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A New Small Molecule C5a Receptor Antagonist Inhibits the Reverse-Passive Arthus Reaction and Endotoxic Shock in Rats[1]

Anna J. Strachan,* Trent M. Woodruff,* Gerald Haaima,[†] David P. Fairlie,[†] and Stephen M. Taylor[2]*

C5a is implicated as a pathogenic factor in a wide range of immunoinflammatory diseases, including sepsis and immune complex disease. Agents that antagonize the effects of C5a could be useful in these diseases. We have developed some novel C5a antagonists and have determined the acute anti-inflammatory properties of a new small molecule C5a receptor antagonist against C5a- and LPS-induced neutrophil adhesion and cytokine expression, as well as against some hallmarks of the reverse Arthus reaction in rats. We found that a single i.v. dose (1 mg/kg) of this antagonist inhibited both C5a- and LPS-induced neutropenia and elevated levels of circulating TNF-α, as well as polymorphonuclear leukocyte migration, increased TNF-α levels and vascular leakage at the site of immune complex deposition. These results indicate potent anti-inflammatory activities of a new C5a receptor antagonist and provide more evidence for a key early role for C5a in sepsis and the reverse Arthus reaction. The results support a role for antagonists of C5a receptors in the therapeutic intervention of immunoinflammatory disease states such as sepsis and immune complex disease. *The Journal of Immunology,* 2000, 164: 6560–6565.

The complement system is an important component of the immunoinflammatory response in host defense. Activation of the complement cascade results in the formation of the anaphylatoxins C3a and C5a and the membrane attack complex which lyses bacterial cells. Inappropriate or excessive complement activation has been associated with a diverse range of immunoinflammatory disease states, including septic shock (1), adult respiratory distress syndrome (2), and immune complex-dependent diseases such as rheumatoid arthritis (3). For this reason, interruption of the complement cascade is an attractive strategy for the development of new anti-inflammatory agents. Such agents currently available include the recombinant soluble receptor inhibitor of complement activation, sCR1 (4, 5), as well as some high m.w. C5a receptor antagonists derived from C5a (6, 7), and these have been shown to be effective in complement-dependent animal models of disease. However, these agents share a number of limitations for their use as prospective drug candidates, notably their large size and complexity as well as unfavorable pharmacokinetic and metabolic profiles, and small molecules are clearly more desirable as general C5a-based drugs.

Specific C5a receptor antagonists can provide evidence of the selective effects of the anaphylatoxin C5a in animal models of immunoinflammatory disease, since the antagonist spares the actions of C3a and the membrane attack complex (C5b-9). To date, only a few recent in vivo studies have been performed with specific C5a receptor antagonists (6–9), and only two of these reports involve agents of low (<1000 Da) m.w. (8, 9). In these studies, the compounds were shown to block the neutropenic (8) and hypotensive (9) effects of endotoxin in rats.

In the present study, we report for the first time some in vivo anti-inflammatory activities of the potent, low m.w. cyclic antagonist AcPhe[L-ornithine-Pro-D-cyclohexylalanine-Trp-Arg] (AcF-[OPdChaWR])[3] of the C5a receptor (10), recently developed in our laboratories, against C5a- and LPS-induced neutropenia, as well as in the reverse-passive Arthus model in the rat peritoneum. This molecule is an analogue of another C5aR antagonist, F-[OPdChaWR], recently reported to show in vivo activity (8), which now has been acetylated to improve its metabolic stability. We demonstrate that AcF-[OPdChaWR] not only inhibits the cellular influx, vascular leakage, and neutropenia in these models, but also suppresses evoked levels of the proinflammatory cytokines TNF-α and IL-6. These results confirm C5a as a key mediator in certain inflammatory reactions.

Materials and Methods

Antagonist preparation

The C5a receptor antagonist AcF-[OPdChaWR] was synthesized previously as described (10). The compound was purified by reversed-phase HPLC and fully characterized by mass spectrometry and proton nuclear magnetic resonance spectroscopy, and the receptor affinity and antagonist potency were determined on intact human polymorphonuclear leukocytes (PMNs) (10).

