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Comparison between Complement and Melittin Hemolysis: Anti-Melittin Antibodies Inhibit Complement Lysis†

Roney O. Laine,‡,§ B. Paul Morgan,‖ and Alfred F. Esser*,‡,§

Laboratory for Structural Biology, Department of Comparative and Experimental Pathology, and Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, Florida 32610

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ABSTRACT: A comparison is made between the hemolytic actions of melittin and the ninth component of complement (C9). Melittin and C9 produce "pores" of similar effective radius in erythrocytes under standardized conditions, and their hemolytic action is suppressed by metal ions at similar concentrations, suggesting a common mechanism. Polyclonal anti-melittin immunoglobulin G (IgG) produced in rabbits retards hemolysis mediated by human C9 in a specific manner. Such antibodies react in several immunoassays with human and monkey C9 but not with C9 from lower animals, and no inhibition of lysis mediated by C9 molecules from these animals is observed. Thus, it is unlikely that anti-melittin IgG reacts with a structural element, such as an amphipathic helix, on human C9 since such structures are also predicted to exist in other C9 molecules. Human C9 and melittin block cross-reactivity in a dose-dependent manner, and anti-melittin IgG recognizes an epitope located between amino acid residues 245 and 390 of human C9 on "Western" blots. Comparison of the melittin and human C9 sequences indicates two regions of complete homology, a tetrapeptide at positions 292–295, and a pentapeptide at positions 527–531 in human C9, corresponding to residues 8–16 in melittin. Inhibition of hemolysis is not caused by blocking of C9 binding to the C5b-8 complex; rather the antibody must dissociate from the bound C9 before lysis ensues, indicating that it interferes with a postbinding event. It is proposed that anti-melittin binds to a conformational epitope on native, folded human C9 and thereby retards unfolding of the molecule, which is required for membrane insertion and hemolysis.

The membrane attack complex (MAC)[1] mediates the cytotoxic effects of complement. It assembles from five water-soluble precursor glycoproteins, C5, C6, C7, C8, and C9, on a target membrane after cleavage of C5 into C5a and C5b and binding of the trimolecular C5b-7 complex. C9, the protein acting last, is responsible for the high efficiency of complement-mediated cytolysis. Although the rough details of the assembly process are understood, the actual mechanism(s) through which the MAC kills cells are still obscure and are a matter of considerable debate (Esser, 1982; Mayer, 1982; Bhakdi & Tranum-Jensen, 1983; Müller-Eberhard, 1984). The primary models for MAC function that are debated are the "leaky patch" model and the "doughnut" model (Esser, 1982). These two models originated from different experimental approaches that were used to study complement-mediated cytolysis. The doughnut model has its roots in the morphological description of the complement lesion as visualized in the electron microscope (Humphrey & Dourmashkin, 1969; Bhakdi & Tranum-Jensen, 1978), whereas the leaky patch model is based on biochemical and biophysical studies aimed at describing the molecular interactions between the terminal complement proteins and target lipid bilayers (Kinsky, 1970; Esser et al., 1979a,b; Sims & Lauf, 1978, 1980). The former envisions formation of protein-walled channels that cause cell death, and the latter postulates strong protein–lipid interactions that reorient lipid bilayers, thereby causing general weakening of the membrane barrier.

We reasoned that a direct comparison between complement-mediated hemolysis and hemolysis resulting from the action of agents that are believed to cause lysis by leaky patch formation might allow a better distinction between the two models for immune lysis. Esser et al. (1979b) had used this approach earlier to compare the efficiency to cause virolysis between channel formers, such as nystatin, and complement. Since formation of nystatin channels across the viral membrane had no effect on infectivity, they concluded that complement

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* Address correspondence to this author at the University of Florida, Box J-145/JHMHC, Gainesville, FL 32610.
‡ Department of Comparative and Experimental Pathology.
§ Department of Biochemistry and Molecular Biology.
‖ Present address: Department of Medical Biochemistry, University of Wales School of Medicine, Cardiff CF4 4XB, U.K.

[1] Abbreviations: MAC, membrane attack complex; EDTA, ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; TBS, Tris-buffered saline; EA, antibody-sensitized erythrocytes; EAC1–8, EA carrying complement proteins C1 through C8; kDa, kilodalton(s). Complement proteins are named in accordance with recommendations in *Bulletin of the World Health Organization* (1968).

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does not inactivate these viruses because of channel formation. These authors also noticed that melittin, the lytic component of bee venom, could lyse viruses in a fashion indistinguishable from that of complement.

Melittin is a water-soluble peptide of 26 amino acids that has frequently been considered to be the prototype of a leaky patch former. The peptide integrates into membranes, thereby disrupting membrane function and causing cell lysis (Sessa et al., 1969). X-ray crystallography (Terwillinger et al., 1982) established an amphipathic, bent α-helical rod structure for the peptide in the crystal, and several reports indicate that melittin can bind as a monomer to membranes and induce hemolysis (Lauterwein et al., 1979; Levin et al., 1982; Dasseux et al., 1984; Hermetter & Lakowicz, 1986). We have now compared hemolysis caused by melittin and by human C9 to search for additional similarities between these two hemolytic systems and have observed that both agents are almost identical in their effects on erythrocytes and show in addition a remarkable immunologic cross-reactivity. Parts of this work have appeared in preliminary form (Esser et al., 1980; Laine & Esser, 1987).

