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Contribution of Anaphylatoxin C5a to Late Airway Responses After Repeated Exposure of Antigen to Allergic Rats

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We attempted to elucidate the contribution of complement to allergic asthma. Rat sensitized to OVA received repeated intratracheal exposures to OVA for up to 3 consecutive days, and pulmonary resistance was then estimated for up to 6 h after the last exposure. Whereas the immediate airway response (IAR) in terms of R_L tended to decrease in proportion to the number of OVA exposures, late airway response (LAR) became prominent only after three. Although premedication with two kinds of complement inhibitors, soluble complement receptor type 1 (sCR1) or nafamostat mesylate, resulted in inhibition of the IAR after either a single or a double exposure, the LAR was inhibited after the triple. Premedication with a C5a receptor antagonist (C5aRA) before every exposure to OVA also inhibited the LAR after three. Repeated OVA exposure resulted in eosinophil and neutrophil infiltration into the bronchial submucosa which was suppressed by premedication with sCR1 or C5aRA. Up-regulation of C5aR mRNA was shown in lungs after triple OVA exposure, but almost no up-regulation of C3aR. Pretreatment with sCR1 or C5aRA suppressed the up-regulation of C5aR expression as well as cytokine messages in the lungs. The suppression of LAR by pretreatment with sCR1 was reversed by intratracheal instillation of rat C5a desArg the action of which was inhibited by C5aRA. In contrast, rat C3a desArg or cytokine-induced neutrophil chemoattractant-1 induced cellular infiltration into the bronchial submucosa by costimulation with OVA, but these had no influence on the LAR. These differences might be explained by the fact that costimulation with OVA and C5a synergistically potentiated IAR, whereas that with OVA and either C3a or cytokine-induced neutrophil chemoattractant-1 did not. C5a generated by Ag-Ab complexes helps in the production of cytokines and contributes to the LAR after repeated exposure to Ag. *The Journal of Immunology,* **2001, 167: 4651–4660.**

Bronchial asthma is considered to be a complex inflammatory disorder of the airways characterized by various pathophysiological features (1). The inhalation of a specific Ag in asthmatic subjects usually results in a dual reaction, consisting of an immediate airway response (IAR)[2] and a late airway response (LAR) (2, 3). Analysis of bronchoalveolar lavage fluid (BALF) and histological finding of bronchial specimens suggested that the infiltration of eosinophils and other inflammatory cells into the bronchial submucosa is a causative mechanism for LAR (4, 5). Various mediators (PGs, cysteinyl-leukotrienes, histamine, and others) released from mast cells after cross-linking of IgE Ab with Ag are important in both responses of bronchial asthma (6). Because the LAR is considered to be a suitable system for studying mechanisms of chronic inflammation in asthma, we attempted to design a reproducible LAR model of significant amplitude by means of repeated Ag challenge of allergic animals (7, 8). It has been suggested that LAR development is usually associated with increased airway responsiveness, but dissociation between the LAR and airway hyperresponsiveness has been noted under some conditions (9). Because the LAR can be inhibited by corticosteroids or by immunization against the allergen (hyposensitization), we attempted to clarify the role of complement activation, as a representative system of innate immunity, in the IAR and LAR occurring after repeated exposure to allergen (10, 11).

It has been suggested that the complement system plays a significant role in bronchoconstriction and the infiltration of inflammatory cells into the lung, as shown using several experimental models for bronchial asthma (12). Recently, several animal studies using C3aR-genetically disrupted mice and naturally defective guinea pigs indicated a role for C3a in airway hyperresponsiveness after Ag exposure without any influence on cellular infiltration (13, 14). However, another potent anaphylatoxin, C5a, remains controversial with respect to its role in allergic asthma, although C5a is important in various human diseases through its diverse actions including chemotactic activity directed to neutrophils, monocytes-macrophages, and eosinophils; direct bronchoconstriction through its receptor on airway epithelial cells; and/or indirect action through the synthesis and release of various cytokines and chemical mediators (15, 16). It was recently suggested that BALFs obtained from asthmatic patients contain C5a/C5a desArg, one of the most prominent neutrophil chemotactic factors (17). Furthermore, a study on experimental animals suggested that IgG immune complexes in the tracheobronchial tree lead to airway hyperreactivity and polymorphonuclear leukocyte influx that are markedly reduced by complement depletion after pretreatment with cobra venom factor (18). There have been conflicting reports regarding changes in the complement cascade in asthmatic patients (1, 12). Because of a paucity of selective inhibitors for the various complement components, a thorough evaluation of the contribution of

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[2] Abbreviations used in this paper: IAR, immediate airway response; LAR, late airway response; BALF, bronchoalveolar lavage fluid; CH50, serum hemolytic complement activity; C5aRA, C5a receptor antagonist; CINC-1, cytokine-induced neutrophil chemoattractant-1; Futhan, nafamostat mesylate; i.t., intratracheally; R_L, pulmonary resistance; sCR1, soluble complement receptor type 1.

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endogenous complement activation to the allergic asthmatic response remains to be performed (1).

Consequently, we attempted to clarify the role of complement activation in airway responses in terms of bronchoconstriction, inflammatory cell infiltration of the bronchial submucosa, the expression of C5a and C3a receptors, and cytokine or chemokine production in the lung using two kinds of complement inhibitors, a low molecular weight and nonselective serine protease inhibitor (nafamostat mesylate; Futhan) and a large and more selective complement inhibitor (soluble complement receptor type 1; sCR1), as inhibitors of complement activation at the C3 and C5 steps (19, 20), instead of complement depletion with cobra venom factor (21), and also by using a C5a receptor antagonist (C5a hexapeptide; NMe-Phe-Lys-Pro-dCha-Trp-dArg) (22).

Materials and Methods

Materials

All experimental protocols were approved by the institutional animal care and use committee of the School of Medicine, Fukuoka University. Male Brown Norway rats (Seakku-Yoshitomi, Fukuoka, Japan) 6–8 wk old and weighing ~250 g were used for the study. A C5a receptor antagonist (C5a hexapeptide; NMePhe-Lys-Pro-dCha-Trp-dArg) and rat cytokine-induced neutrophil chemoattractant-1 (CINC-1) were purchased from the Peptide Institute (Osaka, Japan) (22, 23). sCR1 and Futhan were donated by Avant Immunotherapeutics (Needham, MA) and Torii Pharmaceutical (Osaka, Japan), respectively. Purified rat C5a desArg and C3a desArg were prepared as previously reported and kindly supplied by Dr. T. E. Hugli (Division of Molecular Immunology, La Jolla Institute for Molecular Medicine, La Jolla, CA) (24). *Bordetella pertussis* vaccine (50 μl) containing 6×10^9 heat-killed bacilli was kindly donated by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). Serum hemolytic complement activity (CH50) was determined according to previously described methods (25).

Sensitization of rats

Active sensitization against OVA was performed by s.c. injection of sterile normal saline (1 ml) containing 1 mg OVA (grade II; Sigma, St. Louis, MO) and 200 mg aluminum hydroxide (Sigma). *B. pertussis* vaccine (50 μl) containing 6×10^9 heat-killed bacilli was given i.p. as an adjuvant. Three days later, sterile normal saline (1 ml) containing 1 mg OVA and 200 mg aluminum hydroxide was injected s.c. as a booster. Animals selected for these studies were used 14–28 days after the first injection. The serum IgE concentration was estimated to be 55 ± 3 ng/ml ($n = 3$) before the sensitization and increased to 250 ± 70 ng/ml ($n = 5$) 14 days after the first injection of OVA.

