

Kinetics of apoptosis in the lung of mice with allergic airway inflammation

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Summary

Background Apoptosis has been suggested as a means to facilitate the resolution of eosinophilic inflammation in bronchial asthma. However, the natural course of apoptosis has not been elucidated *in vivo*, and there is no direct evidence for eosinophilic apoptosis within lung tissue.

Objective The purpose of this study is to clarify whether the apoptosis occurs within the lung tissue, and to define the time-course of change in apoptosis ratio during the resolution of pulmonary eosinophilic inflammation.

Methods Ovalbumin (OVA)-sensitized Balb/c mice were challenged with aerosolized OVA. We studied apoptotic cells in the lung of OVA-sensitized mice at 1, 3, 7 and 14 days after OVA challenge by *in situ* detection of DNA fragmentation with deoxynucleotidyl transferase deoxyuridyl triphosphatase nick endlabelling (TUNEL) technique. Apoptotic cells also were identified by electron microscopic analysis in the lung 7 days after OVA challenge.

Results The TUNEL-method revealed that eosinophils localized in the subepithelium of bronchi undergo apoptosis following OVA challenge. Electron microscopy confirmed the presence of apoptotic cells, apoptotic bodies, and macrophages ingesting apoptotic bodies within the lung tissue. The number of apoptotic cells increased concomitantly with the increase in eosinophilic infiltration for 3 days post-challenge. However, both the apoptotic cell counts and the apoptotic ratio continued to increase even after the eosinophil count peaked, indicating rather late induction of apoptosis in the lung. In addition, TUNEL-positive cells were localized in the lung for 14 days post-challenge, indicating prolonged induction of apoptosis after the OVA challenge.

Conclusion Our findings constitute direct evidence of eosinophilic apoptosis *in situ*, and display the kinetics of apoptosis in the lung of the allergic inflammation.

Keywords: apoptosis, eosinophil, bronchial asthma, mice, TUNEL

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Introduction

Characterization of the cellular and chemical events of asthma suggests that inflammatory cells are directly involved in the clinical expression and pathogenesis of this disease [1]. Recently, there has been increasing interest in the involvement of inhibition of eosinophilic apoptosis in

the pathogenesis of asthma [2–4]. Prolonging of the usual life-span of eosinophils (i.e. as short as 4 days) may lead to increased inflammation in asthma. For this reason, the mechanisms which determine and regulate apoptosis have received much attention recently. It has been reported that eosinophils purified from the peripheral blood of asymptomatic healthy atopic patients undergo apoptosis *in vitro*, and that survival *in vitro* of peripheral blood eosinophils for at least 14 days requires continued exposure to granulocyte-macrophage colony-stimulating factor (GM-CSF),

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interleukin (IL)-3, or IL-5 [5], suggesting that these haematopoietins inhibit apoptosis of eosinophils. However, apoptosis in eosinophils that have migrated into the lung tissue has not been evaluated directly *in vivo*. The ratio of apoptotic cells *in situ* to viable migrated inflammatory cells *in situ* may help to address this issue. Eosinophilic apoptosis increases within human sputum after treatment with glucocorticoids [6]. However, it is not clear whether the apoptosis occurs within the lung tissue or in the airway lumen. In our study, we will evaluate eosinophilic apoptosis, using a DNA fragmentation detection technique *in situ* and electron microscopy, to investigate whether it is induced within the lung tissue or in the airway lumen in a mouse model of allergic inflammation.

Materials and methods

Aeroallergen treatment of mice

Eight-week-old male Balb/c mice (20–25 g) were purchased from Charles River Laboratories (Shizuoka, Japan) and were maintained under standard pathogen-free conditions. Sensitization and induction of allergic pulmonary inflammation were performed according to a previously described method [7]. Mice were immunized intraperitoneally on days 0 and 14 of the protocol with 5 µg of ovalbumin (OVA) (Sigma Chemical Co., St Louis, MO, USA) and 100 µg of aluminium hydroxide in saline. Non-sensitized mice received 100 µg of aluminium hydroxide in saline. On day 28, the animals were placed individually in a chamber and were allowed to inhale aerosolized OVA (20 mg/mL) dissolved in saline (non-sensitized mice received saline only) for 30 min delivered by ultrasonic nebulizer. The mice were killed by cervical dislocation at various times after the inhaled OVA challenge (1, 3, 7 and 14 days; each group consists of 4–5 animals). Sensitized animals without OVA challenge which inhaled saline only for 30 min, and were killed 3 days after challenge (four animals). The lungs were excised and frozen quickly with dry ice. Frozen 4 µm thick sections were cut on a cryostat and thaw-mounted on TESTA (3-aminopropyl triethoxysilane)-treated slides. They were air-dried and studied histochemically.

