of int administration of live *S. pneumoniae* and a PAFR antagonist to rabbits resulted in reduced bacteria loads in BALF obtained up to 48 h after inoculation, compared with BALF from animals given pneumococci only [6]. A recent study, however, reported enhanced bacterial outgrowth after intravenous treatment with a PAFR antagonist in a mouse model of pneumococcal pneumonia [10].

The objective of the present study was to obtain more insight into the role of PAFR in the pathogenesis of pneumococcal pneumonia. For this purpose, we compared host responses in PAFR-deficient ($PAFR^{-/-}$) and normal wild-type *(wt)* mice after intranasal (inl) inoculation with live *S. pneumoniae.*

MATERIALS AND METHODS

Animals. $PAFR^{-/-}$ mice were generated in Japan, as described elsewhere [11], and were shipped to the animal facility of the Academic Medical Center in Amsterdam in 1999 (i.e., 3 years before the experiments were conducted). Hence, all $PAFR^{-/-}$ mice used in the present study were born in Amsterdam. $PAFR^{-/-}$ mice were backcrossed 7 times to a C57BL/6 background, making them 99.6% pure C57BL/6. *wt* C57BL/6 mice were obtained from Harlan Sprague Dawley. Both $PAFR^{-/-}$ and *wt* mice were specific pathogen free. All experiments were conducted with 10–12-week-old male mice. Fighting between mice did not occur during the studies described. All experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center.

Induction of pneumonia. Pneumococcal pneumonia was induced as described elsewhere [12, 13]. In brief, *S. pneumoniae* serotype 3 (ATCC 6303) were grown in Todd-Hewitt broth (Difco) for 6 h to mid-logarithmic phase at 37°C, harvested by centrifugation at 1500 *g* for 15 min, and washed twice in sterile isotonic saline. Bacteria were then resuspended in sterile isotonic saline at a concentration of $\sim 1 \times 10^7$ cfu/mL, as determined by plating serial 10-fold dilutions on sheep's blood agar plates. Mice were lightly anesthesized by inhalation of isoflurane (Abott), and 50 μL of bacterial suspension was inoculated inl, corresponding with 5×10^5 cfu of *S. pneumoniae.*

Preparation of lung homogenates. At 24 or 48 h after inoculation, mice were anesthetized by intraperitoneal injection with Hypnorm (Janssen Pharmaceutica) and midazolam (Roche), and blood was obtained from the inferior caval vene. Whole lungs were harvested and homogenized at 4°C in 5 volumes of sterile isotonic saline by use of a tissue homogenizer (Biospect Products), which was carefully cleaned and disinfected with 70% ethanol after each homogenization. Serial 10-fold dilutions in sterile saline were made from these lung homogenates and from blood, and 50-μL volumes were plated onto sheep's blood agar plates and incubated at 37°C. Colony-forming units were counted after 16 h. For cytokine measurements, lung homogenates were lysed in lysis buffer (300 mmol/L NaCl, 15 mmol/L Tris, 2 mmol/L MgCl, 2 mmol/L Triton X-100, and 20 ng/mL pepstatin A, leupeptin, and aprotinin [pH 7.4]) and spun at 1500 *g* at 4°C for 15 min; the supernatant was frozen at −20°C until cytokine measurement.

BAL. The trachea was exposed through a midline incision and was cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott). BAL was performed by instilling two 0.5-mL aliquots of sterile isotonic saline; 0.9–1 mL of BALF was retrieved from each mouse, and total cell numbers were counted from each sample in a hemocytometer (Emergo). BALF differential cell counts were determined on cytospin preparations stained with modified Giemsa stain (Diff-Quick).

Histologic examination. After lungs were fixed in 10% formaline and embedded in paraffin for 24 h, 4-μm–thick sections were stained with hematoxylin-eosin. All slides were coded and scored by a pathologist who did not know the genotype of the mice.

Assays. Levels of the following cytokines and chemokines were measured by use of commercially available ELISAs, in accordance with the manufacturers' recommendations: tumor necrosis factor (TNF)–α and interleukin (IL)–6 (Pharmingen) and IL-1β, macrophage inflammatory protein (MIP)–2, and KC (R&D systems). Limits of detection were 150 pg/mL for TNF-α and IL-1β, 75 pg/mL for IL-6, 47 pg/mL for MIP-2, and 12 pg/mL for KC. Protein concentrations were measured in BALF by use of a commercially available assay (Micro Bicinchoninic Acid Protein Assay; Pierce Biotechnology), according to the recommendations of the manufacturer.

Statistical analysis. Data are shown as means ± SEM, unless otherwise indicated. Comparisons between groups were conducted by use of the Mann-Whitney *U* test. Survival curves were compared by log-rank test. $P < .05$ was considered to be statistically significant.

RESULTS

Protection against pneumococcal pneumonia in $PAFR^{-/-}$ mice. To investigate the involvement of PAFR in the outcome of pneumococcal pneumonia, $PAFR^{-/-}$ and *wt* mice were infected inl with 5×10^5 cfu of *S. pneumoniae* and monitored for 10 days. All *wt* mice died within 85 h after induction of pneumonia. Mortality was delayed and reduced among $PAFR^{-/-}$ mice; 21% survived until the end of the 10-day observation period ($P < .0001$, *wt* vs. $PAFR^{-/-}$ mice; figure 1).

Reduced outgrowth of pneumococci in $PAFR^{-/-}$ mice. To obtain insight into the role of PAFR in early antibacterial defense during pneumococcal pneumonia, we assessed the number of viable bacteria in the lungs 24 and 42 h after inoculation (i.e., at time points before the occurrence of the first deaths). At both time points, the numbers of colony-forming units recovered from

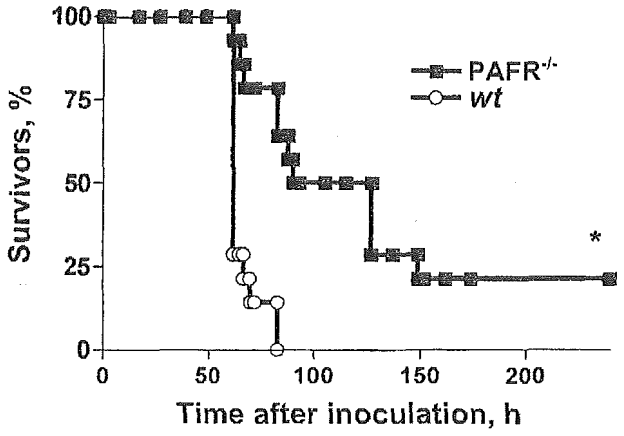

Figure 1. Enhanced survival in platelet-activating factor receptor–deficient ($PAFR^{-/-}$) mice. Survival after intranasal inoculation with *Streptococcus pneumoniae* in wild-type *(wt)* (○) and $PAFR^{-/-}$ (■) mice was assessed twice daily for 10 days ($n = 14$ mice/group). *$P < .05$, vs. *wt* mice.

the lungs of $PAFR^{-/-}$ mice were significantly lower than those recovered from *wt* mice ($P < .05$; figure 2). At 24 h after inoculation, blood cultures were positive for 71% of the *wt* mice and for 14% of the $PAFR^{-/-}$ mice ($P = .03$). At 42 h after inoculation, blood cultures were positive for 83% of the *wt* mice and for 50% of the $PAFR^{-/-}$ mice (*P*, not significant).

Unaltered neutrophil numbers and protein concentrations in BALF of $PAFR^{-/-}$ mice. Neutrophils play a prominent role in host defense against bacterial pneumonia [14, 15]. Because inhibition of PAFR function has been shown to reduce leukocyte influx into the lungs in response to intrapulmonary delivery of killed pneumococci [9], we assessed the number of neutrophils recruited to the alveoli. At 42 h after inoculation, no difference was seen in the number of neutrophils in BALF from *wt* and $PAFR^{-/-}$ mice (figure 3). Moreover, protein concentrations measured in BALF at this time point did not differ between $PAFR^{-/-}$ and *wt* mice (234.3 ± 42.8 and 298.3 ± 68.4 μg/mL, respectively).

Histologic analysis. At 42 h after inoculation, lungs of *wt* mice displayed heavy inflammatory infiltrates characterized by endothelialitis, peribronchial inflammation, and pleuritis. Lung inflammation was clearly less pronounced in $PAFR^{-/-}$ mice (figure 4).

Lung cytokine and chemokine concentrations. Cytokines and chemokines are pivotal mediators of an adequate host response to bacterial infection of the respiratory tract [14, 16]. Therefore, we investigated whether the improved outcome of $PAFR^{-/-}$ mice was associated with a favorable shift in cytokine or chemokine production by measuring the concentrations of TNF-α, IL-1β, IL-6, KC, and MIP-2 in lung homogenates. However, at 24 h after the induction of pneumonia, the pulmonary levels of these protective mediators were lower in $PAFR^{-/-}$ mice than in *wt* mice (all $P < .05$), whereas, at 42 h, all levels were similar in both mouse strains (table 1).