Receptor-binding assay

The apparent binding affinity of AcF-[OPdChaWR] on isolated rat PMNs was determined using a competition binding assay with ^{125}I-C5a as described previously (11).

Neutropenia assay

Female Wistar rats (200–300 g) were anesthetized and a catheter was placed in the femoral vein. (All animal experimentation conducted in this study was performed in accordance with Animal Experimentation Ethics Committee ethical guidelines.) Rats were treated with antagonist (1 mg/kg i.v. in a final volume of 200 μl with 5% ethanol) or 5% ethanol control injection 10 min before C5a or LPS challenge. Rats were given a bolus i.v.

*Department of Physiology and Pharmacology, and [†]Drug, Design and Development Centre, University of Queensland, St. Lucia, Australia

[2] Address correspondence and reprint requests to Dr. Stephen M. Taylor, Department of Physiology and Pharmacology, University of Queensland, St. Lucia 4072, Australia. E-mail address: s.taylor@mailbox.uq.edu.au

[3] Abbreviations used in this paper: AcF-[OPdChaWR], AcPhe[L-ornithine-Pro-D-cyclohexylalanine-Trp-Arg]; PMN, polymorphonuclear leukocyte.

dose of either human recombinant C5a (2 μg/kg; Sigma, St. Louis, MO) or LPS (1 mg/kg; from *Escherichia coli* serotype 055:B5; Sigma), and blood samples were collected into heparinized Eppendorf tubes at regular intervals over a 150-min observation period. PMNs were isolated and counted as previously reported (8) and expressed as a percentage of the PMN concentration before complement challenge. Additionally, whole blood was collected and allowed to clot spontaneously on ice, and serum samples were stored at $-20°C$ until evaluation of cytokine concentrations.

Reverse-passive Arthus reaction

Female Wistar rats (200–250g) were anaesthetized with ketamine (80 mg/kg) and xylazine (12 mg/kg) and a polyethylene catheter was placed in the left femoral vein. A reverse-passive Arthus reaction was induced in the peritoneal cavity by injecting Evans blue (10 mg/kg i.v.), chicken OVA (20 mg/kg i.v.), and rabbit anti-chicken OVA (10 mg/kg i.p.). A group of rats was pretreated with a C5a receptor antagonist, AcF-[OPdChaWR] (1 mg/kg i.v.) 10 min before induction of the Arthus reaction. Rats treated with Evans blue plus antagonist only served as antagonist controls, Evans blue plus chicken OVA only as Ag controls, and Evans blue plus i.p. rabbit anti-chicken OVA served as Ab controls. Rats were placed on a heating pad, and anesthetic was maintained over a 4-h treatment period with periodic collection of blood samples. Blood was allowed to spontaneously clot on ice, and serum samples were collected and stored at $-20°C$. Four hours after induction of the peritoneal Arthus reaction, the peritoneal cavity was lavaged with 25 ml of saline, and the lavage fluid was collected for assessment of the total cell number with a hemocytometer, the differential cell count of a smear was determined using DiffQuick stain, and the OD of the supernatant at 650 nm was measured as an indicator of Evans blue leakage into the peritoneal cavity.

TNF-α measurement

Serum and peritoneal lavage TNF-α concentrations were measured using an ELISA. A 96-well plate (Nunc Maxisorb; Nunc, Roskilde, Denmark) was coated with rabbit anti-rat TNF-α Ab (50 μl of 1 μg/ml in phosphate buffer, pH 6.8; Research Diagnostics), sealed, and incubated for 2 h at 37°C. The plate was washed three times with PBS-Tween 20, and 100 μl of PBS-Tween 20 containing 0.1% BSA was added to each well, sealed, and incubated for 2 h at 37°C. The plate was washed three times with PBS-Tween 20, and samples were diluted 1:100–1:10,000 in PBS-Tween 20-BSA added in duplicate. Recombinant rat TNF-α (PharMingen, San Diego, CA) was diluted from 1 μg/ml to 1 pg/ml in PBS-Tween 20-BSA and added in duplicate to each plate as a standard curve. The plate was sealed and incubated for 2 h at 37°C. The plate was again washed three times with PBS-Tween 20, and 50 μl of murine anti-rat TNF-α Ab (2 μg/ml in PBS-Tween 20-BSA; PharMingen) was added to each well and incubated at room temperature for 30 min. The plate was washed, and 50 μl of biotinylated sheep anti-rat IgG (1 μg/ml; Amersham, Arlington Heights, IL) was added to each well and incubated at room temperature for 30 min. The plate was washed six times with PBS-Tween 20, and 100 μl of streptavidin-peroxidase conjugate (100 ng/ml; Sigma) was added to each well and incubated at room temperature for 30 min. The plate was again washed six times with PBS-Tween 20, and 100 μl of substrate was added to each well (tetramethylbenzidine substrate system; Sigma). Color development occurred over 30 min at room temperature, followed by the addition of 100 μl of 0.5 M H_2SO_4 to each well. Absorbance was read at 450 nm using a 96-well plate reader (Tecan). Concentrations of TNF-α in serum samples were determined by linear regression analysis from the standard curve.

