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FAST TRACK

Pre-neutralization of C5a-mediated effects by the monoclonal antibody 137-26 reacting with the C5a moiety of native C5 without preventing C5 cleavage

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SUMMARY

Complement C5a is aetiologically linked to inflammatory tissue damage in conditions like septicaemia, immune complex diseases and ischaemia-reperfusion injury. We here describe a monoclonal antibody (mAb), 137–26, that binds to the C5a moiety of human C5 and neutralizes the effects of C5a without interfering with C5 cleavage and the subsequent formation of lytic C5b-9 complex. Mouse anti-human C5 mAbs were generated and the reactivity with C5 and C5a was detected by ELISA and surface plasmon resonance. The inhibition of C5a binding to C5a receptor was studied using a radioligand binding assay. The effects of the antibody on C5a functions were examined using isolated neutrophils and a novel human whole blood model of inflammation. Haemolytic assays were used to study the effect on complement-mediated lysis. mAb 137–26 reacted with both solid- and solution-phase C5 and C5a in a dose-dependent manner with high affinity. The antibody competed C5a binding to C5a receptor and inhibited C5a-mediated chemotaxis of neutrophils. Furthermore, the antibody effectively abrogated complement-dependent *E. coli*-induced CD11b up-regulation and oxidative burst in neutrophils of human whole blood. mAb 137–26 was more potent than a C5a receptor antagonist and a previously described anti-C5a antibody. mAb 137–26 did not inhibit complement-mediated lysis, nor did it activate complement itself. Together, mAb 137–26 binds both the C5a moiety of native C5 and free C5a, thereby effectively neutralizing the biological effects of C5a. The antibody may have therapeutic potential in inflammatory diseases where C5a inhibition combined with an operative lytic pathway of C5b-9 is particularly desired.

Keywords complement C5 monoclonal antibody inflammation

INTRODUCTION

The complement system is an important part of a host defense against invading microorganisms. Activation of complement induces a number of biological effects of which many are potent and mediated by the anaphylatoxin C5a. Although these effects are intended to overcome infection, they may be detrimental to the host when the system is activated in an excessive or uncontrolled manner. Activation of complement may contribute to tissue damage and inflammation in a number of clinical conditions, e.g. bacterial sepsis, immune complex diseases, and ischaemia-reperfusion injury [1–3]. Thus, the complement system could act as a double-edged sword in the body. Increasing knowledge about the role of complement in various diseases has underscored its potential as a therapeutic target to reduce tissue injury and inflammation.

Several approaches to inhibit complement have been applied in experimental models and a few are approaching clinics. In principle, specific inhibition of those components in the complement system contributing to tissue damage and inflammation should be targeted to achieve maximal efficacy and minimal, if any, adverse side-effects. A soluble form of complement receptor 1 (sCR1) and a mAb blocking C5 cleavage are examples of different strategies to reduce complement-dependent tissue injury [4,5]. The former blocks activation of C3 and thus the whole complement cascade at an early step, whereas the latter only blocks terminal pathway

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activation. A further discrimination in inhibition of the terminal pathway is obtained by neutralizing C5a while keeping the lytic C5b-9 pathway open. This may be advantageous in gram-negative septicaemia, in particular *Neisseria* infection, where systemic release of C5a may contribute to the irreversible septic shock whereas the lytic pathway may help kill the bacteria [6]. Blocking C5a by mAbs and C5a receptor (C5aR) antagonists has proven to be useful in experimental models of septicaemia, immune complex diseases, and ischaemia-reperfusion injury [7–10].

A number of mAbs to C5a have been described, typically binding to neoepitopes exposed in the C5a fragment after C5 cleavage, but not found in the native C5 molecule [11]. These mAbs bind to C5a after C5 is cleaved into C5a and C5b. We here describe a novel approach of neutralizing C5a by an anti-C5 mAb 137–26 which binds to the C5a moiety of native C5 before cleavage without interfering with the lytic C5b-9 pathway. The antibody also binds C5a even after it is formed.

MATERIALS AND METHODS

Generation of anti-C5 mAbs

Male A/J mice, 7–9 weeks old, were injected subcutaneously with 30 μg of purified human C5 (Advanced Research Technologies, San Diego, CA, USA) in complete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA). At two-week intervals the mice were injected twice subcutaneously with 30 μg of C5 in incomplete Freund's adjuvant. Three days before sacrifice, the mice were injected intraperitoneally with 30 μg of C5 in phosphate buffered saline (PBS). For generation of hybridomas, splenocytes were isolated from immunized mice and fused with SP2/0 myeloma cells. Cells were cultured in a selection medium containing hypoxanthine, aminopterin and thymidine, according to our procedure described earlier [12]. After about 10 days, supernatants from the cell culture were tested for antibody reactivity with purified human C5 by ELISA. Positive hybridomas were then single-cell cloned by a limiting-dilution procedure. The positive hybridomas were expanded for purification of mAbs by protein A chromatography for characterization. Three anti-C5 mAbs used in this study were mAb 137–26 (IgG1), mAb 137–30 (IgG1) and mAb 137–76 (IgG1).

C5 and C5a ELISA

Wells of Immulon II (Dynatech Laboratories, Chantilly, VA, USA) microtest plates were coated overnight with either human C5 or C5a (Sigma, St. Louis, MO, USA) at 0·1 μg/ml (50 μl/well). The nonspecific binding sites in the wells were then saturated by incubation with 200 μl of 2% bovine serum albumin in PBS (PBSB). The wells were then washed with PBST buffer (PBS containing 0·05% Tween 20). Fifty microlitres of culture supernatant from each fusion well or serially diluted purified mAbs were added to each coated well together with 50 μl of PBSB for one hour at room temperature. The wells were washed with PBST. The bound antibodies were then detected by reaction with diluted horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Fc specific) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for one hour at room temperature. The wells were then washed with PBST. Peroxidase substrate solution containing 0·1% 3,3,5,5, tetramethyl benzidine (Sigma) and 0·003% hydrogen peroxide (Sigma) in 0·1 M sodium acetate, pH 6·0, was added to the wells for colour development for 30 min The reaction was terminated by addition of 50 μl of 2 M H_2SO_4 per well. The optical density (OD) was read at 450 nm with an ELISA reader.

