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*J Immunol* 2008; 181:3602-3608; doi: 10.4049/jimmunol.181.5.3602

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Production of C5a by ASP, a Serine Protease Released from Aeromonas sobria

Hidetoshi Nitta,*† Takahisa Imamura,2*, Yoshihiro Wada,§ Atsushi Irie,‡ Hidetomo Kobayashi,¶ Keinosuke Okamoto,‖ and Hideo Baba‡

Aeromonas sobria causes pus and edema at sites of infection. However, the mechanisms underlying these effects have not been elucidated. C5a, the amino-terminal fragment of the complement 5th component (C5), mimics these events. To investigate the involvement of C5a in the pathophysiology of A. sobria infection, we examined release of C5a from human C5 by a serine protease (ASP), a putative virulence factor secreted by this bacterium. C5 incubated with enzymatically active ASP induced neutrophil migration in a dose-dependent manner from an ASP concentration of 3 nM and in an incubation time-dependent manner in as little as 7 min, with neutrophil accumulation in guinea pigs at intradermal injection sites and neutrophil superoxide release. These effects on neutrophils were inhibited by a C5a-receptor antagonist. The ASP incubation mixture with C5 but not C3 elicited little as 7 min, with neutrophil accumulation in guinea pigs at intradermal injection sites and neutrophil superoxide release. These effects on neutrophils were inhibited by a C5a-receptor antagonist. The ASP incubation mixture with C5 but not C3 elicited vascular leakage in a dose- and incubation time-dependent manner, which was inhibited by a histamine H1-receptor antagonist. Together with these C5a-like activities, ASP cleaved C5 to release only one C5a Ag, the m.w. of which was similar to that of C5a. Immunoblotting using an anti-C5a Ab revealed generation of a C5a-like fragment from human plasma incubated with ASP. These results suggest that ASP-elicited neutrophil migration and vascular leakage via C5a production from C5 could occur in vivo, which was supported by that ASP did not affect functions of C5a and neutrophil C5a receptor. Through C5a generation, ASP could be associated with the induction of pus and edema caused by infection with this bacterium. The Journal of Immunology, 2008, 181: 3602–3608.

Aeromonas species are facultative, anaerobic Gram-negative rods that were formerly classified as belonging to the Vibrio group (1) and are widely distributed in aquatic environments (2). In addition to entry through the digestive tract with associated gastroenteritis (3, 4), Aeromonas infection often occurs via skin wounds, causing cellulitis and furunculosis, and advances to more complex illnesses via infection of the fascia, tendons, muscle, joints, and bone (5–7). These infections commonly develop into systemic infections, such as peritonitis, meningitis, hepatobiliary disease, pneumonitis, and sepsis. Aeromonas species release a number of putative virulence factors, including hemolysins, enterotoxins, and proteases (8). We purified one putative virulence factor from the culture supernatant of A. sobria, a 65-kDa serine protease referred to as ASP (A. sobria serine proteinase) (9). This species is predominantly isolated from patients’ blood (10) and is more virulent than other Aeromonas species (11). ASP was recently found to cause vascular leakage (VL) and to lower blood pressure, mostly through activation of the kalilike/kinin system (9), and to produce a pivotal coagulation protease, α-thrombin (12). These effects are likely associated with the induction of shock and disseminated intravascular coagulation, respectively, which are major and fatal complications of sepsis (13). These ASP virulence activities could account for the pathophysiology of generalized A. sobria infections; however, the mechanism of neutrophil accumulation leading to pus formation, seen in cellulitis and furuncles caused by local infections with this bacterium, has not yet been elucidated.

Anaphylatoxin C5a is a 74 aa fragment released from the amino terminus of the C5 convertase (14). C5a is a potent neutrophil chemoattractant (15) that evokes superoxide release and enhances phagocytosis (16–18). As the name anaphylatoxin implies, C5a stimulates mast cells to release histamine, which causes VL (19). These C5a activities mimic early inflammatory processes, such as neutrophil accumulation and edema formation. Thus, C5a is recognized as an important mediator of inflammation.

