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Functional analysis and quantification of the complement C3 derived anaphylatoxin C3a with a monoclonal antibody

R. BURGER, A. BADER, M. KIRSCHFINK, U. ROTHER, L. SCHROD, I. WÖRNER & G. ZILOW *Institute for Immunology, University of Heidelberg, 6900 Heidelberg, West Germany*

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SUMMARY

The C3 fragment C3a belongs to the anaphylatoxins. It has immune regulatory activity and contributes to the pathogenesis of the adult respiratory distress syndrome (ARDS). The low molecular weight (9 kD) of C3a complicates the production of antibodies to C3a. We obtained a monoclonal antibody (designated H13) to human C3a. It reacts with C3a or C3a-desArg and with native C3 but not with C5 or C5a. In immunoblot analysis it reacts with the α- but not with β-chain of C3 and binds to a protein with a mol. wt of about 10 kD present in zymosan-activated sera which is only marginally detectable in non-activated serum and absent in plasma. H13 crossreacts with the analogous proteins of rabbit, guinea pig and sheep. H13 has the capacity to bind ^{125}I-radiolabelled C3a efficiently but fails totally to react with ^{125}I-C5a or with other C3 α-chain fragments. H13 blocks C3a functional activity. It markedly inhibits C3a-induced ^{3}H-serotonin release from platelets *in vitro* and similarly inhibits the C3a-induced extravasation of Evans blue into the skin *in vivo*. H13 does not interfere with the haemolytic activity of C3. An ELISA system was established using H13 which permits quantification of C3a in sera of polytrauma patients. The antibody H13 should facilitate further functional analysis of C3a in experimental systems. It should be useful for quantification of C3a in diagnostic assays and also for application in immunopathology.

Keywords Complement C3 anaphylatoxin C3a monoclonal antibody adult respiratory distress syndrome

INTRODUCTION

Peptides derived from the complement component C3 bind to specific cellular receptors (C3-receptors) and contribute to host-defence and inflammatory reactions by stimulating a variety of cellular functions (Fearon & Wong, 1983; Hugli, 1984; Hartung & Hadding, 1983). C3-fragments exhibit also immunoregulatory functions (Weigle *et al.*, 1983; Weiler *et al.*, 1982). The initial activation of C3 is achieved by proteolytic cleavage of the α-chain resulting in the generation of the biologically active fragments C3a and C3b. The fragment C3a belongs, together with C5a and C4a, to the so-called anaphylatoxins. They represent soluble mediators which trigger a series of biological events after interaction with several cell populations, e.g. granulocytes, macrophages, thrombocytes or mast cells, via C3a-receptors (Hugli, 1984). C3a contributes to the reaction sequence observed in the pathogenesis of ARDS, e.g. in polytrauma or sepsis patients (Rinaldo &

Correspondence: Dr Reinhard Burger, Institute for Immunology, Im Neuenheimer Feld 305, 6900 Heidelberg, W. Germany.

Rogers, 1982; Hyers & Fowler, 1986). Measurement of C3a is of diagnostic value in these patients and an immunological assay is used for this purpose (Hugli & Chenoweth, 1980).

Antibodies selectively recognizing C3a but not other C3 fragments provide important tools for diagnostic assays of C3a *in vitro*. However, the production of specific antisera or monoclonal antibodies (MoAb) to the low molecular C3a (mol. wt. 9 kD) consisting of only 77 amino acids proved to be difficult. In the literature, there is only a single C3a-specific MoAb described (Burger *et al.*, 1982). It reacts with guinea pig C3a but unfortunately fails to react with the human analogue. The present report describes the production and the serological and functional characterization of a monoclonal antibody to human C3a. It has the capacity to block the anaphylatoxic activity of C3a *in vitro* and *in vivo* and has proved to be useful for a quantitative C3a assay.

