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Inhibition of immune-complex mediated dermal inflammation in rats following either oral or topical administration of a small molecule C5a receptor antagonist

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1 Initiation of a peritoneal Arthus reaction by deposition of immune-complexes results in vascular leakage, polymorphonuclear leukocyte (PMN) infiltration, and tumour necrosis factor α (TNFα) and interleukin-6 (IL-6) production. We now demonstrate in rats that oral administration of the C5a receptor antagonist AcPhe[Orn-Pro-D-Cyclohexylalanine-Trp-Arg] (AcF-[OPdChaWR]; 1–10 mg kg^{-1} 30 min prior to immune-complex deposition) inhibits these inflammatory markers in the peritoneal Arthus reaction.

2 Initiation of a dermal Arthus reaction resulted in a significant increase in vascular leakage, PMN infiltration, systemic production of TNFα and pathological changes in the dermis.

3 Pretreatment of rats with AcF-[OPdChaWR] either intravenously (1 mg kg^{-1} 10 min prior to immune-complex deposition) or orally (1–10 mg kg^{-1} 30 min prior to immune-complex deposition) significantly inhibited immune-complex mediated dermal vascular leakage and systemic cytokine production. Topical pretreatment with AcF-[OPdChaWR] (400 μg site^{-1} in 10% dimethyl sulphoxide 10 min prior to immune-complex deposition) also inhibited vascular leakage, as well as histopathological changes associated with a dermal Arthus reaction.

4 Oral administration of 3 mg kg^{-1} AcF-[OPdChaWR] resulted in the appearance of the drug in plasma within 5 min, with peak blood levels ~0.3 μM reached within 20 min. The plasma elimination half-life was ~70 min. The oral activity and bioavailability of AcF-[OPdChaWR], its activity when applied topically to the skin, suggest that small molecule C5a receptor antagonists may have therapeutic utility in dermal inflammatory disorders involving complement activation.

5 This is the first demonstration for either an orally or topically active C5a receptor antagonist, and suggests that small molecule C5a antagonists may have therapeutic utility when given by multiple routes of application.

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Abbreviations: AcF-[OPdChaWR], AcPhe[Orn-Pro-D-Cyclohexylalanine-Trp-Arg]; AcF-[OPGWR], AcPhe[Orn-Pro-Gly-Trp-Arg]; BSA, bovine serum albumin; C5a, complement factor 5a; C5aR, C5a receptor; ELISA, enzyme-linked immunosorbent assay; IL-6, interleukin 6; PBS, phosphate buffered saline; PMN, polymorphonuclear leukocyte; TNFα, tumour necrosis factor α

Introduction

The Arthus model of immune-complex mediated tissue damage involves the passive introduction of an antibody and the corresponding antigen across a vascular barrier. Immune-complex deposition is the initiating factor in a range of immunoinflammatory diseases, known as immune-complex diseases, such as rheumatoid arthritis, psoriasis and systemic lupus erythematosus (Perez & Goldstein, 1987; Terui *et al*., 2000; Weissmann & Korchak, 1984). An Arthus reaction can be initiated experimentally in a number of tissues, including the peritoneum, dermis and lungs. Regardless of the location or the initial causative factor, immune complex formation leads to tissue damage through activation of an inflammatory response.

This inflammatory response involves binding of the immune complex at the Fcγ receptor on phagocytic cells such as tissue-resident macrophages (Heller *et al*., 1999a). Subsequent activation of the classical complement cascade leads to formation of the anaphylatoxins C3a, C4a and C5a, and deposition of the terminal membrane attack complex at the site of immune-complex formation (Morgan, 1995). As polymorphonuclear leukocytes (PMNs) migrate through capillary walls, the endothelial barrier is damaged, resulting in haemorrhage and vascular leakage at the inflammatory locus (Burch *et al*., 1992). Once at the site of immunoinflammation, PMNs cause the release of a milieu of secondary inflammatory mediators, such as TNFα and IL-6, which further promotes the inflammatory process and ensuing tissue damage (Hopken *et al*., 1997; Strachan *et al*., 2000).

The relative importance of a variety of inflammatory mediators in the dermis has been investigated using pharmacological and physiological tools. The importance of binding at the Fcγ receptor on phagocytic cells has been demonstrated, where oedema and vascular leakage were significantly inhibited in the dermis of an Fcγ-

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deficient mouse strain (Baumann *et al*., 2000). Similarly, the requirement for an intact complement system for complete development of a dermal Arthus reaction has been demonstrated using a variety of complement modulators. Serum complement depletion using cobra venom factor, inhibition of serum complement components using either the recombinant soluble complement receptor (sCR1) or a large molecule C5a receptor (C5aR) antagonist, or C5aR deficiency have all been used to demonstrate the central role of complement in the development of the dermal Arthus reaction (Yeh *et al*., 1991, Heller *et al*., 1999a; b). Tissue damage in the dermal Arthus reaction was also hampered by pretreatment of animals systemically with antibodies to P-selectin or I-CAM, CD11b and CD18, TNFα or IL-8 (Rote *et al*., 1996; Santos *et al*., 1998; Smith *et al*., 1996; Warren, 1991; Zhang *et al*., 1995). Similarly, PMN depletion results in a significantly inhibited inflammatory response as a result of immune-complex deposition in the dermis (Scherzer & Ward, 1978). The inflammatory response is also significantly inhibited by a number of nonsteroidal anti-inflammatory agents, including platelet activating factor antagonists, 5-lipoxygenase and COX inhibitors (Boughton-Smith *et al*., 1993; Okamoto *et al*., 1992; Smith *et al*., 1995). Although there is clearly a wide range of mediators involved in the formation of immunoinflammatory tissue damage, there appears to be a common pathway in the dermal Arthus reaction, which shows at least some requirement for the Fcγ receptor, PMN adhesion and migration, and complement activation.

The present study aimed to investigate the specific role of the C5aR in the development of a dermal Arthus reaction in the rat, using a new small molecular weight, specific C5aR antagonist developed in our laboratories (Finch *et al*., 1999). This C5aR antagonist (AcPhe[Orn-Pro-D-Cyclohexylalanine-Trp-Arg]) is a relatively small molecule (MW 896.5), and as demonstrated for the first time in this study, shows pharmacological activity when administered either orally or topically. The immune-complex deposition that occurs in this animal model plays a central role in dermal immune-complex diseases such as psoriasis, for which effective treatment is currently limited. Demonstration of efficacy for this C5aR antagonist provides strong evidence to support the development of this class of pharmacological agent as a new therapeutic approach for the intervention into the underlying immuno-inflammatory reaction that occurs in psoriasis and other inflammatory skin disorders.

Methods

Antagonist preparation

The cyclic C5aR antagonists AcF-[OPdChaWR] and AcF-[OPGWR] were synthesized as previously described (Finch *et al*., 1999). The compound was purified by reverse phase HPLC and fully characterized by mass spectrometry and proton NMR spectroscopy. Peptide binding affinity was characterized at the C5aR in isolated human PMNs as previously described (Finch *et al*., 1997).

