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INHIBITION OF COMPLEMENT-INDUCED [^{14}C] SUCROSE RELEASE BY INTRACELLULAR AND EXTRACELLULAR MONOCLONAL ANTIBODIES TO C9 : EVIDENCE THAT C9 IS A TRANSMEMBRANE PROTEIN

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SUMMARY: Monoclonal antibodies to the terminal component of the human complement pathway, C9, were used to inhibit the complement-induced release of entrapped [^{14}C] sucrose from erythrocyte ghosts. Antibodies were present either outside, or entrapped within the ghosts. Different monoclonal antibodies were demonstrated to inhibit [^{14}C] sucrose release depending on whether the antibody was outside or entrapped within the ghosts. These findings demonstrate that C9 within the membrane attack complex on erythrocyte membranes is an asymmetrical transmembrane protein penetrating into the cytoplasmic space.

The membrane attack complex of complement is made up of the complement components C5b, C6, C7, C8 and C9 in a multimolecular complex (1). However, much controversy exists over the exact structure and the nature of the lesion caused by the complex in biological membranes. Mayer (2) has proposed that the lesion is a rigid protein channel through which ions and small molecules leak, whereas Esser (3) envisages a region of disrupted membrane lipid or 'leaky patch'. Both of these models require that some or all of the components of the membrane attack complex become inserted into the membrane - either to form the protein channel or to disrupt the lipid bilayer. Evidence of insertion of component proteins includes enzymatic stripping studies (4), freeze-fracture electron microscopy of complement-lysed erythrocyte membranes (5) and photolabelling using membrane-restricted photoactive probes (6). A further requirement of the protein channel model is that some or all of the membrane attack complex components span the membrane to form the walls of the putative

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transmembrane channel. However, it remains to be established whether any component occupies a transmembrane position.

We have recently reported the production of a number of monoclonal antibodies to human C9 (7), of which five have been well characterised (8). Four distinct epitopes on the native C9 molecule are recognised by these five antibodies. The aim of this study was to investigate whether complement component C9 occupies a transmembrane position within the membrane attack complex on erythrocyte membranes. In order to establish this, the ability of each monoclonal antibody when present outside, or entrapped within resealed erythrocyte ghosts, to inhibit complement-induced release of a marker molecule ($[^{14}C]$sucrose) was studied.

MATERIALS AND METHODS

$[^{14}C]$sucrose (552 Ci/M) was purchased from Amersham. Antisera to pigeon erythrocytes were raised in guinea pigs by standard methods. Monoclonal antibodies to human C9 were raised as previously reported (7). Human C9 was purified, and C9-depleted serum prepared as previously described (7). Medium A contained 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$ and 10 mM TES pH 7.4. Pigeon erythrocytes were obtained from fresh heparinised pigeon blood and washed and stored in Medium A. Erythrocyte ghosts were made from 100 µl of packed erythrocytes, as previously described (8), entrapping $[^{14}C]$sucrose (10 µl, 2 µCi) plus or minus monoclonal antibody (10 µl, 30 µg). The efficiency of entrappment was determined for $[^{14}C]$sucrose by measuring the percentage uptake in the washed ghosts preparation, and similarly for monoclonal antibody, using a trace amount of $[^{125}I]$-labelled monoclonal antibody. Entrappment of $[^{14}C]$sucrose was about 10%, and of monoclonal antibody, 5-8%. The final concentration of antibody inside the ghosts was thus about 20 µg/ml.

Ghosts (G) were antibody-coated by incubation with a 1/100 dilution of guinea-pig anti-pigeon antiserum in Medium A, for 15 minutes at 37°C. Antibody-coated ghosts (GA) were coated with the complement components up to C8 by incubation with C9-depleted serum diluted 1/10 in Medium A + 1 mM $CaCl_2$, for 5 minutes at 37°C. The GA-C5-8 intermediates thus formed were washed and stored on ice in Medium A. To portions of the GA-C5-8 intermediates, pure C9 was added (final concentration of 10 µg/ml). After a further 5 minutes incubation on ice, the GA-C5-9 intermediates thus formed were washed and resuspended in ice-cold Medium A. The appropriate monoclonal antibody at a final concentration of 20 µg/ml - the same concentration as that attained on entrappment within the ghosts - was added, and the intermediates incubated for a further 5 minutes on ice. The intermediates were then incubated at 37°C, portions being removed at intervals, and the released $[^{14}C]$sucrose in the supernatant measured.

