

EFFECT OF INHALED CYCLOSPORIN A ON THE ALLERGEN-INDUCED LATE
ASTHMATIC RESPONSE AND INCREASED IN AIRWAY
HYPERRESPONSIVENESS IN A GUINEA
PIG MODEL OF ASTHMA

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Oral administration of cyclosporin (CsA), a potent inhibitor of helper T cell function, prevents the allergen-induced late asthmatic response (LAR) and the increase in airway hyperresponsiveness (AH) seen in actively sensitized guinea pigs. The systemic administration of this agent in humans has been associated with serious side effects, therefore, the effects of inhaled CsA were therefor xamined in guinea pigs that were actively sensitized by repeated exposure to nebulized ovalbumin. Respiratory resistance (Rrs) of the animals was measured by an oscillation method and the extent of AH was inferred from the inhaled concentration of histamine required to increase Rrs by 200%. The magnitude of ovalbumin-induced immediate bronchoconstriction after sensitization was similar in CsA-treated and nontreated control animals. However, a LAR was observed in 4/5 control animals but in 0/5 CsA-treated animals. The increase in AH observed 24 hours after antigen exposure in control animals was significantly inhibited by prior CsA inhalation. Significant CsA concentrations were detected by radioimmunoassay in the lungs of CsA-treated animals. Thus, inhaled CsA should be further investigated because it may be useful treating asthma while avoiding side effects.

Key words: airway hyperresponsiveness — cyclosporin A — late asthmatic response

INTRODUCTION

Attention has recently been focused on inflammatory changes in the respiratory tract of patients with chronic asthma, including inflammatory cells such as eosinophils or lymphocytes¹⁾. Clinically, the number of eosinophils or lymphocytes in bronchoalveolar lavage fluid or pulmonary tissue biopsy samples is positively correlated with the severity of asthma²⁾⁻⁶⁾. In paticular lymphocytes are thought to play an important role in chronic asthma. Accordingly, cyclosporin A (CsA), which suppresses the activation of helper T cells⁷⁾⁸⁾, has been considered for treating

patients with asthma. The allergen-induced late asthmatic response (LAR), which occurs 3 to 9 hours after the immediate asthmatic response (IAR), and the continuous airway hyperresponsiveness (AH) that appears approximately 24 hours after the IAR clinically and histologically resemble chronic and intractable asthma. We previously demonstrated that the LAR after exposure to antigen and the ensuing increase in AH can be suppressed by the oral administration of CsA in a guinea pig model of chronic asthma⁹⁾. In clinical trails, the oral administration of CsA showed improvement in the lung function of patients with bronchial asthma¹⁰⁾, or reduces the amount of steroids required by patients with steroid-dependent, intractable asthma and, in some individuals, to result in withdrawal from steroid therapy¹¹⁾. However, the systemic administration of CsA is associated with toxicities such as renal and liver dysfunction, hypertension, and cancer. In an attempt to avoid such side effects, we have now examind

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Abbreviations: **AH** airway hyperresponsiveness; **CsA** cyclosporin A; **H₂₀₀** The inhaled concentration of histamine resulting in a 200% increase in Rrs; **IAR** immediate asthmatic response; **LAR** late asthmatic response; **Rrs** respiratory resistance

the anti-asthma effects of CsA administered topically with an inhaler in the guinea pig model of chronic asthma.

MATERIAL AND METHODS

Sensitization of guinea pigs

This study was carried out in accordance with guidelines for Animal Experimentation, Dokkyo University School of Medicine.

Male Hartley guinea pigs with body masses of approximately 300 g (Doken, Ibaragi, Japan) were sensitized by inhaling ovalbumin (Sigma Chemical Corp., St. Louis, Mo.) from a plastic cylinder (55 cm, diameter; 8 cm, height). The animals were fixed with the nose and mouth toward the center of the cylinder. Ten animals can be exposed simultaneously and equally with this device. Ovalbumin (10 mg/ml) was administered by an ultrasonic nebulizer (model NE-U12; Omuron, Kyoto, Japan) daily for 10 min. On the 9th or 10th day, all animals exhibited asthmatic signs and the respiratory resistance (Rrs) had increased at least twofold as compared to baseline measures before ovalbumin inhalation, indicative of sensitization. Two booster inhalations of ovalbumin (10 mg/ml) were subsequently administered to the guinea pigs for 5 min at weekly intervals. They were randomly divided into two groups for observation of the LAR and AH, and then divided, at random, into control and CsA-treated subgroups. The times required for sensitization of the animals in each group were as follows: LAR control group (n=5) 9.5 ± 0.02 days; LAR CsA-treated group (n=5), 9.4 ± 0.15 days; AH control group (n=5), 9.7 ± 0.11 days; and AH CsA-treated group (n=5), 9.5 ± 0.12 days. There were no significant differences between the control and CsA-treated groups with regard to the number of days required for sensitization.