Polyacrylamide gel electrophoresis and immunoblotting

The reactivity of mAb 137–26 with purified human C5 and recombinant C5a was also determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, under nonreducing condition [13]. The proteins in the gel were stained with either Coomassie Blue for visual inspection or transferred to polyvinylidene difluoride membrane for Western blot analysis [14]. The binding of mAb 137–26 at 1 μg/ml to C5 and C5a on the membrane was detected by incubation with horseradish peroxidase conjugated goat anti-mouse IgG (1 : 5000) (Jackson ImmunoResearch Laboratories). The immunoreactive proteins were identified on film, using enhanced chemiluminescence detection (Supersignal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA).

Complement-mediated haemolysis

For the classical pathway haemolysis, chicken red blood cells (RBC) (5×10^7 cells/ml) in gelatin/veronal buffered saline (GVB^{++}) containing 0·5 mM $MgCl_2$ and 0·15 mM $CaCl_2$ were sensitized with purified rabbit anti-chicken RBC immunoglobulins at 8 μg/ml (Inter-Cell Technologies, Hoperwell, NJ, USA) for 15 min at 4°C. The cells were then washed with GVB^{++}. The washed cells were re-suspended in the same buffer at $1{\cdot}7 \times 10^8$ cells/ml. In each well of a round-bottom 96-well microtest plate, 50 μl of normal human serum (5·2%) was mixed with 50 μl of GVB^{++} of serially diluted mAb 137–26 or a neutralizing anti-C2 mAb 175–62 (IgG1) as a positive control. mAb 175–62 was generated by our laboratory (M.F; unpublished observation). Then 30 μl of the washed sensitized chicken RBCs suspension was added to the wells containing the mixtures. Fifty microlitres of normal human serum (5·2%) were mixed with 80 μl of GVB^{++} to give the serum colour background. An isotype-matched anti-HIV-1 gp120 mAb G3-519 (IgG1) was used as a negative control [12]. The final human serum concentration used was 2%. The mixture was incubated at 37°C for 30 min The plate was shaken on a microtest plate shaker for 15 s. The plate was then centrifuged at 300 × g for 3 min Supernatants (80 μl) were collected and transferred to wells in a flat-bottom 96-well microtest plates for measurement of OD at 405 nm by an ELISA plate reader. The percent inhibition of haemolysis is defined as:

$$100 \times [(OD_{\text{without mAb}} - OD_{\text{serum colour background}}) - (OD_{\text{with mAb}} - OD_{\text{serum colour background}})]/(OD_{\text{without mAb}} - OD_{\text{serum colour background}})$$

For alternative pathway haemolysis, unsensitized rabbit RBCs were washed three times with gelatin/veronal-buffered saline (GVB/Mg-EGTA) containing 2 mM $MgCl_2$ and 1·6 mM EGTA. EGTA was used to inhibit the classical complement pathway. The assay procedures are similar to those of the classical pathway haemolytic assay described above. The final concentration of human serum used in the assay was 10%. Anti-factor D mAb 166–32 (IgG1) specifically neutralzing the alternative pathway was used as a positive control [15,16]. The same isotype-matched control mAb G3-519 was used as a negative control.

Surface plasmon resonance for antibody affinity determination

The affinity equilibrium constant and the binding kinetic constants (association and dissociation) of mAb 137–26 with C5 and C5a were determined by a BIAcore instrument (Pharmacia

Biosensor AB, Uppsala, Sweden). All the binding measurements were performed in HEPES-buffered saline (HBS) (10 mM HEPES, pH 7·4, 150 mM NaCl, 3·4 mM EDTA, 0·005% Surfactant P20) at 25°C. To measure the binding rate constants of C5 and C5a to mAb 137–26, rabbit anti-mouse IgG(Fc) antibodies were immobilized onto a CM5 sensorchip by amine coupling using N-hydroxysuccinimide and N-ethyl-N′-(3-diethylaminopropyl)cardo-diimide. mAb 137–26 was then captured onto the coated sensorchip before the injection of C5 or C5a at different concentrations. Kinetic parameters were obtained by global fitting of kinetic traces under pseudo-first order kinetics using BIAvaluation version 3·0 (Biacore Inc. Piscataway, NJ, USA).

^{125}I-C5a binding assay with purified human neutrophils

Neutrophils were purified from freshly collected, heparinized human whole blood as described earlier [17]. The procedure consists of Dextran T-500 (Pharmacia, Piscataway, NJ, USA) sedimentation, density centrifugation in Histopaque-1077 (Sigma, St. Louis, MO), and hypotonic lysis of RBCs. In the binding assay, mAb 137–26 was serially diluted with a binding buffer (1% BSA in RPMI1640 medium) to give final concentrations of 0·04–640 nM. Ten microlitres of 4 nM ^{125}I-C5a (NEN Life Science Products, Inc., Boston, MA, USA) were added to 40 μl of diluted mAb 137–26 for incubation at room temperature for 15 min Purified recombinant human C5a (Sigma) was used as a positive control, whereas the isotype-matched mAb G3-519 was used as a negative control. For the maximum binding of ^{125}I-C5a, 36 μl of the binding buffer without the antibodies or C5a was used instead. Then 50 μl of neutrophil suspension (2×10^7 cells/ml) was added to the mixture for incubation on ice for 40 min. At the end of the incubation, the mixture from each tube was transferred to the top of 800 μl of a separation buffer (6% BSA in PBS) in another Eppendorf tube. The tubes were centrifuged at 2000× g for 3 min at room temperature. After the supernatant was aspirated, the cell pellet was resuspended in 0·5 ml of de-ionized water to lyse the cells. The cell lysate was then mixed with 3 ml of Ultima Gold scintillation fluid (Packard Instrument, Meriden, CT) for radioactive counting. The percent inhibition of ^{125}I-C5a binding is defined as:

$$[Cpm_{max} - Cpm_{bkg}] - [Cpm_{ca} - Cpm_{bkg}]/[Cpm_{max} - Cpm_{bkg}] \times 100$$

where Cpm_{max} = maximum count per minute without competing agents; Cpm_{bkg} = background cpm without addition of ^{125}I-C5a; Cpm_{ca} = cpm with competing agents.

