Repeated Antigen Inhalation-Induced Reproducible Early and Late Asthma in Guinea Pigs

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ABSTRACT—To develop a model of chronic experimental asthma in guinea pigs, the animal was forced to inhale the mist of a low dose of ovalbumin (OA) adsorbed on fine $Al(OH)_3$ for sensitization once every 4 weeks. The animal was challenged by inhalation with the mist of OA on day 14 after the respective sensitizations. Either the first or the second antigen challenge markedly induced an early asthmatic response (EAR), whereas there was hardly any late asthmatic response (LAR). At the 3rd challenge, LAR also emerged with some severity. These dual responses were consistently observed until the 10th challenge. On the other hand, repeated inhalation/challenge, once every 2 weeks, with OA alone at the same dose tended to lead to the desensitization of the EAR. In addition, LAR was hardly observed throughout the experiments. In both groups, γ_1 and IgE levels in the serum were elevated by the repetitive antigen inhalations, yet no obvious relationship between these antibody levels and the intensity of either EAR or LAR was recognized. The present results indicate that the asthmatic model with reproducible EAR and LAR developed in this study appears to be very beneficial for the investigation of bronchial asthma and for the assessment of anti-asthma drugs.

Keywords: Late asthmatic response, Early asthmatic response, Asthma, Aluminum hydroxide, IgE

The guinea pig has long been preferentially used as a good model of allergic bronchial asthma because the animal shows a very similar or identical sensitive pulmonary response and histological findings of the altered respiratory organ following antigen challenge to those of asthmatic attack patients (1). In addition, the airway smooth muscle of guinea pigs well responds to many biologically active substances such as histamine (2, 3), peptide leukotrienes (p-LTs) (3, 4), serotonin (5, 6), prostaglandin (PG) D_2, $PGF_{2\alpha}$, thromboxane A_2 (7–9) and endothelins (10, 11), which is very similar to that of human beings. For the last decade, among diverse studies in experimental bronchial asthma using guinea pigs, many researchers have extensively focused on the early asthmatic response (EAR) and late asthmatic response (LAR), especially in relation to pulmonary hyperresponsiveness and eosinophil accumulation. Irrespective of sensitization by injection or aerosol inhalation of antigen, most reports have indicated that first, second or third challenge with antigen by inhalation induces not only EAR but also LAR (12–16). However, Everitt and

Moore (17) demonstrated that Dunkin-Hartley and Hartley guinea pigs sensitized by either injection or inhalation of antigen with or without adjuvant, in experiments employing well-documented methods, showed no LAR, although all the animals exhibited EAR when challenged with aerosolized antigen.

On the other hand, it is worthwhile to establish a semi-chronic or chronic experimental model of allergic bronchial asthma for detailed analysis of the disease and the evaluation of the drugs that have been used as a remedy. However, this model has been little studied because repeated antigen challenge causes the pulmonary organ of guinea pigs to become unresponsive (18–23).

Here, we report that repeated inhalations of antigen with an adjuvant, aluminum hydroxide gels [$Al(OH)_3$], for long intervals of time can sensitize guinea pigs to develop highly reproducible pulmonary dysfunction with EAR and LAR in response to every repeated challenge by antigen inhalation.

MATERIALS AND METHODS

Animals

Male, 3-week-old, Hartley guinea pigs weighing 250–300 g were purchased from Japan SLC, Hamamatsu.

Materials

Reagents and their sources were as follows: ovalbumin (OA) and NaCl (Wako Pure Chem., Osaka) and mepyramine maleate (Sigma Chem., St. Louis, MO, USA). The other reagents used were the highest grade of commercial products available.

$Al(OH)_3$ was prepared as previously described (24). In brief, 0.5 N NaOH (100 l) was instilled to 0.5 N $Al_2(SO_4)_3$ (100 l) under vigorous stirring followed by washing 3 times with deionized water. The gels were divided and stored at a concentration of 60 mg/ml of physiological saline in sealed bottles (300 ml/bottle) at 4°C until use.

Anti-OA guinea pig serum was obtained according to the method of Levine et al. (25). In brief, guinea pigs were sensitized by i.p. injection of 10 μg OA/mg $Al(OH)_3$ /animal/time, once every 2 weeks, a total of 10 times. Both the 4-hr and 7-day passive cutaneous anaphylaxis (PCA) titers of the serum were 1 : 500. This serum served as the standard for quantification of γ_1 and IgE antibody levels in sensitized guinea pig sera by PCA and was stored at −80°C until use.

Preparation of OA adsorbed on $Al(OH)_3$ $[OA + Al(OH)_3]$ for sensitization

$OA + Al(OH)_3$ for sensitization was prepared before use. One volume of 2.4 mg OA/ml was instilled to 2 volumes of 60 mg $Al(OH)_3$/ml under stirring to make a concentration of 800 μg OA/40 mg $Al(OH)_3$/ml. A preparation of 16 mg OA/ml for sensitization and challenge and a preparation of 40 mg $Al(OH)_3$/ml for the negative control animals were also prepared.

