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Leucocyte kinesis in blood, bronchoalveoli and nasal cavities during late asthmatic responses in guinea-pigs

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Leucocyte kinesis in blood, bronchoalveoli and nasal cavities during late asthmatic responses in guinea-pigs. T. Nabe, N. Shinoda, K. Yamashita, H. Yamamura, S. Kohno. ©ERS Journals Ltd 1998.

ABSTRACT: Recently, we reported a reproducible model of asthma in guinea-pigs *in vivo*, which developed a late asthmatic response (LAR) as well as an early response. In this study, time-related changes in the occurrence of the LAR and leucocyte kinesis were assessed. Furthermore, the state of the activation of eosinophils that migrated into the lower airways was characterized *in vitro*.

Guinea-pigs were alternately sensitized/challenged by inhalation with aerosolized ovalbumin adsorbed on aluminium hydroxide and ovalbumin alone, once every 2 weeks. At defined times before and after the fifth challenge, airway resistance was measured, blood was drawn and bronchoalveolar lavage (BAL) and nasal cavity lavage (NCL) were performed. Superoxide anion ($\cdot O_2^-$) production of eosinophils was measured with cytochrome *c*.

Occurrence of LAR and considerable increases in circulating eosinophils coincided with each other 5–7 h after the challenge. After 7 h, eosinophil infiltrations into bronchoalveolar spaces were observed. The capacity of eosinophils from the sensitized animals to produce $\cdot O_2^-$ was higher than those from the non-sensitized ones, when eosinophils were stimulated by platelet-activating factor. Although an increased number of eosinophils in the NCL fluid was observed, it was much less than that in the BAL fluid.

Thus, it has been concluded that eosinophilia in the blood and the lung may participate in the occurrence of the late asthmatic response, which is thought to be preferentially evoked in the lower airways in guinea-pigs *in vivo*.
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The late asthmatic response (LAR), one of the features of bronchial asthma in addition to the early asthmatic response (EAR) and bronchial hyperresponsiveness, is observed clinically 3–4 h after allergen inhalation and reaches its maximum 7–8 h later [1]. Many investigators have suggested that the appearance of LAR is related to the degree of lung eosinophilia [2–4]. Thus, it is generally accepted that a possible mechanism for LAR involves the following chain reaction of events: 1) activations of mast cells and T lymphocytes by inhaled antigen result in the release of various chemotactic factors including lipid mediators and cytokines from these cells; 2) these mediators induce blood eosinophilia and neutrophilia, and cause these cells to infiltrate into the airway; 3) these activated granulocytes release or generate various cytotoxic granule proteins, bronchoconstrictive mediators and other substances; and 4) consequently, LAR and airway hyperresponsiveness are induced by these mediators. However, it is still unclear whether LAR is predominantly caused by the activated leucocytes, especially eosinophils.

To date, abundant experimental asthmatic models showing LAR have been developed using various species [5–7]. Recently, we established an asthmatic model in guinea-pigs [8]. Animals were sensitized and challenged by altern-ate inhalations of mists of the respective antigen (ovalbumin (OA)) adsorbed on aluminium hydroxide ($Al(OH)_3$) gels (OA+$Al(OH)_3$) and OA alone, once every 2 weeks. After the inhalation challenge, at the third and 10th OA challenge with reproducibility, EAR and LAR were observed after 10 min and 5–7 h, respectively. An intriguing finding is that these obstructive airway responses in this model preferentially occur in the lower airways rather than in the upper airways. When the antigen mists with diameters of approximately 2.0 µm produced with a hand-made pressure nebulizer were used in this model, approximately 80% of the mists trapped in the whole airways were found in the lung, while most of the inhaled mists were trapped in the nasal cavity when mists were generated with a commercial ultrasonic or pressure nebulizer that produced lar-ger diameter particles [9].

In the present study, detailed time-course changes of leucocyte number in the blood, bronchoalveolar spaces and nasal cavities as well as those of pulmonary function after the fifth antigen inhalation challenge were investigated to analyse how the behaviour of the leucocytes may relate to the occurrence of the LAR, and to confirm that the pulmonary dysfunction preferentially occurred in the lower airways. Furthermore, eosinophils that migrated into bronchoalveolar spaces were purified, and their responsiveness to platelet-activating factor (PAF) was evaluated by monitoring superoxide anion ($\cdot O_2^-$) production to characterize their activation state.

Materials and methods

Animals

Male, Hartley guinea-pigs aged 3 weeks and weighing 250–300 g were purchased from Japan SLC, Hamamatsu, Japan. The animals were housed in an air-conditioned room at a temperature of 23±1°C and 60±10% humidity, illuminated from 08:00–20:00 h, fed a standard laboratory diet and given water *ad libitum*. The first sensitization was started 2 weeks after the purchase.

