Decreased Expression of Th2 Type Cytokine mRNA Contributes to the Lack of Allergic Bronchial Inflammation in Aged Rats

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Sensitized Brown Norway rats are known to develop eosinophilic bronchial inflammation and airway hyperresponsiveness after Ag exposure. However, we have previously observed that sensitized aged rats of the same strain failed to develop such allergic inflammation. In the present study, we investigated age-associated changes of cytokine mRNA expression in bronchoalveolar lavage (BAL) cells. Both young (8- to 10-wk-old) and aged (100- to 120-wk-old) Brown Norway rats were sensitized with OVA, and BAL was performed 24 h after OVA inhalation challenge. Semiquantitative RT-PCR analysis of BAL cells showed that the cells from aged rats preferentially expressed Th1 type cytokine (IFN-γ) mRNA, whereas cells from young animals expressed more Th2 type cytokine mRNAs including those for IL-4 and IL-5. Decreased expression of Th2 type cytokine transcripts in aged animals was further confirmed by quantitative analysis, competitive RT-PCR of BAL cells, and in situ hybridization. The age-associated changes of cytokine profile were not restricted to BAL cells but were a general feature of lymphocytes, as shown by examination of popliteal lymph node draining the site of sensitization. These findings suggest that decreased allergic inflammation in aged animals is attributable to age-dependent impairment of Th2 generation in response to Ag.


B ronchial asthma is based on allergic bronchial inflammation, which is characterized by eosinophil accumulation in the airways (1, 2). Recent evidence suggests that T cell activation may proceed to a certain direction, resulting in a characteristic cytokine profile in allergic reactions (3, 4). Various data obtained in humans and lower animals have indicated that Th2 cells, which produce IL-4 and IL-5, are dominant in allergic bronchial inflammation (4, 5). IL-4 plays a critical role in not only IgE synthesis by B cells (6) but also differentiation of naive T cells to Th2 (7, 8). IL-5 has been reported to induce eosinophil production in bone marrow (9), accumulation of eosinophils in the airways (10), and release of eosinophil cationic proteins that damage the respiratory epithelium (11). On the other hand, IFN-γ, a representative Th1 cell cytokine, is known to have an inhibitory effect on Th2-related responses (8, 12). Thus, the balance between Th1 and Th2 cytokine profiles could modify the immune response at sites of inflammation (3, 13).

The severity of allergic disorders such as bronchial asthma, allergic rhinitis, and atopic dermatitis is considered to decline with age (14, 15). Possible explanations for this change include our previous observation that aged animals failed to produce Ag-specific IgE Ab and to generate eosinophil influx into sites of inflammation in response to Ag stimulation (16). However, the mechanisms remain to be elucidated. In this study, we investigated whether age-associated alterations in the production of cytokines, including IL-4, IL-5, and IFN-γ, were involved in the decreased allergic response of aged rats with OVA-induced asthma. We studied differences of cytokine mRNA expression in the bronchoalveolar lavage (BAL) cells of young and aged rats using the RT-PCR method and found that expression of mRNA for Th2 type cytokines (IL-4 and IL-5) was selectively decreased in the aged rats compared with young animals. We also confirmed histologically that the number of IL-5 mRNA-expressing cells was decreased in the lungs of aged rats using the in situ hybridization technique.

Materials and Methods

Animals
Young (8- to 10-wk-old) and aged (100- to 120-wk-old) male Brown Norway (BN) rats were obtained from Charles River (Tokyo, Japan). The rats were kept at the animal research facility of Hamamatsu University School of Medicine in an air-filtered, specific-pathogen-free environment during these experiments.

Sensitization with OVA and inhalation challenge
Both young and aged rats were sensitized by a s.c. injection into the foot pads of 0.25 mg/kg OVA (Sigma, St. Louis, MO) and alum containing 20 mg/kg Al(OH)3 (provided by Dr. H. Nagai, Gifu Pharmaceutical University, Gifu, Japan) in borate-buffered saline. At the same time, 0.5 ml of Bordetella pertussis vaccine (Wako Pure Chemical Industries, Osaka, Japan) containing 1 × 1010 heat-killed bacilli in PBS (pH 7.4) was administered i.p. as an adjuvant. After 7 days, 0.125 mg/kg OVA in PBS was injected s.c. Fourteen days after the first sensitization, rats were challenged with an aerosol of 5% OVA for 10 min under pentobarbital sodium anesthesia. We also conducted experiments using challenge with 5% BSA (Sigma) to confirm that OVA-induced responses were Ag specific.

