Eosinophilic airway inflammation induced by repeated exposure to cigarette smoke


ABSTRACT: Acute exposure to cigarette smoke causes airway hyperresponsiveness (AHR) in guinea-pigs, which resolves within a few hours. Repeated exposure may have a different effect on the airways.

To address this question, guinea-pigs were repeatedly exposed to cigarette smoke (six cigarettes for 1 h·day⁻¹) for 14 consecutive days. Airway responsiveness to inhaled histamine and differential cell counts in bronchoalveolar lavage fluid (BALF) were evaluated 1 day after the last exposure.

Significant neutrophilia in BALF was observed after 3 days of smoke exposure. Significant eosinophilia in BALF and AHR were observed after 14 days of smoke exposure, but not after 3 or 7 days of smoke exposure. These changes persisted until 3 days after the last exposure and resolved 7 days afterwards. Histologically, the recruited eosinophils were observed predominantly in the airways, but not in the alveoli. Treatment with E-6123, a specific platelet-activating factor receptor antagonist (1 mg·kg⁻¹·day⁻¹ p.o. during smoke exposure) significantly inhibited the eosinophil influx and AHR.

Repeated exposure to cigarette smoke may induce prolonged airway inflammation and airway hyperresponsiveness in guinea-pigs. Platelet-activating factor or platelet-activating factor-like lipids may play a key role in airway hyperresponsiveness, presumably by the induction of eosinophilic airway inflammation.

Airway hyperresponsiveness, a characteristic of asthma, is known to be associated with airway inflammation [1]. In order to clarify the underlying mechanisms of airway inflammation and hyperresponsiveness, several experimental models have been employed, including allergen-challenged, ozone-exposed and cigarette smoke-exposed models [2–7].

Acute exposure to cigarette smoke causes airway hyperresponsiveness in guinea-pigs [4–7]. However, this airway hyperresponsiveness resolves within a few hours [4, 6]. Results obtained from the acute experimental models might not be fully extrapolated to the persistent airway hyperresponsiveness observed in asthmatic subjects.

To establish a prolonged airway hyperresponsiveness model, guinea-pigs were exposed repeatedly to cigarette smoke for 14 consecutive days. This protocol induced airway hyperresponsiveness, lasting at least 3 days; which was concurrent with eosinophilic airway inflammation. These findings raised the possibility that airway hyperresponsiveness induced by repeated exposure may be linked to the eosinophilic airway inflammation. A previous study reported that cigarette smoke increases the levels of platelet-activating factor (PAF)-like lipid(s) in plasma in humans [8]. It is conceivable that PAF or PAF-like lipids may be possible mediators responsible for the eosinophilic airway inflammation and hyperresponsiveness, since PAF has eosinophilic chemotactic activity in vitro [9, 10], and could induce airway hyperresponsiveness in vivo [11–13]. To test the above hypothesis, the effect of a specific PAF receptor antagonist, E-6123 [14, 15], was evaluated in this experimental model.

Materials and methods

Animals

Male Hartley-strain guinea-pigs weighing 500–600 g (Kyudo, Kumamoto, Japan) were randomly divided into several groups (n=5 per group) as follows.

Study protocols

Study 1. Animals were divided into three groups and experiments were performed after a single cigarette smoke exposure (six cigarettes for 1 h), according to the method described below. Measurements of airway responsiveness and bronchoalveolar lavage (BAL) were performed at 1, 6 or 24 h after the exposure. Other animals served as sham-exposed controls.

Study 2. Animals were divided into three groups and exposed repeatedly to cigarette smoke (six cigarettes for 1
h, once daily) for 3, 7 or 14 days. Measurements of airway responsiveness and BAL were performed 1 day after the last exposure. Other animals served as sham-exposed controls.

Study 3. Animals were divided into three groups and exposed repeatedly to cigarette smoke for 14 days. Measurements of airway responsiveness and BAL were performed 1, 3 or 7 days after the last exposure. Other animals served as sham-exposed controls.