Reagents

Reagents and their sources were as follows: OA, bovine serum albumin (BSA) and superoxide dismutase (SOD; copper (Cu), Zinc (Zn) type from bovine erythrocyte) and sodium chloride (Wako Pure Chem., Osaka, Japan); cytochrome *c* (from horse heart) and mepyramine maleate (Sigma Chem., St. Louis, MO, USA); heparin sodium (Takeda Chem. Ind., Osaka, Japan); Percoll (Pharmacia, Uppsala, Sweden), and sodium pentobarbital (Abbott Lab., North Chicago, IL, USA). C-18 PAF was kindly donated by Y. Ashida of Takeda Chem. Ind., Osaka, Japan. The other reagents used were the highest grade of commercial products available.

$Al(OH)_3$ was prepared as previously described [9]. In brief, 0.5 N NaOH (100 L) was instilled to 0.5 N $Al_2(SO_4)_3$ (100 L) under vigorous stirring. After washing three times with purified water, the gels were divided and stored at a concentration of 60 $mg \cdot mL^{-1}$ of physiological saline in sealed bottles (300 $mL \cdot bottle^{-1}$) at 4°C until further use.

Study design

Guinea-pigs were repeatedly sensitized and challenged alternately by inhalation with OA+$Al(OH)_3$ and OA mists once every 2 weeks. Before and 10 min–10 h after the fifth OA inhalation challenge, the airway resistance (Raw) of each guinea-pig was measured and compared with those of the nonsensitized animals, which had been forced to inhale saline mists by the same protocol as used for the sensitized group. In separate experiments, drawings of peripheral blood and bronchoalveolar lavages (BALs) at defined times before and after OA inhalation challenge or saline inhalation in the sensitized or nonsensitized group, respectively, were performed. Time-course changes of leucocyte influx into the nasal cavity after the challenge were also assessed in the sensitized guinea-pig. In addition to these experiments, the *in vitro* responsiveness of purified eosinophils obtained from BAL 1 day after antigen challenge, to PAF was measured and compared with that of nonsensitized guinea-pig eosinophils.

Preparation of OA+Al(OH)$_3$ for sensitization

OA+$Al(OH)_3$ for sensitization was prepared prior to used, as reported previously [9]. One volume of 2.4 mg $OA \cdot mL^{-1}$ was instilled to two volumes of 60 mg $Al(OH)_3 \cdot mL^{-1}$ under stirring to yield a concentration of 800 µg OA· 40 mg $Al(OH)_3^{-1} \cdot mL^{-1}$. OA solution for challenge was also prepared at a concentration of 16 $mg \cdot mL^{-1}$.

Inhalation conditions

As previously described [9], the OA+$Al(OH)_3$ suspension or OA solution (approximately 50 mL) was placed in a 2 L polyvinyl chloride flask, in which the head of a hand-made glassware pressure nebulizer was set through a plug. The stirred suspension was transferred to the nebulizer and circulated with a peristaltic pump (MP-3, Eyela, Tokyo, Japan) at a flow rate of 10 $mL \cdot min^{-1}$. Pressurized air was also supplied to the nebulizer under conditions of 1 atmosphere and a flow of 11.4 $L \cdot min^{-1}$. The mists were first introduced to a 1.6 L polypropylene box to eliminate relatively large gel particle mists before the conscious guinea-pigs were forced to quantitatively inhale them. The median diameters of the OA+$Al(OH)_3$ and OA mists generated with the nebulizer were 2.0 and 2.1 µm, respectively. When guinea-pigs were exposed to a mist of Evans' blue instead of the antigen mists under these inhalation conditions, 79% of the mists trapped in the whole airways were found in the lung and the rest was in the nasal cavity [9].

Sensitization and challenge with antigen

Sensitization and challenge by inhalation of the mists of OA+$Al(OH)_3$ and OA, respectively, were performed according to the method previously described [8]. In brief, guinea-pigs were sensitized by forced inhalation of the OA+ $Al(OH)_3$ mists at a dose of 15 µg OA·750 µg $Al(OH)_3^{-1} \cdot animal^{-1} \cdot t^{-1}$, every 2 weeks for the first two times and then OA mists at doses of 10 µg $OA \cdot animal^{-1} \cdot t^{-1}$ and OA+ $Al(OH)_3$ mists at doses of 15 µg OA·750 µg $Al(OH)_3^{-1} \cdot animal^{-1} \cdot t^{-1}$. These were alternately inhaled by each guinea-pig for challenge and sensitization, respectively, once every 2 weeks until the fifth challenge (week 20). Mepyramine was administered to the animals (10 $mg \cdot kg^{-1}$ *i.p.*) 30 min before each challenge to prevent death from anaphylactic shock. For the negative control, guinea-pigs were forced to inhale physiological saline instead of the antigens once every 2 weeks for 20 weeks.