MATERIALS AND METHODS

Production of monoclonal antibody to C3a. The monoclonal antibodies were obtained by the same procedure as described previously in detail (Burger *et al.*, 1982). Highly purified human C3 was used for immunization of the BALB/c mice. The non-secreting P3-X63-Ag8.653 myeloma line was used for fusion. Ascitic fluid was prepared and stored in aliquots at −70°C as a source of antibodies for most of the described experiments. Ammonium sulphate (40% saturation) precipitated antibodies or protein A-Sepharose purified antibody was used for functional assays of C3a. The antibodies, including irrelevant control antibodies, were adjusted to equal protein concentration after dialysis against PBS. The MoAb to C3a was designated as antibody H13. It was of the IgG2b subclass as shown in an ELISA with subclass-specific antisera (Nordic, Tilburg, Netherlands) and bound protein A efficiently in an ELISA using peroxidase-labelled protein A (Sigma, Deisenhofen).

Antibody binding assay. An ELISA was used for screening and serological characterization as described previously (Burger *et al.*, 1982) using C3 absorbed to PVC-microtitre plates (Dynatech, Plochingen) and peroxidase-conjugated rabbit IgG anti-mouse Ig (Dynatech) as detection reagent. Alternatively, a three-step assay was applied. In this test, binding of monoclonal antibody was detected with biotin-labelled sheep anti-mouse Ig followed by peroxidase-conjugated streptavidin. Both reagents were used in optimal dilutions according to the manufacturer's directions (Amersham, Braunschweig).

Haemolytic assays and intermediates. Preparation of EA, EAC142 and the haemolytic assay for C3 were performed as described (Lachmann & Hobart, 1978).

Protein preparations. Human C3 was prepared from fresh frozen plasma as described (Hammer *et al.*, 1981) and was stored frozen in small aliquots. For functional assays trypsin-generated C3a was used (Bokisch, Müller-Eberhard & Cochrane, 1969). C3 (0·75 mg/ml) was incubated with an optimal concentration (about 35 μg/ml) of trypsin (crystallized three times; Roth, Karlsruhe) for 1 min at 37°C. The reaction was terminated by adding a 3-fold excess (w/w) of soybean trypsin-inhibitor (Serva, Heidelberg). Generation of C3a activity was determined in the ^{3}H-serotonin assay. A mixture of trypsin and trypsin inhibitor was incubated the same way with C3. This mixture served as control in the functional assays both *in vitro* or *in vivo* in order to exclude side effects. Human C3a-desArg was purified from activated serum as described previously (Hugli *et al.*, 1981) and was used for Western Blot analysis.

Functional in vitro *or* in vivo *assays for C3a.* The anaphylatoxic activity of C3a was determined in an *in vitro* ^{3}H-serotonin-release assay using guinea pig platelets as described (Meuer *et al.*, 1981). Thrombin-induced ^{3}H-serotonin-release served as positive control. Activity of C3a *in vivo* was analysed as skin reaction after previous injection of Evans Blue (Cochrane & Müller-Eberhard, 1968). The guinea pigs received by i.v. injection 0·6 ml of Evans Blue (0·5% solution in saline) and 30–60 min later the C3a preparation was injected intradermally. The subsequent extravasation of the dye was evaluated at the inner skin surface after killing the animal 20–30 min later.

Radioimmunoassay for C3a-specificity of MoAb. The ^{125}I-labelled C3a or ^{125}I-labelled C5a from commercially available kits was used (Upjohn/Biosigma, Munich, FRG). Various concentrations of MoAb purified from culture supernatant or, in a simplified assay, of ascites diluted 1×10^{2} or

higher were absorbed to PVC-microtitre plates (25 μl; 3 h, 23°C). After washing four times and saturating the plates with bovine serum albumin (0·1% in PBS) 20 μl of the ^{125}I-radiolabelled C3a or C5a was added. After incubation (3 h, 23°C) the plates were washed three times, dried and the individual wells were cut off using a heated wire. Bound radioactivity was measured in a gamma counter.