Measurement of pulmonary resistance (R_L)

The rats were anesthetized i.p. with urethan (1 g/kg, 25% w/v). The tip of the tracheal tube (5-cm length of polyethylene tubing (PE-240)) was inserted into the trachea through an open tracheostomy. The transpulmonary pressure was determined by monitoring the difference between pressure in the external end of the tracheal cannula and the esophageal cannula using a Statham differential transducer (DP-45; Validyne Engineering, CA). The intrapleural pressure was measured through a water-filled cannula (PE-240) that was placed in the lower third of the esophagus and connected to one port of a differential pressure transducer (DP-45; Validyne). A Fleisch pneumotachograph and a differential transducer were used to monitor the respiratory flow rate (PULMOS-II system; MIPS, Osaka, Japan). R_L was estimated under artificial ventilation with a Harvard Apparatus Rodent Respirator (Millis, MA) at a respiration rate of 65 breaths/min and a tidal volume of 3.5 ml (26).

To evaluate the effects of the drugs on R_L and on the histological features, sCR1 or Futhan was dissolved in sterile saline and administered i.p. 1 h before the challenge, and a C5aRA in sterile saline was administered i.v. 30 min before the challenge.

Experimental design and effects of anticomplementary drugs

Sensitized rats were divided into three groups to study effects of the numbers of OVA exposures, i.e., single, double, and triple, on time course changes in R_L, as shown in Fig. 1. OVA grade V (Sigma) was used for OVA exposure. As an example, in the triple OVA exposure experiment, the sensitized rats were daily challenged by inhalation of OVA aerosol for 2 successive days (days 1 and 2). For this purpose, the inhalation of 0.25% OVA aerosol was accomplished by placing the rats for 20 min on each occasion in a 10-liter Plexiglas chamber connected to an ultrasonic nebulizer, "Comfort-mini" (model-10; Sin-Ei Industries, Ageo, Saitama, Japan). The next day (day 3), the final OVA challenge was performed by intratracheal (i.t.) administration of 0.1 ml of a 1.7% OVA solution to estimate the time course change in R_L as well as the histological changes. In the single OVA exposure, the rats were challenged by i.t. administration of 0.1 ml of a 1.7% OVA solution without any previous inhalation of OVA aerosol. The R_L was measured before the challenge (baseline value). After challenge with OVA, the R_L was measured at 1, 5, 10, 15, 30, 45, and 60 min; thereafter, R_L was examined every 30 min for 6 h. As a control, the same volume of saline (0.1 ml) was administered i.t. To avoid influence of volume on R_L, the volume administered into the trachea was always 0.1 ml. At 6 h after the i.t. administration, the rats were exsanguinated by cutting the abdominal aorta, and then the lungs were fixed in situ for histological examination or removed from the rats for biochemical study. The removed lungs were immediately frozen in liquid nitrogen and stored at −80°C until use.

Bronchoalveolar lavage was performed via the tracheal cannula using 2×10 ml of saline containing 1 mM EDTA. The BALF was centrifuged at $300 \times g$ for 5 min at 4°C, and the cell pellet was resuspended in 1.0 ml sterile saline with 0.2% rat serum. Total cell count was determined by adding 50 μl of the cell suspension to 50 μl trypan blue stain and counting cells under a light microscope. The differential cell count was performed from the smear preparation stained with Diff-Quik (International Reagents, Kobe, Japan) and counting 200 cells at random under ×200 magnification. The cells were identified by standard morphology.

Effects of C5a desArg, C3a desArg, or CINC-1 administered i.t.

To evaluate the effects of C5a desArg, C3a desArg, or CINC-1 administered i.t. on R_L and to examine the histological features, pretreatment with sCR1 was conducted for inhibition of the complement system using two different schedules ("final" and "every sCR1"). Whereas "every sCR1" treatment indicates that they were pretreated with sCR1 before every OVA exposure, final sCR1 treatment indicates that the rats were pretreated only before the final OVA exposure.

Northern hybridization analysis of C5aR and C3aR expression in lungs

Total RNA was extracted from the lungs using TRIzol (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. The RNA (30 μg/lane) was size fractionated by electrophoresis on an agarose-formaldehyde gel, transferred to a nylon membrane (Hybond-N; Amersham, Buckinghamshire, U.K.) and hybridized at 42°C for 16–18 h with a ^{32}P-labeled rat C3aR or C5aR cDNA probe (27, 28), or a ^{32}P-labeled GAPDH probe (Clontech, Palo Alto, CA). After hybridization, the blots were then washed three times with 0.1× SCC-0.1% SDS for 15 min at 65°C. The blots were exposed to x-ray film with an intensifying screen at −80°C and scanned with a laser densitometer (Personal Densitometer SI; Molecular Dynamics, Sunnyvale, CA).

Detection of cytokines and chemokine messages by RT-PCR

Total RNA was extracted from the lungs, and cDNA was prepared using 4 μg total RNA. PCR amplification of cDNA samples was conducted with the following primers: IL-5: sense (5′-TGCTTCTGTGCTTGAACGT TCTAAC-3′), antisense (5′-TTCTCTTTTTGTCCGTCAATGTATTC-3′), product size 298 bp; IL-12: sense (5′-TGCCCTGGAGAAACG-3′), antisense (5′-TGCTTCACACTTCTTCAGGAAAGT-3′), product size 271 bp; GAPDH: sense (5′-TGAAGGTCGGTGTCCAACGGATTTGGC-3′), antisense (5′-CATGTAGGCCATGAGGTCCACCAC-3′), product size 983 bp. Rat IL-4, IFN-γ, and eotaxin primers were purchased from Biosource International (Camarillo, CA) and were used according to the manufacturer's instructions. These cDNA PCR products were 177 bp (IL-4), 399 bp (IFN-γ), and 222 bp (eotaxin) long. To enable appropriate amplification in the exponential phase for each target, PCR amplification of various cytokines and GAPDH transcripts was conducted in separate reactions with different numbers of cycles, but using similar amounts of the corresponding cDNA templates generated in a single reverse transcription reaction, as described elsewhere (29). Each amplification cycle consisted of denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and the last cycle included a final extension at 72°C for 5 min. The number of PCR cycles was optimized to ensure amplification in the exponential phase. Different numbers of cycles were tested for various cytokines (ranging between 25 and 45) and GAPDH (between 20 and 40), and 40 and 35 cycles were chosen, respectively, for further analysis.

PCR-generated DNA fragments were resolved in 2% agarose gels, and visualized by ethidium bromide staining using a digital imaging system (Ultra-Lum, Carson, CA). For quantitative evaluation, ODs of RT-PCR product signals were obtained by scanning with a laser densitometer. The value for each specific target was normalized according to those of GAPDH value to express arbitrary units of relative abundance of the specific messages.

Histological examination

At 6 h after the i.t. administration of either saline or OVA, the rats were exsanguinated by cutting the abdominal aorta. The trachea was joined to a tube with a three-way stopcock connected to a reservoir containing the fixative. The lungs were fixed in situ by i.t. administration of 8% formaldehyde solution given at a pressure of 15 cm H_2O and were then stained with H&E. The number of neutrophils, eosinophils, and mononuclear cells (macrophages and lymphocytes) per unit airway area ($=10^4$ μm^2) was determined by morphometry at ×400 magnification under light microscopy as previously described (30). The morphometric analyses were performed by individuals blinded to the protocol design.

Statistical analysis

Data are reported as means ± SEM. The statistical analysis was performed using the General Linear Models Procedure in SAS (Statistical Analysis System; SAS, Cary, NC). A p value of <0.05 was considered significant.

Results

Effects of i.t. exposure of OVA on R_L

Fig. 1 shows the experimental protocols used in this study. Rats actively sensitized against OVA were divided into three groups to study the effects of the number of OVA exposures, single, double, and triple, on time course changes in R_L. In the double or triple OVA exposure experiments, the sensitized rats were exposed daily to the Ag by inhalation of 0.25% OVA aerosol for 20 min in a Plexiglas chamber connected to an ultrasonic nebulizer for 1 or 2 successive days. Thereafter, R_L was monitored for up to 6 h under anesthesia and artificial ventilation after an i.t. instillation of 0.1 ml 1.7% OVA (the last OVA exposure; day 2 or 3). In the single OVA exposure, the rats were challenged by i.t. administration of 0.1 ml 1.7% OVA solution without any previous inhalation of OVA aerosol (day 1).

