Previous reports suggested that cigarette smoke had a protective effect on the development of hypersensitivity pneumonitis (HP). However, smoking rate in chronic pigeon breeder’s lung (PBL) seemed to be high in our clinical experiences. We developed a murine model of PBL by intranasal instillation with pigeon dropping extracts (PDE) for 4 weeks (short-term exposure) and 17 weeks (long-term exposure) to investigate the effect of cigarette smoke on disease processes. In this model, lung inflammation associated with the production of anti-PDE antibodies and antigen dependent lymphocyte proliferation was induced. Long-term exposure to PDE without cigarette smoking resulted in an increase in lung weight / body weight ratio, total cell number in bronchoalveolar lavage (BAL) fluid, and content of hydroxyproline in the lung compared to short-term exposure. After a short-term exposure, cigarette smoke lessened the lymphocytosis in BAL fluid, and lymphocyte proliferation. On the other hand, after a long-term exposure cigarette smoke increased lung hydroxyproline. These results suggest that a short-term cigarette smoking attenuates lung inflammation, but a long-term cigarette smoking enhances lung inflammation with fibrosis.

Key words: pigeon breeder’s lung, cigarette smoke, lung inflammation, lung hydroxyproline

Introduction

Hypersensitivity pneumonitis (HP) is an immunologically induced lung disease caused by repeated inhalation of various environmental antigens in susceptible individuals. Pigeon breeder's lung (PBL) is one of the common HP caused by inhalation of pigeon-related antigens which are included in pigeon droppings and in other pigeon materials. PBL tends to develop chronic disease with few acute symptoms including fever and chills (insidious onset) as compared to summer type HP.

The clinical outcome of PBL is variable and the responsible factors for its variation remain undetermined. Several articles have reported protective effects of cigarette smoking in developing acute HP. Smokers in bird fanciers produced lower concentrations of antibody to bird-related antigens in serum and bronchoalveolar lavage fluid than non-smokers. Several proinflammatory cytokines such as TNF-α and IFN-γ are crucial in the development of HP, but cigarette smoke is known to downregulate the production of these cytokines. In line with these observations, cigarette smoke decreased pulmonary dendritic cells and suppressed lipopolysaccharide-induced pro-
duction of inflammatory cytokines by inhibiting the activation of activator protein-1 in bronchial epithelial cells. In contrast, short-term cigarette smoking enhanced allergic airway inflammation in mice associated with eosinophilic inflammation, an increased dendritic cells in bronchoalveolar lavage fluid, and increased serum antigen-specific IgE. Cigarette smoke has been reported to directly stimulate alveolar macrophages to produce proinflammatory cytokines such as IL-1 and IL-6. Furthermore, pulmonary inflammation was induced accompanied with an increased amount of infiltration of the dendritic cells in the airways and the lung parenchyma in a mouse model exposed with cigarette smoke for 24 weeks. The effects of cigarette smoke on immune responses are contrasting in the development of chronic disease and it was reported more than 50% of chronic PBL patients were smokers. We hypothesized that cigarette smoke may modify the disease process of PBL leading to chronic disease. In the present study, we have evaluated the effect of cigarette smoke on the development of chronic PBL in a murine model by studying the difference between a short-term and a long-term exposure. Lung weight / body weight ratio, serum antibodies and lymphocyte proliferation in response to pigeon-related antigen, BAL profiles and the expressions of INF-γ, TNF-α and IL-10 mRNA in the lung were evaluated to analyze the inflammatory process. The content of hydroxyproline in the lung and histological findings were also examined to evaluate the fibrotic process.

