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A monoclonal antibody to Clq which appears to interact with $C\bar{1}r_2C\bar{1}s_2$-binding site

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A monoclonal antibody (SB-4) to human Clq was prepared. The equilibrium constant of the antibody for Clq was found to be greater than 10^{10} M^{-1}. It has been shown that the antibody binds to the A-B chain dimer, probably via the B chain of Clq. Pepsin digestion of Clq at pH 4.5, which fragments the globular regions but leaves the collagenous region intact, allowed the demonstration that the antigenic site is located in the collagenous region of the molecule. The effect of the antibody on haemolytic activity has shown that it is capable of inhibiting the formation of EAC1 cells from EAC1q cells plus C1r and C1s but is incapable of inhibiting the C1 activity of preformed EAC1 cells. This indicates that the binding of the antibody to the collagenous portion of the B chain of Clq probably prevents interaction between Clq and the $C\bar{1}r_2$-$C\bar{1}s_2$ complex.

Clq; Monoclonal antibody; $C\bar{1}r_2$-$C\bar{1}s_2$ complex; Antigenic site; Binding site; Complement

1. INTRODUCTION

The classical pathway of complement activation is initiated by the activation of C1. C1 is composed of one molecule of Clq and two molecules each of C1r and C1s. Activation of C1 proceeds by three distinct steps: (i) binding of Clq to the Fc region of antibody in immune complexes, which may lead to a conformational change in Clq; (ii) autocatalytic activation of C1r to $C\bar{1}r$; (iii) activation of C1s to $C\bar{1}s$ by $C\bar{1}r$, resulting in the activated form of C1, $C\bar{1}$. A number of models for the mode of activation have recently been proposed [1–3].

Clq consists of 18 polypeptide chains (6A, 6B, 6C) which form nine disulphide-linked dimers (6A-B, 3C-C). Amino acid sequence data have revealed that each chain consists of an N-terminal collagenous region approx. 80 residues in length, the remainder being globular in structure [4]. The collagen-like regions of one A, one B and one C chain are considered to form a triple helix.

The relationship between the structure and function of Clq has been studied by monoclonal anti-Clq antibodies in several laboratories. Since the epitopes in Clq against which the monoclonal antibodies are directed are different, the effect mediated by these monoclonals varies [5–8]. Here we report a monoclonal antibody which recognises the collagenous region of Clq, it reacts with the A-B chain dimer and inhibits the haemolytic activity of Clq if added before the C1 complex is formed.

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2. MATERIALS AND METHODS

Human citrated plasma was obtained from Oxford Regional Blood Transfusion Service and clotted by addition of $CaCl_2$. DEAE Sephacel and CNBr-activated Sepharose 4B were from Pharmacia. ^{125}I carrier-free and nitrocellulose paper were from Amersham, England. Pepsin was from Sigma and perioxidase

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conjugated rabbit anti-mouse IgG was from Miles, England. Balb/c mice were from the Shanghai Institute of Cell Biology, Chinese Academy of Science. The SP^2/O cell line was kindly provided by Professor R.X. Liu (Chinese Academy of Medical Science, Beijing, China).

2.1. *Preparation of complement components*

C1q was prepared as described by Reid [9]. The collagenous fragment of C1q was prepared by pepsin digestion [10,11]. The concentrations of C1q and of the collagenous fragment were determined by amino acid analysis. Other complement components were prepared by published methods: C2 [12], C4 [13], C1 [14].

2.2. *Monoclonal antibody SB-4*

Balb/c mice were immunised subcutaneously with 40 μg C1q per mouse emulsified in complete Freunds adjuvant twice at 2-week intervals. 10 days after the second immunisation the mice were bled and the sera were tested by enzyme-linked immunosorbent assay (ELISA) [15] for antibodies to C1q. The mouse with the highest serum anti-C1q titre was intravenously injected with 10 μg C1q on 3 consecutive days [16]. On the following day spleen cells were fused with SP^2/O cells [17]. Culture supernatants were screened for antibodies to C1q by ELISA. One hybrid-producing antibody to C1q was cloned by limiting dilution and called Shanghai B-4 (SB-4). Relatively large amounts of antibody were produced by transfer of the hybrid cells into the peritoneal cavities of fresh mice and harvesting the ascites fluid.

2.3. *Purification of antibody and preparation of affinity column*

The monoclonal anti-C1q was precipitated from ascites fluid by addition of Na_2SO_4 to 20% (w/v). After centrifugation the precipitate was dissolved in 25 mM Tris-HCl, pH 7.4, and dialysed extensively against the same buffer. The antibody solution was applied to a column of DEAE-Sephacel (Pharmacia) 1.8 × 12 cm equilibrated with 25 mM Tris-HCl, pH 7.4. Under these conditions the antibody was bound to the column and was eluted by a linear gradient from starting buffer to the same buffer containing 200 mM NaCl. Specific antibody was detected by a binding assay and was found to be pure as judged by SDS-PAGE.