Determination of Rrs

Rrs was determined by the oscillation technique which has been described previously¹²⁾

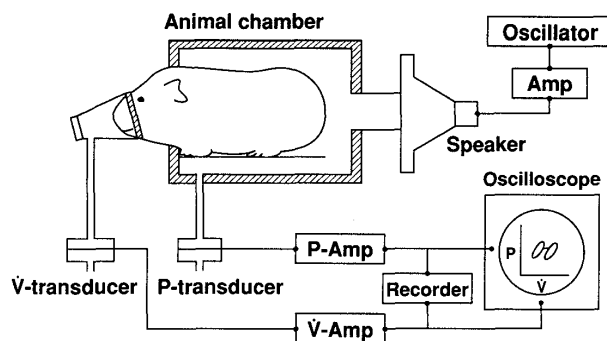


Fig. 1. Schematic illustration of the experimental apparatus for recording changes in Rrs of guinea pigs. P, pressure; V, flow; Amp, amplifier.

(Fig. 1). Animals were placed in a plastic cabinet, which was 10 cm in inner diameter and 18 cm in length, with a wall thickness of 0.5 cm. The animals were fixed by surrounding of the neck with a ring rubber balloon, and a sine wave pressure of 18 Hz was applied from their rear. Pressure was generated with a function oscillator (model LGF-1300; Leader Electronics Corp., Tokyo, Japan) connected to a integrated power amplifier (model KR-2300; Torio, Tokyo, Japan) and a woofer speaker (model 12L-27; Coral Corp., Tokyo, Japan). The amplitude of the oscillatory pressure was adjusted to 7 cmH₂O. The respiratory flow rates of the animals were extracted by a differential transducer (model DP-45; Validyne engineering Corp., Northridge, Calif.) through the screen of the cone-shaped plastic mask, which has an 8-mm hole at the top of the cone with a 200-mesh steel screen, then depicted on an amplifier (model 2G46; Shizume Medical Corp., Tokyo, Japan), and recorded on a polygraph (model WTR751, Graphtec Corp., Tokyo, Japan). The Rrs was derived by measuring the ratio of the internal pressure of the chamber and the respiratory flow rate.

PCA titer measurement

Blood samples were taken from each animals immediately before administration of CsA. Antibody titer was measured by homologous passive cutaneous anaphylaxis (PCA) according to the method described by Watanabe¹³⁾. Two

fold dilutions of each sample were injected intradermally with volume of 0.1 ml into each site of the backs of nonsensitized normal guinea pigs. A mixture of ovalbumin (500 μ g) and 1 ml of 1% Evans Blue in physiological saline was injected intravenously 4 hours after the intradermal injection and 7 days later. The highest dilution producing a more than 8-mm colored spot 30 min after the injection of the antigen was used as the PCA titer. The 4 hours and 7 days-PCA titers were defined as IgG1 and IgE antibody, respectively.

Evaluation of AH after exposure to antigen

Physiological saline was added to a 5-ml ampule of CsA (containing 250 mg of CsA, 3.25 g of polyoxyethylene castor oil, and ethanol) (Sandoz Pharmaceutical Comp. Basel, Switzerland) to a final volume of 50 ml, which was then administered by inhalation with the ultrasonic nebulizer for 1 hour on 7 consecutive days to guinea pigs in the AH CsA-treated group, beginning 7 days after the second booster ovalbumin inhalation. Five milliliter vehicle (3.25 of polyoxyethylene castor oil and ethanol) was diluted with physiological saline to a final volume of 50 ml, and administered to the control group in the same manner. Initial AH of each group was then measured 1 hour after the inhalation of final CsA or dilutions. The animals was then exposed to nebulized ovalbumin (10 mg/ml) for 5 min 3 hours after the determination of the first AH, followed 24 hours later by measuring the second AH by the inhalation histamine. The animals were placed in the animal chamber and forced to inhale physiological saline aerosol for 1 min. The Rrs immediately after inhalation was taken as the baseline value. Histamine was then inhaled at a concentration of 39 μ g/ml for 1 min, and the concentration was then double each subsequent minute, reaching as high as 5000 μ g/ml. The histamine concentration at which the Rrs showed a twofold or greater increase over the baseline value was taken as threshold. By plotting the logarithm of the inhaled histamine concentration on the x

axis and Rrs on the y axis, AH was determined from the log of the histamine concentration (log H_{200}) at which the Rrs achieved 200% of the baseline value.