Detection of apoptotic cells in lung sections

The sections were fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer for 30 min and were incubated in 3% H₂O₂ in absolute methanol for 24 h before staining with terminal deoxynucleotidyl transferase (TdT) deoxyuridyl triphosphate (d-UTP) nick end labelling (TUNEL) technique [8]. Briefly, TdT was used to incorporate biotinylated deoxyuridine at sites of DNA cleavage, with avidin peroxidase used as the detection agent. Positive

controls (deoxyribonuclease [DNase]-treated cell preparations) and thymocytes stained positively, indicating DNA fragmentation. No staining was evident in negative controls (TdT omitted). Some sections were counterstained with Giemsa's solution after TUNEL staining.

Immunocytochemistry for lymphocyte and macrophage analysis

The sections were fixed by acetone at room temperature for 5 min and were subjected to Giemsa's staining or immunocytochemistry for CD4⁺ T lymphocytes or macrophages. After blocking the endogenous peroxidase with 3% H₂O₂, rat anti-mouse CD4 (L3T4) monoclonal antibody or rat anti-mouse macrophage (clone BM-8 or F4/80) monoclonal antibody (PharMingen, San Diego, CA, USA) diluted 1:200 with phosphate-buffered saline (PBS) was used as a primary antibody. Sections were then incubated with biotin-conjugated goat anti-rat IgG (1:500, Tago, Camarillo, CA, USA) at 4 °C for 3 h, followed by avidin-biotin peroxidase reaction (Vectastain ABC Kit, Vector, Burlingame, CA, USA).

Electron microscopy

Seven days after OVA challenge mice were perfusion-fixed via the heart with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer. The lung was removed and post-fixed in the same fixative for 24 h. The lung was washed several times with 0.1 mol/L phosphate buffer, followed by 10% and 20% sucrose dissolved in the same buffer for more than 1 h each time. The tissues were then frozen in liquid nitrogen and cut on a cryostat at a thickness of 20 µm. The section was thawed in 0.1 mol/L phosphate buffer at ≈ 15 °C. Adjacent sections were thaw-mounted on gelatinized slides and were stained with Giemsa's solution to detect the region of interest.

After washing, the tissues were post-fixed for 1 h in 1% OsO₄ in 0.1 mol/L phosphate buffer and then dehydrated. To enhance contrast for electron microscopy, the tissues were stained with 1% uranyl acetate at the 70% alcohol dehydration stage. Serial ultrathin sections were mounted on Formval-coated single-slot (2 × 1 mm) grids and stained with lead.

Analysis of cellular infiltrate

The number of eosinophils, TUNEL-positive cells, CD4⁺ T lymphocytes and macrophages were counted in all medium airways present in each section using a light microscope. These counts reflected the number of cells per unit area (number/mm²) of the subepithelial area of medium airways. Apoptosis results also were expressed as the apoptosis ratio

([TUNEL-positive cells]/[all infiltrating cells including eosinophils, lymphocytes and macrophages counted from adjacent immunostained-sections]). A total of 12 separate fields ($\times 20$ objective and $\times 10$ eyepiece) was examined and data represent the mean $\pm$ SD for groups of four or five mice. Significant differences between experimental groups were assessed using the Scheffe type multiple comparison test. Significance was accepted at $P < 0.05$.

Results

Kinetics of antigen-induced cellular infiltration into the lung

In sensitized mice, only a few eosinophils were detected in the subepithelial area of the bronchus (Fig. 1A). Aerosol challenge of mice with OVA induced a significant increase in eosinophils on day 1 in comparison with mice given aerosolized saline ($P < 0.01$) (Figs 1B, 2A). The number of eosinophils reached a peak on day 3 and decreased slightly thereafter (Fig. 1C,D). On day 14 following OVA challenge, eosinophilic infiltration decreased significantly in comparison with that on day 3 ($P < 0.01$) and day 7 ($P < 0.05$), and there was no significant difference in eosinophil number between sensitized mice and mice on day 14 (Fig. 1E).

Infiltration of T lymphocytes and macrophages was also significantly elevated following OVA challenge ($P < 0.01$). The T lymphocyte infiltration into the subepithelial area reached a peak on day 3 and then decreased on day 14. However, the numbers of T lymphocytes were still significantly higher on day 14 than those of sensitized mice ($P < 0.05$) (Fig. 2B). Macrophage infiltration reached a peak on day 3 and then decreased. The numbers of macrophages on days 7 and 14 were still higher than those of sensitized mice without OVA challenge ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 2C).