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Abbreviations used in this paper: BAL, bronchoalveolar lavage; pLN, popliteal lymph node.
**Harvesting of BAL fluid and cell analysis**

Rats were sacrificed 24 h after OVA challenge by exsanguination under ether anesthesia. The lungs were removed intact from rats, and BAL was performed with 5 × 3 ml of HBSS (Sigma) through a polyethylene tube introduced into the trachea. The BAL fluid was centrifuged at 200 × g for 10 min, and cell pellets were resuspended in 1 ml of HBSS. BAL fluid supernatants were studied for ELISA. The total cell count was determined, and aliquots of the suspensions were used for preparation of cytocentrin slides to assess the cell differentiation. The rest of each cell suspension was re-centrifuged, and the cell pellet was prepared for RNA extraction. In another set of experiments, the lungs were harvested, cut into small pieces, fixed with 4% paraformaldehyde in PBS for 16 h, and immersed in 30% sucrose in PBS for 8 h. Sections were cut, embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan), and stored at −80°C until use for in situ hybridization.

**Popliteal lymph node cell culture**

To investigate age-associated changes in the systemic response of lymphocytes to immunization, popliteal lymph nodes (pLN), which were the draining nodes for the OVA administration site, were excised 2 wk after the first sensitization. pLN cells were prepared at 1 × 10⁷/ml in RPMI 1640 (Sigma) containing 5% FCS (Life Technologies, Gaithersburg, MD). Then 2-ml aliquots of the suspensions were cultured in a 24-well flat-bottom culture plate at 37°C under a humidified atmosphere of 5% CO₂ in air with or without OVA at a final concentration of 1 mg/ml. Cells were washed at 3, 6, 9, 12, 18, and 24 h after the start of incubation; washed twice; and used for preparation of mRNA. Cell viability was tested by trypan blue exclusion and always exceeded 90%. The supernatants of cultured pLN cells of 24 h of incubation were obtained for ELISA study.

**Flow cytometric analysis of popliteal lymph node cells**

An aliquot of the pLN cell suspension was used for analysis of cell surface Ag expression by direct immunofluorescence method with a flow cytometer (Epics Profile II, Coulter, Hialeah, FL). The number of fluorescence-positive cells per 5000 cells was determined. Briefly, pLN cells (1 × 10⁷/ml) were incubated for 30 min in the dark with a saturating concentration of FITC-labeled anti-rat CD5 (OX-19) or PE-labeled anti-rat CD45RA (OX-33) to detect CD5⁺T cells and B cells, respectively. Then the cells were washed twice with HBSS containing 0.5% BSA (Sigma). The mAbs were purchased from PharMingen (San Diego, CA).

**RNA preparation and cDNA synthesis**

Total cellular RNA was isolated from BAL cells and pLN cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (17). The RNA was precipitated by centrifugation at 4°C, washed once with 70% ethanol, air-dried, and suspended in diethylpyrocarbonate-treated water. The amounts of RNA was determined by spectrophotometry, and 2 μg total cellular RNA were used for the first-strand cDNA synthesis with Moloney murine leukemia virus reverse transcriptase (200 U, Life Technologies) and random hexanucleotide (100 nM). After terminating the reaction by heating at 70°C for 5 min, distilled water was added up to 100 μl.

**PCR amplification**

The expression of IL-2, IL-4, IL-5, IL-10, and IFN-γ mRNA was studied by PCR amplification. β-Actin was also amplified as a control for constitutive gene expression. Gene-specific oligonucleotide primers were designed using the published website on the rat cDNAs for the individual cytokines and β-actin obtained from GenBank. The primer sequences were as follows: IL-2, sense, 5'-CATTGTGAGGAGATGATGC-3' ; antisense, 5'-CATTGTGAGGAGATGATGC-3'; IL-4, sense, 5'-ACCTTGGCTGACTTTTACTCAG-3' (408-bp product); IL-4, sense, 5'-ACCTTGGCTGACTTTTACTCAG-3' (352-bp product); IL-5, sense, 5'-GGTTTGAGCGTGGACTCATTCACG-3' (408-bp product); IL-5, sense, 5'-GGTTTGAGCGTGGACTCATTCACG-3'; IL-10, sense, 5'-GCTTTTCTTCGGAAGACTCAG-3' (408-bp product); IL-10, sense, 5'-GCTTTTCTTCGGAAGACTCAG-3'; IFN-γ, sense, 5'-GAAAGCTTAGAAGTCTGAAAGAAC-3' (352-bp product); IFN-γ, sense, 5'-GAAAGCTTAGAAGTCTGAAAGAAC-3'; IL-12, sense, 5'-TGCACAGCACCTTTACGAAGC-3' (352-bp product); IL-12, sense, 5'-TGCACAGCACCTTTACGAAGC-3'.

