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Mechanism of Cytolysis by Complement

(erythrocytes/leaky-patch model/doughnut model)

MANFRED M. MAYER

Department of Microbiology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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ABSTRACT **The attack of complement is directed against the lipid moiety of the cell membrane; a single lesion at the site of fixation of complement proteins C5–C9 is responsible for lysis of a cell. There are two hypothetical models for the generation of this membrane lesion. The first of these, designated the *leaky-patch model*, postulates either direct enzymatic attack or enzymatic generation of a lytic substance by C5-C9. As a result, the phospholipid bilayer of the membrane would be disrupted and a leaky patch permitting passage of water and salt would appear. However, this hole would persist only as long as enzymatic action continues. Thus, the leaky-patch model would not produce a stable hole, and for this reason it is considered an unlikely mechanism.**

The second hypothesis, termed the *doughnut model*, describes a structural concept for creating a hydrophilic passage through the hydrophobic phospholipid bilayer of the membrane. In essence, this would be a rigid and hollow structure, like a doughnut, with a hydrophobic exterior, which is inserted into the phospholipid bilayer of the cell membrane in such a way that its hollow hydrophilic core becomes a channel through which salt and water can exchange freely between the interior of the cell and the extracellular environment. The late-acting complement proteins C5–C9 are the most probable source of the structural components of the doughnut. A combination of the leaky-patch and doughnut models may represent the most likely mechanism.

Recent studies of complement (C) have focused attention on the late-acting components, C5–C9, which make up the cytolytic part of the system (1–8). It has been shown that the $\overline{C4, 2, 3}$ enzyme activates C5 by cleavage (2, 3) and that the resulting C5b fragment combines with C6, which serves as a stabilizer (4). In turn, the C5b, 6 complex combines with C7 (5, 6) yielding C5b, 6, 7, which appears to be the foundation of the lytic attack mechanism (1). After uptake of C8 by this complex, partial membrane damage is produced as evidenced by slow lysis of erythrocytes in the state erythrocyte–antibody–complement components 1–8 (EAC1–8) on incubation at 37° (7). When the terminal complement component, C9, joins the attack system, rapid lysis ensues indicating overt membrane damage. Recent studies of the reaction of EAC1–8 with C9 (8) have reaffirmed the one-hit theory in its strict sense, namely, that a single membrane lesion at the C5–C9 locus suffices for lysis, and this has led to an examination of possible mechanisms for producing this lesion. These are described in the present paper in relation to newer concepts of membrane structure.

Abbreviations: C, C1, C2, C3, etc.—C refers to complement, and the numbers indicate the components of the complement system. The letters "a" or "b", as in C5b, refer to fragments. The "bar" in $\overline{C1}$ or $\overline{C4, 2, 3}$ indicates an enzymically active component or complex. EAC1–5 is a shorthand designation for cells carrying antibody and components C1, C4, C2, C3, and C5. Nomenclature of *Bull. Wld. Hlth. Org.* (1968) **39**, 935–938.

The fluid mosaic model of membrane structure

Physicochemical studies of the proteins and lipids in cell membranes have led to a new concept of membrane structure, termed the fluid mosaic model (9, 10). As in the Davson–Danielli model (11), amphipathic phospholipids are believed to form a double layer within which the hydrophobic tails appose each other, while the hydrophilic heads point outward toward the bulk aqueous phase. The lipid bilayer represents a viscous fluid matrix in which other lipids, as well as globular proteins, lipoproteins, and glycoproteins are embedded. Some of the proteins are believed to penetrate through the entire thickness of the lipid bilayer. It is thought that the proteins are in globular form, with a substantial proportion of alpha-helix, but little or no beta conformation. The membrane surface is thought to be a hydrophilic mosaic of protein and lipid, the former being oriented so that the hydrophilic regions, such as the polar amino acids or the carbohydrate moiety of glycoproteins, face outward toward the bulk aqueous phase. A significant aspect of this model is the element of fluidity, in contrast to the rigidity implicit in earlier models. As a consequence, the lipids and at least some of the proteins can undergo more or less lateral movement within the lipid matrix.