Inhalation conditions

As previously described (24), the $OA + Al(OH)_3$ suspension or OA solution (approx. 50 ml), placed in a 2 l polyvinyl chloride flask in which the head of a hand-made glassware pressure nebulizer was set through a plug, was supplied to the nebulizer and circulated with a peristaltic pump (MP-3; Eyela, Tokyo) at a flow rate of 10 ml/min under stirring. The pressured air was also supplied to the nebulizer under the pressure of 1 atm and rate of 11.4 l air/min. To eliminate mists of any relatively large gel particles, the mists were first introduced into a 1.6 l polypropylene box before the 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 under these inhalation conditions, 79% of the mists trapped in the whole airway was found in the lung and the
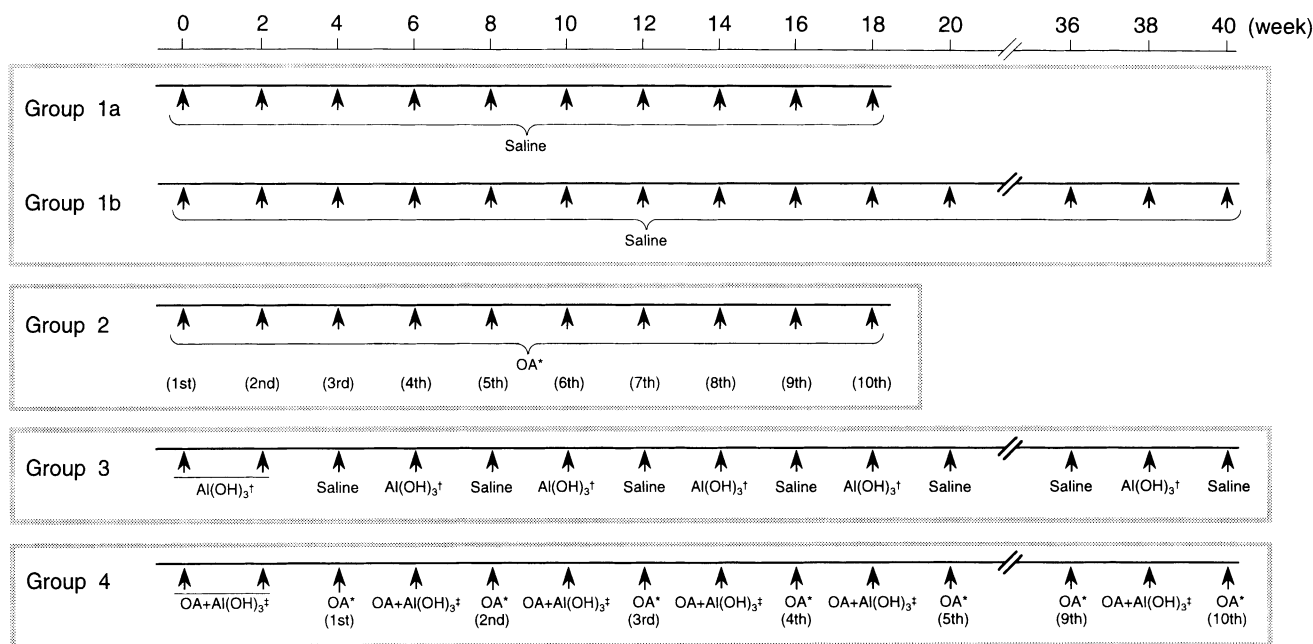