Pharmacokinetics

Female Wistar rats (200–250 g) were anaesthetized with an intraperitoneal injection of ketamine (80 mg kg^{-1}) and xylazine (10 mg kg^{-1}; Lyppard, Australia). A femoral vein catheter was inserted, and rats were dosed either intravenously or orally with AcF-[OPdChaWR] at 3 mg kg^{-1} dissolved in 10% ethanol (20 μl) plus saline (180 μl). A heating pad was used to maintain the body temperature of the rats while blood samples were taken over a period of 3 h. Blood samples were immediately added to tubes containing heparin (1250 IU ml^{-1}), centrifuged (11 000 $\times g$) and stored on ice. The plasma layer of each sample was removed, diluted 1 : 3 with HPLC grade acetonitrile, vortexed (10 s) and centrifuged (11 000 $\times g$). This process produced precipitation of large plasma proteins and complete extraction of AcF-[OPdChaWR] from the plasma.

The upper fluid phase (50 μl) was removed and a deuterated internal standard $^2H_3$CO-F-[OPdChaWR] (50 μl, 5 μg ml^{-1} in 50% acetonitrile/water) was added and the samples were stored at -20°C pending analysis. These samples were transferred to 96 well plates and evaporated to dryness using a GeneVac centrifugal evaporator then reconstituted in the wells with mobile phase (20 μl). Samples were analysed by LCMS using an Agilent 1100 series HPLC equipped with a well plate autosampler coupled with a PE Sciex Qstar Pulsar ESI-TOF mass spectrometer. Samples (10 μl) were injected onto a Phenomenex Luna C18 column 2 $\times$ 50 mm and eluted isocratically with 36% acetonitrile 64% water containing formic acid 0.1% at 200 μl min^{-1}, retention time 2 min. The flow was split 3 : 1 into the mass spectrometer and ions were scanned over the mass range 896–900. Ion chromatograms were extracted 896.2–896.8 for AcF-[OPdChaWR] and 899.2–899.8 for the internal standard and were smoothed twice prior to integration. Concentrations were determined from a standard curve of drug: internal standard peak area ratios. Standards were prepared by adding an appropriate amount of the drug and internal standard to plasma from an untreated rat and were extracted and prepared by the same method as the experimental samples. Additionally, standard solutions made up in mobile phase were compared to the plasma-extracted standards to demonstrate that the drug was being extracted efficiently. The limit of detection in plasma was <10 ng ml^{-1}.

Reverse passive Arthus reaction

All animal experimentation was conducted within the guidelines and with approval from the animal experimentation ethics committee of the University of Queensland, Australia. A reverse passive peritoneal Arthus reaction was induced as previously described (Strachan *et al*., 2000), and a group of rats were pretreated prior to peritoneal deposition of antibody with AcF-[OPdChaWR] by oral gavage (10 mg kg^{-1} dissolved in 10% ethanol/90% saline solution to a final volume of 200 μl) or an appropriate oral vehicle control 30 min prior to deposition of antibody. Female Wistar rats (150–250 g) were anaesthetized with ketamine (80 mg kg^{-1} i.p.) and xylazine (12 mg kg^{-1} i.p.). The lateral surfaces of the rat were carefully shaved and five distinct sites on each lateral surface clearly delineated. A reverse passive Arthus reaction was induced in each dermal site by injecting Evans blue (15 mg kg^{-1} i.v.), chicken ovalbumin (20 mg kg^{-1}

i.v.) into the femoral vein 10 min prior to the injection of antibody. Purified rabbit anti-chicken ovalbumin (RDI, NJ, U.S.A.) was injected (saline only, 100, 200, 300 or 400 μg antibody in a final injection volume of 30 μl) in duplicate at two separate dermal sites on each lateral surface of the rat, giving a total of 10 injection sites per rat. Rats were placed on a heating pad, and anaesthetic 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 dermal Arthus reaction, the anaesthetised rat was euthanazed and a 10 mm^2 area of skin was collected from the site of each Arthus reaction. Skin samples were stored in 10% buffered formalin for at least 10 days before histological analysis using haematoxylin and eosin stain. Additionally, a second set of skin samples were placed in 1 ml of formamide overnight, and the absorbance of Evans blue extraction measured at 650 nm, as an indicator of serum leakage into the dermis (Hopken *et al*., 1997).

Rats were pretreated with the C5aR antagonist, AcF-[OPdChaWR] as the TFA salt, either intravenously (0.3–1 mg kg^{-1} in 200 μl saline containing 10% ethanol, 10 min prior to initiation of dermal Arthus), orally (0.3–10 mg kg^{-1} in 200 μl saline containing 10% ethanol by oral gavage, 30 min prior to initiation of dermal Arthus in rats denied food access for the preceding 18 h) or topically (200–400 μg site^{-1} 10 min prior to initiation of dermal Arthus reaction), or with the appropriate vehicle control. Topical application of the antagonist involved application of 20 μl of a 10–20 mg ml^{-1} solution in 10% dimethyl sulphoxide (DMSO), which was then smeared directly onto the skin at each site, 10 min prior to induction of the Arthus reaction. The saline-only injection site from rats treated with Evans blue only served as antigen controls, the saline-only injection site from rats treated with Evans blue plus topical DMSO only served as a vehicle control, the saline-only injection site from rats treated with Evans blue plus either intravenous, oral or topical antagonist only served as antagonist controls, and Evans blue plus dermal rabbit anti-chicken ovalbumin served as antibody controls. Topical application of the peptide AcF-[OPGWR] which has similar chemical composition and solubility as AcF-[OPdChaWR] but with an IC_{50} binding affinity of >1 mM in isolated human PMNs (unpublished data), served as an inactive peptide control. AcF-[OPGWR] was also dissolved in 10% DMSO and applied topically at 400 μg site^{-1} 10 min prior to initiation of the Arthus reaction.

TNFα measurement

Serum TNFα concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Pharmingen, U.S.A.). Antibody pairs used were a rabbit anti-rat TNFα antibody coupled with a biotinylated murine anti-rat TNFα antibody.

Interleukin-6 measurement

An ELISA method as described previously was used to measure serum and peritoneal lavage fluid interleukin-6 (IL-6) concentrations (Strachan *et al*., 2000). Antibody pairs used were goat anti-rat IL-6 coating antibody (1 μg ml^{-1}, RDI, U.S.A.) coupled with biotinylated goat anti-rat IL-6 antibody (1 μg ml^{-1}, RDI, U.S.A.) as a capture antibody.

Pathology assessment

Skin samples were fixed in 10% buffered formalin for at least 10 days, and stained with haematoxylin and eosin using standard histological techniques. Dermal samples were analysed in a blind fashion for evidence of pathology, and the degree of PMN infiltration was scored on a scale of 0–4. Initiation of a dermal Arthus reaction resulted in an increase in interstitial neutrophils, which was quantified in the following manner. Sections were given a score of 0 if no abnormalities were detected. A score of 1 indicated the appearance of increased PMNs in blood vessels, but no migration of inflammatory cells out of the lumen. A score of 2 indicated the appearance of small numbers of PMNs in the interstitial tissue and a score of 3 was assigned with larger numbers of PMNs present in the tissue and more prominent accumulations of inflammatory cells around blood vessels. A maximal score of 4 indicated a severe lesion was present in dermal sections, with excessive infiltration of PMNs into the tissues and migration of these cells away from blood vessels.

Data and statistical analysis

Standard curves of AcF-[OPdChaWR] in acetonitrile and plasma were plotted as ratios of the drug relative to a deuterated analogue internal standard *versus* concentration. These curves were used to determine the concentrations of AcF-[OPdChaWR] recorded at numerous time points following i.v. or oral dosing, which were graphed using a semi-log scale. The plasma half-lives for oral and i.v. administration of AcF-[OPdChaWR] were calculated for individual experiments using Rstrip software (Micromath Inc.) and expressed as geometric means and ranges shown in brackets.