DISCUSSION

S. pneumoniae is the most frequently isolated pathogen in community-acquired pneumonia [17]. In the United States alone, >500,000 cases of pneumococcal pneumonia are reported each year, with a fatality rate of 5%–7%. In recent sepsis trials, *S. pneumoniae* emerged as an important causative pathogen, especially in the context of pneumonia [18]. In the United States, the mortality rate of 40,000 deaths/year caused by *S. pneumoniae* is higher than that caused by any other bacterial pathogen [19]. Because infections caused by *S. pneumoniae* are increasingly difficult to treat as a result of the emergence of antibiotic-resistant strains, it is clear that respiratory-tract infection by *S. pneumoniae* represents a major health care problem. Fundamental research has elucidated an important mechanism by which the pneumococcus interacts with cells lining the respiratory tract to cause tissue invasion. In particular, the PC component that prominently features in the pneumococcal cell wall specifically binds to PAFR expressed on human respiratory ep-

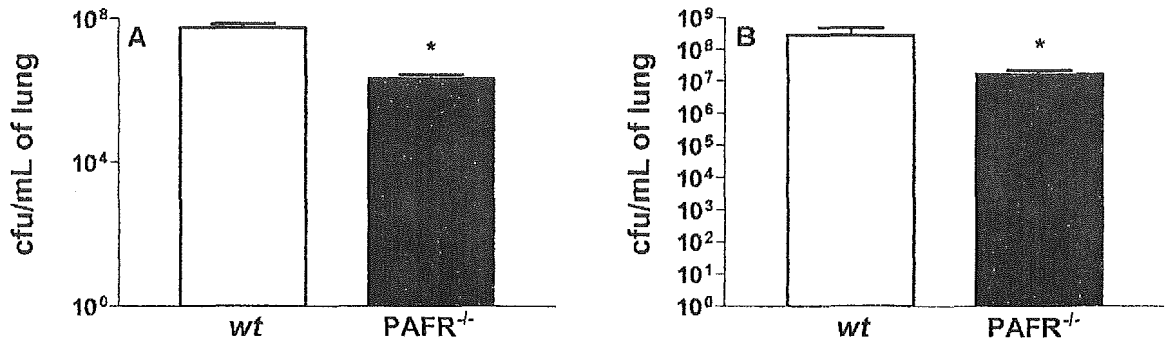

Figure 2. Decreased nos. *Streptococcus pneumoniae* organisms in lungs of platelet-activating factor receptor–deficient ($PAFR^{-/-}$) mice. Pneumococci in lungs of wild-type *(wt) (white bars)* and $PAFR^{-/-}$ *(black bars)* mice were measured 24 *(A)* and 42 *(B)* h after inoculation with *S. pneumoniae*. Data are mean ± SEM ($n = 7$ mice/group/time point). *$P < .05$, vs. *wt* mice.

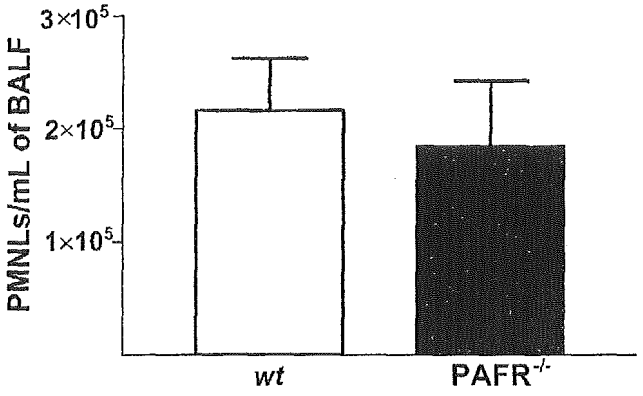

Figure 3. Mean ± SEM granulocyte influx in bronchoalveolar lavage fluid (BALF) 48 h after intranasal inoculation of *Streptococcus pneumoniae* in wild-type *(wt)* and platelet-activating factor receptor–deficient ($PAFR^{-/-}$) mice (n = 8 mice/group). PAFR deficiency does not influence recruitment of polymorphonuclear leukocytes (PMNLs) into alveoli during pneumococcal pneumonia. *$P < .05$, vs. *wt* mice.

ithelial cells, which facilitates bacterial entry into these cells [6]. In addition, the capacity of pneumococci to transcytose to the basal surface of rat and human endothelial cells is dependent on PAFR [7]. Although, to our knowledge, an interaction between pneumococci and the murine PAFR has not been formally demonstrated, here we provide compelling evidence that this mechanism is important for the virulence of pneumococci during murine respiratory tract infection in vivo. Using $PAFR^{-/-}$ mice, we have demonstrated that PAFR is used by *S. pneumoniae* to induce lethal pneumonia, as reflected by greatly reduced mortality, attenuated bacterial outgrowth in the lungs, and diminished dissemination of the infection in $PAFR^{-/-}$ mice.

The favorable outcome of $PAFR^{-/-}$ mice can not be explained by an enhanced innate immune response to *S. pneumoniae.* Indeed, even the local levels of protective cytokines and chemokines were lower in $PAFR^{-/-}$ mice early after the inoculation, suggesting that the initiation of the production of these mediators depends, at least in part, on the early interaction between pneumococci and PAFR. Alternatively, the absence of PAF signaling itself may have contributed to this finding, because inhibition of PAF has been found to attenuate the production of cytokines, especially TNF-α, induced by lipopolysaccharide (LPS) [1–3]. Similarly, the attenuated inflammatory response in lung tissue of $PAFR^{-/-}$ mice can be explained by either the absence of an interaction between pneumococcal PC and PAFR, the absence of endogenous PAF activity, or the presence of lower bacteria loads in the lungs of $PAFR^{-/-}$ mice, providing a less potent proinflammatory stimulus to the direct environment. Of note, neutrophil influx and protein concentrations in the BALF were similar in $PAFR^{-/-}$ and *wt* mice, which contradicts the results of earlier investigations, demonstrating that local administration of a PAFR antagonist diminished leukocytosis and increased protein concentrations in the cerebrospinal fluid and BALF of rabbits given *S. pneumoniae* intracisternally or int, respectively [9].

To our knowledge, 2 earlier studies investigated the effect of PAFR antagonists on the outgrowth of pneumococci in models of pneumonia. In the first study [6], a PAFR antagonist administered int together with *S. pneumoniae* reduced the number of colony-forming units recovered from BALF obtained up to 48 h after inoculation in rabbits, compared with BALF from rabbits administered bacteria only. In the second study [10], mice that received another PAFR antagonist intravenously had higher bacteria loads than did control mice. The 2 types of data indicate differences that remain to be explained, although spe-

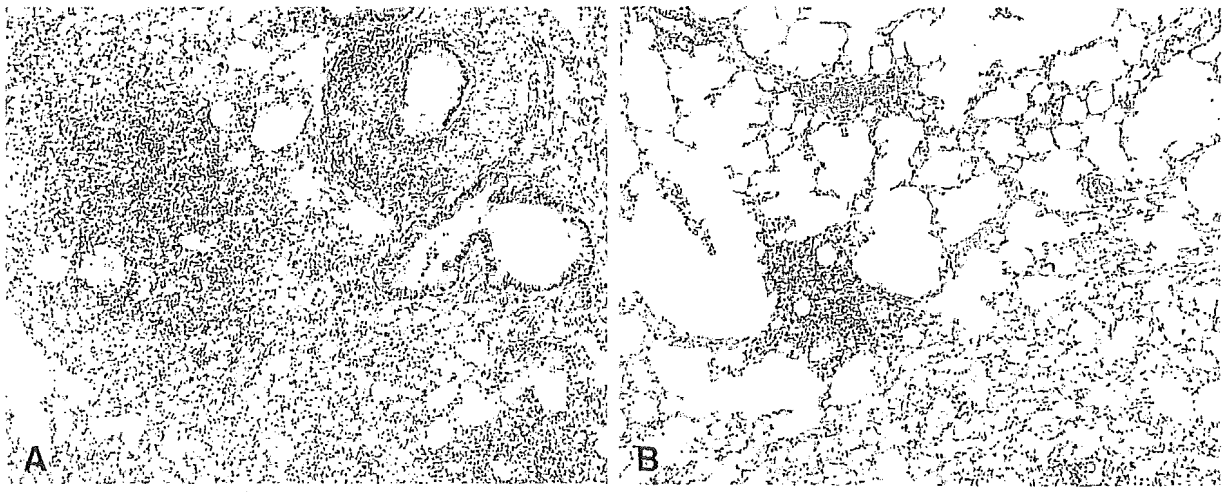

Figure 4. Histopathologic analysis of lungs, 42 h after inoculation with *Streptococcus pneumoniae*, shows heavy inflammatory infiltrates characterized by endothelialitis, peribronchial inflammation, and pleuritis. Lung inflammation was clearly less pronounced in platelet-activating factor receptor–deficient mice *(B)* than in wild-type mice *(A).* Representative slides are shown (hematoxylin-eosin staining; original magnification, ×33).

Table 1. Cytokine and chemokine concentrations in lung homogenates of wild-type *(wt)* and platelet-activating factor receptor–deficient ($PAFR^{-/-}$) mice inoculated with *Streptococcus pneumoniae*.

Cytokine/ chemokine	24 h after inoculation		42 h after inoculation	
	wt	$PAFR^{-/-a}$	*wt*	$PAFR^{-/-}$
TNF-α	2.1 ± 0.4	0.9 ± 0.2	1.8 ± 0.3	2.7 ± 1.1
IL-1β	8 ± 0.8	3.3 ± 1.1	4.3 ± 0.8	6.4 ± 2.1
IL-6	5.3 ± 0.6	1.7 ± 0.7	4.3 ± 0.7	3.6 ± 1.2
KC	8.8 ± 0.5	5.8 ± 0.6	9.6 ± 0.9	7.8 ± 1.6
MIP-2	7.0 ± 1.5	4.3 ± 0.6	29.4 ± 10.6	8.1 ± 1.8

NOTE. Data are mean ± SEM nanograms of each cytokine or chemokine per milliliter of lung homogenate (n = 8 mice/group). IL, interleukin; MIP, macrophage inflammatory protein; TNF-α, tumor necrosis factor–α.

[a] $P < .05$, vs. *wt* mice.

cific properties of the PAFR antagonist may have played a role. Nonetheless, the present data obtained with $PAFR^{-/-}$ mice, together with earlier data [6, 9], are consistent with the hypothesis that PAFR is used by *S. pneumoniae* in vivo to cause severe pneumonia.