Exposure time of the guinea-pigs to antigen mists for quantitative inhalation was calculated from the following three values: 1) mist concentrations of OA or OA+$Al(OH)_3$, which were calculated by using Evans' blue in place of OA [9]; 2) the rate for the trapping of these Evans' blue mists in the lung following inhalation [9]; and 3) respiratory volume for 1 min of the individual guinea-pigs, which was measured by a two-chambered, double-flow plethysmograph system as described below.

Pulmonary function measurements

Raw before and 10 min to 10 h after the fifth antigen challenge by inhalation in the conscious guinea-pig was measured by a two-chambered, double-flow plethysmograph system (Pulmos-I; M.I.P. S., Osaka, Japan). In brief, the animal was placed with its neck extending through the partition of a two-chambered box. The specific Raw (Raw × thoracic gas volume) was measured through detection by the respective sensors of airflow supplied to the front and rear chambers according to the method described by PENNOCK *et al.* [10]. Raw was calculated by dividing the specific Raw by the individual tidal volume in place of the thoracic gas volume because the functional residual capacity could not be detected in the system.

Peripheral blood drawing and counting of leucocytes

One hour before and 1 h to 7 days after the fifth OA inhalation challenge, peripheral blood was drawn in the presence of 100 U·mL^{-1} of heparin from the abdominal vein of the sensitized guinea-pig under pentobarbital anaesthesia (40 mg·kg^{-1} *i.p.*). Similarly, blood samples from the nonsensitized negative control animals were also drawn under anaesthesia at the same time-points after the last saline inhalation. The total leucocyte number was determined by staining with Turk's solution (Nacalai Tesque, Kyoto, Japan). For determining the numbers of differentiated leucocytes, the blood specimen was centrifuged on the Settling chamber (Neuro Probe, Cabin John, MD, USA) at 50×g for 30 s at 4°C after hypotonic treatment, and then the settled leucocytes were stained with Diff-Quik® solution (International Reagents, Kobe, Japan), followed by the microscopic counting of a total of least 500 cells.

BAL and cell counts

Following the drawing of blood the sensitized and the nonsensitized guinea-pigs were exsanguinated from the abdominal aorta. After perfusion of the lung with calcium ion (Ca^{2+})-free BSA-containing Tyrode's solution *via* the pulmonary artery and isolation of the lung with the trachea. BAL was performed with the physiological solution (5×10 mL). Hypotonic lysis was performed in order to remove occasional contaminating erythrocytes. The total leucocyte number was determined by staining with trypan blue solution (Nacalai Tesque, Kyoto, Japan) followed by counting under the microscope. The differential cell count was done as described above.

Nasal cavity lavage (NCL) and cell count

After the blood drawing and BAL, NCL was carried out, by the modified method, as previously reported [11], in some of the sensitized guinea-pigs. In brief, after ligation of the trachea, one end of the silicone tubing, with the other end being connected to an air pump, was positioned on the right nostril. Under reduced pressure (-0.19 atmospheres when the nostril was completely plugged) by the air pump, 10 mL of Ca^{2+}-free BSA-containing Tyrode's solution was aspirated from the left nostril *via* tubing. The recovery of the fluid was more than 90%. The cell counts were performed in the same manner for the BAL study.

Purification of alveolar eosinophils

Purification of alveolar eosinophils, which had been obtained from the nonsensitized and the sensitized guinea-pigs 1 day after the respective saline and OA inhalations by BAL, was performed as previously described [12]. After the hypotonically-treated BAL cells were incubated in a polystyrene culture flask (75 cm^2) at 37°C for 1 h in 5% CO_2 and 95% air, suspended nonadherent cells were stratified on discontinuous (50, 60 and 70%) Percoll layers. Following centrifugation at 360×g for 30 min at 4°C, eosinophils were recovered from the bottom of the centrifuge tube. Purities of eosinophils obtained from the nonsensitized and the sensitized guinea-pigs were 74±1.9 and 77±7.2% (n=3), respectively. The other cells were mononuclear cells (23±0.4 and 21±6.4%, respectively) and neutrophils (3±1.5 and 3±0.9%, respectively). Cell viabilities were more than 97% in all experiments. The suspended eosinophils were used for the experiment to determine superoxide anion ($\cdot O_2^-$) production.