RESULTS AND DISCUSSION

The experimental approach in this study was to use monoclonal antibodies outside and entrapped within resealed erythrocyte ghosts to inhibit the complement-induced release of a marker molecule ($[^{14}C]$sucrose, molecular

radius 0.44 nm). It was therefore first necessary to demonstrate the production of stable intermediates with the membrane attack components C5-8 and C5-9 on their surfaces. Antibody-coating of ghosts with an anti-pigeon erythrocyte antiserum caused no detectable release of entrapped [^{14}C]sucrose. Coating the ghosts with the complement components up to C8 (GA-C5-8) caused release of less than 10% of the entrapped marker, and incubation of GA-C5-8 intermediates at 37°C for 60 minutes released less than 20% of the marker. The ghosts coated with components C5-9 (GA-C5-9) were stable at 0°C, releasing about 15% of the entrapped marker over a 60 minute period, but on incubating these intermediates at 37°C there was a rapid onset of marker release, which was complete within 15 minutes (Figure 1).

Of the five monoclonal antibodies studied, three, C9-36, C9-42 and C9-47, inhibited [^{14}C]sucrose release when present outside the GA-C5-9 intermediates (Figure 2). C9-47 caused the largest inhibition of marker

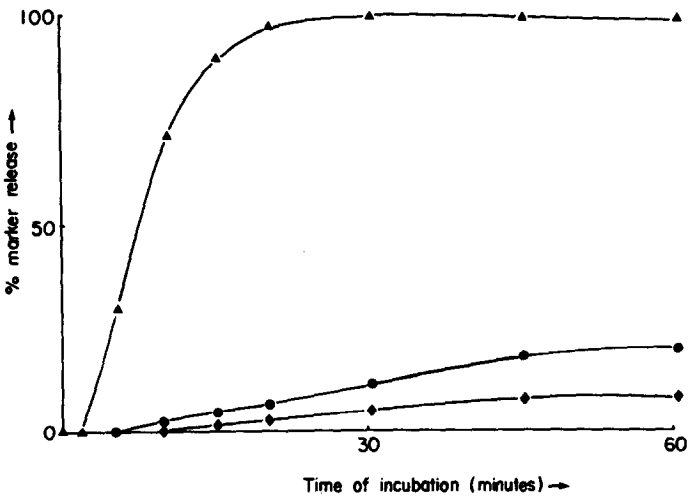

Figure 1.
Release of [^{14}C]sucrose from ghost intermediates at 37°C. The intermediates were incubated at 37°C, portions removed at intervals, and the percentage release of [^{14}C]sucrose measured in the supernatant. Antibody-coated ghosts (GA) released less than 10% of entrapped marker during a 60 minute incubation (◆—◆). Ghosts coated with the complement components up to C8 (GA-C5-8) released less than 20% of entrapped marker over the same period (●—●). Ghosts coated with the complete membrane attack complex (GA-C5-9) released marker rapidly at 37°C (▲—▲). All points are the means of triplicate determinations.

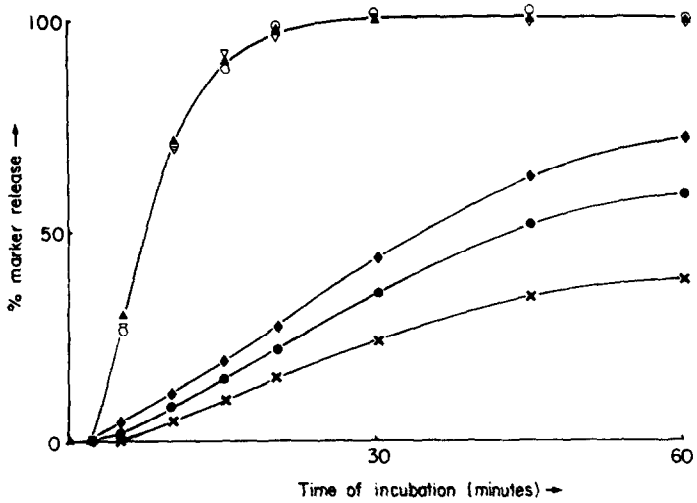

Figure 2.
Effects of external monoclonal antibodies on complement-induced [^{14}C]sucrose release.
GA-C5-9 intermediates were incubated with each monoclonal antibody for 5 minutes on ice, prior to incubating at 37°C. Portions were removed at intervals, and the released [^{14}C]sucrose measured. All the points are the means of triplicate determinations.
(▲= No Antibody, ○= C9-8, ▽= C9-34, ◆= C9-36, ●= C9-42, ✕= C9-47).

release after 60 minutes of incubation (62% inhibition). C9-42 inhibited marker release by 41% over the same period, and C9-36 by 28%. Antibodies C9-36 and C9-47 had previously been shown to compete for the same or closely related epitopes on native C9 (7). The large differences in the inhibition of marker release caused by these two antibodies implies that they recognise distinct epitopes on C9 within the membrane attack complex. Monoclonal antibodies C9-8 and C9-34 caused no detectable inhibition of marker release when present outside the GA-C5-9 intermediates. The three antibodies C9-36, C9-42 and C9-47 had previously been shown to bind to C9 after its insertion into the membrane attack complex on cells, whereas, binding was not demonstrated for antibodies C9-8 and C9-34 (9).