An affinity column was prepared by binding approx. 7.5 mg antibody to 1 g CNBr-activated Sepharose 4B (Pharmacia) following the manufacturer's instructions. 10 ml human serum made 2.5 mM with DFP was applied to the anti-C1q monoclonal antibody Sepharose column, at a flow rate of 10 ml/h. The column was washed with 140 mM NaCl, 20 mM EDTA, 25 mM Tris-HCl, pH 7.4, followed by the same buffer with 500 mM NaCl until the A_{280} approached zero. The bound C1q was eluted with 140 mM NaCl, 50 mM diethanolamine, pH 11.5, and fractions were immediately neutralised with solid glycine.

2.4. *Immunoblotting*

12% SDS polyacrylamide gels [18] were run and proteins were blotted onto nitrocellulose as in [19]. After treatment with antibody, specific binding was detected using peroxidase-conjugated rabbit anti-mouse IgG followed by development with 4-chloro-1-naphthol and H_2O_2 [19].

2.5. *Serology*

To measure the affinity of binding of SB-4 to C1q, fixed amounts of antibody were mixed with varying amounts of antigen until the antigen-antibody interaction had come to equilibrium (24 h). The amount of free antibody present at equilibrium in each mixture was then determined using a solid-phase radioimmunoassay [19]. The affinity was calculated from a plot of free antibody concentration vs total antigen added [19].

2.6. *C1q haemolytic assay*

Sheep erythrocytes coated with rabbit antibody (EA) were prepared as described by Mayer [20]. C1q-depleted serum (C1qD) was prepared by passing human serum through an SB-4 monoclonal anti-C1q column. Haemolytic assays were performed as follows.

EA cells (1×10^7) with different amounts of purified C1q in 150 μl $DGVB^{2+}$ were incubated at 0°C for 30 min followed by 2 min at 37°C. 100 μl C1qD 1/500 in $DGVB^{2+}$ was added and incubated for 30 min at 4°C followed by 2 min at 37°C. The mixture was centrifuged at 1000 × g for 10 min. The supernatant was removed and the cells resuspended in 100 μl $DGVB^{2+}$. 100 μl $DGVB^{2+}$ containing excess human C4 and C2 was added and incubated at 37°C for 15 min. Finally 200 μl of 1/40 guinea pig serum in GVB-EDTA was added and incubated at 37°C for 30 min. 1 ml cold saline was added to stop the reaction and the A_{412} of the supernatant determined after centrifugation at 1000 × g for 10 min to remove unlysed cells.

Z values were determined [20] and the amount of C1q giving approx. 75% lysis (i.e. $Z = 1.31$) was chosen for subsequent experiments. This was found to be 2.6 ng per tube for inhibition assays. Various concentrations of SB-4 monoclonal anti-C1q or MRC OX7 monoclonal anti-Thy1 [21] were added at various stages during the assay and in all cases C1q at 2.6 ng/tube was used. Antibody was incubated: (i) with C1q prior to addition to EA cells; (ii) with EAC1q cells prior to addition of C1qD; (iii) with EAC1 cells prior to addition of C4 and C2; for 15 min at 37°C and the assay continued as described above. Percentage inhibition of haemolysis was calculated and plotted vs antibody added.

3. RESULTS AND DISCUSSION

The SB-4 antibody affinity column gave a good yield of C1q (0.6 mg from 10 ml outdated plasma) and the breakthrough peak from the affinity column was useful as a reagent in the haemolytic assay of C1q.

Western blot analysis of the antibody is shown in fig.1. Lanes 3,4 show a blot stained with amido black and lanes 1,2 the same samples incubated with SB-4, followed by perioxidate-conjugated rabbit anti-mouse IgG. Bound antibody has been visualised by development with chloronaphthol. Tracks 1,3 contain reduced C1q and tracks 2,4 contain unreduced C1q. It can be seen that the antibody binds to the A-B chain dimer of C1q under

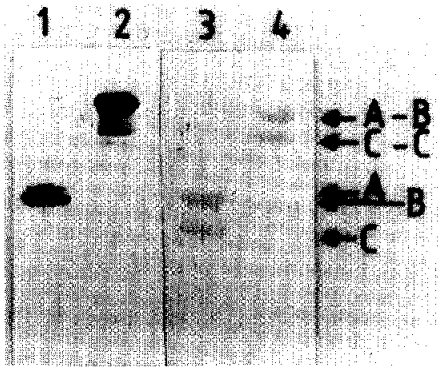

Fig.1. Western blots of C1q with SB-4. Tracks: 1, reduced C1q detected using SB-4; 2, unreduced C1q detected using SB-4; 3, reduced C1q stained with amido black; 4, unreduced C1q stained with amido black.