$\cdot O_2^-$ production

Production of $\cdot O_2^-$ was measured by the reductive method using cytochrome *c* [13]. Briefly, Tyrode's solution (50–100 µL) containing 10 µM cytochrome *c* and 2 µM PAF or vehicle (Tyrode's solution) was preincubated at 37°C for 5 min. The reaction was started by adding an equal volume of purified eosinophils (2×10^5 eosinophils·mL^{-1}) to the mixture. After 60 min the reaction was stopped by adding ice-cold SOD solution to a final concentration of 76 U·mL^{-1}, and then the mixture was centrifuged at 1,700×g at room temperature for 10 min. Absorbances at 540 and 550 nm of the obtained supernatant were measured. $\cdot O_2^-$ production was calculated from the difference in the absorbance between the two wavelengths with an extinction coefficient of 19.1×10^3·M^{-1}·cm^{-1} as pmol of cytochrome *c* reduced·10^5 eosinophils^{-1}.

Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA). If a significant difference was detected, the individual group difference was determined by Bonferroni's multiple test. A p-value of less than 0.05 was considered to be statistically significant.

Results

Time-course changes of R*aw*

Time-course changes of *R*aw following the fifth OA inhalation challenge to guinea-pigs, which had been alternately sensitized and challenged repeatedly, are shown in figure 1.

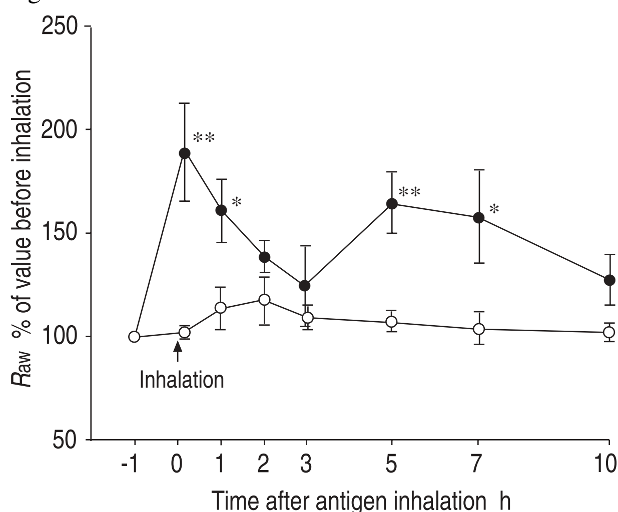

Fig. 1. – Time-course of the airway resistance (*R*aw) before and after the fifth antigen inhalation challenge in sensitized guinea-pigs. Each point represents the mean±SEM of five animals. ❍: nonsensitized; ●: sensitized. *,**: p<0.05, p<0.01, compared to the nonsensitized group.

Table 1. – Time-course of changes in numbers of total leucocytes, monocytes and lymphocytes in the peripheral blood of nonsensitized and sensitized guinea-pigs before and after the respective fifth saline and antigen inhalation challenge

Time after inhalation	Number of cells ×10⁶ cells·mL⁻¹					
	Leucocytes		Monocytes		Lymphocytes	
	Nonsensitized	Sensitized	Nonsensitized	Sensitized	Nonsensitized	Sensitized
-1 h	6.17±0.28	5.71±0.51	0.91±0.10	0.93±0.14	0.73±0.14	0.79±0.15
1 h	6.42±0.79	5.48±0.39	0.96±0.11	0.98±0.09	0.85±0.10	1.08±0.21
3 h	5.58±0.58	7.27±0.46	0.83±0.10	1.08±0.12	0.70±0.15	0.60±0.09
5 h	6.58±0.69	8.07±0.55	1.16±0.20	1.07±0.09	0.82±0.14	0.67±0.07
7 h	6.40±0.44	7.31±0.56	1.14±0.17	0.91±0.17	0.81±0.19	1.08±0.17
10 h	6.05±0.42	4.97±0.39	1.14±0.14	0.57±0.06	0.80±0.19	0.78±0.09
1 day	5.84±0.71	4.82±0.34	1.09±0.17	0.90±0.11	0.79±0.23	0.88±0.10
3 days	5.77±0.81	4.24±0.37	1.02±0.18	0.70±0.14	0.72±0.11	1.03±0.15
7 days	6.50±0.61	4.27±0.40*	1.01±0.16	0.62±0.06	0.80±0.17	0.70±0.12

Values are presented as mean±SEM of nine (nonsensitized) and nine to 12 (sensitized) animals. *: $p<0.05$, compared to the nonsensitized group.

Significant increases in Raw corresponding to EAR were seen at 10 min and 1 h after the challenge followed by a gradual decline at 2 and 3 h. At 5 h, Raw was significantly elevated again and was sustained for at least the following 2 h, which is considered to be the LAR. In the nonsensitized group, which had been treated with aerosolized saline, no obvious change of Raw was observed at any time.

Time-course changes in leucocytes in peripheral blood

Time course changes in the numbers of total leucocytes, monocytes and lymphocytes, and eosinophils and neutrophils of the sensitized group after the OA challenge are shown in table 1 and figure 2, respectively, in comparison with those of the nonsensitized group after saline inhalation.