Materials and Methods

Animals
Specific pathogen-free C57BL/6J mice were purchased from Sankyo Medical animal supply (Sankyo Labo. Co., Tokyo Japan). Female mice, weighting 18-24g, were housed in plastic cages and were fed standard mouse chow and water at Tokyo Medical and Dental University. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

Instillation with pigeon dropping extracts (PDE)
PDE was obtained according to the previously described method. Briefly, fresh pigeon dropping were stirred with a 20 volume of phosphate buffered saline solution (pH 7.4) for 24 h followed by dialysis against distilled water and lyophilized. PDE contains large amounts of endotoxin, whose biological activities might play a role in the initiation of PBL. Freeze-dried PDE was dissolved with sterile 0.9% saline and adjusted to 0.2 mg/ml. Under light anesthesia with inhalation of diethyl ether, PDE dissolved in 40 ml of 0.9% saline (8 mg) was applied at the tip of the nose of mice and they inhaled PDE involuntarily. Instillation was conducted 3 days per week for an indicated period of time. Control mice were administrated 40 ml of physiologic saline solution in the same manner including anesthesia.

Cigarette smoking
Mice were put into the capsules toward the cylinder with Hamburg type (Borgwalt Co.) filled with cigarette smoke of 10 filtered commercial cigarettes (Marlboro, Philip Morris Products, SA) once a day. Exposure was done 5 days per week from 1 week before the initiation of instillation with PDE.

Experimental design
Three groups of mice were studied. (1) control mice instilled with saline solution (group C). (2) mice instilled with PDE (group P). (3) mice instilled with PDE and exposed to cigarette smoke (group P+S). Each group was divided into two subgroups; instilled for either 4 weeks (short-term exposure) and for 17 weeks (long-term exposure).

Bronchoalveolar lavage (BAL)
Plastic cannula was inserted into the trachea. BAL samples were obtained by washing the whole lungs with 1.0 ml aliquots of 0.9% saline three times. After centrifugation, BAL cell pellets were washed and resuspended in RPMI and total cell counts were examined. Cytospin preparations were fixed and stained using Diff-Quick staining, and differential cell counts were made.

ELISA assay for anti-PDE IgG antibody
Antibody against PDE in sera was measured by an enzyme-linked immunosorbent assay. Each well of polystyrene plates (Immulon 2; Dynatech Laboratories; Alex-andria, VA) was coated with a 100 ml of 1mg/ml PDE in carbonate buffer (pH 9.6) at 4°C overnight. After washing the wells three times with phosphate buffered saline solution (pH 7.4) containing 0.05% polysorbate-20 (Tween-20) (PBST), each well was treated with 0.05% bovine serum albumin-PBST to block the free binding surface of the wells. Our previous study showed that the optical dilution to evaluate anti-
bodies was 1: 400 for the sera. Samples in 100 µl quantities were added to each well and incubated at 37 °C for 1h. After washing the wells with PBST, 100 µl of a 1:1000 diluted solution of rabbit anti-mouse IgG coupled to horseradish peroxidase was added and incubated at room temperature for 1h. After washing the wells again, the substrate was added, and color was developed and measured at 490-nm and 620-nm.

Quantification of cytokine mRNA expression in the lung
RNA was extracted from the left lung and INF-γ, TNF-α and IL-10 mRNA were quantified by a real time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)27. Amplification of cDNA was performed by ABI PRISM 7700 Sequence Detection System using Taqman real time RT-PCR kit (Applied Biosystems) with specific probe and primers according to the manufacture’s instructions. The results were expressed as the ratio of the experimental mice to the control mice.

Lymphocyte proliferation
BAL cells were resuspended in RPMI and 1x10^5 cells were plated into wells of a 96-well plate in the presence or in the absence of pigeon sera. We have not employed PDE as the antigen for lymphocytes proliferation test because crude PDE contains cytotoxic components to lymphocytes, but we have successfully applied pigeon sera to human BAL cells. After 5 day-culture with or without pigeon sera, [3H] thymidine was added to the culture for the last 24 h. Cell proliferation was measured by an automated liquid scintillation counter. The results were expressed as stimulation index, which is the geometric mean counts per minute of stimulated cultures with pigeon sera divided by the geometric mean counts per minute of unstimulated cultures as control (without pigeon sera).