It is probable that the lytic attack mechanism of the complement system acts on the lipid matrix of the cell membrane, rather than on its proteins, since liposomes made up of lipids and glycolipids can be lysed by complement (12, 13). Furthermore, it is known from studies with erythrocytes, as well as Krebs ascites tumor cells (14), that complement produces a hole in the cell membrane that is large enough to permit free exchange of salts and water between the interior of the cell and the surrounding fluid, but that is not large enough to permit release of the macromolecular contents of the cell. Due to the Donnan effect, there is a net uptake of salt and water through this hole, and the resultant swelling then produces large discontinuities in the membrane through which macromolecules leave the cell. This swelling is a secondary phenomenon of no direct concern to the models to be discussed and, therefore, the present paper is limited to consideration of the structural and chemical requirements for the formation of the primary hole in the fluid lipid matrix of the membrane.

The one-hit theory

In developing a plausible model for the cytolytic attack mechanism account must be taken of the restriction imposed by the one-hit theory. This theory was developed during the 1950's from kinetic and statistical studies (15), and recently it has been shown by direct chemical analysis of C2 that a single molecule of this component suffices for lysis of an erythrocyte under optimal experimental conditions (16). When initially formulated, and until about 10 years ago, it was my interpretation of the one-hit theory that a single lesion in the membrane of a cell actually suffices for lysis. However, this view became questionable when it was found that $C\bar{1}$ cleaves the C2 molecule (17) and that the $C\overline{4,2}$ convertase cleaves C3 (18), thus producing a shower of C3 fragments some of which become bound to the cell membrane. If enzymatic cleavage at a single site on the membrane can produce a large number of fragments, which then bind at numerous other membrane sites, it could no longer be assumed, *a priori*, that the one-hit theory *necessarily* indicates a single membrane lesion to be sufficient for lysis of a cell. Conceivably, each of the fragments produced in a shower originating from a single enzymatic site might give rise to a membrane lesion.

The one-hit theory has again received close attention in recent studies of the reaction of C9 with EAC1–8 in which it was shown by kinetic experiments, including temperature profile studies, that C9 becomes bound to these cells by a nonenzymic process (8). It was found that the reaction conforms to the one-hit theory and that its efficiency is so high that input of only 1–2 molecules of C9 per cell suffices for lysis of EAC1–8 under optimal conditions. It was also shown that the fixation of C9 on EAC1–8 is very firm, even though it is probable, in light of the nonenzymic fixation mechanism, that C9 becomes bound noncovalently. It is very likely, therefore, that the C9 molecule is restricted to its site of initial fixation, i.e., it would not tend to "wander" over the cell surface by a process of repeated dissociation and association.

It is also necessary to consider the possibility that one of the late-acting complement components is an enzyme, which produces a substance capable of damaging the cell membrane. Conceivably, the molecules of such a substance could diffuse from their site of generation and attack the cell membrane at numerous remote loci. However, this possibility can be ruled out for a number of reasons. Firstly, attempts to demonstrate a lytic substance in the aqueous phase after complement action have not been successful*. Secondly, the cytolytic action of complement is largely restricted to the cell on which the antibody and complement components are fixed. Except for the possibility of transfer of the C5b, 6, 7 complex to other cells, which is a minor effect, bystander cells are not destroyed by complement. Thirdly, cells in the state EAC1–9 will undergo lysis in a milieu containing only sodium chloride and buffer solutes, i.e., in the absence of possible substrates in the bulk aqueous phase on which one of the late-acting complement components could act enzymatically (8). As regards the possibility of releasing a lytic substance by enzymatic action on membrane constituents, for example, formation of lysolecithin by phospholipase A, it is likely that such a lytic substance would diffuse in the plane of the membrane but would not tend to diffuse into the bulk aqueous phase.

Thus, one is led to the conclusion that the one-hit theory is applicable not only in terms of certain individual complement components, but with respect to the final event, the production of the membrane lesion, which causes lysis of the cell. It is clear that the lytic process takes place at the site where the late-acting complement components are fixed and that there is no subsequent "shower" activity. The present hypothetical models have been designed to comply with this restriction.

