

Contribution of Anaphylatoxins to Allergic Inflammation in Human Lungs

Masayoshi Abe^{*,1}, Hiroshi Hama⁴, Takayuki Shirakusa², Akinori Iwasaki², Nobuhumi Ono⁴, Nobuhiro Kimura³, Tony E. Hugli⁵, Noriko Okada⁶, Takeshi Katsuragi¹, and Hidechika Okada⁶

¹Department of Pharmacology, ²Second Department of Surgery, and ³First Department of Internal Medicine, School of Medicine, and ⁴Department of Drug Information, School of Pharmaceutical Science, Fukuoka University, Fukuoka, Fukuoka 814–0180, Japan, ⁵Torrey Pines Institute for Molecular Studies, San Diego, CA92121, U.S.A., and ⁶Department of Biodefense Medicine, Nagoya City University Graduate School of Medical Sciences, Nagoya 467–8601, Japan

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Abstract: The contribution of complement activation to allergic asthma remains controversial. In order to elucidate the role played by the complement split products, anaphylatoxins C3a and C5a, we evaluated their effects on production of cysteinyl-leukotrienes (cysLTs) by human lung fragments following an anaphylactic reaction. The lung tissues obtained from two patients with lung cancer showed C5aR-, C5L2R-, and C3aR-mRNA expression. When the chopped lung fragments passively sensitized with human IgE were incubated with anti-human IgE antibody, a significant amount of cysLTs was generated in comparison with the control (without anti-IgE antibody). The co-addition of human C5a at doses of 0.1 to 10 ng/ml to the anti-IgE antibody potentiated cysLT production. The response was bell-shaped in distribution, significant, and peaked at a C5a concentration of 1 ng/ml. The co-addition of human C3a up to 1,000 ng/ml seemed to increase cysLT production, but not to any significant extent. A novel C5a receptor complementary peptide, acetylated peptide A, dose-dependently inhibited cysLT production by the human lung fragments following the anaphylactic reaction in the presence of 1 ng/ml C5a. However, this peptide did not inhibit cysLT production in the presence of 100 ng/ml C3a. It is suggested that the anaphylatoxin C5a potentiates cysLT production in human lung tissues and contributes to allergic inflammation in disorders such as asthma, thus acetylated peptide A may be useful for suppressing allergic inflammation in the lungs.

Key words: Allergic asthma, C5a inhibitor, Cysteinyl-leukotrienes, Anaphylatoxin

Bronchial asthma is one of the most prevalent health problems in industrialized countries and is considered to be a complex inflammatory disorder of the airways characterized by various pathophysiological features (17). Although various mediators and cytokines may be involved in asthma inflammation, recent studies suggest that cysteinyl-leukotrienes (cysLTs) are one of the most important mediators in this disease (19). Very recently, it was suggested that complement activation products, especially anaphylatoxins C3a and C5a, contribute to the inflammation of asthma (1, 4, 14, 21), and several animal studies using C3aR-genetically disrupted mice and naturally defective guinea pigs indicated a role for C3a in airway hyperresponsiveness after allergen challenge without any demonstrable influence on

cellular infiltration (4, 14). In contrast, it has been reported that another anaphylatoxin, C5a, significantly contributes to immediate and late airway responses in association with submucosal infiltration of inflammatory cells including eosinophils (1, 21). Taken together, C5a may not only play an important role in the pathophysiology of allergic asthma, but its role may be different from that of C3a in the pathophysiology of this disorder.

Although reports have been accumulating concerning the roles of C3a and C5a in asthma as it affects animal models, the precise participation of these anaphylatoxins in human disorders remains obscure. Several potent antagonists against C5a receptor have been developed to ameliorate various refractory disorders (3, 20, 22), but there are considerable species differences in

*Address correspondence to Dr. Masayoshi Abe, Department of Pharmacology, School of Medicine, Fukuoka University, Fukuoka, Fukuoka 814–0180, Japan. Fax: +81–92–865–4384. E-mail: abemasa@fukuoka-u.ac.jp

Abbreviations: AcPep A, acetylated Peptide A; C3aR, C3a receptor; C5aR, C5a receptor (CD88); C5L2R, C5a-like receptor 2; cysLTs, cysteinyl-leukotrienes.

the primary structure of anaphylatoxins and their receptors (9, 11). In order to elucidate the role of anaphylatoxins in human asthma, we attempted to create an experimental model using human lung tissues. Using this model, we compared the effects of two anaphylatoxins, C3a and C5a, on cysLT generation by human lung tissues following an anaphylactic reaction.

