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Important role of neutrophils in the late asthmatic response in mice

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ABSTRACT

Aims: Neutrophils have been found increasingly in the lungs of patients with severe asthma; however, it is unclear whether the neutrophils contribute to the induction of the airway obstruction. We determined using a murine model whether neutrophils are involved in the late asthmatic response (LAR), and analyzed mechanisms underlying the antigen-induced airway neutrophilia.
Main methods: BALB/c mice sensitized by ovalbumin (OVA) + $Al(OH)_3$ were challenged 4 times by intratracheal administration of OVA. Airway mechanics were measured as specific airway resistance.
Key findings: Induction of the LAR after the 4th challenge coincided with airway neutrophilia. In contrast, eosinophil infiltration was established prior to the 4th challenge. A treatment with an anti-Gr-1 monoclonal antibody (mAb) before the 4th challenge selectively suppressed increases in the neutrophil number and myeloperoxidase (MPO) level in bronchoalveolar lavage fluid (BALF), and attenuated the magnitude of LAR by 60–70%. Selective suppression of eosinophilia by anti-IL-5 mAb had little effect on the LAR. The increases in neutrophil number and MPO level were partially inhibited by an anti-CD4 mAb treatment. The $CD4^+$ cell depletion also significantly inhibited increases in neutrophil chemoattractants, IL-17A, keratinocyte-derived chemokine (KC) and macrophage inflammatory protein (MIP)-2 in BALF. However, blockade of FcγRII/III failed to suppress the neutrophilia.
Significance: These data suggest that neutrophils are key inducers of the LAR, and that the antigen-induced neutrophilia is partially dependent on activated $CD4^+$ cells that are involved in the production of IL-17A, KC and MIP-2.

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Introduction

Asthma is characterized by an airway obstructive response that is based on chronic airway inflammation (Djukanovic et al., 1990). Eosinophils as well as $CD4^+$ T cells are major inflammatory cells that migrate into the lungs of asthmatic patients (Djukanovic et al., 1990), while neutrophils have been regarded as minor effector cells in the pathogenesis of asthma. There has been mounting evidence suggesting that neutrophils are increased in the lungs of some patients with chronic asthma and in some patients who have died after exacerbation of asthma (Sur et al., 1993; Hahy et al., 1995; Lamblin et al., 1998; Wenzel et al., 1997; Foley and Hamid, 2007). However, the functional role of neutrophils in the pathogenesis of asthma is still unclear and remains controversial.

The late asthmatic response (LAR), which is induced several hours after an allergen challenge, is regarded as one of the characteristic phenotypes of asthma (Varner and Lemanske, 2000). We have established a murine asthma model in which there is a late phase increase in airway resistance (Nabe et al., 2005). In this model, ovalbumin (OVA)-sensitized BALB/c mice were intratracheally challenged four times with the allergen. The LAR was induced after the 4th but not the 1st through 3rd challenges. Accumulation of eosinophils and $CD4^+$ T cells had been induced in the lungs by the time before the 4th challenge (Nabe et al., 2005). In contrast, neutrophilia was induced during the 2nd and 3rd challenges, but then disappeared prior to the 4th challenge. The 4th challenge induced a recurrence of neutrophilia (Nabe et al., 2005). Based on these findings, we hypothesize that the cellular mechanism for the development of the LAR is as follows. The 1st through 3rd challenges are responsible for establishing the airway inflammation that is characterized by infiltrations of eosinophils and $CD4^+$ T cells. Subsequently, the 4th challenge induces neutrophil infiltration into the airway, which contributes importantly to the induction of the LAR.