Immunoblotting analysis. SDS-PAGE and immunoblotting were performed as described (Ruppel, Diesfeld & Rother, 1985; Towbin, Stachelin & Gordon, 1979) with some modifications. Gradient gels (5–18%) were used. Cytochrom C (Boehringer, Mannheim) and proteins from commercially available kits served as mol. wt markers (Bio-Rad, Munich). The separated proteins were electrophoretically transferred to nitrocellulose paper. Part of the paper was stained for protein with Ponceau S (Sigma, Deisenhofen). After saturation of the paper with BSA (1% solution) individual nitrocellulose strips were incubated with the monoclonal antibodies at an appropriate dilution. Bound antibody was detected by incubation with biotinylated sheep anti-mouse Ig followed by streptavidin-peroxidase (Amersham, Braunschweig) according to the manufacturer's directions. 4-Chloro-1-naphthol (Sigma) was used as substrate.

ELISA for quantification of C3a. The following buffers were used for this assay: Buffer A, 0·01 M potassium phosphate, 0·15 M NaCl, pH 7·4; Buffer B, 2% BSA solution in buffer A; Buffer C, buffer A containing 1% BSA and 0·1% Tween 20; Buffer D, 1% BSA in buffer A. Wells of PVC microtitre plates (Costar, No. 2595) were incubated (5 h, room temperature) with 50 μl of rabbit anti-human C3a (Behring, Marburg), diluted 1:1000 in buffer A. After saturation of the plates with buffer B (4°C, overnight) and washing three times with buffer C, the C3a containing sample (50 μl, diluted in buffer D) was added and incubated for 1 h at room temperature. After washing three times, the wells were incubated with MoAb H13 (ascites, diluted 1:1500 in buffer D) followed by washing and incubation (1 h) with biotin-labelled rabbit antimouse Ig (1:500 in buffer D; Dianova, Hamburg) and, subsequently, after washing three times, for 1 h with peroxidase-conjugated streptavidin (1:1000; Amersham, Braunschweig). After the final washing, the substrate ABTS was added and the optical density was measured in an ELISA reader (Dynatech, Plochingen). Before the assay, native C3 was removed from the plasma samples by precipitation with 16% polyethylene glycol 4000 (Merck, Darmstadt) to prevent it interfering with this assay. Alternatively, immunoabsorption with Sepharose coupled with a mixture of two monoclonal antibodies (Burger *et al.*, unpublished) recognizing the C-terminal segment of the C3 alpha-chain (MoAb H 206) or the beta-chain (MoAb H11) was performed.

RESULTS

Serological characterization of MoAb H13. The growing hybrids were screened by ELISA using purified native human C3 as antigen. About 15% of the hybrids reacted in the initial screening. A number of hybridomas was selected for detailed analysis. Ascitic fluid was prepared after cloning twice by limiting dilution and used for ELISA. The MoAb H13 reacted up to high dilutions (1×10^6) with C3 but failed to react with purified C5 (Fig. 1). Similarly, H13 reacted with C3a-desArg purified from activated serum up to high dilutions (1×10^5) but it showed no reactivity with several other purified C3 fragments or genetically engineered C3 segments expressed in *E. coli*. It failed to react with highly purified human C3d (kindly provided by Dr Martinez, Heidelberg), with C3g (a gift of Professor Lachmann, Cambridge) or with the C3-derived leucocyte mobilizing factor (Rother, 1972) partially purified from activated serum. We recently used C3 cDNA clones for production of a number of bacterial hybrid proteins which contain the C-terminal half of the alpha-chain of C3 (Ma *et al.*, 1985). None of these hybrid proteins reacted in ELISA or dot-blot analysis with the MoAb H13.