C5a-induced chemotaxis of human neutrophils

Human neutrophils were isolated as described above. The assays were performed using the ChemoTx plate system with membrane pore size of 5 μ in diameter (Neuro Probe, Gaithersburg, MD, USA). As a positive control, a cyclic peptidic C5aR antagonist, AcPhe[L-ornithine-Pro-D-cyclohexylanine-Trp-Arg] (abbreviated as AcF-[OPdChaWR]), was synthesized according to the method described earlier [18]. Serially diluted mAb 137–26, the isotype-matched control mAb G3-519, or AcF-[OPdChaWR] (final concentration, 0·3–100 nM) was mixed with 1 nM C5a in the wells of the plate. This concentration of C5a was found to give the maximum chemotaxis of neutrophils in the assay. For controls, PBSB with or without C5a was used to measure the C5a-induced and spontaneous migration of neutrophils, respectively. Fifty microlitres of neutrophil suspension ($3{\cdot}6 \times 10^6$ cells/ml) were added to the top of the framed filter for incubation in 5% CO_2 at 37°C for 45 min After incubation, the quantity of neutrophils migrated to the lower chambers was measured by a nonradioactive cell proliferation assay using a tetrazolium compound MTS (Promega, Madison, WI, USA). The number of chemotactic neutrophils was represented by the optical density of soluble formazan product measured at 490 nm using an ELISA plate reader.

Human whole blood model of inflammation

The model has been described in detail previously [19]. It was used to study the activation of neutrophils and monocytes by C5a produced as a result of complement activation by *E. coli* in anticoagulated human whole blood. All equipment (tubes, tips, etc.) and solutions used were endotoxin-free according to information from the manufacturers. Burst-test kit with opsonized *E. coli* strain LE392 (ATCC 33572), was obtained from ORPEGEN (Pharma, Heidelberg, Germany). The oxidative burst and CD11b expression was examined in whole human blood obtained from voluntary healthy blood donors. The blood was anticoagulated with lepirudin (Refludan®, Hoechst, Frankfurt am Main, Germany). Lepirudin was tested not to interfere with complement activation. All incubations were performed at 37°C. The samples were preincubated for 4 min with antibodies, peptides or PBS until PBS (baseline samles) or *E. coli* (test samples) was added. mAb anti-C5a antibody 561 (IgG2a), a kind gift from Prof J. Köhl, Hannover [20] and the C5aR antagonist AcF-[OPdChaWR] were used as positive controls. The *E.* coli concentrations used in the CD11b experiments and oxidative burst experiments were 1×10^7 bacteria/ml blood and 1×10^8 bacteria/ml blood, respectively. The baseline sample (T-0) was processed immediately. Subsequent incubations for CD11b detection and oxidative burst assay were performed for 10 and 20 min, respectively. After incubation, 100 μl of blood was used for the flow cytometric assays. The oxidative burst was measured using the substrate dihydrorhodamine123 and performed as described in the Burst-test procedure. For the measurement of the CD11b expression the whole blood was fixed with paraformaldehyde and then stained with anti-CD11b PE (Becton Dickinson, San Jose, CA, USA) and the nuclear dye LDS-751 (Molecular Probes, Inc., Eugene, OR, USA). Oxidative burst and CD11b expression were measured as median fluorescence intensity (MFI) using a FACSCalibur flow cytometer (Becton Dickinson). All experiments were performed 3–5 times.

Complement activating potency

To examine the potential of complement activation by mAb 137–26 after complexed with C5, the antibody was incubated with 150 μl of pooled, normal human serum obtained from five blood donors in each of two experiments. The isotype-matched antibody mAb G3-519 was used as a negative control and heat aggregated IgG (HAIGG) (Gammaglobulin Kabi, Uppsala, Sweden) as a positive control. All incubations were performed at 37°C for 1 h. EDTA was then added at a final concentration of 10 mM to stop further complement activation and the serum samples were then stored at −70°C until being analysed. Samples were analysed for complement activation products described below.

Enzyme immunoassays for complement activation products

C1rs-C1inhibitor complexes (C1rs-C1inh), C4bc, C3bc and the terminal sC5b-9 complex (TCC) were quantified by ELISAs principally as described in detail earlier [21–24]. Each sample was

analysed in triplicate. The monoclonal antibodies used in the C1rs-C1inh and C4bc assays were a kind gift from Prof C.E. Hack, Amsterdam, the Netherlands.

Data presentation

Data were obtained from 2 to 5 independent experiments for each assay and were presented either as mean ± standard deviation or as one representative of the experiments performed.

RESULTS

mAb 137–26 binds human C5 and C5a with high affinity

mAb 137–26 was tested for reactivity with purified human C5 and recombinant C5a by ELISA. mAb 137–26 reacted with C5 in a dose-dependent manner with high potency (Fig. 1a). Another anti-C5 mAb 137–76, specific for the β-chain of human C5, also reacted with C5 in the same assay (Fig. 1a). mAb 137–26, but not mAb 137–76, reacted with C5a in a dose-dependent manner with high potency (Fig. 1b). In order to preclude the possibility that mAb 137–26 binds to an induced epitope on C5 as a result of immobilization of the antigen on ELISA plates, we measured the affinity equilibrium constants and the binding kinetic constants (association and dissociation) of the antibody with solution-phase C5a and C5 by surface plasmon resonance using BIAcore. mAb 137–26 has a high binding affinity for both C5 and C5a, with equilibrium dissociation constants K_D in subnanomolar range (Table 1). The relatively lower binding affinity of mAb137–26 to C5 than C5a could be attributed to some denatured C5 molecules in the preparation which was purified from pooled human plasma. The antibody also binds effectively to C5 and C5a on Western immunoblots (data not shown). Taken together, the results indicate that mAb 137–26 binds to a common epitope found on C5a and C5.