Fig. 1. Schedule for sensitization and challenge with the mist of antigen or vehicle to the guinea pig. *, 10 μg ovalbumin (OA)/animal/time; †, 750 μg $Al(OH)_3$/animal/time; ‡, 15 μg OA + 750 μg $Al(OH)_3$/animal/time. The ordinal number shown in parentheses represents the time of challenge.
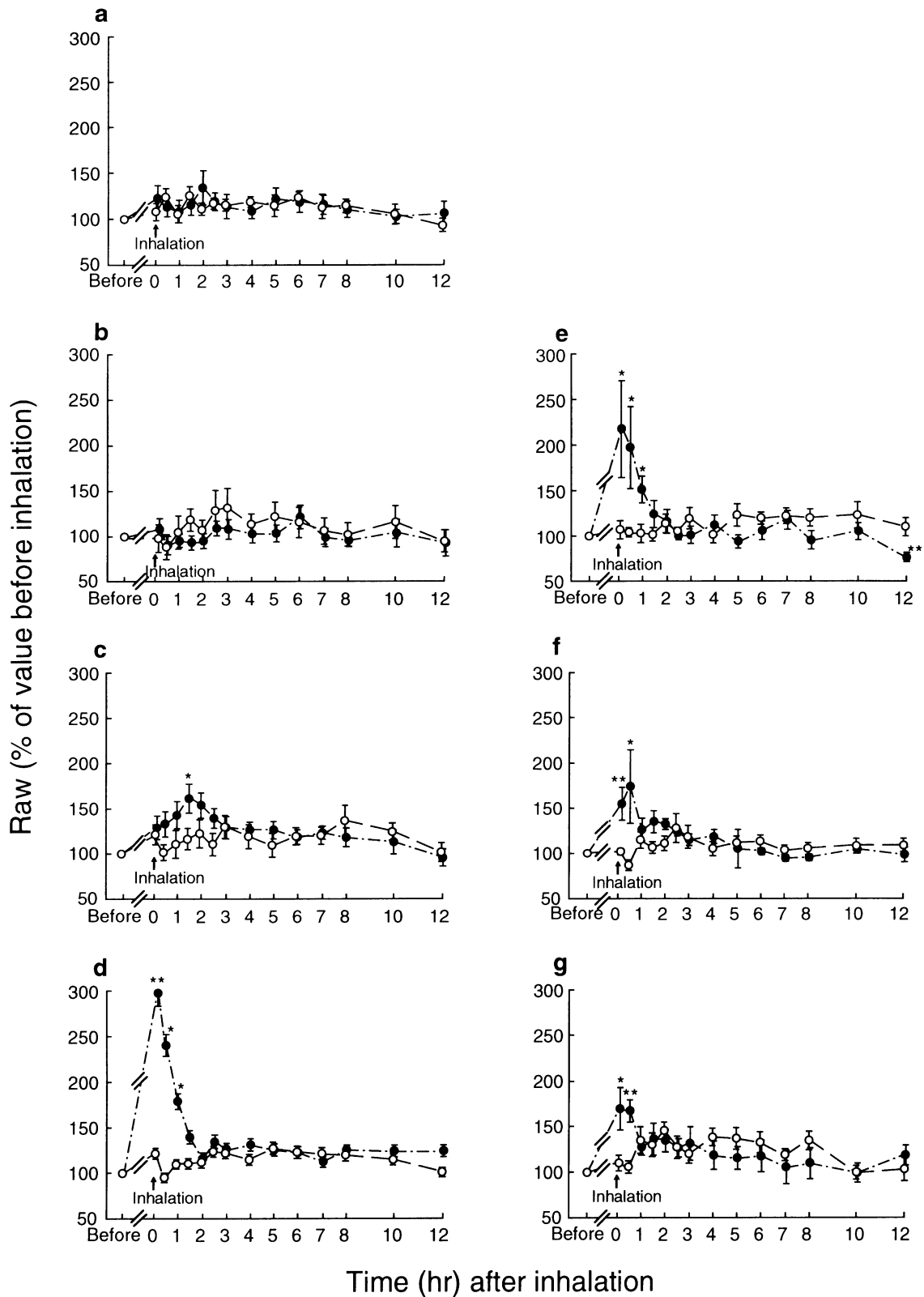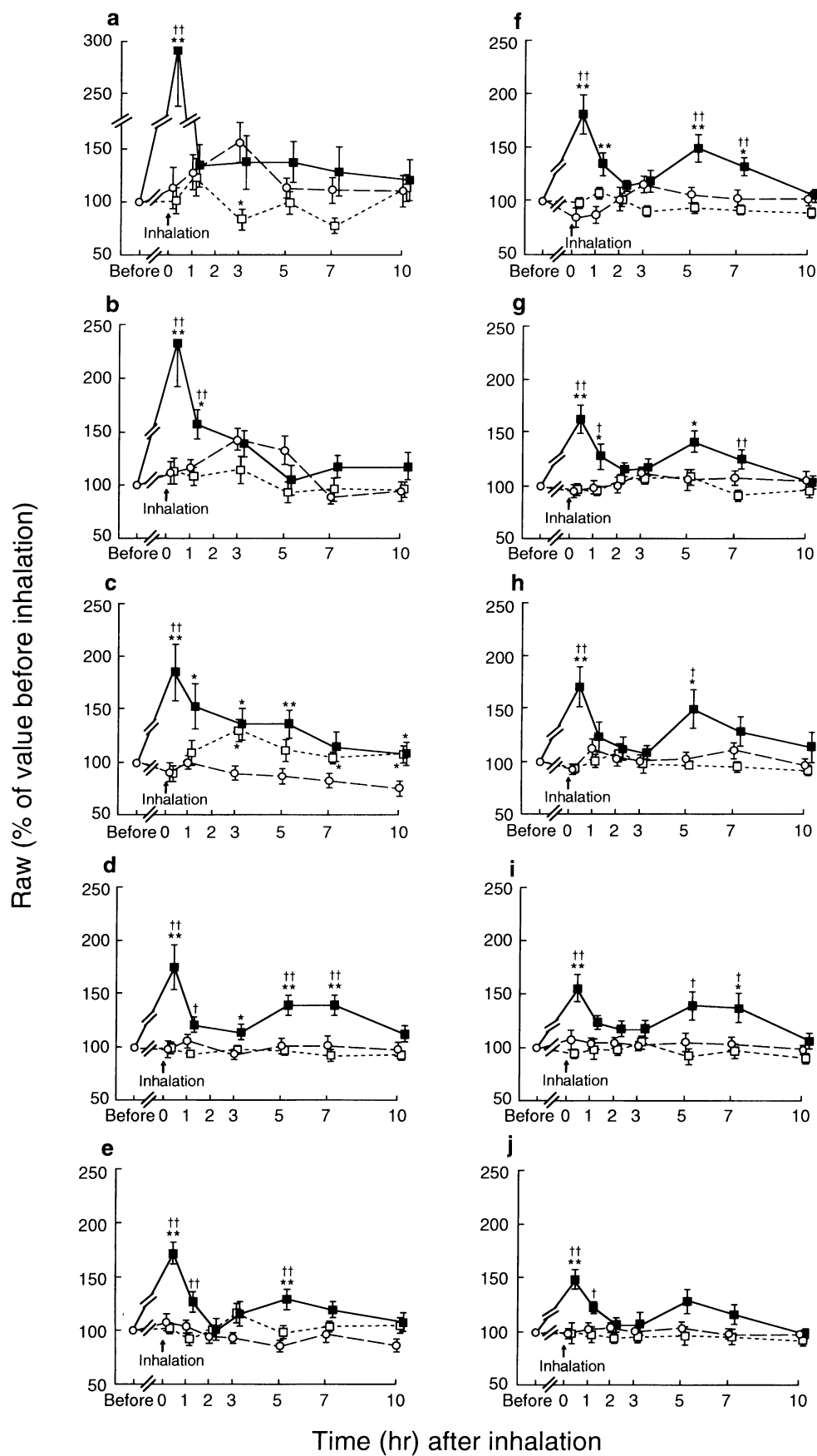
Fig. 2. Time course changes of airway resistance (Raw) by the 1st (a), 2nd (b), 3rd (c), 4th (d), 6th (e), 8th (f) and 10th (g) inhalation of ovalbumin (OA) in sensitized guinea pigs. Group 1a (open circle): Inhalation of physiological saline, once every 2 weeks. Group 2 (closed circle): Inhalation of 10 μg OA/animal, once every 2 weeks. Guinea pigs were treated with mepyramine (10 mg/kg, i.p.) 30 min before each inhalation. Each point represents the mean±S.E. of 5 to 10 animals. Significantly different from Group 1a: *P<0.05, **P<0.01.