The C3a-specificity of MoAb H13 was confirmed by a direct radioimmunoassay using commercially available ^{125}I-labelled C3a and ^{125}I-C5a. The MoAb was immobilized to microtitre plates and binding of the radiolabelled anaphylatoxins was determined. This assay proved to be sensitive and showed low background values (70–150 ct/min). MoAb H13 had a marked binding capacity for ^{125}I-C3a but failed to bind ^{125}I-C5a (Fig. 2). Almost one-third of the radioactivity added

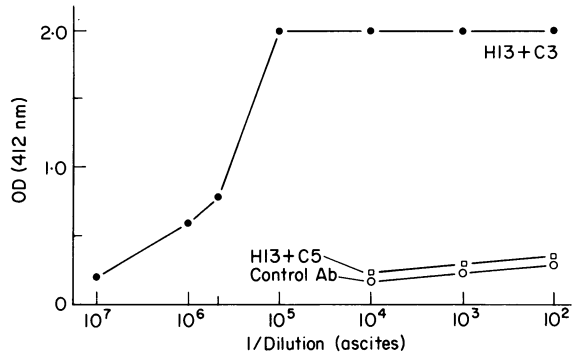

Fig. 1. Reactivity of MoAb H13 with purified human C3. Ascites was used at the indicated dilutions. C3 or C5 were attached to PVC plates and antibody binding was determined by ELISA. The ordinate shows the optical density (OD). (●) H13+C3; (□) H13+C5; (○) Control Ab.

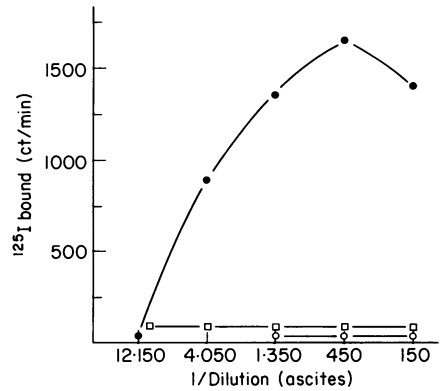

Fig. 2. MoAb binds ^{125}I-C3a but not ^{125}I-C5a. Microtitre plates were incubated with ascites at the indicated dilutions, saturated with BSA, and binding of the radiolabelled anaphylatoxins was determined. The ordinate shows bound ct/min per microtitre well. (●) H13+^{125}I-C3a; (□) H13+^{125}I-C5a; (○) Control Ab+ ^{125}I-C3a. 100% C3a 4801 ct/min; C5a 7624 ct/min.

was absorbed at optimal antibody concentrations. All other antibodies to C3 or to irrelevant antigens showed only background values with ^{125}I-C3a or ^{125}I-C5a. Incubation of the microtitre plates with diluted ascites was feasible for attachment of sufficient amounts of antibody in this assay (Fig. 2).

The MoAb H13 was tested for its applicability in Western blot analysis against purified C3, purified C3a and against C3a-containing serum samples (Fig. 3). Purified C3 was reduced with 2-mercaptoethanol (0·1 M) and its subunits separated by SDS-PAGE were demonstrated by immunoblotting with MoAb H13. The antibody reacted with C3 and the alpha-chain of C3 (mol. wt 110 kD) but did not react with the beta-chain or with purified C5. It reacted intensively with the purified C3a (Fig. 3; panel e). It should be mentioned that in some experiments after separation under reducing conditions only a weak reactivity of C3a for H13, if any, remained (panel c). This indicates a contribution of the disulphide bridges in C3a for the expression of the corresponding antigenic determinant. Whole serum or plasma was analysed similarly by Western blot. In fresh plasma (0·01 M EDTA) H13 reacted with C3, and did not react with any low-molecular weight material. Complement activation was induced by incubation (30 min, 37°C) of serum with aggregated immunoglobulin or with zymosan. In the activated serum, MoAb H13 reacted clearly