Study 4. Animals were divided into four groups as follows: 1) sham exposure with vehicle treatment, 2) sham exposure with E-6123 treatment, 3) repeated exposure with vehicle treatment, and 4) repeated exposure with E-6123 treatment. Animals were exposed repeatedly to cigarette smoke for 14 days. Other animals served as sham-exposed controls. Measurements of airway responsiveness and BAL were performed 1 day after the last exposure. E-6123 (1 mg·kg⁻¹·day⁻¹) or vehicle was orally administered 3 h before the exposure to cigarette smoke or sham each day. The dose of E-6123 was based on previous reports [14, 15].

Study 5. Three animals were exposed repeatedly to cigarette smoke for 14 days and were used for histopathological examination 1 day after the last exposure. Three animals served as sham-exposed controls.

Exposure to cigarette smoke

Animals were placed in a 125 L chamber made of plexiglas and exposed to diluted cigarette smoke in an awake and unrestrained state. The chamber had three holes of 3–5 mm in diameter. An exhaust hole on the top of the panel was used as a fresh air inlet. With constant vacuum flow, the smoke stream was drawn into the chamber and mixed with fresh air. One cigarette took 5 min to burn and was then immediately detached from the chamber. The visible smoke disappeared over the next 5 min. Animals were exposed to six cigarettes for 1 h·day⁻¹. The cigarettes were purchased from Japan Tobacco Co. (Tokyo, Japan). According to the manufacturer's specifications, each cigarette contained 2.7 mg of nicotine and 26 mg of tar.

Measurement of total pulmonary resistance

Guinea-pigs were anaesthetized i.p. with 50 mg·kg⁻¹ pentobarbital sodium. Animals were intubated via tracheostomy and mechanically ventilated with a respirator (Model No. 680, Harvard Apparatus, South Natick, MA, USA) at a constant tidal volume of 7 mL·kg⁻¹ and a rate of 60 breaths·min⁻¹. To evaluate pleural pressure, a fluid-filled catheter was introduced into the oesophagus at a point such that the maximal amplitude of pressure was obtained. The animals were placed supine in a body plethysmograph and the plethysmograph airflow was measured with a Fleisch pneumotachograph (TV-132T; Nihon Kohden, Tokyo, Japan) and a differential pressure transducer (TP-602T, Nihon Kohden). The plethysmograph was made of plexiglas and had 2.80 L of dead space (customized; Chest Medical, Tokyo, Japan). Transpulmonary pressure (Ptp) was estimated as the difference between the oesophageal and airway opening pressure, measured by a differential pressure transducer (TP-603T; Nihon Kohden). Total pulmonary resistance (RL) was calculated from Ptp and airflow.

Measurement of airway responsiveness

Airway responsiveness to histamine was determined by inhalation of an increasing concentration of histamine administered via the endotracheal tube. Histamine aerosols (output, 1.5 mL·min⁻¹) were generated by an ultrasonic nebulizer (TUR-3200; Nihon Kohden) placed in-line with the ventilator. Dose-response curves were constructed as follows: saline aerosol was given for 15 breaths and the subsequent Rt value was used as a baseline. The histamine aerosol was administered for 15 breaths, separated by 5 min intervals. The concentration of histamine was increased for each series of 15 breaths. Rt was monitored for 5 min after each nebulization and the maximum value was plotted against histamine concentration. To achieve a constant-volume history, hyperinflations (triplicate of tidal volumes) were obtained between each histamine challenge. The challenge was halted when Rt exceeded 200% of baseline. The provocative concentration of histamine required to produce a 200% increase in Rt (PC200) was calculated by log-linear interpolation from individual animals.