IL-6 measurement

An ELISA method as described above also measured serum and peritoneal lavage fluid IL-6 concentrations. Briefly, a 96-well plate was coated with goat anti-rat IL-6 (1 μg/ml; Research Diagnostics), followed by the addition of PBS-Tween 20-BSA to block nonspecific binding sites. Serum samples and recombinant rat IL-6 (1 μg/ml-1 pg/ml; Research Diagnostics) were diluted in PBS-Tween 20-BSA and added to the plate in duplicate and incubated for 2 h. The plate was washed, and biotinylated goat anti-rat IL-6 Ab (1 μg/ml; Research Diagnostics) was added to each well. Streptavidin-peroxidase conjugate was added to each well, followed by substrate, and color development was allowed to occur for 30 min before addition of 0.5 M H_2SO_4. Absorbance was read at 450 nm.

Data and statistical analyses

Receptor-binding affinities were determined for C5a and AcF-[OPdChaWR] to isolated human and rat PMNs. Analysis of dose-response curves was performed using nonlinear regression analysis (GraphPad
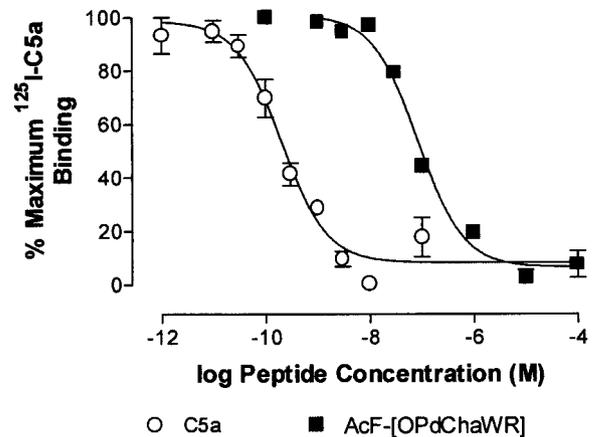

FIGURE 1. C5a and antagonist-binding affinity to rat and human PMNs. Rat PMNs were isolated from whole blood and incubated with 50 pM ^{125}I-C5a in the presence of either C5a or AcF-[OPdChaWR]. Both C5a and antagonist inhibited binding of ^{125}I-C5a binding to isolated PMNs to give a relative binding affinity value. IC_{50} values were obtained from each experiment and the means were determined. Data are shown as a percentage of maximal binding of ^{125}I-C5a (mean $\pm$ SEM, $n = 3$–4).

Prism 2.0; GraphPad, San Diego, CA). IC_{50} values (molar concentration of peptide required to cause 50% inhibition of maximal response) were determined from these dose-response curves, and the mean $-\log IC_{50}$ values calculated from individual curves was used for statistical analysis. PMN concentrations following C5a or LPS challenge were expressed as means $\pm$ SEM for each treatment group ($n = 3$–6). Results derived from the Arthus model were expressed as means $\pm$ SEM ($n = 3$–5) for each treatment group. Mean values for each group were compared with Ab control values using Student's t test, and statistical significance was assessed at $p \leq 0.05$.