Although the total leucocyte numbers of the nonsensitized animal were not influenced by saline inhalation for 7 days, the OA inhalation challenge to the sensitized guinea-pig tended to increase total leucocyte numbers at 3–7 h after challenge, compared with the value at the previous time point. This number reached its maximum at 5 h with 8.07×10^6 cells·mL^{-1}. Thereafter, the number gradually reduced up to day 3 (table 1).

Saline inhalation did not alter eosinophil numbers in the blood of the nonsensitized guinea-pigs. Although the unchallenged sensitized guinea-pigs had a relatively small number of eosinophils in the blood compared to the nonsensitized animals, these animals responded to the antigen challenge with an increase in the granulocyte number at 1 and 3 h, followed by a drastic rise ($p<0.01$) until 5 h, at which time the mean percentage of eosinophils in the total leucocyte number was 7.5%. This was followed by a gradual decrease up to day 1 and then remained at about 0.13×10^6 cells·mL^{-1} thereafter (fig. 2a). The neutrophil number of the sensitized group changed with essentially the same time-course as the total leucocyte number. It tended to increase from 3–7 h after the challenge, and subsequent decreases from 1–3 days were observed. On day 7, the number was significantly lower than that of the nonsensitized animals (fig. 2b).

As regards the numbers of mononuclear cells, monocytes and lymphocytes of the nonsensitized and the sensitized groups, no obvious changes were seen at any time in comparison with the respective values before the antigen or saline inhalation (table 1).

Time-course changes of leucocytes in BAL fluid (BALF)

Time-related changes of infiltrated leucocytes into bronchoalveolar spaces of the nonsensitized and the sensitized guinea-pigs following the respective saline and OA challenges are shown in table 2 and figure 3.

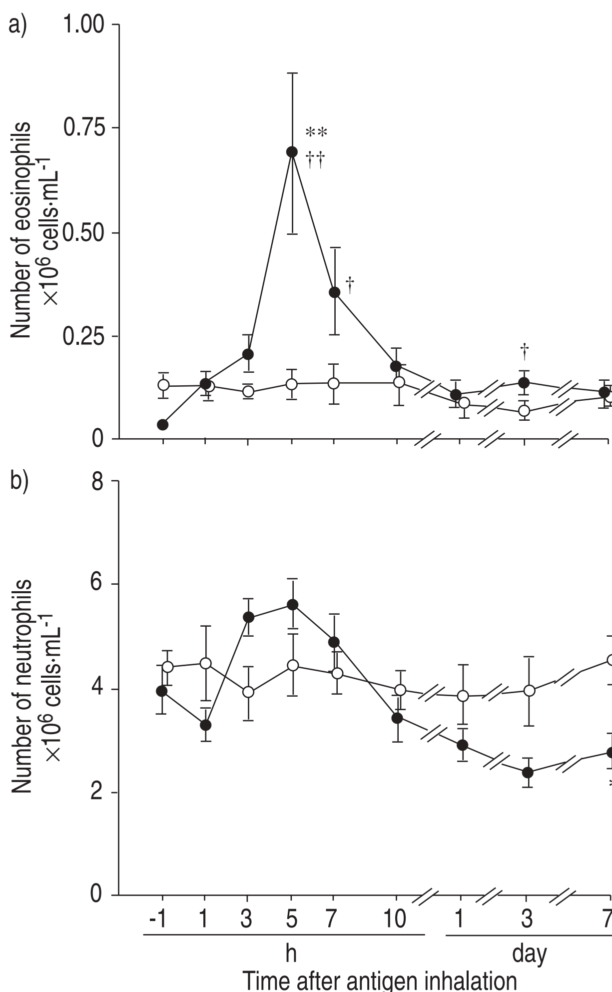

Fig. 2. – Time-course of: a) eosinophil and b) neutrophil numbers in the peripheral blood of sensitized guinea-pigs before and after the fifth antigen inhalation challenge. Each point represents the mean±SEM of nine to 12 animals. ○: nonsensitized; ●: sensitized. *,**: $p<0.05$, $p<0.01$, compared to the nonsensitized group. †,††: $p<0.05$, $p<0.01$, compared to the sensitized group prior to the challenge.