PCR amplification was performed by adding 2.5-μl aliquots of each cDNA sample to 22.5 μl of reaction mixture (50 mM KCl, 10 mM Tris-HCl (pH 8.8), 0.1% Triton X-100, 0.4 mM concentrations each of dNTP, 3.0 mM MgCl₂ for IL-5, and 1.5 mM for other cytokines, 0.2 μM forward and reverse gene-specific primers) containing 1.25 U of Taq DNAopolymerase.
merase (Nippongene, Tokyo, Japan). Amplification was done in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT) under the following conditions: denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 2 min, with final extension at 72°C for 4 min. We determined the optimum number of cycles for each primer set so that the specific product was amplified during the exponential phase of amplification. Based on the results of preliminary studies, 40 cycles were used for IL-2 and IL-4, whereas IL-5, IL-10, and IFN-γ were run for 32 cycles, and β-actin was amplified for 26 cycles. Negative control reactions performed without RT yielded no detectable fragments with any primer pair.

**Results**

**Time course study of cellular differentiation and cytokine mRNA expression in BAL cells after OVA challenge in young rats**

To determine the optimal conditions, we conducted preliminary time course study of cellular differentiation and cytokine mRNA levels in BAL cells after OVA inhalation challenge in young rats (Table I).

BAL analysis revealed that neutrophils significantly increased, with a peak at 6 h after OVA provocation, whereas the number of eosinophils was elevated with a peak 48 h later. The number of lymphocytes gradually increased 48 h after inhalation challenge. Control animals, which underwent BAL 24 h after saline inhalation without OVA, failed to exhibit increased number of eosinophils, although there were mild increases in the number of neutrophils and macrophages. Exposure to BSA did not cause any significant changes in BAL findings compared with saline inhalation group.

Semi-quantitative RT-PCR analysis of BAL cells showed that the expression of IL-4 and IL-5 mRNA increased up to 24 h after Ag challenge and then decreased at 48 h (Table II). Whereas transcripts of IL-10 and IFN-γ reached a plateau at 24 through 48 h, there was a nonsignificant change of IL-2 mRNA levels after OVA provocation. The BSA inhalation failed to induce significant changes in cytokine mRNA expression compared with saline inhalation (Table II). On the basis of these data, we performed subsequent analysis of BAL cell cytokine mRNAs at 24 h after OVA inhalation challenge.

**BAL cell population differences between young and aged rats**

We confirmed that there was no increase in the number of eosinophils in aged rats 24 h after OVA challenge (Fig. 1), a finding consistent with our previous observation (16). The number of eosinophils in aged rats was approximately one-tenth of that seen in young rats (Fig. 1). The number of neutrophils and macrophages was also higher in the young group, whereas there was no significant difference of lymphocytes between the two groups (Fig. 1).

**BAL cell cytokine expression differences between young and aged rats**

**Expression of mRNA.** Fig. 2 shows cytokine mRNA expression in BAL cells from young and aged rats at 24 h after Ag exposure.

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**Table II. Time course study of cytokine mRNA expression in BAL cells after inhalation challenge in young rats**

<table>
<thead>
<tr>
<th>Time After Challenge (h)</th>
<th>OVA Exposure</th>
<th>Saline Exposure</th>
<th>BSA Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td><strong>mRNA expression (ratio/β-actin)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>0.11±0.08</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.06±0.01</td>
<td>1.00±0.10*</td>
<td>0.95±0.11*</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.10±0.02</td>
<td>0.34±0.04*</td>
<td>1.19±0.22*</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.14±0.02</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.05±0.01</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Cytokine mRNA expression levels in BAL cells from young rats after inhalation challenge were analyzed by semi-quantitative RT-PCR as described in Materials and Methods. Results are the mean ± SEM from six independent experiments. *, p < 0.05 compared with before inhalation challenge. †, p < 0.05 compared with saline and BSA exposure.
Expression of IL-4 and IL-5 mRNAs was significantly higher in the young group than in the aged group, whereas IFN-γ mRNA was higher in the aged rats. The expression of IL-2 and IL-10 mRNAs did not differ between the groups.

