The bactericidal mechanism of the complement membrane attack complex



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# Abstract

The complement system is an important innate immune defense, containing about 30 secreted and membrane-bound factors. To fight bacterial infections, these factors collaborate to opsonize bacteria, attract phagocytic cells and to lyse Gram-negative bacteria via membrane-insertion of the membrane attack complex (MAC). The bactericidal MAC is generated via a specific assembly sequence initiated by the labile C5b, and followed by C6 and C7 to form the membrane-associated C5b-7 complex. The assembly is complete after incorporation of C8 and multiple C9 molecules, the complement factors that traverse the membrane. Although the assembly has been studied in detail, it is still controversial how insertion of the MAC leads to bacterial killing. Especially, since fully assembled MACs can been found in the envelope of Gram-positive and complement-resistant Gram-negative bacteria. Unraveling the bactericidal mechanism of the MAC might provide new insights into the MAC-resistant phenotypes, and can be a target for new therapeutic interventions. This review describes the assembly of the MAC in detail, including recently determined 3D structures of the complement proteins. This data emphasizes the time- and sequencespecific assembly of the MAC. Second, the characteristics of the membrane lesions in model systems, e.g. erythrocytes and artificial lipid bilayers, are discussed. Finally, models of the bactericidal mechanism of the MAC are reviewed and new directions in MAC research are proposed.

## Laymen's summary

Bacterial infections can result in severe diseases. Anti-bacterial therapies are quite effective, however a rising number of therapy-resistant bacteria have been found in patients. For this reason, there is an urgent need for new therapies that tackle resistant bacteria. One way to challenge the bacteria is to boost the host immune system, that naturally attacks the bacteria. An important example is the complement system. The complement system resides in all body fluids, and when activated can form pores in the surface of the bacteria by the formation of the membrane attack complex. As a result, crucial bacterial functions are deregulated and the bacteria is killed. Unfortunately, complement-resistant bacteria can withstand the lethal actions of the membrane attack complex. Understanding the mechanism how these bacteria are protected against the membrane attack complex, might provide a new lead in developing anti-bacterial therapies. These therapies would specifically target the bacterial anti-complement defenses, and will allow effectively bacterial killing by the natural complement system. This review summarizes data about the membrane attack complex, and about its interaction with complement-resistant bacteria and with bacteria that are killed by the complement system. Hereby, research goals are proposed that could help fight bacterial infections.

## The complement system

Bacteria that invade across the host epithelial barriers can cause severe diseases. The infection will be countered by the immune system, of which the early responses are effected by the innate immune system. One important aspect of the innate immune defense is the bactericidal activity of serum. This bactericidal action is an effect of the complement system, which consists of more than 30 proteins, either membrane-bound as receptors, or secreted in serum and body fluids.<sup>1</sup> Functionally, the complement system can be divided into three parts. The system can mark (opsonize) pathogens for phagocytic cells, it can stimulate inflammatory reactions and it can kill Gram-negative bacteria by the surface-deposition of the membrane attack complex (MAC). The importance of the complement system is seen in patients with genetic deficiencies in complement components who have a predisposition for recurrent bacterial infections.<sup>2</sup>

Activation of the complement system occurs via three different pathways; the Classical pathway, the Lectin pathway or the Alternative pathway (**Figure 1A**). Briefly, in the Classical pathway the complement factor C1q binds to antibody-antigen complexes or to various surface structures. In the Lectin pathway, the mannose binding lectin (MBL) and ficolins recognize specific carbohydrate patterns on bacterial surfaces. The Alternative pathway is an amplification step in the cascade, but can also be stimulated by various bacterial factors.<sup>3</sup> Although the three pathways differ in their mode of activation, they converge into a common sequence via the assembly of C3 convertases at bacterial surfaces. (**Figure 1A**) C3 convertases cleave the complement factor C3 into the pro-inflammatory factor C3a and into the labile C3b which covalently interact with nearby surfaces. Deposition of C3b is an important step in the complement activation. Besides opsonizing the bacteria, C3b also initiates the formation of C5 convertases that will cleave C5 into C5a and C5b (**Figure 1A**). Eventually, the assembly sequence of C5b, C6, C7, C8 and multiple C9 molecules leads to the bacterial surface-deposition of the MAC, and can directly induce bacterial lysis.<sup>4</sup>

Sequences in the complement system are tightly controlled to only target the pathogen, hence healthy host cells remain unaffected. In serum are complement regulatory factors present that prevent over-activation and limit the diffusion range of complement activation. Examples are Factor H and I that abolish C3b activity, while the assembly of the MAC is inhibited by the S-protein and clusterin.<sup>4</sup> In addition, host cells have several transmembrane proteins (*e.g.* CD59) that prevent the assembly of the MAC on the cell membrane.<sup>4</sup> Unfortunately, bacteria have also acquired escape mechanisms that result in complement resistance.<sup>5</sup> In addition, it has been known for a long time that Gram-positive bacteria are not sensitive to the complement MAC.<sup>6</sup>

Since the discovery of the bactericidal effect of serum and the formation of MAC lesions on target membranes, studies have addressed the membrane-disrupting mechanism of the MAC. However, only limited data is available about how complement-resistant Gramnegative bacteria and Gram-positive bacteria withstand the bactericidal activity of the MAC. The purpose of this review is to give an overview and discussion points about the bactericidal effect of the MAC. First, the assembly of the membrane attack complex is described in detail, including recently clarified 3D structures of complement proteins. Second, functional data obtained with model systems are discussed. This is followed by an overview of the bactericidal mechanism of the MAC and future research directions.



**Figure 1. Overview of the complement system cascade. (A)** The complement system recognizes target cells (*e.g.* bacteria) via three different pathways. The Classical pathway is activated by antigen-antibody (Ag-Ab) complexes, the Lectin pathway recognizes surface carbohydrates and the Alternative pathway reacts to specific surfaces, and provides an amplification step in complement activation. All pathways lead to the formation of C3 convertases (C4b2a or C3bBb) that cleave C3 into C3a and C3b. C3b interacts with nearby cell surfaces, and can further react with factor B to form new C3 convertases. In addition C3b can be incorporated in existing C3 convertases to generate C5 convertases (C4b2a3b or C3bBbC3b). Following cleavage by C5 convertases, C5a is released from C5 and acts as an anaphylatoxin, while C5b initiates the membrane attack complex assembly. C5b reacts with C6 and C7, and the resulting C5b-7 complex interacts with membranes. A subsequent assembly and membrane insertions of C8 and multiple C9 molecules results in membrane lesions. Regulators of the complement cascade are shown in black. Figure adapted from ref <sup>4</sup>. (B) Electron microscopy images of membrane attack complex lesions in erythrocyte membrane attack complex inserted in the light colored membrane is shown in the bottom image, marked by a black arrow. Adapted from ref <sup>7</sup>.