PAF functions as a proinflammatory mediator in models of severe bacterial infection. Indeed, high PAF levels were detected in the lungs of rats after systemic injection of LPS [20] and in the BALF of patients with sepsis [21]. Inhalation of aerosolized PAF provoked inflammatory cell influx in the interstitium and alveoli [22, 23]. Finally, pretreatment with PAFR antagonists strongly diminished the pulmonary changes elicited by systemic or intrapulmonary administration of LPS, including increased pulmonary vascular leak and edema [24–27]. Together, these data suggest that PAF promotes inflammatory responses to bacteria, especially in the lung. A proinflammatory role for PAF in the pulmonary compartment is further supported by recent findings in $PAFR^{-/-}$ mice, revealing strongly reduced lung injury and respiratory failure induced by aspiration of acid [28]. Theoretically, these proinflammatory properties would make PAF a potentially protective mediator during pneumonia [14, 16]. Such a protective role of PAF in host defense against respiratory tract infection indeed was found in a model of pneumonia caused by *Klebsiella pneumoniae*, a bacterium that does not express PC, using the same type of $PAFR^{-/-}$ mice that were used in the present study [29]. The present investigation clearly establishes that the absence of PAFR overshadows this potential PAF-mediated increase in antibacterial defense, most likely through a function that is unrelated to its interaction with PAF (i.e., through its interaction with pneumococcal PC). These data may also apply to other pathogens that express PC, although this needs to be investigated in future studies.

It has been shown that *S. pneumoniae* needs PAFR to enter epithelial cells. Indeed, our study confirms this by showing that $PAFR^{-/-}$ mice are less likely to develop invasive disease and have improved host defense during pneumococcal infection. Thus, PAFR antagonism appears to be protective. However, the blockage of the proinflammatory properties of PAF by this strategy might be detrimental in acute inflammation.

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Platelet-Activating Factor Receptor Develops Airway Hyperresponsiveness Independently of Airway Inflammation in a Murine Asthma Model[1]

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Lipid mediators play an important role in modulating inflammatory responses. Platelet-activating factor (PAF) is a potent proinflammatory phospholipid with eosinophil chemotactic activity in vitro and in vivo. We show in this study that mice deficient in PAF receptor exhibited significantly reduced airway hyperresponsiveness to muscarinic cholinergic stimulation in an asthma model. However, PAF receptor-deficient mice developed an eosinophilic inflammatory response at a comparable level to that of wild-type mice. These results indicate an important role for PAF receptor, downstream of the eosinophilic inflammatory cascade, in regulating airway responsiveness after sensitization and aeroallergen challenge. *The Journal of Immunology,* **2004, 172: 7095–7102.**

Bronchial asthma is a complex disease of the lung characterized by reversible airway obstruction, chronic airway inflammation, and airway hyperresponsiveness (AHR)[3] to nonspecific stimuli. The progression of airway inflammation involves several cell types, including $CD4^+$ Th2 cells, eosinophils, and mast cells (1). The immunopathogenic role of Th2 cells is suggested by the roles of their products, such as IL-4, IL-5, and IL-13 in the recruitment and activation of the primary effector cells of the allergic response, eosinophils and mast cells. Activation of these cells results in the release of many inflammatory mediators that seem to induce AHR individually or coordinately (2, 3), although the precise molecular mechanisms predisposing to the development of AHR in asthmatics are largely unknown. The hypothesis that airway inflammation is responsible for AHR is based on the finding of a significant relationship between the parameters of airway inflammation and AHR (4, 5) and on the observation that inhaled steroids reduce both airway inflammation and AHR (6, 7). However, a number of studies in asthmatic patients have cast doubt on the requirement of airway inflammation for AHR (see review in Ref. 8). In addition, dissociation of AHR from airway inflammation has also been reported in some mouse models of asthma, because IL-5-deficient BALB/c mice partially developed AHR by OVA sensitization/challenge in the absence of airway inflammation (9). Conversely, IL-10-deficient C57BL/6 mice failed to develop AHR even in the presence of robust airway inflammation (10).

Platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent phospholipid mediator with various biological activities besides platelet activation (11). PAF acts by binding to a G protein-coupled seven-transmembrane receptor (12–16). PAF has long been implicated in the pathophysiological mechanisms of asthma (17), because exogenous PAF closely mimics many of the clinical features of asthma, including AHR (18, 19), bronchoconstriction (18), tracheal fluid secretion (20), and airway microvascular leakage (21) in animals and humans. PAF is detected in bronchoalveolar lavage (BAL) fluid from asthmatic patients but not from nonallergic subjects (22). Eosinophils and mast cells activated in asthmatic airways may be the cellular origins of PAF, because these cells are known to produce PAF in response to various stimuli in vitro (23, 24). Furthermore, PAF was reported to be a potent chemotactic factor for eosinophils (25) and to induce eosinophil degranulation in vitro (26). By using PAF receptor-deficient ($pafr^{-/-}$) and PAF receptor-overexpressing mice, we have previously demonstrated that PAF plays a critical role in anaphylaxis and acute injury in the lung (27, 28), suggesting that PAF mediates early-phase responses of allergy and inflammation in the tissue. However, the importance of PAF in the development of the allergen-induced AHR and chronic inflammation associated with asthma has not yet been investigated in $pafr^{-/-}$ mice. To define the role of PAF in the late-phase responses of allergy, we used an established murine asthma model, where mice were immunized with aluminum hydroxide adjuvant-adsorbed OVA and challenged with aerosolized OVA. In this study, we describe that $pafr^{-/-}$ mice, sensitized and challenged with OVA, displayed reduced AHR despite a significant eosinophilic airway inflammatory response. PAF may contribute to AHR in asthmatics independently of the eosinophilic airway inflammation.

Materials and Methods

Mice

$pafr^{-/-}$ mice were produced on a mixed C57BL/6 × 129/Ola genetic background as described previously (27). In the present study, $pafr^{-/-}$ mice and

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[3] Abbreviations used in this paper: AHR, airway hyperresponsiveness; PAF, platelet-activating factor; BAL, bronchoalveolar lavage; LT, leukotriene; PAS, periodic acid-Schiff; R_L, total lung resistance; $EC_{200}R_L$, effective concentration of methacholine required to double the basal R_L.

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the corresponding wild-type ($pafr^{+/+}$) control mice have been backcrossed for 6–10 generations onto a BALB/c genetic background. The animals were maintained in a light-dark cycle with light from 7:00 a.m. to 8:00 p.m. at 22°C. Mice were fed with a standard laboratory diet and water ad libitum. All of the mice in this study were used under a protocol approved by the University of Tokyo Ethics Committee for Animal Experiments.

During the course of the backcrossing, we selected mice homozygous for the intact allele of the group IIA phospholipase A_2 gene that is linked to the PAF receptor gene on murine chromosome 4 (29, 30). The genetic distance between these genes is ~4.3 cM. Both C57BL/6 and 129/Ola inbred mice are deficient in group IIA phospholipase A_2 due to a congenital disruption of the gene, whereas BALB/c inbred mice have an intact gene for group IIA phospholipase A_2 (29, 31). Thus, our selection was able to exclude the possible effects of group IIA phospholipase A_2 deficiency, which may cause an abnormal metabolism of PAF, on the phenotypes of $pafr^{-/-}$ mice. For genotyping by PCR, genomic DNAs were isolated from tail biopsies. The PCR for PAF receptor alleles was performed with 10 pmol of each primer and 2.5 U of KOD Dash DNA polymerase (Toyobo, Osaka, Japan) in a 50-μl final volume. The PCR profile involved a 2-min denaturation step at 94°C, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 65°C for 2 s, and extension at 74°C for 30 s. The primers were as follows: forward, 5′-TATGGCTGACCTGCTCTTCCT GAT-3′, and reverse, 5′-TATTGGGCACTAGGTTGGTGGAGT-3′, for detecting the intact PAF receptor allele; and forward, 5′-GCCTGCTTGCCG AATATCATGGTGGAAAAT-3′, and reverse, 5′-AGGATGCAGTAGC CACAATGGATAC-3′, for detecting the disrupted PAF receptor allele. The former set of primers amplified a 287-bp DNA fragment, and the latter PCR product consisted of ~900 bp. The PCR for group IIA phospholipase A_2 alleles was performed with 10 pmol of each primer and 1.0 U of Ex *Taq*DNA polymerase (Takara, Kyoto, Japan) in a 40-μl final volume. The PCR profile involved a 2-min denaturation step at 94°C, followed by 35 cycles of denaturation at 95°C for 30 s, and annealing and extension at 65°C for 3 min. The primers were as follows: forward, 5′-TGTACCTGT CCTTCACAGAGCTGAC-3′, and reverse, 5′-TCCACTTTTCTCCAGG CGCTTGTAGC-3′, producing 673- and 674-bp DNA fragments from genomic DNA with the intact or mutant allele, respectively. *Hin*fI digestion of the PCR products produced polymorphic fragments of 291, 267, and 115 bp with the intact allele or 368, 191, and 115 bp with the mutant allele. The DNA fragments were detected by 2% agarose gel electrophoresis, and ethidium bromide staining.

Experimental design

Male and female mice at the age of 8–15 wk were used. Within each experimental group, the sex ratio and the backcross generation were equal, and the age did not differ significantly.