Quantification of IL-4 and IL-5 expression in BAL cells. Accurate quantitative analysis of IL-4 and IL-5 transcripts in BAL cells was done by competitive RT-PCR using internal standard cDNA and specific primers. Data obtained by this method confirmed that young rats exhibited significantly higher levels of both cytokine mRNAs than aged rats (Fig. 3).

ELISA analysis for IL-4 and IFN-γ in BAL fluids. ELISA analysis confirmed that the protein levels of IL-4 in BAL fluid supernatants were significantly higher in young rats than aged rats (young vs aged, 18.36 ± 2.19 vs 3.26 ± 0.43, p < 0.01). However, we could not detect IFN-γ with ELISA in BAL fluid supernatants from both groups.

Histological localization of IL-5 mRNA expression

We performed in situ hybridization to detect localization of the cells expressing IL-5 mRNA. There was a substantial number of IL-5-positive cells in the peripheral airways of lungs from the young rats (Figs. 4, A and B). In contrast, there were few IL-5-positive cells in the lungs of the aged rats (Figs. 4, C and D). The quantitative analysis of the in situ hybridization showed that the number of IL-5 mRNA-positive cells per length of basement membrane was significantly larger in the tissue from young rats than those from aged rats (young vs aged, 40.74 ± 4.58 vs 6.35 ± 0.73, p < 0.01).

Ag-specific cytokine expression by cultured pLN cells

Expression of cytokine mRNA. To further analyze the age-related differences of cytokine expression shown by Ag-stimulated lymphocytes, we tested in vitro cytokine mRNA expression by pLN cells. These pLN cells showed no significant difference in the proportion of OX-19-positive cells between young and aged animals (young vs aged, 49.0 ± 4.0% vs 40.8 ± 1.3%) by flow cytometric analysis. In addition, May-Giemsa staining of pLN cells revealed that there were few eosinophils among these cells in both groups of animals.

Sensitized pLN cells were incubated in the presence or absence
of OVA, and cytokine mRNAs were studied by semiquantitative RT-PCR analysis. When the cells were cultured without OVA, there was no significant increase of IL-5 mRNA expression during the 24-h incubation period (Fig. 5A). However, incubation with OVA caused a significant increase of IL-5 mRNA expression in the young rats, but not in the aged rats. In contrast, there was no age-dependent difference in the expression of $\beta$-actin mRNA (Figs. 5A and 6).

In the case of IL-4 and IFN-$\gamma$, freshly harvested cells expressed the mRNAs at a certain level in both groups (Figs. 5, B and C). However, when the cells were incubated with OVA, enhanced expression of IL-4 mRNA was seen in the young rats, and increased IFN-$\gamma$ mRNA expression was observed in the aged rats after 24 h of incubation (Figs. 5, B and C). In contrast, the cells lost their expression of these mRNAs during incubation without OVA.

**Discussion**

BN rats are known as a high IgE responder strain (19, 20) and are used as a model of bronchial asthma because sensitized BN rats are reported to show airway hyperresponsiveness and eosinophilic inflammation after Ag exposure (21, 22). Studies of these rats have revealed that activated CD4$^+$ T cells play an essential role in the development of allergic inflammation by producing Th2 cytokines such as IL-4 and IL-5 (23, 24). In contrast, Sprague Dawley rats, which have been documented to fail to develop airway hyperresponsiveness and pulmonary eosinophilia, preferentially generate Th1 type cytokines (25). Taken together, these results suggest that the direction of CD4$^+$ T cell differentiation (Th1 or Th2) in response to Ag stimulation is critical in the development of allergic bronchial inflammation.

We previously reported that OVA-sensitized aged rats failed to exhibit Ag-specific eosinophilic bronchial inflammation with decreased airway reactivity to Ag exposure. In addition, serum OVA-specific IgE levels were also significantly lower in aged rats than in young ones when both groups were sensitized in the same way (young vs aged, 0.698 ± 0.10 vs 0.446 ± 0.06 (absorbance unit), $p$, 0.05) (16). In the present study, we focused on age-related changes of cytokine profile in BN rats. We observed that mRNAs for Th2 type cytokines (IL-4 and IL-5) were decreased in BAL cells from aged rats, whereas the level of a Th1 type cytokine (IFN-$\gamma$) was higher in the aged rats than in the young rats after Ag challenge. Furthermore, our in vitro study of cytokine profile in

**Table III. Concentration of IL-4 and IFN-$\gamma$ in supernatant of cultured pLN cells**

<table>
<thead>
<tr>
<th></th>
<th>IL-4 (pg/ml)</th>
<th>IFN-$\gamma$ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA (+)</td>
<td>9.32 ± 0.93*</td>
<td>43.3 ± 32.6†</td>
</tr>
<tr>
<td>OVA (-)</td>
<td>2.27 ± 0.43*</td>
<td>15 &gt;</td>
</tr>
<tr>
<td>Aged</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA (+)</td>
<td>564.2 ± 101.6†</td>
<td>15 &gt;</td>
</tr>
<tr>
<td>OVA (-)</td>
<td>2 &gt;</td>
<td>15 &gt;</td>
</tr>
</tbody>
</table>

*a The concentration of each cytokine in supernatant was determined by ELISA.