rest was in the nasal cavity (24).

Sensitization and/or challenge with antigen

Sensitization and challenge with OA or OA+Al(OH)$_3$ in all the experiments were performed by inhalation of the mist. Five groups of guinea pigs were employed for the experiments, one for each of the following sensitizing/challenging schedules: Group 1a (n = 6): Inhalation of physiological saline, once every 2 weeks for 18 weeks (negative control of Group 2); Group 1b (n = 13): Inhalation of physiological saline, once every 2 weeks for 40 weeks (negative control of Group 4); Group 2 (n = 5 − 10): Inhalation of 10 μg OA/animal, once every 2 weeks; Group 3 (n = 7): Inhalation of 750 μg Al(OH)$_3$ /animal, every 2 weeks for the first 2 times and then alternate inhalations of physiological saline and 750 μg Al(OH)$_3$/animal, once every 2 weeks; Group 4 (n = 13): Inhalation of 15 μg OA + 750 μg Al(OH)$_3$/animal, every 2 weeks for the first 2 times and then alternate inhalations of 10 μg OA/animal (for challenge) and 15 μg OA + 750 μg Al(OH)$_3$/animal (for sensitization), once every 2 weeks (Fig. 1).

The guinea pigs of each group were treated with mepyramine, an antihistaminic, at a dose of 10 mg/kg (i.p.) 30 min prior to the following respective inhalations: Group 1a, the 3rd to 10th saline inhalations; Group 1b, the saline inhalations corresponding to OA inhalation challenges of Group 4; Group 2, the 3rd to 10th OA inhalations; Group 3, saline inhalations; Group 4, OA inhalation challenges.

Exposure time (min) with antigen mists to guinea pigs for quantitative inhalation was calculated from the following 3 values: 1) mist concentrations of OA or OA + Al(OH)$_3$, which was calculated by using Evans' blue in place of OA (24); 2) the rate of these Evans' blue mists trapped in the lung following inhalation (24); and 3) respiratory volume for 1 min of individual guinea pigs, which was measured by a two-chambered, double-flow plethysmograph system as described in the next section.

Measurement of the pulmonary function

The airway resistance (Raw) before and after antigen challenge by inhalation in the conscious guinea pig was measured by a two-chambered, double-flow plethysmo-graph system (Pulmos-I; M.I.P.S., Osaka). 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, cmH$_2$O/sec) was measured by the detection of the respective sensors of air flow equipped to the front and rear chambers according to the method described by Pennock et al. (26). Raw [cmH$_2$O/(ml/sec)] was calculated by dividing the specific Raw by the individual tidal volume (ml) in place of the thoracic gas volume (ml) because the functional residual capacity (ml) could not be detected in the system.

PCA

The levels of γ_1 and IgE antibodies against OA in the serum from the sensitized guinea pigs (Groups 2 and 4) were assessed by 4-hr and 7-day PCA, respectively, according to the method of Ovary et al. (27) and Levine et al. (25).

Statistical analyses

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 probability value (P) of less than 0.05 was considered to be statistically significant.