# Assembly of the MAC

The complement cascade can result in the deposition and the membrane-insertion of the MAC. Electron microscopy (EM) images have showed that the MAC forms characteristic round membrane lesions in target membranes (**Figure 1B**).<sup>7</sup> The sequential assembly of C5b, C6, C7, C8 and multiple C9 molecules reveals important aspects about these membrane lesions. Moreover, the assembly elicits how the complement system limits collateral damage to nearby cells.

### Marking the pathogen

At the beginning of the complement cascade, the target surface is marked by the covalent attachment of the C3 convertase. C3 convertase cleaves the complement factor C3 into the small C3a fragment which consists of an anaphylatoxin domain, and into the large C3b fragment. The C3b fragment proceeds the complement cascade by interacting covalently with the target cell.<sup>4</sup> Structural studies that solved the 3D structure of C3 and its fragments gave insight into the specificity of this complement reaction. C3b undergoes a major conformational change upon the release of the C3a fragment (**Figure 2A**).<sup>8</sup> Its thioester-like domain (TED), that is shielded in proximity of the C3a fragment, relocates to the periphery.

Hereby, a thioester moiety is exposed that will react with molecules nearby the C3 convertase. When a high concentration of C3b is deposited on the target surface, the C3b fragments react with C3 convertases and form C5 convertases.<sup>9</sup>

After marking the pathogen by the C3 and C5 convertases, the assembly of the MAC is initiated by C5, the first structural protein in the MAC. C5 is activated in a similar way as C3, its homolog. C5 convertases cleave C5 into the C5a fragment, a potent anaphylatoxin, and a large C5b fragment that recruits the other MAC complement factors.<sup>4</sup> Structural studies have shown that, analogous to C3b, C5b undergoes a major conformational change in its TED (**Figure 2B**).<sup>10,11</sup> In contrast to C3b, the C5b fragment lacks a thioester moiety and does not react with nearby surfaces. However, the exposed C5b structure renders the protein unstable, and in the absence of C6 the ability to form a MAC will decay 50% in 2.3 minutes.<sup>12</sup> Interestingly, also a 50% decay of 30.3 minutes has been reported, but this was linked to a high level of C3b on the target cell.<sup>13</sup> Weak ionic interactions between C3b and C5 exist, and this likely prolongs the activity of C5b, limits its diffusion and thus increases the specificity of the complement cascade.<sup>14</sup>



**Figure 2. Major conformational changes in C3b and C5b reveal time-dependent complement activation.** (**A**) The 3D structures of C3 and C3b determined by X-ray crystallography. C3 convertases cleave C3 into the minor C3a and the major C3b fragment. Hereby, a distinct conformational change can be observed in the C3d domain of C3b (also known as the thioester-like domain). The exposed thioester will covalently react with nearby molecules. (**B**) The structures of C5 and of C5b in complex with C6 (C6 is not shown in the figure). Analogous as seen with its homolog C3, the C5d domain (thioester-like domain) is exposed in the periphery of the C5b fragment, after the release of the small C5a fragment. The C5d domain lacks a reactive thioester, but consists of a C6-binding domain. Adapted from ref <sup>10</sup>.

### Assembly of the C5b-8 receptor

Assembly of the MAC is continued by C6, the complement factor that binds and stabilizes C5b. The protein has remarkable similarities with the subsequent MAC proteins C7, C8a- $\gamma$ , C8 $\beta$  and C9 (**Figure 3**). They all possess a central membrane attack complex and perforin (MACPF) domain surrounded by modules. Recently, the 3D structures of C6, and of C6 in complex with C5b (C5b-6) have been determined.<sup>15,11,10</sup> Unbound C6 is an extended protein, wherein the MACPF domain resides in the center, and the complement control proteins (CCPs) and the Factor I modules (FIMs) are located at the periphery (**Figure 4A**, **left**). The structure of C6 in complex with C5b shows that C6 undergoes rotational conformational changes in the CCP and the FIM modules. These domains enwrap around the exposed TED in C5b, at the outer part of the protein (**Figure 4A**, **right**). This is in agreement with biochemical data showing that the CCPs in C6, and to a lesser extent the FIMs, are required to bind C5b.<sup>13</sup> In contrast with the short half-life of C5b, shows the C5b-6 complex a stable character in solution, conflicting with the specific time-dependent assembly of the MAC.<sup>16</sup> Interestingly, weak ionic interactions between C5 and C7 have been found.<sup>17</sup> This affinity might help C5 to attract C7 in serum, thereby increasing the velocity of the next step in the complement cascade.



Figure 3. Similarities between the complement factors in the membrane attack complex. Schematic representation of the domains in C6, C7,  $C8\alpha$ - $\gamma$ , C8 $\beta$  and C9. Note that C8 $\gamma$  has no domain similarities with the other complement factors, but is non-covalently associated with C8 $\alpha$ . Abbreviations. TS: thrombospondin 1 module; LR: LDL receptor module; MACPF: membrane attack complex and perforin domain; CH1 and CH2: transmembrane helices; EGF: EGF receptor module; CCP: complement control protein module; FIM: Factor I module. Adapted from ref<sup>10</sup>.

Assembly of the MAC is followed by the interaction between C5b-6 and C7, a homolog of C6 (**Figure 3**). The 3D structure of C7 has not been determined, but EM images have given us an indication of its structure (**Figure 4B, top**).<sup>18</sup> The model of C7 resembles the extended structure of unbound C6. After incorporation of C7 in C5b-6, the complex has a tendency to aggregate, presumably because hydrophobic regions are exposed.<sup>19</sup> As a consequence, the metastable C5b-7 complex can associate with nearby membranes in a very limited time-frame.<sup>20,21</sup> The complex only associates with the outer leaflet of the lipid bilayer, and is thereby not sufficient to generate membrane lesions.<sup>22</sup> In **Figure 4B**, **bottom**, an EM image of the C5b-7 complex inserted in a liposome is shown. The complex comprises of a membrane-arising stalk that extends into two or three braching arms.<sup>23,24</sup> However, C5b-7 complexes associated with liposomes might represent dimers, and it is not clear if a C5b-7 dimer can be found in the fully assembled MAC.<sup>18</sup> Immuno-EM of the membrane-bound C5b-7 revealed that one of the arms represents C5b, while C7 resides in the stalk.<sup>24</sup> Furthermore, biochemical studies identified that C6 and C7 are, compared to C5b, proximate to the phospholipids.<sup>18,25,26</sup> These data suggest that in the C5b-7 complex, the MACPF domain containing proteins C6 and C7 form the membrane-associated stalk that branches into a C5b-containing arm (**Figure 5A**).