Table 2. – Time-course of changes in numbers of total leucocytes and mononuclear cells (MNC) in the bronchoalveolar lavage fluid obtained from nonsensitized and sensitized guinea-pigs after the respective fifth saline and antigen inhalation challenge

Time after inhalation	Number of cells ×10⁶ cells·animals⁻¹ Leucocytes Non-sensitized	Leucocytes Sensitized	MNC Non-sensitized	MNC Sensitized
-1 h	11.7±2.7	12.5±1.7	10.7±2.7	11.5±1.6
1 h	11.3±1.3	10.7±1.5	10.5±1.3	9.5±1.4
3 h	11.2±2.5	15.6±2.4	10.5±2.5	14.7±2.4
5 h	12.0±1.4	15.4±2.9	11.1±1.3	14.4±2.7
7 h	12.2±2.2	21.1±3.5	11.2±2.2	17.5±2.7
10 h	11.5±1.8	17.0±2.5	10.8±2.0	13.4±2.8
1 day	10.4±1.9	14.9±2.1	9.5±1.9	11.4±1.9
3 days	10.2±1.5	19.7±4.0*	9.3±1.5	15.6±3.2
7 days	10.1±1.6	12.1±1.8	9.2±1.4	10.6±1.7

Values are presented as mean±SEM of nine (nonsensitized) and nine to 12 (sensitized) animals. *: $p<0.05$, compared to the nonsensitized group.

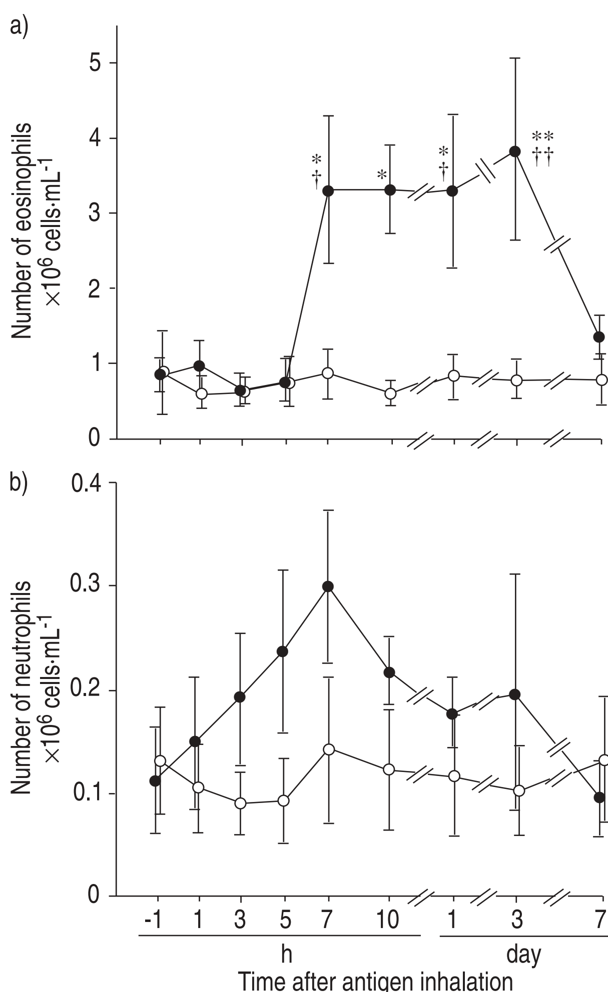

Fig. 3. – Time-course of: a) eosinophil and b) neutrophil numbers in the bronchoalveolar lavage fluid of sensitized guinea-pigs, before and after the fifth antigen inhalation challenge. Each point represents the mean±SEM of nine to 12 animals. ❍: sensitized; ●: nonsensitized. *,**: $p<0.05$, $p<0.01$, compared to the nonsensitized group. †,††: $p<0.05$, $p<0.01$, compared to the sensitized group prior to the challenge.

When the nonsensitized guinea-pig was forced to inhale aerosolized saline, the total number of leucocytes in the BALF was not altered. Yet, the total leucocyte number in the OA-challenged, sensitized animals tended to increase with time, from 3 h to 1 day. By day 3, the elevation was significant, mainly reflecting the increase in the number of mononuclear cells (table 2).

On the other hand, the eosinophil number in BALF of the challenged sensitized group did not increase relative to the prechallenge number until 5 h. The granulocytes showed an abrupt and significant infiltration into the BALF at 7 h, followed by a sustained accumulation in the inflamed tissue up to day 3. Seven days after the challenge, the number of leucocytes had decreased (fig. 3a). Neutrophils tended to migrate into the space more quickly than eosinophils. Infiltration was already evident at 1 h and infiltration peaked at 7 h following exposure. From 10 h to 7 days after the challenge, the neutrophil number gradually diminished (fig. 3b). Saline inhalation by nonsensitized guinea-pigs caused no apparent changes in any granulocyte number in BALF (fig. 3).

Time-course changes of leucocytes in NCL fluid (NCLF)

Table 3 shows changes of leucocyte numbers in NCLF of sensitized guinea-pigs at various times after the aerosol OA exposure.