Bronchoalveolar lavage

Animals were killed by exsanguination. The lung was gently lavaged three times with normal saline via the tracheal cannula at a pressure of 2.45 kPa. Total cell counts were determined under light microscopy with a standard haemocytometer. The lavage fluid was centrifuged at 200 xg for 10 min at 4°C. The cell pellet was resuspended in normal saline to obtain a suspension of 10⁶ cells·mL⁻¹. Cytospin preparations (Cytospin 3, Shandon, Pittsburgh, PA, USA) were made and the cells were visualized with a modified Wright-Giemsa stain (Diff-Quick, Baxter, McGaw Park, IL, USA). Differential counts on 500 cells were performed in a blind fashion under light microscopy.

Histopathological assessment

To avoid possible traumatic damage due to histamine-induced bronchoconstriction or BAL, histopathological assessment in airway and lung tissue was made in separate animals. Animals were anaesthetized i.p. with 50 mg·kg⁻¹ pentobarbital sodium and exsanguinated. The cervical trachea and the lungs were removed en bloc. A cannula was introduced into the proximal portion of the trachea and the lungs were distended with buffered formalin applied at a constant pressure of 2.45 kPa. Both main bronchi and the parenchyma of the right caudal lobe were dissected out and embedded in paraffin. Sections (5 mm
CIGARETTE SMOKE AND EOSINOPHILIC INFLAMMATION

Histopathological assessment (light microscopy) was performed in a blind fashion on randomized sections.

Drugs

Pentobarbital sodium was obtained from Abbott (North Chicago, IL, USA). Histamine diphosphate was obtained from Sigma Chemical Co. (St Louis, MO, USA). (S)-(−)-6-(2-chlorophenyl)-3-cyclopropanecarbonyl-8,11-dimethyl-2,3,4,5-tetrahydro-8H-pyrido[4',3':4,5]thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine (E-6123) was provided by Eisai Pharmaceutical (Tokyo, Japan) and was dissolved in 100% ethanol at a concentration of 10 mg·mL⁻¹, then diluted in 0.9% saline to a final concentration of 1 mg·mL⁻¹.

Data analysis

PC200 values are expressed as log (PC200 × 100). Other values are expressed as the arithmetic mean and standard error (SEM). PC200 values were compared by one-way analysis of variance (ANOVA) with the Bonferoni correction. Changes in the BAL cell counts were compared by the Kruskall-Wallis H-test followed by the Mann-Whitney U-test. A level of p<0.05 was accepted as statistically significant.

Results

Time course of airway responsiveness and pro-inflammatory cell counts in BALF after a single exposure to cigarette smoke

Figure 1 illustrates the effects of a single exposure to cigarette smoke on airway responsiveness and pro-inflammatory cell profiles in BALF (BALF). Compared with sham-exposed controls, there was no significant changes in airway responsiveness at 1, 6 or 24 h after smoke exposure (fig. 1a). The number of eosinophils in BALF also showed no significant change after exposure (fig. 1b). Significant neutrophilia was observed 1 h after exposure (fig. 1c). There was no significant change in the number of macrophages and lymphocytes in the BALF (data not shown).

Effect of repeated exposure to cigarette smoke on airway responsiveness and pro-inflammatory cell counts in BALF

Figure 2 illustrates the effects of repeated exposure to cigarette smoke on airway responsiveness and cell profiles in BALF. Compared with sham-exposed controls, there was no significant change in airway responsiveness after 3 or 7 days of repeated smoke exposure. However, after 14 days of repeated exposure, airway responsiveness increased significantly (fig. 2a). Significant eosinophilia in BALF was also noted after 14 days, but not after 3 or 7 days (fig. 2b). Significant neutrophilia had already been observed after 3 days of exposure, with no further increase after this (fig. 2c). There was no significant change in the number of macrophages and lymphocytes in the BALF (table 1).

