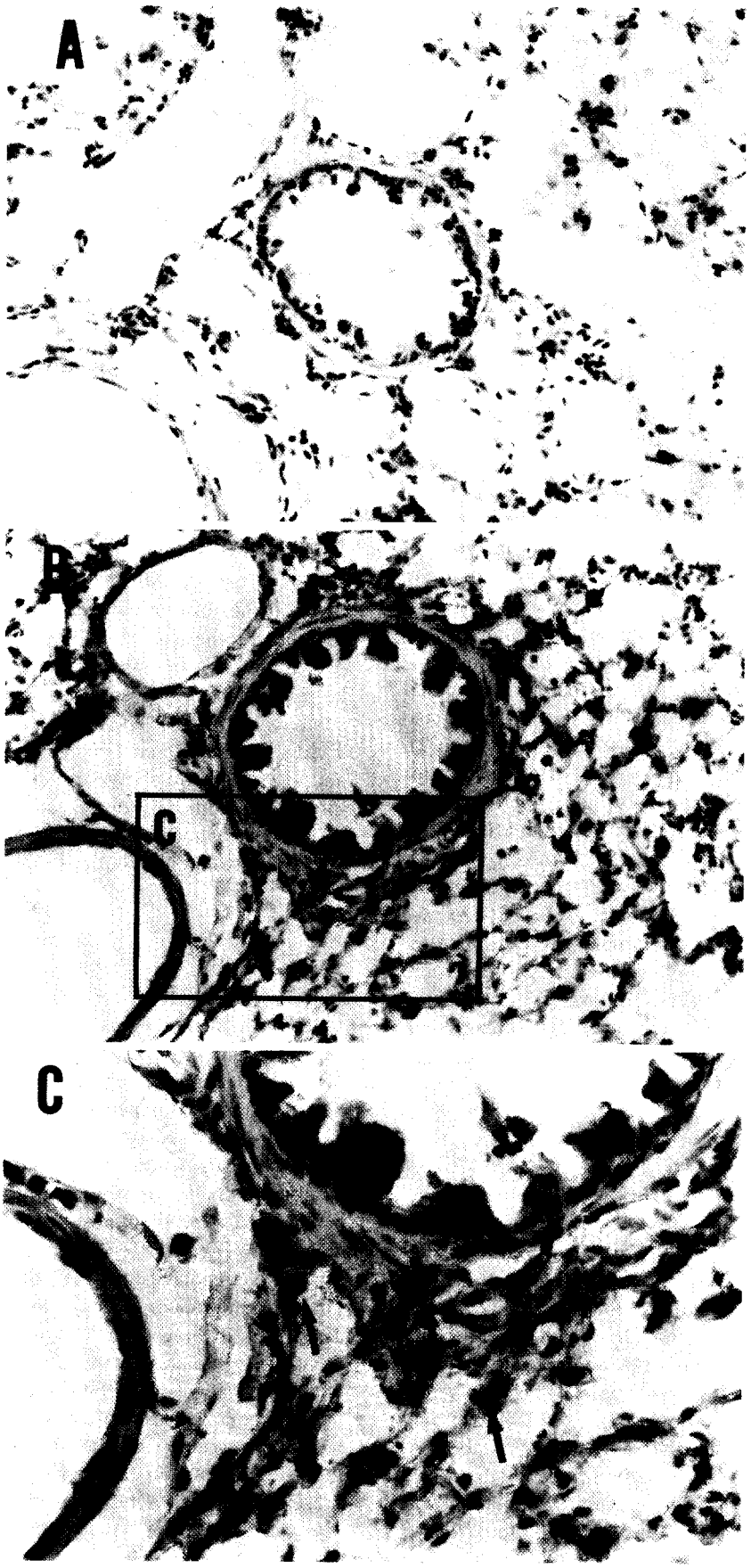
The data were expressed as the means ± S.E. The statistical significance of the data was evaluated by means of Dunnett's multiple range test.