Results

Receptor-binding affinity of AcF-[OPdChaWR]

Human recombinant C5a demonstrated competitive inhibition of ^{125}I-C5a binding with comparable affinity to both isolated human and rat PMNs, with $-\log IC_{50}$ values of 9.25 $\pm$ 0.09 ($IC_{50} = 0.5$ nM) and 9.75 $\pm$ 0.17 ($IC_{50} = 0.2$ nM), respectively. The antagonist peptide AcF-[OPdChaWR] similarly demonstrated competitive inhibition of ^{125}I-C5a binding to isolated rat PMNs, with a $-\log IC_{50}$ of value 7.11 $\pm$ 0.33 ($IC_{50} = 70$ nM) (Fig. 1). The receptor-binding affinity of AcF-[OPdChaWR] in human PMNs has been previously reported with a $-\log IC_{50}$ value of 6.57 $\pm$ 0.05 ($IC_{50} = 20$ nM) (10) and was not significantly different from that obtained for rat PMNs (Fig. 1) .

Inhibition of C5a and LPS inflammatory activity

Intravenous treatment of rats with human C5a resulted in a rapid and transient decrease in circulating PMNs (neutropenia), with the concentration of PMNs reaching a minimum of 18 $\pm$ 6% prestimulation controls levels at 5 min, then returning to pretreatment values within 60 min (Fig. 2). Similarly, i.v. LPS (1 mg/kg) resulted in a rapid decrease in circulating PMNs which reached a minimum value of 31 $\pm$ 4% at 30 min and returned to pretreatment values by 150 min (Fig. 2). Pretreatment of rats with a single dose (1 mg/kg i.v.) of AcF-[OPdChaWR] 10 min before C5a or LPS challenge significantly inhibited both C5a- and LPS-induced neutropenia (Fig. 2). Intravenous administration of the same doses of C5a and LPS produced significant elevations in serum TNF-α levels, reaching a maximum value of 30 $\pm$ 3 ng/ml ($n = 6$) at 30 min for C5a and 54 $\pm$ 11 ng/ml ($n = 6$) at 90 min for LPS (Fig. 2).
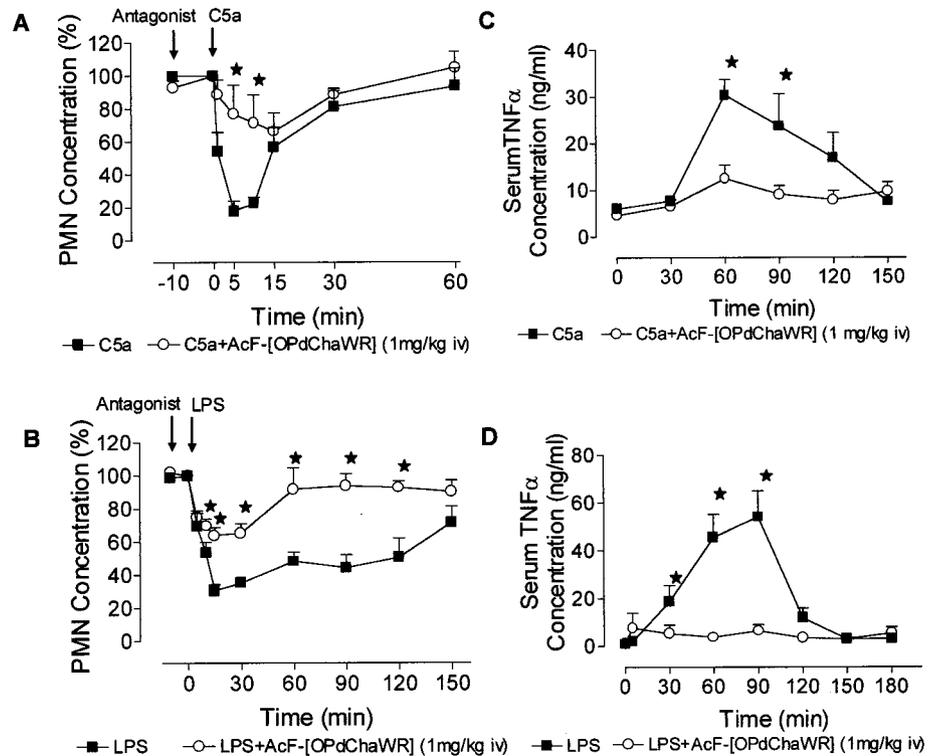
FIGURE 2. Inhibition of C5a- and LPS-induced neutropenia and TNF-α expression with a C5a receptor antagonist. Intravenous C5a (2 μg/kg) or LPS (1 mg/kg) resulted in a rapid and transient neutropenia (*A* and *B*) and a significant increase in detectable circulating TNF-α (*C* and *D*). Pretreatment of rats with i.v. C5a receptor antagonist (1 mg/kg) inhibited neutropenia and elevated cytokine expression. Data are shown as means $\pm$ SEM (*n* = 3–6).