**Figure 4. Structures of the MAC proteins C6, C7 and C8.** (A) *Left*: Rotational conformational changes in C6 after its interaction with C5b, as determined by X-ray crystallography. *Right*: The structure of the C5b-6 complex. Adapted from ref <sup>10</sup>. (B) Upper image depicts a model of unbound C7 based on electron microscopy images. Bottom image shows the C5b-7 complex incorporated into a liposome. The arrow points to the stalk of the C5b-7 complex that extends from the liposomes into branched arms. From ref <sup>18</sup>. (C) 3D structure of C8. The C8y subunit is colored green, C8β is shown in blue and C8α in red. In C8β and C8α, the membrane attack complex and perforin (MACPF) domains are indicated by dark colors. Arrows point at the transmembrane helices (TMH1 and TMH2) in C8α.Image adapted from ref <sup>27</sup>.

Incorporation of C8 in the membrane-associated C5b-7 complex is required to generate functional membrane lesions (**Figure 5B**). C8 consists of three different subunits: C8a, C8 $\beta$  and C8 $\gamma$ . C8a and C8 $\beta$  have similar domains as C6, C7 and C9 (**Figure 3**), while C8 $\gamma$  is a small protein that non-covalently associates with C8a.<sup>28</sup> In the MAC, C8 $\beta$  is the protein that interacts with the C5b-7 complex and C8a- $\gamma$  binds, and thereby incorporates, C9 molecules into the MAC.<sup>29,30</sup> X-ray crystallography has solved the crystal structure of C8 (**Figure 4C**).<sup>27</sup> C8 $\beta$  and C8a interact with each other via the N-terminal part of the MACPF domains, whereby the lower part of the MACPF domains diverges into a 22 degrees rotated structure.<sup>27</sup> This rotation is probably the start of the round membrane lesions that characterize the fully assembled MAC. The C5b-8 complex has a deeper membrane insertion than the preluding C5b-7 complex, and allows access for multiple C9 molecules.<sup>22</sup> It is believed that the MACPF domains of C8 traverse the target membrane via transmembrane helices (TMH). This is postulated since the TMHs in C8 are longer and more hydrophobic compared to the TMHs in C6 and C7.<sup>31</sup> Consistently, biochemical studies indicate that in the C5b-8 complex the C8a subunit resides most in the hydrophobic region of the membrane.<sup>32,25</sup>



Figure 5. Cartoon describing the, hypothetical, assembly of the C5b-9 complex in a membrane bilayer. (A) The complex formed by C5b (red), C6 (green) and C7(blue) associates with the target membrane, but does not disrupt the integrity of the lipid bilayer. In electron microscopy this complex usually comprises of two branches. (B) Incorporation of the C8 heterotrimer results in membrane lesions, that allow permeability to small molecules and ions. C8 $\beta$  (yellow) binds the C5b-7 complex at a yet unknown location, and is inserted in the lipid bilayer. C8 $\gamma$  (orange) is a small protein associated with C8 $\alpha$ . C8 $\alpha$  (pink) is deeply inserted into the target membrane and provide a docking site for C9 molecules. (C) The assembly of the membrane attack complex (MAC) is followed by C9 (purple). This protein traverse the whole membrane. Studies have determined that the protein reach the lumen of the target cells with both his transmembrane helices, as here hypothesized by a n-shaped structure. (D) In the fully assembled MAC, multiple C9 molecules self-polymerize, and might form an amphipathic pore structure.

### Circular membrane insertion by multiple C9 molecules

The last step in the MAC assembly is the incorporation of multiple C9 molecules. C9 is required to form the characterizing circular and extensive membrane lesions. Certain characteristics make C9 an efficient membrane disrupting protein. The first is its ability to self-polymerize into a ring-like structure. Even in the absence of the C5b-8 complex, C9 can polymerize, in the presence of metal ions or when incubated at high concentrations at temperatures above 46°C.<sup>33,34,35</sup> The resulting poly(C9) ring consists of 12-18 C9 molecules, and can be inserted into lipid bilayers during its polymerization.<sup>34,36</sup> The abovementioned conditions likely affect inhibitory regions that prevent self-polymerization in C9, *e.g.* a short N-terminal region in C9 (first 16-23 amino acids) and a Ca<sup>2+</sup> binding domain.<sup>37,38</sup> Since the membrane-inserted poly(C9) structure resembles the MAC lesions, it has been proposed that the self-polymerized C9 ring is also present in the MAC.<sup>33</sup> Another interesting feature of C9 is its careful incorporation into the MAC. When C9 is incubated with the membrane-bound C5b-8 complex at 4°C, only one C9 molecule can bind to the complex.<sup>39</sup> The incorporated C9 might traverse the whole bilayer, since this C9 conformation can be captured by an antibody from the lumen-side of a vesicle (**Figure 5C**).<sup>40</sup> When the C5b-9<sub>1</sub> complex is incubated at

37°C, the incorporated C9 undergoes a conformational change that allows more C9 molecules in the MAC complex.<sup>39</sup> In addition, incorporation of C9 might be a time-dependent process. When unsaturated C5b-9 complexes are incubated at 37°C for only a few minutes, they may lose their ability to accept more C9 molecules.<sup>41</sup> Although this could be an important aspect in generating stable membrane lesions, contradicting results have been found.<sup>42,43</sup>

EM studies and cross-linkage studies with membrane-restricted probes support the theory that C9 is localized in the membrane-lining MAC tubule.<sup>24,44,25,32</sup> Likely, C9 elongates the C5b-8 structure starting from the C8a MACPF domain and self-polymerizes into a ring via its MACPF domain, as schematically indicated in **Figure 5D**.<sup>31</sup> This is consistent with data showing that the TMH1 and TMH2 in C9 are implicated in its self-polymerization.<sup>45,46,47</sup> However, not all data are clear as the orientation of the C9 TMHs traversing the lipid bilayer is still under debate. The N-terminal part of C9 (amino acids 1-245, **Figure 3**) is exposed in the MAC from inside the lumen, while the region between the two TMHs (region 305-324) resides at the outer side of the membrane.<sup>48,49</sup> This suggests that the TMH1 traverses the membrane from inside the lumen toward the C5b-8 complex and the TMH2 inserts into the membrane towards the lumen, as like a n-shaped structure. However, a completely inverse orientation has been suggested by studying the structure of membrane-free poly(C9) structures.<sup>46</sup>

Overall, the terminal pathway of the complement system reveals a time-dependent assembly that limits the membrane damage of nearby cells. Since the discovery of poly(C9) it is clear that the ring-like membrane lesions are caused by C9, and that C5b-8 acts predominantly as a receptor that marks the target membrane.