mAb 137–26 does not inhibit C5 activation

The effect of mAb 137–26 on C5 cleavage was studied by haemolytic assays. mAb 137–26 and the isotype-matched control mAb G3-519 did not inhibit the classical pathway haemolysis of sensitized chicken RBCs, whereas the positive control, anti-C2

Table 1. Binding affinity of mAb 137–26 to C5 and C5a

	k_{on}($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (M)
C5	$1{\cdot}42 \times 10^{5}$	$6{\cdot}97 \times 10^{-5}$	$4{\cdot}92 \times 10^{-10}$
C5a	$3{\cdot}70 \times 10^{6}$	$2{\cdot}25 \times 10^{-4}$	$6{\cdot}09 \times 10^{-11}$

k_{on}, kinetic association constant; k_{off}, kinetic dissociation constant; K_D, equilibrium dissociation constant = k_{off}/k_{on}

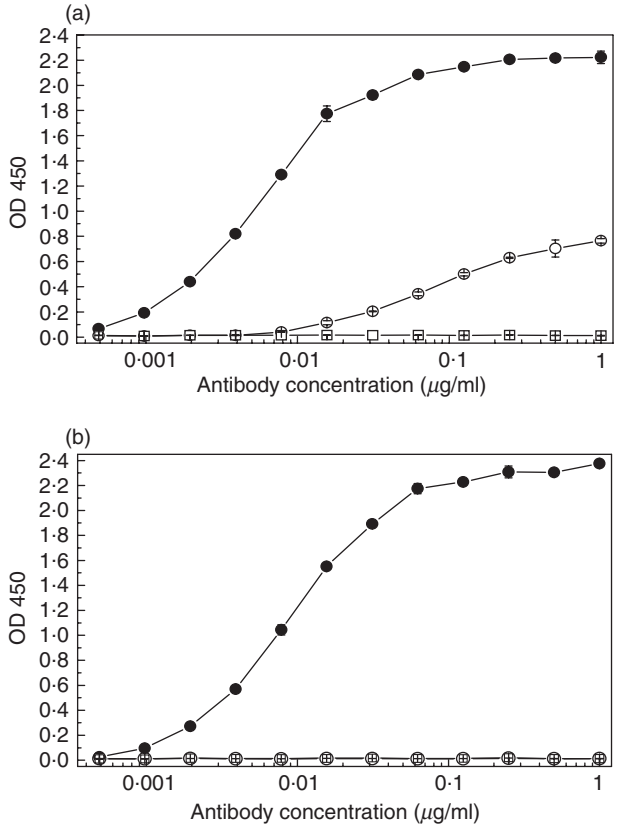

Fig. 1. Reactivity of mAb 137–26 with (a) human C5 and (b) human C5a in ELISA. mAb 137–26 (●) reacts with both C5 and C5a, whereas the C5 β-chain specific antibody, mAb 137–76 (○), reacts with C5 only. Isotype-matched control mAb G3-519 (□) does not react with C5 and C5a.

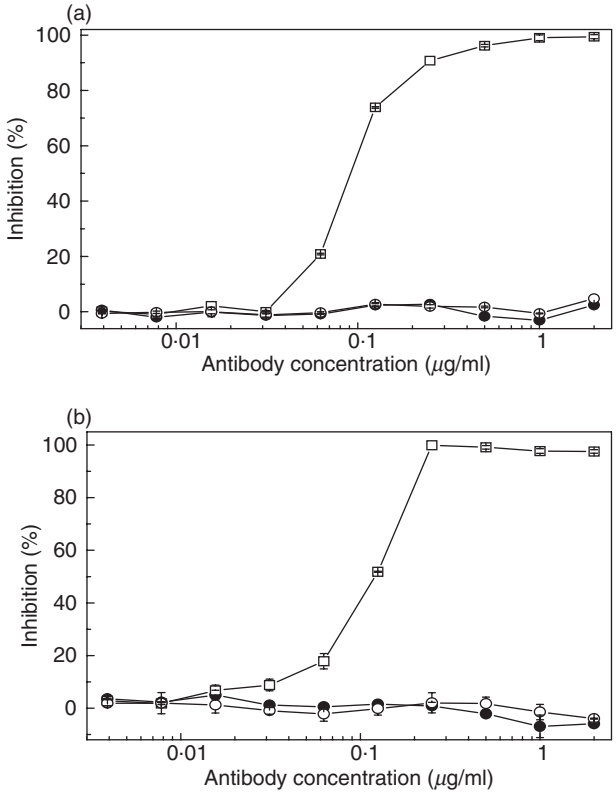

Fig. 2. Effects of mAb 137–26 on complement-mediated haemolysis (a) *via* the classical pathway in sensitized chicken red blood cells and (b) the alternative pathway in unsensitized rabbit red blood cells. mAb 137–26 (●) does not inhibit haemolysis *via* either the classical or the alternative complement pathway. In contrast, the anti-C2 mAb 175–62 (□, panel a) inhibits haemolysis *via* the classical pathway and the anti-factor D mAb 166–32 (□, panel b) inhibits haemolysis *via* the alternative pathway. Isotype-matched control mAb G3-519 (○) has no effect.

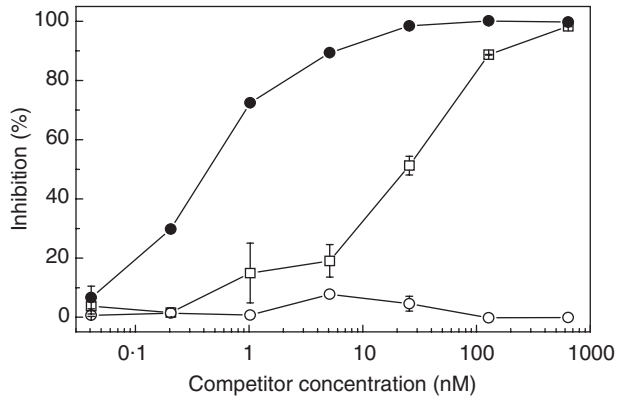

Fig. 3. Inhibition of C5a binding to C5aR on human neutrophils by mAb 137–26. ^{125}I-human C5a (0·4 nM) were incubated with purified human neutrophils, in the presence or absence of varying concentrations of the inhibitors, mAb 137–26 (●) or recombinant human C5a (□). The concentration for 50% inhibition (IC_{50}) for mAb 137–26 and C5a are 0·45 and 30 nM, respectively. Isotype-matched control mAb G3-519 (○) has no effect.