Materials and Methods

Materials. Approval from ethics committees and medical product agencies were obtained from the Medical School of Fukuoka University. Recombinant human C5a was purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.) and human C3a was purified as previously reported (13). Human purified IgE and goat anti-human IgE antibody were purchased from Chemicon International (Temecula, Calif., U.S.A.). A complementary peptide of C5a named as Pep A (ASGA-PAPGPAGPLRPMF) is a complementary peptide to amino acids 33 to 53 of human C5a (6, 10, 12). Its N-terminal alanine was acetylated and named as acetylated Pep A, as reported elsewhere (Asai et al., in submission).

Chopped lung fragments and cysLT generation. Human lungs resected from patients with lung cancer were used in this study. A small portion of tissue was obtained from the resected lung immediately after the surgical resection. Pleura, large blood vessels, and bronchi were dissected and cut into small pieces (approximately 1 cm) with scissors. The chopped lung fragments were washed with Tyrode's buffer and periodically kept under a vacuum using a 50 ml disposable syringe to further remove blood. The chopped lungs were then centrifuged at $200\times g$ for 10 min at 4 C. This process was repeated one more time and the chopped pieces were cut into smaller pieces (2–3 mm) with scissors. These lung fragments were incubated with purified human IgE (final concentration = 10 $\mu\text{g/ml}$, Chemicon International) at 22 C overnight (usually for 15–16 hr). The fragments were then washed with Tyrode's buffer and centrifuged at $200\times g$ for 1 min at 4 C. This was repeated three times. Each 300 mg sample of lung fragments was weighed and placed into a siliconized tube on ice. Each incubation mixture with or without anti-human IgE antibody (final concentration, 7.75 $\mu\text{g/ml}$, Chemicon International) in Tyrode's buffer was incubated at 37 C for 30 min. After addition of cold ethanol, the incubation mixture was centrifuged at $3,000\times g$ for 30 min at 4 C and the supernatant was stored at -80 C until analyzed.

Assay of cysLTs. CysLTs (LTC_4 , LTD_4 , and LTE_4) in the supernatants were measured by immunoassay using

precolumn extraction/reversed-phase high-performance liquid chromatography (RP-HPLC) according to a previously reported method (16). Briefly, ethanol was added to the aliquots including $^3\text{H-LTE}_4$ as an internal standard to give a final concentration of 15%. After adjusting to pH 3.0–3.5, the samples were loaded onto a Sep-Pak cartridge (Waters Corporation, Milford, Mass., U.S.A.). Methanol-eluted fractions passed through a minicolumn were concentrated under reduced pressure using a Speed Vac Concentrator (Savant Instruments, Holbrook, N.Y., U.S.A.). After resuspension with 150 μl of HPLC solvent A, 75 μl of the concentrated fraction were injected onto a Novapak C18, 5 μm column ($0.39\times 15\text{ cm}$) (Waters Corporation). Each LTC_4 , LTD_4 , and LTE_4 fraction was collected using a Model 201 fraction collector (Gilson, Villiers le Bel, France) and then evaporated under reduced pressure. The residues in the LTC_4 and LTD_4 fractions were analyzed by enzyme immunoassay (EIA) using a Cysteinyl-leukotriene EIA kit (Cayman Chem., Ann Arbor, Mich., U.S.A.) and that in the LTE_4 fraction, using a Leukotriene E_4 EIA kit (Cayman). LTC_4 , LTD_4 , and LTE_4 values were normalized based on the recovery rates of $^3\text{H-LTE}_4$ ($31.6\pm 1.0\%$, $n=25$). The sum of LTC_4 , LTD_4 , and LTE_4 was regarded as the total amount of cysteinyl-leukotrienes (cysLTs). The HPLC system consisted of a Model 600 controller, a 717 autosampler (Waters), and a Novapak C18 column. The fractions collected by the fraction collector were kept at 4 C using a Thermo NESLAB (RTE 7, Gilson). We used solvent A (acetonitrile/methanol/water/acetic acid, 30:12:58:0.03, vol/vol) containing 0.03% ethylenediamine-*N, N, N'*, *N'*-tetraacetic acid (EDTA-free acid; Dojindo, Kumamoto, Japan) and solvent B, which consisted of acetonitrile/methanol/water/acetic acid (68:12:20:0.01, vol/vol) containing 0.001% EDTA. All solvents were adjusted to pH 5.6 with sodium hydroxide (Nacalai Tesque, Kyoto, Japan). The mobile phase was begun with solvent A and was then changed to solvent B at 20 min. The flow rate was 1 ml/min.