## **Characterization of the MAC in model systems**

Most studies concerning the assembly of the MAC and the resulting membrane lesions have been conducted in model systems, *e.g.* erythrocytes and artificial lipid bilayers. The advantage of these systems is the ease to characterize the membrane lesions compared to bacterial membranes. However, these model systems have no endogenous trigger to activate the complement cascade. Therefore, erythrocytes of species (*e.g.* rabbit, sheep) different from the complement-containing serum (*e.g.* human, Guinea pig) are used and coated with antibodies. Since erythrocytes are lysed (hemolysis) by small membrane lesions which cause ion effluxes, experiments have also been conducted with erythrocyte ghosts. These erythrocytes are already lysed and subsequently resealed in the presence of several differently sized markers. In this way, there is no influence of the osmotic pressure, while the diffusion of markers gives an indication of the membrane lesion size. On artificial lipid bilayers, the MAC is assembled by using the purified complement factors C5b-6, C7, C8 and C9. Both systems can be used to determine the stability and the size of the membrane lesions. However, it should be noted that killing bacteria is a more complex process, as will be discussed in the next section.

### Membrane lesions of the MAC: pores or leaky patches

Since the first MAC studies, two models have been proposed about the nature of the membrane lesions. One of them is the leaky patches model, which states that the rigid lipid bilayer of the target cells is disorganized by insertion of the MAC. The resulting membrane lesions are unstable and fluctuate in time and size. Alternatively, it has been proposed that the MAC forms stable pores in the membrane with a hydrophilic core of uniform size (**Figure 6**).<sup>50</sup> Accumulating data have shown that both models are valid, reliant on the experimental conditions.

The most straightforward evidence that the MAC can from leaky patches is proven by C5b-8 complexes. Incubating erythrocyte ghosts with high concentrations of C5b-6, C7 and C8 leads to functional membrane lesions.<sup>51</sup> The membrane lesions have a size around 0.7 nm (size of sucrose), and can release ions across artificial lipid membranes.<sup>51,42,21,52,53</sup> The C5b-8 membrane lesions are time- and concentration-dependent, and are rapidly fluctuating.<sup>54</sup> These features match the characteristics of leaky patches. However, further research indicates that rather than C5b-8 monomers, C5b-8 dimers or aggregates are required to generate the functional membrane lesions.<sup>41,55,39</sup> In contrast, the pore model is supported by studies analyzing C9. As discussed in the previous section, C9 can selfpolymerize into poly(C9) under specific conditions. When this polymerization is initiated in the presence of artificial lipid bilayers, stable and uniform ion conductance can be observed.<sup>56</sup> Furthermore, the assembly of poly(C9) on protein-stripped erythrocytes and on liposomes results in large membrane lesions with a diameter size of 9 nm.44,54 These data underline the limitations of the model systems in MAC research. Functional membrane lesions can be generated in different ways and in different sizes, however it cannot provide information about potential bactericidal activity of the membrane lesions. Nevertheless, interesting insights have been obtained about the composition and the membrane-disrupting capacity of the MAC.



**Figure 6. Models about the character of membrane attack complex lesions.** *Left*: the cartoon proposes that the membrane attack complex (MAC) (indicated by the white cylinder) induces membrane lesions via leaky patches. The hydrophobic region of the phospholipids cluster around the membrane-inserted part of the MAC. The distortion of the lipid bilayer breaches the impermeability of the membrane. Due to the instability of this configuration the membrane lesions are constantly fluctuating. *Right:* the cartoon shows the MAC pore model. The MAC inserts as a stable transmembrane pore in the lipid bilayer. The amphipathic MAC ring has an internal hydrophilic core that allows the passage of hydrophilic molecules through the membrane. Figure adapted from ref <sup>57</sup>.

#### Membrane disrupting capacity of C9

One of the interesting findings in characterizing the MAC membrane lesions is the athrombin cleaved form of C9; C9n. C9n consists of the C9a (amino acids 1-244) and the C9b (amino acids 245-537) fragments that are associated by disulfide-bonds. C9n has similar capacity to lyse erythrocytes as C9, but it loses much of its ability to polymerize.<sup>58</sup> Inducing the formation of poly(C9) with C9n fragments results in arc-like self-polymerizations instead of ring-like structures.<sup>59</sup> This challenged the vision that ring structures are required to produce maximum membrane damage in erythrocytes. More importantly, it was shown that the C9b fragment, isolated from C9n, can associate and distort lipid bilayers.<sup>52,53</sup> Consequently, erythrocytes are lysed when incubated in the presence of C9b, but not when incubated in the presence of uncleaved C9.60 In artificial lipid bilayers, ion conductance measured by C9b membrane lesions resemble that of the MAC, in contrast with conductance induced by poly(C9).<sup>53</sup> These data support the notion that the MAC does not need to form whole ring-like structures for efficient membrane lesion formation. More importantly, the C9b fragment provides information about the working mechanism of C9. The C9a part of C9 acts as a safe switch and, when C9a is cleaved off or when conformation changes are induced in the C9b fragment, C9 can disrupt the target membranes without the need for other complement proteins. This is an important aspect in targeting Gram-negative bacteria, as discussed in the next section.