Evaluation of the LAR after exposure to antigen

Animals in control and CsA-treated groups were exposed to either dilutions or CsA, respectively, as described for evaluation of AH. Following 1 hour after the final exposure, to protect the guinea pig from the histamine-induced anaphylactic shock during the IAR, the histamine H1-receptor antagonist, diphenhydramine hydrochloride (30 mg per kilogram body mass) (Sigma Chemical Corp., St Louis, Mo.) was administered intraperitoneally 15 min before exposure to antigen according to the method described by Hutson. Animals then inhaled ovalbumin (20 mg/ml) for 10 min, and the changes in Rrs were measured for up to 9 hours.

Measurement of CsA

CsA in blood and lung tissue homogenates was measured with a RIA kit (Cyclotrack; Incster Corp., Stillwater, MN, USA)¹⁴⁾. This RIA contains a primary mouse antibody against CsA. The antibody has been mixed with donkey anti-mouse and normal mouse serum to form an immuno complex. CsA in a homogeneous lung solution with 3 ml physiological saline and 250 μ l of the whole blood samples were extracted by addition of 1 ml of 95% methanol, stirred and centrifugally separated at 2000 $\times$ g for 5 min.

For the assay, 50 μ l of the supernatants were incubated with 100 μ l of the tracer, iodohistaminylsuccinyl cyclosporin C [¹²⁵I], followed by 1 ml of the performed immune complex for 1 hr at 25°C. After the incubation, the samples were centrifuged for 20 min at 1000 $\times$ g and radioactivity of the separated precipitate was counted in a gamma counter (Aloka Co., Well-type scintillation counter ARC950, Tokyo, Japan). The CsA concentration (ng/ml) in the sample was inferred on a CsA dose-radioactivity curve of the standard serums containing CsA (10, 40, 80, 120, 240, and 500 ng/

ml). The level of sensitivity of the assay was 20 ng/ml. The concentrations of CsA in the lung were expressed per unit mass (ng/g). The weight of used each lung ranged from 0.2746 to 0.7039 g.

Statistical analysis

Data are expressed as mean $\pm$ SEM. Difference between groups were assessed by Student's *t*-test for data of LAR and AH, by Wilcoxon-Mann-Whitney test for that of concentration of CsA. *P* value of <0.05 was considered statistically significant.

RESULTS

PCA titer

PCA titer as IgG1 in each sample serum were

as follows: AH control group ($n=5$), 240 ± 73 ; AH CsA-treated group ($n=5$), 253 ± 66 ; LAR control group ($n=5$), 263 ± 70 ; LAR CsA-treated group ($n=5$), 249 ± 62 . No significant differences in IgG1 were observed between each group. PCA titer as IgE in each sample serum were as follows: AH control group, 162 ± 46 ; AH CsA-treated group, 150 ± 34 ; LAR control group, 168 ± 51 ; LAR CsA-treated group, 173 ± 58 . No significant differences in IgE were also observed between each group. Therefore, all the animals were sensitized to the same extent.

Changes in AH after exposure to antigen

The effect of histamine concentration on Rrs was determined before and 24 hours after inhalation of ovalbumin in sensitized guinea pigs in both control (Fig. 2A) and CsA-treated groups (Fig. 2B). The results are summarized in Figure