Dermal vascular leakage was quantitated from the optical density of skin punches (100 μl of the Evans blue extracts) expressed as a percentage of the absorbance of plasma (100 μl) collected from the same rat. Optical density values and serum TNFα concentrations are expressed as mean $\pm$ s.e.mean, and compared to dermal Arthus control values using a Student's *t*-test, with a parametric Newman–Keuls post-test and statistical significance assessed at $P<0.05$. Histological scores of a pathology index are expressed as mean $\pm$ s.e.m., and statistical significance between groups was assessed using a Student's *t*-test and a *post hoc* nonparametric Dunn's test, with significance defined when $P<0.05$.

Results

Pharmacokinetic analysis of AcF-[OPdChaWR]

Drug standards and extracted plasma samples from AcF-[OPdChaWR]-treated animals were analysed by automated HPLC-mass spectrometry. The initial HPLC phase separated the components of the extracted plasma sample with very high specificity and the second mass spectrometry phase identified and quantified the molecular ion of AcF-[OPdChaWR] present in the sample. The slope of AcF-[OPdChaWR] standard curves in both plasma and aceto-

nitrile/water were not significantly different ($P>0.05$), which indicated that the acetonitrile extraction procedure used in these experiments resulted in quantitative recovery of the compound. The plasma derived standard curve was used to calculate the plasma concentration of AcF-[OPdChaWR] in each sample from the drug-treated groups. The coefficient of variation for 10 injections of the 10 μM drug standard was 3.9% demonstrating the precision of the analysis.

The plasma concentrations of AcF-[OPdChaWR], when administered to the rat intravenously and orally at a dose of 3 mg kg^{-1}, are shown in Figure 1. In the case of i.v. dosing, the concentration of the antagonist detectable in circulation rapidly decreased during the distribution phase, with a distribution half life of 26 min (3–34 min, $n=4$). The slower elimination phase had a half life of 69 min (43–79 min, $n=6$). After oral administration of 3 mg kg^{-1} AcF-[OPdChaWR] the plasma concentration of AcF-[OPdChaWR] in circulation peaked around 20 min at 0.32 ± 0.082 μM ($n=6$) and absorption and elimination half-lives were 26 min (15–47 min, $n=6$) and 70 min (64–82 min, $n=6$) respectively.

Initiation of a peritoneal Arthus reaction in the rat led to an increase ($P<0.05$) in vascular leakage and PMN infiltration within 4 h when compared to antibody control values (Figure 2). TNFα and IL-6 levels in the peritoneal lavage fluid were both significantly elevated 4 h after initiation of immune-complex mediated injury ($P<0.05$) when compared to antibody control values (Figure 2). All of these markers of inflammation were completely inhibited by pretreatment of the rat with AcF-[OPdChaWR] at 10 mg kg^{-1} by oral gavage 30 min prior to immune complex deposition, with no significant difference from antigen control values (Figure 2). Additionally, initiation of a peritoneal Arthus reaction led to a significant elevation of circulating TNFα and IL-6 levels ($P<0.05$ when compared to antibody control values), which reached a maximum of 22.2 ± 3.1 ng ml^{-1} ($n=6$) at 3 h for TNFα and 129.3 ± 10.4 ng ml^{-1} ($n=6$) for IL-6 at 2 h post immune-complex deposition (Figure 3). Pretreatment of rats with 10 mg kg^{-1} AcF-[OPdChaWR] by oral gavage 30 min prior to

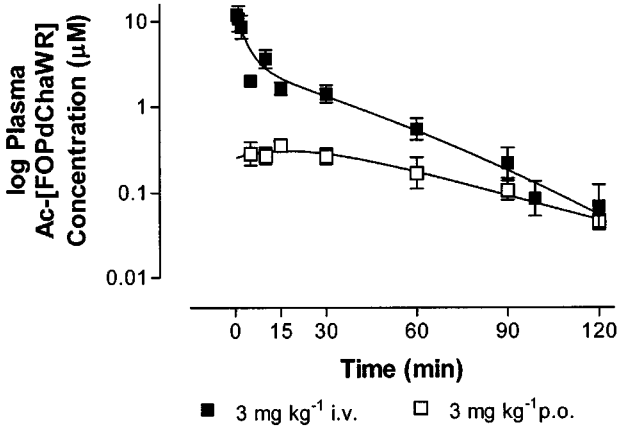

Figure 1 Intravenous and oral plasma pharmacokinetics of AcF-[OPdChaWR] in the rat. Rats were anaesthetized, a femoral vein catheter inserted, and were dosed with AcF-[OPdChaWR] either intravenously or orally (3 mg kg^{-1}). Plasma samples were collected at regular intervals prior to and after AcF-[OPdChaWR] administration, and the concentration of AcF-[OPdChaWR] in circulation determined using liquid chromatography-mass spectroscopy. Data are shown as mean$\pm$s.e.mean ($n=3-6$).

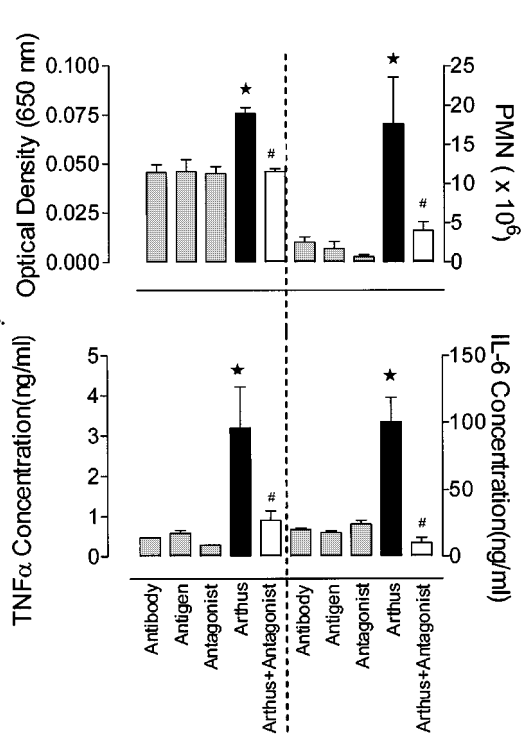

Figure 2 Inhibition of peritoneal reverse passive Arthus reaction by oral AcF-[OPdChaWR]. A single dose of AcF-[OPdChaWR] (10 mg kg^{-1} p.o.) blocked vascular leakage (measured by increased optical density for Evans blue), influx of PMNs, and elevation of both TNFα and IL-6 in the peritoneal cavity 4 h after induction of immune complex formation. Data shown are means$\pm$s.e.mean ($n=3-6$). *$P<0.05$ compared to antibody control values, # indicates a P value <0.05 when compared to values obtained following initiation of an Arthus reaction (ANOVA with Newman–Keuls post-test pairs comparison).

initiation of a peritoneal Arthus reaction resulted in no significant elevation in circulating TNFα or IL-6 at any time point, with maximum levels of 8.4 ± 3.2 ng ml^{-1} ($n=4$) at 3 h and 11.4 ± 6.1 ng ml^{-1} ($n=4$) at 2 h post antibody injection respectively (Figure 3).