#### The MAC has high similarity to other pore-forming proteins

Most complement factors in the MAC contain a MACPF domain. This domain was named after the similarities in sequences between the MAC proteins and perforin in that region. Perforin is a known pore-forming protein that induces apoptosis in dysfunctional target cells. Interestingly, there are analogies between perforin and the MAC proteins. Under the right conditions, perforin can self-polymerize into a ring structure.<sup>56</sup> These perforin-induced rings resemble the MAC ring structure, as both are high molecular weight structures and resistant to salts and SDS, probably because of disulfide bonds and hydrophobic interactions.<sup>61,56</sup> Perforin polymerization is mediated by specific residues in the MACPF domain that have also been suggested to be involved in complement protein polymerization.<sup>62</sup> Moreover, similar to C9, self-polymerization and membrane-association of perforin depends on Ca<sup>2+</sup> levels that induces the relocation of hydrophobic residues.<sup>56,63</sup> The similarities of the MACPF domains in C9 and perforin, and the fact that both proteins can form membrane-disrupting pores, indicate that the MACPF domains are specifically involved in that process.

The MACPF domains became even more interesting, when the 3D structure of the MACPF domains in the complement proteins were determined. The MACPF 3D structure showed striking similarities with that of cholesterol-dependent cytolysin (CDC) proteins.<sup>64,65</sup> CDC proteins are pore-forming proteins secreted by Gram-positive bacteria, that have a well-characterized working mechanism. First, CDC proteins recognize the target membrane via interactions with cholesterol. Then, the proteins self-polymerize into a membrane-associated ring-structure, the pre-pore state. Finally, the CDC protein undergoes a major conformation change, resulting in the insertion of its TMHs in the target membrane.<sup>66,67</sup> Importantly, one study showed that the TMHs in the MACPF domains and the TMHs in the CDC proteins are functionally similar.<sup>68</sup> Molecular techniques have proven that the TMH2 in both C8a and C9 can partially replace the function of the TMH2 in the CDC protein of its are functionally similar.<sup>68</sup> This proves that although the sequences of the MACPF domains are distinct from that of the CDC proteins, they share a similar structural and functional mechanism to insert into target membranes.

In overall, these data support the existence of a similar pore-forming mechanism for the MAC, perforin and CDC proteins. However, it should be taken in consideration that in contrast with perforin and the CDC proteins, the MAC consists of additional proteins which, with exception of C5b, also contain a MACPF domain. Consequently, differences in the membrane disrupting process can be expected between the complement proteins and CDC proteins. Nevertheless, these findings impose exciting information that membrane disruption, although sequential different, might follow a common structural mechanism between the MACPF and CDC families.

#### Size heterogeneity of the MAC

As discussed at the beginning of this section, there is evidence that several mechanisms exist for the MAC to form membrane lesions. In concordance with this, the size and composition of the membrane lesions are heterogeneous by nature. This is best seen by the influence of C9 concentrations in generating C5b-9 complexes. When the MAC is generated in the presence of low C9 concentrations, the lesions are small and are seen as arc-like structures in EM images. Increasing the C9 concentration leads to larger lesion sizes and ring-like membrane lesions.

Thus, how are the MACs generated in the physiological situation? In serum, the ratio of C8 and C9 is 1:2.<sup>41</sup> At this low C9 ratio only small lesion sizes would be generated. However, it has been shown that, dependent on the serum doses, the consumption of C8 and C9 are not equal. At low serum doses, most of C8 is incorporated in the MAC resulting in an average of two to three C9 molecules per C5b-8. In contrast, at high serum doses only a moderate amount of C5b-8 complexes are formed while most of C9 is incorporated into the MAC. These C5b-9 complexes have an average of six to eight C9 molecules per C5b-8.<sup>41</sup> Unfortunately, this is not the only problem in characterizing the composition of MAC in

model systems. There are also practical problems to determine the ratio of C9 per C5b-8 in the MAC. The sedimenting behavior of the MAC reveals high molecular weight structures and aggregates, which provide doubtful numbers.<sup>41</sup> Even when the incorporation of C9 in premade C5b-8 complexes is measured, the MAC composition differs from three to sixteen C9 molecules per C5b-8.<sup>70,71,42</sup>

To complicate matters even more, it has been proposed that the MAC might consist of two C5b-8 complexes, the  $(C5b-8)_2C9$  structure.<sup>72</sup> Studies showed that two independent C5b-6 structures can be cross-linked together in the MAC, and that twin channels are observed in EM images.<sup>73,23,74,41</sup> This would result in a reduction of the C9 to C5b-8 ratio by 50%. However, nowadays the possibility that the MAC consists of a  $(C5b-8)_2C9$  composition has been dismissed. When the concentration of C5b-8 on erythrocytes ghosts increases, only a steep increase in lesion size is observed. In contrast, increasing the concentration of C9 gives a sigmoidal curve for an increase in marker size release. This indicates that multiple C9 molecules are collaborating (*e.g.* its polymerization), but no influence of C5b-8 collaboration was found.<sup>75,69</sup> As discussed before, there are several ways in which the MAC can generate membrane lesions and the possibility that the MAC consists of a (C5b-8)<sub>2</sub>C9 structure in the physiologic situation should be taken in consideration.

This section gave an overview of the many ways in which MAC membrane lesions can behave. The MAC has a heterogeneous range of size and composition, which generates a lot of controversy about this topic. Characterization of the membrane lesions in model systems revealed multiple mechanisms for membrane lesion generation, but they cannot give definitive answers regarding the MAC on bacterial target membranes.

# **Bactericidal and non-bactericidal MACs**

To understand the bactericidal mechanism of the MAC, it is first important to discuss the bacterial envelopes. Gram-negative bacteria are composed of an inner membrane (IM), lining the cytoplasm, and an outer membrane (OM) lining the extracellular space (**Figure 7**). The OM (7.5 nm) is a barrier that protects against the extracellular environment, and consists mainly of lipopolysaccharide (LPS). In the OM, outer membrane proteins (OMPs) facilitate the transport of nutrients and ions through pores. Between the two membranes a thin peptidoglycan layer (2.5 nm) extends into the interstitial periplasm (10 nm), and is necessary to maintain the turgor pressure of the cytoplasm. The harsh environment of the periplasm may be compared to that of lysosomes.<sup>76,77</sup> Gram-positive bacteria are composed of only one membrane surrounded by a thick multilayer of peptidoglycan.<sup>76</sup> Both types of bacteria can be surrounded by a capsule when they are in the non-exponential phase. The capsule prevents activation of the immune system and might protect against complement-mediated lysis.<sup>78</sup>

Differences between the envelope composition of Gram-positive and Gram-negative bacteria have important consequences for their complement sensitivity. In the extensive MAC literature, the MAC has only been found bactericidal towards Gram-negative bacteria. For this reason, the MAC is studied almost exclusively in Gram-negative bacteria. This is an intriguing topic, especially since the MAC (16 nm) cannot traverse the whole length of the Gram-negative envelope.<sup>77</sup>



**Figure 7. Schematic of the bacterial envelopes.** *Left*: Gram-positive bacteria consist of one lipid bilayer, which is surrounded by a thick multilayer of peptidoglycan. *Right*: Gram-negative bacteria have a more complex structure. The inner membrane lays beneath the periplasm, wherein a small peptidoglycan layer resides, to maintain the turgor pressure. The outer membrane forms a protective shell, and is decorated with LPS. Abbreviations; IMP: integral membrane protein; OMP: outer membrane protein; LPS: lipopolysaccharide. Figure adapted from ref.<sup>76</sup>.