3. Results

3.1. Effects of suplatast on airway hyperresponsiveness

In the first series of experiments, we examined whether suplatast and ketotifen inhibited the expression of airway hyperresponsiveness in guinea pigs. To this end, animals immunized by repetitive ovalbumin inhalation were tested

Fig. 5. Antigen-induced accumulation of $CD4^+$ T cells in lung tissue. The lungs were removed from normal animals or immunized animals 24 h after ovalbumin challenge. The lung tissue sections were immunolocalized with monoclonal antibody for $CD4^+$ T cells. (A) Normal group (×200); (B) immunized group (×200); (C) immunized group (×400). Arrow: $CD4^+$ T cells.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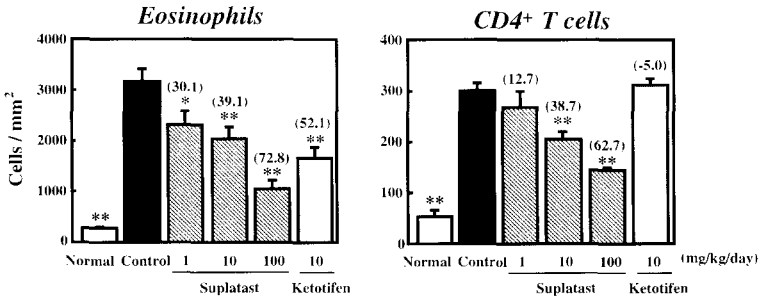

Fig. 6. Effects of suplatast and ketotifen on the accumulation of eosinophils and CD4$^+$ T cells in bronchioles of guinea pigs. The lung tissues obtained 24 h after antigen challenge from immunized guinea pigs were stained with Luna for eosinophils or were immunostained for CD4$^+$ T cells. Suplatast or ketotifen was given orally from day 0 to day 14. Eosinophils and CD4$^+$ T cells were counted blind on three bronchioles that were selected at random. Each column and vertical bar represents the mean ± S.E. (eosinophils, $n = 8$; CD4$^+$ T cells, $n = 5$). Values in parentheses indicate % inhibition. * $P < 0.05$, ** $P < 0.01$, as compared with the control.

for responsiveness against acetylcholine before and after ovalbumin challenge. Ovalbumin challenge of immunized control animals resulted in a marked increase in ΔlogPC200 even if they were treated with anti-histamine 30 min prior to antigen challenge, while no increase in ΔlogPC200 was observed when immunized animals were challenged with saline inhalation (Fig. 1). The antigen-induced airway hyperresponsiveness was hardly suppressed by oral administration of suplatast (100 mg/kg) or ketotifen (100 mg/kg) 1 h prior to antigen challenge (Fig. 2). In contrast, hyperresponsiveness was effectively suppressed by suplatast (1, 10 and 100 mg/kg per day) in a dose-dependent manner as well as by ketotifen (10 mg/kg per day), when they were given orally from day 0 to day 14. Given that suplatast selectively suppresses IgE antibody synthesis in mice (Yanagihara et al., 1993), we measured serum levels of ovalbumin-specific antibodies. As shown in Table 1, ovalbumin inhalation resulted in the production of anti-ovalbumin IgG antibody, while inducing no anti-ovalbumin IgE antibody production. Suplatast did not suppress the production of anti-ovalbumin IgG antibody.

3.2. Effects of suplatast and ketotifen on cellular infiltration into the airway lumen

There was a marked increase (4.9-fold) in the total number of cells in the bronchoalveolar lavage fluid collected from immunized control guinea pigs 24 h after ovalbumin challenge, when compared with the bronchoalveolar lavage fluid obtained from normal animals (Fig. 3). This was largely due to an increase in the number of eosinophils (18.5-fold), lymphocytes (5.5-fold), and macrophages (2.5-fold). The increase in the numbers of eosinophils and macrophages was significantly suppressed by suplatast or ketotifen. Lymphocyte accumulation was suppressed by suplatast even at the dose of 10 mg/kg per day, although this dose of ketotifen was ineffective.

3.3. Histological studies on the accumulation of eosinophils and CD4$^+$ T cells in airway wall

We further examined the effects of suplatast and ketotifen on the infiltration of eosinophils and CD4$^+$ T cells into lung tissues 24 h after ovalbumin challenge. There was a marked accumulation of eosinophils in the bronchiolar wall and submucosa (Fig. 4) and of CD4$^+$ T cells in submucosa (Fig. 5). The numbers of eosinophils and CD4$^+$ T cells located in a cuff outside these tissues were counted, and the results are shown in Fig. 6. Suplatast inhibited the increase in both eosinophils and CD4$^+$ T cells in a dose-dependent manner, whereas ketotifen suppressed the increase in eosinophils, but not that in CD4$^-$ T cells.