Sensitization and challenge protocol

Eosinophilic pulmonary inflammation was induced according to the method of Foster et al. (32) with slight modifications. Briefly, mice were sensitized on days 0 and 14 by i.p. injection of 50 μg of OVA (grade V; Sigma-Aldrich, St. Louis, MO)/1 mg of aluminum hydroxide (Imject Alum; Pierce, Rockland, IL) in 200 μl of 0.9% sterile saline (Otsuka, Tokyo, Japan). Nonsensitized mice received only 1 mg of aluminum hydroxide in 0.9% pyrogen-free saline. On day 23, the sensitized mice were exposed three times at 1-h intervals to an aerosol of OVA (10 mg/ml) in 0.9% saline for 30 min. The nonsensitized mice received saline only. The aerosol with a mass median diameter of 3.8 μm was generated at 20 l/min by a nebulizer (Pariboy; Pari, Starnberg, Germany) into a plastic desiccator of 19 liters, whose internal pressure was maintained at atmospheric pressure by an aspirator. The aerosol challenge protocol was then repeated every second day thereafter for 8 days. Mice were studied 16–22 h after the last aerosol challenge.

Serum and BAL fluid samples

For the collection of blood and BAL fluid, mice were anesthetized with 1.5 g of urethane per kilogram of body weight by an i.p. injection at a volume of 10 ml/kg, and placed in the supine position. The blood was taken by cutting the femoral vein and artery. The blood sample was collected in a serum separator tube coated with a coagulant (Seraquick Super; Azwell, Osaka, Japan), and then allowed to clot at room temperature for 1 h. The serum was recovered by centrifugation at 2,000 rpm for 10 min at room temperature. After a subsequent centrifugation at 12,000 rpm for 10 min at 4°C in a microcentrifuge, the supernatant was stored at −80°C until use.

Once bleeding had ceased, the trachea of a tracheostomized mouse was cannulated with an 18-gauge metal cannula with a beveled tip. After opening of the thorax by a wide incision of the diaphragm, the lung was lavaged twice with 1 ml of Ca^{2+}- and Mg^{2+}-free PBS containing a proteinase inhibitor mixture (Complete; Roche, Mannheim, Germany) at room temperature. The initial lavage was instilled and retrieved one time, whereas the second lavage was instilled twice. This procedure allowed for a greater number of lung washes with less diluent. In total, ~1.6 ml of BAL fluid was consistently recovered. The sample was centrifuged at 1000 rpm for 10 min at 4°C, and the supernatant was collected and stored at −80°C. The cell pellet was resuspended in 200–250 μl of cold saline containing 0.1% fatty acid-free BSA (Serologicals Proteins, Kankakee, IL). After an appropriate dilution (2- to 20-fold) of the cell suspension with Turk solution (Mutoh Chemical, Tokyo, Japan), the total cell number was counted with a hemocytometer. Slides of BAL fluid cells were prepared by placing 3×10^5 cells into a cytocentrifuge (Cytospin 3; Shandon, Pittsburgh, PA) at 350 rpm for 2 min, and staining with Diff-Quik (International Reagents, Kobe, Japan). The percentages of eosinophils, lymphocytes, macrophages/monocytes, and neutrophils were determined by counting their number in randomly selected areas, and dividing these numbers by the total cell count (at least 300 cells).

Determination of Ab levels in serum

The total IgE, and OVA-specific IgE and IgG1 levels in appropriately diluted sera were measured by ELISA as previously described (27). The lower limit of detection for total IgE was 50 ng/ml.

Determination of cytokine and cysteinyl leukotriene levels in BAL fluid

The concentrations of cytokines in the BAL fluid were determined using murine ELISA kits obtained from Endogen (Woburn, MA) for IL-4 and IL-5, and R&D Systems (Minneapolis, MN) for IL-13. Whole-lung samples were homogenized on ice using a rotor/stator type tissue homogenizer (Physcotron; Microtec, Chiba, Japan) for 40 s in 8 ml of PBS containing the proteinase inhibitor mixture per gram of lung tissue. After centrifugation at 18,000 × *g* for 10 min, the resulting supernatants were stored at −80°C until use. The lower limits of detection for IL-4, IL-5, and IL-13 were 5.0, 5.0, and 1.5 pg/ml, respectively. The total level of cysteinyl leukotrienes (LTC_4, LTD_4, and LTE_4) in the BAL fluid was evaluated by an enzyme immunoassay kit from Amersham (Piscataway, NJ). The lower limit of detection was 15 pg/ml.

Lung histology

After the blood collection, the lungs were removed and fixed in 10% phosphate-buffered formalin. From the paraffin-embedded right and left lobes of lung, three sections of 3-μm thickness were prepared at the upper, middle, and lower positions of each lobe, and stained with either H&E or periodic acid-Schiff (PAS). A semiquantitative scoring system was used to grade the size of lung infiltrates in the H&E-stained sections, where +5 signifies a widespread infiltrate around the majority of vessels and bronchioles, and +1 signifies a small number of inflammatory foci. The total lung score represents the sum of the scores of both lobes. The goblet cell hyperplasia in the PAS-stained sections was graded by a semiquantitative scoring system (0 = <5% goblet cells in airway epithelium; 1 = 5–25%; 2 = 25–50%; 3 = 50–75%; 4 = >75%) as performed by McMillan et al. (33). The sum of the airway scores from right lobe was divided by the number of airways examined (16–29 per mouse), and expressed as PAS score in arbitrary units. For both semiquantitative scoring, randomized and blinded slides were graded by S.I.

Measurement of airway responsiveness

A separate group of mice was anesthetized with a mixture of ketamine and pentobarbital (35 mg/kg each) by i.p. injection. A metal cannula was inserted into the trachea of a tracheostomized mouse. The total lung resistance (R_L) of a mechanically ventilated mouse was measured as previously described (34). Saline and methacholine (acetyl-β-methylcholine chloride; Wako, Osaka, Japan) were inhaled at a positive end-expiratory pressure of 3 cmH_2O. At the start of the protocol, two deep inhalations (3-fold the tidal volume) were delivered to standardize the volume history. All animals were then challenged with the saline aerosol for 2 min. The aerosol was generated with an ultrasonic nebulizer (Ultra-Neb100; DeVilbiss, Somerset, PA) and delivered through the inspiratory line into the trachea. Measurements of 10-s duration were made during the tidal ventilation beginning 1 min after the administration of the saline aerosol. This represented the baseline measurement. Subsequently, each dose of the methacholine aerosol was administered for 2 min in a dose-response manner (0.3125, 0.625, 1.25, 2.5, 5.0, 10, and 20 mg/ml saline). During the experiments, oxygen gas was continuously supplied to the ventilatory system. Airway responsiveness was assessed as the effective concentration of methacholine

Table I. *Total cell and differential counts obtained from BAL fluid*[a]

Treatment	n	Total Cell Counts ($\times 10^5$)	Differential Counts (% Total Cells)			
			Eosinophils	Lymphocytes	Macrophages	Neutrophils
pafr$^{+/+}$ SAL	4	1.6 ± 0.1	0.0 ± 0.0	2.7 ± 1.5	97.2 ± 1.5	0.1 ± 0.0
pafr$^{-/-}$ SAL	4	2.0 ± 0.1	0.0 ± 0.0	2.6 ± 1.2	97.0 ± 1.4	0.3 ± 0.2
pafr$^{+/+}$ OVA	7	40.9 ± 6.3	69.1 ± 3.0	17.7 ± 2.3	12.2 ± 1.9	1.0 ± 0.2
pafr$^{-/-}$ OVA	7	47.3 ± 4.1	63.5 ± 1.4	21.0 ± 2.0	13.7 ± 1.3	1.7 ± 0.5

[a] Data are the means ± SEM. SAL, Saline-immunized and saline-aerosolized treatment; OVA, OVA-immunized and OVA-aerosolized treatment. After BAL, the recovered cells were counted, and a portion of the cells were centrifuged onto microscope slides using a cytocentrifuge. The slides were stained with Diff-Quik, and differential cell counts were obtained. For the total cell counts and the percentages of the designated cell types, no significant differences were found between *pafr*$^{+/+}$ OVA and *pafr*$^{-/-}$ OVA mice (*t* test).

required to double the basal R_L ($EC_{200}R_L$), which was calculated by interpolation.

Binding assay for muscarinic receptors

Each membrane fraction was prepared from four lung tissues of two male and two female mice as previously described (35). The binding assays were performed in triplicate using 100 μg of membrane protein.

Statistical analysis

Mann-Whitney's *U* test (for nonparametric analysis) or unpaired *t* test (for parametric analysis) was used to determine the levels of difference between two groups. A value of $p < 0.05$ was considered to have statistical significance. For four groups, the difference was evaluated by ANOVA. When the ANOVA showed significant differences, pairwise comparisons were tested by Bonferroni-Dunn posthoc test, where $p < 0.0083$ was considered to be significant. All statistical calculations were performed with StatView-J, version 5.0 (Abacus Concepts, Berkeley, CA). The values for all measurements were expressed as the mean ± SEM.

Results

Serum Ig levels

In both *pafr*$^{+/+}$ and *pafr*$^{-/-}$ mice, aeroallergen challenge was associated with a significant increase in the serum levels of total and OVA-specific IgE, compared with their respective saline-treated controls (data not shown). However, there were no significant differences between *pafr*$^{+/+}$ and *pafr*$^{-/-}$ mice, when either the total or OVA-specific IgE level was compared. Similarly to the IgE levels, we found no difference in the OVA-specific IgG1 levels between *pafr*$^{+/+}$ and *pafr*$^{-/-}$ mice (data not shown).

Inflammatory cell recruitment in BAL fluid

The recovery of cells from the BAL fluid of saline-aerosolized *pafr*$^{+/+}$ and *pafr*$^{-/-}$ mice revealed a predominance of alveolar macrophages in both groups, without any significant difference between the numbers (Table I). Aerosol challenge of mice with OVA induced a drastic increase in the total cell number compared with mice given aerosolized saline (Table I). Differential cell counts revealed that the infiltrates in both genotypes were mainly composed of eosinophils. However, the total numbers of cells and the proportions of eosinophils, lymphocytes, macrophages/monocytes, and neutrophils did not differ between *pafr*$^{+/+}$ and *pafr*$^{-/-}$ mice given OVA (Table I). These data imply that *pafr*$^{-/-}$ mice were capable of recruiting significant numbers of inflammatory cells into the airway lumen after OVA challenge in a manner similar to *pafr*$^{+/+}$ mice.