Each of the monoclonal antibodies were also entrapped within ghosts, and the effect on complement-induced [^{14}C]sucrose release studied. Of the five antibodies, only one, C9-34, inhibited marker release when entrapped within the ghosts (Figure 3). Marker release after 60 minutes of incubation was inhibited by 51%. None of the antibodies shown to inhibit marker release when present outside the ghosts caused any inhibition when entrapped

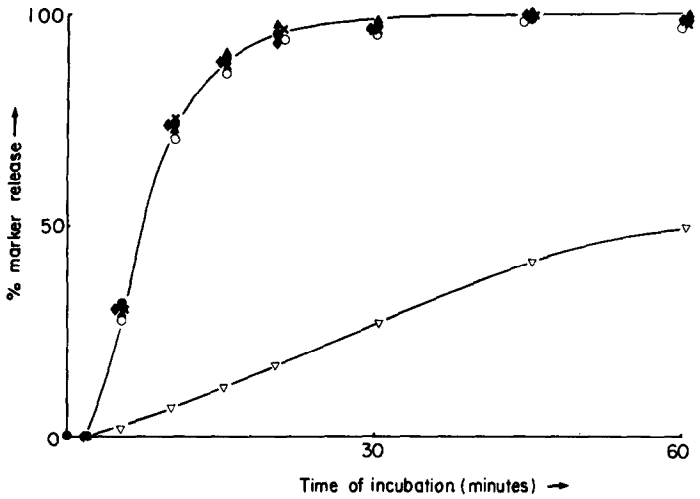

Figure 3.
Effects of entrapped monoclonal antibodies on complement-induced [^{14}C] sucrose release.
GA-C5-9 intermediates entrapping each monoclonal antibody were incubated at 37°C. Portions were removed at intervals, and the released [^{14}C] sucrose measured. All the points are the means of triplicate determinations. (▲= No Antibody, ○= C9-8, ▽= C9-34, ◆= C9-36, ●= C9-42, ✕= C9-47).

within the ghosts, and conversely, C9-34 which inhibited marker release when entrapped, caused no inhibition when present outside the ghosts.

Further evidence that the monoclonal antibodies were inhibiting marker release by binding to C9 outside or inside the ghosts was provided by the preincubation of antibody with C9. Preincubation of antibody with an excess of pure C9 prior to entrapping within ghosts, or adding to GA-C5-9 intermediates, caused a complete loss of antibody-induced inhibition of marker release (data not shown).

These results provide for the first time, firm evidence that C9 within the membrane attack complex on erythrocyte membranes is a transmembrane protein, exposed at the inner and outer surfaces of the membrane. A recent brief report by Whitlow et al (10) described the use of cross-linking reagents entrapped within erythrocyte ghosts to demonstrate that C9 was exposed at the inner membrane surface. However, the possibility of reagent leakage through the complement-damaged membrane as a cause of the C9 cross-linking observed was not ruled out. No such explanation of our findings is possible, as we have demonstrated that antibodies which

block marker release when present inside the ghosts do not block when present outside, and vice versa. Our results also demonstrate that C9 is asymmetrically distributed across the membranes, with different epitopes exposed at the inner and outer surfaces. This finding offers intriguing possibilities for using the monoclonal antibodies to map the C9 molecule in its native state and after insertion into the membrane attack complex as a means of unravelling the conformational changes occurring on insertion.

The use of specific antibodies to the other components of the membrane attack complex should reveal whether C9 is the only component occupying a transmembrane position, or whether other components are also transmembrane.

In summary, we have demonstrated, using a novel application of monoclonal antibodies that:

a) Complement-induced marker release from erythrocyte ghosts can be inhibited by monoclonal antibodies to C9.

b) C9 occupies a transmembrane position in the membrane attack complex on erythrocyte membranes, with different epitopes exposed at the inner and outer membrane surfaces.

These findings support the hypothesis that the complement lesion in the erythrocyte membrane is a transmembrane protein channel made up in whole or in part by C9 molecules (2).

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