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Generation of a monoclonal antibody to mouse C5 application in an ELISA assay for detection of anti-C5 antibodies

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We have generated a monoclonal antibody with specificity for the fifth component of mouse complement (C5). This antibody precipitates the two chains of C5 from normal mouse serum and inhibits C5-dependent hemolysis in a functional complement test.

In this study we describe its application in an enzyme-linked immunoadsorbent assay (ELISA assay) for the detection of anti-C5 antibodies in serum. Monoclonal anti-C5 coupled to wells of an ELISA plate specifically binds C5 from unfractionated normal mouse serum. This subsequently serves as antigen to bind anti-C5 serum antibodies. By this approach we have circumvented the need for extensive purification of C5 from serum which would be required if C5 was directly coupled to ELISA plates as antigen. Serum antibodies from C5-immunized mice bound with high avidity to wells containing normal serum as antigen source in amounts representing 1 μg to 250 ng C5. There was no antibody binding to wells containing C5-deficient serum as antigen source. The immune reaction was detected by development with enzyme-coupled goat-anti mouse Ig antibodies specific for various mouse Ig subclasses. This method allows the qualitative characterization of immune responses to mouse C5 which is an ideal model for a natural self antigen in studies of immunological tolerance.

KEYWORDS: complement C5, monoclonal antibody, ELISA assay.

INTRODUCTION

The fifth component of complement (C5) is a glycoprotein consisting of two disulfide-linked polypeptide chains present in serum in a concentration of 50–80 μg ml^{-1} [1, 2].

A genetic defect resulting in the absence of C5 from serum has been described

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for about 40% of inbred strains of mice[3, 4]. C5 mRNA is translated in cells from C5 deficient strains but the product is not secreted[5]. Absence of C5 from the circulation prevents tolerance induction to this self protein so that deficient mice produce anti-C5 antibodies upon immunization.

Two congenic strains of mice which differ only at the C5 locus[6] have been used to study tolerance to C5 as a natural self protein. B10D2/nSn (C5-sufficient) and B10D2/oSn (C5-deficient) were used in reciprocal cell transfers into irradiated secondary hosts[7]. These studies suggested that tolerance in the C5 sufficient strain is not operating at the level of the B cells because B cells from N mice and T cells from O mice cooperate to produce anti-C5 antibodies.

So far immune responses to C5 could only be measured by a functional inhibition assay of complement-mediated lysis. This assay is very sensitive but has the disadvantage of not allowing qualitative comparisons between anti-C5 antibodies. For studies of tolerance to C5 it would, for instance, be very important to determine Ig subclasses of anti-C5 secreted under different conditions. For this purpose an enzyme-linked immunoadsorbent (ELISA) assay is much more suitable.

However, this approach was complicated by the fact that purification of mouse C5 to a degree that it can be directly used as antigen for an ELISA without causing problems with increased backgrounds is a tedious and time-consuming task. We have therefore generated a monoclonal antibody to C5. Coupled to a plate, this antibody will selectively bind C5 from unfractionated mouse serum and so enable assay of anti-C5 antibodies from test sera without the need to purify the antigen source.

MATERIALS AND METHODS

Mice

B10D2/nSn (N) and B10D2/oSn (O) mice were obtained from the Jackson Laboratory, Bar Harbor, ME.

Source of C5

Serum from NN male mice was used as C5 source. Serum was precipitated with 10 × the volume of distilled water saturated with CO_2 according to the method described by Cinader *et al.*[3] and the precipitate was dissolved in $\frac{1}{10}$ of the original serum volume. A minimum C5 concentration of 50 μg ml^{-1} serum was taken as the basis for calculations mentioned in the text.

Immunizations

Mice were injected in the hind footpads with 50 μl C5 in complete Freund's adjuvant (CFA) each (equalling about 25 μg C5 per injection). Two injections in CFA were given at an interval of 10 days.