Quantification of hydroxyproline
Content of hydroxyproline in the right lung was determined as described in detail elsewhere28. Briefly, lungs were homogenized with cold TCA followed by hydrochloric acid. Chloramine-T was added, followed by perchloric acid and p-demethyl-aminobenzaldehyde. Color change was assessed by spectrophotometry and estimated on a standard curve of hydroxyproline.

Histological evaluation
In the mice which had not been lavaged, the left lung was perfused with 2% paraformaldehyde through the left main bronchus to be fixed in 2% paraformaldehyde-PBS. The sections were embedded in paraffin, cut in 5 µm-thick sections, and stained with hematoxylin and eosin. The sections were evaluated under light microscopy.

Statistics
Statistical analysis was done with post-hoc test using Scheffe’s F test or Mann-Whitney nonparametric unpaired analysis using a statistical software (Statcel 2). Values are expressed as the mean ±SEM. Values of p < 0.05 were considered as significant.

Results
Lung weight / body weight ratio
Lung weight / body weight ratio was estimated before BAL. A short- term exposure to PDE induced an increase in lung weight / body weight ratio, and a long-term exposure to PDE caused a more prominent increase (Figure 1). There were no significant changes of lung weight / body weight ratio between the mice instilled with saline solution (0.0078±0.0009) and exposed to cigarette smoke alone (0.0071±0.0006) after a short-term exposure.

BAL
The recovery rate of BAL fluid in all groups was more than 80%. Total cells in BAL fluid were significantly (p < 0.0001) increased after a long-term exposure com-
pared to those after a short-term exposure (Figure 2). There was no significant difference in the total cells in BAL fluid between group P and group P+S both in a short-term and a long-term exposure.

Instillation with PDE also resulted in an increase in the percentage of lymphocytes both in a short-term and a long-term exposure. The percentage of lymphocytes in group P+S was significantly less than group P in a short-term exposure, whereas no significant difference was detected between them in a long-term exposure (Figure 3).

Production of anti-PDE IgG antibody

Significantly higher titer of anti-PDE IgG antibody was detected in group P than in group C both in a short-term and a long-term exposure. Group P+S seemed to produce less titer of the antibody than group P both in a short-term and a long-term exposure, however no significant difference was demonstrated (Figure 4).

Cytokine mRNA expression in the lung

The ratio of mRNA expression of IFN-γ, TNF-α and IL-10 in the lung compared with the controls after a long-term exposure was demonstrated in Figure 5. Instillation of PDE upregulated the expression of these cytokines more than 100 times than controls. Cigarette smoke attenuated the increased expression of these three cytokines.

Lymphocyte proliferation

Proliferation of lymphocyte in BAL fluid induced by pigeon sera was shown in Figure 6. The antigen-dependent lymphocyte proliferation was not demonstrated in group C with a stimulation index of 1.06 (mean, n=2). In group P after a short-term and a long-term exposure, higher lymphocyte proliferation was induced with a stimulation index of more than 2.0. Cigarette smoke inhibited the induction of lymphocyte proliferation in a short-term exposure. However, in a
CIGARETTE SMOKING IN PIGEON BREEDER’S LUNG MODEL

**Discussion**

PBL is a form of HP caused by hypersensitivity reactions to inhaled pigeon-related antigens and the clinical outcome of PBL is variable. Although many factors such as cigarette smoking, as well as the duration of inhaled antigen exposure, the dosage of exposed antigens, and individual susceptibility and responsiveness to the inhaled antigen may contribute to the out-

**Histological findings**

Repetitive intranasal instillation with PDE resulted in inflammatory changes in the lung (Figure 8). A short-term exposure caused inflammatory changes in predominantly peribronchiolar and perivascular region characterized by infiltration with lymphocytes. Inflammatory changes in group P+S were less severe compared with group P. After a long-term exposure, inflammatory changes in peribronchiolar and perivascular region were less prominent compared to a short-term exposure and alveolar macrophages infiltrating into alveolar space were observed. In group P+S after a long-term exposure, increased numbers of alveolar macrophages in the alveolar space were observed compared to group P.