Pretreatment of rats with a single dose of the C5a receptor antagonist (1 mg/kg i.v. 10 min before challenge) completely blocked both C5a- and LPS-induced elevation of serum TNF-α (Fig. 2). To eliminate the possibility that the recombinant human C5a used in these experiments may have contained trace levels of LPS affecting the interpretation of the results, the C5a stock solution was boiled at 110°C for 30 min and then injected into a rat (2 μg/kg i.v.) (12). The neutropenic activity was lost (data not shown), demonstrating that the recombinant human C5a used in this study was free of pharmacologically active levels of contaminating LPS.

Inhibition of cellular influx into the peritoneum by AcF-[OPdChaWR]

The injection of Ab into the peritoneum resulted in a large cellular influx after 4 h, 46% of the cells being PMNs, which represented about a 10-fold increase in the peritoneal PMN cell content over controls (Fig. 3). Accompanying this PMN influx was vascular leakage of serum, as demonstrated by the increased levels of Evans blue in the peritoneal lavage fluid (Fig. 3). Neither the administration of Ag alone, Ab alone, nor C5a antagonist alone affected cellular influx or leakage of Evans blue into the peritoneal cavity. Pretreatment of rats with a single i.v. dose (1 mg/kg) of the C5a receptor antagonist AcF-[OPdChaWR] at 10 min before induction of the Arthus reaction completely blocked both the PMN influx and the increased leakage of serum, as monitored by Evans blue.

Inhibition of the expression of TNF-α and IL-6 by AcF-[OPdChaWR]

Treatment of rats with Evans blue and either the C5a antagonist, Ag, or Ab alone caused no significant change in baseline serum or peritoneal lavage, TNF-α, or IL-6 concentrations. Induction of the reverse-passive Arthus reaction in the peritoneal cavity caused a significant increase in circulating TNF-α concentrations at 3 h, which returned to pretreatment values within 4 h of the Arthus reaction induction (Fig. 4). The peritoneal Arthus reaction also

caused an increase in IL-6 concentrations in the serum, which reached statistical significance at 1–3 h and returned to pretreatment values within 4 h of induction (Fig. 4). In addition, the peritoneal lavage fluid of rats undergoing an Arthus reaction demonstrated significantly elevated TNF-α and IL-6 levels 4 h after induction (Fig. 5). Pretreatment of rats with the C5a receptor antagonist at 1 mg/kg i.v. 10 min before induction of the Arthus reaction blocked the elevation in cellular influx and cytokine expression at the site of inflammation, as well as the elevation of circulating cytokine levels when compared with Ab control values (Figs. 3–5). This result demonstrates complete suppression of cytokine formation levels by the C5a antagonist with the dose and route of administration used.