RESULTS

Change of Raw induced by antigen

The respective time courses of Raw at the first to 10th inhalations of OA alone (Group 2) are shown in Fig. 2. At the 1st and 2nd inhalations, no significant variances of Raw were observed (Fig. 2: a and b). However, the 3rd inhalation induced a moderate but significant increase of Raw 1.5 hr after the provocation (Fig. 2c). Furthermore, at 10 min after administration of the 4th inhalation, Raw reached three times the value before the inhalation (Fig. 2d). This increase is considered to correspond to EAR. Through the 5th and 6th inhalations, the EAR was still consistently observed, but the response was somewhat deteriorated (Fig. 2e). With further inhalations, the response was greatly reduced: There was minimum elicitation of EAR at the 8th and 10th inhalations (Fig. 2: f and g). Throughout the repetitive challenges by the

Fig. 3. Time course changes of airway resistance (Raw) by the 1st (a), 2nd (b), 3rd (c), 4th (d), 5th (e), 6th (f), 7th (g), 8th (h), 9th (i) and 10th (j) inhalation of ovalbumin (OA) in sensitized guinea pigs. Group 1b (open circle): Inhalation of physiological saline, once every 2 weeks. Group 3 (open square): Inhalation of 750 μg Al(OH)$_3$/animal, every 2 weeks for the first 2 times and then alternate inhalations of physiological saline and 750 μg Al(OH)$_3$/animal, once every 2 weeks. Group 4 (closed square): Inhalation of 15 μg OA + 750 μg Al(OH)$_3$/animal, every 2 weeks for the first 2 times and then alternate inhalations of 10 μg OA/animal (for challenge) and 15 μg OA + 750 μg Al(OH)$_3$/animal (for sensitization), once every 2 weeks. Guinea pigs were treated with mepyramine (10 mg/kg, i.p.) 30 min before the challenge. Each point represents the mean ± S.E. of 7 to 13 animals. Significantly different from Group 1b (*P < 0.05, **P < 0.01) or from Group 3 (†P < 0.05, ††P < 0.01).

antigen inhalation, no discernible increase of Raw corresponding to the LAR was recognized.

Figure 3 shows the results of the time course changes of Raw following respective OA inhalation challenges during alternate repeated inhalations of OA+Al(OH)₃ for sensitization and OA for challenge (Group 4). The first antigen challenge, which was taken on day 14 after the second inhalation of OA+Al(OH)₃ in the consecutive first two sensitizations, induced striking EAR. However, no detectable LAR was recognized, although slight eleva-

tion of Raw was maintained from 1 to 10 hr after the challenge (Fig. 3a). Similar results of the time course change of Raw were obtained at the second challenge: There was still no LAR between 5 and 10 hr after the challenge (Fig. 3b). However, at the 3rd challenge, comparable differences from the former two challenges in the time course change of Raw were observed; Raw was sustained at considerably elevated levels until 5 hr after the provocation, which followed transiently increased Raw (EAR), and LAR is thought to be involved in this. LAR
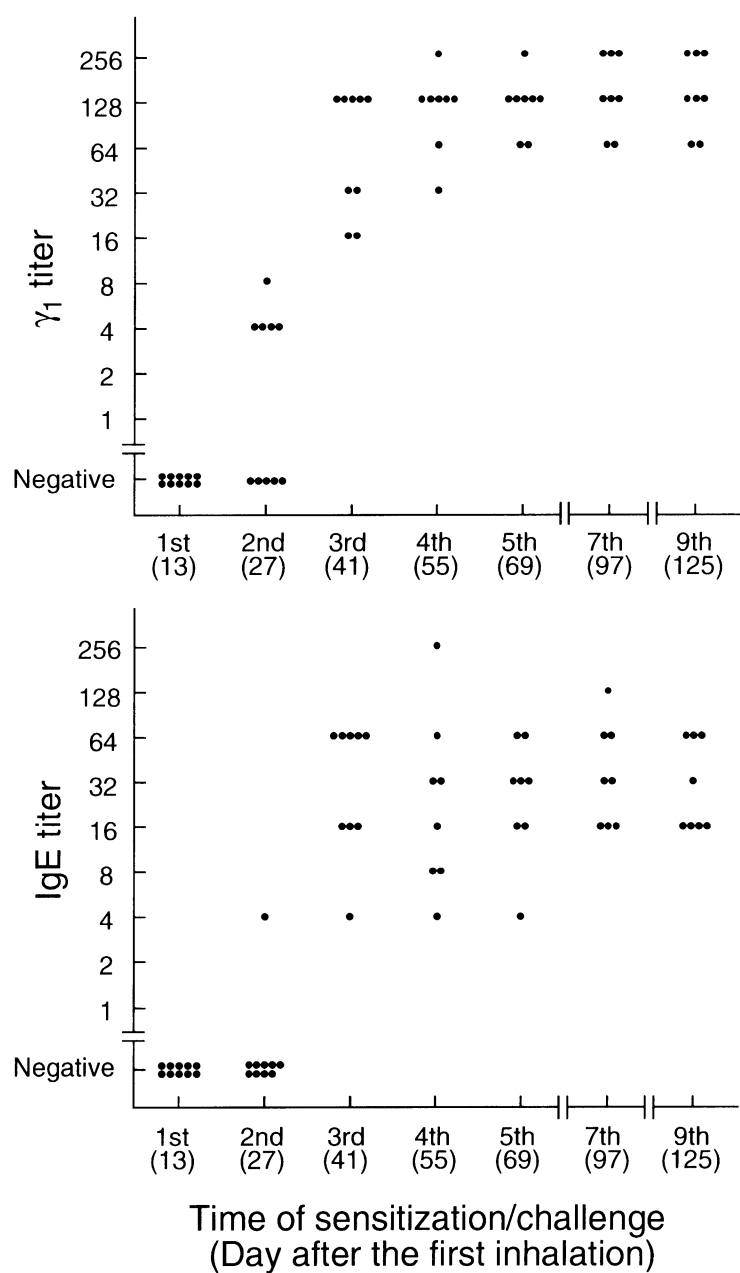

