Inhibition of the formation of the complement membrane-attack complex by a monoclonal antibody to the complement component C8 α subunit

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The effect of nine monoclonal antibodies to complement component C8 on the interaction of C9 with preformed cell-surface C5b–8 complexes and on the functional insertion of C8 into the membrane-attack complex (MAC) was investigated. None of the antibodies prevented C9 insertion into a preformed C5b–8 complex. One antibody (F1) directed to the C8 α subunit clearly inhibited formation of a functional MAC. It is proposed that this antibody prevents the C8 α subunit unfolding and distorting the bilayer to allow C9 insertion.

INTRODUCTION

The assembly of the membrane-attack complex (MAC), which is composed of the five terminal complement components, is initiated by proteolytic cleavage of C5 to C5a and C5b, followed by the sequential addition to C5b of C6, C7, C8 and C9 (Muller-Eberhard, 1988). The obligatory and specific sequential steps of MAC formation suggest that each intermediate complex expresses binding (receptor) sites for the components that precede and follow it in the sequence. The binding of C8 (a trimer of α , β and γ subunits; Sodetz, 1988) to membrane-bound C5b–7 is mediated via its subunit (Monahan & Sodetz, 1981; Brickner *et al.*, 1985; Stewart *et al.*, 1987). Although the primary role of the C5b–8 complex is to allow membrane insertion and polymerization of component C9 (Podack *et al.*, 1982), the nature of the molecular interaction between C8 and C9 still remains poorly understood. Solution binding studies have shown that the α -subunit of C8 forms a stable complex with C9 (Stewart & Sodetz, 1985). Despite this observation, attempts to establish a direct interaction between C8 and C9 on target membranes by cross-linking experiments have failed (Monahan *et al.*, 1983). Tschopp *et al.* (1982a,b) and Esser *et al.* (1985) have shown that C9 inserts into either lipid bilayers made leaky by reducing surface pressure or into small unilamellar vesicles. These findings indicate that lipid bilayer perturbation by C5b–8 (McClosky *et al.*, 1986) might play a major role in the insertion of C9 (Stanley *et al.*, 1986; Stanley, 1988).

In an attempt to study the role played by C8 in the formation of the MAC, monoclonal antibodies against the three polypeptide chains of C8 were raised and characterized (Abraha *et al.*, 1988). In the present paper we report the results of studies carried out using the

monoclonal antibodies against C8 to interfere with the function of C8 in MAC formation.

EXPERIMENTAL

Mouse monoclonal antibodies to human C8 were described previously (Abraha *et al.*, 1988) and were affinity purified on protein A using a Bio-Rad Maps II kit (Bio-Rad Laboratories, Watford, Herts., U.K.). Human C8+C9+C8-depleted normal human serum (NHS), C9-depleted NHS and C5b–9 complexes were prepared as previously described (Morgan *et al.*, 1983; Abraha *et al.*, 1988; Tomlinson *et al.*, 1989).

Preparation of pigeon erythrocyte ghosts, loading with [14 C]sucrose (Amersham International Ltd., Amersham, Bucks., U.K.) and sensitizing of cells and ghosts with anti-erythrocyte antibodies were as previously described (Morgan *et al.*, 1984). In experiments to test the effect of anti-C8 monoclonal antibodies on the ability of cell-surface C5b–8 to allow insertion of C9, antibody-sensitized erythrocytes or ghosts were incubated with a 1:10 dilution of C9-depleted serum at 37 °C for 10 min, followed by a wash. Erythrocytes (approx. 10^8) or erythrocyte ghosts (approx. 10^8) were then incubated with each of the anti-C8 antibodies (40 μ g/ml final concn.) in a final volume of 100 μ l for 10 min on ice, followed by the addition of pure C9 (10 μ g/ml final concn.) and incubation at 37 °C. After 15 min incubation, erythrocytes were diluted to 1 ml with Medium A (see below), pelleted and the release of haemoglobin (A_{540}) estimated. In experiments with erythrocyte ghosts, aliquots (50 μ l) were removed after 0 and 15 min incubation and release of [14 C]sucrose was measured. The conditions were chosen such that, when the anti-C9 monoclonal antibody M47 was substituted for the anti-

Abbreviations used: C8 etc., complement component C8 etc.; MAC, membrane-attack complex; Medium A, Tes-buffered saline (full composition and pH value is given in the text); NHS, normal human serum; E, fresh washed pigeon erythrocytes; A, guinea-pig anti-(pigeon erythrocyte) antibodies; G, pigeon erythrocyte ghosts; MAb, monoclonal antibody.