Th2 cytokine and cysteinyl leukotriene levels in BAL fluid

We assessed the levels of the Th2 cytokines IL-4, IL-5, and IL-13 in the BAL fluid (Table II). In saline-treated mice of either genotype, the levels of the Th2 cytokines were near or below the limit of detection. Aeroallergen-challenged *pafr*$^{+/+}$ and *pafr*$^{-/-}$ mice showed elevated levels of the Th2 cytokines in the BAL fluid compared with their respective nonsensitized controls. Although there were trends toward higher levels of all three Th2 cytokines in *pafr*$^{+/+}$ mice compared with *pafr*$^{-/-}$ mice, these differences did not reach statistical significance ($p = 0.10$ for IL-4, $p = 0.20$ for IL-5, and $p = 0.12$ for IL-13; Mann-Whitney's *U* test). Whole-lung homogenates of the allergen-challenged mice also contained similar levels of IL-5 and IL-13 in both genotypes ($p = 0.87$ for IL-5, and $p = 0.07$ for IL-13; Mann-Whitney's *U* test). Next, the total level of cysteinyl leukotrienes (LTC_4, LTD_4, and LTE_4) in the BAL fluid was evaluated (Table II), because these lipid mediators are also involved in airway inflammation in mice as well as humans (36–38). In saline-treated mice of either genotype, the levels of cysteinyl leukotrienes were below the limit of detection. Aeroallergen challenge resulted in comparable elevation of the cysteinyl leukotriene levels in *pafr*$^{+/+}$ and *pafr*$^{-/-}$ mice ($p = 0.38$, *t* test).

Lung histology

The lung tissue fixed after OVA inhalation revealed dense peribronchial and perivascular accumulation of inflammatory cells as well as gross alterations in the structural integrity of the airway walls (Fig. 1, *B* and *D*). However, semiquantitative grading of the

Table II. *Th2 cytokine and cysteinyl leukotriene levels in lung*[a]

Treatment	BAL Fluid (pg/ml)					Lung Homogenate (pg/g lung)		
	IL-4	IL-5	IL-13	Cysteinyl leukotrienes	n	IL-5	IL-13	n
pafr$^{+/+}$ SAL	12.0 ± 3.8	5.0 ± 0.0	1.5 ± 0.0	15 ± 0	4	ND	ND	
pafr$^{-/-}$ SAL	9.1 ± 0.9	5.0 ± 0.0	2.2 ± 0.5	15 ± 0	4	ND	ND	
pafr$^{+/+}$ OVA	66.4 ± 15.4	53.8 ± 19.9	248.0 ± 45.2	239 ± 45	10	393 ± 32	2152 ± 203	13
pafr$^{-/-}$ OVA	32.7 ± 5.7	20.7 ± 6.0	156.4 ± 30.2	192 ± 26	10	388 ± 27	1747 ± 101	14

[a] Data are the means ± SEM. *pafr*$^{+/+}$, Wild-type mice; *pafr*$^{-/-}$, PAF receptor-deficient mice; SAL, saline-immunized and saline-aerosolized treatment; OVA, OVA-immunized OVA-aerosolized treatment. BAL fluid and whole-lung homogenate were centrifuged, and the resulting supernatant was subjected to ELISA. There was no significant difference between *pafr*$^{+/+}$ OVA and *pafr*$^{-/-}$ OVA mice (Mann-Whitney's *U* test) in any mediators. The lower limits of detection for IL-4, IL-5, IL-13, and cysteinyl leukotrienes were 5.0, 5.0, 1.5, and 15 pg/ml, respectively.

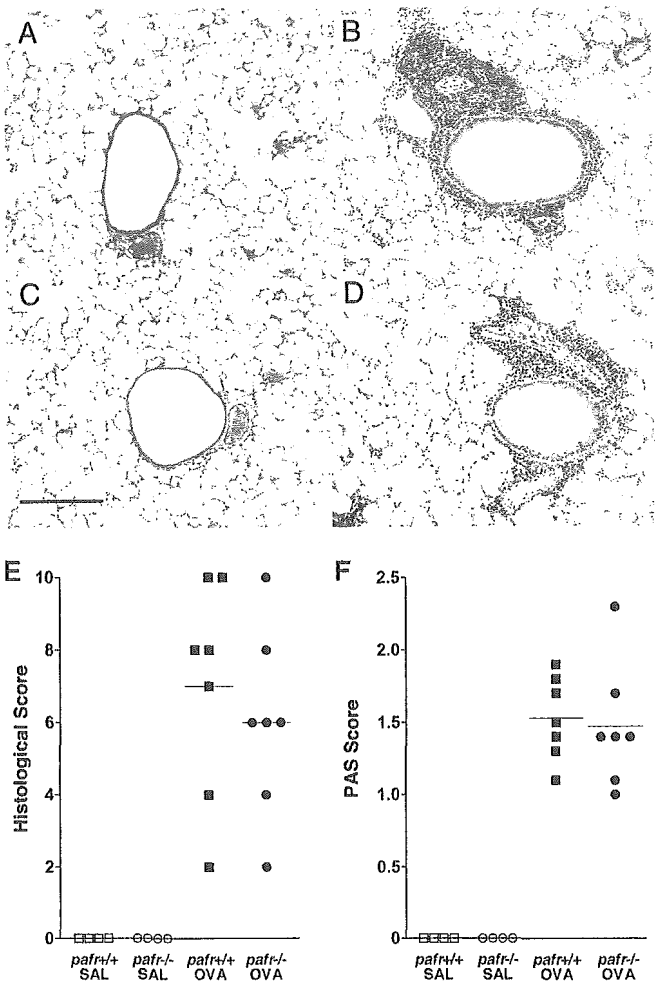

FIGURE 1. Histological analysis of lung sections. *A*, Nonsensitized $pafr^{+/+}$ mice exposed to an aerosol of saline. *B*, Sensitized $pafr^{+/+}$ mice exposed to OVA. *C*, Nonsensitized $pafr^{-/-}$ mice exposed to saline. *D*, Sensitized $pafr^{-/-}$ mice exposed to OVA. The H&E-stained sections shown are representative of six lung sections per mouse, from four or seven mice in each saline-treated or OVA-treated group, respectively. Scale bar, 200 μm. *E* and *F*, Assessments of lung inflammation. The stained sections were semiquantitatively scored as described in *Materials and Methods*, and scores for individual mice are presented. Bars depict means of groups. After aeroallergen challenge, $pafr^{-/-}$ mice develop lung inflammation at a comparable level to $pafr^{+/+}$ mice, as determined in sections stained with H&E (*E*). The levels of OVA-induced mucus production in $pafr^{+/+}$ and $pafr^{-/-}$ mice are identical, as determined in sections stained with PAS (*F*).

sections failed to elucidate a significant difference in the degree of airway inflammation between $pafr^{+/+}$ and $pafr^{-/-}$ mice (p = 0.34, Mann-Whitney's U test; Fig. 1*E*). Similarly, as shown in Fig. 1, *A* and *C*, the histological findings after saline treatment were unremarkable, with no observable differences between $pafr^{+/+}$ and $pafr^{-/-}$ mice (score: 0 in *E*). Excessive production of airway mucus glycoproteins by goblet cells in airway epithelium is a consistent finding in the lung of asthmatics. Semiquantification of goblet cells stained with PAS revealed similar mucus scores in $pafr^{-/-}$ mice compared with $pafr^{+/+}$ mice (Fig. 1*F*). Taken together, these results suggest that airway inflammation and goblet cell hyperplasia fully occurs in the absence of the PAF signaling.

Airway responsiveness

To assess aeroallergen-induced physiologic changes, both baseline R_L and airway responsiveness to an inhaled spasmogen, methacholine, were determined. Several aeroallergen-challenged mice had an increased baseline R_L compared with mice treated with saline. However, when analyzed as a group, aeroallergen-challenged mice exhibited no significant difference in the basal R_L in either saline-treated $pafr^{+/+}$ mice (0.55 ± 0.06 vs 0.39 ± 0.05 $cmH_2O/ml/s$; p = 0.09, ANOVA with Bonferroni-Dunn test) or saline-treated $pafr^{-/-}$ mice (0.50 ± 0.06 vs 0.40 ± 0.04 $cmH_2O/ml/s$; p = 0.30) (Fig. 2*A*). The inhalation of methacholine showed that $pafr^{+/+}$ mice aerosolized with OVA developed AHR compared with $pafr^{+/+}$ mice treated with aerosolized saline, because OVA-treated $pafr^{+/+}$ mice required a significantly lower dose of methacholine to achieve a 100% increase of the baseline R_L ($EC_{200}R_L$) than saline-treated $pafr^{+/+}$ mice ($\log EC_{200}R_L$ = −0.15 ± 0.09 vs 1.01 ± 0.12; $p < 0.0001$, ANOVA with Bonferroni-Dunn test) (Fig. 2*B*). Likewise, aerosol challenge of $pafr^{-/-}$ mice with OVA induced a significantly greater responsiveness to methacholine challenge compared with $pafr^{-/-}$ mice given saline ($\log EC_{200}R_L$ = 0.55 ± 0.16 vs 1.13 ± 0.08; p =