C5a is a C5 split product released by cleavage of the peptide bond at the carboxy-terminal side of the Arg74 residue (14), and ASP has been shown to cleave peptide bonds at the carboxy-terminal side of Arg residues (9, 12). We demonstrated previously that ASP-induced VL was partially inhibited by an antihistamine drug (9), suggesting C5a production by this bacterial protease in vivo. To study the mechanism of neutrophil accumulation at sites of A. sobria infection in humans, we investigated the ability of ASP to produce C5a from human C5.

Materials and Methods

Materials

Phenol red-free HBSS was purchased from Nissui Pharmaceutical. Evans blue was obtained from Merck. Recombinant human C5a and N,N-diethyl-9,9-biiodrindiumtrinitrate (lucigenin) were purchased from Sigma-Aldrich. Human C5 and C3 were purchased from Calbiochem. fMLP was purchased from C sophisticate.
purified from Peptide Institute. Polyclonal anti-human C5a goat IgG and HRP-conjugated rabbit IgG against goat IgG were purchased from R&D Systems and Nichirei, respectively. Cobra venom factor (CVF) was purchased from Quidel Corporation. The complement C5a receptor antagonist, N-methyl-Phe-Lys-Pro-o-cyclohexylalanine-n-cyclohexylalanine-n-Arg, was synthesized as described previously (20). Other chemicals were purchased from Wako Pure Chemical Industries. Normal human plasma was prepared by centrifugation of a mixture of nine volumes of freshly drawn blood from healthy volunteers and one volume of 3.8% (weight to volume ratio) sodium citrate.

**Purification of ASP**

ASP was initially purified from the culture supernatant of *A. sobria* according to the method reported previously (21). The ASP sample was analyzed by SDS-PAGE (10% polyacrylamide gel) under reducing or non-reducing conditions. The enzyme preparations proved to be homogenous, showing a single band on SDS-PAGE under both conditions, representing a protein of molecular mass 65 kDa (9).

**Neutrophil chemotaxis assay**

To isolate neutrophils, heparinized human venous blood (10 U/ml) from healthy donors was mixed with one-quarter volume of 6% Dextran 200,000 dissolved in 0.9% NaCl and kept at room temperature for 40 min. The leukocyte-rich supernatant was placed in a half volume of Ficoll-Paque PLUS (GE Healthcare BioSciences AB) (22), and after centrifugation at 300 × g at 4°C for 20 min, sedimented cells were used. The proportion of polymorphonuclear cells in the cell preparations was >95% when measured by Turk’s stain solution. The neutrophil viability was >98% when determined by the Trypan blue dye exclusion method.

Chemotactic activity was measured in 48-well chambers (Neuro Probe) using polyvinylpyrrolidone-free polycarbonate membranes 10 μm thick with 3-μm pores (Neuro Probe). Aliquots of 90 μl of C5 (350 nM) were incubated at 37°C with 10 μl of ASP at various concentrations for 60 min, or with 100 nM ASP for various periods. After addition of 1 μl of 10 mM diisopropyl fluorophosphate (DFP), a serine protease-specific inhibitor, to terminate the reaction. A volume of 30 μl of the mixture was placed in the lower chamber. PBS, ASP (100 nM), and C5 alone were used as negative controls, and recombinant C5a (0.1–100 nM) and FMLP (100 nM) were used as positive controls. A volume of 50 μl of neutrophils resuspended at 2 × 10⁶ cells/ml in phenol red-free HBSS containing 3% BSA was placed in the upper chamber, separated from the lower chamber by a polycarbonate membrane. To examine C5a receptor dependency, aliquots of 1 ml of the neutrophil suspension were incubated with 10 μl of the C5aR antagonist (100 μM) or PBS for 10 min at 37°C before being placed in the upper chamber. After incubation for 90 min at 37°C, the membrane was removed, rinsed with PBS, fixed in methanol, and stained with Giemsa solution (1/20 dilution, v/v). Cells were counted in 5 high-power fields (×400 magnification) chosen at random, and chemotactic activity was determined by counting the mean number of migrated neutrophils.