No changes were observed in the number of total leucocytes, mononuclear cells and neutrophils at any time in comparison with the respective numbers before the challenge. Although only $3±1×10^2$ eosinophils·animal⁻¹ were found in the fluid of the sensitized unchallenged guinea-pigs subsequent antigen exposure tended to induce a small eosinophil influx into the nasal cavity, with the maximum ($70±40×10^2$ cells·animal⁻¹) observed at 3 h.

PAF-induced ·O_2^--production of eosinophils

Figure 4 shows the 1 µM PAF-stimulated ·O_2^--production of eosinophils that migrated into bronchoalveolar spaces of nonsensitized and sensitized guinea-pigs 1 day after respective saline and OA inhalations.

Table 3. – Time-course of changes in numbers of total leucocytes, mononuclear cells (MNC), eosinophils and neutrophils in the nasal cavity lavage fluid obtained from sensitized guinea-pigs after the fifth antigen inhalation challenge

Time after inhalation	Number of cells ×10⁴ cells·animals⁻¹ Leucocytes	MNC	Eosinophils	Neutrophils
-1 h	3.23±1.09	2.54±1.03	0.03±0.01	0.65±0.37
1 h	2.98±0.41	2.45±0.47	0.25±0.19	0.29±0.15
3 h	3.32±1.49	2.07±1.07	0.70±0.40	0.63±0.40
5 h	2.05±0.42	1.77±0.39	0.15±0.06	0.12±0.04
7 h	2.88±1.11	2.34±0.89	0.07±0.03	0.47±0.31
10 h	1.85±1.18	1.71±1.15	0.03±0.03	0.11±0.07
1 day	2.37±0.88	2.02±0.67	0.11±0.08	0.24±0.14
3 days	1.91±0.60	1.50±0.36	0.09±0.09	0.33±0.26
7 days	1.95±0.74	1.44±0.62	0.01±0.01	0.50±0.21

Values are presented as mean±SEM of four to five animals.

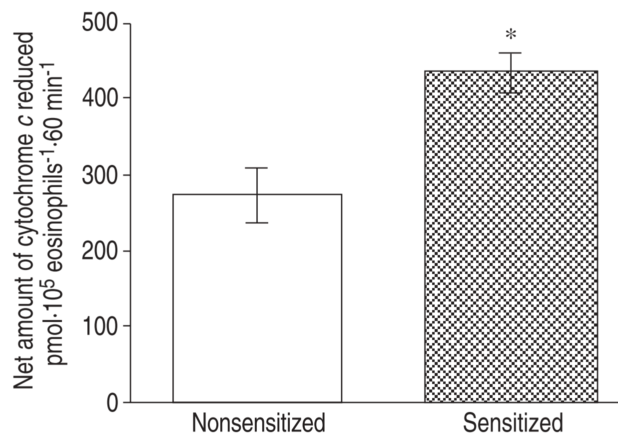

Fig. 4. – Platelet-activating factor (1 µM)-induced superoxide anion production of bronchoalveolar eosinophils obtained from nonsensitized and sensitized guinea-pigs. Spontaneous amounts of cytochrome *c* reduced by eosinophils from the nonsensitized and the sensitized guinea-pigs were 35±6 and 34±1 pmol·10^5 eosinophils^{-1}, respectively. Each column represents the mean±SEM of three experiments. *: $p<0.05$, compared to the nonsensitized group.

Although the spontaneous amounts of $\cdot O_2^-$-production were not different between the two groups, the net amount of the PAF-induced $\cdot O_2^-$-production of eosinophils from the sensitized guinea-pigs was significantly larger than that from the nonsensitized animals. Both the spontaneous and the PAF-induced $\cdot O_2^-$-generation in this experiment were completely abolished by pretreatment of the cells with 76 U·mL^{-1} SOD (data not shown).

Discussion

In the present study, we examined the time-course changes of leucocyte numbers in peripheral blood, BALF and NCLF after antigen challenge in our asthmatic model. This has been produced by repetitive antigen inhalations, to analyse the relationship between the appearance of the LAR and leucocyte kinesis and to confirm that the asthmatic response preferentially occurred in the lower airways. In addition, the *in vitro* responsiveness of bronchoalveolar eosinophils to PAF was evaluated to characterize their activation states. EAR and LAR were seen biphasically at 10 min and 1 h and at 5 and 7 h, respectively, after the fifth challenge, which is similar to the observations of a previous report [8]. Furthermore, a transient but obvious increase in eosinophil numbers in the circulation was induced 5 h after the challenge ($p<0.01$), followed by significant infiltrations of these cells into the bronchoalveolar space 7 h to 3 days after the challenge. Regarding leucocyte migration into the nasal cavity, only a minimal increase in eosinophil numbers was observed. Furthermore, the ability of eosinophils from sensitized guinea-pigs to produce $\cdot O_2^-$ stimulated by PAF was significantly higher than that of eosinophils from nonsensitized animals. Our findings suggest that infiltration of eosinophils, some of which were in activated states, may contribute to the appearance of the LAR. In addition, the pulmonary obstructive response observed in this experimental asthma is preferentially evoked in the lower airways.