Materials and Methods

Proteins. Human complement proteins C8 and C9 were isolated from Cohn fraction III as described by Esser and Sodetz (1988). Natural bee venom melittin was purchased from Sigma and further purified as described by Quay and Condi (1983). A sample of synthetic melittin was the gift of Dr. F. Prendergast (Mayo Clinic).

Sera. Human serum was prepared from fresh frozen plasma (Civitan Regional Blood Center, Gainesville, FL) by addition of $CaCl_2$ to a final concentration of 20 mM and incubation for 2 h at 37 °C. Sera depleted in C8 or C9 were prepared as described (Esser & Sodetz, 1988). Sera from monkeys, bovine, rabbits, guinea pigs, rats, and mice were obtained from the Laboratory of Animal Medicine, University of Florida.

Preparation of Antibodies to Melittin. Antisera to melittin were prepared in several New Zealand White rabbits by using differently prepared or different sources of antigen. All immunization schedules were similar; first, injection of antigen (0.5 mg per rabbit) in complete Freund's adjuvant, followed 4 weeks later by two booster injections (0.5 mg per rabbit) in incomplete Freund's adjuvant at intervals of 2 weeks. The first antiserum was produced by injecting melittin that had been cross-linked with glutaraldehyde to the carrier hemocyanin, whereas all subsequent antisera were produced with non-cross-linked carrier-free melittin (0.5 mg per rabbit). The latest antiserum was produced with synthetic melittin. All sera behaved qualitatively similar although their titers were not identical. Polyclonal immunoglobulin G (IgG) from such sera was isolated as published previously (Dankert et al., 1985). Antibodies were also prepared by immunoaffinity chromatography on solid-phase melittin (Kaetzel & Dedman, 1987). For this purpose purified melittin was coupled to cyanogen bromide activated Sepharose 4B (Pharmacia), and 126 mg of anti-melittin IgG was applied to the column. After extensive washing with 0.5 M NaCl in 10 mM Tris buffer, pH 7.2, the column was eluted with 0.1 M glycine hydrochloride (pH 2.3) and antibody-containing fractions were dialyzed against Tris-buffered saline (TBS; 10 mM Tris, 0.15 M NaCl, pH 7.2). We also received a sample of affinity-purified anti-melittin IgG prepared against melittin coupled to hemocyanin from Dr. J. R. Dedman (University of Texas Medical School, Houston, TX).

Hemolytic Assays. Hemolytic activity of C9 was assayed by a standard method using EAC1-8 as indicator cells as described by Dankert et al. (1985). These cells were prepared from sheep erythrocytes and human complement proteins. The effects of solutes or antibodies on C9-mediated lysis of EAC1-8 cells, however, were measured by continuously monitoring forward light scattering (Fischer, 1967) of the stirred cell suspension in a spectrophotometer with thermostated cuvette holders. Hemolysis mediated by C9 from different animals was initiated by adding 40 μL of animal serum, 100-fold diluted and containing 10 mM EDTA, as a source of C9 to 1.25×10^7 EAC1-8 cells.

Hemolysis induced by melittin was measured according to the same technique. Inhibition of hemolysis by metal ions was assessed by following the procedure of Boyle et al. (1979), and the effect of increasing size of the buffer ions on lysis was measured as described by Aubert and Motais (1975).

Kinetics of Antibody Binding. To measure the kinetics of anti-melittin IgG binding to EAC1-8, 6.7 μM anti-melittin IgG (containing ^{125}I-anti-melittin IgG as a tracer) was incubated with 0.3 nM human C9 at 4 °C for 30 min before addition of 2.5×10^7 EAC1-8 cells. Five minutes later the cells were rapidly warmed up to 37 °C, aliquots were withdrawn at different time points and pelleted, and hemoglobin concentration in the supernatant and radioactivity associated with the pellet (after an additional washing step) were determined. Alternatively, the cells were washed twice at 4 °C to remove unbound antibody and C9 and then warmed to 37 °C; hemolysis and antibody binding were determined as described above.

Immunoblotting and ELISA Methods. C9 or C9 proteolyzed by α-thrombin or trypsin (Dankert & Esser, 1986) and sera from different species were electrophoresed on polyacrylamide gels in SDS under nonreducing conditions as detailed by Laemmli (1970). The separated proteins were then transferred to nitrocellulose sheets according to the procedure of Towbin (1979), incubated with anti-melittin or anti-C9 IgG, and visualized with a secondary antibody conjugated to alkaline phosphatase (HyClone Laboratories, Logan, UT) following the general procedures of Blake et al. (1984) and Johnson et al. (1984).