#### Complement factors are sufficient to kill Gram-negative bacteria

In Gram-negative bacteria, the complement cascade encounters first the LPS-bearing OM. It is known that some complement-resistant Gram-negative bacteria have developed mechanisms to prevent the assembly of the MAC at this points.<sup>5</sup> In the absence of these resistance factors, the C5b-9 complex can be fully assembled and inserted into the OM. As a result the integrity of the OM is affected. Insertion of the MAC allows small molecules to passage towards the periplasm, and phospholipids and LPS are shed from the OM.79,80,81 However, insertion of the MAC in the OM is not specific for its bactericidal effect. (Figure 8, left) In complement-resistant Gram-negative bacteria the MAC has been found inserted in the OM, and as a result the OM lost phospholipids and its permeability increased.<sup>82,83,80</sup> In more direct ways, studies have shown that the incorporation of C5b-9 complexes exclusively in the OM is not lethal, even not for complement-sensitive bacteria. To prove this one study assembled C5b-9 complexes in liposomes. The C5b-9 complex bearing liposomes were subsequently fused with complement-sensitive bacteria. While the C5b-9 complexes were found in the target bacteria, and small hydrophilic molecules could reach the periplasm, no increase in bacteriolysis was detected.<sup>84</sup> Another study showed that complement-treated donor bacteria could spontaneous transfer their assembled C5b-9 complexes to untreated bacteria. As a result, the untreated bacteria acquired the MACs into their OM, but showed no bactericidal effect.<sup>85</sup> Collectively, these data provide evidence that OM damage is not sufficient to kill bacteria.

Bacterial proteins in the OM allow transport of ions, indicating that the OM is not a strict barrier.<sup>76</sup> Incorporation of the MAC increases the permeability of the OM for larger molecules. Although, the increase in membrane permeability is not lethal per se, it is possible that certain serum factors can gain access to the periplasm and the IM. These factors might execute the bactericidal effects of the MAC. For this reason, it has been investigated if lysozyme assists MAC-mediated bacterial killing. Lysozyme can degrade the peptidoglycan layer that is located in the periplasm. The peptidoglycan layer maintains the turgor pressure, but when degraded might be unable to prevent bacteriolysis. However,

there is no strict requirement for lysozyme in MAC-mediated bacterial killing as lysozyme free-serum is bactericidal to sensitive bacteria.<sup>86</sup> Moreover, it has been shown that treating complement-sensitive bacteria with only purified complement factors kills the bacteria.<sup>87</sup> This proves that the complement factors are sufficient for the bactericidal effect, but that this process is more extensive than only the incorporation of the MAC in the OM.

### **Characteristics of the bactericidal MAC**

The complement factors have to breach more than the OM to kill bacteria. Other steps required for the bactericidal effect of the MAC have been elucidated by comparing complement-resistant and complement-sensitive bacteria.

As mentioned previously, the MAC can be found on both complement-sensitive and complement-resistant bacteria. But, that does not mean that the composition of the MAC is similar on sensitive and resistant bacteria. One of the ways to characterize the fully assembled MAC is to identify high molecular weight structures that are resistant to SDS denaturation.<sup>88</sup> These fully assembled structures, however, are not specific for the bactericidal effect. SDS-resistant MAC complexes were not present in all complement-sensitive bacteria.<sup>89,90,91</sup> Furthermore, some studies found these structures in the absent of a bactericidal effect.<sup>92,93</sup>

In contrast with the composition of the MAC and its insertion in the OM, specific bactericidal characteristics were found in the way the MAC inserts into the membrane. Studies have used two different ways to test the stability of the C5b-9 complexes. When complement-treated bacteria are exposed to high salt concentration, hydrophilic interactions are disrupted while unexposed hydrophobic interactions remain stable. Via this method, it has been shown that the MAC elutes in higher quantities in complement-resistant bacteria than in complement-sensitive bacteria.94,95 This result indicates that bactericidal MACs are inserted in more hydrophobic regions. Another method to determine membrane insertion of the MAC is to subject the bacteria to proteases. A limited proteolytic cleavage of the bacterial envelope will release the proteins that are not inserted in the hydrophobic bacterial membranes. In line with the above data, the complement proteins inserted in complementsensitive bacteria were more resistant to proteolytic cleavage.<sup>96,6,95,97</sup> In overall, these studies indicate that the bactericidal MAC inserts more in the hydrophobic membranes of the Gram-negative bacteria compared to non-bactericidal MACs. Conversely, one study contradicts this conclusion. They did not find differences between resistant and sensitive bacteria in eluting MACs via the above mentioned methods.<sup>92</sup> There are explanations why elution of the MAC might not be specific for the bactericidal effect. In the previous paragraph it was argued that the MAC can be inserted in the OM without being a specific predictor of bacterial killing. Moreover, it is possible that C5b-9 complexes or poly(C9) structures assemble around other hydrophobic regions in the bacterial envelope. Especially poly(C9) structures have been noted as highly resistant to proteolytic cleavage.98

More consistent characteristics have been found by looking at the IM of the target bacteria. The IM provides a strict barrier for small molecules and ions, to regulate important cellular functions in the bacteria, *e.g.* oxidative phosphorylation for energy metabolism. In complement-sensitive bacteria this barrier is damaged by the complement factors (**Figure 8, right**). It has been shown that small molecules can pass the IM of complement-sensitive bacteria after serum treatment.<sup>86,99,100,82</sup> Small holes in the IM might already be the cause of bacterial death, since colicins, which are bacterial toxins, make small pores in the IM and are sufficient to kill the Gram-negative bacteria.<sup>101</sup> Another feature of the bactericidal MAC is that it affects the energy metabolism in the bacteria. The oxygen consumption of the bacteria is decreased after serum-treatment, indicating defective energy metabolism.<sup>102,103</sup> Another marker is a rapid rise in intracellular ATP in the bacteria, followed by a decrease of ATP below standard levels.<sup>104</sup> This interesting phenomenon was further investigated by studies showing that the bactericidal activity of the MAC can partially be prevented by uncoupling of the oxidative phosphorylation.<sup>105,104,89</sup> It is yet unclear how the energy

metabolism of the bacteria can accelerates its own killing process. One possibility is that MAC-mediated killing requires proton transport towards the periplasm, as will be discussed. In conclusion, it is clear that the bactericidal effect of the MAC is characterized by IM damage. How complement factors might affect the integrity of the IM is discussed in the next paragraph.