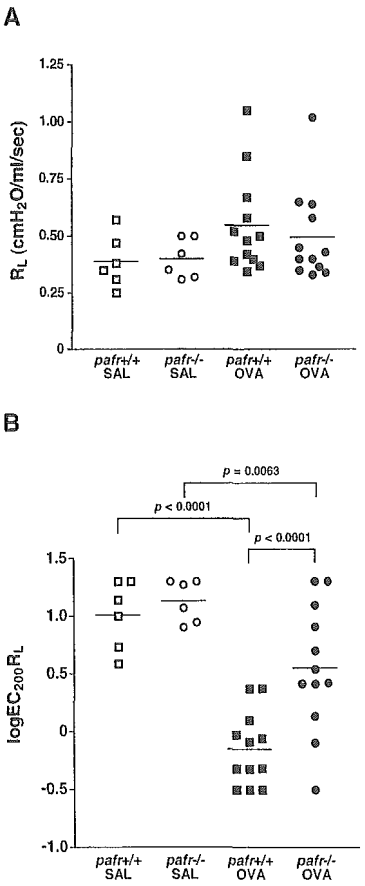

FIGURE 2. PAF receptor-regulated development of AHR in sensitized and aeroallergen-challenged mice. *A*, Baseline R_L. SAL, Saline-immunized and saline-aerosolized treatment; OVA, OVA-immunized and OVA-aerosolized treatment. Values for individual mice are presented. Bars depict means of groups. Before methacholine inhalation, no significant differences were observed among the four groups despite a nonsignificant trend toward an increased baseline R_L in the aeroallergen-challenged groups. *B*, Airway responsiveness to methacholine. Airway responsiveness was assessed by $EC_{200}R_L$. The logarithmic values of $EC_{200}R_L$ for individual mice are presented. Bars depict the means of the groups. $pafr^{-/-}$ OVA mice have significantly lower responsiveness to methacholine than $pafr^{+/+}$ OVA ($p < 0.0001$, ANOVA with Bonferroni-Dunn test). OVA-challenged $pafr^{+/+}$ and $pafr^{-/-}$ mice were significantly more responsive than their respective saline-treated controls ($p < 0.0001$ and p = 0.0063, respectively).

0.0063), but lower responsiveness than $pafr^{+/+}$ mice given OVA ($p < 0.0001$). These findings indicate that aeroallergen-induced AHR develops in $pafr^{-/-}$ mice to a significantly lower degree than $pafr^{+/+}$ mice.

Because methacholine is an agonist of muscarinic acetylcholine receptors, we examined the muscarinic receptor-binding activities of lung tissues from OVA-treated mice using a radiolabeled antagonist, [*N-methyl*-^{3}H]scopolamine. The lung membranes of $pafr^{+/+}$ and $pafr^{-/-}$ mice aerosolized with OVA bound similar amounts of this nonselective antagonist (Fig. 3), indicating comparable expression of muscarinic receptor-binding activity (B_{max} = 42.7 ± 2.7 and 44.8 ± 1.8 fmol/mg protein, respectively; $n = 3$; $p = 0.54$, t test). The binding was saturable with similar calculated K_d values of 237 ± 15 and 197 ± 19 pM ($n = 3$; $p = 0.16$, t test) in $pafr^{+/+}$ and $pafr^{-/-}$ mice, respectively.

Discussion

The murine asthma model recapitulates many of the features of human asthma, including the abundant eosinophilic and lymphocytic infiltration. PAF is chemotactic for eosinophils as well as macrophages/monocytes and neutrophils, all of which are also able to produce PAF (13, 14). Thus, it was reasonable to assume that PAF receptor may contribute to the induction of the airway inflammation associated with asthma. Unexpectedly, however, our studies indicate that the lack of PAF receptor did not alter the recruitment of inflammatory cells (i.e., total numbers of cells, or proportions of eosinophils, lymphocytes, macrophages/monocytes, and neutrophils) in the BAL fluid in this asthma model (Table I). Consistently, we found no significant histological differences in the degree of inflammation in the lung between $pafr^{+/+}$ and $pafr^{-/-}$ mice (Fig. 1). These data strongly suggest that PAF is dispensable for the airway inflammation, at least under our murine asthma model. Alternate chemoattractants, such as chemokines and leukotrienes (39–42), may recruit inflammatory cells to the airways. Indeed, BAL fluids from $pafr^{+/+}$ and $pafr^{-/-}$ mice contained comparable levels of cysteinyl leukotrienes, which are reported as important mediators for airway inflammation (36–38) (Table II). The present observations are consistent with our previous studies of thioglycolate-elicited peritoneal exudate macrophages (43), casein-elicited peritoneal exudate neutrophils (44), and acid-elicited neutrophils in the lung (28), where no differences were detected in cell numbers and differentials between $pafr^{+/+}$ and $pafr^{-/-}$ mice. However, another study of $pafr^{-/-}$ mice demonstrated diminished eosinophil recruitment in a murine model of allergic pleurisy where the s.c. sensitized mice were challenged once with OVA by intrapleural injection (45). The sensitization/challenge protocol of the pleurisy model is substantially different from that of the asthma model regarding route of Ag sensitization/challenge and frequency of Ag challenge; in this study, the i.p. sensitized mice were repeatedly challenged with OVA aerosols. Therefore, the lack of any differential recruitment of inflammatory cells in $pafr^{-/-}$ airways is likely due to the nature of the chronic inflammatory responses in the asthma model.

Elevated serum IgE levels have been reported to be important in the development of asthmatic responses (46, 47). Mice passively sensitized with IgG1 as well as IgE were reported to develop AHR and airway inflammation after allergen challenge (48). IL-4 and IL-5 are thought to be central to the development of asthmatic symptoms, because IL-5 regulates the differentiation, recruitment, and activation of eosinophils (49), and IL-4 drives IgE synthesis by B cells (50). Another Th2 cytokine, IL-13, is also hypothesized to play a pivotal role in the pathogenesis of asthma by activating B cells, eosinophils, and airway smooth muscle cells (51). OVA sensitization/challenge of $pafr^{-/-}$ mice resulted in serum Ab responses (data not shown) and airway Th2 responses (Table II). In all of these aspects, however, they were indistinguishable from $pafr^{+/+}$ controls. These findings suggest that a deficiency in PAF receptor did not affect the ability to induce humoral immune responses or Th2-directed cytokine responses to Ag.

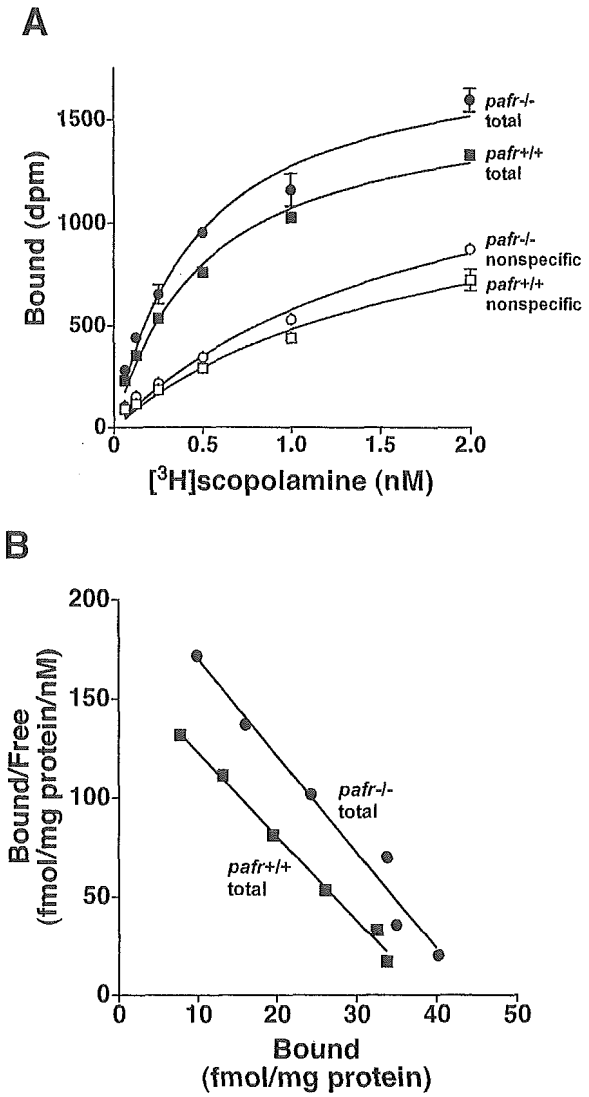

FIGURE 3. Muscarinic cholinergic receptor expression in lungs from sensitized and aeroallergen-challenged mice. *A*, [*N-methyl*-^{3}H]Scopolamine binding to lung membrane fractions. The lungs were excised and homogenized in a buffer, and the membrane fraction was prepared by ultracentrifugation. Aliquots of the resuspended membranes from $pafr^{+/+}$ and $pafr^{-/-}$ mice were incubated with increasing concentrations of [*N-methyl*-^{3}H]scopolamine for the detection of total binding. Nonspecific binding was determined by incubation in the presence of 20 μM methacholine. After incubation, free and bound antagonists were separated by rapid filtration on glass microfiber filters. Each point is the mean ± SEM of triplicate determinations, and the data are representative of three independent experiments. *B*, Scatchard analysis of the specific binding of [*N-methyl*-^{3}H]scopolamine to the lung membrane fractions shown in *A*. The specific binding was calculated as the difference between the total and nonspecific values. The data of $pafr^{+/+}$ and $pafr^{-/-}$ mice are shown. The mean values of K_d and B_{max} from the three independent experiments had no statistical significance between $pafr^{+/+}$ and $pafr^{-/-}$ mice given OVA (see *Results*).