Time course of airway responsiveness and pro-inflammatory cell counts in BALF after repeated exposure to cigarette smoke

Figure 3 illustrates the time course of airway responsiveness and cell profiles in BALF after the last exposure in animals exposed for 14 days. The cigarette smoke-induced airway hyperresponsiveness persisted until 3 days after the last exposure and resolved 7 days later (fig. 3a). Similarly, the eosinophilia and neutrophilia persisted until 3 days after the last exposure and returned to baseline values 7 days later (fig. 3b and 3c). There was no significant change in the number of macrophages and lymphocytes in the BALF (data not shown).
Effect of E-6123 on airway hyperresponsiveness and increases in pro-inflammatory cell numbers in BALF induced by repeated exposure to cigarette smoke

Figure 4 illustrates the effects of E-6123 treatment on airway hyperresponsiveness, eosinophilia and neutrophilia induced by 14 days of repeated exposure to cigarette smoke. E-6123 treatment itself had no effect on airway responsiveness in sham-exposed animals. E-6123 treatment, however, significantly inhibited cigarette smoke-induced airway hyperresponsiveness (fig. 4a). E-6123 treatment had no effect on the number of eosinophils and neutrophils in sham-exposed animals. The cigarette smoke-induced eosinophilia and neutrophilia were significantly suppressed by treatment with E-6123 (fig. 4b and c).

Histopathological findings of eosinophil infiltration in tissues of airways and alveoli

Compared with sham-exposed controls, eosinophils were observed frequently in the mucosa of the main bronchi of cigarette smoke-exposed animals (fig. 5a and b). In contrast, eosinophils were scarce in the alveoli of sham- and smoke-exposed animals (fig. 5c and d). Neutrophils could not be evaluated because of difficulties in the identification of their cell bodies in the tissue stained with haematoxylin and eosin.

Table 1. Effects of repeated exposure to cigarette on the number of macrophages and lymphocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sham</th>
<th>1 day</th>
<th>3 days</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^5 cells·mL^-1</td>
<td>2.28±0.34</td>
<td>2.64±0.13</td>
<td>2.56±0.29</td>
<td>2.58±0.35</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^5 cells·mL^-1</td>
<td>0.17±0.04</td>
<td>0.15±0.04</td>
<td>0.16±0.09</td>
<td>0.25±0.07</td>
</tr>
</tbody>
</table>

Fig. 2. – Effect of repeated exposure to cigarette smoke on airway responsiveness and pro-inflammatory cell counts in bronchoalveolar lavage fluid (BALF). a) There was no significant change in airway responsiveness after 3 or 7 days of smoke exposure, compared with the sham-exposed controls. After 14 days of exposure, the airway responsiveness increased significantly. b) Significant eosinophilia in BALF was noted after 14 days of smoke exposure, but not after 3 or 7 days of smoke exposure. c) Significant neutrophilia was observed 3 days of exposure, but the cell numbers remained unchanged on the following days of exposure. PC200: provocative concentration of histamine required to produce a 200% increase in pulmonary resistance. *: p<0.05; **: p<0.01.

Fig. 3. – Time-course of airway responsiveness and pro-inflammatory cell counts in bronchoalveolar lavage fluid (BALF) after cessation of repeated exposure to cigarette smoke. a) Cigarette smoke-induced airway hyperresponsiveness persisted 3 days after the last exposure but was diminished by 7 days. b) Eosinophilia and c) neutrophilia persisted 3 days after the last exposure but had returned to the baseline value by 7 days. PC200: provocative concentration of histamine required to produce a 200% increase in pulmonary resistance. *: p<0.05; **: p<0.01.
Discussion

The present study demonstrated that 14 days of repeated exposure to cigarette smoke induced airway hyperresponsiveness which persisted for at least 3 days after the last exposure in guinea-pigs.