**Superoxide assay**

Superoxide released from neutrophils was measured according to the method of Matthews et al. (23), with minor modifications. In brief, neutrophils (1 × 10⁶ cells) suspended in 100 μl HBSS (pH 7.4) containing 3% BSA and 100 μM lucigenin, were incubated in 96-well plates at 37°C for 30 min. Cells were then stimulated by adding 7.8 μl of C5 (1 mg/ml) and 3.3 μl of ASP (0, 0.3, 1, or 3 μM) and incubated at 37°C. As controls, 1 μl of C5a (1 or 10 μM) or 3.3 μl of ASP (3 μM) was added to the cell suspension. Chemiluminescence produced in the cell suspension at 37°C was measured every 30 s for 12 min with a Wallac 1420 ARVO Multilabel Counter (PerkinElmer). Data were expressed as cpm and means ± SD in triplicate assays are shown. To study C5a receptor dependency, neutrophils (1 × 10⁶ cells/100 μl) were preincubated with the C5aR antagonist at 1 μM at 37°C for 10 min before the assay.

**Neutrophil accumulation and VL assay**

These experiments were performed according to the criteria for animal experiments of the Kumamoto University Animal Experiment Committee and were approved by the Committee. To investigate the ability to accumulate neutrophils in vivo, guinea pigs (350–450 g in body weight, both sexes) were anesthetized by i.m. injection of ketamine (80 mg/kg body weight), followed by intradermal injection of 0.1 ml of ASP (30 nM) treated with DFP (1 mM), ASP (30 nM) alone, or C5 (350 nM) incubated with or without ASP (30 nM) at 37°C for 1 h, into the clipped flank. After 6 h, the guinea pigs were euthanized by exsanguination under ether anesthesia, and sample-injected skin samples were excised. These tissues were fixed in formalin for 24 h and embedded in paraffin. Sections 2 μm thick were stained with H&E and observed with a microscope. Neutrophils, determined morphologically, were counted in five high-power fields (HPF) chosen at random (magnification ×400) and the means/HPF ± SD (n = 3) are shown.

C5 (350 nM) was incubated at 37°C with various concentrations of ASP for 30 min, or with 10 nM ASP for various periods, followed by treatment with 1 mM DFP. C3 (5 μM) was incubated at 37°C with 100 nM ASP for various periods, followed by treatment with 1 mM DFP. To assess vascular leakage activity, guinea pigs anesthetized with ketamine were administered Evans blue (2.5% solution in 0.6% saline) i.v. (30 mg/kg body weight), followed by intradermal injection of 50 μl of test sample (dissolved in PBS) into the clipped flank. After 10 min, the guinea pigs were euthanized by exsanguination under ether anesthesia, and blue-dyed skin tissues were excised and incubated in 3 ml of formamide at 60°C for 48 h. VL activity was determined by quantitatively measuring extracted Evans blue by absorption at 620 nm, as described previously (24). Activity was expressed in terms of μg of dye extracted. The activity of the buffer was subtracted from the activity of each sample. Diphenhydramine (30 mg/kg body weight) was injected i.p. 1 h before intradermal injection of samples into the guinea pigs.

**Flow cytometric analysis**

Neutrophils (1 × 10⁶ cells) suspended in 100 μl HBSS (pH 7.4), containing 3% BSA, were incubated with either of chymotrypsin (0.1 mg/ml) or ASP (100 nM) at 37°C for 60 min, and placed on ice. Then, cells were washed and incubated with FITC-conjugated anti-C5a-receptor mAb (S erotec) at 4°C for 30 min. C5a-receptor Ag was quantified by flow cytometry with FACScan (Becton Dickinson). FITC-conjugated nonspecific mouse Ab was used as a negative control.