Injection of saline-only in the dermis resulted in no elevation in vascular leakage when compared to antigen-only control values while injection of antibody in antigen-loaded rats resulted in a dose-dependent increase in dermal vascular leakage. The elevation in vascular leakage was significant ($P<0.05$) when compared to saline injection controls, with values of $6.6\pm1.02\%$, $10.0\pm1.2\%$ and $17.6\pm0.8\%$ ($n=4$) of the circulating plasma seen with 200, 300 and 400 μg of antibody respectively (Figure 4). Treatment of rats with intravenous AcF-[OPdChaWR] (0.3–1 mg kg^{-1}) 10 min prior to initiation of a dermal Arthus reaction resulted in a significant ($P<0.05$) reduction in vascular leakage at both the 300 and 400 μg dermal sites (Figure 4A). Similarly, treatment of rats with oral AcF-[OPdChaWR] at dose levels of 1 and 10 mg kg^{-1} significantly ($P<0.05$) reduced the elevation in vascular leakage seen with intradermal injection of 300 and 400 μg antibody (Figure 4B). Topical application of AcF-[OPdChaWR] (200–400 μg dermal site^{-1} in a volume of 20 μl) also significantly ($P<0.05$) inhibited the immune-complex mediated elevation in vascular leakage initiated by both 300 and 400 μg of antibody dermal site^{-1} (Figure 4C).

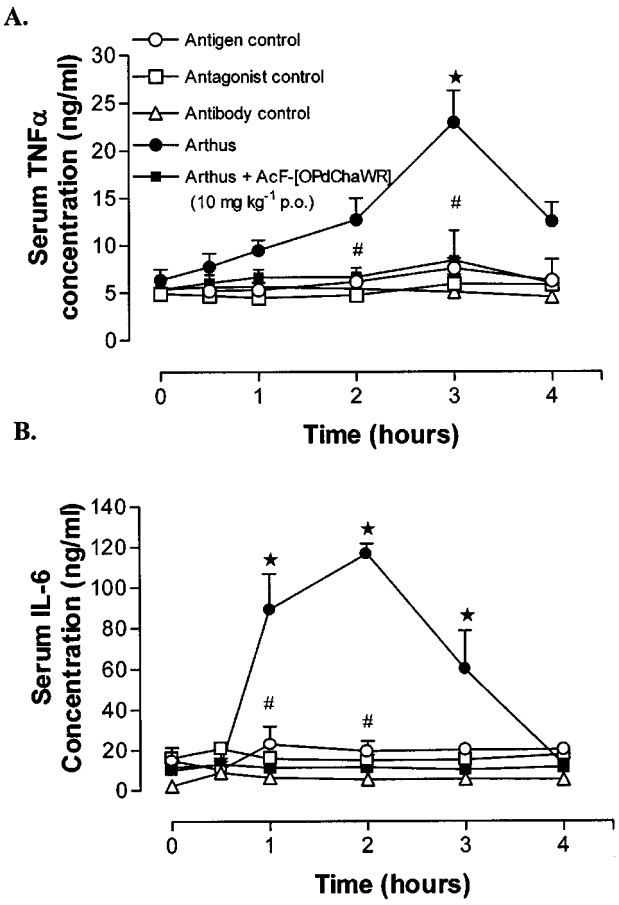

Figure 3 Inhibition of immune complex-induced elevation in circulating cytokines by oral AcF-[OPdChaWR] in the rat. Initiation of a peritoneal Arthus reaction led to a significant increase in circulating TNFα (A) and IL-6 (B) levels, which were inhibited by pretreatment of rats with AcF-[OPdChaWR] (10 mg kg^{-1} p.o.). Data are shown as mean ± s.e.mean ($n = 3-5$). *indicates a P value <0.05 when compared to antibody control values, # indicates a P value <0.05 when compared to values obtained following initiation of an Arthus reaction (ANOVA with Newman–Keuls post-test pairs comparison).

Topical application of neither 10% DMSO alone (20 μl dermal $site^{-1}$) nor the inert C5a antagonist analogue AcF-[OPGWR] in 10% DMSO (400 μg dermal $site^{-1}$ in a volume of 20 μl) produced any reduction in vascular leakage at any dermal Arthus reaction site (Figure 4D). Intradermal injection of antibody alone in the absence of intravenous antigen also resulted in no significant elevation in Evans blue leakage at any antibody dose (Figure 4D), demonstrating the absolute requirement for interaction of antibody with antigen in the dermis for development of an Arthus reaction.

The dermal Arthus reaction was achieved in rats which had received an intravenous injection of antigen and intradermal injection of the corresponding antibody at 0, 100, 200, 300 and 400 μg $site^{-1}$ in duplicate on the lateral surface of each rat, giving a fixed total of 2 mg of antibody given to each rat in this study. Intradermal injection of this total amount of antibody resulted in a significant ($P<0.05$) elevation in serum TNFα levels, reaching a maximum level of 20.5 ± 2.4 ng ml^{-1} ($n = 6$) in circulation 3 h after initiation of the dermal Arthus reaction, then returning to pretreatment values within 4 h (Figure 5). Pretreatment of rats with AcF-[OPdChaWR] intravenously at 0.3–1 mg kg^{-1} resulted in a significant dose-dependent reduction in this dermal Arthus-induced rise in circulating TNFα (Figure 5A). Similarly, pretreatment of rats with AcF-[OPdChaWR] at 1–10 mg kg^{-1} by oral gavage dose-dependently inhibited this rise in serum TNFα (Figure 5B). Conversely, pretreatment of dermal sites with AcF-[OPdChaWR] topically resulted in no inhibition of this rise in serum TNFα at any time point (Figure 5C). Pretreatment of dermal sites with neither DMSO alone, nor the inactive peptide AcF-[OPGWR] resulted in any reduction in the rise in serum TNFα following initiation of a dermal Arthus reaction (Figure 5D).

Treatment of rats with antagonist alone either intravenously, orally or topically at any dose resulted in no change in the pathology index of skin samples (Figure 6). Treatment of skin samples with the topical vehicle alone (DMSO) or the inactive C5a analogue AcF-[OPGWR] (400 μg dermal $site^{-1}$) alone also resulted in no change in the pathology index, nor did they significantly inhibit the Arthus-induced increase in pathology index (Figure 6D). Pretreatment of rats with AcF-[OPdChaWR] intravenously or orally at any dose did not result in a significant inhibition of immune complex-induced increase in the pathology index of skin samples (Figure 6B–C). Conversely, pretreatment of rats with AcF-[OPdChaWR] topically (400 μg dermal $site^{-1}$) resulted in a complete inhibition in the pathology index of skin samples similar to dermal Arthus control skin sample values (Figure 6D). AcF-[OPdChaWR] levels in plasma were not detectable (<10 ng ml^{-1}) following topical administration.

Discussion

The reverse passive Arthus reaction in rats is a well-characterized acute model of the immune complex-mediated inflammation that is clinically relevant to a range of immuno-inflammatory diseases in humans. Immune complex formation within the peritoneal cavity and the dermis of a rat leads to an influx of PMNs and other inflammatory cells, local and systemic elevation of inflammatory cytokines (TNFα, IL-6), and vascular leakage at the site of inflammation (Hopken *et al*., 1997). All of these inflammatory markers are effectively inhibited by a single oral or i.v. dose of AcF-[OPdChaWR] regardless of the site of tissue injury. The multiple anti-inflammatory effects of AcF-[OPdChaWR] in this model, together with reported studies using C5aR knockout mice (Hopken *et al*., 1996; 1997) or a large polypeptide C5aR antagonist that also inhibits PMN influx (Heller *et al*., 1999a), suggest a key role for C5a in the Arthus reaction and provide a compelling argument for the development of C5aR antagonists to treat immune complex diseases (Hopken *et al*., 1996; 1997; Strachan *et al*., 2000).