Immunization for generation of monoclonal antibodies

A B10D2/oSn mouse was immunized in the hind footpads with C5 four times at intervals of 3 days. The first injection was given in CFA, the three consecutive ones in PBS.

Fusion and screening for monoclonal antibodies

One day after the last C5 injection, poplietal, inguinal and para-aortic lymph nodes were removed and cells were fused in a 1:1 ratio with the myeloma X63-Ag8.653 using PEG 4000[8]. Cells were immediately plated in 96-well flat-bottom culture plates (Costar) at 3×10^4 cells/well with 1×10^5 cells/well o peritoneal feeder cells in HAT selection medium. Supernatants from wells with growing hybridoma cells were tested for inhibition of lysis in the complement assay. Hybridoma cells which produced supernatants that were >30% inhibitory were subcloned at 1 cell/well and subsequently expanded in tissue-culture flasks.

Haemolytic complement assay for mouse C5

Fifty microlitres appropriately diluted C5-containing serum, 50 µl of C5-deficient serum diluted $\frac{1}{4}$ as a source of all other complement components and 30 µL of a 1% rabbit red-blood-cell (rabbit RBC) solution sensitized with antibody to rabbit RBC were incubated in a 96-well microtitre plate for 4 h at 37°C. The plates were spun and the supernatant was flicked off. The degree of lysis was determined from the concentration of unhaemolysed cells after addition of water and read as optical densities at 415 nm. Haemolytic activity was expressed as:

$$\text{Percentage specific lysis} = \frac{\text{experimental O.D.} - \text{background O.D.}}{\text{maximal O.D.} - \text{background O.D.}} \times 100$$

Maximal lysis values were obtained by adding water to rabbit RBC. For evaluation of background lysis C5-containing serum was replaced by C5-deficient or heat-inactivated serum.

Inhibition of mouse C5—screening test for hybridoma supernatants

To test for the presence of anti-C5 antibodies in hybridoma supernatants, 50 µl of supernatant was added to the mixture of C5 containing serum, C5-deficient serum and sensitized rabbit RBC described above. Control wells received medium instead of hybridoma supernatant.

ELISA for detection of anti-C5 antibodies

Wells of flexible round-bottom 96-well plates (Costar) were coated with 2 µg of

monoclonal antibody BB5.1 purified by affinity chromatography on protein A–Sepharose (Pharmacia) and diluted in borate buffer pH 8·5. All incubations were done for 2 h at 37°C. After blocking of uncoated sites with 10% foetal-calf serum–phosphate-buffered saline (FCS–PBS), serum from N was added as C5 antigen source and incubated. The plates were then washed thoroughly with PBS containing 0·05% Tween before appropriately diluted test antisera were added. Binding of antibodies was made visible with alkaline-phosphatase coupled goat-anti-mouse-Ig subclass-specific antibodies and their substrate (Southern Biotechnology). Results are expressed in arbitrary units of optical densities at 415 nm.

Immunoprecipitation of mouse C5 and gel electrophoresis

Normal and C5 deficient mouse sera were precipitated with 37·5% ammonium sulphate, resuspended to the original serum volume in PBS and labeled by the iodogen method[9]. Labeled samples were pre-adsorbed with 50 µl anti-mouse-Ig affinity gel (Cappel) and 50 µl protein A–Sepharose (Pharmacia) for 30 min at room temperature. They were then precipitated with 40 µl monoclonal antibody culture supernatant (about 10 µg antibody per ml) or 40 µl of polyclonal antiserum, followed by addition of 50 µl protein A–Sepharose and another incubation period of 1 h. Precipitated samples were washed in PBS, resuspended in sample buffer, boiled for 5 min in the presence of 2-mercaptoethanol and electrophoresed on a 7% sodium dodecyl sulphate–polyacrylamide (SDS–PAGE) gel according to the description by Laemmli[10].

Human C5

Human C5 was isolated from plasma as previously described[11].