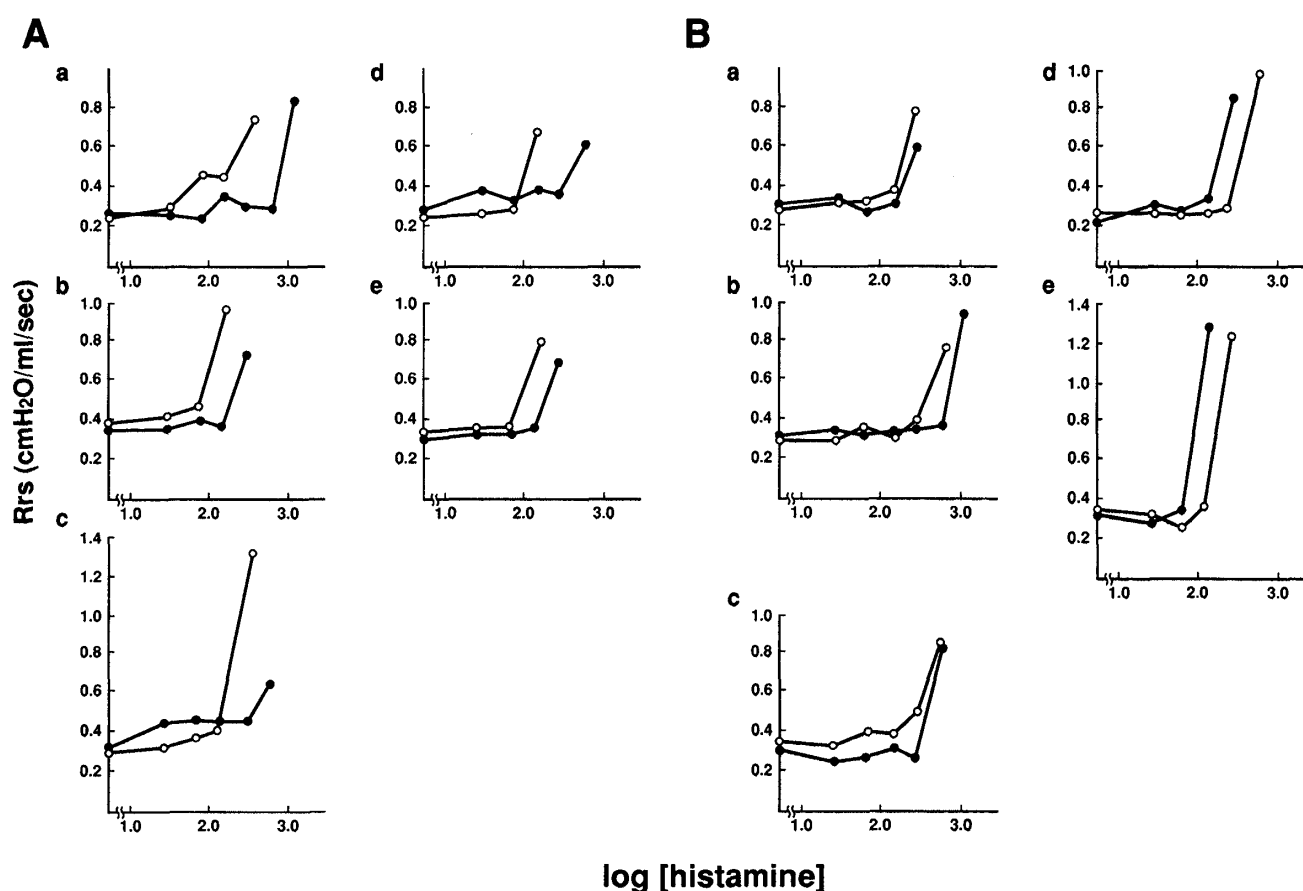


Fig. 2. Histamine dose-response curves for Rrs before (closed circles) and 24 hours after (open circles) antigen challenge in each control (A) (a, b, c, d, e) and CsA-treated (B) (a, b, c, d, e) guinea pigs.

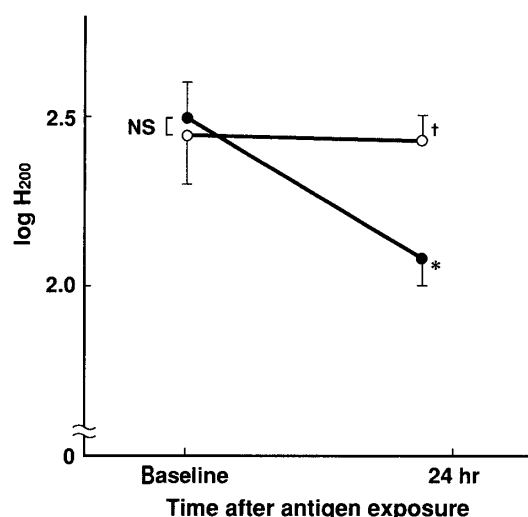


Fig. 3. Effect of antigen challenge on AH (log H 200) in control (closed circles) and CsA-treated (opened circles) guinea pigs. Values are means $\pm$ SEM (n=5). * p < 0.05 vs. before antigen challenge, + p < 0.05 vs. control.