Immune-complex mediated inflammatory disorders such as psoriasis and rheumatoid arthritis are diseases primarily at the site of immune-complex deposition, but also involve a systemic component thought to be mediated by a change in circulating phagocytic cell functioning (Johnson *et al*., 1986). Although it is widely accepted that immune complex deposition leads to increased cytokine expression at the local site of inflammation, the systemic expression of these proinflammatory cytokines following initiation of a dermal Arthus reaction has not been as widely recognised as an inflammatory marker (Heller *et al*., 1999b; Hopken *et al*., 1997; Kohl & Gessner, 1999). We have

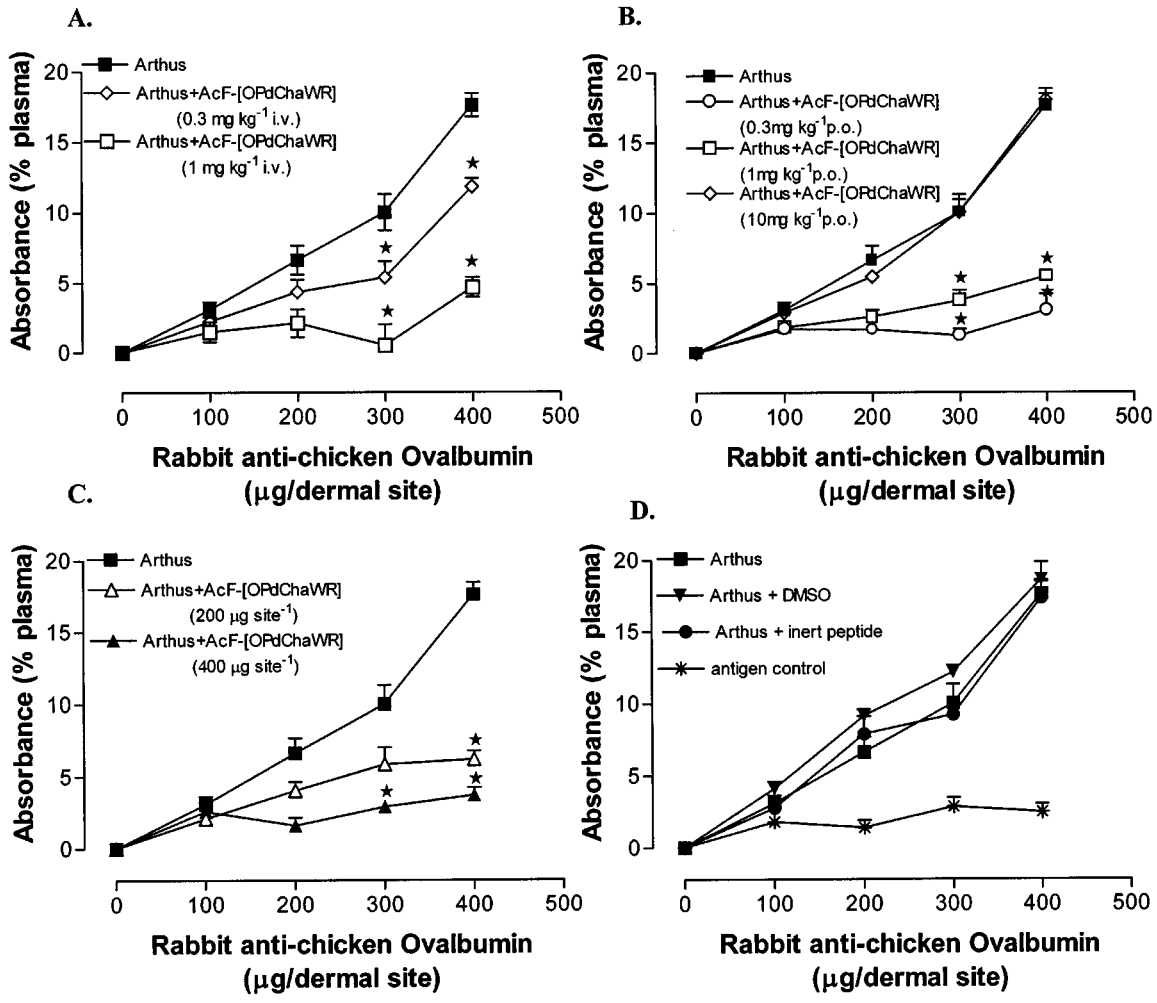

Figure 4 Inhibition of the vascular leakage associated with a dermal Arthus reaction by intravenous (A), oral (B) and topical (C) AcF-[OPdChaWR], and appropriate topical controls (D). Optical density of dermal punch extracts are shown following intradermal injection of rabbit anti-chicken ovalbumin at 0–400 μg site^{-1} following pretreatment with AcF-[OPdChaWR] intravenously, orally or topically. Data are shown as absorbance at 650 nm as a percentage of the plasma absorbance, as mean values $\pm$ s.e.mean ($n=3-6$). *indicates a P value <0.05 when compared to Arthus control values (ANOVA with Newman–Keuls post-test pairs comparison).

recently reported effective inhibition of the systemic upregulation of both TNFα and IL-6 following initiation of a peritoneal Arthus reaction by a C5aR antagonist, when given i.v. at a single dose (1 mg kg^{-1}) (Strachan *et al*., 2000). This is in agreement with results seen with a large molecule C5aR antagonist in a mouse peritoneal Arthus reaction (Heller *et al*., 1999a). We now show that cellular influx and vascular leakage into the peritoneum, as well as local and systemic inflammatory cytokine production are effectively inhibited by a single oral dose of AcF-[OPdChaWR].

Serum samples were collected at regular intervals following induction of the dermal Arthus reaction, and the circulating levels of TNFα were measured. Circulating levels of this cytokine were significantly elevated indicating that the dermal Arthus reaction is also not necessarily limited to the site of inflammation, but can involve a systemic component. This systemic induction of TNFα was inhibited by i.v. or oral pretreatment of rats with the C5aR antagonist, indicating a requirement for C5a in cytokine production. However, this elevation of systemic TNFα associated with a dermal Arthus reaction was not inhibited by topical application of the C5aR antagonist, despite the suppression of local inflammatory events. This result suggests that the production of TNFα is in large part a systemic event, and that distribution of pharmacologically effective levels of the antagonist when applied topically is restricted to the dermis. Inhibition of both the local and systemic inflammatory markers of immune complex-mediated inflammation by an orally active agent indicates that AcF-[OPdChaWR] is a potential anti-inflammatory drug candidate for treatment of immunoinflammatory disorders, and that local application may be an appropriate therapeutic route for the treatment of certain dermal immunoinflammatory conditions.