Electron microscopy of complement lesions

The action of complement on cell membranes is accompanied by the appearance of characteristic lesions observable by electron microscopy (19). When the surface of the membrane lies flat on the electron microscope grid, a dark central portion surrounded by a light ring is seen. The dark central portion, as well as the surrounding ring, may be irregular in appearance. It has been said that the dark central portion is a depression in the membrane surface that has become filled with the negative stain, while the surrounding ring appears to be a relatively raised edge (19). An alternative interpretation might be that the dark central portion is a hydrophilic region, while the light ring is hydrophobic. In the case of lesions produced by human complement, the dark central portion has a diameter of 10–11 nm (100–110 Å), while with guinea pig complement the internal diameter of the lesion is 8.5–9.5 nm (85–95 Å). Presently available electron microscope images do not indicate whether the lesion penetrates the entire thickness of the cell membrane. It would be of interest to examine membranes treated with complement by the freeze-etch technique in which the lipid bilayer of the membrane is cleaved along its inner hydrophobic plane, thus revealing the interior aspect of the membrane for electron microscopic examination.

With respect to the chemical characteristics of the lesions, it is noteworthy that treatment of membranes bearing lesions with trypsin, or with buffer at pH 2.5, did not affect the appearance of the lesions, except for sharpening it. Also, complement lesions on *Escherichia coli* lipopolysaccharide were not removed by treatment with Pronase. On the other hand, complement lesions on erythrocyte membranes fixed with formalin could be removed partly by treatment with alcohol–ether or completely by extraction with chloroform–methanol (19). These observations have been interpreted to mean that the lesions are located in the lipid layer of the membrane and that they may represent bubbles or micelles (19).

There has been some uncertainty as to the correlation between the number of complement lesions observed by electron microscopy and the number of holes calculated from the one-hit theory. Initially, evidence was presented indicating a rather good correlation (20). However, subsequent studies showed that, in certain reactions, the number of electron microscope lesions is substantially greater than that predicted from the one-hit theory (19). As a result of the recognition that clusters of lesions are formed when experimental conditions are chosen so that $C\bar{1}$ becomes a limiting factor (21), it is now clear that the numerical correspondence between electron microscope lesions and predicted holes is indeed good, provided experimental conditions leading to clustering are avoided. This means that the electron microscopic lesion seen after complement action is not an epiphe-

* This statement refers to lytic substances of low molecular weight such as lysolecithin.

nomenon but represents the damaged membrane site responsible for lysis†. Also, these results uphold the one-hit theory in its strict sense, which implies that the lytic agent is formed and acts where the late-acting complement components are bound.

First hypothesis: the leaky-patch model

One of the oldest concepts of complement hemolysis is the hypothesis that complement contains phospholipase A, which hydrolyzes lecithin producing lysolecithin, a hemolytic substance (23). In response to recent attempts at reviving this hypothesis (24), it has been pointed out that the erythrocyte membrane, the antibody globulin, and the serum serving as a source of complement do not contain enough lecithin to yield a quantity of lysolecithin sufficient for lysis (25). That quantity equals about 10^8 molecules of lysolecithin per cell (25). It is interesting, and probably significant, that lysis by other lytic agents like saponin, sodium oleate, or sodium desoxycholate, also requires an enormous quantity of lysin, about 10^9–10^{10} molecules per cell (see ref. 26)‡.

However, in light of the one-hit theory, such high values are not really applicable to complement hemolysis. Thus, when a lytic substance like sodium desoxycholate is added to an erythrocyte suspension it leaves the aqueous phase and penetrates into the lipid matrix of the entire cell membrane which, in the case of the sheep erythrocyte, has a surface of 26×10^6 nm^2 (19). By contrast, the complement lesion has a surface area of about 80 nm^2. Hence, if 10^9 molecules lyse a cell on penetrating into an area of 26×10^6 nm^2, it might be argued that about 3000 molecules should suffice for an area of the size of the complement lesion.