Fig. 4. 4-hr (γ_1) and 7-day (IgE) homologous passive cutaneous anaphylaxis titers of the serum from guinea pigs sensitized and challenged by inhalation with ovalbumin (Group 2). Sera were drawn on the 13th day after each antigen inhalation.

could not be clearly distinguished from the EAR because of the possible prolongation of the EAR (Fig. 3c). The 4th antigen challenge induced time course changes of Raw with apparently separated EAR and LAR: The respective peak Raws were seen 10 min in the former and 5 or 7 hr in the latter after the challenge (Fig. 3d). These biphasic responses persisted at every antigen challenge on and after the 4th one at least until the 10th inhalation (on day 280 after the first sensitization), although the EAR seemed to be slightly deteriorated with repetition of the

challenge and the LAR occurred with variable intensities (Fig. 3: e−j).

In the control groups (Groups 1a, 1b and 3) that had been treated with either aerosolized saline or Al(OH)$_3$, no obvious change of Raw was induced by inhalation of physiologic saline at any time (Fig. 2 and 3).

Throughout the above experiments, there were no significant differences among the groups when Raw at the time before the respective challenges were compared: mean±S.E. of Raw values 1 hr before the 10th challenge

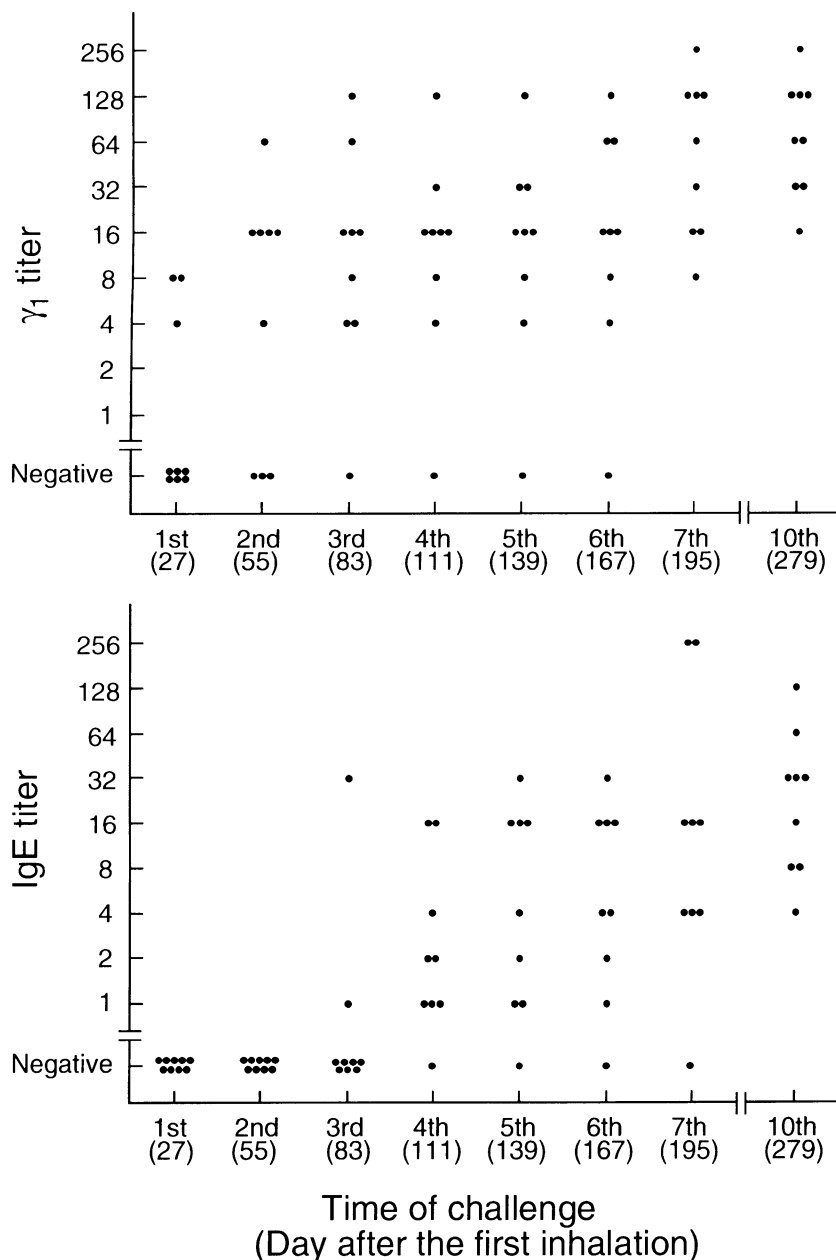**Fig. 5.** 4-hr (γ_1) and 7-day (IgE) homologous passive cutaneous anaphylaxis titers of the serum from guinea pigs sensitized by inhalation with ovalbumin (OA) + Al(OH)$_3$ and challenged by inhalation with OA (Group 4). Sera were drawn on the day before the respective challenges.