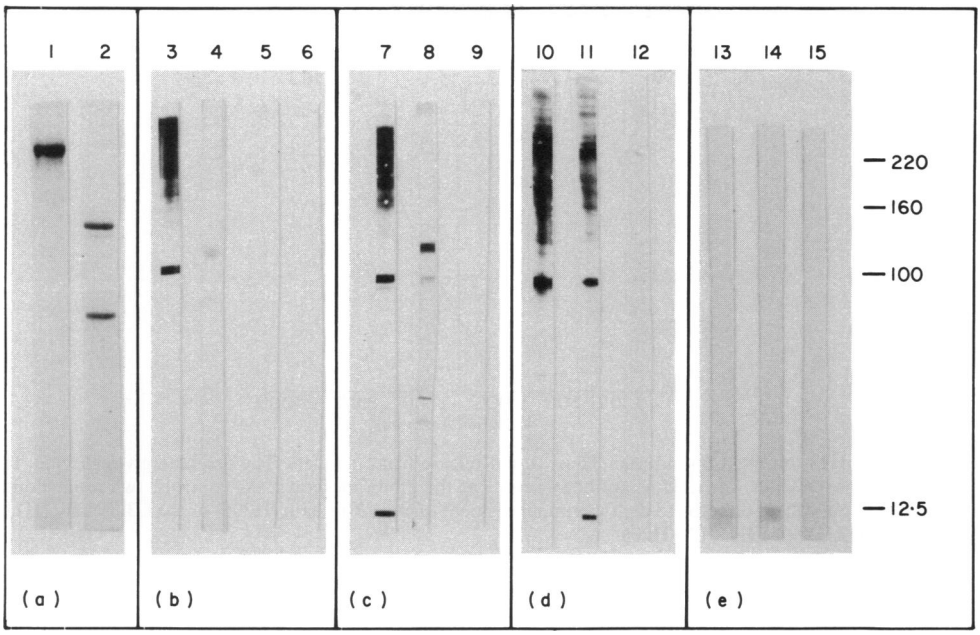

Fig. 3. Western blot analysis. Gradient gels were used (except in panel (a). (a) Coomassie blue staining of purified C3, lane 1 non-reducing (NR), lane 2 reducing (R) conditions, showing the α- and β-chains. (b–e) show immunoblots, ascites is used at a dilution of 1/5000. (b) Lane 3 (NR), C3 is stained after reaction with H13 as a broad band (these gels are overloaded in order to demonstrate the C3a generated as shown in (c) and (d). The antibody reacts with an additional undefined C3 fragment (mol. wt about 100 kD which is almost undetectable by protein staining. Lane 4 (R), staining of the C3 α-chain by H13. Lane 5 (NR) and 6 (R), no staining of C3 with control ascites. (c) After trypsin-treatment of C3 an additional 10 kD band (C3a) is stained (lane 7, NR) which is virtually undetectable under reducing conditions (lane 8). Lane 9, no staining of C5 with H13. (d) H13 reacts with a 10 kD protein in activated serum (lane 11) which is absent in EDTA-plasma (10). No staining of activated serum with control antibody (12). (e) Lane 13, protein stain of purified C3a/C3a-desArg, lane 14, staining of C3a (NR) with H13 but not with control antibody (15).

with a protein with a mol. wt of 10 kD. The same protein was very weakly detectable in fresh, non-activated serum (not shown).

The cross-reactivity of MoAb H13 with C3a of other species was determined. Western blot analysis of activated serum from sheep and guinea pig revealed the staining of an analogous 10 kD protein band as in activated human serum (results not shown). The antibody also reacted with C3 in rabbit serum. We previously described the monoclonal antibody 103 to guinea pig C3a (Burger *et al.*, 1982). Cross-blocking studies were performed in the ELISA-system in order to determine whether the two antibodies react with the same or adjacent determinants of C3a. Biotin-labelled antibody H13 was used for this purpose followed by peroxidase labelled avidin. Preincubation of guinea pig C3 with Ab 103 almost completely blocked the binding of the biotinylated antibody H13.