Enzyme-linked immunosorbent assays (ELISAs) were used to measure melittin-specific antibodies and to titer cross-reactivity with human C9 (Engval & Perlmann, 1971). Melittin and C9 were bound to microtiter plates (Falcon Microtest III, Becton Dickinson) at a concentration of 0.25 μg/well, and changes in absorbance of the reaction mixture (50 μL/well) were followed directly in a kinetic microplate reader (Molecular Devices Inc., Palo Alto, CA). For the competition ELISAs, anti-melittin IgG (20 μg/mL) was preincubated with various concentrations of either C9 (0.01-2.4 mg/mL) or melittin (0.01-12 pg/mL), and then the mixture was added to melittin- or C9-coated wells, respectively. Goat anti-rabbit IgG conjugated with horseradish peroxidase (HyClone Laboratories, Logan, UT) and *o*-phenylenediamine were used to detect positive wells.

Results

Apparent Size of Melittin and Complement Channels. Erythrocytes (E) are impermeable to cations, and therefore, any disruption of the cell's membrane barrier that leads to cation flux results in colloid osmotic lysis. One can conveniently estimate the equivalent pore size of channels or leaks by measuring the rate or time required to achieve 50% hemolysis ($t_{50\%}$) in buffers prepared from ions with increasing Stokes' radius (Albert & Motais, 1975). We reasoned that a comparison between the relative effects of different buffer ions on $t_{50\%}$ of complement and melittin hemolysis could reveal

Table I: Effect of Buffer Solutes on Melittin and C9 Hemolysis

buffers, 300 mOsm, pH 7.0	lysis half-times (arbitrary units)[a] E_SAC1-7 + C8,9	E_S + melittin	E_RAC1-7 + C8,9	E_R + melittin
Na^+Cl^-	1	1	1	1
choline(1+)	1-2	1-2	0.5-1	0.5-1
$^+PyrCH_2CH_2Pyr^+$	5-7	7-8	5-7	2-4
phosphate(2-)	3-4	4-8	4-5	2-4
succinate(2-)	3-5	4-8	5-8	5-8
citrate(3-)	10-15	29-31	8-10	12-15
EDTA(3-)	9-12	29-31	10-13	11-14

[a] Hemolysis for all cells in NaCl was adjusted experimentally to 15 (±1) min and then set to 1 in arbitrary units; the indicated values for other solutes are the range obtained in four different experiments.

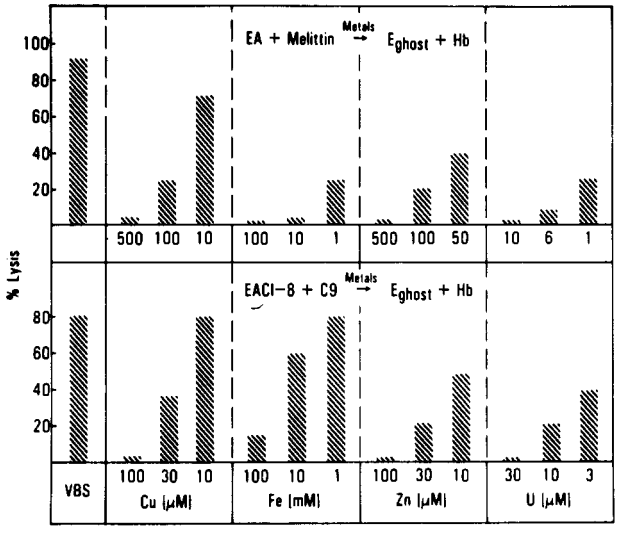

FIGURE 1: Influence of metal ions on melittin- and C9-mediated hemolysis. Top panel: Antibody-coated erythrocytes (EA) in veronal-buffered saline (VBS) were incubated with an amount of melittin sufficient to lyse 90% of the cells after 30 min. The metals were present during the incubation in the form of ions at the indicated concentrations. At the end of the incubation time the cells wer centrifuged and the hemoglobin concentration in the supernatant was measured by absorbance at 412 nm. Bottom panel: EAC1-8 in VBS were incubated with an amount of human C9 sufficient to lyse 80% of the cells after 60 min. The metal ions were present during the incubation, and hemolysis was measured as indicated above.

whether or not both lytic agents produce holes of similar sizes. Sheep (E_S) and rabbit (E_R) erythrocytes are incubated with melittin in isotonic buffered NaCl to yield a $t_{50\%}$ value of ≈15 min. Similarly, E_S or E_R were incubated with antibody (A) and complement (C) components to produce EAC1-8. Such cells were then incubated in isotonic buffered NaCl with amounts of purified human C9 sufficient to produce lytic rates with similar $t_{50\%}$ (≈15 min). Hemolysis was measured spectrophotometrically at 620 nm by continuously recording changes in forward light scattering. Table I lists the $t_{50\%}$ values of hemolysis for both agents and cell types in buffers of increasing size. The delay in hemolysis produced by melittin and complement parallel each other quite well, indicating that the holes produced are of similar effective size.