RESULTS

Fusion of lymph node cells from a C5-immunized B10D2/oSn mouse with the myeloma X63-Ag8.653 resulted in hybridoma growth in 20% of the culture wells. Six hybridomas produced supernatant that was > 30% inhibitory in the C5 haemolytic assay. These were subcloned by limiting dilution culture and subsequently expanded. Two of the six hybridomas secreted antibody of the IgG2b class but proved to be unstable. The remaining four stable hybridomas were of the IgG1 class and one of them, termed BB5.1, will be described in detail.

Inhibition of C5-mediated lysis by monoclonal antibody BB5.1

Supernatant of hybridoma BB5.1 strongly inhibited haemolytic activity in the complement assay as shown in Fig. 1. A $\frac{1}{5}$ dilution of supernatant reduced lysis mediated by a concentration of approximately 12·5 ng C5 (serum dilution of $\frac{1}{200}$) from 85% to 20%.

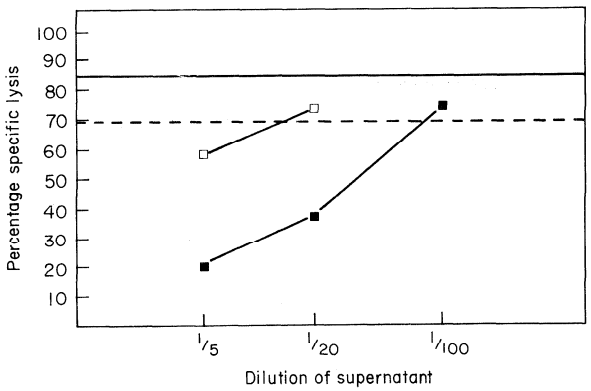

Fig. 1. Supernatant from hybridoma BB5.1 was tested for inhibition of a C5-dependent complement test. Mouse C5 containing serum was diluted 1/200 (equalling a final concentration of 12·5 ng C5 in the test). The horizontal line (NN serum) represents percentage specific lysis in the absence of hybridoma supernatant. The stippled horizontal line (C5 human) shows lysis by purified human C5 (final concentration 3 μg). Inhibition of lysis by BB5.1 supernatant is shown by the closed symbols. Reconstitution of lysis by addition of 3 μg human C5 is shown by the open symbols.

It has been shown earlier that purified human C5 can render complement deficient mouse serum haemolytically active[2]. Addition of human C5 to the assay containing inhibitory hybridoma supernatant indeed reconstituted haemolytic activity. This indicates that the inhibitory activity was directed against C5. It should be mentioned that none of the monoclonal antibodies to mouse C5 reacted with human C5 as measured in an ELISA (data not shown). Human C5 proved to have reduced lytic capacity compared with mouse C5 in this assay. It had to be added in concentrations 240-fold higher than the estimated C5 concentration in mouse serum to achieve comparable levels of lysis which might be due to loss of lytic function during purification.

Immunoprecipitation of mouse C5 (see Fig. 2)

Specificity of BB5.1 for mouse C5 was proved by immunoprecipitation. SDS–gel electrophoresis was performed with mouse sera after precipitation with BB5.1 or polyclonal anti-C5. BB5.1 precipitated the two chains of C5 (α-chain, 125,000 kDa and β-chain, 83,000 kDa) out of C5-sufficient mouse serum [lane (c)]. No corresponding bands were detected in precipitates of C5-deficient serum [lane (a)]. The same reaction pattern was observed with polyclonal anti-C5 [lane (d) vs lane (b)].

Detection of anti-C5 serum antibodies using BB5.1 in an ELISA assay

In the course of attempts to establish an ELISA assay for detection of anti-C5 antibodies we encountered considerable problems with high background values due to even minute immunoglobulin contamination in C5 preparations from mouse serum which were used as antigen coupled to wells of a microtitre plate.