Assay of anaphylatoxins. C3a/C3a desArg and C5a/C5a desArg concentrations in the supernatant were measured by Cytometric Bead Array (BD Biosciences Pharmingen, San Diego, Calif., U.S.A.) according to the manufacturer's instruction. Two-color flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, Calif., U.S.A.).

Expression of complement receptors in the lung tissues. Total RNA was extracted from a portion of the lungs resected from the two patients with lung cancer, and cDNA was prepared using reverse transcriptase. PCR amplification of cDNA samples was conducted

with the following primers: C5aR: sense (5'-TTGTTC-ACGTCCATTGTACAG-3'), antisense (5'-GGCCAA-GCCGGCCCCTCGGAA-3') (12); C5L2R: sense (5'-CTGGCAGTGCCCATTTGCCCGT-3'), antisense (5'-CACCCCGCACGCCCGCTGAAC-3') (5,18); C3aR: sense (5'-TCGCTGGCTCACTTGGCTCTC-3'), antisense (5'-CATCCCTACATTGCGATGATT-3') (2, 8). For glyceral aldehyde phosphate dehydrogenase (GAPDH) as a control, we used sense (5'-TGGAATCCCATCACCATCT-3'), antisense (5'-GTCTTC-TGGGTGGCAGTGAT-3') (7). The PCR was performed with the following reagents: 0.5 µg sample cDNA, 500 nmol/liter primers, 200 µmol/liter dNTPs, 20 mmol/liter Tris HCl, 1.2 mmol/liter MgCl₂ and 2.5 units *Taq* DNA polymerase (TaKaRa) in a 100 µl reaction solution. The program was 94 C for 1 min, 65 C for 1 min and 72 C for 1.5 min for 40 cycles. With the GAPDH gene, 0.25 µg cDNA was used and 28 cycles were performed. Products were analyzed on a 2.5% agarose gel.

Statistical analysis. Data are reported as the means ± S.E.M. The statistical analysis was performed using analysis of variance (ANOVA); and the Tukey-Kramer test was used for multiple comparisons. A value of $P < 0.05$ was considered significant.

Results and Discussion

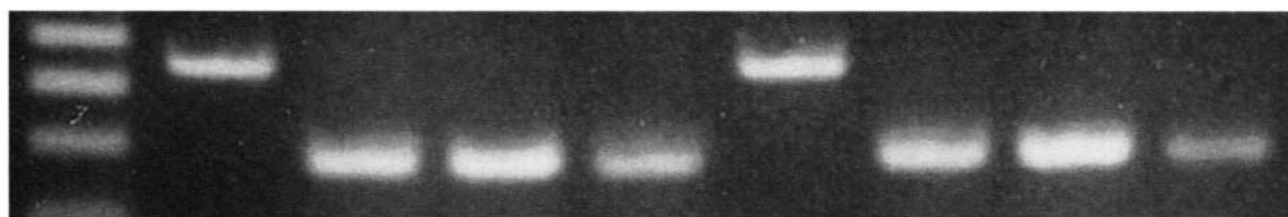
In order to evaluate expression of C5aR-, C5L2R-, and C3aR-mRNAs, we isolated total RNA from portions of the surgically resected lung tissues which appeared normal and were distant from the tumor lesions of the two patients (2, 5, 7, 8, 12, 18). Lung tissue of both patients showed expression of C5aR-, C5L2R-, and C3aR-mRNAs as well as constitutively

expressed GAPDH-mRNA, as shown in Fig. 1.