In this study, we examined the association of neutrophils with the induction of the LAR. Since Gr-1 is known to be predominantly expressed on neutrophils, we used RB6-8C5, an antibody against Gr-1 (Egan et al., 2008), to deplete neutrophils. However, because it is

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known that Gr-1 is also expressed on other leukocytes such as eosinophils and mononuclear cells (MNC) in mice (Lopez, 1984; Fleming et al., 1993; Nagendra and Schlueter, 2004), we used a flow cytometer to examine the composition of the Gr-1$^+$ cells that infiltrated the lung. Since our initial results indicated that eosinophils were the other Gr-1$^+$ cells, we assessed their role by examining the selective suppression of eosinophilia using an anti-IL-5 monoclonal antibody (mAb). Additionally, whether the airway neutrophilia and production of neutrophil chemoattractive cytokines and chemokines are dependent on antigen-induced CD4$^+$ cell activation was investigated using an anti-CD4 mAb. Also, whether FcγRII/III activation by antigen–antibody immune complexes was involved in the neutrophilia was assessed by using an anti-FcγRII/III mAb.

Materials and methods

Sensitization and challenge

Six-week-old BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). The mice were sensitized by intraperitoneal (i.p.) injections with OVA (Grade V, Sigma Chemical Co., St. Louis, MO) adsorbed to $Al(OH)_3$, which was made according to our previously reported method (Nabe et al., 2005), at a dose of 50 μg OVA adsorbed to 2 mg $Al(OH)_3$/0.5 ml of PBS/animal on days 0, 14 and 28. The sensitized mice were challenged on days 35, 36, 37 and 40 under inhalation anesthesia with isoflurane (Abbott Japan, Tokyo, Japan) with 2% OVA at a volume of 25 μl by intratracheal administration, as reported previously (Ho and Furst, 1973). As a negative control group, mice that had been sensitized with OVA $Al(OH)_3$ i.p. were treated with 4 mock airway challenges with PBS alone (Sensitized-Non-Challenged, S-NC).

This animal study was approved by the Experimental Animal Research Committee at Kyoto Pharmaceutical University.

Measurement of pulmonary function

Two different indicators of airway resistance, specific airway resistance (sRaw: $cmH_2O \times ml/(ml/s)$) and/or enhanced pause (Penh). "Penh" has been known to be a unit-less index, which can be obtained in laboratory animals using whole body plethysmography (Hamelmann et al., 1997) (Buxco Electronics, Troy, NY). It is the unique indicator of airway responsiveness and resistance that can be obtained in conscious unrestrained animals and therefore is freed from the bias imposed by anesthesia or restraining stress. However, it was also suggested that Penh could not be a reliable parameter of airway resistance (Bates et al., 2004). Thus, we used not only Penh but also "specific airway resistance (sRaw: $cmH_2O \times ml/(ml/s)$)", which can be obtained by double-flow plethysmography (Pennock et al., 1979; Flandre et al., 2003) (Pulmos-I. II. III, M.I.P.S., Osaka, Japan). These parameters were measured 1 h before and at various times (10 and/or 30 min, and 1, 2, 3 and 4 h) after the 4th OVA challenge. Both systems are non-invasive technologies that make it possible to assess pulmonary function longitudinally over a prolonged time course.

To increase the reliability and reproducibility of the plethysmography data collected, all measurements were made in an environment controlled for both temperature (21–24 °C) and humidity (45–65%).

Treatment with mAbs

In accordance with a previously reported method, a single intraperitoneally administered dose (150 μg/animal) of either an anti-Gr-1 mAb (RB6-8 C5, eBioscience, San Diego, CA), an isotype-matched control Ab, rat IgG2b (eBioscience) or rat IgG (Sigma, St. Louis, MO), or PBS was given 18 h prior to the 4th antigen intratracheal challenge (Czuprynski et al., 1994).