4. Discussion

The present study was designed to examine the effect of suplatast on the development of airway hyperresponsiveness in an animal model. It has been reported that guinea-pigs develop an augmented airway responsiveness to non-specific stimulation when they receive repetitive provocation by inhaled antigen (Arimura et al., 1994). Indeed, ovalbumin challenge of guinea-pigs immunized twice with aerosolized ovalbumin resulted in an augmented responsiveness to inhaled acetylcholine, which was accompanied by a marked increase in the number of eosinophils, lymphocytes and macrophages in bronchoalveolar lavage fluid as well as in lung tissues. It was found that single treatment with suplatast (100 mg/kg) 1 h prior to antigen challenge failed to inhibit the development of airway hyperresponsiveness. Suplatast potentially has the ability to inhibit the antigen-induced acute response by inhibiting the release of chemical mediators from mast cells. In the present experiments, however, antihistamine treatment was necessary to prevent acute death of the animals, suggesting that higher doses of suplatast may be required to inhibit

the development of airway hyperresponsiveness. On the other hand, chronic treatment with suplatast (10 and 100 mg/kg per day) significantly inhibited the airway hyperresponsiveness induced by antigen challenge. This suggests the possibility that suplatast prevents immunization of animals from producing specific IgE antibody, since suplatast has been shown to selectively suppress the production of IgE antibody in mice without affecting that of IgM and IgG antibodies (Matsuura et al., 1992). However, guinea pigs immunized with aerosolized antigen produced high titers of specific IgG antibody but little specific IgE antibody, and suplatast did not suppress specific IgG production. These findings suggest that specific IgG antibody, not IgE antibody, mediates the release of chemical mediators by antigen-induced mast cell activation in animals immunized under the present conditions. Therefore, the inhibitory effect of suplatast on IgE production is not linked to the suppression of airway hyperresponsiveness.

The important aspect is the observation that suplatast treatment significantly suppressed the accumulation of eosinophils in airway lumens and lung tissue. As with suplatast, chronic treatment with ketotifen also inhibited the airway hyperresponsiveness and accompanying eosinophil accumulation. Although there is no direct evidence that eosinophils elicit the airway hyperresponsiveness in this model, eosinophils that migrated to the site of inflammation have been shown to play a causative role in tissue damage by releasing cytotoxic granules (Capron, 1992). Ketotifen has been reported to be capable of inhibiting the activities of platelet activating factor (Mazzoni et al., 1985) and leukotrienes (Fink et al., 1986), both of which are potent inducers of eosinophil accumulation. Suplatast effects on the eosinophil migration induced by these factors remain to be elucidated. However, Yamaya et al. (1995) found that suplatast prevents the murine peritoneal eosinophilia induced by antigen-stimulated Th2 cells producing interleukin-4 and interleukin-5. More important, the present study showed that suplatast treatment inhibited the infiltration of $CD4^+$ T cells as well as that of eosinophils into lung tissues. It has been reported that, in asthmatic patients, eosinophil infiltration is associated with activated $CD4^+$ T cells (Azzawi et al., 1990). Moreover, there is compelling evidence that $CD4^+$ T cells migrating into the site of allergic inflammation belong to the population of Th2 cells producing interleukin-4 and interleukin-5 (Robinson et al., 1993). Recently, interleukin-5 was demonstrated to (1) promote the differentiation of eosinophils (Sanderson et al., 1985); (2) prolong the survival of eosinophils (Yamaguchi et al., 1988); (3) synergize with platelet activating factor and with leukotriene in the migration of eosinophils (Yamaguchi et al., 1988); and (4) prime eosinophils for increased activities (Saito et al., 1988). All these findings support a significant role of interleukin-5 in the pathogenesis of the allergic inflammation that underlies airway hyperresponsiveness. This was also shown in guinea pigs by Van Oosterhout et al. (1993) who demonstrated the role of interleukin-5 in airway eosinophilia and in the development of airway hyperreactivity. In addition, interleukin-4 has been suggested to facilitate the infiltration of eosinophils, lymphocyte, and macrophages into the site of inflammation by inducing the expression of vascular cell adhesion molecule-1 on endothelial cells (Thornill et al., 1990). We are currently investigating the effect of suplatast on interleukin-4 and interleukin-5 mRNA expression in lung tissue taken after antigen challenge. Although the precise mechanism by which suplatast inhibits the airway hyperresponsiveness and underlying allergic inflammation remains to be elucidated, our findings suggest strongly that suplatast may contribute to the therapy of the asthmatic patient by its ability to impair the recruitment of eosinophils and $CD4^+$ T cells to the site of allergic inflammation.

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