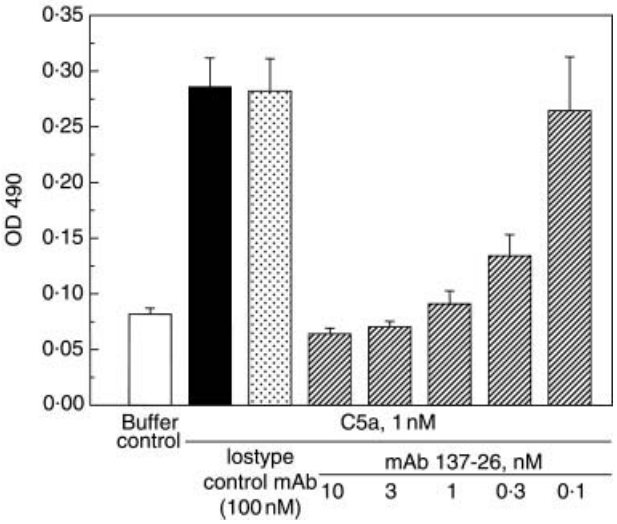

Fig. 4. Inhibition of C5a-induced chemotaxis of human neutrophils by mAb 137–26. C5a at 1 nM was used to induce chemotaxis of human neutrophils. Varying concentrations of mAb 137–26 were added for blocking of C5a. The degree of neutrophil migration was represented by the optical density of the formazan product as described in the text.

mAb 175–62, effectively inhibited the haemolysis (Fig. 2a). mAb 137–26 did not inhibit the alternative pathway haemolysis of unsensitized rabbit RBCs, whereas the positive control, anti-factor D mAb 166–32, effectively inhibited the haemolysis (Fig. 2b).

mAb 137–26 inhibits the binding of C5a to human neutrophils

mAb 137–26 was tested for inhibition of C5a binding to C5aR on human neutrophils by a radioligand binding assay. The results show that mAb 137–26 is more potent than unlabelled C5a in inhibiting the binding of ^{125}I-C5a to purified human neutrophils (Fig. 3). For the binding assay with 0·4 nM of ^{125}I-C5a, the concentration for 50% inhibition (IC_{50}) for mAb 137–26 and C5a are 0·45 nM and 30 nM, respectively.

mAb 137–26 inhibits C5a-induced chemotaxis of human neutrophils

The activity of mAb 137–26 to inhibit C5a-mediated chemotaxis was tested in an assay using human neutrophils. The antibody at a concentration higher than 1 nM completely inhibited the chemotaxis of human neutrophils induced by 1 nM C5a (Fig. 4). Furthermore, mAb 137–26 is more potent than the C5aR antagonist AcF-[OPdChaWR] in inhibiting C5a-induced chemotaxis of neutrophils (Fig. 5). Consistent with the data from the competitive radioligand study described above, the results indicated that mAb 137–26 is a potent neutralizing antibody against C5a.

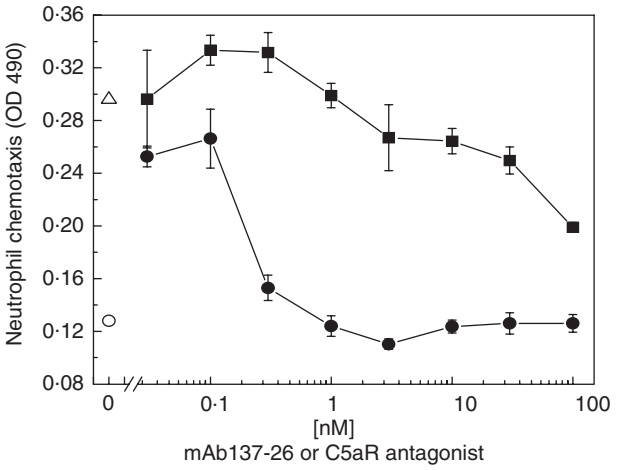

Fig. 5. Relative potency of inhibition by mAb 137–26 and C5aR antagonist AcF-[OPdChaWR] in C5a-induced chemotaxis of human neutrophils. C5a at 1 nM was used to induce chemotaxis of human neutrophils. Chemotaxis of neutrophils in the presence (△) or absence of C5a (○) was used as control. Varying concentrations of mAb 137–26 (●) and AcF-[OPdChaWR] (■) were added for blocking C5a. The degree of neutrophil migration was represented by the optical density of the formazan product described in the text.

mAb 137–26 inhibits CD11b expression and oxidative burst in whole blood

The effect of mAb 137–26 on complement-dependent *E. coli*-induced up-regulation of CD11b and oxidative burst was examined in a novel whole human blood model of inflammation [19]. We first compared the effect of mAb 137–26 with two other mAbs to C5, one (mAb 137–30) binding to native C5 and blocking the cleavage into C5a and C5b and the other (clone 561) which binds both C5a and C5 [20]. Neutrophil CD11b expression was reduced by more than 90% by all three antibodies, in contrast to the isotype-matched control antibody G3-519 which had no effect (Fig. 6a). Notably, the potency of mAb 137–26 in reducing neutrophil CD11b expression was 10 times higher than the anti-C5 mAb 137–30 and anti-C5a mAb 561. Similarly, all three anti-C5/C5a antibodies efficiently reduced neutrophil oxidative burst by more than 80%, in contrast to the control antibody G3-519 which had no effect (Fig. 6b). In contrast to the effect on CD11b expression, the anti-C5 mAb 137–30 was the most potent in reducing neutrophil oxidative burst. An efficient effect of mAb 137–26 in reducing monocyte CD11b expression and oxidative burst was also observed, although less pronounced than for neutrophils, amounting approximately 50% reduction in both markers, which