Functional analysis of MoAb H13. The ability of MoAb H13 to interfere with the anaphylatoxic function of C3a *in vitro* or *in vivo* was investigated. C3a was generated by trypsin-digestion of purified C3. It efficiently induced the release of ^{3}H-serotonin from platelets of normal guinea pigs but not from platelets of C3a-receptor negative guinea pigs (Bitter-Suermann & Burger, 1986). As shown in Fig. 4, in the presence of MoAb H13 the C3a-induced ^{3}H-serotonin release is completely inhibited whereas control antibodies known to react with different sites of the C3 molecule had no effect.

Similarly, the activity of C3a *in vivo* as measured by skin reactivity was inhibited. Intradermal injection of an optimal concentration of C3a caused an extravasation of Evans Blue into the skin (diameter around the injection site about (1·6–1·8 cm). Preincubation of C3a with H13 abolished its

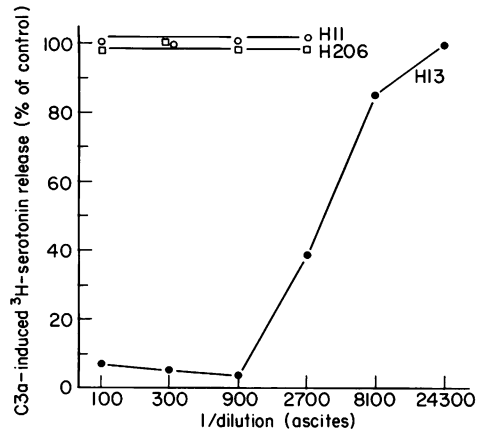

Fig. 4. MoAb H13 inhibits C3a-induced ^{3}H-serotonin release from platelets. The ordinate gives specific ^{3}H-serotonin release. Ascites was used as antibody source at the dilutions shown. As controls, two monoclonal antibodies (unpublished) directed to the C-terminal segment of the C3 α-chain (H206) or the C3 β-chain (H11) were used. (●) H13; (○) H11; (□) H206.

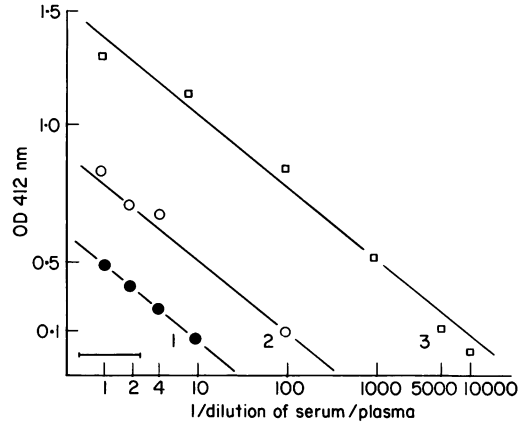

Fig. 5. MoAb H13 permits quantification of C3a by an ELISA. An indirect ELISA was used (see text for detail). Normal plasma (1) containing 0·01 M EDTA, plasma from a polytrauma patient (2) or zymosan-activated normal serum (3) were titrated. Control: no serum or plasma added.

capacity to induce extravasation of the dye, whereas control antibodies had no inhibitory effect. No skin reaction was observed after injection of MoAb H13 alone, and as expected, C3a-receptor negative animals failed to react upon injection of C3a.

The cleavage site for the C3-convertases is located at amino acid 77 of the C3-alpha-chain. An antibody to the adjacent minor C3a fragment might interfere with the cleavage, i.e. activation of C3. However, preincubation of purified C3 with MoAb H13 did not lead to an inhibition of C3 haemolytic function as revealed by an unimpaired haemolysis upon incubation with the EAC142 intermediate and C5–C9. In controls, other MoAb to the C3b moiety of C3 efficiently inhibited haemolytic activity of C3 (unpublished results).