After passive sensitization of the chopped lung fragments with human IgE, the fragments were stimulated by adding anti-human IgE antibody. A significant amount of cysLTs was generated by the addition of the antibody in comparison with the control (without anti-IgE antibody), as shown in Fig. 2A. While the concentrations of C3a/C3a desArg and C5a/C5a desArg were 158 ± 1 ng/ml and 7.3 ± 3.0 ng/ml ($n=3$) in the supernatants of the control, those were 115 ± 6 ng/ml and 6.5 ± 2.4 ng/ml ($n=3$) after the stimulation with anti-IgE antibody. Consequently, it was suggested that the reaction of IgE and anti-IgE antibody did not produce endogenous C3a or C5a from the chopped lung fragments. However, the co-addition of human intact C5a at doses of 0.1 to 10 ng/ml potentiated cysLT production in a bell-shaped distribution pattern with the maximum level at 1 ng/ml C5a. However, the effect of adding human C3a, even up to 1,000 ng/ml was not significant, although it increased cysLT production to some extent (Fig. 2B). Use of either anaphylatoxin alone did not stimulate cysLT production by the lung fragments (data not shown). As for the sources of these anaphylatoxins, the C5a used was recombinant protein expressed in *E. coli*, but the C3a was a purified protein of human origin (13). We used a C5a concentration of 1 ng/ml in the following anaphylactic reaction model because this dose significantly increased cysLT production and correlated well with the reported concentration detected in sputum from asthmatic patients, median value, 1.293 ng/ml (15). In contrast, the reported median value of C3a in sputum of asthmatic subjects was 4.590 ng/ml (15). We chose to use a C3a concentration of 100 ng/ml for the anaphylactic reaction model because we required a sufficient amount of C3a to compare the

Case 1

Case 2



GAPDH C5aR C5L2R C3aR GAPDH C5aR C5L2R C3aR

Fig. 1. Expression of C5a receptor-, C5L2 receptor-, C3a receptor-, and GAPDH-mRNAs in lung tissues obtained from two patients with lung cancer.