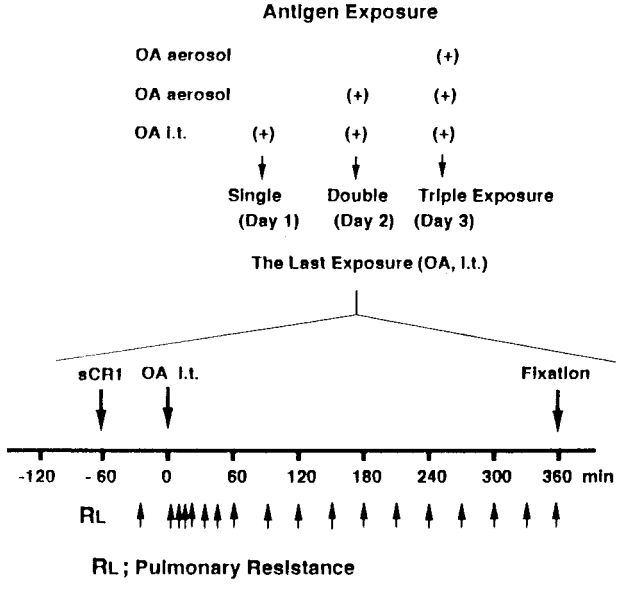

FIGURE 1. Experimental schedules for the exposure of actively sensitized rats to OVA (OA) for up to 3 consecutive days. Certain rats were exposed to the Ag by inhalation of OVA aerosol. The final challenge was conducted by an i.t. instillation of 0.1 ml of a 1.7% OVA solution, and R_L was then estimated for up to 6 h after the last exposure.

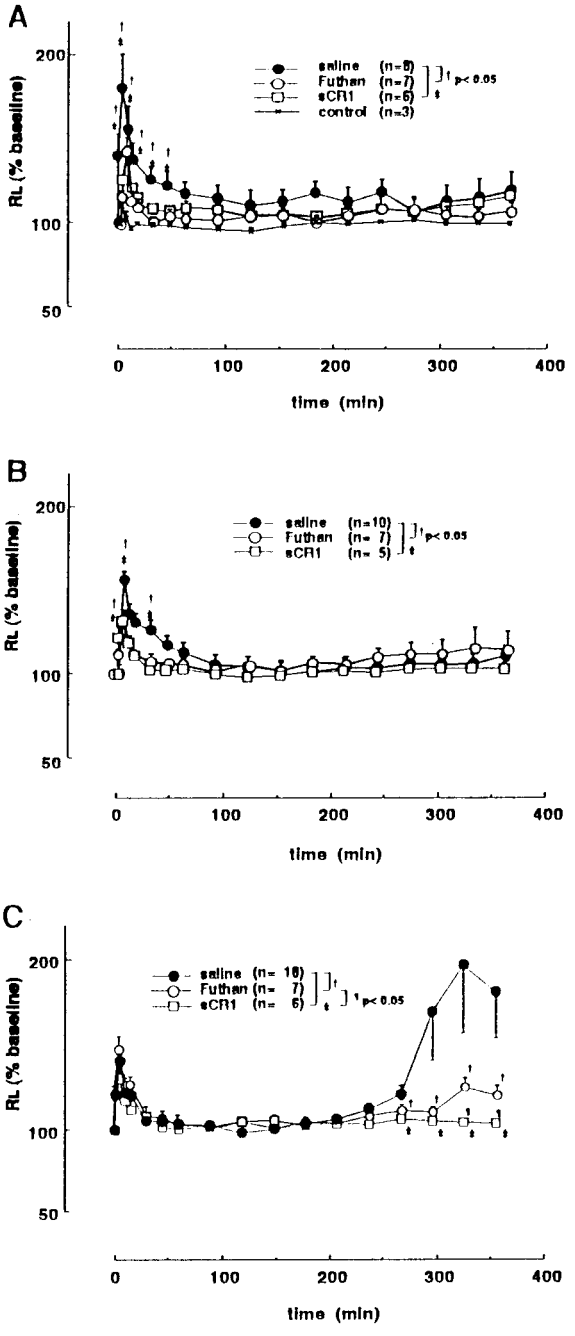

FIGURE 2. *A*, Time course of R_L changes after the first OVA exposure to actively sensitized rats (day 1) without (saline 1 ml/kg i.p.) or with pretreatment with sCR1 (10 mg/kg i.p.) or Futhan (1 mg/kg i.p.) 1 h before the challenge. The control consisted of sensitized rats given 0.1 ml saline i.t. instead of OVA. The baseline value of saline was 0.204 ± 0.003 cm H_2O/ml/s. *B*, Time course of R_L changes after the second OVA exposure of actively sensitized rats with or without pretreatment with either sCR1 or Futhan 1 h before every OVA exposure (day 2). The rats inhaled OVA aerosol the previous day. The baseline value of saline was 0.206 ± 0.003 cm H_2O/ml/s. *C*, Time course of R_L changes after the third exposure of actively sensitized rats to OVA with or without pretreatment with sCR1 or Futhan 1 h before every OVA exposure (day 3). The rats inhaled OVA aerosol for the last 2 consecutive days. The baseline value of saline was 0.217 ± 0.003 cm H_2O/ml/s. The baseline value of each group differed from that of the saline group by <10%. Data are expressed as percent baseline (R_L value before challenge = 100%) values and are presented as means ± SEM. Numbers in parentheses indicate the number of animals used. †, $p < 0.05$ comparison of saline and Futhan, ∗, $p < 0.05$ comparison of saline and sCR1; ¶, $p < 0.05$ comparison of Futhan and sCR1.

As shown in Fig. 2*A*, when the rats were given 0.1 ml saline i.t., no significant changes in R_L were observed (control). On the other hand, i.t. instillation of 0.1 ml 1.7% OVA into sensitized rats (saline) resulted in a rapid increase in R_L, a form of IAR, to nearly 2 times the baseline value within 10 min of challenge. This response tended to decrease in proportion to the number of OVA exposures, as shown in Fig. 2, *B* and *C*. In contrast, rats that received the triple OVA exposure showed a prominent LAR after the triple challenge with OVA, as shown in Fig. 2*C*. We therefore evaluated the effects of two complement inhibitors (Futhan and sCR1) on the R_L. Pretreatment with Futhan (1 mg/kg i.p.) 1 h before the challenge significantly inhibited IAR by the single or double OVA exposure, but not by the triple exposure. However, by the third OVA exposure (day 3), Futhan suppressed LAR. Next, the effects on R_L of pretreatment with a more selective complement inhibitor, sCR1, were evaluated using various doses (5, 10, 20, and 30 mg/kg) and two different routes of administration, namely i.v. or i.p. These preliminary experiments suggested that pretreatment with sCR1 at a low dose of 5 mg/kg only partially inhibited the LAR but that a dose of 10 mg/kg was as effective as 30 mg/kg in inducing a complete inhibition. Moreover, i.p. administration of 10 mg/kg was as effective as i.v. administration. Consequently, an sCR1 dose of 10 mg/kg administered i.p. was selected for the following experiments. Pretreatment with sCR1 also significantly inhibited IAR by a single or double OVA exposure, but not by a triple OVA exposure, as shown in Fig. 2. In rats pretreated with sCR1, the LAR was completely inhibited after the triple OVA exposure (Fig. 2*C*). Table I summarizes the maximum magnitudes of IAR and LAR according to the different OVA exposures and the influence of pretreatment with two kinds of complement inhibitors on these responses. sCR1 was more potent than Futhan in inhibiting the LAR on day 3.