AHR is a cardinal feature of asthma. PAF has been reported to induce AHR in animals and humans (18, 19), although the mechanisms are not fully understood. Moreover, we previously reported

that transgenic mice overexpressing PAF receptor showed AHR to inhaled methacholine under physiological conditions (52). To determine whether a lack of PAF receptor has an effect on the development of airway dysfunction, AHR was assessed in $pafr^{+/+}$ and $pafr^{-/-}$ mice. We found that $pafr^{-/-}$ mice had airway responsiveness similar to $pafr^{+/+}$ mice after saline-aerosolized treatment (Fig. 2*B*), indicating that the basal airway responsiveness is not different between $pafr^{+/+}$ and $pafr^{-/-}$ mice. Following Ag challenge, $pafr^{-/-}$ mice developed significantly increased airway responsiveness compared with their saline-treated controls. Furthermore, their responsiveness proved to be significantly lower than that of $pafr^{+/+}$ mice given OVA. Thus, PAF receptor is critical for the development of AHR following repeated aeroallergen challenge in sensitized mice, and AHR develops by PAF receptor-dependent and -independent pathways.

As reviewed by Drazen et al. (2) and Gali (3), two pathways involving mast cells and eosinophils have been elucidated to mediate aeroallergen-induced AHR. Recently, Hogan et al. (9) proposed a novel pathway to the development of AHR intimately mediated by $CD4^+$ T cells independently of IL-4 and IL-5, although the details of this pathway remain unknown. The relative contribution of these three cellular pathways to the induction of AHR is likely to be dependent on a number of factors including the strain of mouse, the choice of Ag, and the protocols for Ag sensitization and challenge, which may account for the apparent conflict observed among the mouse asthma models used by different investigators (9, 32, 39, 53–56). In the present study, we induced AHR in BALB/c mice with the procedure of Foster and coworkers (9, 32, 54, 57, 58) who have provided corroborative evidence of the important role of IgE, eosinophils, Th2 cytokines, and $CD4^+$ T cells. They immunized and boosted mice with OVA by i.p. injection of aluminum hydroxide-absorbed OVA, followed by repeated exposure to aerosolized OVA.

It is interesting that after such a strong sensitization/challenge procedure, little or no obligatory role of mast cells in AHR was observed in mast cell-deficient W/W^v mice (56, 59, 60). Consistently, AHR occurred normally with the sensitization/challenge procedure in IL-4-deficient BALB/c mice (9). Thus, in our study, the mast cell-dependent pathway could be excluded from the possible cellular mechanisms leading to the induction of AHR. Hence, it still remains unclear whether PAF is involved in the mast cell pathway. By using other procedures for sensitization/challenge to yield relatively attenuated airway responses, an even more pronounced contribution of PAF receptor to the mast cell pathway may be observed (56, 60).

$pafr^{-/-}$ mice, which have BALB/c genetic background, showed partially but significantly attenuated AHR despite a robust airway inflammation with infiltration of eosinophils and lymphocytes, indicating dissociation of AHR from airway inflammation in the mice. As described above, $CD4^+$ T cells regulate two distinct pathways that have been proposed to regulate aeroallergen-induced AHR; one is dependent on eosinophils, and another acts independently of IL-4 and IL-5. In BALB/c mice, the latter pathway is reported to play a major role in the development of AHR without eosinophilic inflammation and morphologic changes in the airways (9). Thus, it is possible that dissociation between AHR and airway inflammation observed in $pafr^{-/-}$ BALB/c mice is due to the involvement of PAF in the latter pathway. However, PAF also may be responsible for the development of AHR through the former (eosinophil) pathway. Although this lipid mediator was shown to be dispensable for eosinophil recruitment in this asthma model, it is possible that the infiltrated eosinophils in $pafr^{-/-}$ mice are not fully activated at the site of inflammation because of the lack of PAF stimulation. This is reminiscent of the results obtained in the murine acute lung injury model using $pafr^{-/-}$ mice in that PAF was essential for the activation of neutrophils but not for their recruitment (28).

The alternative possible target of PAF is smooth muscle. Our data demonstrate that the deficiency of PAF receptor is not associated with a detectable change in either the expression level (B_{max}) or ligand affinity (K_d) of muscarinic receptors in the lung, as measured by the nonspecific antagonist [*N-methyl*-^{3}H]scopolamine (Fig. 3). Although change of a minor pool of receptors cannot be ruled out, it is likely that the impaired muscarinic cholinergic response is due to a postreceptor event. PAF increases the susceptibility of smooth muscle to cholinergic stimulation, possibly by modulating the function of M_3 muscarinic receptor, a primary receptor for smooth muscle contraction (61). Indeed, we reported that the AHR to methacholine in transgenic mice overexpressing PAF receptor is mediated by a pathway sensitive to a PAF receptor antagonist (52). Similarly to the present data, the muscarinic receptor-binding activities (B_{max} and K_d) of the PAF receptor transgenic mice were indistinguishable from those of wild-type control mice (35). Because PAF receptor mRNA was detected in airway smooth muscle in human peripheral lung (62), it is possible for PAF to modulate the M_3 receptor-evoked smooth muscle contraction at the level of intracellular signal transduction. Whereas M_3 receptor on smooth muscle cells couples to phosphoinositol turnover through $G_{q/11}$ (61), PAF receptor is capable of coupling to $G_{i/o}$ and $G_{q/11}$ (14), suggesting a stimulatory cross talk between the intracellular signals from the two distinct receptors (63).

In most cases, AHR is strongly associated with airway inflammation (64–67), and anti-inflammatory drugs are currently used for bronchial asthma (6, 7). However, the PAF-mediated AHR appears to be independent of inflammation, because $pafr^{-/-}$ mice showed a reduction of AHR without diminishment of airway inflammation as shown in this study. This notion is further supported by our previous findings that the PAF receptor-overexpressing mice had AHR without obvious inflammatory responses (52). Recombinant plasma-type PAF acetylhydrolase abrogated airway responsiveness and inflammation concomitantly in a mouse asthma model (65). The apparent discrepancy between our data and those of the report may be attributed to the difference of sensitization procedure. In addition, it is notable that substrates for PAF acetylhydrolase and agonists for PAF receptor do not overlap completely (14, 68).

In summary, the present study demonstrates an important role for PAF receptor in the development of AHR after allergic sensitization/challenge in mice despite the normal expression density and ligand affinity of muscarinic cholinergic receptor. Furthermore, the airway inflammation was not affected by the absence of PAF receptor, suggesting that, as a complement anaphylatoxin C3a (69), PAF only acts downstream of the airway inflammation in bronchial asthma.

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特集：高齢者感染症の背景と Pathogenesis

8．老年病に合併する感染症の病像と治療のキーポイント

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特集 高齢者感染症の背景と Pathogenesis

8. 老年病に合併する感染症の病像と治療のキーポイント

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高齢者においては各臓器の機能や免疫能が低下しており易感染性である一方，初期症状がはっきりしないことが多いために診断に苦慮し，重症化することもある。高齢者においては，いかなる状態変化にあたっても，常に感染症の存在を念頭におく必要がある。治療にあたっては抗生物質による治療はもちろんのこと，栄養状態の改善をはかり，体内薬物動態，肝・腎機能，薬物相互作用に留意して副作用の軽減につとめなくてはならない。治療後の身体機能，ADL の回復のために必要な社会的資源の活用を積極的に行うことも望まれる。

Key Words：高齢者／感染症／体内薬物動態／栄養状態

I はじめに

本邦の高齢者人口は急速に増加している。総人口に占める 65 歳以上の割合は 1975 年には 7.9%（887 万人）であったものが，2000 年には 17.2%（2,187 万人）となっており，2025 年には 27.4%（3,312 万人）になると推定されている。20 世紀に入ってから，衛生環境の改善，抗菌剤の開発・進歩，ワクチン製剤の利用などにより，感染症に起因する死亡率が減少したことが高齢化の一因と考えられる。しかし，高齢化の進展に伴い，これまで制圧可能と思われていた感染症が再び注目を浴びるに至っている。本稿では高齢者に合併する感染症の病像，および治療のキーポイントについて概説する。

II 高齢者における感染症の病像

高齢者はその長い人生の中で多くの感染症に罹患しており，十分な感染抵抗性を有しているかのように思える。しかし実際には，加齢により各臓器の機能や免疫能の低下が生じており，また糖尿病や担癌状態などの基礎疾患の併存により易感染性となっている。時には副腎皮質ステロイドや化学療法剤の投与など，医原性の要因が易感染性を助長する。このため通常は起因菌とならないような口腔内常在菌による感染症や，結核，帯状疱疹などに罹患しやすい状態である。ひとたび感染が成立すれば容易に敗血症状態に至る。各種の抗生物質が投与されており，耐性菌感染症であることが多い点も治療を難しいものにする。

一方，初期症状がはっきりしないということが高齢者においてはむしろ特徴的なこととされる。本人も「何となく調子が悪い」という程度の自覚しかないことがあり，また家族もこれに気付かず，発見・診断がおくれることがある。近年では独居のケースも稀では無く，たまたま訪問したヘルパーが意識不明の状態で倒れているところを発見して救急車で搬送するという事態も経験される。結果として，必要な抗菌剤治療の開始が遅れ，不幸にも急性呼吸窮迫症候群（Acute Respiratory

Clinical manifestations and therapeutic keypoints of infectious diseases in the elderly
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Distress Syndrome：ARDS）や他臓器不全（Multiple Organ Failure：MOF）を合併して重症化する例も多く見受けられる。表1に高齢者で感染症を考慮すべきとされる非特異的な症状をまとめた。高齢者の場合，混乱，食欲不振，転倒，局所神経兆候，尿失禁といった，極めて非定型的な症状で発症したり，またこれが唯一の症状であったりする。慢性関節リウマチや変型性関節症などのために消炎鎮痛剤を常用していたり，副腎皮質ステロイドを服用しているケースもあり，全く発熱をみない例も多い。Trivalle ら[1]は院内で発熱を来した高齢者についてまとめ，発熱時の平均直腸温は38.1℃であったが，このうち38.5℃以上を示した例はわずか8%に認めたのみであったと報告している。さらに基礎体温自体の低下を指摘する意見もある。Castle ら[2]はナーシングホームに居住する高齢者における口腔内基礎体温が成人と比較して低下していることを報告し，高齢者に対しては発熱と考える基準を成人より低く設定することで感染症の診断能が向上すると述べている。白血球数やその分画（核の左方移動），血清CRP（C反応性蛋白）値は感染症の傍証として有用であるが，高齢者においては重症感染症であっても白血球数が上昇しない例もみられ，注意が必要である。血清CRP値も発症時点ではまだ上昇していないことがあり，またステロイド使用例では上昇しにくい。