**Figure 8. Characteristics of the bactericidal and the non-bactericidal membrane attack complexes in Gram-negative bacteria.** For colors in the membrane attack complex (MAC) see Figure 5. *Left:* The MAC can still be assembled in the outer membrane (OM) of Gram-negative bacteria without inducing bactericidal effects. Phospholipids might be shed of the OM, and the OM can become permeable for small molecules. The MAC might elute by high salt concentrations and can be digested by proteases. *Right:* When the incorporated MAC is bactericidal, the inner membrane is breached and shows permeability to small molecules. The oxygen consumption and the ATP levels in the bacteria are decreased. How the complement proteins damage the inner membrane is yet unclear. Abbreviations; IMP: integral membrane protein; OMP: outer membrane protein.

#### Models describing IM damage by the complement proteins

There are two different models that can describe how the IM is damaged during MACmediated bacterial killing. One suggests that the whole C5b-9 complex inserts into the IM of Gram-negative bacteria. The second model proposes that only C9 molecules are required to mediate IM damage, and that the MAC just acts as a transporter of C9 molecules.

There are three options how the whole C5b-9 complex can insert into the IM. One option is that C5b-9 assembles at the IM. For this, all the complement factors have to transfer, through the OM-inserted C5b-9 (twin) channels, towards the periplasm. However, the complement cascade is triggered at the OM, and the time-restricted assembly of the C5b-9 complexes makes it unlikely that they will assemble at the IM. Moreover, it has consistently been shown that when C5b-8 complexes are complemented with C9 molecules, the bacteria are effectively killed.<sup>106,82,91,107</sup> Thus, the combination of C5b-8 complexes inserted in the OM and of C9 molecules, is sufficient to kill Gram-negative bacteria. Another option is that the C5b-9 complexes are inserted in specialized regions in the bacterial envelope, adhesion zones, wherein the membrane are at close proximity.<sup>99</sup> At these adhesion zones, bacterial proteins span both the IM and the OM. When the MAC assembles at these points, the C5b-9 complex might traverse the whole bacterial envelope (**Figure 9**, **left**). This might be reinforced by studies showing that certain antibodies can induce a complement-sensitive phenotype in Gram-negative bacteria.<sup>108,109,110</sup> This effect is mediated

at an early stage of complement activation, before the generation of C5 convertases.<sup>108</sup> This indicates that the location of C5b-9 complex assembly is important. However, the insertion of the MAC in specialized regions has yet to be proven. The third option how the MAC inserts into the IM is that the complex behaves differently in double membrane systems. Incorporation of C9 into the C5b-8 complex might differ from the circular self-polymerization in model systems. Although purely speculative, the environment of the periplasm combined with the two hydrophobic membranes might allow a C9 polymerization that reaches deeper into the bacterial envelope.



**Figure 9. Two models describing how the complement factors can mediate inner membrane damage in Gram-negative bacteria.** For the colors of the membrane attack complex (MAC) see Figure 5. *Left*: This model describes that the whole C5b-9 complex is inserted in the bacterial inner membrane. Normally, the bacterial envelop is too large for the MAC to reach the inner membrane. However, this might be possible at specialized adhesion zones, where also bacterial proteins crosses both membranes. *Right*: This model proposes that the MAC that is inserted in the bacterial outer membrane facilitates the transport of C9 (purple) towards the periplasm. Here, certain triggers might activate the C9 molecules. The activated C9 molecules insert into the inner membrane of the bacteria and induce bacteriolysis. Abbreviations; IMP: integral membrane protein; OMP: outer membrane protein.

The model that C9 molecules insert into the IM, while the C5b-9 complex only provides access to the bacterial envelope, is supported by direct data (**Figure 9**, **right**). As already discussed, the C9 protein has interesting abilities, *e.g.* its self-polymerization and hydrophilic to amphipathic transition. These features are controlled by the N-terminal part of the protein, and need triggers in order to activate. Activation of C9 by the C5b-8 or the C5b-9 complexes, inserted in the OM, likely results in incorporation of C9 into the OM membrane. Nevertheless, C9 can be activated by other triggers as well, and for this reason studies have examined whether the environment of the periplasm can activate C9 molecules. To address this, it has been tried to transfer C9 molecules into the periplasm without the help of other complement factors.<sup>89,103</sup> When bacteria are incubated in a high concentration of EDTA and sucrose, an osmotic shock is induced and the OM becomes porous. When this osmotic pressure is induced in the presents of C9, the complement factor is shocked into the periplasm. Inducing this C9 shock procedure into complement-sensitive bacteria reduced the oxygen consumption and survival of the target bacteria.<sup>89,103</sup> Interestingly, the transfer of C9 into the periplasm of complement-resistant bacteria did not affect the bacterial survival.<sup>89,103</sup> However, it cannot be excluded that high EDTA concentrations or the osmotic

shocking procedure itself activate the C9 molecules. Therefore, a more elegant approach to transfer C9 into the periplasm was tested. Gram-negative bacteria were transformed with a plasmid encoding for the C9 protein. To ensure that the protein would be transferred into the periplasm, a periplasm signal peptide was cloned in front of the C9 protein.<sup>111</sup> As a result, complement-sensitive bacteria were lysed by C9 proteins introduced in the periplasm. Consistently, complement-resistant bacteria were not susceptible to periplasmic C9 molecules.<sup>111</sup> Besides, studies have showed that the activated form of C9 can insert into IM vesicles and into bacteria stripped of their OM and peptidoglycan layers.<sup>79,60</sup> Collectively, it can be concluded that the periplasmic entry of C9 results into bactericidal effects, likely because of its insertion into the IM, while complement-resistant bacteria can withstand this attack. Nevertheless, it still has to be proven that C9 can enter the periplasmic space through the OM membrane lesions, caused by the C5b-9 complexes. This was addressed by two studies that incorporated C5b-9 complexes exclusively in the OM of complementsensitive bacteria. They accomplished that by either fusion of C5b-9 bearing liposomes with acceptor bacteria, or by allowing the spontaneous transfer of C5b-9 complexes from donor bacteria. Both studies incubated the C5b-9 bearing acceptor bacteria with C9 molecules, but the bacterial survival was not reduced, indicating that C9 was not able to enter the periplasm.<sup>84,85</sup>

### **Future directions**

Although there is quite some understanding of the killing process, there is still a need to clarify the steps in the process of MAC-mediated bacterial killing. Bacteria that resist fully assembled MACs might have acquired resistance phenotypes at the last step of the complement mediated killing process. Further understanding of this last step might allow systematic approaches to identify common resistant phenotypes.