Effect of Metal Ions on Complement and Melittin Lysis. Previous experiments by Boyle et al. (1979) had indicated that C9-mediated lysis of EAC1-8 was inhibitable by metal ions, and Esser et al. (1980) and Bashford et al. (1986) had provided evidence that melittin-mediated lysis of erythrocytes and killing of Lettré cells, respectively, were also inhibitable by metal ions. We have now compared the effects of metal ions on complement- and melittin-mediated hemolysis of the same target cells under standardized conditions. The results of these experiments (Figure 1) demonstrate that erythrocytes from two

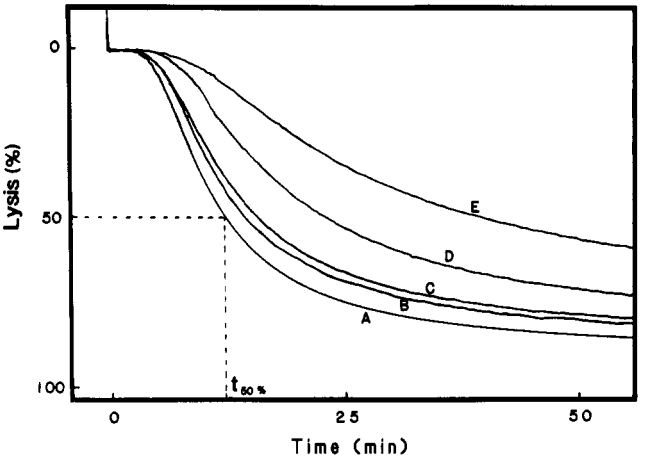

FIGURE 2: Effect of anti-melittin on lysis of EAC1-8 by human C9. Forward light scattering of 1.25×10^7 cells suspended by continuous stirring in a total volume of 2 mL of TBS at 37 °C was monitored at 620 nm. Addition of C9 (0.3 pmol) caused a decrease in light scattering (curve A), indicating hemolysis. The time required to lyse 50% of the cells is indicated as $t_{50\%}$. Addition of 0.3 pmol of C9 that was preincubated with either or 6.0 nmol (curve C), 15 nmol (curve D), or 30 nmol (curve E) of anti-melittin IgG resulted in progressive retardation of lysis. Incubation with 30 nmol of nonimmune IgG, corresponding to the highest amount of specific anti-melittin IgG, caused a small amount of inhibition (curve B).

Table II: Inhibition of C9-Mediated Lysis of EAC1-8 by Anti-Melittin IgG

serum C9 source	lysis half-time, $t_{50\%}$ (s), anti-melittin IgG +	−	$\Delta t_{50\%}$
human	820	580	240
monkey	840	560	280
bovine	980	840	140
rat	580	440	140
mouse	900	760	140
guinea pig	180	160	20
	lysis half-time, $t_{50\%}$ (s), nonimmune IgG +	−	$\Delta t_{50\%}$
human C9	880	740	140

different species are protected by such ions from lysis by both agents to almost identical degrees.

Immunologic Cross-Reactivity between Melittin and Human C9. Encouraged by these results, we searched for more direct structural similarities between melittin and C9. The amphipathic α-helix formed by melittin is thought to be required for lysis (Terwilliger et al., 1982). Thus, we produced antibodies against melittin with the hope that such antibodies might detect similar features in C9 that are necessary for lysis. Figure 2 demonstrates that purified polyclonal anti-melittin IgG inhibits in a concentration-dependent manner C9-mediated lysis of EAC1-8. All inhibitory activity can be removed by passing the antibodies through a column of immobilized melittin (data not shown). Table II list $t_{50\%}$ values of EAC1-8 lysis by C9 present in sera from different animals and the effect of melittin-specific IgG on lysis half-times. Because nonimmune IgG has a slight effect on $t_{50\%}$, we also calculated $\Delta t_{50\%}$ values by subtracting $t_{50\%}$ values recorded with nonimmune IgG from the $t_{50\%}$ values recorded in the presence of anti-melittin IgG. It is evident that anti-melittin IgG affects only human and monkey C9 but not C9 from lower animals.

Inhibition of C9-mediated hemolysis could result either from blocking of C9 binding or from interference with a postbinding