The cells were incubated with or without OVA for 48 h. Data are expressed the mean ± SEM from four independent experiments. $*, \; p < 0.01$. OVA (+), cultured with OVA; OVA (-), cultured without OVA.
OVA-stimulated pLN cells showed that cells from aged rats preferentially expressed Th1 type cytokine in response to Ag stimulation, whereas young rats predominantly expressed Th2 type cytokines. These findings were consistent with our previous in vitro experiments that BCG-primed splenocytes from aged Sprague Dawley rats produced more IFN-γ than those from the young animals in response to BCG restimulation (26). Based on these findings, it is suggested that age-related changes of cytokine profile can be attributed to the decreased allergic reaction with aging.

IL-5 is considered to be a key effector cytokine in eosinophilic infiltration of the allergic inflammatory sites (9, 27). This would be consistent with our previous observation that pretreatment of young rats with an anti-IL-5 Ab suppressed pulmonary eosinophilia (16). The present study further indicated that IL-5 mRNA expression in BAL cells and the number of IL-5-positive cells in the lungs were decreased in aged animals along with the failure to elicit allergic inflammation. Although previous reports have indicated that IL-5 is detected not only in CD4+ T cells (known to be the major source in allergic inflammation (4, 28)) but also in mast cells (29) and eosinophils (30), we did not assess exactly how each of these cell types contributed to IL-5 mRNA expression in the present study. However, our in vitro experiments with Ag-primed pLN cells, which contained few eosinophils and mast cells, provided evidence that an age-associated decline of the capacity in lymphocytes to express IL-5 mRNA was involved in the decrease of IL-5 mRNA expression in BAL cells from aged rats.

IL-2 is known to be essential for the proliferation and clonal expansion of Ag-specific T cells (31). On the other hand, IL-10 was originally considered to be a predominately Th2 type cytokine in mice (3), although its role in allergic reaction remains unclear. We found no significant changes in the expression of IL-2 and IL-10 mRNA in BAL cells from young and aged rats, suggesting that these cytokines are not intimately involved in age-associated alterations of allergic inflammation.

Some investigators have suggested that cytokines produced by CD8+ T cells have suppressive effect on allergic inflammation (32). In this regard, we have previously demonstrated that there was no difference in the CD4:CD8 ratio of BAL cells from young and aged rats (16). Thus, it seems unlikely that a change in the CD4:CD8 balance was involved in the producing different cytokine profiles of the young and aged animals, although this observation by no means excludes the possibility that aged rats show functional dominance of CD8+ T cells.

It has been widely recognized that the alterations in immune system during the aging process are mainly characterized by quantitative and qualitative changes of lymphocytes, especially T cells (33, 34). There is a large body of literature showing that T cells from aged humans and rodents have a decreased ability to proliferate and to yield clonal expansion (31, 33), which appear to be partially caused by the impairment of cytokine-dependent signals transduction in aged T cells (35). With respect to age-related alterations of cytokine profiles, Hobbs et al. (36) have reported that IFN-γ and IL-4 transcripts were selectively suppressed in aged animals.

The molecular mechanism underlying age-associated changes of the cytokine profile has proved elusive. The early decision toward Th1 and Th2 cells in immune response depends on the balance between IL-12 or IFN-γ (39, 40), which favors Th1 responses, and IL-4 or IL-10, which favors Th2 responses (41). Thus, Th1 differentiation begins with the production of IL-12 by macrophages and the subsequent induction of IFN-γ production by NK cells (40). However, the sources of the initial IL-4 needed to induce Th2 development have not been clearly defined, although candidate sources of IL-4 include NK CD4+ T cells, CD4+ memory T cells, eosinophils, and mast cell/basophil lineage. It is therefore conceivable that dysfunction of IL-4 production by these cells during sensitization may be involved in age-associated changes of the cytokine profile. Taken together, our findings suggest that decreased Th2 type cytokine might be responsible for the suppression of allergic reactions in aged animals.

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