感染症により糖尿病や心不全，呼吸不全など他の併存疾患が増悪することもよく経験される。糖尿病の存在は感染症のコントロールを困難にし，他方，感染症は糖尿病のコントロールを悪化させる。

以上から，高齢者においてはいかなる状態変化にあたっても，常に感染症の存在を念頭におく必要があると考えられる。また，症状の軽重は重症度を必ずしも反映しないため，慎重な重症度評価を行うことが必要である。

表1　高齢者にみられる感染症の不定症状

せん妄
混乱，不穏，性格変化
倦怠感
食欲低下
発熱がないこともある
転倒
体重減少
尿失禁
肺炎
咳嗽，喀痰がないこともある
腹膜炎
腹膜刺激症状がないこともある
髄膜炎
項部硬直がないこともある
拘縮のため評価困難なことがある

Ⅲ　高齢者における感染症治療の考え方

1）栄養補給

高齢者においては免疫能の低下から感染症のリスクが増大することが知られている。そしてその免疫能に対して様々な栄養素が関与していることがわかっている。しかし栄養剤の投与が明らかな臨床的有用性を示したとする報告はあまりない。高齢者は歯科的障害，うつ，痴呆，脳血管障害，癌などのため，食事の摂取が不足して栄養状態が悪化しやすい。また，一人暮らしの高齢者では食事の準備もできず，栄養価の低い安価な食物に頼る傾向がある。日光を浴びることの少ないADL（日常生活動作）の低下した高齢者ではビタミンDの合成が不足しがちである。

高齢者では蛋白やカロリーなど，全般的な栄養状態が低下するケースが多い。急性期病院や慢性ケア施設に入所した高齢者においては，その約過半数（～65%，報告による）がすでに低栄養状態である。またSullivan ら[3]によれば，栄養状態の良好な高齢者においても入院による栄養状態の悪化，院内死亡率の上昇が認められるとされている。

ビタミンや微量元素が免疫能の維持に重要な役割を果たしていることもおそらく間違いない。とくにビタミンA，B_{12}，E，および亜鉛，セレニウムは欠乏症の頻度が高いにもかかわらず免疫の維持に重要である。Chandra ら[4]は栄養状態の良好な高齢者を対象としてマルチビタミン／微量元素

表2 チトクローム P450 (CYP) の分類

ファミリー	サブファミリー	アイソザイム	特徴
CYP1	CYP1A	CYP1A1	アリールハイドロカーボン誘導で発現
		CYP1A2	ヒト常在型，肺に多く，肝臓に少ない
CYP2	CYP2C	CYP2C9	ヒト常在型
		CYP2C19	20%の日本人で欠損，臨床的意義大
	CYP2E	CYP2E1	アルコールで誘導
	CYP2D	CYP2D6	遺伝的多型あるが，日本人で欠損少ない
CYP3	CYP3A	CYP3A4	30%の薬物が基質，臨床的意義大
		CYP3A7	CYP3A4 と類似
CYP4	CYP4A	CYP4A1	プロスタグランジンなどの脂肪酸代謝と関連

製剤を投与した群と投与しない群とに無作為に分け，12カ月間観察し，非投与群において有意なビタミン欠乏を認め，罹病期間の延長，抗生物質投与期間の延長を報告している。

2）薬物治療

高齢者において，薬物の体内動態は一般成人と比較して大きく変化している。高齢者に対する薬物治療を行う上では，このことを考慮したうえで投与量，投与間隔を調整する必要が生じる。

まず，高齢者では体内の総水分量が成人に比して減少しており，逆に体内脂肪量は増加している。したがって，脂溶性薬物はより体内に長く残存する。

薬剤の相互作用も成人に比べて生じやすいので注意が必要である。とりわけ多くの薬剤を服用している高齢者の場合，その可能性は高いものとなる。代表的な例としては，erythromycin と terfenadine または astemizole(致死的不整脈)，aminoglycoside と furosemide（聴器毒性の増強），quinolone と鉄やアルミニウム，マグネシウム，カルシウムといった多価イオンを含む薬剤（消化管からの薬物の吸収を阻害して抗生剤の効果を減弱する），metronidazole と warfarin(ワーファリンの代謝が阻害されて抗凝固能が亢進する）などがあげられる。1日1回ないし2回の経口投与が可能な薬剤は体内薬物動態が比較的安定している。こうした薬物を用いることで，薬剤による有害作用を減らし，コストを削減し，服薬コンプライアンスを改善し，結果的に治療効果を上げる可能性がある。特に高齢者は多剤を服用していることが多いため，服薬回数，服薬量はコンプライアンスに極めて重大な影響を与える。入院患者においては，在院日数が減り，副作用なく外来治療に移ることができると指摘されている。服薬コンプライアンスを上げるうえで家族の協力が必須と考えられる例も認められる。高齢者ではむしろ服薬コンプライアンスが高いとする報告も散見されている[5]。

表3 CYP3A4 を阻害する薬剤ほか

エリスロマイシン
ジョサマイシン
ミデカマイシン
クラリスロマイシン
イトラコナゾール
ミコナゾール
フルコナゾール
シメチジン
エチニルエストラジオール
クロトリマゾール
ジルチアゼム
グレープフルーツジュース

薬物の排泄の上でもっとも重要と考えられるのが肝臓における代謝と腎臓における排泄である。

肝臓での薬物代謝に重要な役割を果たすのはチトクローム P450 (CYP) という酵素である。CYP は分子量 5,000 前後のヘム蛋白で，肝細胞内小胞体膜に存在し，これにより薬物は酸化的代謝をうけて極性を獲得し，腎・胆道系へ排泄されやすく

なる。CYPは1種類ではなく，表2に示すような性質の異なるファミリーを形成しており，おのおののファミリーはさらにサブファミリー，アイソザイムに分類されている。加齢に伴いCYPによる代謝能力は低下するため，薬物の排泄が遅延する。CYP3A4はこの中でも主要な薬物代謝酵素であり，CYP3A4を阻害する薬剤（表3）を使用する際には相互作用に十分な注意が必要である。

また腎機能も薬物の体内動態を決定する重要な因子である。高齢者の場合，クレアチニンの数値のみでは腎機能の単純な比較はできない。なぜなら，加齢に伴って筋肉量が減少し，クレアチニンそのものの産生量が減少するためである。理想的には，糸球体濾過率（Glomerullar fillutration rate：GFR），またはクレアチニンクリアランス(Ccr)を算出することが望ましい。Ccrの算出方法としては，以下の式が有名である。

$$Ccr\ (mL/分) = \frac{U \times V}{P} \times \frac{1.48}{A}$$

（U：尿中クレアチニン濃度 [mg/dL]，P：血中クレアチニン濃度 [mg/dL]，V：1分間あたりの尿量 [mL/分]，A：体表面積 [m^2]，1.48：日本人の平均体表面積 [m^2]）

畜尿の煩雑さを考慮すれば，Crockcroft-Gaultの式[6]も有用である。

$$Ccr\ (mL/分) = \frac{(140 - 年齢) \times 体重\ [kg]}{P\ [mg/dL] \times 72}$$

（女性ではさらに0.85をかける）

得られたCcrをもとにして投与量を決定するが，とくに注意が必要な薬剤としては，digoxin，aminoglycosideなどがあげられる。Muhlbergらは高齢者薬物動態を調査した諸研究から，血中濃度が治療域にある高齢者と中毒を起こしかねない血中濃度をもった高齢者のCcrを比較し，40mL/分を投与量調節が必要なカットオフ値とすることを報告している[7]。いずれにせよ，高齢者においてはクレアチニンの値に惑わされることなく，実際の腎機能を評価した上で投与量を調節する必要があると考えられる。経験的には成人常用量の半量程度で十分なことが多い。

感染症の存在を疑ったならば，喀痰，尿，便，血液，カテーテル，褥瘡部など感染を示唆する可能な限りの検体を採取した上で適切な抗生剤をempiricalに開始する。原因菌が推定され，感受性が判明した時点で抗生剤を変更する必要がある。その際，より抗菌スペクトラムの狭い抗生剤を選択することになる。投与中に薬剤の副作用を逐次監視し，相互作用の有無をチェックし，新たな状態の変化があれば，治療下に発症した新たな感染症や，重感染の存在を疑ったり，潜行する薬剤副作用を評価する必要がある。また，こうしたときには，肺塞栓や不整脈，心不全，コントロール不良の糖尿病などに対する注意を払う必要がある。高齢者では抗生剤治療に対する反応が遅いこともあり，また感染症自体が改善したという確かな証拠が存在するにもかかわらず，身体機能の回復には時間がかかることがある。ADLの低下，すなわち身の回りの事や歩行に介助を要するようになったり，着衣や食事や入浴も自分一人ではできなくなってしまうこともある。幸いにしてADLの低下を来さなかった場合でも，退院先(とくに自宅)に必要にして十分な介護力があるかどうかも綿密に調査して，社会的資源を利用する必要に迫られることがある。

Ⅳ　おわりに

高齢者に発症する感染症の病像とその治療法について概説した。高齢者において，感染症の診断，治療に苦慮する場面は極めて多いと思われる。感染症の存在を疑うことが第一の出発点である。高齢者の身体的，精神的，社会的キャパシティーに応じた柔軟な治療を心掛けることが求められる。

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分子呼吸器病

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