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C8 antibodies, approx. 50 % inhibition of marker release was observed (Morgan *et al.*, 1984).

In experiments to test the effect of anti-C8 monoclonal antibodies on the functional insertion of C8 into the MAC, aliquots (10 μ l) of NHS were preincubated with the monoclonal antibodies (40 μ g/ml final concn.) for 20 min at room temperature (20 °C) before addition to antibody-sensitized erythrocytes or ghosts. Samples were taken at 0 and 15 min of subsequent incubation at 36 °C, and the release of haemoglobin or [14 C]sucrose was measured. Again conditions were chosen such that incubation of NHS with the same amount of the anti-C9 monoclonal antibody M47 results in approx. 50 % inhibition of marker release.

Throughout the experiments erythrocytes and ghosts were resuspended and incubated in Medium A, which contained 140 mM-NaCl, 5 mM-KCl, 2 mM-MgCl₂ and 10 mM-Tes, adjusted to pH 7.4 with NaOH.

Dot-blot immunoassay was as previously described (Abraha *et al.*, 1988), and protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

RESULTS

IgG monoclonal antibodies against each of the three polypeptide chains of C8 were used in functional studies to examine their effect on the assembly of the MAC. Screening experiments were carried out to examine whether any of the antibodies added to preformed cell-surface C5b-8 intermediates would inhibit the functional insertion of C9 and whether any of the antibodies added to soluble C8 would prevent its functional incorporation during MAC formation. Throughout the study the release of either haemoglobin from sensitized intact pigeon erythrocytes or [14 C]sucrose from resealed sensitized erythrocyte ghosts was used as an index of MAC formation.

In the first experiment sensitized pigeon erythrocyte intermediates (EAC1-8) were prepared and incubated with each of the anti-C8 antibodies. The release of markers was subsequently assessed upon addition of C9. None of the antibodies affected haemoglobin release from intact erythrocytes (Table 1). The same result was obtained when measuring [14 C]sucrose release from erythrocyte-ghost intermediates (GAC1-8) treated in the same way (results not shown). Thus none of the anti-C8 antibodies prevented the functional interaction of C9 with preformed cell-surface C5b-8.

In the second experiment, aliquots of whole human

Fig. 1. Effect of increasing concentration of anti-C8 antibodies on the formation of a functional MAC

serum were preincubated with each of the antibodies. The complement cascade was then activated by adding either sensitized intact erythrocytes or sensitized erythrocyte ghosts. It was found that antibody F1 caused consistent inhibition of marker release (Table 1). Similar results were obtained when measuring [14 C]sucrose release from sensitized erythrocyte ghosts (results not shown).

The effects of F1 were examined further by incubating NHS with different amounts of F1 and examining the effect on haemolysis. Fig. 1 shows that whereas F1 increasingly blocked haemolysis when its concentration was increased, L6, an anti-C8 α antibody that binds to a

Table 1. Effect of anti-C8 monoclonal antibodies on haemoglobin release from pigeon erythrocytes

Results (means $\pm$ S.E.M. for four experiments) are expressed as a percentage of haemoglobin release when no MAb is present.