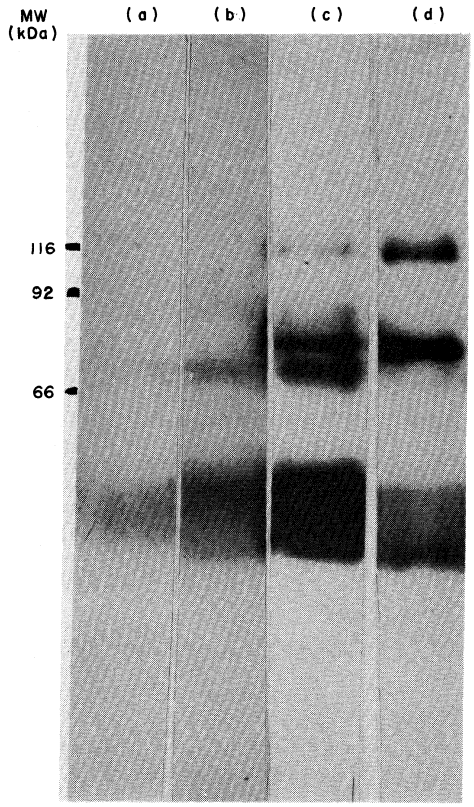

Fig. 2. Immunoprecipitation of mouse C5. Mouse sera were labeled with Na[^{125}I] (Amersham) and precipitated as described in Materials and Methods. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis was performed under reducing conditions in a 7% polyacrylamide gel. Sera in Lane A and C were precipitated with monoclonal BB5.1 supernatant, in lane B and D with polyclonal anti-C5 antiserum. Lane (a), O serum (C5-deficient); Lane (b), O serum; Lane (c), N serum (C5-sufficient); Lane (d), N serum. Molecular weight standards were obtained from BioRad.

One main incentive to generate a monoclonal antibody to C5 was the assumption that it could be used as a tool specifically to focus C5 from serum to the microtitre plate thus circumventing the need for extensive purification of C5.

For the following experiments shown in Fig. 3(a), (b) BB5.1 was coupled to microtitre wells and N serum was added as antigen source. Polyclonal anti-C5 antiserum derived from an O mouse which was immunized twice with C5 in CFA was tested in three dilutions. Binding was detected using alkaline-phosphatase-coupled anti-mouse-Ig antibodies specific for IgG2a and 2b.

The test antiserum showed strong anti-C5 activity measurable up to a dilution of $\frac{1}{1000}$ [Fig. 3(a)].

Background values of wells which either received no N serum as C5 source or no test antiserum are represented in the figure by the shaded area. Provided the plates were thoroughly washed after addition of C5-containing serum to remove unbound