Mice given aerosolized saline developed no significant inflammatory cell infiltration in their lung during the whole period of post-challenge.

Number of apoptotic cells in the lung

Almost no TUNEL-positive cells were detected in the subepithelial area of sensitized mice given aerosolized saline (Fig. 1F). One day after OVA challenge, a few TUNEL-positive cells were detected where eosinophils and T lymphocytes showed infiltration (Fig. 1G). TUNEL-positive cells were scattered in the subepithelial area, along with a number of inflammatory cells. They increased on days 3 and 7 ($P < 0.01$), and appeared to be localized to the subepithelium where eosinophils were concentrated (Figs 1H,I). The number of TUNEL-positive cells reached a peak on day 7 and then decreased ($P < 0.05$) (Fig. 2D). However,

a measurable amount of TUNEL-positive cells could still be identified on day 14 (Fig. 1J).

Identification of apoptotic cell sources

Adjacent thin sections on day 7 revealed that TUNEL-positivity correlated well with eosinophilic infiltration (Figs 3A,B). Some TUNEL-positive cells were identified as eosinophils when stained with Giemsa's solution (Fig. 4).

Change of apoptosis ratio following OVA challenge

The apoptosis ratio ([TUNEL-positive cells]/[all infiltrating cells including eosinophils, lymphocytes and macrophages counted from adjacent immunostained sections]) was relatively constant (1.2 to 1.3%) from day 1 to day 3 (Fig. 5). There was a significant ($P < 0.01$), twofold increase in the ratio to 2.8% by day 7. The ratio remained high on day 14 (1.9%).

Electron microscopic analysis

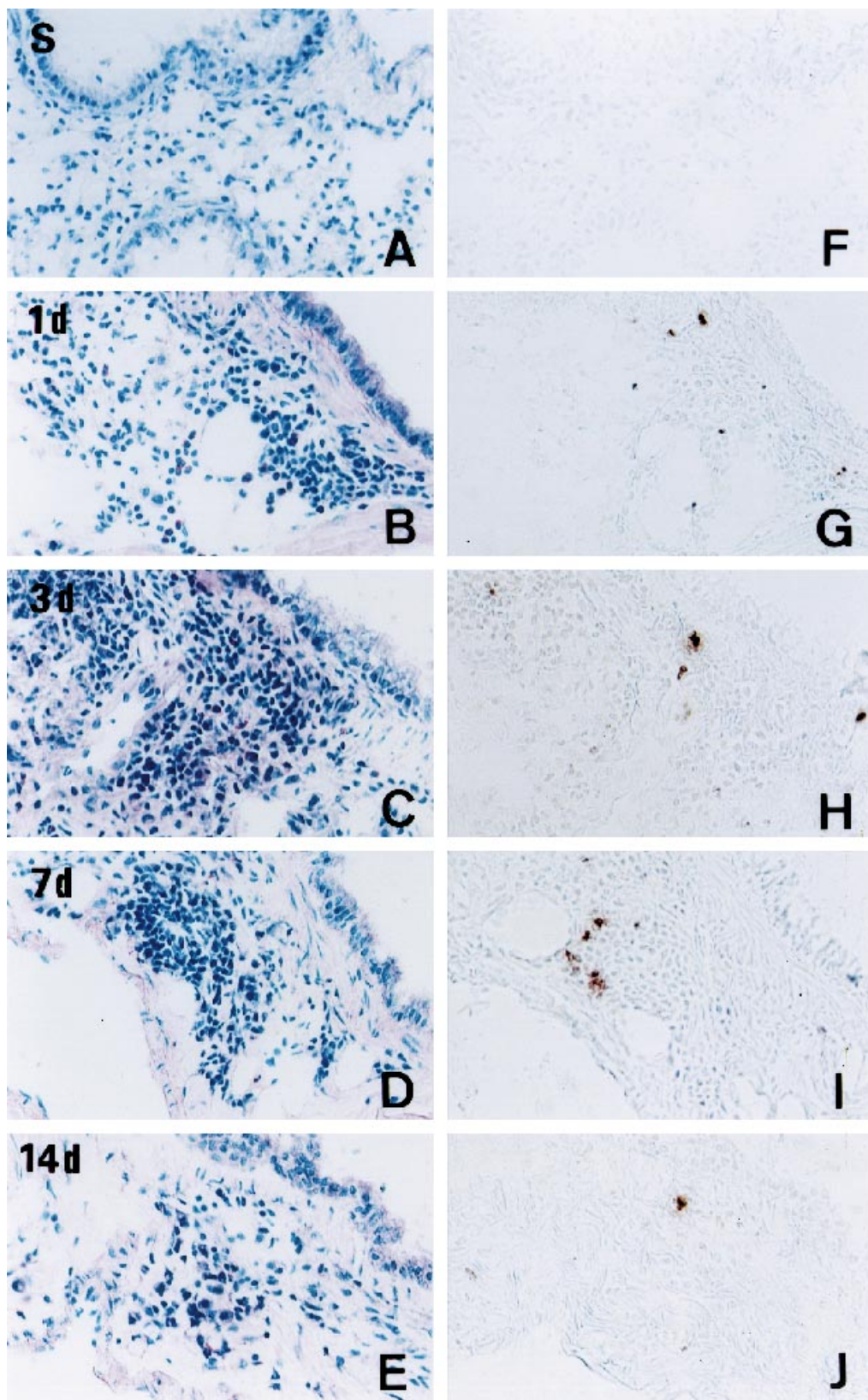
Morphological assessment with electron microscopy revealed typical conventional features of apoptotic cells that had migrated into lung tissue (Fig. 6). They included nuclear chromatin condensation (Figs 6A,C), apoptotic bodies (Fig. 6B), and macrophages containing apoptotic bodies (Figs 6A,B). Some cells contained a segmented nucleus which showed chromatin condensation and many granules, suggesting that the apoptotic cell was an eosinophil (Fig. 6C).