	No MAb	Control IgG (X63)	Anti-C8 β		Anti-C8 α					Anti-C8 γ	
			A2	B1	D5	E2	F1	K1	L6	J1	M1
Addition of MAb after C5b-8 formation before adding C9	100	95 $\pm$ 6	96 $\pm$ 4	99 $\pm$ 2	87 $\pm$ 2	91 $\pm$ 1	94 $\pm$ 6	91 $\pm$ 9	85 $\pm$ 6	91 $\pm$ 9	89 $\pm$ 6
Addition of MAb to whole serum before adding to antibody-coated cells	100	99 $\pm$ 1	83 $\pm$ 7	83 $\pm$ 5	89 $\pm$ 5	84 $\pm$ 8	32 $\pm$ 12	74 $\pm$ 5	98 $\pm$ 2	68 $\pm$ 3	91 $\pm$ 9

different, but overlapping, epitope (Abraha *et al.*, 1988), and X63, a non-specific IgG, had no effect at concentrations up to 200 $\mu\text{g}/\text{ml}$. When the concentration of IgG reached 300 $\mu\text{g}/\text{ml}$, X63 and L6 had a small (approx. 20%) inhibitory effect, but at this concentration the inhibition by antibody F1 was much more pronounced.

The ability of each of the anti-C8 monoclonal antibodies to interact with preformed C5b-9 complexes was shown by dot-blot immunoassay. The ratio of binding to C8 compared with binding to C5b-9 containing an equivalent molar concentration of C8 was, for each antibody: A2, 1.48; B1, 1.16; D5, 1.30; E2, 0.94; F1, 1.85; K1, 1.17; L6, 1.22; J1, 1.23; M1, 1.14 (mean for duplicate experiments in each case).

DISCUSSION

We have previously prepared ten monoclonal antibodies to human complement component C8 (Abraha *et al.*, 1988). The nine IgG antibodies used in the present study comprise two anti-C8 β antibodies, two anti-C8 γ antibodies and five anti-C8 α antibodies. The latter were classified in four overlapping epitope groups by competitive binding assay. All of the antibodies used in the present study have been shown to react with preformed C5b-9 by dot-blot immunoassay and in some cases by immunofluorescence (Sewry *et al.*, 1987). Preliminary studies using $(\text{NH}_4)_2\text{SO}_4$ fractions of ascites fluid containing anti-C8 monoclonal antibodies suggested that several of the antibodies might be capable of interfering with MAC formation (Abraha *et al.*, 1986; Abraha, 1988; Luzio *et al.*, 1987), but the present experiments using affinity-purified monoclonal antibodies showed that none can inhibit the insertion of C9 into preformed C5b-8 complexes. One anti- α antibody, coded F1, clearly inhibited the release of markers when incubated with serum prior to the activation of the complement cascade. In separate experiments to examine the effect of the anti-C8 antibodies on marker release from sensitized erythrocyte ghosts preincubated with C9-depleted NHS (GAC1-8), no effects of any antibodies on marker release were seen (Abraha, 1988), though it should be noted that marker release in such experiments is always low, approximately double that observed from sensitized ghosts (GA) alone.

An attractive hypothesis for the mechanism of action by which the F1 antibody blocks the release of markers when incubated with normal human serum prior to the initiation of complement activation is that it prevents the unfolding of C8 α and its subsequent insertion into the target membrane. F1 inhibition of MAC formation is unlikely to be simply a steric effect, because L6, which binds to a different but overlapping epitope, has no equivalent blocking effect (Fig. 1). The fact that the epitope on the C8 α -subunit recognized by antibody F1 is available for binding both in free C8 and in MAC complexes, suggests that F1 does not simply bind to and block the domain of the C8 molecule that mediates interaction between C8 α and C9. Dot-blot experiments have shown that antibody F1 does not bind to any terminal complement component other than C8 α (Abraha *et al.*, 1988), thus providing evidence that its inhibition of marker release cannot be due to an effect on C9 or C8 β .

The proposed hypothesis for F1 action is consistent

with present knowledge of the primary structure of terminal complement components, since these have considerable sequence similarity, and it is likely that the mechanism by which they unfold and become inserted into the target membrane is the same (Stanley & Luzio, 1988). The proposed mechanism for F1 action is analogous to the explanation of the ability of the anti-C9 antibody M34 to prevent unfolding and insertion of C9 into target membranes (Stanley *et al.*, 1986; Marazziti *et al.*, 1988), though the latter antibody has been shown to act from the intracellular side of the plasma-membrane bilayer. Our data is consistent with antibody F1 preventing the C5b-8-induced disturbance of the phospholipid bilayer that may be necessary for the functional insertion of C9 into target membranes (Stanley *et al.*, 1986; Stanley, 1988).

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