3. Whereas the AH of the control group increased significantly after exposure to antigen

[log H₂₀₀: 2.51 ± 0.11 before and 2.10 ± 0.09 24 hours after antigen exposure (p < 0.05)]. That of the CsA-treated group was not significantly affected (log H₂₀₀: 2.45 ± 0.15 before and 2.43 ± 0.06 24 hours after antigen exposure). No significant difference in AH before antigen exposure was observed between control and CsA-treated group whereas there was significant difference in AH 24 hours after the exposure between two groups (p < 0.05). Thus, the increase in AH following exposure to antigen was significantly inhibited by CsA.

Changes in Rrs after exposure to antigen

The transition in Rrs after the inhalation of ovalbumin in sensitized guinea pigs is shown in Fig. 4. Rrs is expressed as percent of baseline value. The IAR after antigen exposure was reflected in Rrs values of $188.0 \pm 30.4\%$ and $170.2 \pm \%$ of baseline values in control and CsA-treated groups, respectively, with a ten-

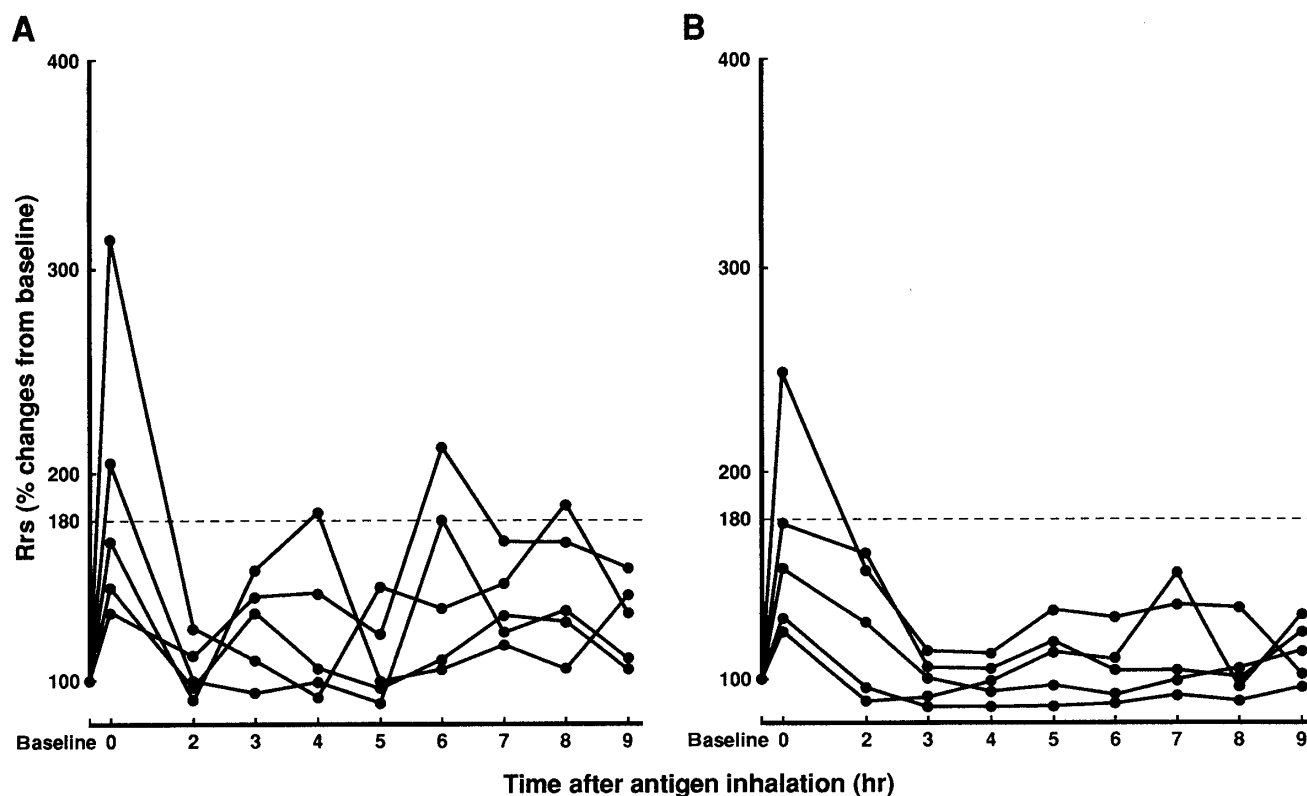


Fig. 4. Changes in Rrs after antigen challenges in control (A) (n=5) and CsA-treated (B) (n=5) guinea pigs. Rrs is expressed as percent of baseline value. An increase in Rrs to 180% or more between 3 to 9 hours after antigen challenge indicates a LAR.