Histological studies

Fig. 3, *A* and *D*, show representative histological findings in bronchial tissue at 6 h after i.t. administration of 0.1 ml saline (control). When the rats received various numbers of OVA exposures up to a total of three, the degree of infiltration of inflammatory cells (eosinophils and neutrophils) into the bronchial submucosa increased in proportion to the incidence of exposure (Fig. 4). When rats received the triple OVA exposure, an extremely high infiltration of inflammatory cells was recognized in the bronchial submucosa (Fig. 3, *B* and *E*). The infiltrating cells were counted by a morphometric analysis, and the findings are summarized in Fig. 4. Differentiation of the infiltrated cells is shown in Fig. 4*B*. To assess systemic complement activation in this model, when the CH50 values of rats given saline i.t. (control) and rats given a triple OVA exposure were compared (on day 3), the value for former group was 36.3 ± 1.2 U/ml and that of the latter was 41.9 ± 0.6 U/ml (mean ± SEM, each $n = 4$). This result suggested that systemic complement activation did not occur after up to three repeated i.t. OVA exposures. Pretreatment with Futhan slightly suppressed cellular infiltration into the bronchial submucosa after OVA exposure (data not shown). In contrast, pretreatment with sCR1 significantly suppressed this infiltration after each OVA exposure (Fig. 4*A*). Fig. 3, *C* and *F*, show representative histological findings in the bronchial submucosa after the triple OVA exposure with sCR1 pretreatment before every exposure, which indicate remarkable suppression of cellular infiltration by sCR1. Fig. 3, *G* and *H*, show cytological finding of BALFs from control and rats, respectively, that received the triple OVA exposure. BALF from control rats contained predominantly macrophages (>90%), as shown in Fig. 4, *C* and *D*. BALF from the rats that received the triple OVA exposure showed predominance of eosinophils and significantly more cells than those from the control. Pretreatment with sCR1 led to significant reduction of cell number recovered in BALF. The BALF data did not exactly confirm those of the morphometric analysis. The percent of eosinophils after sCR1 treatment seemed to be higher in the BALF than that in the morphometry, but this difference was statistically insignificant between both methods (Fig. 4, *B* and *D*).

Because the complement inhibitors suppressed the potentiation of LAR after repeated allergen exposures, we next examined whether these effects were in part due to a suppression of C5a production. As shown in Fig. 5*A*, pretreatment with the C5aRA (1 mg/kg i.v.) inhibited R_L change for up to 6 h after the first exposure to OVA plus 10 ng C5a desArg to sensitized rats pretreated with sCR1 (10 mg/kg i.p.). When the C5aRA (1 mg/kg) was administered i.v. to rats before every OVA exposure (a total of three times), the R_L time course after the third exposure revealed a significant suppression of the LAR, but not of the IAR, as shown in Fig. 5*B* and Table I. Histological analysis also showed a reduced cellular infiltration into the bronchial submucosa by pretreatment with C5aRA (Fig. 3*I* and Fig. 4*A*).

C5aR and C3aR mRNA expression in the lungs

The rats were divided into five groups; group 1, control; group 2, sensitized and saline-challenged; group 3, triple OVA-exposed; group 4, triple OVA-exposed pretreated with sCR1; and group 5, with C5aRA. After total RNA was extracted from each lung in these five groups, C5aR and C3aR mRNA expression was studied by Northern hybridization. The results showed significantly up-regulated expression of C5aR in the lungs of rats subjected to a triple OVA exposure (group 3, $n = 9$) compared with group 1 or 2 (Fig. 6, *A* and *B*). Pretreatment with sCR1 or C5aRA significantly suppressed the up-regulation of C5aR mRNA in the lungs (groups 4 and 5). In contrast, up-regulation of C3aR mRNA in the lungs was hardly detected by Northern hybridization (Fig. 6, *A* and *C*).

Table I. *Effects of OVA exposure on levels of IAR and LAR and the influence of pretreatment with Futhan, sCR1, or C5aRA on those levels*[a]

	IAR				LAR			
Day	Saline	Futhan	sCR1	C5aRA	Saline	Futhan	sCR1	C5aRA
1	190 ± 11 (8)	142 ± 6 (7)	142 ± 5 (6)		126 ± 9 (8)	112 ± 5 (7)	116 ± 4 (6)	
2	156 ± 6 (10)	128 ± 4 (7)	132 ± 4 (5)		117 ± 6 (10)	114 ± 10 (7)	104 ± 4 (5)	
3	141 ± 5 (18)	147 ± 9 (7)	132 ± 5 (6)	141 ± 3 (6)	207 ± 41 (18)	138 ± 8 (7)	108 ± 2 (6)	110 ± 2 (6)

[a] Maximum levels of IAR and LAR after OVA exposure were summarized. The effects of pretreatment with two kinds of complement inhibitors (Futhan and sCR1) or C5aRA on the maximum level of IAR and LAR were compared with effects of using saline pretreatment only. Days 1, 2, and 3 indicate single, double, and triple OVA exposures, respectively (see Fig. 1). The rats were pretreated with saline (1 ml/kg i.p.), Futhan (1 mg/kg i.p.), or sCR1 (10 mg/kg i.p.) 1 h before but with C5aRA (1 mg/kg i.v.) 30 min before every OVA exposure. After the sensitized rats were given 1.7% OVA solution (0.1 ml) i.t. at the last OVA exposure, R_L was monitored for up to 6 h. Each value was divided by the value obtained before the challenge (baseline value, 100%) and expressed as a percent of the baseline level. The variation among mean baseline values in each group was <10%. Numbers in parentheses indicate the number of animals used.

Cytokines and eotaxin mRNA expression in the lungs

Total RNA was extracted from each lung of the five groups of rats, and mRNA expression for IL-4, IFN-γ, IL-12, IL-5, and eotaxin was then examined by RT-PCR. Fig. 7*A* shows representatives of IL-4, IFN-γ, and GAPDH mRNA expression in lungs. The lungs from group 3 showed significantly elevated expression of IL-4 and IFN-γ mRNA and increased levels of eotaxin and IL-5 mRNAs in comparison with lungs from groups 1 and 2, as shown in Fig. 7, *B–F*. Pretreatment with sCR1 and C5aRA (groups 4 and 5) resulted in a significantly reduced expression of IL-12 in comparison with group 3 and reduced levels of IL-4 and IFN-γ, IL-5, and eotaxin.