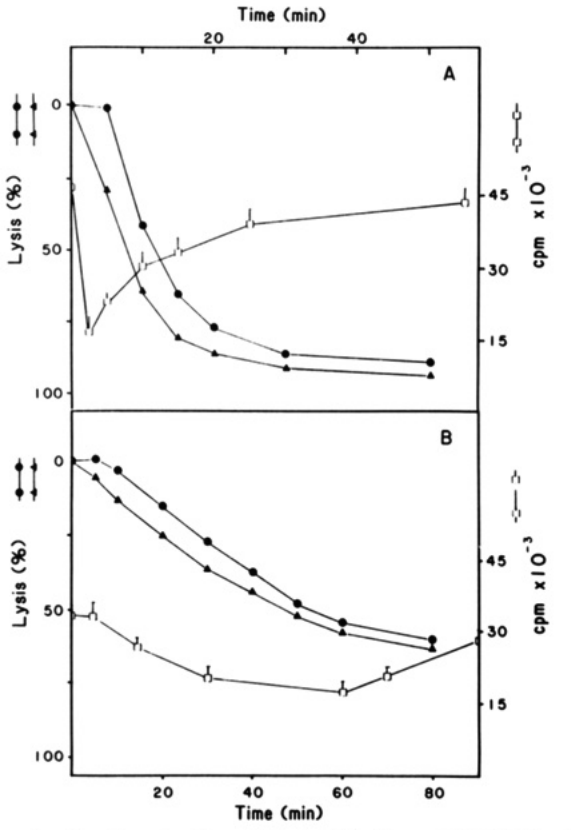

FIGURE 3: Kinetics of anti-melittin IgG binding to EAC1–8 in the presence of C9. Panel A: EAC1–8 cells were incubated at 4 °C with human C9 and with (●) or without (▲) ^{125}I-anti-melittin IgG. After being warmed to 37 °C, aliquots were withdrawn and centrifuged at the indicated time points, and binding (open symbols) of radioactive antibody to the cell pellet and hemolysis (A_{412} of the supernatant; closed symbols) were measured. Panel B: Preincubation at 4 °C was performed as described above, but cells were washed once in the cold to remove unbound C9 and radioactive IgG before warming to 37 °C. ^{125}I-IgG binding and hemolysis were measured as described for panel A.

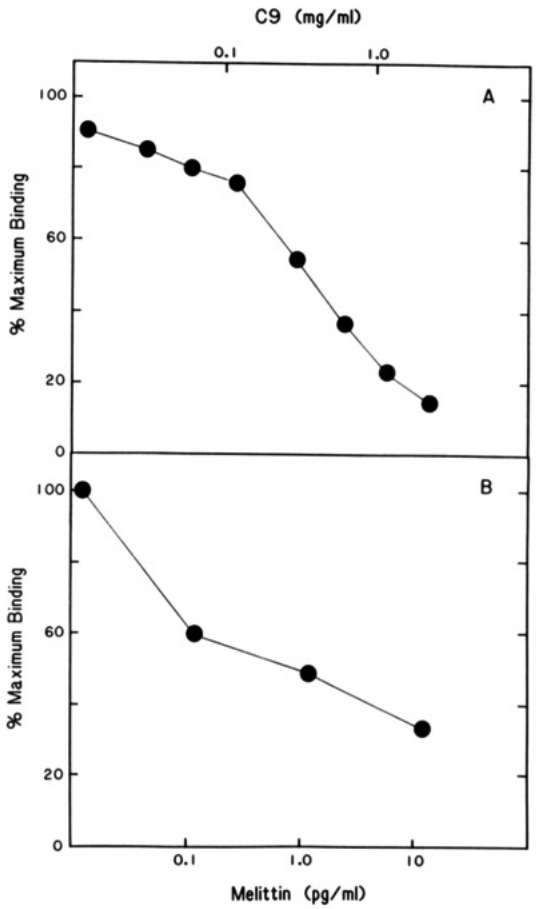

FIGURE 4: Inhibition of anti-melittin IgG binding to C9- or melittin-coated microtiter plates. Panel A: Plates were coated with melittin, and anti-melittin IgG was added to achieve maximal binding as measured by ELISA using anti-rabbit IgG coupled to horseradish peroxidase. An identical amount of anti-melittin IgG was preincubated with the indicated concentrations of human C9, and the extent of binding was measured as before. Panel B: Microtiter plates were coated with human C9, and blocking of anti-melittin IgG binding by increasing concentrations of melittin was measured as described above.

event, such as C9 membrane insertion, by the added antibodies. To distinguish between these possibilities, we compared the kintics of binding of radioiodinated anti-melittin IgG with the rate of hemolysis. The assay was performed in two ways: (a) excess free C9 was present continuously during the assay (Figure 3A), or (b) the efficiency of only bound C9 was determined (Figure 3B). The results clearly demonstrate that C9 binding to EAC1–8 cells is not inhibited bt the antibody but that ^{125}I-anti-melittin IgG actually binds to EAC1–8 in the cold presumably as a C9–IgG complex. When excess IgG and C9 are washed away at lower temperatures and the cells are then warmed to 37 °C, the antibody is released before hemolysis sets in (Figure 3A), suggesting that it interferes with a postbinding event. Conducting the assay in the continuous presence of free C9, however, produces, after an initial drop, again an increase in membrane-bound ^{125}I-IgG that is not seen in the absence of excess C9.

The inhibitory effect of anti-melittin IgG on lysis elicited by human C9 resulted from immunologic cross-reactivity between melittin and C9. As shown in Figure 4A, polyclonal anti-melittin IgG reacts strongly in ELISA with melittin adsorbed to polystyrene microtiter wells, and this reaction can be inhibited in a dose-dependent manner by preincubation of the antibody with monomeric native human C9. The reverse experiment where C9 was fixed to the plate and fluid-phase melittin was used during the preincubation gave the same result (Figure 4B).