Quantification of C3a by an ELISA with MoAb H13. Quantification of C3a in plasma of ARDS patients is of diagnostic importance and of prognostic value. The commercially available assay uses ^{125}I-labelled C3a. For obvious practical reasons, an assay for C3a which does not require radioactive material is of advantage for routine diagnosis. Therefore, we attempted to use MoAb

H13 for the establishment of an assay for C3a based on the ELISA system. These experiments should mainly reveal whether MoAb H13 can principally be used for an ELISA, e.g. whether it has sufficient affinity, etc. The following assay was used (for details see Materials and Methods): the IgG-fraction of a polyclonal anti-C3a antiserum (Behring, Marburg) was immobilized to PVC-microtiter plates. After incubation with the C3a-containing plasma samples the bound C3a was detected by incubation with MoAb H13. Before the assay, native C3 was removed from the C3a-containing sample by precipitation with polyethyleneglycol to prevent its binding to the anti-C3a antibodies. Binding of MoAb H13 was demonstrated with biotin-labelled rabbit anti-mouse Ig sandwich reagent followed by peroxidase-conjugated streptavidin and the substrate ABTS. As positive controls, either zymosan-activated normal serum or plasma samples from polytrauma patients were used. A previous C3a determination using the commercial C3a assay revealed elevated C3a levels in these samples. The result of the ELISA is shown in Fig. 5. Titration of the zymosan-activated serum revealed strong reactivity up to a high serum dilution. The plasma samples from a normal control individual (136 ng C3a/ml; normal range: up to 220 ng/ml) showed only a weak reaction whereas patient plasma with a moderately elevated C3a-level (448 ng C3a/ml) gave an increased reactivity in this ELISA. Therefore, the results of the ELISA correlate obviously with the results obtained in the assay using ^{125}I-labelled C3a. According to the titration of the control plasma, the sensitivity of the assay is in the range of about 10–40 ng/ml. This is about the same range as the commercial C3a-radioimmunoassay which according to the manufacturer has a sensitivity of 40 ng/ml.

DISCUSSION

The complement component C3 has a marked functional versatility: C3a derived fragments mediate a variety of biological effects by triggering a number of cellular effector functions. C3a attracted particular interest. It plays a well-documented role as one of the three anaphylatoxins (Hugli, 1984). Recently, it was shown that C3a functions as a immunoregulatory molecule (Weigle *et al.*, 1983). Finally, it was demonstrated that C3a is involved, together with C5a, in the pathogenesis of ARDS (Rinaldo & Rogers, 1982; Hyers & Fowler, 1986).

Antibodies to C3a would obviously be helpful for analysis of its biological functions, especially *in vitro*, and permit also quantification of C3a. Polyclonal antisera to C3a are available. However, there is no report about monoclonal antibodies to human C3a. The reason is probably the weak immunogenicity of C3a due, on the one hand, to its low-molecular weight and, on the other hand, to the conserved primary structure of C3a which is relatively similar within the various species (Hugli, 1981; 1984). Monoclonal antibodies to C3a might be of advantage for various experimental systems especially where C3a has to be distinguished from other biologically active C3-fragments. Some of the controversial results on C3-functions in the literature might result from cross-contamination of C3 fragment preparations with peptides of similar physicochemical properties. Monoclonal antibodies as site-specific reagents help to avoid these pitfalls.

The data described in this report demonstrate that MoAb H13 is directed to human C3a or the C3a-desArg peptide, respectively. This conclusion is based on the combined application of serological methods and of functional test systems for analysis of C3a. MoAb H13 reacts with a C3a or a C3a-desArg determinant which is present also on native C3. Under serum condition, the ubiquitous serum carboxypeptidase (SCPN/SCPB or anaphylatoxin inactivator) rapidly cleaves off the C-terminal arginin required for the biological functions of C3a and generates the circulating desArg-form of C3a and of the other anaphylatoxins. Unfortunately, so far there is no antibody available to any neo-antigenic determinants conceivably exposed upon cleavage of C3a from the C3 molecule. Such antibodies would have the advantage that uncleaved C3 would not compete for binding to the antibody and thereby would not interfere in the various experimental or quantitative assay systems.