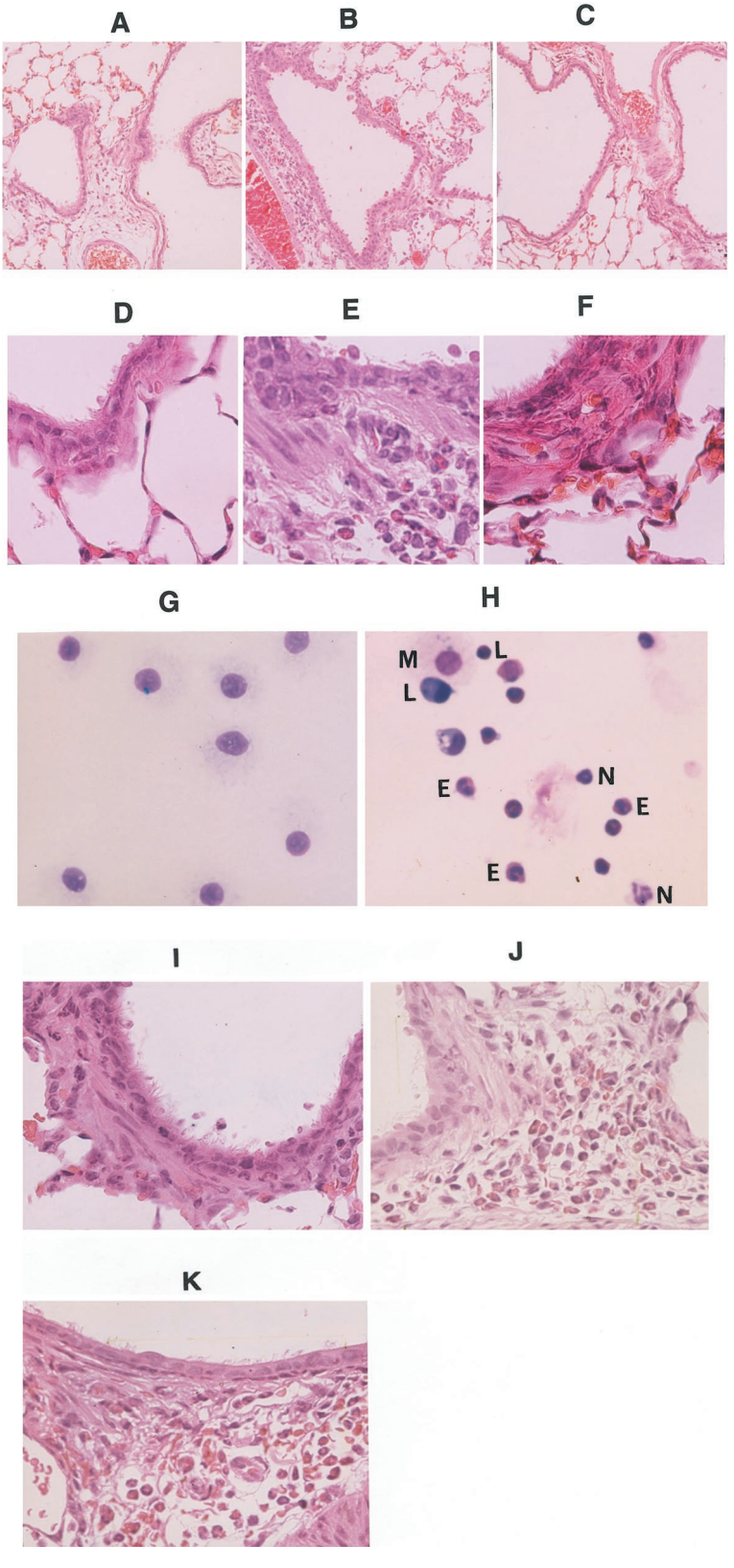

FIGURE 3. Histological and cytological findings in bronchial tissue and BALF after repeated OVA exposure of actively sensitized rats and the effects of pretreatment with sCR1 or C5aRA. *A*, Control (0.1 ml saline i.t.); *B*, triple OVA exposure (day 3); *C*, triple OVA exposure with pretreatment with sCR1 before every OVA exposure. ×125 magnification by light microscopy, H&E. *D–F*, Higher magnifications (×500) of *A*, *B*, and *C*, respectively. *G*, Cytology of control (0.1 ml saline i.t.). *H*, That of triple OVA exposure (day 3). E, N, L, and M indicate eosinophils, neutrophils, lymphocytes, and macrophages, respectively. ×500 magnification, Diff-Quik staining. *I*, Triple OVA exposure with pretreatment with the C5aRA before every OVA exposure (day 3). i.t. administration of C5a desArg (10 ng; *J*) or C3a desArg (200 ng) (*K*) to rats (day 2) that received a double OVA exposure with pretreatment with sCR1 before each (every sCR1). ×500 magnification.

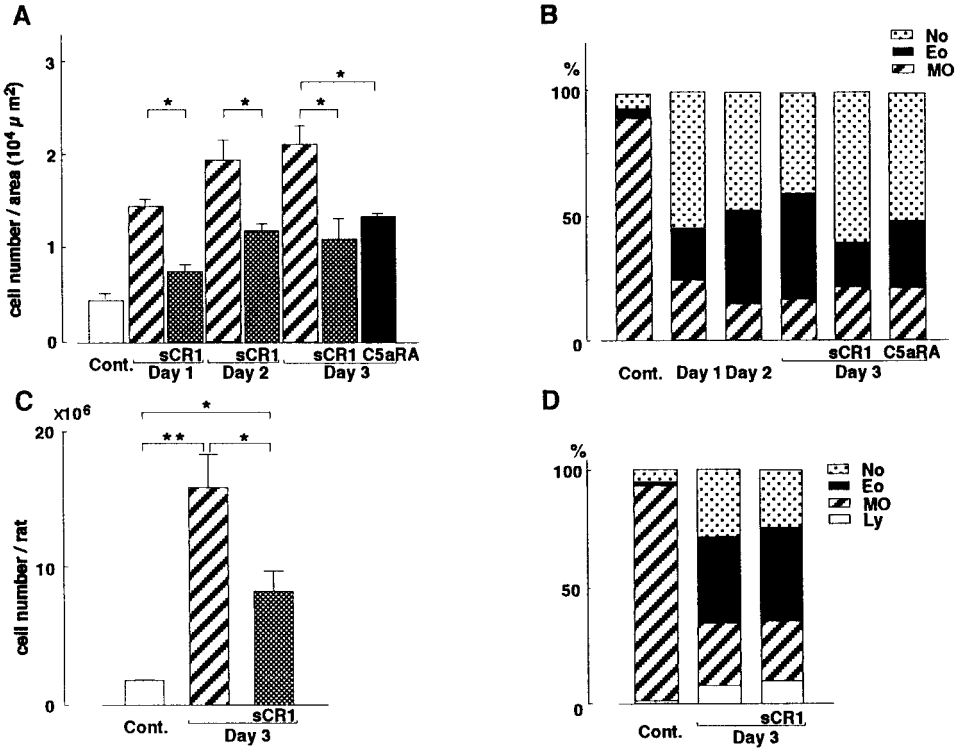

FIGURE 4. Morphometric analysis of infiltrated inflammatory cells in the bronchial submucosa and the influence of pretreatment with sCR1 (10 mg/kg, i.p.) or C5aRA (1 mg/kg i.v.). *A*, The number of total inflammatory cells is expressed as the number of cells per unit area (=10^4 μm^2). *B*, Cellular differentiation of the infiltrating inflammatory cells shows levels of neutrophils (No), eosinophils (Eo), and mononuclear cells (MO) after each challenge. Data are presented as means ± SEM of two fields per six rats. *C*, The number of total cells recovered in BALF from control rats (Cont.) (saline i.t., $n = 3$), rats receiving triple OVA exposure ($n = 4$), and rats pretreated with sCR1 ($n = 3$). *D*, Cellular differentiation of cells in BALF shows levels of neutrophils, eosinophils, macrophages, and lymphocytes. *, $p < 0.05$; and **, $p < 0.01$.

Reconstitution experiments with C5a desArg, C3a desArg, and CINC-1

We next examined whether i.t. addition of purified rat C5a desArg reversed the suppression of LAR by pretreatment with sCR1. In this experiment, administration of sCR1 was performed in two different manners; one schedule called for administration before every OVA exposure (every sCR1), whereas with the other, sCR1 was administered only at the last OVA exposure (final sCR1). With this approach, we were able to evaluate the autologous desensitization of C5a in terms of its biological activity. Fig. 8 shows the effects of C5a desArg with final sCR1 (A) and every sCR1 (B) on the time course of R_L. As seen in Fig. 8, the LAR reappeared after the addition of 10 ng C5a desArg under both conditions by costimulation with OVA. The former treatment (A) resulted in smaller increases in the LAR level than the latter (B) after the double and triple OVA exposure. Table II summarizes these results. Fig. 3*J* shows that the reappearance of the LAR in rats on the every sCR1 schedule (day 2) caused by i.t. administration of 10 ng C5a desArg plus OVA was associated with cellular infiltration into the bronchial submucosa. In contrast, i.t. addition of 200 ng C3a desArg plus OVA to rats on the every sCR1 schedule (day 2) did not influence the time course of R_L changes (Fig. 8*B*). However, histological examination suggested an infiltration of eosinophils and neutrophils in the bronchial submucosa similar to that observed with C5a desArg plus OVA (Fig. 3*K*).