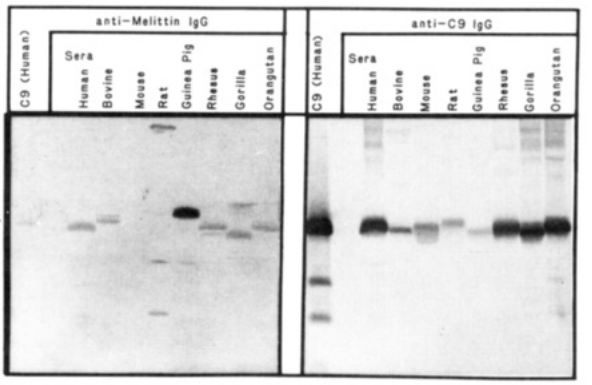

FIGURE 5: Binding of anti-melittin IgG and anti-human C9 to C9 from different species on Western blots. Purified human C9 (0.2 μg) and sera (0.2 μL) from the indicated species were electrophoresed in SDS and transferred to nitrocellulose sheets as described under Materials and Methods. Binding of anti-melittin IgG (left panel) and anti-human C9 (right panel) was detected with alkaline phosphatase conjugated anti-rabbit IgG.

Epitope Mapping. "Western" blotting was used in an attempt to identify the epitope in C9 that is recognized by anti-melittin IgG. As shown in Figure 5 (left panel), antibodies prepared against synthetic melittin react strongly with purified human C9 and with C9 contained in human and monkey sera but did not cross-react with proteins of similar molecular

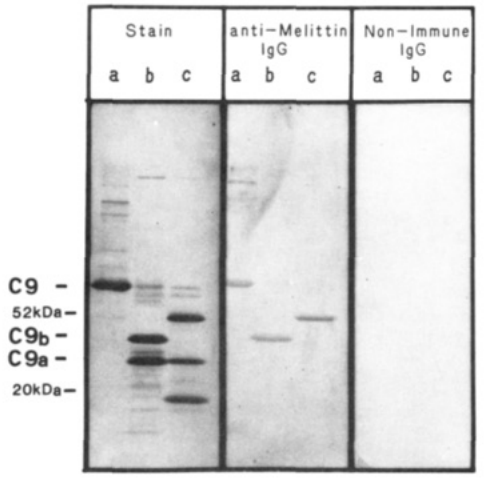

FIGURE 6: Localization of the anti-melittin epitope on C9 by Western blotting of C9 and fragments. Human C9 (lanes a) and fragments derived from digestion with α-thrombin (lanes b) or trypsin (lanes c) were electrophoresed in SDS and transferred to nitrocellulose sheets as described under Materials and Methods. The left panel shows a Coomassie blue stained sheet indicating the location of C9 and the thrombin and trypsin fragments. Binding of anti-melittin IgG to C9, the C9b thrombin fragment, and the amino-terminal trypsin fragment is visualized by alkaline phosphatase staining (center panel). The right panel is a control with nonimmune IgG.

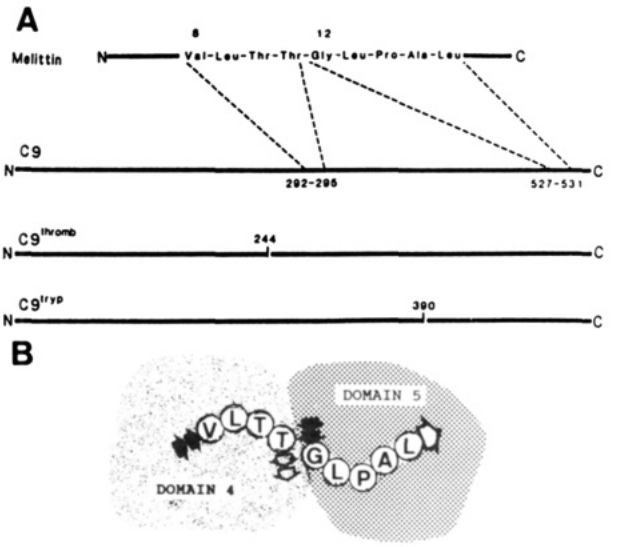

FIGURE 7: Panel A: Location of identical peptide segments in melittin and human C9 and the major cleavage sites of α-thrombin and trypsin. Panel B: Hypothetical folding of domains 4 and 5 in native C9.

weight in sera from lower animals although polyclonal anti-human C9 IgG cross-reacts with C9 from all species (Figure 5, right panel). Reduction and alkylation of sulfhydryl groups in C9 abrogated recognition by anti-melittin. Identical results were obtained with purified IgG isolated from immune sera elicited by biological melittin, and independent of whether or not a carrier protein was used and what type; anti-melittin IgG produced in another laboratory (Kaetzel & Dedman, 1987) was likewise effective (data not shown). When C9-derived proteolytic fragments were transferred to nitrocellulose and tested with anti-melittin IgG, the C9b carboxy-terminal fragment produced by α-thrombin and the 52-kDa amino-terminal fragment produced by trypsin react positively with anti-melittin IgG (Figure 6). The results from these experiments locate the epitope in a region between residues 245 and 390 in the C9 sequence. A computer search indeed found a tetrapeptide (-VLTT-; residues 292–295) that is identical with residues 8–11 in melittin but also a pentapeptide (-GLPAL-; residues 527–531) with complete identity to residues 12–16 in melittin (Figure 7). However, it should be noted that no cross-reactivity with the carboxy-terminal 20-kDa trypsin fragment of human C9 that contains this pentapeptide was detectable on immunoblots. According to a structural model of C9 (Stanley & Herz, 1987), the tetrapeptide is located in domain 4 and the pentapeptide in domain 5 of C9. In mouse C9 the corresponding tetrapeptide is -VLRS- and the equivalent pentapeptide is missing because of a deletion of the last 14 amino acids (Stanley & Herz, 1987); there is, however, a very similar pentapeptide in mouse C9 (-PALIS-; residues 445–449) that is identical with residues 14–18 in melittin.