For the dermal Arthus reaction, topical administration of AcF[OPdChaWR] was as efficacious as administration of the drug by oral or i.v. routes. Circulating levels of AcF[OPdChaWR] were not detectable after topical administration, while levels of the drug in plasma reached a peak of up to 0.3 μM around 20 min following oral administration of a single 3 mg kg^{-1} dose. We have previously shown that AcF[OPdChaWR] is an insurmountable antagonist on PMNs (Paczowski

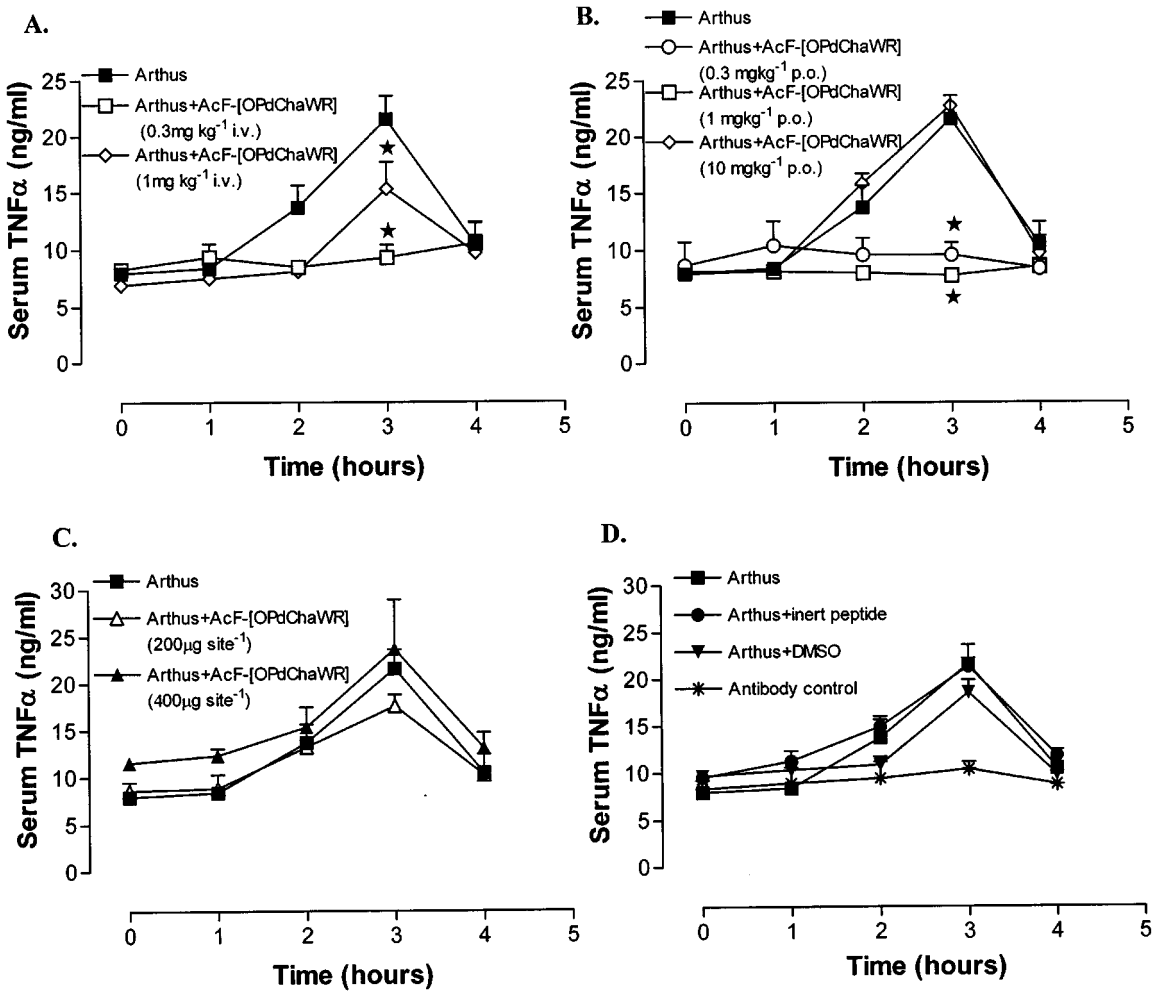

Figure 5 Inhibition of the rise in circulating TNFα associated with a dermal Arthus reaction by intravenous (A), oral (B) and topical (C) AcF-[OPdChaWR], and appropriate topical controls (D). Serum TNFα concentrations are shown at regular intervals after initiation of a dermal Arthus reaction, with group of rats pretreated with AcF-[OPdChaWR] intravenously, orally or topically. Data are shown as mean values ± s.e.mean ($n = 3-6$). *indicates a P value of <0.05 when compared to Arthus control values (ANOVA with Newman–Keuls post-test pairs comparison).

et al., 1999), with activity in the very low nanomolar range (Finch *et al.*, 1999; Paczowski *et al.*, 1999). Prolonged (>24 h) inhibition of C5a-induced neutropenia and block of C5a binding to the PMN C5a receptor is seen following a single oral dose of AcF[OPdChaWR] at times after administration when the drug is beneath the pharmacologically effective concentrations and the levels of detection in the plasma (Strachan *et al.*, in preparation). Taken together, the data indicate that these properties of AcF[OPdChaWR] enhance and prolong its activity *in vitro* and *in vivo* and suggest that the drug binds tightly to the receptor and dissociates only slowly, i.e., is a pseudoirreversible antagonist. This property of AcF[OPdChaWR] would also facilitate its activity following topical or oral administration, since plasma or tissue concentrations of the drug would not necessarily reflect its pharmacological activity. The relatively high levels of the drug in the dermis after topical administration or in the mucosal circulation following oral administration would effectively block regional PMN C5aR, and in the case of the dermis, endothelial C5aRs (Foreman *et al.*, 1994) which are involved in local PMN margination. To confirm this hypothesis and the proposed mechanism for the prolonged effect of AcF[OPdChaWR] *in vivo*, studies are under way to determine the rate of dissociation of the drug from its receptor.

We have previously reported that a single intravenous dose of AcF-[OPdChaWR] (1 mg kg^{-1}) significantly inhibited the induction of TNFα in the circulation by LPS or C5a (Strachan *et al.*, 2000). This blockade of LPS-induced and immune complex-induced elevation in circulating TNFα levels by AcF-[OPdChaWR] suggests a pivotal early position for C5a involvement relative to TNFα in the inflammatory cascade. Inhibition of proinflammatory cytokine formation may be a principal means by which C5a antagonists suppress a variety of immunoinflammatory diseases in animal models. In agreement with this hypothesis, C5a causes the secretion of numerous cytokines from monocytes/macrophages *in vitro*, which has been shown to be inhibited by C5aR antagonists (Haynes *et al.*, 2000). The blockade of TNFα production by a C5a antagonist may be particularly significant in view of reports of clinical efficacy in the treatment of rheumatoid arthritis for agents which antagonise TNFα (Charles *et al.*, 1999; Joosten *et al.*, 1999; Ohshima *et al.*, 1999).