Discussion

Evidence for eosinophilic apoptosis in vivo

This is the first report to show direct evidence for eosinophilic apoptosis *in situ* during allergic inflammation in mice. Previous studies have shown that few apoptotic cells are detected in tissue samples [9,10], although a number of free apoptotic cells are observed in samples from the airway lumen [6,11,12], suggesting that there is rapid removal of apoptotic cells by phagocytes in the tissue [6]. We detected apoptosis using the TUNEL method, which is sensitive and has been used widely as an *in situ* detection method for DNA fragmentation [13], although the results of a recent study have suggested that DNA fragmentation is not necessarily the primary event in apoptosis [14]. We also confirmed the presence of apoptosis by ultrastructural observation.

Our results demonstrate that eosinophils that migrate into lung tissue undergo apoptosis within the subepithelium following OVA challenge. A previous study which addressed apoptotic eosinophils from the airways of



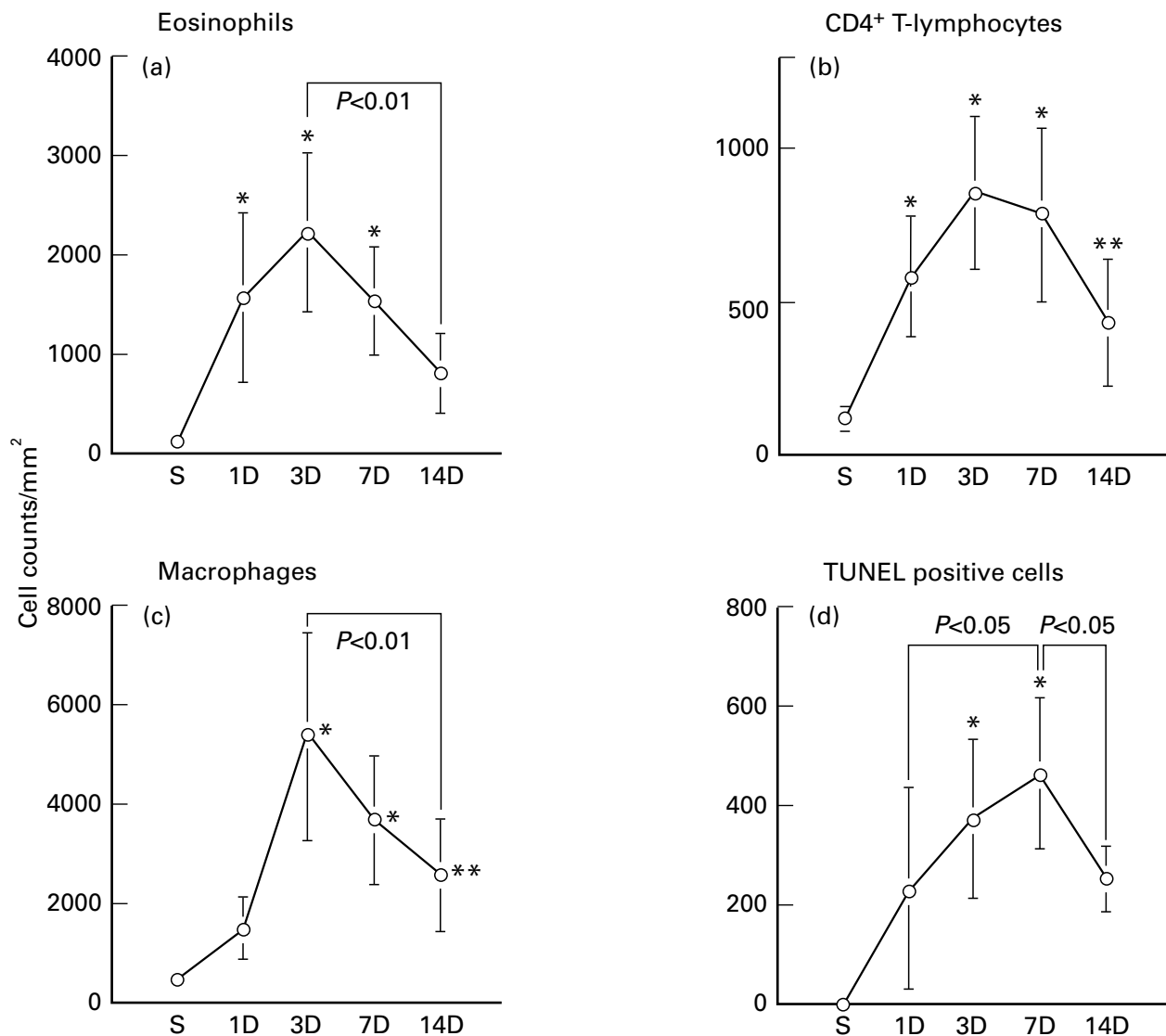


Fig. 2. Time course of cell infiltration (eosinophil, CD4 positive T-lymphocyte, and BM-8 positive macrophage) into the subepithelium of the bronchi, and kinetics of the expression of TUNEL-positive cells in the same area of sensitized mice following a single OVA challenge. Eosinophils, T lymphocytes and macrophages peaked on day 3. TUNEL-positive cells increased after OVA challenge and peaked on day 7. OVA: Ovalbumin; * $P < 0.01$, ** $P < 0.05$, compared to that on the sensitized mice without OVA challenge.

asthmatic patients on corticosteroid treatment did not disclose whether eosinophilic apoptosis occurred within lung tissue [6]. In the present study we observed that, under electron microscopy, many free apoptotic cells as well as apoptotic body-containing macrophages were localized in

the tissue during the late phase of eosinophilia after OVA challenge. Together with the results of TUNEL-staining, the present study showed that eosinophils undergo apoptosis and then were phagocytosed by macrophages within the lung tissue, but not in the airway lumen.

Fig. 1. Time course of infiltration of inflammatory cells (A-E: Giemsa's staining), and expression of TUNEL-positive cells (F-J), in sensitized mouse lung following ovalbumin challenge. Two adjacent sections were prepared from sensitized lung without challenge (S) and following challenge at the indicated interval. In the sensitized lung, no TUNEL-positive cells are seen (F). A few TUNEL-positive cells were apparent on day 1 (G), where eosinophils infiltrated (B). TUNEL-positive cells time-dependently increased in number from day 3 (H) to day 7 (I), then decreased on day 14 (J). The number of eosinophils and other cells such as macrophages peaked on day 3 (C). Bar = 100 μ m. TUNEL: terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labelling.