The bactericidal mechanism of the MAC has been studied mostly in Gram-negative bacteria. It is clear that the last step before bacterial killing is IM damage caused by the complement factors. In this review two models have been described how IM damage can be generated. One of the model states that the C5b-9 complex traverses both the OM and the IM. This fundamental question can be examined in double-membrane liposomes, wherein the MAC might disrupt the inner membrane.<sup>112</sup> In fact, data from a recent EM study suggests that pneumolysin, a CDC protein, can disrupt two subsequent bilayers in multilamellar liposomes.<sup>113</sup> The second question would be if adhesion zones, whereby the OM and IM are separated by only a small periplasm, exist in Gram-negative bacteria. High resolution fluorescence imaging might be able to capture these locations. For the model whereby C9 is solely responsible for the IM damage, it is most important to prove that C9 can enter the periplasm of the target bacteria via the C5b-9 pores. Sensitive (pH activated) labeling systems or readouts should be developed to identify the presence of C9 in the periplasm. Moreover, it needs to be clarified which factors in the periplasm can activate C9 molecules. It has been reported that proteins in the periplasm can induce and prevent disulfide bond formation, which are required for C9 polymerization.<sup>111,114</sup> In addition, bacterial oxidative phosphorylation accelerates the MAC killing process, and this might be linked to proton secretion into the periplasm. It is interesting to test if these factors are necessary for bactericidal killing. Genetically enforcing these factors in complement-resistant bacteria might even allow a complement-sensitive phenotype. When the C9 (in)activating factors have been identified, a more systemic approach might reveal how complementresistant bacteria can withstand fully assembled MACs. Common mechanisms of complement-restriction might represent new therapeutic targets

Another interesting topic is the most common MAC-resistant phenotype, *i.e.* Grampositive bacteria. In these bacteria it is assumed that the assembly of the MAC get stuck in the peptidoglycan layer.<sup>1</sup> Hence, it is proposed that the thick peptidoglycan layer is the factor that determines their complement resistance. However, the interaction of the MAC with Gram-positive bacteria have not been studied extensively. There are two reports that

show that the C5b-9 complexes are assembled on Gram-positive bacteria.<sup>6,93</sup> These C5b-9 complexes have stable hydrophobic interactions, since they cannot be eluted by high salt concentrations.<sup>6</sup> At which component in the bacterial envelop, the stable MACs are assembled is unknown. However, it has been shown that the MAC depositions on Grampositive have specific localizations and this can differ between bacterial strains.<sup>93</sup> This indicates that although the bacteria prevent MAC assembly at most of its surface, there are places were the MAC can be assembled. Why the MAC cannot kill Gram-positive bacteria has not been examined, and this might be a topic for future research. One possibility is that the peptidoglycan layer prevents the MAC to reach the bacterial membrane. When the thickness of the peptidoglycan layer is reduced, the MAC might be able to reach and insert into the membrane. Another option is that the peptidoglycan layer inactivates C9. When C9 molecules are activated at a distance of the bacterial membrane, they will self-polymerize into a poly(C9) ring and lose their detergent capacity. For this, it is possible to test if C9b fragments, which have less tendency to polymerize into a ring structure, are able to reach the bacterial membrane. Alternatively, mutagenesis of the C9 molecule might decrease its susceptibility for activation, and this stable C9 construct may allow passage through the thick peptidoglycan layer towards the bacterial membrane. Another approach is to transform the Gram-positive bacteria with a secreting C9 plasmid, which introduces the C9 protein in front of the bacterial membrane, analogous to the transformation experiment in Gramnegative bacteria.<sup>111</sup> A different hypothesis is that the Gram-positive bacteria are not killed by MACs, even when they are inserted in the bacterial membrane. Pores in the Grampositive bacteria may not be lethal due to the protective function of the peptidoglycan layer. Another complement-resistant option is that Gram-positive membranes contain proteins that prevent the insertion of the MAC. This is an interesting option, since Gram-positive bacteria secrete CDC proteins which possess a structure similar to the MACPF domain.<sup>64,65</sup> Bacterial proteins in the membrane might capture the complement proteins and prevent their membrane insertion.

# Conclusion

The bactericidal mechanism of the MAC is a complex process. First, the complement factors have to recognize the target bacteria and activate the complement cascade. After marking the pathogen by deposition of C5 convertases there is a time-restricted sequence initiated by C5b, resulting in the membrane attachment of C5b-7. Further incorporation of C8 and C9 molecules lead to permeabilization of the target membrane. From model systems we know that these C5b-9 complexes are heterogeneous in composition, and form both pores and other membrane disrupting structures. Nevertheless, this membrane attack is not successful against Gram-positive bacteria and complement-resistant Gram-negative bacteria. Comparing bactericidal and non-bactericidal MACs gave us insight into the requirements for bacterial killing. Disruption of the OM in Gram-negative bacteria is not sufficient in killing the bacteria, nor is the assembly of the MAC on Gram-positive bacteria. The bactericidal effects are characterized by IM damage, which is likely executed by the complement factor C9. C9 has to be activated before it can self-polymerize and insert into target membrane via an amphipathic conformation. However, it is yet unclear if this protein targets the IM via incorporation into the C5b-9 complex, or rather ends up into the periplasm via C5b-9 pores and disrupts the inner lipid bilayer by itself. Moreover, the special roles for the periplasm and the bacterial energy metabolism in this process have yet to clarified. When the complete bactericidal mechanism of the MAC is determined, then new approaches to boost the complement system might be developed. Especially, the complement-resistance of bacteria that allow the full assembly of the MAC may be elucidated by systemic approaches.

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