In view of the finding that the reversal in the LAR was achieved with C5a desArg during complement inhibition with sCR1, we next attempted to restore the LAR by the i.t. addition of a chemokine, CINC-1. Results of this reconstitution experiment showed that CINC-1 at amounts up to ~100 times higher (100 pmol = 785

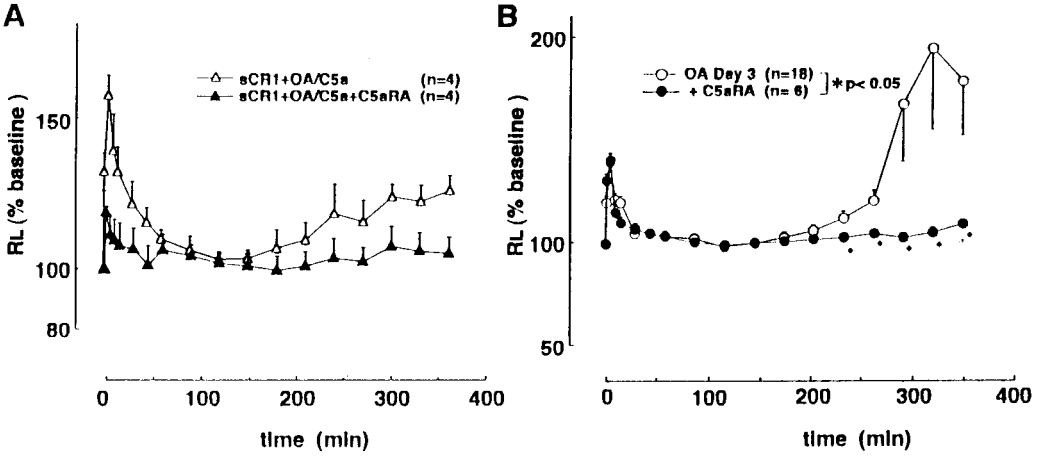

FIGURE 5. Effects of pretreatment with C5aRA on the time-course of R_L after OVA (OA) exposure. *A*, Effects of C5aRA on the time course of the R_L change after the first exposure of actively sensitized rats to OVA plus 10 ng C5a desArg (day 1) when pretreated with sCR1 (10 mg/kg, i.p.) 1 h before challenge. *B*, Effects of pretreatment with or without C5aRA on the time course of R_L after triple exposure to OVA (day 3). C5aRA (1 mg/kg) was administered i.v. 30 min before every OVA exposure. Data are expressed as percent baseline values and are presented as means ± SEM ($n = 6$).

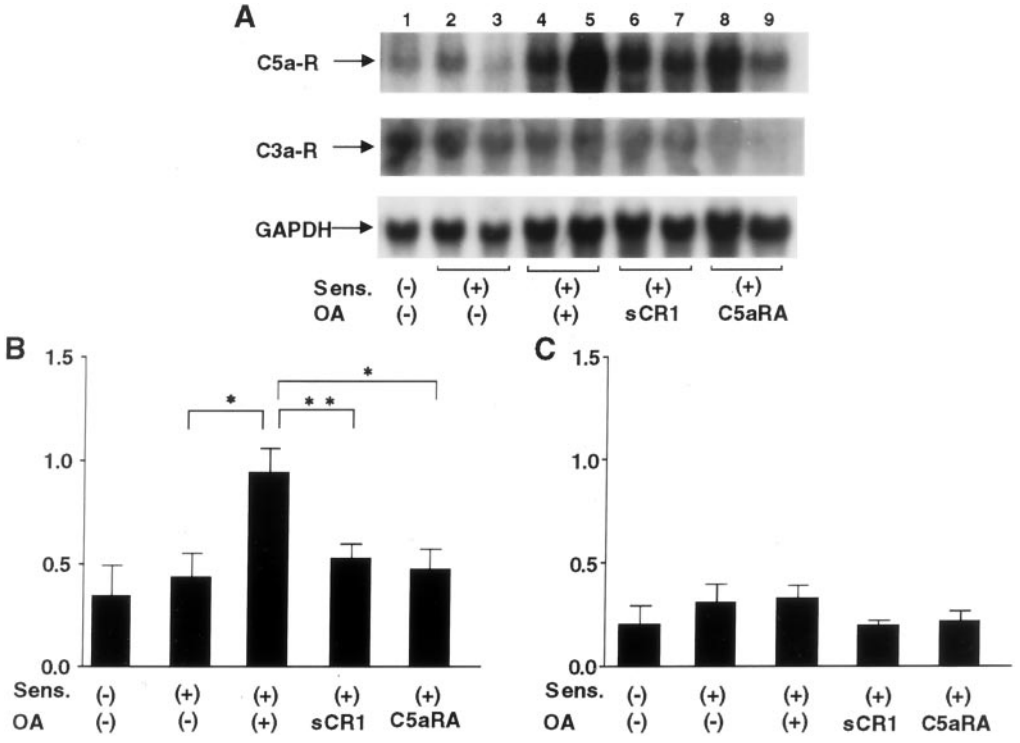

FIGURE 6. Northern hybridization analysis of C5aR and C3aR mRNA expression in lungs after triple exposure to OVA (OA) and the effects of pretreatment with sCR1 or C5aRA (day 3). *A*, Representatives of five independent experiments. *Lane 1*, nonsensitized, saline-challenged rat; *lanes 2* and *3*, sensitized (Sens.), saline-challenged rats (independent duplicate samples); *lanes 4* and *5*, sensitized, triple OVA-challenged rats; *lanes 6* and *7*, rats pretreated with sCR1 before every OVA challenge; *lanes 8* and *9*, rats pretreated with C5aRA before every OVA challenge. *B*, Densitometric analysis of C5aR mRNA expression ($n = 9$) and *C*, C3aR mRNA expression ($n = 7$), presented as ratios to GAPDH mRNA. *, $p < 0.05$; **, $p < 0.01$.