It is along these general lines that one can formulate a hypothetical mechanism, called the *leaky-patch model*, which postulates that the lytic attack of the complement system involving components C5–C9 generates a few thousand molcules of a lytic agent at a site on the cell membrane. These molecules would penetrate into the membrane at this locus and disrupt the lipid bilayer, thus causing a leaky patch. However, the above estimate of 3000 molecules per lesion is far too low because the molecules of lytic agent will diffuse in the plane of the membrane away from their site of generation and, therefore, if the leaky patch is to persist, new molecules of lytic substance must be synthesized to replace those that diffuse away. It is important to recognize, hence, with respect to the characteristics of this model, that maintenance of the leaky patch will require continuous generation of lytic agent.

Next, let us consider the source of the substrate. Since EAC1–9 will undergo lysis in a medium containing only sodium chloride and buffer solutes, i.e., in the absence of possible substrates in the bulk aqueous phase, the needed substrate would have to come from the complement components or from the membrane. As to the complement, it would be necessary to assume that a single molecule, or at most a few molecules, of one of the late-acting complement components, could supply many thousands of substrate molecules. Since this seems most unlikely, it is more reasonable to assume that the membrane supplies the substrate. However, in this case, we must face a new difficulty, namely, the fact that erythrocytes are not susceptible to lysis by phospholipase A because the membrane phospholipid is not accessible to the enzyme (27). On the other hand, erythrocytes can be lysed by phospholipase A in the presence of a basic polypeptide from *Naja naja* venom, which damages the cell membrane (27). Accordingly, if we postulate that the late-acting complement components, C5–C9, contain a phospholipase, it would be necessary to assume, also, that another one of these components is a substance that renders membrane phospholipids accessible to attack by this enzyme. It might be hypothesized further, that the other three complement proteins serve to bind and localize the enzyme and the membrane modifier in the correct orientation on the cell surface.

The leaky-patch hypothesis need not be restricted to mechanisms that generate a lytic substance. It is also conceivable that a leaky patch might be produced by action of a phospholipase that does not produce a lytic agent, for example, phospholipase C. Presumably, it would be necessary to postulate, also, that one of the late-acting complement components is a modifying agent that renders membrane phospholipds accessible to the enzyme. Furthermore, as in the lysolecithin-type case, continuous enzymatic action would be needed to maintain the leaky patch.

Attempts to demonstrate lipolytic action by complement have yielded contradictory results (25, 28, 29). The problem is a difficult one because the requisite biochemical methods are far less sensitive than hemolytic tests and, therefore, it may be necessary to await the isolation of highly purified complement components in substantial quantity before this issue can be put to a definitive test.

Regardless of the manner in which the leaky patch is produced, whether by generation of a lytic substance or by direct enzymatic attack, the crucial element of the leaky-patch model is the concept that it represents a small disrupted patch in the phospholipid bilayer. Since the bilayer is in a fluid state, the leaky patch must be regarded as a hole devoid of rigid structure. Consequently, it would be expected that leaky patches would not be of uniform size and that their size would change with time. However, as noted, the electron microscope lesions are quite uniform in size.

Furthermore, the leaky-patch mechanism would not be expected to produce stable holes§. Since the lytic agent will spread through the membrane by diffusion, a rapid generating mechanism would be required to furnish a steady supply of lytic substance to maintain the leaky patch. It is open to question whether such a steady state could be maintained for long. In order to obtain direct evidence on this point, we have recently studied the behavior of cells that had suffered the primary complement damage but that were prevented

† The report that EAC1-5 carry lesions visible by electron microscopy (22) has been denied (13).

‡ The dose–response curve of hemolysis by desoxycholate is sigmoidal, with a very large maximal slope, which indicates that its lytic action is a multi-hit process. This suggests the need for cooperative action among numerous molecules of desoxycholate, which means that solvation of a single molecule of desoxycholate, here and there, into the cell membrane will not produce disruption of the phospholipid bilayer. Only if many molecules of desoxycholate solvate into the phospholipid bilayer in close proximity to one another will they break up the membrane.