in Groups 1a, 1b, 2, 3 and 4 were 1.24±0.07 (n=6), 1.15±0.05 (n=13), 1.17±0.14 (n=5), 1.10±0.04 (n=7) and 1.07±0.07 (n=13) cmH$_2$O/(ml/sec), respectively.

γ_1 and IgE antibody levels in the serum during sensitization/challenge with antigen

The levels of γ_1 and IgE antibodies in the serum during the course of the repetitive sensitizations/challenges with inhaled OA+Al(OH)$_3$ and/or OA (Groups 2 and 4) were quantified by measuring their titers of 4-hr and 7-day PCA, respectively.

Figure 4 represents the results in the group sensitized/challenged with OA without adjuvant (Group 2), which were obtained from the serum on day 13 after each antigen inhalation. None of the guinea pigs following the first sensitization were found to be positive for either 4-hr or 7-day PCA. At the second antigen inhalation (on day 27 after the first sensitization), 5 and 1 out of 10 animals showed elevated levels of respective specific γ_1 and IgE antibodies in the serum. At the 3rd (on day 41 after the first sensitization), significant levels of these antibodies were detected in all animals with values of more than 16× for 4-hr PCA and 4× for 7-day PCA. These antibody titers in the serum were not decreased but rather slightly increased by further inhalations of OA at least until the 10th inhalation.

Figure 5 shows γ_1 and IgE antibody levels in the serum from the Group 4 guinea pigs during the course of sensitization with OA+Al(OH)$_3$/challenge with OA. The serum was obtained on the day before the respective challenges with OA for the PCA titers. The sera from a few animals at the first challenge (on day 27 after the first sensitization) were positive for 4-hr PCA, but all sera were negative for 7-day PCA. At the second challenge (on day 55 after the first sensitization), the sera of 6 out of 9 animals were 4-hr PCA positive. However, none of these sera showed any specific IgE antibody against OA. By further inhalations of OA+Al(OH)$_3$ and OA, both the γ_1 and IgE antibody levels in the serum were elevated: All animals were positive for 4-hr PCA at the 7th challenge (on day 195 after the first sensitization) and for 7-day PCA at the 10th challenge (on day 279 after the first sensitization), respectively. By repeating sensitization /challenge, no decrease in the level of either γ_1 or IgE antibody was recognized.

DISCUSSION

In the past, studies have not been directed to develop experimental asthma using guinea pigs, in which the animal is sensitized and challenged repeatedly with antigen on a long term basis, although these animals are frequently used as a good model for the analyzing asthma and the assessment of drugs for its remedy. One reason for this is the easy development of tolerance with repeated antigen challenges in this species (18–23). If such a model with experimental asthma with chronicity could be made, it would contribute much to asthma research. Therefore, we examined whether semi-chronic or chronic experimental asthma in the guinea pig can be established by repeated inhalations with a small defined dose (10 μg/animal /time) of antigen (OA) with or without the adjuvant, Al(OH)$_3$, at certain intervals. In the present experiments, the time course changes of Raw and the levels of γ_1 and IgE in the serum during long-term sensitizations /challenges were investigated.

Repeated alternate inhalations of OA+Al(OH)$_3$ for sensitization and OA for provocation at 2-week intervals can cause the appearance of LAR at a certain stage (4 months after the first sensitization) in addition to EAR in the guinea pig. The LAR was sustained with reproducibility and was constant until at least the 10th inhalation challenge, while EAR slightly decreased with the time of inhalation. On the other hand, repeated inhalations of OA alone at the same dose in similar sensitization/challenge conditions also produced EAR with an intensity that was more rapidly decreased with the time of inhalation than that in the case of the sensitization /challenge with OA+Al(OH)$_3$/OA, but no obvious LAR was observed at any sensitizing/challenging time.

In general, the sensitization by not only injection but also inhalation with antigen without adjuvant has been reported to stimulate only the production of cytophilic γ_1 antibodies (28). However, the present sensitization/challenge by inhalation with a small dose of OA alone induced detectable levels of IgE as well as γ_1 on the day before the 3rd sensitization/challenge, although the appearance of the latter seemed to slightly precede the former. On the other hand, comparatively different from that with OA alone, the appearance of these antibodies in the serum in the group sensitized/challenged with OA+Al(OH)$_3$/OA was delayed: Three out of 9 animals were positive for 4-hr PCA and all animals were negative for 7-day PCA in the serum on the day before the first challenge. However, similar to the case with OA alone, both the number of animals positive for 4-hr and 7-day PCA and their titers were increased by the repetition of sensitization/challenge. Eventually, all sera on the day before the 10th challenge were positive for either PCA.