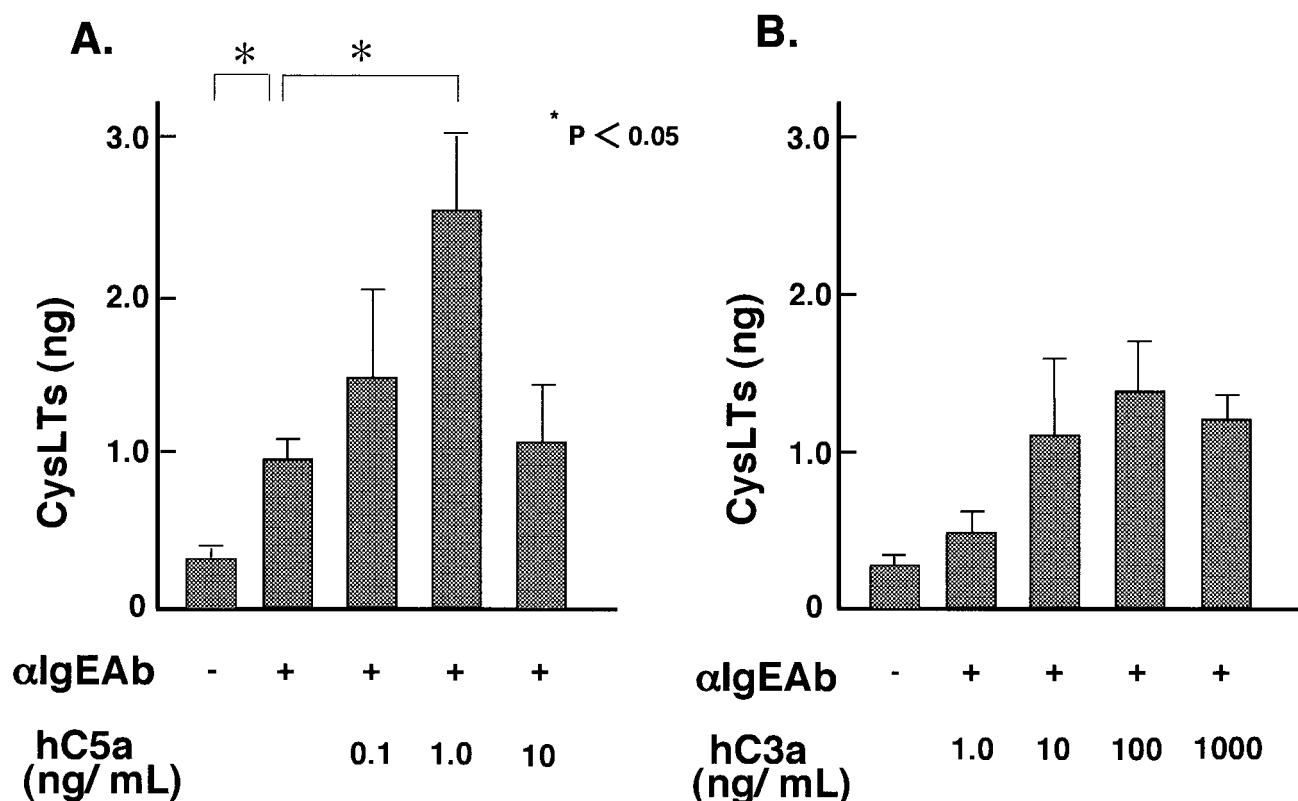


Fig. 2. Effects of addition of C5a at 0.1–10 ng/ml (A, $n=10$) and C3a at 1–1,000 ng/ml (B, $n=10$) on cysLT production by human chopped lung fragments following the anaphylactic reaction. The lung fragments were incubated with or without human IgE at 22 C for 15 hr for passive sensitization. After washing with Tyrode's buffer, the passively sensitized lung fragments were stimulated with anti-human IgE antibody (7.75 μ g/ml) at 37 C for 30 min. After the termination of the reaction, cysLTs in the supernatants were assayed by purification with column chromatography and EIA. * $P<0.05$.

effects of these anaphylatoxins in this *in vitro* anaphylactic reaction. Since the co-addition of C5a potentiated cysLT production following the IgE and anti-IgE antibody reaction, complement activation may contribute to allergic inflammation in lungs especially during the effector phase of asthma (1, 3, 21). A novel C5a inactivator, acetylated peptide A, dose-dependently inhibited cysLT production by the human lung fragments following the IgE and anti-IgE antibody reaction in the presence of 1 ng/ml C5a, as shown in Fig. 3 (10). The addition of acetylated peptide A at 1 μ g/ml or more significantly inhibited cysLT production and at these doses the cysLT amount seemed to be less than the control, but not to a significant extent. However, this peptide did not inhibit cysLT production following stimulation with IgE and anti-IgE antibody in the presence of 100 ng/ml C3a. Since the co-addition of C3a did not significantly potentiate cysLT generation when compared with IgE and anti-IgE antibody alone, it remains to be determined whether acetylated peptide A precisely antagonized C3a activity. This peptide has been shown to inhibit the action of C5a in human neutrophils, in a

human cell line, and in an *in vivo* C5a-induced rat shock model (10). Since this novel C5a inhibitor appears to inhibit the C5a reaction in human lung tissues, it may be useful for suppressing allergic inflammation in human lungs.