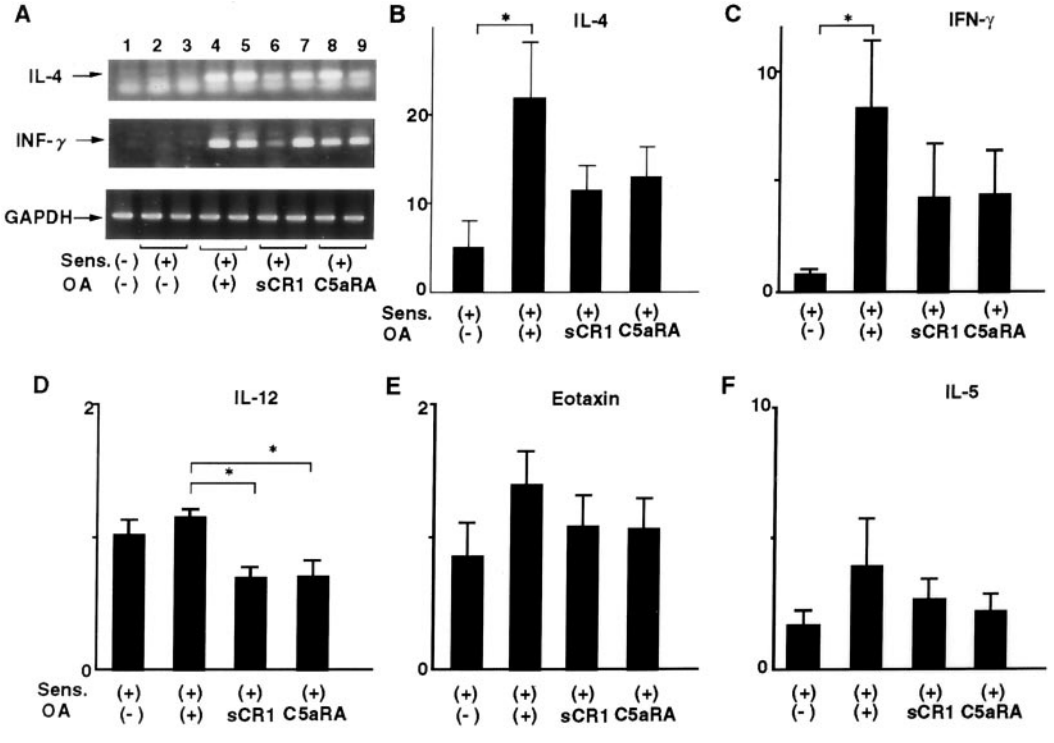

FIGURE 7. Cytokine (IL-4, IFN-γ, IL-12, IL-5) and eotaxin mRNA expression in lungs after triple exposure to OVA (OA) (day 3) and the effects of pretreatment with sCR1 and C5aRA. *A*, Representatives of three independent experiments. *Lane 1*, a nonsensitized, saline-challenged rat; *lanes 2* and *3*, sensitized (Sens.), saline-challenged rats (independent duplicate samples); *lanes 4* and *5*, sensitized, triple OVA-challenged rats; *lanes 6* and *7*, rats pretreated with sCR1 before every OVA challenge; *lanes 8* and *9*, rats pretreated with C5aRA before every OVA challenge. Densitometric analysis of IL-4 (*D*), IFN-γ (*C*), IL-12 (*D*), eotaxin (*E*), and IL-5 (*F*) mRNA expression (each $n = 6$). Each mRNA level was expressed relative to the level of the respective GAPDH mRNA and the relative value of the control (nonsensitized, saline-challenged rats) was standardized to 1.0.

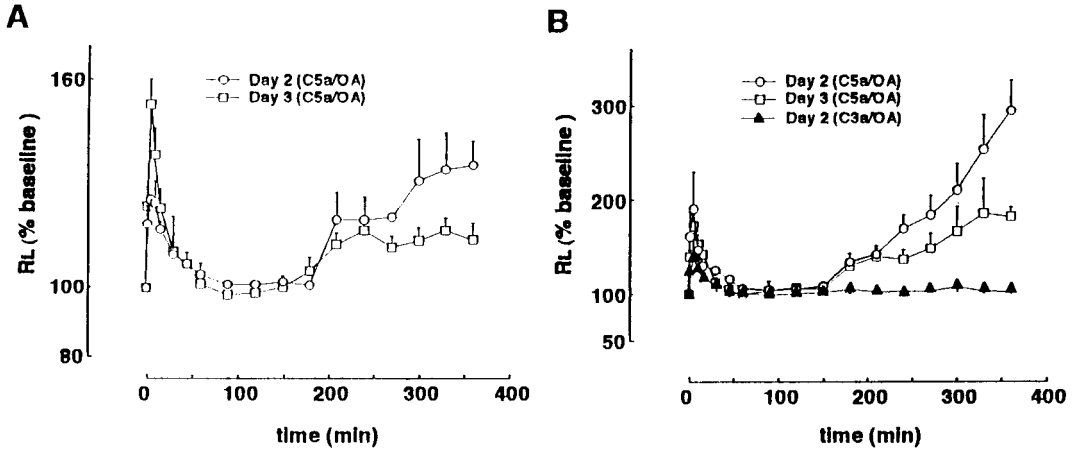

FIGURE 8. Reversal of the R_L suppression after treatment with sCR1, by i.t. addition of 10 ng C5a desArg (○, □) or 200 ng C3a desArg (▲) in combination with OVA. The rats were pretreated with sCR1 according to two different schedules: *A*, pretreated only before the final OVA exposure (final sCR1); and *B*, pretreated before every OVA exposure (every sCR1). Data are expressed as percent baseline values and are presented as means ± SEM (n = 4). Different vertical scales were used for *A* and *B*.

ng) than that of C5a desArg did not restore the magnitude of the LAR under conditions of pretreatment with sCR1 after single or double OVA exposure (Fig. 9*A*). These results suggested that the neutrophil chemotactic factor did not induce an LAR after costimulation with the Ag. We next compared cellular infiltration into the bronchial submucosa after OVA plus CINC-1 stimulation with that after exposure to OVA plus C5a desArg. As shown in Fig. 9*B*, a morphometric analysis showed that OVA plus CINC-1 stimulation resulted in the accumulation of as many inflammatory cells into the bronchial submucosa as stimulation with OVA plus C5a desArg.

Comparison of IAR levels induced by i.t. administration of C5a desArg, C3a desArg, and CINC-1 alone or in combination with OVA

C5a desArg (10 ng) administered i.t. induced an IAR, but neither C3a desArg (200 ng) nor CINC-1 (785 ng) alone could induce a significant IAR in comparison with saline administered i.t., as summarized in Table III. Whereas the combined administration of OVA plus C5a desArg induced a significantly higher IAR than did OVA alone, OVA plus C3a desArg or CINC-1 did not affect the IAR level in comparison with OVA alone.

Discussion

Previous clinical studies focusing on allergic diseases have suggested that systemic complement activation is unlikely to contribute to LAR because there is no change in the CH50, C3, or C4 level in serum of asthmatic subjects after challenge with an allergen (31). Similarly, in this study, we did not detect any decrease in serum CH50 levels 6 h after the third i.t. Ag exposure compared with the control (saline i.t.), suggesting that there was no definite involvement of systemic complement activation during LAR development in this model. After the three repetitions of Ag challenge, we noted that the LAR was associated with a cellular infiltration of the bronchial submucosa that consisted predominantly of eosinophils and neutrophils. Similarly, in a previous report on dermal challenge with allergens in human allergic subjects, it was shown that a triple, but not a double, repetition of Ag exposure led to augmented LAR and increased cellular infiltration in the dermis (11). We then attempted to assess the involvement of complement activation in the airway responses after repeated Ag challenge using two complement inhibitors, Futhan and sCR1 (19, 20). Both inhibitors suppressed IAR after single or double exposure to OA but not after triple exposure. In contrast, both inhibitors suppressed LAR and cellular infiltration after the third exposure. When the contribution of C5a to LAR was examined using a C5aRA, LAR, and cellular infiltration of the bronchial submucosa were simultaneously inhibited (22). Taken together, these results suggest that endogenous production of C5a through local complement activation in the airway system after an Ag-Ab reaction might be causally related to the development of LAR. Because LAR has been considered to be intimately associated with symptomatic asthma and to be related to cellular infiltration of the bronchial submucosa and because drugs that inhibit LAR may have a potent anti-inflammatory effect in the treatment of asthma, the results of the present study suggest that, in future, anticomplementary drugs may prove to be effective in antiasthmatic therapy (32).

Table II. *Influence of previous complement inhibition on levels of IAR and LAR after i.t. administration of OVA plus C5a desArg (10 ng)*[a]

	IAR		LAR	
Day	Every sCR1	Final sCR1	Every sCR1	Final sCR1
2	198 ± 35	130 ± 3	298 ± 33	139 ± 9
3	178 ± 19	157 ± 10	203 ± 27	122 ± 1

[a] The effects of costimulation with OVA plus C5a desArg (10 ng) on the maximum levels of IAR and LAR using two different schedules of sCR1 pretreatment. Every sCR1 indicates rats pretreated with sCR1 before every OVA exposure, and final sCR1 indicates rats pretreated only before the last OVA exposure. Each value is shown as a percent of the baseline level, and each maximum value of the IAR and LAR was expressed as mean ± SEM (n = 4).