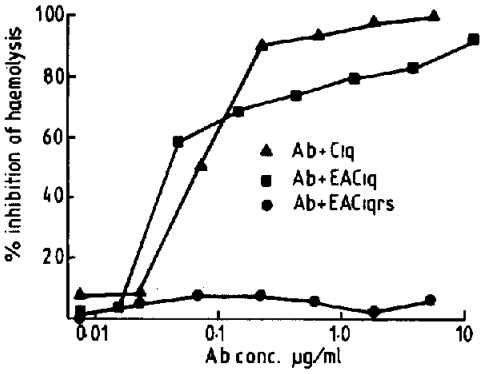

Fig.2. Inhibition of binding of SB-4 to C1q-coated microtitre plates by C1q (●) and the collagenous region of C1q (■). A theoretical curve based on the assumption that the equilibrium constant of the antibody is ∞ is also shown (○). To calculate this curve the antibody concentration has been taken to be 3.0 nM and the number of accessible antigenic sites, per C1q molecule, equal to 3.

non-reducing conditions. After reduction C1q A and B chains are not well separated, but close inspection indicates that SB-4 is probably binding to the B chain.

Fig.2 shows the result of adding varying quantities of pure C1q to a fixed amount of ^{125}I-labelled antibody and determining the fraction of antibody that remains uncomplexed with antigen at equilibrium. Preliminary experiments showed that equilibrium was fully established within 24 h. Also plotted is the theoretical relationship between % binding and the concentration $[A_o]$ of C1q on the assumption that the antibody binds irreversibly to the antigen. C1q has up to six antigen sites per molecule but it is probable that steric hindrance prevents all of these binding antibody simultaneously. The data in fig.2 were obtained using a nominal antibody concentration of 4.75 nM. Given that 60–70% of this is active antibody [21] and that, as fig.2 shows, 50% neutralization of the antibody occurred at a C1q concentration of 0.7–0.8 nM, it appears that 3–4 of the C1q antigenic sites are simultaneously accessible to antibody. The departure of the experimental data from the theoretical curve obtained assuming irreversible binding is too small to allow a precise evaluation of the antibody affinity but it is estimated to be in excess of 10^{10} M^{-1} [19].

C1q was digested with pepsin to yield a fragment which was shown by SDS-PAGE and amino acid analysis to be the collagenous region of C1q [10]. When binding assays were performed using this material it was found to be almost as effective as C1q in inhibiting the binding of SB-4 to C1q-coated plates (fig.2). This indicates that the epitope recognised by SB-4 resides within the collagenous region of C1q.

A C1q haemolytic assay was developed in which monoclonal antibody could be added to various stages in the reaction to test whether inhibition of activity occurred. Addition of either no antibody or anti-Thy1 monoclonal antibody (MRC OX-7) at

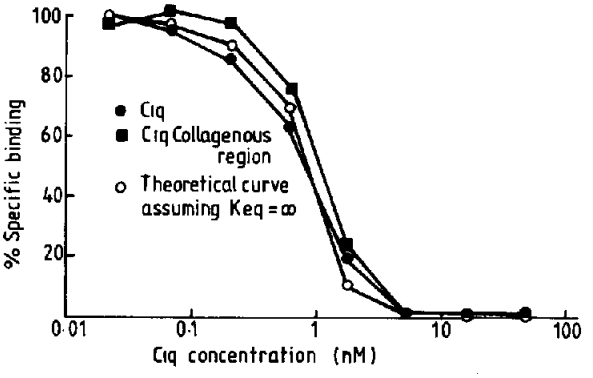

Fig.3. Inhibition of C1 haemolytic activity by SB-4 anti-C1q monoclonal antibody. The inhibition of haemolysis is shown when SB-4 was added at various stages during the assay; (▲) antibody added to C1q before addition of EA cells; (■) antibody added after formation of EAC1q cells; (●) added after formation of EAC1qrs but before addition of C4 and C2.

any stage in the haemolytic assay did not inhibit lysis of the target erythrocytes. When various amounts of SB-4 were mixed with C1q before addition of EA cells 50% inhibition of activity was observed with an antibody concentration of around 0.1 μg/ml. A similar result was obtained when antibody was added after the formation of EAC1q cells. However, when antibody was added to EAC1qrs cells prior to addition of C4 and C2, no inhibition of lysis occurred, even at levels 100-fold higher than that which caused 50% inhibition when the antibody was added before C1r and C1s.

The SB-4 monoclonal antibody thus appears to bind to, or mask, the site(s) in the C1q molecule which are important for binding the $C1r_2$-$C1s_2$ complex. As judged by the Western blotting data this site appears to involve the collagen-like portion of the B chain.

These results support models of C1 in which it is proposed that $C1r_2$-$C1s_2$ is closely associated with collagen-like regions of C1q in the C1 complex [1]. Activation of C1 by immune aggregates in plasma results in the rapid removal of $\overline{C1r}$ and $\overline{C1s}$ by the C1 inhibitor which thus leaves the collagen-like regions of C1q free to interact with the widespread C1q receptor which has the potential to fulfil a number of effector functions [22] such as cell-mediated cytotoxicity, inhibition of Il-1 production and stimulation of oxidative metabolism. The SB-4 monoclonal antibody may be a useful tool in the study of the interaction between C1q and its receptors.

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