In this model, significant eosinophilia in BALF was observed, paralleled by an increase in airway hyperresponsiveness. The histopathological study revealed that recruited eosinophils were observed predominantly in mucosal tissue in the airways, but not in the alveoli, indicating the development of eosinophilic airway inflammation. Previous
in vivo

siveness and inflammation release PAF in response to various stimuli [22]. Finally, activity [8]. Secondly, PAF has strong eosinophil chemotactic ac-
smoke increases the levels of PAF-like lipids in plasma [7, 16, 17]. In the present study, we have established the prolonged model of eosi-
nophilic airway inflammation and hyperresponsiveness, which is different from the acute exposure models. Acute exposure to cigarette smoke is known to cause airway hyperresponsiveness in guinea-pigs [4–7]. However, this airway hyp-erresponsiveness resolves within a few hours and does not accompany eosinophilic airway inflammation. Acute ex-posure models might not fully address the relationship between eosinophilic airway inflammation and hyperresponsiveness in humans. The present protocol may provide one of the experimental models to investigate how the eosinophilic airway inflammation could contrib-
ute to prolonged airway hyperresponsiveness.

In asthmatics, a recent report has revealed that cigarette smoking enhances airway responsiveness to histamine as well as eosinophil and neutrophil activities, thus causing resistance to inhaled steroid therapy [33]. In this regard, the present experimental model may be adequate to investi-
gate the effect of cigarette smoking on airway inflamma-
tion and airway hyperresponsiveness in asthmatics. In another point of view, the presented model would be more accurately a model for airway hyperresponsiveness associated with chronic obstructive pulmonary disease (COPD). It is well known that COPD is associated with cigarette smoking. Mild-to-moderate airway hyperrespon-
siveness is frequently observed in patients with COPD [34]. Recently, it has been revealed that eosinophilic air-
way inflammation is a significant finding in patients with chronic bronchitis [35–37]. Although the exact mecha-
nisms for the development of eosinophilic airway inflamma-
tion and hyperresponsiveness in COPD is known, the results of this investigation might suggest one of the possi-
ble mechanisms.

Several investigators have reported that chronic passive smoking induced airway hyperresponsiveness in guinea-pigs [38, 39]. In contrast, KARLSSON et al. [40] reported that chronic active exposure to cigarette smoke induced hyper-
responsiveness to tussive stimuli, but not to a bronchocon-
strictor, histamine. In this study, the animals were exposed to cigarette smoke in a chamber where the total body is exposed. This model might be concerned with the one of passive smoking rather than active smoking. In this regard, another point needs to be considered. While the previous studies of passive smoking used the sidestream of cigarette smoke, the present model used the mainstream, which may rather resemble active smoking models. It is possible that both exposure pattern and source of smoke have considerable effects on the results of these investiga-
tions. To elucidate this issue, further studies are needed.

This study was started based on previous experience about acute exposure models [7, 41, 42]. When anaes-
ethetized guinea-pigs were acutely exposed to less diluted smoke through the tracheostomy tube for 10 min, marked bronchoconstriction occurred within a few minutes [41, 42]. When exposing animals to more diluted smoke for only 3 min, it did not cause direct bronchoconstriction, but induced significant airway hyperresponsiveness to inhaled
histamine [7]. It has been established that the effect of cigarette smoke on airway responsiveness is dose-dependent [4–6]. In this study, the smoke was extremely diluted in a large chamber, and acute exposure did not induce airway hyperresponsiveness in spite of a 1 h of exposure duration. Therefore, it was assumed that the condition of exposure may be milder than that elicited in previous studies. Although the best method to estimate the burden of smoke exposure may be to measure the amount of carboxyhemoglobin (HB-CO) in blood immediately after exposure, no data on HB-CO were available in this study.

In summary, repeated exposure to cigarette smoke induced prolonged airway hyperresponsiveness with eosinophilic airway inflammation in guinea-pigs. These changes were significantly inhibited by pretreatment with E-6123, a specific platelet-activating factor receptor antagonist. Intrinsic platelet-activating factor may play an important role in airway hyperresponsiveness induced by repeated exposure to cigarette smoke, possibly by the induction of eosinophilic airway inflammation in guinea-pigs.

Acknowledgements: The authors are grateful to Eisai Pharmaceutical for the donation of E-6123.

References