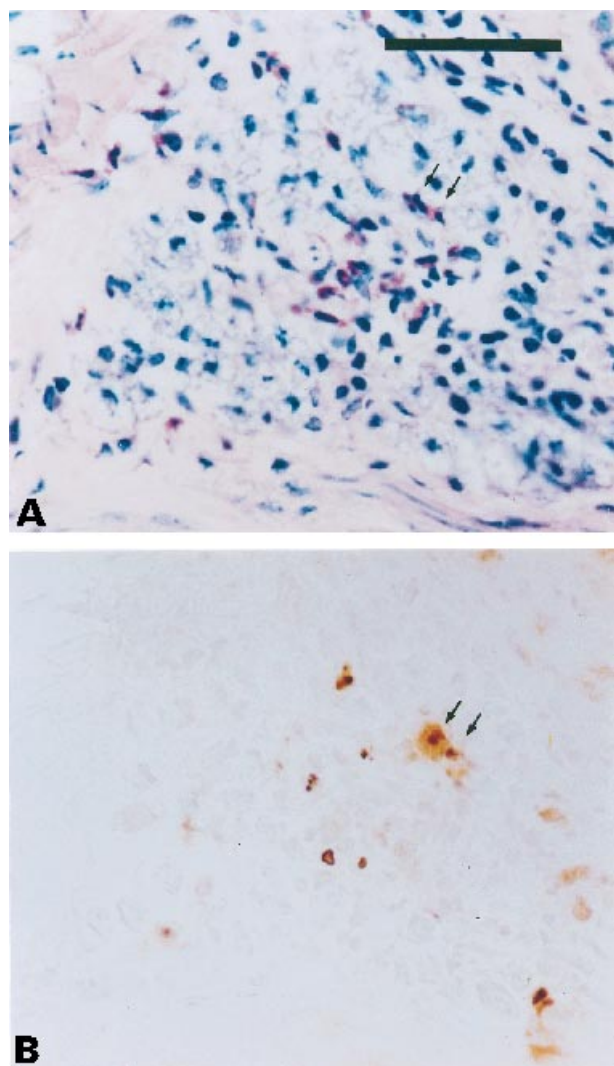


Fig. 3. Photomicrographs of mouse lung sections obtained from sensitized mouse 7 days after challenge stained by Giemsa's solution (A) and TUNEL method (B). TUNEL-positivity corresponds eosinophils in adjacent sections (Arrows). Bar = 100 μ m.

The present study demonstrated that only a few TUNEL-positive cells could be stained as eosinophilic. Eosinophilic staining may be abolished after inducing apoptosis in cells in association with nuclear condensation. Our electron microscopic study revealed that the organelles more or less showed degeneration in the cytoplasm when nuclear condensation progressed. In addition, our preliminary study reveals that the cultured human eosinophils undergoing apoptosis are Giemsa stain-negative (data not shown). Accordingly, it is possible that some non-eosinophilic TUNEL-positive cells as well as eosinophilic TUNEL-positive cells were eosinophils, although we could not

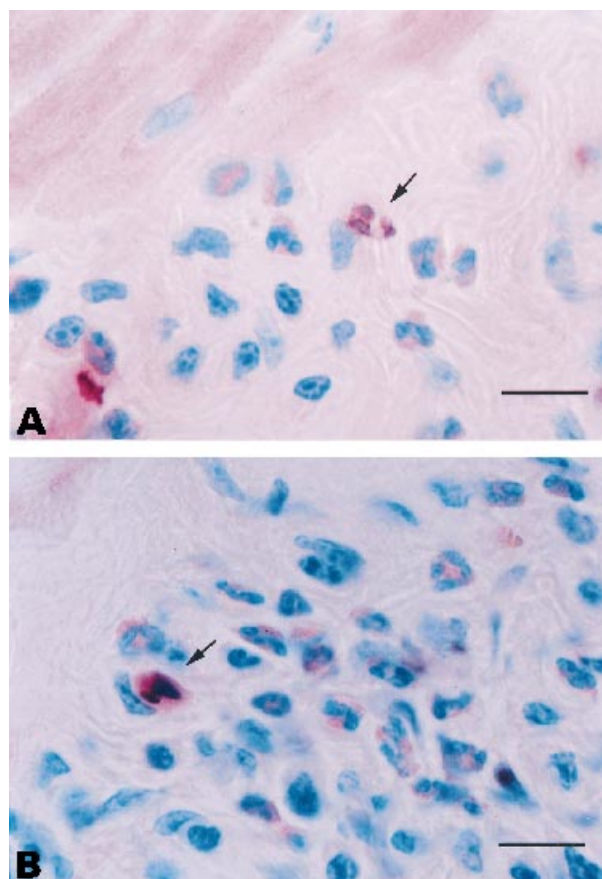


Fig. 4. The TUNEL-staining sections followed by counterstaining with Giemsa's solution obtained from sensitized mouse 7 days after challenge. A: An eosinophil, which has a fragmented nucleus, is TUNEL-positive, indicating apoptosis (arrow). Other eosinophils are not TUNEL-positive. B: A TUNEL-positive cell with pinkish-brown cytoplasm is present in the cluster of cells including eosinophils (arrow). The origin of this cell is not clear, but is most likely an eosinophil. Bar = 10 μ m.

exclude the possibility that inflammatory cells other than eosinophils were TUNEL-positive.