Discussion

An acute elevation of circulating C5a causes a number of rapid physiological responses, including up-regulation of endothelial P-selectin (13) and neutrophil ICAM expression, (14), resulting in a rapid adhesion of neutrophils to the vascular endothelium. This response can be mimicked by an i.v. injection of human recombinant C5a in the rat, resulting in a rapid decrease in levels of circulating PMNs (1, 8, 9). C5a is degraded by serum peptidases (15) and the C5aR is rapidly internalized (16) with consequent desensitization to subsequent challenges with C5a; therefore, the neutropenia response observed is transient (8). Similarly, i.v. LPS in rats results in activation of the serum complement system and a significant up-regulation of circulating levels of endogenous C5a (1). In this way, the neutropenia response to exogenously administered human C5a can be mimicked in the rat by i.v. administration of LPS, by the resulting up-regulation of endogenous rat C5a. Pretreatment of the rat with the C5a receptor antagonist, AcF-[OPdChaWR], which binds potently to both rat and human PMN C5aRs, significantly inhibited the neutropenic response to both human C5a and endogenous rat C5a.
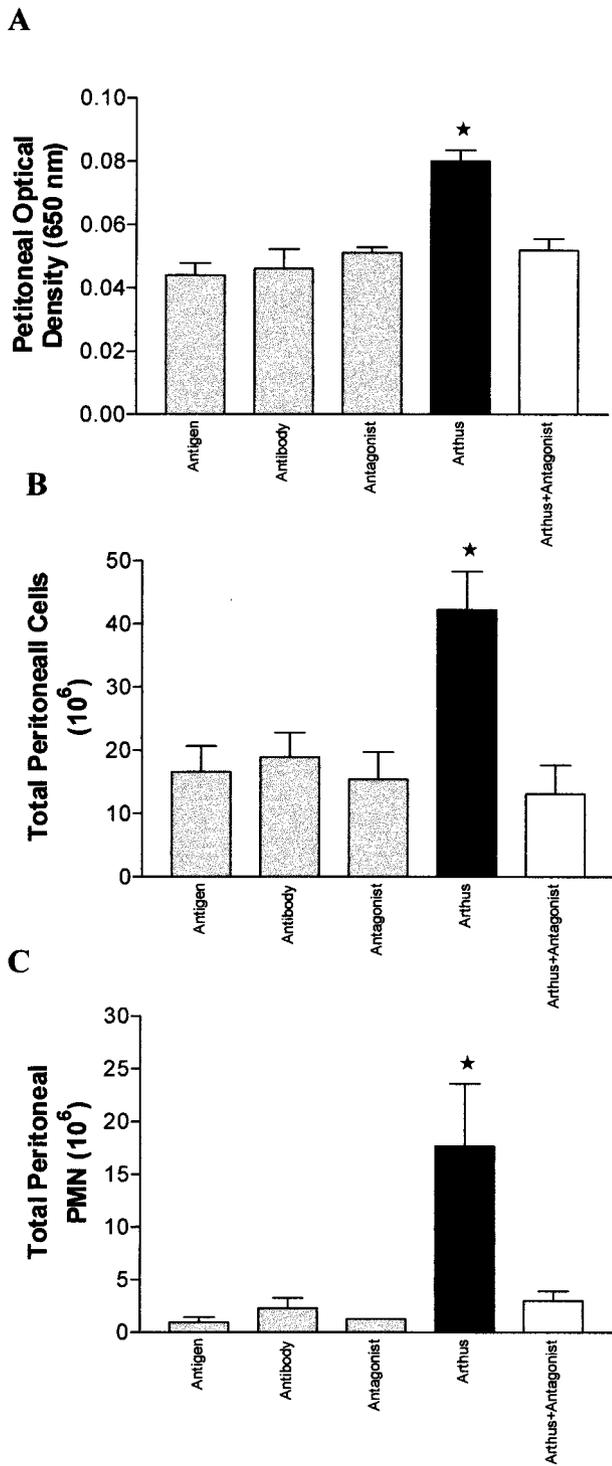
FIGURE 3. Reverse-passive Arthus-induced vascular leakage and cell migration. Initiation of a peritoneal Arthus reaction resulted in a significant increase in optical density (*A*), total cell number (*B*), and total PMN number (*C*) in the peritoneal lavage fluid 4 h after induction of immune complex formation. Data are shown for Ab, Ag, and antagonist control rats, Arthus-treated rats, and rats pretreated with antagonist (1 mg/kg i.v.) before initiation of a peritoneal Arthus reaction. The peritoneal cavity was lavaged 4 h after Arthus reaction induction, and total cell number was counted, the OD of the supernatant was recorded, and total PMN number was determined from a differential cell count. Data are shown as means ± SEM ($n = 3$–5). *, $p \leq 0.05$ vs Ab control.
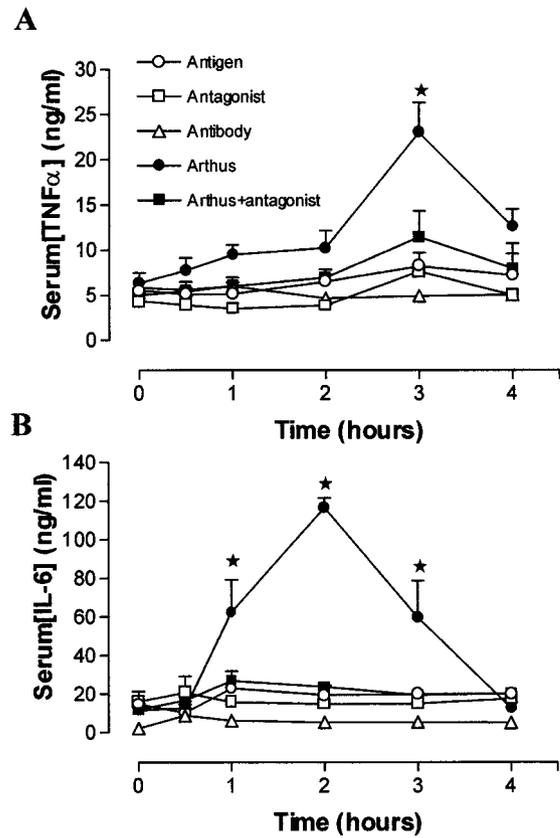