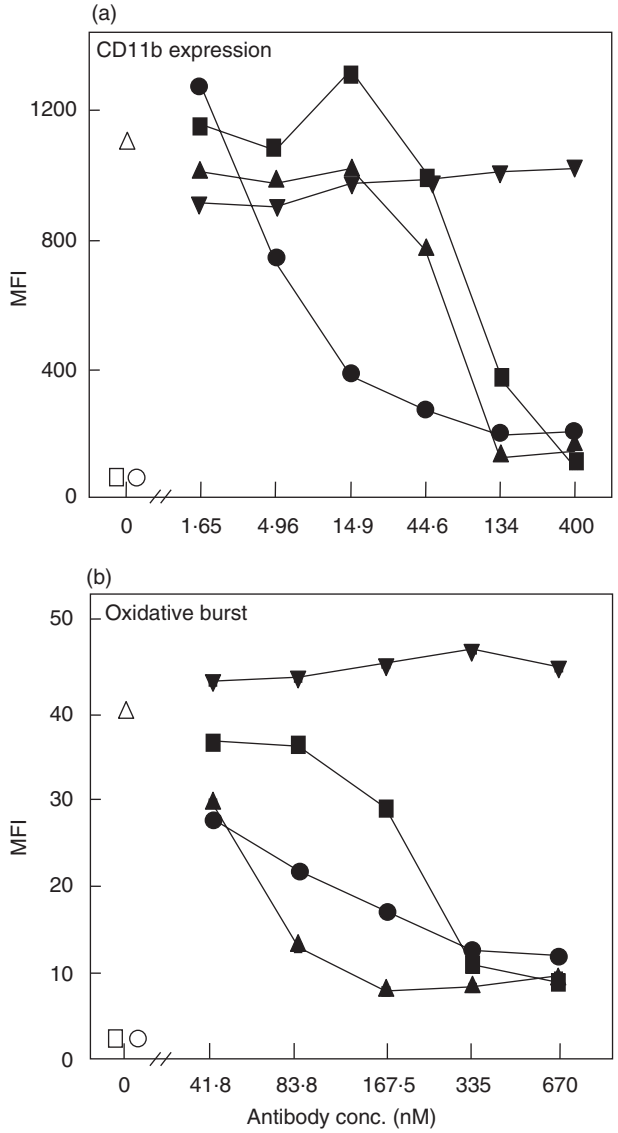

Fig. 6. Inhibition of (a) CD11b expression and (b) oxidative burst by anti-C5 mAbs in human whole blood. *E. coli* was incubated in lepirudin-anticoagulated whole human blood at 1×10^7 bacteria/ml for CD11b expression and 1×10^8 bacteria/ml for oxidative burst. Neutrophils and monocytes were separately gated and examined and the neutrophil results presented. mAb 137–26 (●) abrogated very efficiently CD11b expression with a substantial higher potency than the anti-C5 mAb 137–30 (▲) and the anti-C5a mAb 561 (■), whereas the isotype-matched control mAb G3-519 (▼) had no effect. The effect of mAb 137–26 on oxidative burst was intermediate between mAbs 137–30 and 561. T-0 = baseline sample (□). T-10 = whole blood incubated only with PBS (○). Pos. ctr. = whole blood incubated with *E. coli* (open triangle △). The experiment was repeated four times with identical results.

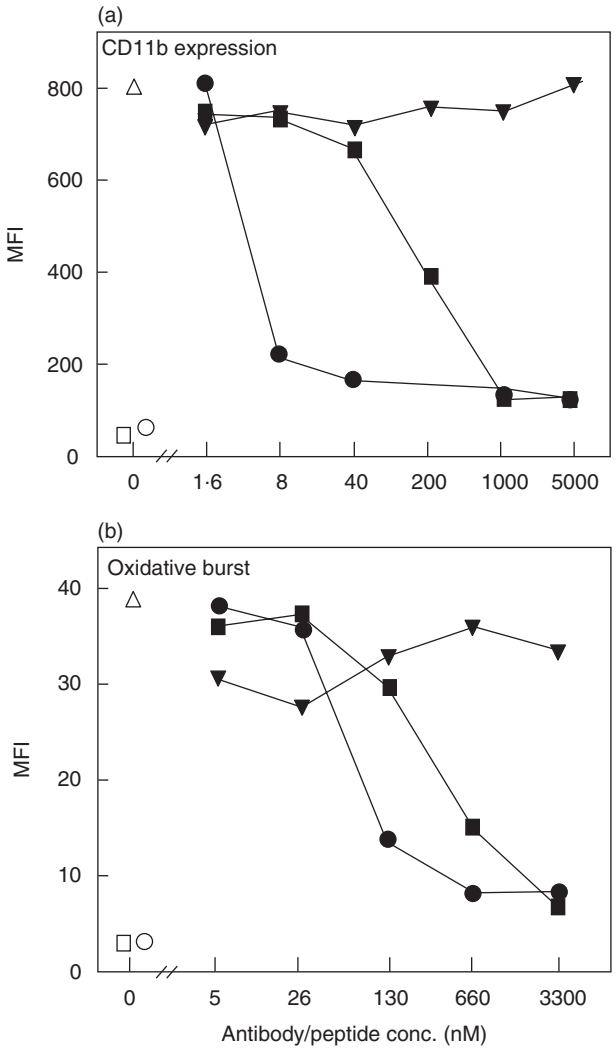

Fig. 7. Inhibition of (a) CD11b expression and (b) oxidative burst by mAb 137–26 and the C5aR antagonist AcF-[OPdChaWR] in human whole blood. The experiment was performed as described in Fig. 6. Both mAb 137–26 (●) and the C5aR antagonist (■) inhibited neutrophil CD11b expression and oxidative burst efficiently, but mAb 137–26 was substantially more potent, particularly in inhibition of CD11b expression. Irrelevant control peptide (▼) has no effect. Other symbols are as described in Fig. 6.

was also the case for the other anti-C5 antibodies (results not shown).