In preliminary experiments, when the mists of 10 μg OA/animal were inhaled once every 2 weeks, 8 out of 12 guinea pigs of Group 2 died within 10 min after the 3rd inhalation of the antigen. The majority of Group 4 also died at the 1st challenge. Therefore, the guinea pigs of each group were treated with mepyramine at a dose of 10 mg/kg (i.p.) 30 min before the respective challenges.

Consequently, no guinea pigs died of anaphylactic shock under the mepyramine treatment in both sensitized groups (Group 2 and 4) at any challenge, suggesting that histamine released from mast cells largely contributes to the occurrence of EAR in a stage during repetitive challenges, although it is not clear whether 10 mg/kg of the drug completely antagonized the action of histamine released.

Deterioration of EAR observed especially in the OA repetitive inhalation group is not likely to be proportional to the serum levels of γ_1 or IgE in both cases of the present sensitization methods in accordance with another report (23). Andrew et al. (23) suggested that the desensitization in EAR is due to the decreased releasability of histamine in low concentrations of challenging antigen. In the present experiments, mepyramine was used for prevention of sudden death, so that the possibility can be excluded. However, other chemical mediators such as arachidonate metabolites responsible for the EAR might be decreased in the same manner. Fleisch et al. (29) showed that the amounts of not only histamine but also p-LTs (called slow reacting substance of anaphylaxis in the past) released anaphylactically from the guinea pig lung (30, 31) decrease with increasing age. This is more likely in the present case, since a considerable time period is necessary for performing these experiments.

Our recent experiment showed that no deaths are observed in Group 4 at the 4th challenge under conditions where treatments without mepyramine are performed: Although the intensity of EAR was enhanced, LAR was unchanged. These observations suggest that repetitive challenges may lead to the decrease of histamine releasability from mast cells or the downregulation of H_1-receptors and that histamine might not participate in causing LAR. Furthermore, different from other guinea pig models (12, 14, 16, 17), it would become possible to evaluate newly developed antihistaminic or inhibitors of histamine release if asthmatic responses were observed without mepyramine treatment.

The mechanism of the occurrence of LAR still remains to be elucidated. Okada et al. (32) reported that silica, which is known to stimulate macrophages to release cytokines including interleukin (IL)-1 (33), and IL-1 potentiates antigen-induced LAR when simultaneously administered at the time of sensitization with antigen. Because of the elevated γ_1 and IgE levels in the serum induced by them, they suggested that the substantial amounts of these antibodies are required for a significant magnitude of LAR. However, Terashi et al. (34) suggested that IgE is not associated with LAR because animals passively sensitized with the antibody could not elicit significant LAR. Thus, there is a controversy about whether there is actually a correlation between levels of cytophilic

antibodies and the magnitude of LAR. As demonstrated in the present experiments, in the group in which sensitization/challenge was carried out by inhalation with OA without adjuvant (Group 2), hardly any detectable LAR was observed even though all the animals presented high levels of not only IgE but also γ_1 in their sera. Therefore, it is strongly suggested that neither IgE nor γ_1 is directly linked to the occurrence of LAR. This seems to be further supported by the present results obtained from the experiments in which OA+Al(OH)$_3$ was used for the sensitization: The correlation between serum levels of γ_1 or IgE and the intensity of LAR was hardly recognized.

On the other hand, it is not known why LAR was hardly observed at any time of the challenge in the group sensitized/challenged with OA alone, even though several reports have indicated that LAR occurs at the challenge following 1 to 3 times sensitization with antigen for a relatively short period of time (12–16). In contrast, it is interesting that the LAR was elicited by repeated sensitizations/challenges with OA+Al(OH)$_3$/OA, although serum levels of γ_1 and IgE were quite high in either sensitization/challenge method (Groups 2 and 4). The reason for this is also unclear so far. Johns et al. (35) have pointed out that the late response observed in the other reports includes some responses that occurred in the upper airways. We reported that the challenging antigen mists generated with the hand-made glassware pressure nebulizer used in the present study is fine (2.1 μm) compared to those generated with other two nebulizers, the DeVilbiss nebulizer (2.7 μm) and an ultrasonic nebulizer (4.7 μm). Resultantly, when guinea pigs were forced to inhale the mists, approx. 80% of the trapped mists in the airways was found in the lung, but no more than 50% and 20%, respectively, of the other two-generated mists arrived in the lung (24). These observations suggest not only that the LAR observed in the OA+Al(OH)$_3$/OA group was largely reflected by responses at the lung, but also that the present LAR was elicited with the result that the inhaled Al(OH)$_3$ together with antigen had activated some immune systems.

Taken together, these results strongly suggest that the present method of repeated sensitizations/challenges with antigen+Al(OH)$_3$/antigen is very beneficial for the analysis of developing asthma, especially LAR and the evaluation of the drugs to be used or having been used for the treatment of the disease. Now, we are currently investigating in this model the hyperresponsiveness to some chemical mediators, the types of migrated leukocytes into the airway, the contribution of proinflammatory cytokines, the contribution of the upper and the lower airways to Raw and so on.

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