Results of Northern hybridization revealed that C5aR mRNA was up-regulated in lungs after the triple OVA exposure, whereas C3aR up-regulation was hardly detected, similar to the results of a previous report using LPS stimulation (27). However, we did not have a definite explanation for the different regulation between C5aR and C3aR mRNAs. This up-regulation of C5aR was inhibited by pretreatment with sCR1 or C5aRA.

OVA exposure resulted in increased expression of cytokine and chemokine mRNAs including IL-4, IL-5, eotaxin, and IFN-γ; however, complement inhibition or the blocking of C5aR resulted in decreased levels of these cytokines and chemokine in the lungs. The IL-12 message from lungs after the third exposure to Ag was reduced after pretreatment with a complement inhibitor or C5aRA

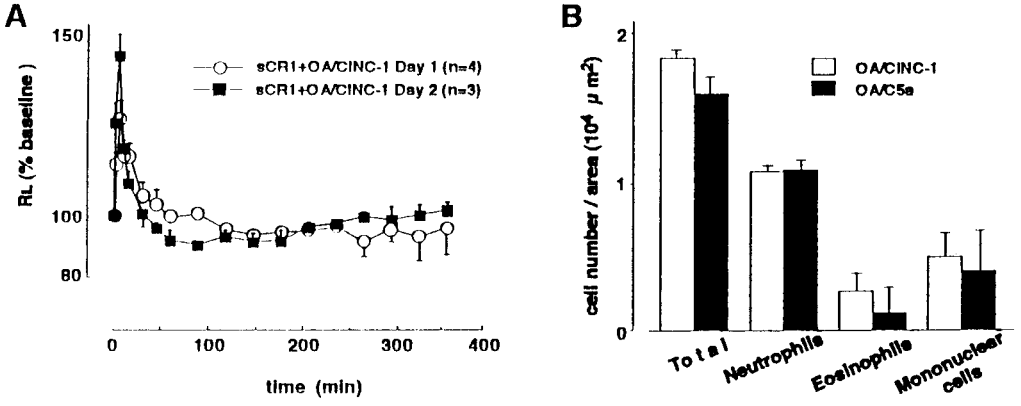

FIGURE 9. *A*, Effects of CINC-1 on the time course of R_L change after OVA exposure. CINC-1 (785 ng) was administered i.t. with OVA to rats that were pretreated according to the every sCR1 schedule on day 1 (○), or on day 2 (■). Data are expressed as percent baseline values and are presented as means ± SEM. Numbers in parentheses indicate the number of animals used. *B*, Morphometric analysis of the infiltrated inflammatory cells in the bronchial submucosa following i.t. exposure to OA plus 10 ng C5a desArg or OA plus 785 ng CINC-1 on day 1. Data are presented as means ± SEM of two fields per five rats.

which was consistent with a previous report showing that the blocking of C5aR resulted in reduced production of IL-12 by monocytes-macrophages (33). Because IL-12 may suppress type 2 cytokines, inhibition of C5aR expression and the resultant IL-12 suppression may be harmful during initiating and propagating phases in atopic asthma. However, in the effector phase, inhibition of complement activation or the blocking of C5aR seemed to partially suppress type 2 cytokines and chemokine message levels, including those of IL-4, IL-5, and eotaxin, after Ag exposure.

We next examined whether the i.t. addition of C5a desArg could restore the LAR when complement was inhibited by pretreatment with sCR1. Results of this reconstruction experiment showed that the suppression of LAR was reversed by the i.t. addition of a low dose of C5a desArg (<1 pmol) and that, additionally, cellular infiltration reappeared in the bronchial submucosa. On the other hand, the airway response to exogenously added C5a desArg differed according to the protocol used for pretreatment. The response to C5a desArg was much higher when sCR1 was given before every OVA exposure than when given only with the final exposure. Because under the every sCR1 conditions endogenous production of C5a would be continuously suppressed compared with production under the final sCR1 conditions, the difference in the airway response to exogenously added C5a desArg might be explained by the contact or lack of contact of airway tissues with C5a endogenously produced during the previous challenge with the Ag and would arise via a mechanism for the autologous desensitization to C5a (34, 35). In contrast, i.t. instillation of 20 times more C3a desArg in combination with OVA did not induce an LAR in spite of an accumulation of eosinophils in the bronchial submucosa. When the i.t. addition of a potent neutrophil chemokine, CINC-1 (a member of the IL-8 family), in combination with OVA was examined in terms of its ability to induce an LAR, CINC-1 (up to 100-fold more than C5a desArg) was incapable of LAR induction when used in combination with the Ag, thus suggesting that C5a plays a specific role in the LAR in this model (36). Nevertheless, CINC-1 plus OVA stimulation resulted in the accumulation of as many granulocytes in the bronchial submucosa as were noted with C5a desArg plus OVA. The results that C3a desArg and CINC-1 were able to induce cellular infiltration but without significant LAR may suggest a dissociation between cellular infiltrate and LAR (9). When we compared these three stimulants with respect to their ability to induce an IAR, C5a desArg (10 ng) administered i.t. was most successful, whereas neither C3a desArg (200 ng) nor CINC-1 (785 ng) alone had any significant effect. Although the combined administration of OVA plus C5a desArg produced a significantly higher IAR than using OVA alone, the combination of OVA plus either C3a desArg or CINC-1 did not. Because the augmention of IAR using OVA plus C5a desArg is partially inhibited by histamine and cysteinyl-leukotriene receptor antagonists, as previously reported from this laboratory, this potentiation can be partly attributed to increased histamine release and cysteinyl-leukotriene production (37). Consequently, it is speculated that the contribution of C5a to the LAR may involve both its anaphylatoxic ability to stimulate mediator release and its potent chemotactic activity (15, 37).

Table III. *Effects of i.t. administration of stimulants and costimulation with OVA on the IAR*[a]

Stimulants	Peak Height of IAR (% baseline)
Saline	111 ± 2 (5)
OVA alone	177 ± 8 (7)
C5a alone	167 ± 12 (6)
C3a alone	117 ± 3 (3)
CINC-1 alone	108 ± 2 (3)
C5a/OVA	202 ± 10* (7)
C3a/OVA	172 ± 9 (4)
CINC-1/OVA	161 ± 8 (7)

[a] Maximum values of R_L within 30 min after the i.t. administration of saline, OVA, C5a (10 ng C5a desArg), C3a (200 ng C3a desArg), CINC-1 (785 ng) alone, or OVA plus one of the latter three were compared in rats not pretreated with sCR1 (on day 1). Each value is expressed as a percent of the baseline level, and each maximum value is expressed as a mean ± SEM. The number of rats used is indicated in parentheses. Challenge with OVA plus C5a significantly increased the IAR level as compared with use of either OVA or C5a alone. *, $p < 0.05$.

In conclusion, it is suggested that C5a contributes to the development of LAR after repeated Ag-Ab reaction based on the following evidence: 1) suppressed pulmonary resistance and infiltration of inflammatory cells by complement inhibition or the blocking of C5aR; 2) up-regulation of C5aR mRNA in the lungs and its down-regulation by complement inhibition or the blocking of C5aR; 3) increased levels of cytokine and chemokine messages, and their down-regulation by complement inhibition or the blocking of C5aR; 4) reappearance of LAR after the addition of C5a desArg; 5) potentiation of Ag-induced IAR by costimulation with C5a desArg. However, these results are in contrast to very recently reported animal studies using C3aR-genetically disrupted mice and

C3aR-naturally defective guinea pigs (13, 14). Both studies indicated that animals with a disruption or defect in C3aR exhibited decreased bronchoconstriction without affecting cellular infiltration in comparison with wild-type strains, which suggested a role for C3a in airway hyperresponsiveness after Ag exposure. Therefore, it is possible that the anaphylatoxin C5a plays a different role from that of C3a in allergic asthma. Consequently, therapeutic interruption of complement activation, especially involving a blocking of the C5a or C3a receptor, may provide a novel and effective therapeutic intervention in a subgroup of asthmatic subjects.

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