dency toward decrease in the magnitude by the drug but not significantly different. Four of the five animals in the control group also exhibited a delayed increase in Rrs to 180% or more after 3 to 9 hours. None of the CsA-treated animals showed such a LAR. The maximum value of Rrs between 3 and 9 hours after antigen exposure was compared between each animals. The mean maximal values of Rrs of the control animals ($187.4 \pm 37.3\%$) increased significantly from that of CsA-treated animals ($122.1 \pm 24.6\%$) ($p < 0.05$), indicating that CsA significantly inhibited the development of LAR (Fig. 5).

CsA concentration in blood and pulmonary tissue

The CsA concentration in blood of the control group ($n=10$) ranged from 20 to 38 ng/ml, whereas that of the CsA-treated groups was 122.3 ± 10.1 ng/ml ($n=5$) and 107.2 ± 12.4 (n=5) approximately 9 and 24 hours after antigen challenge (Fig. 6A). All the concentration in lung extracts of controls before expres-

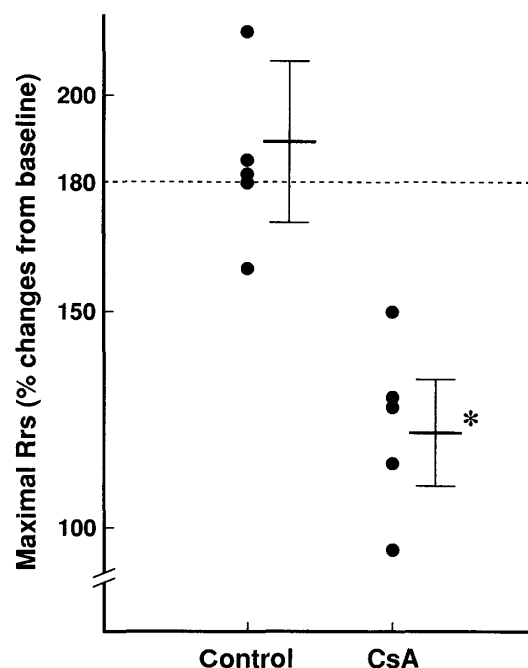


Fig. 5. The maximum value of Rrs measured between 3 and 9 hours after antigen challenge in each animals is plotted for Rrs at the late-phase responses. * $p < 0.05$ vs. control.

sion per unit mass ($n=10$) were less than 27 ng/ml, one of which was below the level, 20 ng/ml,