**SDS-PAGE**

Aliquots of 10 μl of ASP (100 nM) were incubated with 90 μl of human C5 (350 nM) at 37°C. At various time points, samples of 10 μl of the mixture were withdrawn, followed by addition of 1 μl of DFP (10 mM) to terminate the reaction. Then, the samples, dissolved in SDS-buffer and boiled for 5 min, were analyzed by SDS-PAGE under reducing conditions using 15% polyacrylamide gels. A Silver Stain II kit (Wako Biochemicals) was used for protein staining.

**Immunoblotting**

To detect C5a produced from C5 by ASP, aliquots of 5 μl of ASP (0.1 μM) were incubated with 45 μl of C5 (350 nM in PBS) at 37°C. At various time points, samples of 10 μl of the mixture were withdrawn, followed by addition of 1 μl of DFP (10 mM) to terminate the reaction. To obtain a glycosylated C5a positive control, samples of 5 ml of plasma were incubated with 1 U of CVF at 37°C for 30 min, followed by addition of 5 μl of a carboxypeptidase N inhibitor (3 mM), 3,3'-diaminobenzidine hydrochloride, and aliquots of 2 μl of the plasma were used. To detect C5a produced in human plasma by ASP, samples of 45 μl of citrated human plasma were incubated with 5 μl of ASP (1.7 μM) at 37°C. At various times points, samples of 2 μl were withdrawn, followed by addition of 0.5 μl of DFP (10 mM) to terminate the reaction. C5 and plasma, incubated with ASP, were analyzed by SDS-PAGE under reducing conditions using 15% polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (Immobilon Transfer Membranes; Millipore). The membranes were incubated with anti-human C5a goat IgG (×1000 dilution), followed by HRP-conjugated anti-goat IgG rabbit IgG (×1000 dilution). The bands were visualized by ECL (Amersham Biosciences).

**Statistics**

Statistical analysis was performed using the unpaired Student’s *t* test. Values are expressed as means ± SD (n = 3).

**Results**

Production of neutrophil chemotactic activity from C5 by ASP

To determine whether ASP can produce C5a-like activity from human C5, we incubated C5 with ASP and examined the mixture for neutrophil chemotactic activity, a representative biological activity of C5a. ASP produced neutrophil chemotactic activity...
activity from C5 at its plasma concentration (25) in a dose-dependent manner at concentrations starting at 3 nM and reached a plateau at 30 nM, above which the activity of ASP-treated C5 was comparable to that of recombinant C5a at 10 nM (Fig. 1A). Neutrophil chemotactic activity production by ASP began at 7 min and peaked at 60 min, and the C5a receptor antagonist, which had no agonist activity at the concentration used, completely inhibited the neutrophil chemotactic activity of ASP-treated C5 but not that of fMLP (Fig. 1B). Nontreated C5 and ASP alone exerted marginal activity, and DFP-inactivated ASP did not produce activity from C5 (Fig. 1, A and B). These results indicate that enzymatically active ASP produced C5a receptor-dependent neutrophil chemotactic activity from human C5.

To confirm the chemotactic activity produced from C5 by ASP in vivo, we injected ASP-treated C5 into guinea pig skin and investigated neutrophil accumulation at the sites of injection. Marked neutrophil accumulation was induced by intradermal injection of ASP-treated C5 (20.8 ± 10.9/HPF) but not by C5 incubated with DFP-inactivated ASP (3/10 HPF) or C5 alone (0/10 HPF) (Fig. 2), indicating that ASP-treated C5 is capable of inducing neutrophil accumulation in vivo. The finding that ASP alone caused slight but significant neutrophil accumulation (6.9 ± 5.5/HPF) (Fig. 2) agreed with the observation that an intradermal injection of the protease into guinea pigs induced histamine-dependent VL (9), one of the biological activities of C5a, demonstrating the ability of ASP to release C5a from guinea pig C5.