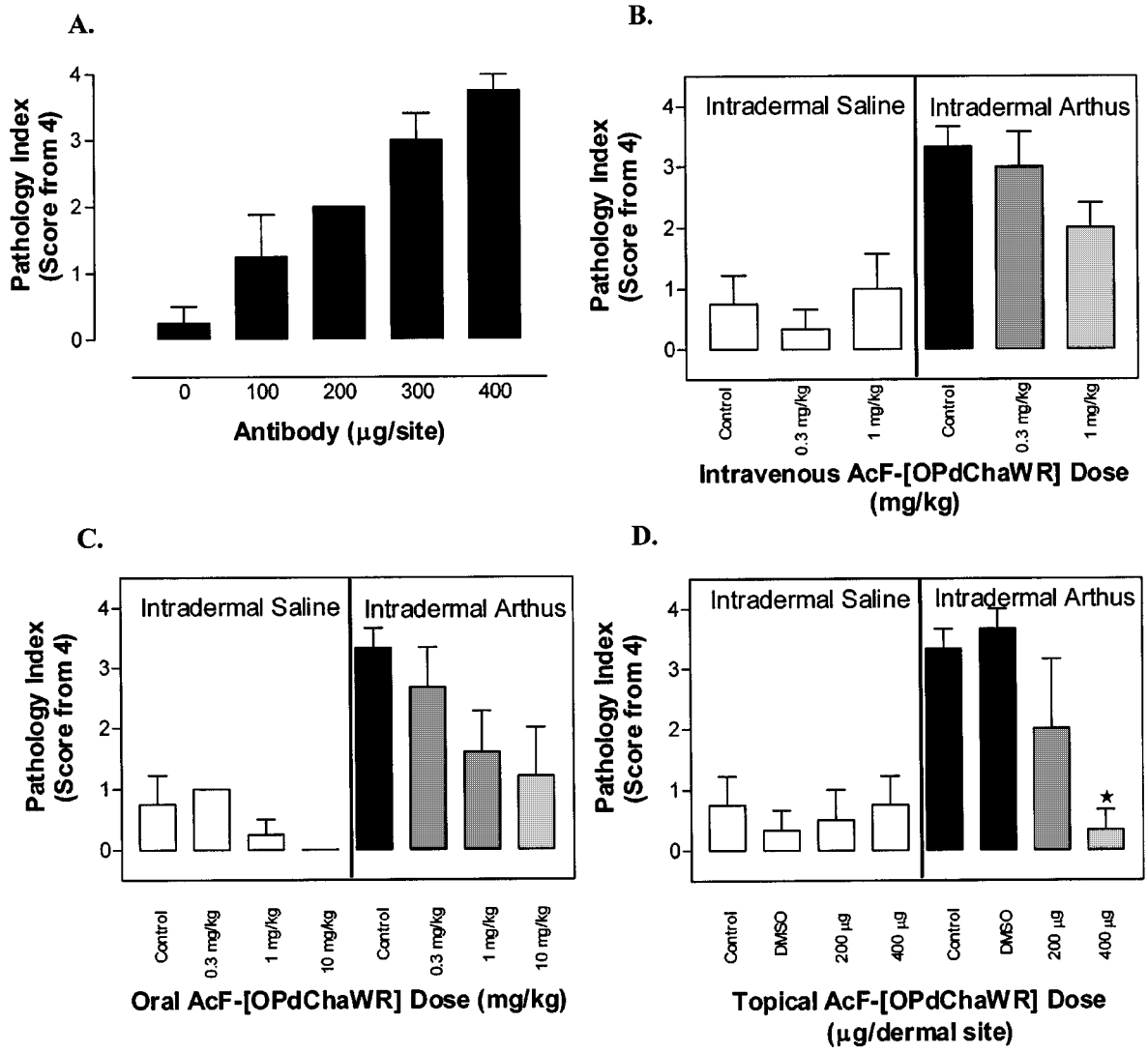

Figure 6 Reduction of the pathology index associated with a dermal Arthus reaction by intravenous, oral and topical AcF-[OPdChaWR]. Intradermal injection of increasing amounts of antibody leads to a dose-responsive increase in the pathology index scored by dermal samples (A). Data are shown for dermal samples intradermally injected with saline or 400 μg site^{-1} antibody ($n=5$) in rats pretreated with AcF-[OPdChaWR] intravenously (B) ($n=3$), orally (C) ($n=3$) and topically (D) ($n=3$). Data are shown as mean values $\pm$ s.e.mean. * $P<0.05$ when compared to Arthus control values using a non-parametric t-test.

Vascular leakage and inflammatory cell infiltration are universal components of a wide range of inflammatory disease states including dermal immune complex-mediated conditions, such as psoriasis. The dermal Arthus model demonstrates a major upregulation of these components. Current therapies for the treatment of dermal immunoinflammatory disorders include topical and systemic steroids and retinoids, vitamin D_3 and phototherapy (Fogh & Kragballe, 2000; Guenther, 2000). However, these therapies, alone or in combination, have both acute and chronic side effects, and do not always provide adequate relief (Ashcroft *et al.*, 2000). The present study suggests there may be an absolute requirement for C5a for full development of the key immunoinflammatory events in the dermis of the rat, and that this inflammation can be blocked using a C5aR antagonist given intravenously, orally or topically. The results of the present study suggest that C5aR antagonists may be suitable drug candidates for intervention in certain dermal immunoinflammatory disorders, when given by a variety of routes.

In summary, we have shown for the first time the pharmacological activity of a small molecule C5aR antagonist following either oral or topical administration. As such, AcF-[OPdChaWR] (or an analogue) is a novel drug candidate with potentially greater usefulness in the clinical setting than other large molecule C5a-inhibiting agents currently under investigation.

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References

ASHCROFT, D.M., PO, A.L., WILLIAMS, H.C. & GRIFFITHS, C.E. (2000). Systematic review of comparative efficacy and tolerability of calcipotriol in treating chronic plaque psoriasis. *Br. Med. J.,* **320,** 963 – 967.

BAUMANN, U., KOHL, J., TSCHERNIG, T., SCHWERTER-STRUMPF, K., VERBEEK, J.S., SCHMIDT, R.E. & GESSNER, J.E. (2000). A codominant role of Fc gamma RI/III and C5aR in the reverse Arthus reaction. *J. Immunol.,* **164,** 1065 – 1070.

BOUGHTON-SMITH, N.K., DEAKIN, A.M., FOLLENFANT, R.L., WHITTLE, B.J. & GARLAND, L.G. (1993). Role of oxygen radicals and arachidonic acid metabolites in the reverse passive Arthus reaction and carrageenin paw oedema in the rat. *Br. J. Pharmacol.,* **110,** 896 – 902.

BURCH, R.M., CONNOR, J.R., BATOR, J.M., WEITZBERG, M., LAEMONT, K., NORONHA-BLOB, L., SULLIVAN, J.P. & STERANKA, L.R. (1992). NPC 15669 inhibits the reversed passive Arthus reaction in rats by blocking neutrophil recruitment. *J. Pharmacol. Exp. Ther.,* **263,** 933 – 937.

CHARLES, P., ELLIOTT, M.J., DAVIS, D., POTTER, A., KALDEN, J.R., ANTONI, C., BREEDVELD, F.C., SMOLEN, J.S., EBERL, G., DEWOODY, K., FELDMANN, M. & MAINI, R.N. (1999). Regulation of cytokines, cytokine inhibitors, and acutephase proteins following anti-TNFα therapy in rheumatoid arthritis. *J. Immunol.,* **163,** 1521 – 1528.

FINCH, A., VOGEN, S., SHERMAN, S., KIRNARSKY, L., TAYLOR, S. & SANDERSON, S. (1997). Biologically active conformer of the effector region of human C5a and modulatory effects of N-terminal receptor binding determinants on activity. *J. Med. Chem.,* **40,** 877 – 884.

FINCH, A.M., WONG, A.K., PACZKOWSKI, N.J., WADI, S.K., CRAIK, D.J., FAIRLIE, D.P. & TAYLOR, S.M. (1999). Low-molecular-weight peptidic and cyclic antagonists of the receptor for the complement factor C5a. *J. Med. Chem.,* **42,** 1965 – 1974.

FOGH, K. & KRAGBALLE, K. (2000). Recent developments in vitamin D analogs. *Curr. Pharm. Des.,* **6,** 961 – 972.

FOREMAN, K.E., VAPORCIYAN, A.A., BONISH, B.K., JONES, M.L., JOHNSON, K.J., GLOVSKY, M.M., EDDY, S.M. & WARD, P.A. (1994). C5a-induced expression of P-selectin in endothelial cells. *J. Clin. Invest.,* **94,** 1147 – 1155.