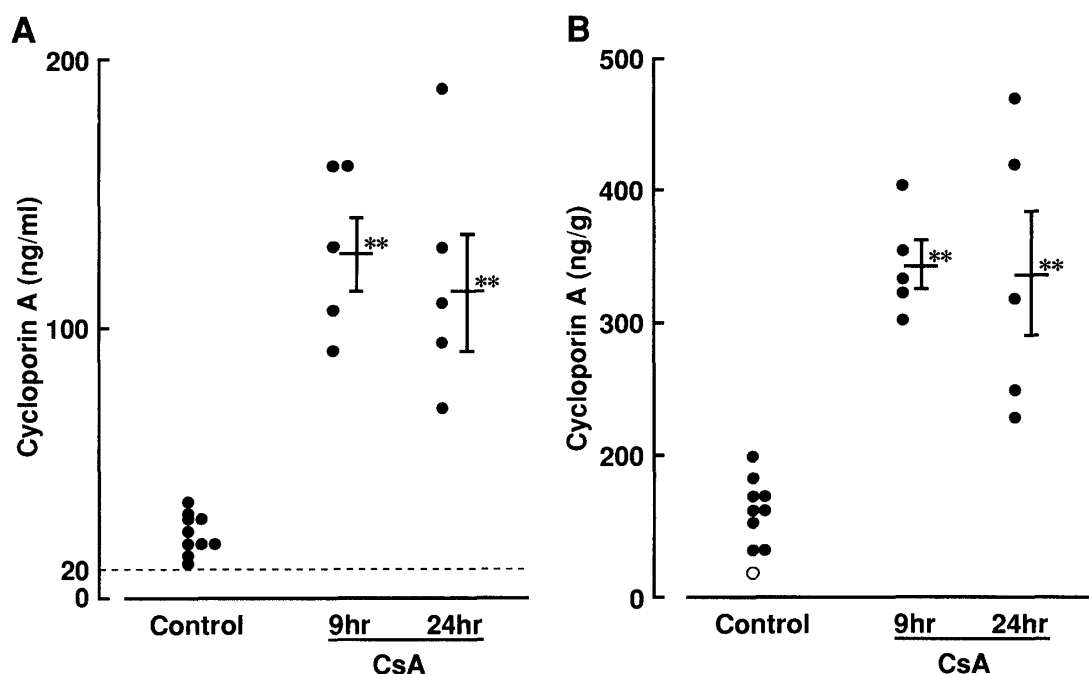


Fig. 6. CsA concentrations in whole blood (A) and lung tissue (B) of control ($n=10$) and CsA-treated guinea pigs 9 hours ($n=5$) and 24 hours ($n=5$) after the final administration of the drug. A dot shows CsA concentration of each sample. Mean value of each group was also shown with SEM. ** $p < 0.01$ vs. control.

of sensitivity of the assay. The concentrations in the lung tissue for the CsA-treated groups was 352.3 ± 31.4 ng/g ($n=5$) and 339.3 ± 80.9 ng/g ($n=5$) approximately 9 and 24 hours, respectively, after the final administration of the drug (Fig. 6B). Thus, the lower limit of detection of CsA in the extracted solution is considered to be from about 30 ng/ml.

DISCUSSION

In guinea pigs actively sensitized by repeated inhalation of a ovalbumin, we have shown that inhaled CsA suppressed both expression of the LAR and the increase in AH after antigen challenge.

We employed a guinea pig model of asthma in the present study that has previously been described and established⁽⁹⁾⁽¹⁵⁾⁽¹⁶⁾. This model can reproduce a constant and significant increase in AH and LAR. In LAR study, according to the method described previously^{(17)–(19)}, we have used a H1-receptor antagonist to allow the administration of a high dose of antigen to the airway to permit the development of consistent LAR, since the development and magnitude of a LAR has been described to dependent on the size of antigen stimulus in human subjects⁽²⁰⁾.

Because Rrs includes the resistance of upper and lower airways, upper airway may effect the changes in Rrs measured by the oscillation technique. By using the technique, Iijima⁽²¹⁾ have previously reported the increase in Rrs in the late phase, which was correlated with inflammatory changes in lower airway in the guinea pigs. Further, Okada⁽²²⁾⁽²³⁾ has already described that the changes in the Rrs are sufficient to indicate LAR in guinea pigs by reasoning by reasoning the changes in the immediate and the late phase more closely reflect the changes in the resistance of the lower airway. Therefore, we decided to measure changes in Rrs for asthmatic response by the oscillation technique.

The mechanism, underlying the IAR is thought to be IgE-mediated mast cell activation