Induction of neutrophil superoxide release by ASP-treated C5

To study the C5a-associated effect on neutrophils further, we examined ASP-treated C5 for the ability to elicit superoxide release. ASP produced superoxide-releasing activity from C5 in a dose-dependent manner, which was comparable to the activity of recombinant C5a and was completely blocked by a C5a receptor antagonist (Fig. 3). Nontreated C5 did not induce superoxide release. Chemiluminescence from neutrophils elicited by ASP-treated C5 was markedly reduced in the presence of superoxide dismutase (300 U/ml) (data not shown). These results indicated that ASP-treated C5 could induce superoxide release from neutrophils in a C5a receptor-dependent manner, similar to C5a.
measured in guinea pigs treated with or without diphenhydramine. C3 (5 μM) incubated with ASP at 100 nM. Samples were analyzed by SDS-PAGE under reducing conditions using 15% polyacrylamide gels (A). Lane a, C5 alone; Lanes b–e, C5 incubated with ASP for 2, 7, 20, or 60 min, respectively. ASP was incubated with C5 (B) or human plasma (C) at 37°C for various time periods, followed by addition of DFP. Samples were analyzed by SDS-PAGE under reducing conditions using 15% polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. C5a fragments were analyzed by immunoblotting. Lane f, C5; Lanes g–j, C5 incubated with ASP for 2, 7, 20, or 60 min, respectively; Lane k, plasma treated with CF; Lane l, plasma alone; Lanes m–p, plasma incubated with ASP for 60, 90, 120, or 180 min, respectively; Lane q, plasma treated with CVF.

**FIGURE 5.** Cleavage of complement C5 and production of C5a by ASP. Aliquots of 10 μl of ASP (100 nM) were incubated with 90 μl of human C5 (350 nM) at 37°C. At various time points, samples of 10 μl of the mixture were withdrawn, followed by addition of 1 μl of DFP (10 mM). Samples were analyzed by SDS-PAGE under reducing conditions using 15% polyacrylamide gels. Lane a, C5 alone; Lanes b–e, C5 incubated with ASP for 2, 7, 20, or 60 min, respectively. ASP was incubated with C5 (B) or human plasma (C) at 37°C for various time periods, followed by addition of DFP. Samples were analyzed by SDS-PAGE under reducing conditions using 15% polyacrylamide gels transferred onto polyvinylidene fluoride membranes. C5a fragments were analyzed by immunoblotting. Lane f, C5; Lanes g–j, C5 incubated with ASP for 2, 7, 20, or 60 min, respectively; Lane k, plasma treated with CF; Lane l, plasma alone; Lanes m–p, plasma incubated with ASP for 60, 90, 120, or 180 min, respectively; Lane q, plasma treated with CVF.

**FIGURE 6.** Effect of ASP on neutrophil C5a-receptor. A, Neutrophils (2 × 10^6/ml) were incubated with a protease at 37°C for 60 min. Then, C5a-receptor on neutrophils was quantified by flow cytometry. Gray and black histograms indicate neutrophils treated with FITC-conjugated anti-C5a-receptor or nonspecific Abs, respectively. Solid and dashed lined histograms indicate neutrophils incubated with ASP (100 nM) or chymotrypsin (0.1 mg/ml), respectively, followed by treatment with anti-C5a-receptor Ab. B, Neutrophils (2 × 10^6/ml) were incubated with ASP (100 nM) or chymotrypsin (100 nM) at 37°C for 60 min. After addition of DFP (1 mM), chemotactic activity of protease-treated neutrophils for C5a (10 nM) was measured. (–), in the absence of protease; Chym, chymotrypsin.

**FIGURE 4.** Production of VL activity from C5 by ASP. A, C5 was incubated with various concentrations of ASP at 37°C for 30 min, followed by 1 mM DFP addition. VL activity was then measured in guinea pigs. ○, untreated ASP; ●, DFP-inactivated ASP; ∗, p < 0.01 vs C5 alone (3.36 ± 1.24 μg). B, C5 (350 nM) or C3 (5 μM) was incubated with ASP (10 nM) at 37°C for various times, followed by 1 mM DFP. VL activity was measured in guinea pigs treated with (upper right) or without (upper left) diphenhydramine. C3 (5 μM) was incubated with ASP (100 nM) at 37°C for various times, followed by 1 mM DFP (lower). BK, bradykinin 1 μM; HIS, histamine 10 μM. C, C5 was incubated with ASP (10 nM) at 37°C for various periods, followed by addition of 1 mM DFP. VL activity was then measured in guinea pigs treated with or without diphenhydramine. ○, untreated; △, diphenhydramine-treated. ∗, p < 0.01 vs C5 alone.