Putative Amphipathic Structures in Mouse and Human C9. Because anti-melittin IgG did not cross-react with mouse C9, it was of interest to see whether or not the latter contains predicted amphipathic structures. As shown in Table III, the predicted (Eisenberg et al., 1986) amphipathic secondary structures of human and mouse C9 are quite similar.

Table III: Predicted Amphipathic α-Helices in Mouse and Human C9[a]

mouse C9 sequence amino acids	human C9 sequence amino acids	mouse C9 sequence amino acids	human C9 sequence amino acids
	35–43	331–339	334–342
43–51	44–52		353–361
	131–139	401–409	401–409
193–201	200–208	434–442	432–440
250–258		476–484	473–481
273–281	280–288		

[a] Amphipathic α-helices are predicted for 9 amino acid segments in mouse and human C9 by using the algorithm and the normalized hydrophobicity values of Eisenberg et al. (1984).

DISCUSSION

In this study we have provided further evidence that complement protein C9 can lyse red blood cells by a mechanism that is very similar to if not identical with the mechanism used by the amphipathic peptide melittin. Not only are the apparent "hole" sizes produced by the two lytic agents very similar under standardized conditions, external agents, such as metal ions, inhibit both lysins to an equal degree. These observations are consistent with the hypothesis that C9 and melittin affect the bilayer structure of lipids that provide the barrier against free solute flow across the plasma membrane. As discussed in detail by Esser (1982) and Bashfort et al. (1986), modulation of the lipid structure could be a common feature in the mechanism of lytic toxins. Previous studies have indeed demonstrated that chaotropes, such as KSCN (Giavedoni & Dalmasso, 1976), surfactants, such as deoxycholate (Stolfi, 1968) and A_2C (Shin et al., 1981), and metal ions (Götze et al., 1968; Boyle et al. 1979, Bashford, et al., 1986) that influence membrane structure modulate the hemolytic action of complement. At the present time, however, it is difficult to provide more than a phenomenological comparison between different toxins since the exact molecular details and requirements for lysis are still largely unknown.

These difficulties became apparent again in our attempts to provide a structural basis for the similarities between melittin and complement-mediated hemolysis. Investigators studying the mechanism of melittin-mediated hemolysis generally agree with the notion that the amphipathic α-helical conformation of the peptide is a required structural element for function [for review, see Kaiser and Kezdy (1984)]. C9 is thought to contain several of these structures, and one could argue that these amphipathic elements likewise play a role in

complement lysis (Shiver et al., 1986; Stanley et al., 1986). To provide direct experimental evidence for this possibility, we decided to produce antibodies against melittin in the hope that antibodies could be elicited that specifically recognize an amphipathic helix. The feasibility of this approach was demonstrated by Khalil et al. (1986), who showed that a monoclonal antibody against a peptide forming a putative amphipathic α-helix in human apolipoprotein A-1 recognized peptides with different amino acid sequences provided that they still had the possibility to form an amphipathic helix. As demonstrated here, anti-melittin antibodies recognize human C9 specifically. To assure that the observed cross-reactivity was specific for anti-melittin antibodies, we did not use carrier proteins for immunization, and by using synthetic melittin we verified that melittin was indeed the immunogen and not a contaminant in the biologically derived material. Thus, we are confident that the observed cross-reactivity is between anti-melittin IgG and human C9. While these experiments were in progress, two reports appeared demonstrating that anti-melittin antibodies could be used to identify calmodulin-binding domains on calmodulin target proteins (Kaetzel & Dedman, 1987) and also to detect phospholipase stimulatory proteins in cells (Clark et al., 1987). Apparently the antigenic sites on melittin are also present on other proteins.