Late and prolonged induction of eosinophilic apoptosis

In our analysis of the kinetics of apoptotic cells in lung tissue, we showed that the percentage and absolute numbers of apoptotic cells, which do not usually exceed 1.5% of the total inflammatory cells in the sensitized mouse lung before challenge, are increased to 2.8% in the subepithelial area of mouse lung 1 week post-challenge but not in samples from mice prior to that time. The ratio of 1.5% corresponded well with the apoptosis ratio in the thymus and lymph nodes of normal mice (data not shown). The number of apoptotic cells apparently is very small (at most 2.8%) so that most

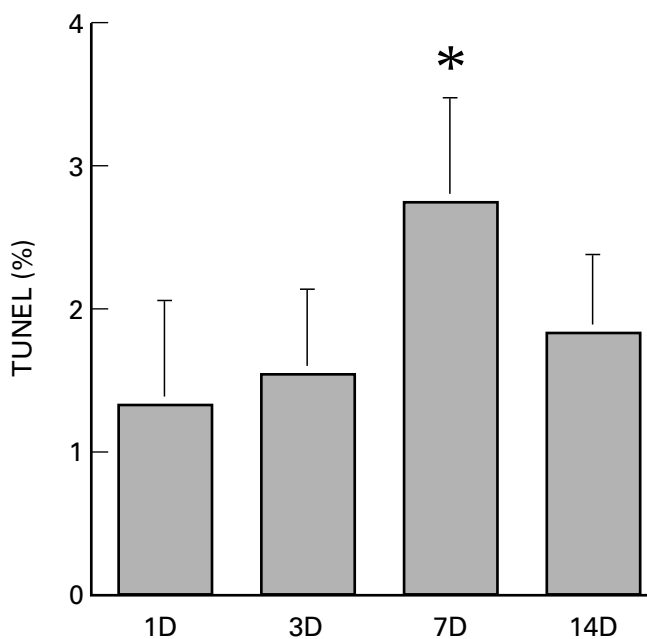


Fig. 5. The apoptotic ratio based on TUNEL-positive cells/all infiltrating cells in three adjacent sections of the lungs in the same lung sections obtained from sensitized mice following OVA challenge. An increase in the apoptosis ratio occurred on day 7 compared to that on day 1 or day 3. * $P < 0.01$.

cells after allergen challenge are not apoptotic and that apoptosis is a rare phenomenon. However, this ratio is rather significantly high, because the surviving cell number can decrease to half within 3 days, if the cells would undergo apoptosis by 3 h after receiving death signals. Previous *in vitro* study has demonstrated that in apoptosis induced by anti-Fas antibody, the total dying process occurs within 3.5 h [14,15]. Also, studies on the effect of Fas-ligation on eosinophil apoptosis have recently been reported [16–18]. The number of eosinophils presented in this study appeared to decrease to about half during the period of day 3 and 7 following allergen challenge, in good agreement with the result from the calculation mentioned above.

After OVA challenge, an increase in apoptotic cells was observed concomitantly with an increase in eosinophilic