**FIGURE 5.** Cleavage of complement C5 and production of C5a by ASP. Aliquots of 10 μl of ASP (100 nM) were incubated with 90 μl of human C5 (350 nM) at 37°C. At various time points, samples of 10 μl of the mixture were withdrawn, followed by addition of 1 μl of DFP (10 mM). Samples were analyzed by SDS-PAGE under reducing conditions using 15% polyacrylamide gels (A). Lane a, C5 alone; Lanes b–e, C5 incubated with ASP for 2, 7, 20, or 60 min, respectively. ASP was incubated with C5 (B) or human plasma (C) at 37°C for various time periods, followed by addition of DFP. Samples were analyzed by SDS-PAGE under reducing conditions using 15% polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. C5a fragments were analyzed by immunoblotting. Lane f, C5; Lanes g–j, C5 incubated with ASP for 2, 7, 20, or 60 min, respectively; Lane k, plasma treated with CVF; Lane l, plasma alone; Lanes m–p, plasma incubated with ASP for 60, 90, 120, or 180 min, respectively; Lane q, plasma treated with CVF.

**FIGURE 6.** Effect of ASP on neutrophil C5a-receptor. A, Neutrophils (2 × 10^6/ml) were incubated with a protease at 37°C for 60 min. Then, C5a-receptor on neutrophils was quantified by flow cytometry. Gray and black histograms indicate neutrophils treated with FITC-conjugated anti-C5a-receptor or nonspecific Abs, respectively. Solid and dashed lined histograms indicate neutrophils incubated with ASP (100 nM) or chymotrypsin (0.1 mg/ml), respectively, followed by treatment with anti-C5a-receptor Ab. B, Neutrophils (2 × 10^6/ml) were incubated with ASP (100 nM) or chymotrypsin (100 nM) at 37°C for 60 min. After addition of DFP (1 mM), chemotactic activity of protease-treated neutrophils for C5a (10 nM) was measured. (–), in the absence of protease; Chym, chymotrypsin.

**Generation of VL activity from C5 by ASP**

Next, to investigate mast cell degranulation activity, we examined ASP-treated C5 for VL activity. Enzymatically active ASP generated VL-inducing activity from C5 in a dose-dependent manner starting at a concentration of 3 nM (Fig. 4A). ASP also generated VL-inducing activity from C5 in an incubation time-dependent manner and diphenhydramine, a histamine H1 receptor antagonist, abolished the VL activity completely (Fig. 4, B and C), indicating that ASP-treated C5-elicited VL was mediated by histamine. Thus, ASP-treated C5 can induce mast cell degranulation, and released histamine causes VL, mimicking an effect of anaphylatoxin, similar to C5a. We also examined ASP-treated C3 for VL activity and found that human C3 (5 μM) incubated with ASP at 100
C5a production by Aeromonas serine protease

FIGURE 7. Effect of ASP on C5a. Recombinant C5a (10 nM) was incubated with ASP (100 nM) or chymotrypsin (0.1 mg/ml) at 37°C for 60 min. Then, neutrophil chemotactic activity of C5a treated with each protease was measured. (-), in the absence of protease; Chym, chymotrypsin.

nM produced no significant VL activity, even after 1-h incubation (Fig. 4B). It is unlikely that ASP produces C5a, another anaphylatoxin, from human C3 at the plasma concentration.