FIGURE 4. Time course of reverse-passive Arthus-induced elevated serum cytokine levels. Initiation of a peritoneal Arthus reaction resulted in significantly elevated serum TNF-α (*A*) and IL-6 (*B*) concentrations in the serum which had resolved within 4 h. Data are shown for Ab, Ag, and antagonist control rats, Arthus-treated rats, and rats pretreated with antagonist (1 mg/kg i.v.) before initiation of a peritoneal Arthus reaction. Serum was collected periodically over the 4-h treatment period, and serum cytokine levels were measured using ELISA. Data are shown as means ± SEM ($n = 3$–5). *, $p \leq 0.05$ vs Ab control.

C5a causes the release of TNF-α and ILs from monocytes in vitro (17, 18), but this effect in vivo has not been described previously. The i.v. administration of C5a caused an increase in circulating levels of TNF-α, and this increase was blocked by pretreatment with the C5a antagonist. We have recently shown that an analogue of AcF-[OPdChaWR] is effective in inhibiting the neutropenia induced by endotoxin and C5a in a model of acute endotoxic shock (8). In the present study, the new compound AcF-[OPdChaWR] blocks both neutropenia as well as the elevation of TNF-α levels following LPS, and this strongly suggests that C5a is a key mediator for both of these effects.

The peritoneal Arthus reaction involves an acute localized inflammatory reaction that is also characterized by PMN migration to the inflammatory locus, vascular leakage, and cytokine production at the site of inflammation (19–21). The acute inflammatory events in this model of immune complex disease can be inhibited by intervention in the complement system (22, 23). Recombinant soluble human complement receptor type 1, which blocks activation of the complement cascade, inhibits neutrophil accumulation and margination, tissue immunoreactivity to C3b, and formation of the membrane attack complex in a dermal Arthus reaction (24). The Arthus reaction can also be limited by cobra venom factor depletion of systemic complement activity (25), supporting the
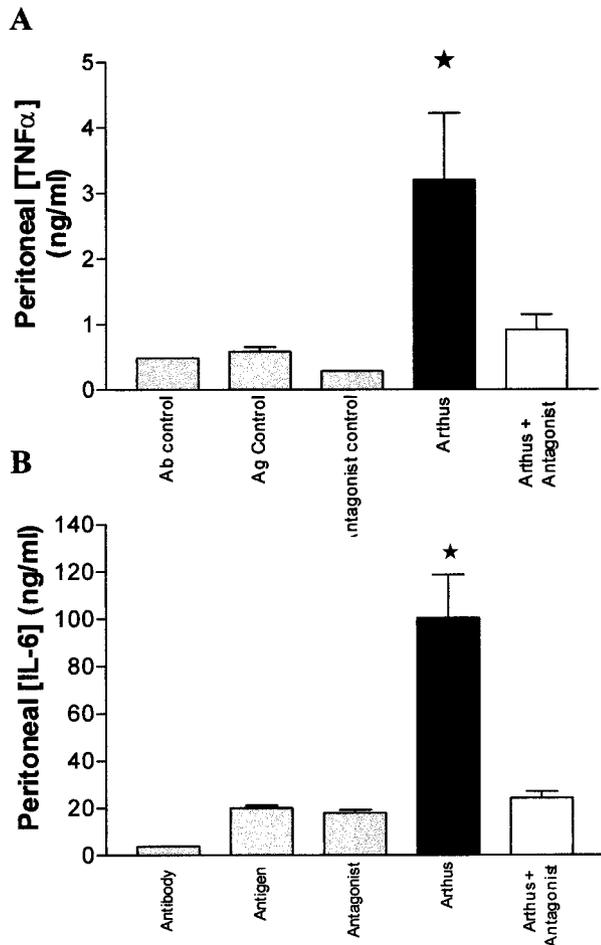
A