In close accordance with other guinea-pig models [5, 14], the present results demonstrated that the increase of eosinophil number in BALF was seen 7 h after challenge, while the LAR decreased with time. Since LAR was already induced at 5 h, eosinophil increase in the bronchoalveoli neither proceeds nor coincides with LAR. It has been reported from histological analyses that the cell influx into bronchoalveolar spaces follows accumulation within bronchial and bronchiolar tissues [14, 15]. Furthermore, as shown in the present study, the number of circulating eosinophils elevated abruptly from 3–5 h, suggesting that the increased number of cells is likely to have largely accumulated in the lung tissue at 5 h. On the other hand, neutrophilia in BALF is reported not to be related to the occurrence of LAR. HUTSON *et al.* [16] demonstrated that antineutrophil antibody did not inhibit the appearance of the response even when the number of circulating neutrophils was decreased almost completely.

Abundant experimental studies have focused on leucocyte infiltration into lung tissue or bronchoalveolar spaces assessing histology or BAL cell counts. In addition, an obvious increase in the eosinophil population was observed in the peripheral blood of asthmatic subjects [17, 18]. BARTON *et al.* [19] have examined the time-course changes in the circulating leucocyte number in a guinea-pig model of asthma at 6–12 h intervals after antigen inhalation: the increase in eosinophil population was not seen at 6 h, but only occurred 24 h post-challenge. Other studies also demonstrated that the release of eosinophils from bone marrow into blood occurred 1–2 days after antigen challenge in sensitized guinea-pigs [20, 21]. As shown in the present experiment, the number of circulating eosinophils markedly increased at 5 h in our model. The reason for the time difference in eosinophil recruitment in the blood is not clear, however, different sensitization and challenge methods may be responsible. For instance in the study by BARTON *et al.* [19] sensitized guinea-pigs with OA without adjuvant by a noninhalation method and challenged employing a single antigen inhalation. In contrast, in the present model of experimental asthma, T-lymphocytes and other inflammatory cells whose characteristics had been altered by repeated antigen activation, might rapidly respond to the fifth antigen challenge. Supporting this consideration, COLLINS *et al.* [22] suggested the presence of a rapidly mobilizable bone marrow pool of eosinophils from the results that *i.v.* injection of interleukin (IL)-5 to guinea-pigs caused a rapid (<1 h) and dramatic increase in the number of circulating eosinophils, corresponding to a reduction in the number of bone marrow eosinophils.

It has been reported that incubation of guinea-pig eosinophils [23] as well as human eosinophils [24] with IL-5 *in vitro* gave the eosinophils the ability to enhance biological responses to stimuli such as PAF and phorbol myristate acetate. Considering these reports and the present enhanced ability of PAF-induced $\cdot O_2^-$-production of the eosinophils obtained from the sensitized animal, it is further suggested that the eosinophils were primed *in vivo* by being exposed to IL-5 which had been produced in the sensitized animal by antigen inhalation challenge.

On the other hand, numbers of circulating eosinophils and neutrophils of the sensitized guinea-pig before and 1–7 days after the challenge, respectively, tended to be smaller than those of the nonsensitized animal. These phenomena might be due to the difference in the accumulation ratio of these granulocytes in the bone marrow pool, the peripheral blood and tissues between the two groups,

which is considered to be induced by activations of immune component cells after repeated sensitizations/challenges.

Meanwhile, results in the present study have revealed that only the influx of eosinophils into the nasal cavity increased from 0.3×10^3 cells·animal^{-1} to 7.0×10^3 cells·animal^{-1} 3 h after the inhalation challenge, suggesting that some allergic responses occurred in the nasal cavities. However, this level is comparatively small when you consider the data in our recently reported new model of allergic rhinitis in guinea-pigs using Japanese cedar pollens as antigen, which are restrictively trapped in the nasal cavity after inhalation; in that model, approximately 200×10^3 cells·animal^{-1} of eosinophils migrated into the nasal cavity at 2–4 h after the challenge with pollen [11]. In addition, the abundant influx of eosinophils into the bronchoalveolar space was markedly higher than the modest increase of the inflammatory cells into the nasal cavity, suggesting that the obstructive response observed in this experimental asthma is preferentially evoked in the lower airways.

In conclusion, since peripheral eosinophil recruitment and lower airway eosinophilia coincided with the appearance of the late asthmatic response, leucocytes may be involved in this phenomenon to a certain extent. Furthermore, in the present inhalation challenge, the airway obstructive response in guinea-pigs *in vivo* is preferentially induced in the lower airways, not in the nasal cavity.

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