by antigen⁽²⁴⁾. Although the mechanism to develop LAR is not clear yet, the pathogenesis has recently been considered to be related to inflammatory changes in the airway accompanied with infiltration of inflammatory cells, particularly eosinophils⁽¹⁷⁾, and it has recently been suggesting that T lymphocytes play an orchestrating role in the inflammatory reaction by cytokines⁽²⁵⁾⁽²⁶⁾, which is thought to occur summarily in patients with chronic asthma⁽²⁾⁽²⁷⁾. Actually, T-lymphocytes and their products are characteristic of chronic asthma and correlate with disease severity⁽²⁸⁾. And, Frew⁽²⁵⁾ has observed that T-lymphocytes accumulated within the bronchial walls of guinea pigs during the progression of the LAR. Thus, a T-lymphocytes suppressant, such as CsA has attracted attention as an anti-asthma therapy as logical. In the present study, inhaled CsA could inhibit LAR and the expected increase in AH after antigen exposure, which is the same as the results of previous studies employing oral administration of CsA⁽⁹⁾⁽²⁹⁾. Fukuda⁽²⁹⁾ has previously observed oral administration of CsA suppressed antigen-induced LAR and AH along with inhibition of eosinophil infiltration in the airway in guinea pig model of asthma. Similarly, we observed a decrease in eosinophils in bronchi of inhaled CsA-treated guinea pigs at late phase after antigen exposure in a preliminary study (data not shown). Hence, inhibitory effects of inhaled CsA on LAR and AH might be partially due to regulation of T-lymphocytes activation, since CsA has been shown to suppress activation of guinea pig T lymphocytes and their production of cytokines⁽³⁰⁾. Histamine, in addition to IAR, has also been reported to contribute to late nasal response after antigen challenge⁽³¹⁾, and thus we are unable to exclude a reduction of the effect of this mediator from activated mast cells as a factor in development of LAR in present study. On the other hand, inhaled CsA has no significant inhibitory effect on IAR in vivo, which has been shown in studies with oral administration of the drug⁽⁹⁾⁽²⁹⁾. However, it has recently been shown that CsA also

inhibits IgE-mediated mediators such as histamine, leukotriene C4 and prostaglandin D2 release from human mast cells³²⁾³³⁾. It has not been known whether CsA in vivo show the same effect as that in vitro. We have no means to explain the discrepancy between the in vivo and in vitro studies because we did not measure the concentrations of these mediators in the lung tissue.

CsA has proved an effective treatment in clinical trials conducted in patients with chronic asthma¹⁰⁾. This finding is consistent with a study showing that CsA inhibited the activation of T lymphocytes from individuals with glucocorticoid-resistant asthma³⁴⁾. Although hypertrichosis and hypertension were observed as side effects in the clinical trials¹⁰⁾, no severe reactions were apparent. However, there remains the possibility of severe side effects, such as renal dysfunction, infection, and cancer, from long-term CsA administration. Our observation that the administration of CsA by inhalation suppressed the respiratory reactions in asthmatic guinea pigs leads to CsA levels not only in the lung but also blood. The whole blood concentration of CsA was comparable to the low end of the accepted therapeutic range for oral CsA therapy in humans. Although the blood level in human cannot be directly compared to those in guinea pigs, the level in guinea pigs would be expected to cause the same side effects as CsA given orally. Thus, our data is not enough to indicate any advantage of inhaled CsA over orally administered drug. But an administration by inhalation may be the first step to prevent the side effects associated with the systemic administration of the CsA, and we should investigate further the effect of reduced dose of inhaled CsA on the pulmonary response, the inhalation method, and the direct toxicity of the drug to the respiratory mucosa.

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モルモット喘息モデルにおける遅発型喘息反応 (LAR) 及び 気道過敏性亢進に対する吸入サイクロスポリン A の効果

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以前、我々は、慢性喘息モデルである能動感作モルモットの抗原暴露後の LAR 及び気道過敏性の亢進が強力な helper T cell 抑制作用を有するサイクロスポリン A (CsA) の経口前投与によって抑制されることを認め、臨床的有用性を示唆した(日本胸部疾患学会誌, 29, 1089~1095, 1991). 今回は、更に CsA の気道内吸入局所投与による抗喘息効果の検討を行った. 正常雄 Hertley 系モルモットに対して卵白アルブミン (OA) の連続反復吸入暴露によって能動感作した後、CsA (5mg/ml) を 1 日 1 回 1 時間、連続 7 日間吸入させた後、OA 抗原吸入を行い、経時的に呼吸抵抗 (Rrs) をオッシレーション法を用いて測定した. CsA 非処置のコントロール群 (n=5) では、抗原暴露 3~9 時間後に、LAR が 80% 出現したのに対して、CsA 処置群 (n=5) では、認められなかった. また、24 時間後の吸入ヒスタミンに対する気道過敏性の検討では、コントロール群 (n=5) で認められた気道過敏性の亢進が CsA 処置群 (n=5) では、有意に抑制された ($p < 0.05$). 以上より CsA は、経口投与と同様に吸入投与によっても抗原暴露後の LAR 及び気道過敏性の亢進を抑制を示したことから、CsA の臨床的有用性及び全身投与による副作用を回避する手段として吸入投与が有用である可能性が示唆された.