B

FIGURE 5. Reverse-passive Arthus-induced elevation of peritoneal cytokine levels. Initiation of a peritoneal Arthus reaction resulted in significantly elevated serum TNF-α (*A*) and IL-6 (*B*) concentrations in the peritoneal lavage fluid 4 h after induction of immune complex formation. Data are shown for the Ab, Ag, and antagonist control rats, Arthus-treated rats, and rats pretreated with antagonist (1 mg/kg i.v.) before initiation of a peritoneal Arthus reaction. The peritoneal cavity was lavaged 4 h after Arthus reaction induction, and cytokine levels were measured using ELISA. Data are shown as means $\pm$ SEM (n = 3–5). *, $p \leq 0.05$ vs Ab control.

requirement for complement in this inflammatory reaction. Furthermore, these inflammatory effects have previously been demonstrated to be C5a dependent, in that genetic deletion of the C5a receptor in mice showed impaired inflammatory responses to immune complex formation in both the peritoneum and skin (23). Mice deficient in C5a receptors showed significantly reduced neutrophil influx, TNF-α and IL-6 expression, and vascular leakage at the site of inflammation when compared with wild-type littermates. The Arthus reaction appears to be also dependent on adhesion and migration of PMNs to the site of immune complex formation (26). Pretreatment with a P-selectin Ab inhibits Arthus-induced swelling, neutrophil accumulation, and endothelial P-selectin expression, despite complement deposition at the site of dermal immune complement formation (27). The present study now demonstrates that a small molecule C5a receptor antagonist inhibits a wide range of the immunoinflammatory events associated with immune complex formation and is in agreement with a preliminary finding utilizing a large m.w. C5a analogue antagonist (6).

Although it is widely accepted that immune complex deposition leads to increased cytokine expression at the local site of inflam-

mation (20, 23), the systemic expression of these proinflammatory cytokines has not previously been described. In the present study, serum samples were collected at regular intervals following induction of the peritoneal Arthus reaction, and the circulating levels of TNF-α and IL-6 were measured. Both of these proinflammatory cytokines were significantly elevated in circulation, indicating that the Arthus reaction is not necessarily limited to the site of inflammation, but can also involve a systemic component. Both the local and systemic induction of cytokines were significantly inhibited by pretreatment of rats with the C5a receptor antagonist, again indicating a requirement for C5a in cytokine production in the Arthus reaction.

Proinflammatory cytokine production is a universal component of a wide range of disease states including immune complex-mediated conditions such as nephritis (28), arthritis (29), and acute graft rejection (30). An agent which can inhibit the production of cytokines such as TNF-α and IL-6 both locally and systemically may have wide therapeutic applications in the prevention and treatment of these and other disease states. The present study indicates that a C5a receptor antagonist is very effective in inhibiting cytokine production when administered before direct challenge with exogenous or endogenous C5a (by LPS administration), as well as in an acute immune complex disease model. The application of a C5a receptor antagonist in other disease states involving complement activation and up-regulation of local and systemic cytokines needs to be further explored both as a preventative and therapeutic agent. The availability of small molecule C5a receptor antagonists now allows for the elucidation of the role of C5a in immunoinflammatory diseases as well as the development of orally active agents as prospective drugs for treating inflammatory diseases.

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