**Lung hydroxyproline content**

Lung collagen was quantified by measuring the content of hydroxyproline in the lung. In a short-term exposure, instillation with PDE with or without cigarette smoke did not alter the lung hydroxyproline content compared with the controls (group P: 1.10 ± 0.08; group P+S: 1.10 ± 0.05). On the other hand, group P in a long-term exposure showed an increase in hydroxyproline compared to a short-term exposure (Figure 7). In addition, a significant increase was found in group P+S compared to group P after a long-term exposure, showing that cigarette smoke enhanced the fibrotic process in the lung.

**Fig. 5.** Cytokine mRNA expression in the lung compared with controls after a long-term exposure. Vertical line represents the ratio of mRNA expression compared with controls. Closed bar: mice instilled with PDE (group P); hatched bar: mice instilled with PDE and exposed to cigarette smoke (group P+S). Data are expressed as the mean ± SEM for 4 animals in each group. * p<0.05 (Mann-Whitney test).

**Fig. 6.** Lymphocyte proliferation in BAL fluid induced by pigeon sera. S.I.: stimulation index, mean counts in cells stimulated with pigeon sera divided by those unstimulated (without pigeon sera). Closed bar: mice instilled with PDE (group P); hatched bar: mice instilled with PDE and exposed to cigarette smoke (group P+S); 4W: after 4 weeks; 17W: after 17 weeks. Data are expressed as the mean ± SEM for 4 animals in each group. * p<0.05 (Scheffe’s F test).

long-term exposure, cigarette smoke did not alter the induction, showing no significant difference between group P and group P+S (Figure 6).
Fig. 8. Microscopic photograph of the lung (Hematoxylin-eosin stain). Upper left: mice instilled with PDE (group P) after a short-term exposure (original magnification x 4); upper right: mice instilled with PDE and exposed to cigarette smoke (group P+S) after a short-term exposure (original magnification x4); middle left: group P after a long-term exposure (original magnification x4); middle right: group P after a long-term exposure (original magnification x10); bottom left: group P+S after a long-term exposure (original magnification x 4); bottom right: group P+S after a long-term exposure (original magnification x10).
come of the clinical course\textsuperscript{5,6}, each factor has not been fully analyzed because of the limitations of the retrospective studies in humans. To better understand the detailed pathogenesis of PBL, we developed an animal model of PBL using mice. In the present study we demonstrated that repetitive intranasal instillation of PDE induced lung inflammation associated with lymphocytosis in BAL fluid, the production of anti-PDE antibody, and a positive lymphocyte proliferation in the presence of pigeon sera. Long-term exposure to PDE resulted in an increase in lung weight / body weight ratio and in the total cell number in BAL fluids compared to short-term exposure. In addition, increased amounts of hydroxyproline after a long-term exposure suggested the development of fibrotic changes. These findings are similar to the pathological features of PBL in humans.

It has been shown that HP occurs more frequently in nonsmokers than smokers, and several investigations have demonstrated that cigarette smoke had a protective effect on the development of HP both in human and animal models\textsuperscript{7-9}. Previous studies reported that smokers in bird fanciers produced lower concentrations of IgG antibody against bird related antigens in sera and BAL fluids than non-smokers\textsuperscript{10-12}. In contrast, short-term cigarette smoking was reported to enhance allergic airway inflammation in mice associated with eosinophilic inflammation, increase the dendritic cells in BAL fluid, and increase serum antigen-specific IgE\textsuperscript{20}. The present study using mice showed that exposure to cigarette smoke had a tendency to reduce the production of anti-PDE IgG antibody. Cigarette smoke also lessened lymphocytosis in BAL fluid and histological inflammatory changes after a short-term exposure. The molecular mechanisms involved in the above result of our study are not clear. It has been reported that several proinflammatory cytokines such as TNF-\(\alpha\)\textsuperscript{13-15} and IFN-\(\gamma\)\textsuperscript{16, 17} play crucial roles in the development of HP and cigarette smoke downregulate the production of these cytokines\textsuperscript{18, 19}. In this study, exposure to cigarette smoke resulted in a decrease in mRNA expression of these proinflammatory cytokines in a short-term and a long-term exposure. Our results are consistent with the attenuations of lung inflammation induced by cigarette smoke in the early phase of PBL. However, the enhanced lung inflammation by cigarette smoking after a long-term exposure is inconsistent with the reduced mRNA expression of these cytokines. Cigarette smoking was reported to directly stimulate alveolar macrophages to produce other proinflammatory cytokines such as IL-1 and IL-6\textsuperscript{23}. Further studies will be necessary to elucidate the molecular mechanism of the effect of cigarette smoke on the pathogenesis of PBL.