Liberation of C5a from C5 by ASP
To determine whether ASP can cleave C5, human C5 at its plasma concentration was incubated with ASP for various periods, and generated fragments were analyzed by SDS-PAGE. ASP cleaved C5 even in 2 min at several sites, and released a fragment with a molecular mass similar to that of C5a (10.5 kDa) at 7 min, the concentration of which increased in an incubation time-dependent manner (Fig. 5A). To investigate C5a release by its antigenicity, we performed immunoblotting for ASP-digested C5 using an Ab specific for C5a. Only one spot appeared at 2 min, and it showed a steep increase until 20 min, increasing gradually thereafter (Fig. 5B). This spot migrated to the same position as C5a produced in CVF-treated plasma (Fig. 5B), and its m.w. was similar to the fragment (Fig. 5A). Taken together, these findings indicate that ASP cleaves C5 and releases C5a. ASP liberated C5a from human plasma in an incubation time-dependent manner (Fig. 5C), suggesting that C5a production by ASP occurs in vivo.

Effect of ASP on neutrophil C5a-receptor and C5a
C5a exerts its effects through binding to C5a-receptor on cells; accordingly, degradation of either C5a or the receptor by ASP attenuates C5a effects. Cysteine proteinases of the protozoan Entamoeba histolytica (26) and the major periodontal disease causative agent Porphyromonas gingivalis (27) have been shown to degrade C5a and the receptor, respectively. To address this issue, we investigated the effect of ASP on C5a receptor or C5a. Neutrophils after incubation with ASP reduced neither C5a-receptor Ag nor chemotactic activity for C5a, whereas they reduced the receptor Ag and chemotactic activity for C5a by treatment with chymotrypsin (Fig. 6). Chymotrypsin also reduced the neutrophil chemotactic activity of C5a but the C5a chemotactic activity did not reduce after incubation with ASP (Fig. 7). These results indicate that ASP does not affect interaction between C5a and the receptor on neutrophils.

Discussion
The anaphylatoxin C5a is a byproduct of the complement system activated through any of three pathways and released from C5 via proteolytic cleavage by a C5-convertase (C4b3b2a or C3bBb3b) (28, 29). The present study showed that ASP is a newly recognized C5a-releasing enzyme. C5 incubated with ASP acquired representative C5a biological activities as follows: 1) neutrophil chemotactic activity both in vitro (Fig. 1, A and B) and in vivo (Fig. 2), 2) neutrophil superoxide releasing activity (Fig. 3), and 3) histamine-dependent VL activity (Fig. 4). The dependency of these activities on the C5a receptor (Figs. 1B and 3) supported the suggestion that C5a was present in C5 fragments produced by ASP. In fact, a C5 fragment with a m.w. similar to that of C5a appeared after incubation with ASP (Fig. 5A), in conjunction with generation of C5a-like activities, and ASP released only one C5a Ag that also had a m.w. similar to that of C5a (Fig. 5B). To exert C5a biological activities through binding to the receptor, the Arg residue at the carboxy terminus of C5a is indispensable (30); hence, ASP should cleave C5 at the carboxy-terminal side of Arg74, which is consistent with the substrate specificity of this enzyme (9, 12, 31). Rapid production of C5a (Fig. 5B) by ASP with emergence of C5a biological activities (Figs. 1B and 4B) may indicate ASP preference for C5 as a substrate. However, the incapability of ASP to produce VL activity from human C3 (Fig. 4B) appears to be inconsistent with that C3a is released by cleavage at the carboxy-terminal side of Arg77 (32). ASP belongs to the kexin family and preferentially cleaves peptide bonds following two basic residues (31). Different from C5, C3 has an ArgArg sequence at the upstream of Arg77 (32) and ASP possibly cleaves the peptide bond at the carboxy-terminal side of this paired basic residues before cleaving at that of Arg77, accordingly, C3a may not be released. The observation that ASP produced C5a in human plasma (Fig. 5C) suggests the occurrence of the ASP action on C5 in vivo despite interference by many plasma proteins, including protease inhibitors. Furthermore, the results that ASP did not affect functions of C5a and the receptor (Figs. 6 and 7) exclude a possibility that ASP disturbs interaction of C5a and the receptor, and together with induction of neutrophil accumulation by direct ASP injection into guinea pig skin (9), support relevance of the ASP virulence activity via C5a production.