GUENTHER, L. (2000). Tazarotene combination treatments in psoriasis. *J. Am. Acad. Dermatol.,* **43,** S36 – S42.

HAYNES, D.R., HARKIN, D.G., BIGNOLD, L.P., HUTCHENS, M.J., TAYLOR, S.M. & FAIRLIE, D.P. (2000). Inhibition of C5a-induced neutrophil chemotaxis and macrophage cytokine production in vitro by a new C5a receptor antagonist. *Biochem. Pharmacol.,* **60,** 729 – 733.

HELLER, T., GESSNER, J.E., SCHMIDT, R.E., KLOS, A., BAUTSCH, W. & KOHL, J. (1999a). Cutting edge: Fc receptor type I for IgG on macrophages and complement mediate the inflammatory response in immune complex peritonitis. *J. Immunol.,* **162,** 5657 – 5661.

HELLER, T., HENNECKE, M., BAUMANN, U., GESSNER, J.E., ZU VILSENDORF, A.M., BAENSCH, M., BOULAY, F., KOLA, A., KLOS, A., BAUTSCH, W. & KOHL, J. (1999b). Selection of a C5a receptor antagonist from phage libraries attenuating the inflammatory response in immune complex disease and ischemia/reperfusion injury. *J. Immunol.,* **163,** 985 – 994.

HOPKEN, U.E., LU, B., GERARD, N.P. & GERARD, C. (1997). Impaired inflammatory responses in the reverse arthus reaction through genetic deletion of the C5a receptor. *J. Exp. Med.,* **186,** 749 – 756.

HOPKEN, U., MOHR, M., STRUBER, A., MONTZ, H., BURCHARDI, H., GOTZE, O. & OPPERMANN, M. (1996). Inhibition of interleukin-6 synthesis in an animal model of septic shock by anti-C5a monoclonal antibodies. *Eur. J. Immunol.,* **26,** 1103 – 1109.

JOHNSON, W.J., DIMARTINO, M.J. & HANNA, N. (1986). Macrophage activation in rat models of inflammation and arthritis: determination of markers of stages of activation. *Cell. Immunol.,* **103,** 54 – 64.

JOOSTEN, L.A., HELSEN, M.M., SAXNE, T., VAN DE LOO, F.A., HEINEGARD, D. & VAN DEN BERG, W.B. (1999). IL-1 α beta blockade prevents cartilage and bone destruction in murine type II collagen-induced arthritis, whereas TNFα blockade only ameliorates joint inflammation. *J. Immunol.,* **163,** 5049 – 5055.

KOHL, J. & GESSNER, J.E. (1999). On the role of complement and Fc gamma-receptors in the Arthus reaction. *Mol. Immunol.,* **36,** 893 – 903.

MORGAN, B.P. (1995). Physiology and pathophysiology of complement: progress and trends. *Crit. Rev. Clin. Lab. Sci.,* **32,** 265 – 298.

OHSHIMA, S., SAEKI, Y., MIMA, T., SASAI, M., NISHIOKA, K., ISHIDA, H., SHIMIZU, M., SUEMURA, M., MCCLOSKEY, R. & KISHIMOTO, T. (1999). Long-term follow-up of the changes in circulating cytokines, soluble cytokine receptors, and white blood cell subset counts in patients with rheumatoid arthritis (RA) after monoclonal anti-TNF α antibody therapy. *J. Clin. Immunol.,* **19,** 305 – 313.

OKAMOTO, H., IWAHISA, Y. & TERASAWA, M. (1992). Suppression of the Arthus reaction by Y-24180, a potent and specific antagonist of platelet-activating factor. *Agents Actions,* **35,** 149 – 158.

PACZKOWSKI, N.J., FINCH, A.M., WHITMORE, J.B., SHORT A.J., WONG, A.K., MONK, P.N., CAIN, S.A., FAIRLIE, D.P. & TAYLOR, S.M. (1999). Pharmacological characterization of antagonists of the C5a receptor. *Br. J. Pharmacol.,* **128,** 1461 – 1466.

PEREZ, H.D. & GOLDSTEIN, I.M. (1987). Polymorphonuclear leukocyte chemotaxis in systemic lupus erythematosus. *J. Rheumatol.,* **13,** 53 – 58.

ROTE, W.E., DEMPSEY, E., MAKI, S., VLASUK, G.P. & MOYLE, M. (1996). The role of CD11/CD18 integrins in the reverse passive Arthus reaction in rat dermal tissue. *J. Leukoc. Biol.,* **59,** 254 – 261.

SANTOS, L.L., HUANG, X.R., BERNDT, M.C. & HOLDSWORTH, S.R. (1998). P-selectin requirement for neutrophil accumulation and injury in the direct passive Arthus reaction. *Clin. Exp. Immunol.,* **112,** 281 – 286.

SCHERZER, H. & WARD, P.A. (1978). Lung and dermal vascular injury produced by preformed immune complexes. *Am. Rev. Respir. Dis.,* **117,** 551 – 557.

SMITH, R.J., CHOSAY, J.G., DUNN, C.J., MANNING, A.M. & JUSTEN, J.M. (1996). ICAM-1 mediates leukocyte-endothelium adhesive interactions in the reversed passive Arthus reaction. *J. Leukoc. Biol.,* **59,** 333 – 340.

SMITH, W.G., SHAFFER, A.F., CURRIE, J.L., THOMPSON, J.M., KIM, S., RAO, T. & ISAKSON, P.C. (1995). Characterization of 5-lipoxygenase inhibitors in biochemical and functional in vivo assays. *J. Pharmacol. Exp. Ther.,* **275,** 1332 – 1338.

STRACHAN, A.J., WOODRUFF, T.M., HAAIMA, G., FAIRLIE, D.P. & TAYLOR, S.M. (2000). A new small molecule C5a receptor antagonist inhibits the reverse-passive Arthus reaction and endotoxic shock in rats. *J. Immunol.,* **164,** 6560 – 6565.

TERUI, T., OZAWA, M. & TAGAMI, H. (2000). Role of neutrophils in induction of acute inflammation in T-cell-mediated immune dermatosis, psoriasis: a neutrophil-associated inflammation-boosting loop. *Exp. Dermatol.,* **9,** 1 – 10.

WARREN, J.S. (1991). Disparate roles for TNF in the pathogenesis of acute immune complex alveolitis and dermal vasculitis. *Clin. Immunol. Immunopathol.,* **61,** 249 – 259.

WEISSMANN, G. & KORCHAK, H. (1984). Rheumatoid arthritis. The role of neutrophil activation. *Inflammation,* **8,** S3 – S14.

YEH, C.G., MARSH, JR. H.C., CARSON, G.R., BERMAN, L., CONCINO, M.F., SCESNEY, S.M., KUESTNER, R.E., SKIBBENS, R., DONAHUE, K.A. & IP, S.H. (1991). Recombinant soluble human complement receptor type 1 inhibits inflammation in the reversed passive arthus reaction in rats. *J. Immunol.,* **146,** 250 – 256.

ZHANG, Y., RAMOS, B.F., JAKSCHIK, B., BAGANOFF, M.P., DEPPELER, C.L., MEYER, D.M., WIDOMSKI, D.L., FRETLAND, D.J. & BOLANOWSKI, M.A. (1995). Interleukin 8 and mast cell-generated tumor necrosis factor α in neutrophil recruitment. *Inflammation,* **19,** 119 – 132.

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