The expression of CD80 and CD86 on alveolar macrophages has been shown to increase in HP patients compared with normal subjects as a result of active immune reactions, and to decrease in smokers compared to nonsmokers leading to the less immune reactivity\textsuperscript{29,30}. The low level of the expression of co-stimulatory molecules on alveolar macrophages of smokers may be one reason for the protective effect of cigarette smoke in developing HP\textsuperscript{30,31}. In line with these observations, cigarette smoke decreased pulmonary dendritic cells and suppressed lipopolysaccharide-induced production of inflammatory cytokines by inhibiting the activation of activator protein-1 in bronchial epithelial cells\textsuperscript{20, 21}. In contrast, pulmonary inflammation was induced accompanied with an increased amount of infiltration of the dendritic cells in the airways and the lung parenchyma in a mouse model exposed with cigarette smoke for 24 weeks\textsuperscript{24}. We have examined the expression of co-stimulatory molecules on alveolar macrophages by flow cytometry and showed no significant decrease in the expression of CD80 and CD86 on alveolar macrophages of mice exposed to cigarette smoke for 4 weeks in a preliminary study. These results suggest that the effect of cigarette smoke on decreased inflammation in the lungs is not due to the decreased expression of co-stimulatory molecules on antigen presenting cells in the airways. However, since we have not examined the expression of co-stimulatory molecules on dendritic cells in the thoracic lymph nodes, our results are not conclusive\textsuperscript{25}.

The protective effects of cigarette smoke seen in a short-term exposure were no longer observed in a long-term exposure. The lung weight / body weight ratio and the total cell numbers of the BAL fluid tended to be increased compared to unexposed mice, and a significant increase in lung hydroxyproline was induced by cigarette smoke. These observations are consistent with our previous study of 32 chronic PBL patients showing the high rate of smokers, accounting for 62.5% at the time when the sensitization begun\textsuperscript{26}. To our knowledge, there had been no reported animal model of chronic HP showing the effect of cigarette smoke exposure for the extended period of experimental time (long-term exposure). Our study is likely to be more closely related to chronic PBL in humans than previous studies in sense of long-term sensitization\textsuperscript{27}. In a clinical study of chronic farmer’s lung, Ohtsuka et al. reported that cigarette smoke might promote an insid-
ious form of the disease that led to deterioration of the clinical outcome\(^{30}\). Our results also suggest that cigarette smoke attenuates the inflammation in the lung at the early phase of PBL but enhances lung inflammation and lung fibrosis at the later phase. Cigarette smoke may induce the production of some profibrotic cytokines such as TGF-\(\beta\) after a long-term exposure, and may upregulate the expression of matrix metalloproteinase, such as MMP-9, observed in COPD patients\(^{30}\). However, in this study, direct effect of cigarette smoke on the process of lung inflammation and fibrosis has insufficiently been examined, because we have not prepared smoking alone group without instillation of PDE in the experimental design.

In conclusion, this study has demonstrated that a long term repetitive intranasal instillation of PDE induced lung inflammation associated with an increase of lung hydroxyproline. Although a short-term cigarette smoke attenuated lung inflammation, a long-term cigarette smoke enhanced lung inflammation with more lung hydroxyproline. This is the first report in which an increase in lung hydroxyproline by a long-term cigarette smoke was documented in an animal model of PBL. Further studies are required to unveil the mechanisms involved in an enhancement of lung fibrosis by cigarette smoke.

References

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