A 30-kDa serine proteinase from the house dust mite Dermatophagoides farinae (33) and cysteine proteinases from Porphyromonas gingivalis (34, 35), the major pathogen of periodontal disease, have been reported to generate C5a from human C5; these conclusions, however, were based only on their ability to produce neutrophil chemotactic activity, and the molecule responsible was not identified as C5a. We demonstrated ASP-produced C5a not only by generation of C5a receptor-dependent biological activities but also by the identity of the responsible fragment as C5a based on molecular size and antigenicity. Moreover, the chemotactic activity of the fragment was shown by neutrophil accumulation at ASP-treated C5-injected skin sites of the guinea pig (Fig. 2) and the ASP ability to release C5a from C5 was also shown by C5a Ag production from human plasma (Fig. 5C). ASP is likely the C5a-releasing microbial protease that the activity is well confirmed under semiphysiological conditions.

Oral intake of histamine-rich foods induces gastrointestinal symptoms in humans, such as diarrhea and flatulence (36). Activated, subsequently degranulated intestinal mast cells, which are increased in a mouse model of diarrhea induced by oral ingestion of Staphylococcus aureus peptidoglycan and histamine, are involved in the induction of diarrhea (37). Accordingly, mast cell activation by ASP-produced C5a, represented by histamine-dependent VL (Fig. 4, A–C), could be associated with gastroenteritis caused by A. sobria infection, a common cause of the disease (3, 4). In addition, the release of histamine from mast cells by ASP-produced C5a, together with kinin, another VL-inducing factor generated by this protease (9), could evoke edema in aeromonad-infected wounds and lungs (3) and may lead to the onset of acute respiratory distress syndrome (38). C5a neutrophil chemotactic activity causes neutrophil accumulation in the sites of infection, often.
manifested as pus. Furthermore, C5a elicits neutrophil superoxide release (39), as observed in this study (Fig. 3), and enhances the release of neutrophil elastase in response to LPS in infection by Gram-negative bacteria (40), with these neutrophil mediators inflicting tissue damage. Taken together with the thrombin-producing activity of ASP (12), the ability of C5a to induce expression of tissue factor, the initiator of the blood coagulation, in monocytes (41) and endothelial cells (42) may suggest a link of ASP C5a generation to the development of disseminated intravascular coagulation, which is one of the deadly complications of sepsis that occurs in as many as 40% of patients and often advances to multiple organ failure (43).

The complement system represents a key component in humoral defense against invading microorganisms. Activation of the complement system and induction of inflammatory reactions by C5a appear to be detrimental to A. sobria. However, the observation that C5a was released by ASP following production of fragments larger than the anaphylatoxin (Fig. 5A) suggests ASP cleavage at the C5b domain before the protease releases C5b by cleaving off the C5a domain from C5, indicating generation of impaired C5b, which is probably incapable of initiating subsequent formation of the membrane attack complex. Moreover, exposure of neutrophils to C5a at concentrations occurring in the plasma of sepsis patients can lead to neutrophil dysfunction and paralysis of signaling pathways (44), which may be promoted by decreased C5a receptor content in neutrophils with a concomitant decrease in chemotactic responsiveness to C5a, as shown in the cecal ligation and puncture-induced rat sepsis model (45). Thus, ASP C5a generation may impair the complement system and desensitize neutrophils, thereby allowing the bacterium to escape from attack by the complement system and neutrophils and to grow and spread.

In conclusion, the results of the present study indicated that ASP released from A. sobria can produce C5a at the site of infection or in the circulation, which is likely to be associated with the pathophysiology of diseases caused by infection with this bacterium, including elevated levels of C5a in sepsis (46). Therefore, C5a is a new target for the therapy of infectious diseases, particularly sepsis, and anti-C5a Ab and C5a receptor antagonists, in addition to ASP-specific inhibitors, may be developed as drugs for diseases associated with A. sobria infection.

Acknowledgments
We thank Tatsuko Kubo for her technical assistance.

Disclosures
The authors have no financial conflict of interest.

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