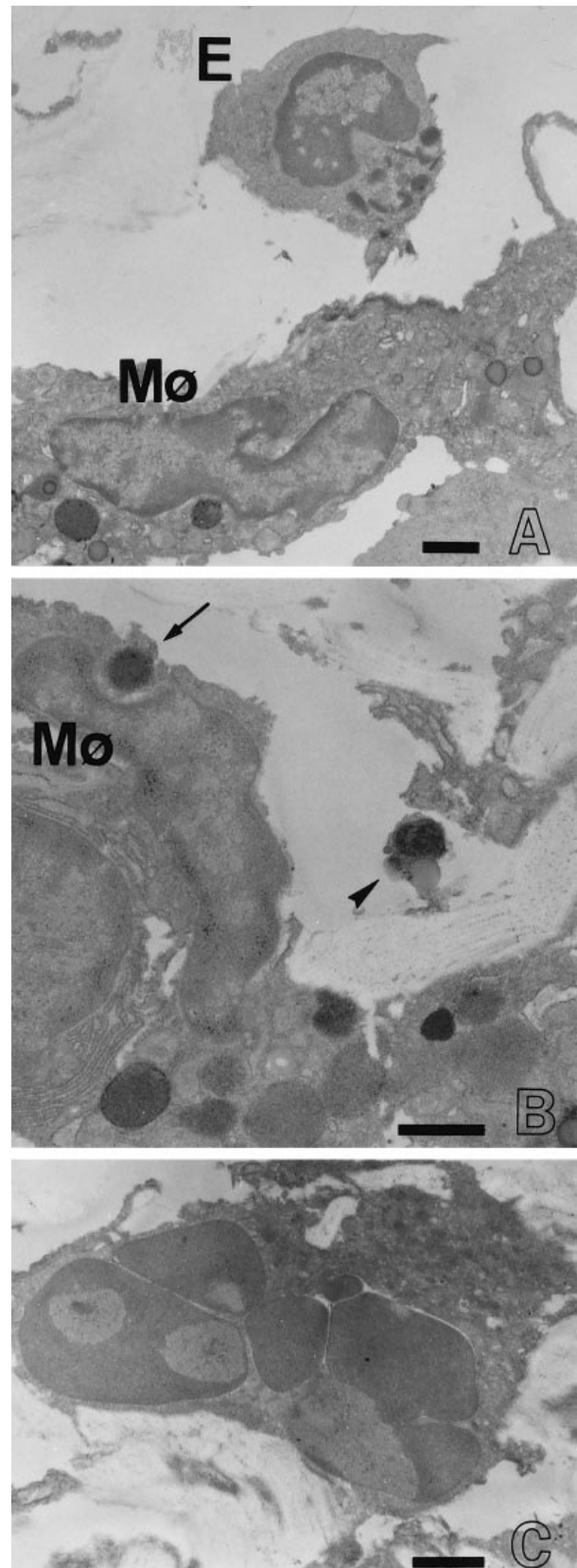


Fig. 6. Electron photomicrographs of apoptotic cells localized in the subepithelium of the bronchus obtained from a sensitized mouse 7 days after challenge. A: An eosinophil showing nuclear condensation and a macrophage containing apoptotic bodies in the cytoplasm are closely associated. B: A free apoptotic body (arrowhead) and apoptotic bodies phagocytosed by a macrophage. One apoptotic body is about to be ingested by a macrophage (arrow). C: An apoptotic granulocyte which has a segmented nucleus showing chromatin condensation. Bar = 1 μ m.

infiltration up to 3 days post-challenge. However, the apoptotic cell counts continued to increase after the eosinophil count peaked, indicating a late increase of apoptosis in the lung. In addition, TUNEL-positive cells were localized in the lung up to 14 days following the challenge, indicating prolonged induction of apoptosis following a single OVA challenge. The apoptotic ratio of total inflammatory cells or eosinophils was not different at day 1 or day 3 after OVA challenge, but increased at day 7 and at 2 weeks. This suggests that the apoptotic signalling changed after 3 days post-challenge.

Thus, one explanation is that apoptosis was suppressed during the early period and induced later. Another explanation is that the apoptotic signalling was more active later. It is likely that apoptosis was suppressed during the early period, because *in vitro* studies have demonstrated that haematopoietins such as GM-CSF, IL-3, or IL-5 inhibit apoptosis of eosinophils [5,19–21]. Such characterization of cell kinetics may account for the prolonged eosinophilic inflammation in bronchial asthma, although tissue eosinophilia is likely the result of both increased production of eosinophils in the bone marrow and decreased apoptosis [22].