We then compared the effect of mAb 137–26 with that of the C5aR antagonist AcF-[OPdChaWR]. On a molar basis mAb 137–26 was approximately 100 times more potent in reducing the CD11b expression and 5 times more potent in reducing oxidative burst (Fig. 7a,b).

mAb 137–26 does not activate complement

To investigate whether mAb 136–26 would activate complement after forming a complex with C5, we incubated the antibody and controls in human serum and measured complement activation products from the classical pathway (C1rs-C1 inhibitor complexes and C4bc), as well as activation of C3 (C3bc) and the terminal pathway (TCC). In contrast to heat-aggregated immunoglobulins which showed a substantial and dose-dependent activation revealed by all four activation markers, mAb 137–26 had no complement activating capacity (Fig. 8).

DISCUSSION

C5a is the most potent proinflammatory mediator of the complement system. It is formed through enzymatic cleavage of C5 by C5

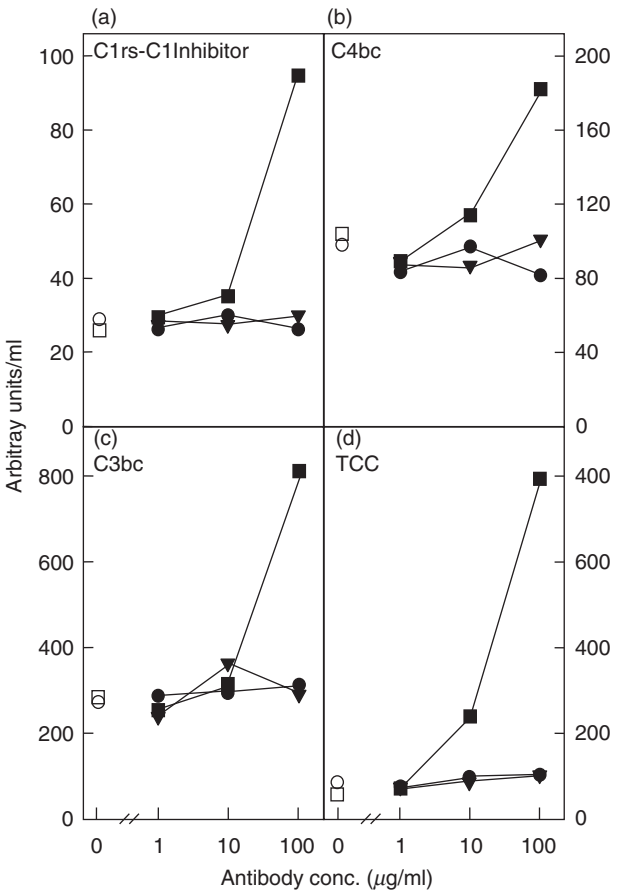

Fig. 8. Complement activation potential of mAb 137–26. A possible complement activation capacity of mAb 137–26 (●) was investigated by incubating with human serum at 37°C for 1 h. The isotype-matched control mAb G3-519 (▼) was used as a negative control and heat-aggregated immunoglobulins HAIGG (■) as a positive control. Detection of the complement activation products (a) C1rs-C1Inhibitor complexes, (b) C4bc, (c) C3bc and (d) TCC revealed a dose-dependent classical complement activation by HAIGG, whereas mAb 137–26 and mAb G3-519 did not activate complement. T-0, baseline serum sample (□); T-60, serum incubated with PBS for 60 min (○). The experiment was repeated once with identical results.

convertases formed after activation of C3 through either the classical, the lecitin or the alternative pathway. C5a is a potent stimulator of neutrophils and monocytes [25,26], induces chemotactic migration of neutrophils [27], eosinophils [28], basophils [29], and monocytes [30], activates endothelial cells to express adhesion molecules essential for sequestration of activated leucocytes, which mediate tissue inflammation and injury [31–33]. C5a also mediates inflammatory reactions by causing smooth muscle contraction, increasing vascular permeability, inducing basophil and mast cell degranulation and inducing release of lysosomal proteases and oxidative free radicals [34]. Furthermore, C5a modulates hepatic acute-phase gene expression and augments the overall immune response by increasing the production of TNFα, IL-1, IL-6, and IL-8 [1–3].

C5a binds to C5aR (CD88), with very high affinity [34]. More recently, an orphan receptor called C5L2 has been reported to bind C5a and C5a desArg [35]. The functions of C5L2 remain to be studied. C5aR belongs to a superfamily of seven-transmembrane-domain, G protein-coupled receptors. It is broadly expressed on neutrophils, monocytes, basophils, eosinophils, hepatocytes, lung smooth muscle and endothelial cells, and renal glomerular tissues [36–41]. Since the biological functions of C5a are mainly receptor-mediated, the alternative strategy of blocking C5a would be to block the C5aR. However, if C5a is blocked before receptor binding, any receptor-mediated effect will be neutralized, even if there are several different C5aRs present. Furthermore, possible receptor-independent effects will be abolished as well. In support of this argument, we found in the present study that at the same molar concentrations mAb 137–26 inhibited chemotaxis, up-regulation of CD11b and oxidative burst of human neutrophils more efficiently than the C5aR inhibitor AcF-[OPdChaWR].

Our data show that mAb 137–26 recognizes a unique epitope shared by C5 and C5a. The antibody binds to the C5a moiety of C5 before its being activated. It also binds to C5a that has already been formed. It neutralizes C5a with high potency by inhibiting its binding to C5aR. This property is very beneficial in view of the fact that C5a, once formed, could bind very rapidly with high affinity to C5aR on various cells, thereby activating signal transduction cascade which results in inflammation and tissue injury. The high neutralizing capacity of mAb 137–26 is further supported by its more potent inhibition of C5a-mediated effects than mAb 561 which also binds both C5 and C5a [20]. mAb 561 did not compete the binding of mAb 137–26 to human C5 in ELISA (unpublished data), indicating that these mAbs recognize distinct epitopes on human C5.