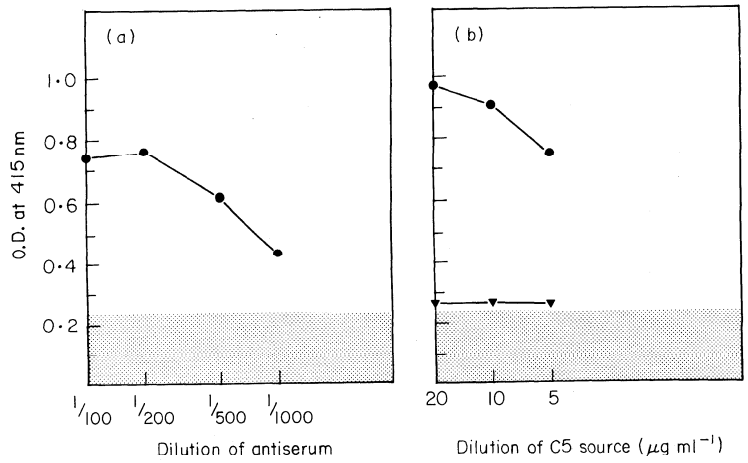

Fig. 3. Enzyme-linked immunoadsorbent assay (ELISA assay) for detection of anti-C5 antibodies. Two micrograms of protein A–affinity-purified monoclonal antibody BB5.1 were coupled to wells of an ELISA plate. In (a), N serum was added in a concentration equalling 10 μg C5 ml^{-1} (500 ng per well). Polyclonal anti-C5 anti-serum from an O mouse immunized twice with C5 in CFA was added in four dilutions. In (b), N (●—●) or O (▼—▼) serum was added in three dilutions equalling 20, 10 and 5 μg C5 ml^{-1} (1 μg, 500 ng and 250 ng per well). A constant dilution (1/200) of polyclonal anti-C5 antiserum was tested on these wells. Binding reactions were developed with alkaline-phosphatase-coupled goat anti-mouse-IgG2a and 2b. The shaded areas signify background values obtained from wells which either received no C5 antigen or no anti-C5 antiserum. Results are expressed in arbitrary units of O.D. at 415 nm.

mouse immunoglobulin, we have not encountered problems with high background values.

This is further illustrated in Fig. 3(b). In this experiment we have titrated C5-containing N serum as antigen source and C5-deficient O serum as control and added a constant amount of the test antiserum. A concentration of N serum equalling 5 μg ml^{-1} (250 ng final) C5 was still sufficient to allow detection of anti-C5 activity. The same dilutions of C5-deficient control serum did not result in binding of anti-C5 antibodies.

DISCUSSION

In this paper we describe a monoclonal antibody specific for mouse C5. This antibody inhibits lysis in a C5-dependent complement assay and precipitates C5 from normal serum. Among the many possibilities for further studies with this monoclonal antibody we were specially interested in using it as a tool to set up a rapid convenient ELISA assay for quantitative and qualitative detection of anti-C5 antibodies in mouse serum. Coupled to wells of an ELISA plate it specifically binds C5 from serum without the need for prior purification of C5. Serum antibodies from C5 immunized mice bound with high avidity to wells containing C5 in concentrations between 1 μg and 250 ng. There was no antibody binding to wells containing C5-deficient serum as antigen source. We have performed parallel tests to compare

anti-C5 activity measured in an ELISA with inhibition in the functional complement test (data not shown). In our hands the two tests were concordant—both inhibition and binding in an ELISA were seen up to a $\frac{1}{1000}$ dilution of the anti-serum. Harris *et al.*[7] who used the inhibition assay as their readout system for anti-C5 stated that an extremely strong antiserum will produce detectable inhibition at dilutions up to $\frac{1}{2400}$. In most experiments, dilution of their test antisera ranged between $\frac{1}{25}$ and $\frac{1}{50}$. So we can assume that the ELISA assay is at least comparable if not superior to the inhibition assay as far as sensitivity is concerned.

Antibodies of all isotypes with the exception of IgG1 (the isotype of BB5.1) can be detected in this assay. In a number of experiments we have screened for antibodies of the IgG1 class using extensively purified C5 directly coupled to the plate as antigen. IgG1 antibody titres were generally lower than IgG2a and 2b, but there was no serum which exclusively had either IgG1 or IgG2a and 2b (data not shown). So we assume that detection of IgG2a and 2b antibodies gives a representative measurement of an IgG antibody response.

It is not known yet what part of the C5 protein is recognised by BB5.1. However, it is clear that the determinant is not exclusively expressed on the haemolytically active molecule. Our serum preparations used as antigen source in the ELISA were stored at 4°C and had lost haemolytic activity in the complement test, but were still able to bind anti-C5 antibodies.

In addition to the application described in this paper the monoclonal anti-mouse-C5 antibody could be employed in affinity column purification of mouse C5. An analogous method for purification of human C5 with relatively high yields has been described by Wetsel *et al.*[12]. It remains to be seen if a single step affinity isolation would purify C5 to an extent that it could be directly coupled to wells of an ELISA plate.

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