One can speculate about the antigenic site recognized by the antibody. Its inhibitory effect for C3a might be interpreted as reaction with a determinant close to the C-terminus bearing the biological activity (Hugli, 1984). However, steric effects might easily occur because the small size of

this molecule. Inhibition of C3a by an antibody does therefore not necessarily reflect binding to a site closely adjacent to the C-terminus. Similarly, the cross-blocking observed with the two C3a-recognizing antibodies H13 and 103 (Burger *et al.*, 1982) in a competitive assay using guinea pig C3 might simply reflect sterical blockade and does not necessarily indicate reactivity with the same molecular site. A specificity of H13 for the C-terminus of C3a itself is also unlikely because of its highly conserved structure within the various species. In addition, the fact that MoAb H13 does not interfere with the haemolytic function of C3 supports the assumption that the corresponding C3a determinant is not adjacent to the C3a C-terminus representing the cleavage site for the C3 convertases. This failure to inhibit C3 haemolytic activity demonstrates that a general steric blocking of the C3 molecule by the antibody reaction with C3a does not occur. It should be mentioned that an antibody to guinea pig C5a exists which binds to C5a, but does not block its activity (Rasokat, Burger & Hadding, 1981).

There are several obvious areas of application for an antibody like H13. Firstly, it should be useful in experimental systems for analysis of C3a function *in vivo* and *in vitro*, e.g. with regard to C3a receptor interaction. Such an application is facilitated through the cross-species reactivity of MoAb H13 which reacted with C3a of the three species tested including animals for which experimental shock models are established. This extensive cross reactivity of the monoclonal antibody was somewhat unexpected, since conventional antisera to C3a from different species fail to crossreact over the species barrier. Reactivity (in Ouchterlony analysis) was only observed with the corresponding antigen (Hugli, 1981). Secondly, in immunopathology, MoAb H13 may represent an important control reagent for distinction of native C3 remaining in the tissue, from the biologically active C3b or C3b-derived fragments generated by C-activation. Native C3 is H13-positive whereas C3b fragments would only react with monoclonal antibodies to C3b/C3d determinants but not with the C3a-specific H13. In a model system using FACS analysis, H13 proved to discriminate efficiently between non-specifically deposited C3 on human lymphocytes and C3b bound after treatment with an therapeutically utilized antibody to the CD3-antigen (T3) together with human complement (Sugita *et al.*, 1986). MoAb H13 was similarly used as a control reagent to demonstrate C3 activation and deposition on microorganisms, e.g. mycoplasma (H.W.L. Ziegler-Heitbrock & R. Burger, unpublished). Thirdly, the most important application of MoAb H13 might be its use for C3a quantification in ARDS-patients. It might provide an equivalent or, due to its efficient binding capacity, a probably better reagent than conventional antisera for the present C3a assay based on ^{125}I-labelled C3a. For practical purposes, C3a assays independent of radioactive material are preferred. An ELISA for determination of C5a was described previously based on the use of a heterologous antiserum (goat) to C5a (Kunkel *et al.*, 1983). Figure 5 reveals that H13 is useful for the establishment of a C3a-assay based on the ELISA system. Pathological C3a-containing plasma samples containing only weakly evaluated C3a levels when tested in the C3a-radioimmunoassay were also positive in the ELISA. The purpose of this pilot study with the C3a-ELISA was mainly to demonstrate the principal applicability of H13 for the ELISA-system. Before considering an application of this antibody in diagnostic assays a more complete comparison with existing assay systems is of course required. In particular, a larger number of patient or normal plasma has to be tested to permit appropriate statistical analysis. In addition, the influence of other parameters on the assay has to be determined, e.g. effects of anticoagulants or drugs, origin and handling of the plasma etc. Additional, more elaborate experimental variations in the assay conditions would presumably improve the sensitivity of the ELISA.

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