Our results demonstrate the kinetics of inflammatory cell apoptosis for 2 weeks after a single OVA challenge of sensitized mice, possibly reflecting the events during a single asthma attack. Both corticosteroid and theophylline therapy may suppress airway inflammation of asthma by acting on inflammatory cells, including eosinophils [6,23–26]. However, the effects of these drugs on apoptosis *in situ* have not been established. The animal model of asthma which showed late and prolonged progression of eosinophilic apoptosis may be useful to develop new therapeutic strategies for bronchial asthma concerning optimal time for starting drugs, the selection of drugs, and the minimum period of administration.

Significance of eosinophilic apoptosis in bronchial asthma

Apoptosis is a physiological form of cell death [27] and is believed to be directly involved in the resolution of inflammation [28]. It has been shown both *in vitro* and *in vivo* that neutrophils undergo apoptosis, a process that leads to their ingestion as senescent, but intact cells by macrophages, without the release of phlogistic cell contents [29]. An *in vitro* study has suggested that apoptotic eosinophils also are removed from the tissue without releasing inflammatory mediators following ingestion by macrophages [2], one of the important mechanisms in the resolution of inflammation [30]. Our electron microscopic study has shown apoptotic bodies, some of which are ingested in the macrophages. Thus, this may represent the ultimate fate of inflammatory cells including eosinophils that migrate into lung tissue following OVA challenge. We also observed free

TUNEL-positive cells in the subepithelium. Some of these may have leaked into the airway and may have been detected as free apoptotic cells, without ingestion by macrophages, in bronchoalveolar lavage fluid [6]. The mechanisms for phagocyte recognition of cells undergoing apoptosis are under investigation [31–33].

In conclusion, our findings suggest that eosinophilic apoptosis is associated with the natural resolution of eosinophilia in allergic inflammation in mice. It is also possible that eosinophils might undergo cytolytic degeneration as has been reported recently by Cheng *et al.* [34]. However, the present model can be useful in the evaluation of anti-asthma drugs *in situ* with respect to their contribution to the regulation of apoptosis.

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