However, it appears that our anti-melittin antibodies do not cross-react with human C9 just because they detected an amphipathic helix per se. This conclusion is based on the observation that anti-melittin IgG recognized only human and monkey C9 but not C9 in sera from lower animals. As shown in Table III, mouse C9 contains almost as many predicted amphipathic α-helical structures as human C9, but no cross-reactivity with mouse C9 nor inhibition of mouse C9 mediated lysis was observed. Nevertheless, we do believe that also in our system anti-melittin antibodies are effective probes of tertiary structure as they are in the case of calmodulin-binding proteins (Kaetzel & Dedman, 1987). This contention is supported by the fact that there are two segments in human C9 that are present in melittin (Figure 6), and it is conceivable that C9 is folded in such a way as to simulate the structure of melittin as sketched in Figure 7B. Whether or not the predicted antigenic site is folded in form of a helix is, of course, unknown, nor do we have as yet direct experimental evidence that the epitope in human C9 recognized by anti-melittin is a conformational epitope composed of amino acids 292–295 and 527–531. However, several observations are consistent with this hypothesis. First, anti-melittin IgG detects a human C9 fragment that contains the homologous tetrapeptide. Second, in the nonreactive mouse C9 both parts of the epitope are different; i.e., the equivalent tetrapeptide contains a positive charge not present in the human tetrapeptide, and the pentapeptide is entirely missing or a pentapeptide with a different sequence is present but is located in a different segment. Third, anti-melittin IgG reacts only with folded human C9 since reduction and alkylation abrogate reactivity. Fortunately, our prediction can be tested experimentally by synthesizing peptides that contain the identical segments together with the amino- and carboxy-terminal flanking residues, i.e., peptides 287–297 and 523–533, and by assaying whether or not (a) anti-melittin reacts with such peptides, (b) antibodies directed against such peptides affect C9 function, and (c) such anti-peptide antibodies block C9 recognition by anti-melittin. These studies are presently in progress.

With respect to the mechanism of inhibition of lysis by anti-melittin IgG it is evident that inhibition did not result from prevention of C9 binding to the C5b-8 site on the target cell. It is clear that anti-melittin bound together with C9 on the target cell but that it had to be released before lysis proceeded. Using the cartoon shown in Figure 7B and the C9 model of Stanley and Herz (1987) as a guide, we predict that the contact region between the putative domains 4 and 5 provides the epitope for anti-melittin IgG and that the antibody retards unfolding or separation of these domains since lysis proceeds after release of the antibody. We agree with Stanley and Herz (1987) that domain 4 is involved in the unfolding process leading to hemolysis but prefer that unfolding between domains 4 and 5 and not between domains 3 and 4 as suggested by them is crucial for function. This view is supported by the fact that several epitopes present on domain 5 are affected strongly when C9 changes from its solution structure to its membrane-bound form (Laine & Esser, 1987).

A further interesting fact is our observation that anti-melittin IgG binding to the target membrane increases after an initial drop (Figure 3A). To reconcile the results that anti-melittin antibodies can bind to C9 in solution and must dissociate to allow functional expression, and yet also bind to C9 when associated with a membrane, we propose that two forms of C9 exist on the target membrane after lysis. Earlier experiments by several investigators, notably Borsos and his colleagues [reviewed by Boyle and Borsos (1982) and Boyle (1984)], suggested the existence of lytic and nonlytic or aborted MACs. Thus, when anti-melittin IgGs bind to C9 in solution, they retard expression of the lytic C9 form, possibly by preventing C9 insertion, but later they bind again to C9 molecules that have not unfolded but are associated with the target and may not have inserted.

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Demembranated Muscle Fibers Catalyze a More Rapid Exchange between Phosphate and Adenosine Triphosphate than Actomyosin Subfragment 1†

R. Bowater‡ and J. Sleep*
Cell Biophysics Unit, 26-29 Drury Lane, London WC2B 5RL, England
Received July 22, 1987; Revised Manuscript Received February 10, 1988

ABSTRACT: The rate of ATP $\rightleftharpoons$ P_i exchange, that is, the incorporation of medium P_i into ATP during the net hydrolysis of ATP, has been measured for rabbit psoas muscle fibers, myofibrils, and actomyosin subfragment 1 (acto-S1). The maximum exchange rate in fibers at saturating $[P_i]$ is 0.04 s^{-1} per myosin head at 8 °C, pH 7, and an ionic strength of 0.2 M. The dependence of the rate on P_i concentration can be approximated by a hyperbola with an apparent dissociation constant (K_m) of 3 mM. Myofibrils catalyze ATP $\rightleftharpoons$ P_i exchange with a similar K_m but at a slightly lower rate. In contrast, the soluble acto-S1 system, in which ATP hydrolysis is not coupled to tension generation, catalyzes exchange at a rate 500 times lower than that of fibers at low P_i concentration, and the K_m for P_i is greater than 50 mM. The difference between the ATP $\rightleftharpoons$ P_i exchange of fibers and of acto-S1 is discussed in terms of a model in which P_i binds to a force-generating state AM′·ADP and, due to mechanical constraint, the average free energy of this state is higher in the fiber than in acto-S1.

The kinetics of the hydrolysis of ATP by myosin subfragment 1 in the presence of actin are understood in moderate detail in solution. However, in intact muscle, movement of the myosin cross bridge while bound to actin results in work production, and the nature of the coupling between the chemical reaction and cross-bridge movement is less clear. Work on the thermodynamics of myosin and actomyosin (AM) ATPase (Goody et al., 1977; Cardon & Boyer, 1978; White & Taylor, 1976; White, 1977) led to the conclusion that the release of P_i from AM·ADP·P_i to give AM·ADP (the state formed by adding ADP to AM) has a dissociation constant, K_d, of about 300 M. The physiological concentration of P_i in unfatigued muscle is about 0.1 mM, and the free energy change associated with P_i release is about 35 kJ [RT ln

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‡ Present address: Division of Physical Biochemistry, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, England.

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