§ By analogy, it should be noted, as is well known, that erythrocyte ghosts prepared by hypotonic hemolysis possess an intact membrane. Thus, after the explosive rupture in the hypotonic medium, the discontinuities in the phospholipid bilayer of the membrane disappear.

from proceeding to the secondary stage and lysis by suspending them in an isotonic salt solution containing 25% albumin for about 0.5 hour[¶]. When such cells were sedimented by centrifugation and suspended again in isotonic buffered-salt solution without albumin, they lysed to the same extent as a control suspension of cells, which was never transferred to albumin. This means that the primary holes persisted during the time the cells were kept in albumin. Hence, the primary holes are stable, and this would argue against the leaky-patch model.

In summary, I believe that these difficulties do not necessarily rule out the leaky-patch model, but they are serious enough to have led to consideration of a different concept involving a lesion possessing rigid structure.

Second hypothesis: the doughnut model

The difficulties of our first model are due to diffusion, which tends to dissipate the leaky patch. If diffusion of the lipids were blocked by a wall of protein surrounding the leaky patch, dissipation would be avoided and the hole would be stable. For example, let us assume for the sake of argument, that the cell membrane contains integral proteins that penetrate the lipid bilayer and that are arranged in a cylindrical fashion, the central area containing a pocket of lipid. Furthermore, let us assume that C5–C9 become fixed at such a site and produce a leaky patch in the central lipoidal area. The resulting hole would be stable without the need for continuous enzymatic action.

The introduction of the concept of a rigid cylindrical barrier surrounding the leaky patch leads directly to a general hypothesis, called the *doughnut model*, that postulates that a stable hole can be produced by creating or assembling a rigid doughnut-shaped structure, with a hydrophobic outside and a hydrophilic inside, which floats in the lipid bilayer of the membrane in such a manner that its hydrophilic annular space becomes a channel connecting the interior of the cell with the extracellular environment.

Although I have introduced this general concept by suggesting that the cell membrane might contain integral proteins arranged in the shape of a hollow cylinder and that C5–C9 might create a hole inside this structure, this specific idea is not tenable for two reasons. Firstly, in the case of guinea pig C2 and C9 it has been shown that the lytic efficiency of these factors is very high, approaching 100%. This means that only 1–2 molecules are required for lysis of a cell and, consequently, C5–C9 would have to be fixed selectively at the postulated hollow cylindrical sites. This, of course, is unlikely. Secondly, liposomes made of lipid and glycolipid can be lysed by complement and typical lesions are seen by electron microscopy (13). Hence, the concept of a cylindrical barrier to diffusion of the lipids can be invoked only if we assume that the complement proteins, rather than membrane proteins, supply the constituents of the barrier.

There is no *a priori* reason why several of the late-acting complement components could not be arranged to form a doughnut with an appropriate distribution of hydrophobic and hydrophilic regions. Thus, the outside of the doughnut would have to be composed of nonpolar polypeptides associated with lipid, while its interior would need to contain polar polypeptides, possibly associated with carbohydrate. The general make-up of this doughnut would be similar to the structure of the ionophorous antibiotic, valinomycin (30). Also, by way of analogy, glutamine synthetase (31) from *E. coli* has been shown to comprise 12 subunits that are arranged in two stacks and that exhibit a hollow core. Thus, the idea that the late-acting complement components make up a multisubunit macromolecule possessing a hollow structure is not without precedent.

Indeed, in studies with human complement proteins it has been shown that a macromolecular aggregate, comprising C5–C9, is assembled on the surface of erythrocytes undergoing lysis by complement (1), though a shape other than that of a doughnut was proposed. However, since the combined molecular weight of complement components C5–C9 is almost 600,000 (1), it is evident that they possess sufficient mass to form a doughnut-like structure of adequate size, namely, one possessing a hole of 10 nm (100 Å) in diameter and about the same thickness as the membrane, i.e., about 7.5–9 nm (75–90 Å).