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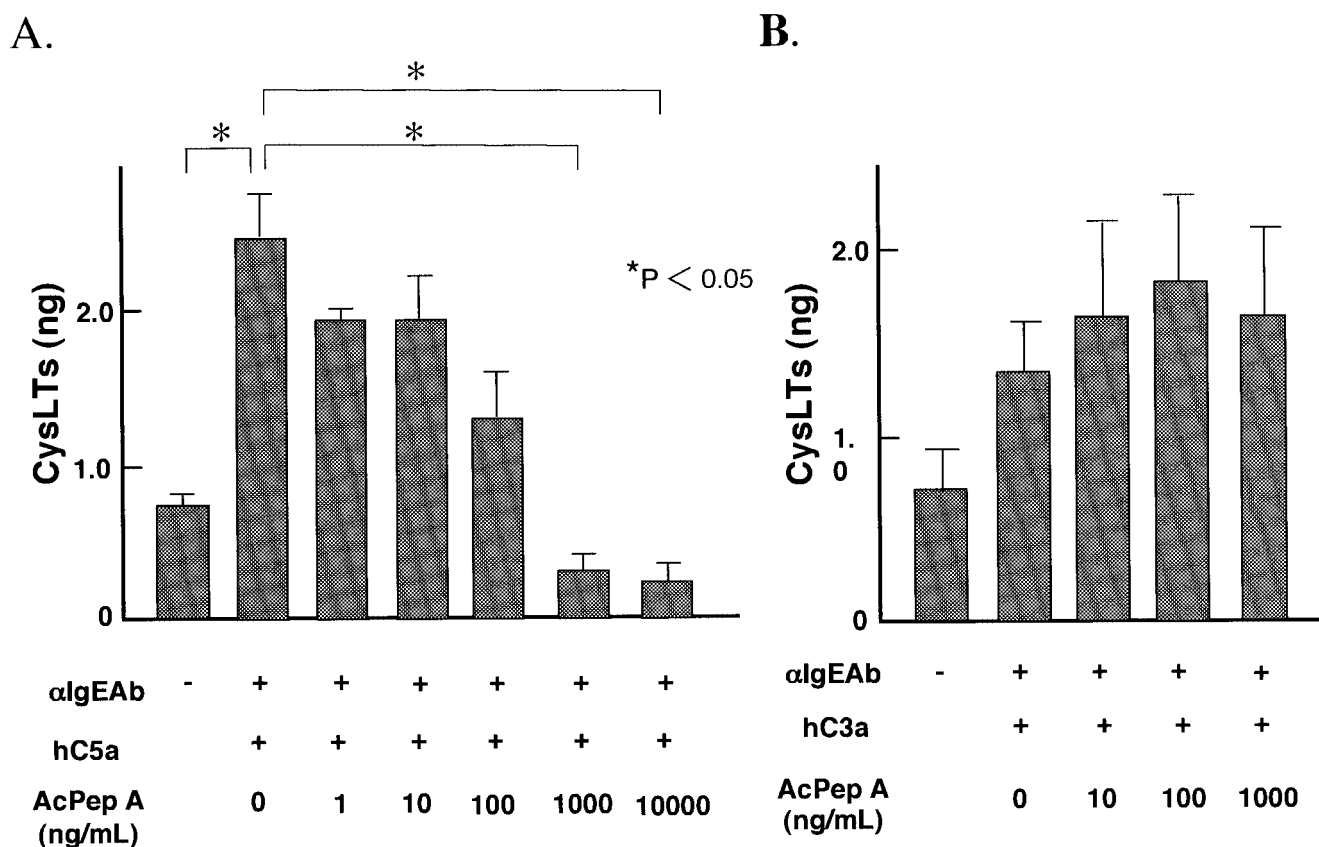


Fig. 3. Effects of acetylated peptide A on cysLT production by human chopped lung fragments following the anaphylactic reaction. The passively sensitized lung fragments were stimulated with or without anti-human IgE antibody (7.75 μ g/ml) in the presence of C5a at 1 ng/ml (A, $n=5$) or C3a at 100 ng/ml (B, $n=4$) at 37 C for 30 min. Various doses of acetylated peptide A were added to the incubation mixture before stimulation with anti-IgE antibody. * $P<0.05$.

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