The anti-IL-5 mAb-producing hybridoma cells, TRFK-5, were kindly donated by Prof. Kohtaro Fujihashi (University of Alabama at Birmingham, Birmingham, AL). The hybridoma cells were cultured and grown, and then intraperitoneally injected in severe combined immunodeficiency mice (Clea Japan, Tokyo, Japan) that had been treated with pristane (Sigma). Ascites was collected 10–14 days after the injection. IgG was purified by protein G affinity chromatography (GE Healthcare, Uppsala, Sweden), followed by desalting using PD-10 column chromatography (GE Healthcare). The purified anti-IL-5 mAb or rat IgG was intraperitoneally administered a single time at 3 h prior to the 1st antigen challenge at doses of 50, 100 and 200 μg/animal.

A cell line producing rat IgG2b mAbs that recognize the murine CD4 (YTS191.1.2) molecule were kindly provided by Prof. Osami Kanagawa (Washington University, St. Louis, MO). The anti-CD4 mAb was produced and purified as described above. The purified anti-CD4 mAb or rat IgG was intraperitoneally administered a single time at 18 h prior to the 4th antigen challenge at a dose of 0.6 mg/animal.

Anti-FcγRII/III mAb-producing hybridoma cells, 2.4G2, were purchased from American Type Culture Collection (Manassas, VA). The anti-FcγRII/III mAb was produced and purified as described above. The purified anti-FcγRII/III mAb or rat IgG was intraperitoneally administered a single time at 1.5 h prior to the 4th antigen challenge at a dose of 1 mg/animal.

Analysis of cells recovered by bronchoalveolar lavage (BAL)

Mice were sacrificed by lethal intramuscular injection using 50 μl of a mixture of ketamine (25 mg/ml) and xylazine (10 mg/ml). The pulmonary circulation was perfused using 5 ml PBS, with the lungs then lavaged via a tracheal catheter using two aliquots of 0.8 ml PBS containing 2% FBS. Total leukocyte numbers were determined by a particle counter and size analyzer (Z2, Beckman Coulter, Brea, CA) after treatment with ACK lysis buffer to remove any contaminating erythrocytes. For the differential cell counts, cells were transferred onto a glass slide by centrifugation in a cell settling chamber (Neuro Probe, Gaithersburg, MD). Subsequently, cells were then stained with Diff-Quik solution (Sysmex International Reagent, Kobe, Japan).

The numbers of CD4$^+$ and CD8$^+$ cells in BALF were measured by a flow cytometry as previously reported (Nabe et al., 2005). In brief, after incubation with anti-mouse FcγRII/III mAb (clone 2.4G2, BD Biosciences, San Diego, CA) to block binding of subsequent antibodies to FcγRII/III, cells were incubated with PE-conjugated anti-mouse CD8α mAb (clone 53–6.7) and Cy-Chrome-conjugated anti-mouse CD4 mAb (clone H129.19) (both from BD Biosciences). After washing, the stained cells were fixed with 4% paraformaldehyde, and then analyzed using a FACSCalibur (BD Biosciences) and Cell Quest software (version 3.3, BD Biosciences).

Flow cytometric analyses of Gr-1$^+$ cells

Composition of Gr-1$^+$ cells in the BAL fluid (BALF) was analyzed using flow cytometry and morphological observation. In brief, BAL cells were first incubated with the anti-mouse FcγRII/III antibody for 20 min at 4 °C. The cells were then incubated with FITC-labeled anti-Gr-1 mAb (RB6-8 C5, eBioscience) or FITC-labeled rat IgG2b (eBioscience) at 5 μg/ml for 20 min at 4 °C. After washing three times with PBS supplemented with 2% FBS, the stained cells were fixed with 4% paraformaldehyde for 12–18 h, and then analyzed using FACSCalibur and Cell Quest software. Dot plot for Gr-1$^+$ cells was expressed by FL1 and FL2 after precise compensation.

For analysis of the Gr-1$^+$ cell morphology, the cells were sorted using a FACSCalibur, centrifuged onto a glass slide, and then stained with Diff-Quik.

Histological studies

In separate experiments from BAL study, the lung was isolated before or 4 h after the 1st or 4th challenge. The isolated lung was fixed

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