The doughnut model would be in accord with the observation that the electron microscope lesions are uniform in size. Furthermore, it would be easy to explain why the holes produced by human complement have an apparent diameter of 10–11 nm (100–110 Å), while those made by guinea-pig complement measure about 8.5–9.5 nm (85–95 Å). If the rings seen by electron microscopy actually represent the postulated doughnut-shaped structure lying embedded in the matrix of the cell membrane, and if they were made up of the late-acting complement components, or fragments thereof, the difference in size between the lesions produced by human complement and those made by guinea-pig complement may simply reflect a difference in molecular dimensions of the complement components of these two species.

Furthermore, the characteristic appearance of the complement lesions, as seen by electron microscopy after negative staining, can be interpreted in accord with this model, namely, that the dark central portion represents a hydrophilic region, while the surrounding ring is the hydrophobic exterior of the doughnut.

The presence of complement proteins in the postulated doughnut-shaped structure might be disputed on the grounds that treatment with proteolytic enzymes did not destroy the typical complement lesions, whereas application of lipid solvents removed them (19). However, there are alternative interpretations. For example, it is possible that the lesions contain a protein that resists proteolytic attack because susceptible peptide bonds are not accessible to the active site of the proteolytic enzyme, possibly because the protein is associated with lipid. Conversely, it would be expected that treatment with lipid solvents would remove the lesions, whatever their chemical nature, since they represent structures that lie embedded in a lipid matrix.

In pursuing the implications of the doughnut model in the future it will be important to seek information on the precise locus and shape of the C5–C9 macromolecular aggregate. It will be necessary to determine whether these components are loosely adsorbed like peripheral membrane proteins, or whether they are firmly associated with the membrane lipids like integral membrane proteins (9, 10). Furthermore, whether

[¶] It is noteworthy that dextran, molecular weight 75,000, does not block hemolysis by lysolecithin, even though it balances the colloid osmotic pressure (25). This is due to the fact that this lysin produces massive breakdown of the membrane structure at the concentration needed for lysis.

the complement proteins penetrate the entire thickness of the membrane will be an important question. Also, the possibility must be considered that integral membrane proteins may associate with complement proteins, thus becoming part of the doughnut. In this context, it is of interest that EAC1–8 bind C9 very firmly, even though the bonds are noncovalent. This suggests the formation of hydrophobic bonds. If so, it is tempting to speculate that the C9 molecule may undergo a conformational change in its reaction with EAC1–8 through which interior hydrophobic regions of the C9 molecule are exposed and become accessible to hydrophobic bonding with lipoidal constituents of the cell membrane. Such conformational changes may play an important role in the process of assembly of the postulated doughnut and its penetration into the lipid matrix of the membrane.

In addition, the role of enzymes must be considered in the processes of assembly and penetration. It is known that the first step of formation of the attack mechanism, namely, the activation of C5 by cleavage, which yields C5b, is an enzymatic process mediated by $\overline{C4, 2, 3}$ (2, 3). Beyond this point there is no definitive evidence on enzymatic activity. As in the leaky-patch model, it might be assumed that one of the late-acting complement proteins is a phospholipase that creates a leaky patch, and that this facilitates penetration of the complement proteins forming the doughnut. Indeed, I suspect that a combination of the leaky patch and the doughnut concepts may provide the most likely model, since a leaky patch produced by a lytic agent would become stabilized by attachment and penetration of a doughnut made up of complement components. On the other hand, it is conceivable that a complement doughnut possessing an appropriate arrangement of hydrophobic and hydrophilic regions might create a stable hole without benefit of a lipolytic enzyme. Obviously, these are questions requiring detailed study of the properties of the late-acting complement components, of their mode of interaction, of their possible enzymic properties and, most significantly, of the manner in which they become arranged on or within the membrane.

In summary, while these discussions are necessarily conjectural, the concepts and models I have described will be useful in future experimental explorations and, accordingly, they should be regarded as working hypotheses from which informative experiments on the cytolytic action of complement can be developed.

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