There are two principally different ways of inhibiting C5 functions, namely by mAbs blocking cleavage of C5, like mAb 137–30 and mAb 137–76, and 5G1·1 [42], or by neutralizing C5a, e.g. by mAb 137–26, without inhibiting the cleavage of C5. In the latter case the ability to form lytic C5b-9 is maintained. Our data clearly demonstrate that mAb 137–26 inhibits the pro-inflammatory effects of C5a, but does not inhibit C5b-9-mediated haemolysis. Although most bacteria are cleared by phagocytosis at the level of C3 activation, C5b-9 is an important killing mechanism of some gram-negative bacteria like *Neisseriae*, and may in these cases be an important defense mechanism against bacterial sepsis [6]. Consistent with the findings of this study, we have recently showed in a whole blood model of meningococcal sepsis with *Neisseria meningitides* that mAb 137–26 inhibits C5a-mediated oxidative burst of neutrophils and monocytes (Tom Mollnes *et al.,* unpublished observation). Moreover, in that study we showed that the antibody did not inhibit the formation of fluid-phase SC5b-9, as detected by a quantitative ELISA, and preserved C5b-9-mediated bacteriocidal activity. These results are consistent with the current finding that mAb 137–26 does not inhibit complement-mediated haemolysis.

C5a is involved in the systemic inflammatory response syndrome (SIRS) as manifested by acute respiratory distress syndrome (ARDS) and multiorgan failure (MOF) [43–45]. C5a has also been shown to play an important role in the development of tissue injury, and particularly pulmonary injury, in animal models of septic shock [46]. In sepsis models using rats, pigs and nonhuman primates, anti-C5a antibodies administered to the animals before treatment with endotoxin or E. *coli* resulted in decreased tissue injury, as well as decreased production of IL-6 [46–48]. More importantly, blockade of C5a and C5aR with neutralizing polyclonal antibodies has been shown to significantly improve

survival rate [7], prevent MOF [49], and ameliorate dysfunction of the coagulation and fibrinolysis system [50] in a caecal ligation/puncture model of sepsis in rats and mice. Interestingly, the growth of aerobic bacteria, cultured from the spleen and liver of septic animals, was significantly reduced following neutralization of C5a [7]. It is interpreted that the generation of C5a during sepsis suppresses polymorphonuclear function, and this leads to decreased bacterial clearance by mechanisms, such as leukotriene $(LT)B_4$ or IL8, perhaps by heterologous desensitization [51]. Together, inhibition of the C5a/C5aR pathway in sepsis could be an attractive therapeutic approach.

Immunoglobulin G-containing immune complexes (IC) contribute to the pathophysiology in a number of autoimmune diseases, such as systemic lupus erthyematosus and rheumatoid arthritis [52,53]. Elevation of plasma C5a levels correlates with the severity of the disease state [54,55]. Recent studies show that C5aR deficient mice are protected from tissue injury induced by IC [56,57]. The results are consistent with the observation that a small peptidic C5aR antagonist inhibits the inflammatory response caused by IC deposition [58]. Therefore, there is mounting evidence that C5a plays an important aetiological role in IC diseases.

C5a is thought to be a major mediator in myocardial ischaemia-reperfusion injury [59]. Complement inhibition reduced myocardial infarct size in mice [60], and treatment with anti-C5a antibodies reduced injury in a rat model of hindlimb ischaemia-reperfusion [61]. Reperfusion injury during myocardial infarction was also markedly reduced in pigs that were retreated with a monoclonal anti-C5a IgG [9]. A recombinant human C5aR antagonist reduces infarct size in a porcine model of surgical revascularization [62]. Patients undergoing extra-corporeal circulation like cardiopulmonary bypass and haemodialysis, suffer from a systemic inflammation due to biomaterial-induced alternative complement pathway activation [63–65]. C5a causes increased capillary permeability and oedema, bronchoconstriction, pulmonary vasoconstriction, leucocyte and platelet activation and infiltration to tissues, in particular the lung [66]. Administration of an anti-C5a monoclonal antibody was shown to reduce cardiopulmonary bypass and cardioplegia-induced coronary endothelial dysfunction [67].

Complement inhibition is a therapeutic strategy which is being tested clinically, as a result of the increasing knowledge of the role of complement in tissue damage and the promising results from experimental animal models. As in any clinical treatment the primary goal is '*primare non nocere*' (do no harm). The use of mAbs as pharmaceuticals could lead to *in vivo* complement activation when the mAbs binds to their antigens [68]. mAbs to soluble proteins, like mAb 137–26, could theoretically generate immune complexes, particularly if they react with a repeating epitope. In the present study we show that mAb 137–26 does not activate complement in human serum after it is complexed with C5. This attentuates the concern that therapeutic application of the antibody would lead to complement activation.

An important principle in complement inhibition is to balance the beneficial effects obtained by the inhibition, with preservation of sufficient functional activity for microbial protection and tissue renovation. Blocking at the level of C5 does not inhibit C3 activation and thereby preserves the crucial defense functions like opsonization of bacteria, immune complex clearance and immune response to foreign antigens. Whether C5 cleavage should be blocked or only C5a should be inhibited, depends on the pathophysiology of the actual condition. Although C5a plays a major role in inflammation and tissue injury of bacterial sepsis and immune complex disease, C5b-9 could be involved in tissue injury in other conditions. C5b-9 stimulates platelets to express CD62P (P-selectin) which is involved in inflammatory reactions found in cardiopulmonary bypass [15,69]. Inhibition of C5b-9 formation reduces renal ischaemia/reperfusion injury in a mouse model [70]. In such conditions, complete inhibition of C5 activation should be more effective than C5a blockade alone. On the other hand, in cases where it is important to leave the lytic pathway open and block the inflammatory effects of C5a, e.g. in Neisserial or other gram-negative septicaemia, mAb 137–26 could be a suitable candidate.

Taken together, this study indicates that the novel anti-C5/C5a mAb 137–26 is a potent inhibitor of C5a functions. This antibody could be useful for treatment of C5a-mediated clinical conditions, such as bacterial sepsis, immune complex diseases and certain ischaemia-reperfusion injuries. Selective inhibition of complement targets for specific clinical conditions is an attractive strategy to maximize clinical benefits and to reduce potential side-effects.

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