IMMUNOBIOLOGICAL FUNCTIONS OF THE COMPLEMENT SYSTEM

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INTRODUCTION

Complement is a multimolecular, self-assembling system which acts as an important mediator in a variety of immunopathological processes (1-6).

Early immunologists showed that the destruction of certain microorganisms like *Vibrio cholerae* required specific antibody and a non-specific thermolabile factor present in normal serum which was called initially **alexin** (to keep the denomination used by Buchner for the substance involved in the bactericidal activity of normal serum) and for which Ehrlich and Morgenroth proposed the word **complement**, now abreviated as C.

Up to 1926, only 4 components were recognized in the complement system and almost nothing was known concerning its immunobiological functions, besides specific hemolysis and bacteriolysis. Today, 11 proteins are known to integrate the system and the spectrum of C activities has been considerably enlarged to include such effects as phagocytosis enhancement, chemotaxis for PMN-leukocytes and monocytes, increase in vascular permeability, contraction of smooth muscle, involvement in blood coagulation, generation of kinins, etc. It is the purpose of this paper to briefly review the present knowledge of the composition of this extremely interesting biological system, as well as its mode of activation and its participation as a mediator in some important immunobiological phenomena.

THE PROTEINS OF THE COMPLEMENT SYSTEM AND ITS REACTION SEQUENCE

It is presently accepted that the complement system comprises 11 protein components designated by C1q, C1r, C1s, C2, C3 and so on up to C9, the number of the component indicating the sequence in which they act, with the exception of C4, which acts after C1 and before C2.

These eleven proteins are subunits of three macromolecular assemblies: (i) the C1q,r,s macromolecule which represents the recognition and alarm system; (ii) the $C\overline{4b,2a,3b}$ enzyme, known as C5 convertase, responsible for the assembly of the membrane attack complex; and (iii) the cytolytic attack element itself which comprises complement proteins C5b to C9.

Activation of the recognition and alarm element

The recognition component of the complement system is C1q, a collagen-like glycoprotein of 400,000 daltons[1] which combines with determinants of the Fc region of IgM and of certain subclasses of IgG antibody, leading to the activation of $C\overline{1s}$ to the enzymatic state C1s,whose natural substrates are C4 and C2.

Assembly of the membrane attack element

The assembly of this element is initiated when $C\overline{1s}$ cleaves C4 into C4a (8,000 m.w.) and C4b (198,000 m.w.), as well as C2 into C2a (80,000 m.w.) and C2b (37,000 m.w.). In the course of immune lysis, only a small proportion of C4b combine with receptors (S′) on the erythrocyte surface. Most of it remains in the fluid phase and becomes inactive, because of the short half-life of the binding site of the fragment.

[1] The molecular weights given throughout this text refer to human C.

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Bound C4b provides the receptor for C2a and a new enzyme is formed, $\overline{C4b,2a}$ or C3 convertase, which cleaves C3 into C3a (8,900 daltons) and C3b (ca. 170,000 daltons), the latter being able to fix onto numerous receptors (S") of the cell surface. However, only those C3b fragments that are bound in the proper orientation, adjacent to the $\overline{C4b,2a}$ enzyme, are able to participate in the lytic process, by forming another enzyme – the C5 convertase, a trimolecular complex ($\overline{C4b,2a,3b}$), which cleaves C5 into C5a (16,500 daltons) and C5b (ca. 165,000 daltons). Bound C5b decays rapidly to an inactive state, unless stabilized by combining with C6.

The cytolytic element

The C5b,6 complex reacts with C7 to form another trimolecular complex, C5b,6,7 which is able to bind to erythrocytes even in the absence of antibody (Reactive lysis; cf. 7,8). The half-life of this complex in the fluid phase is, however, very short (30 sec. at 37°). Therefore, only a small proportion of nascent C5b,6,7 becomes embedded in the phospholipid bilayer of the cell membrane in a stable form and is able to react with C8 and C9 to provide the final "needle", a decamolecular complex – $C5b,C6,C7,C8,(C9)^6$, of about 1,000,000 daltons m.w. (9), which pierces the membrane, generating "transport pores" or channels through which salt, water and other small molecules can readily flow, but not macromolecules such as proteins and nucleic acids. The biomembrane acquires then the characteristics of a semipermeable membrane, and as a consequence of a Donnan effect, is explosevily ruptured liberating the hemoglobin content of the red cell.

Two remarks may be appropriate before we come to the next item of this presentation and go into the discussion of some important biological activities of the complement system, which, by the way, do not involve the entire sequence C1 to C9, as described above and diagrammatically represented in table 1 and figure 1.

1. Indirect, as well as direct evidences suggest that complement cytolysis is a "one-hit" process, a single hole in the membrane being sufficient for lysis (10), cf. figures 2 and 3.

2. During the sequential action of the C components on the cell membrane, i.e., in the so-called "complement cascade", two important amplification effects occur: one single $\overline{C1s}$ can generate a series of sites carrying $\overline{C4b,2a}$ and, in turn, each $\overline{C4b,2a,3b}$ can generate a cluster of C5b-9 lytic sites (S‴).

ACTIVATION OF COMPLEMENT BY THE ALTERNATIVE PATHWAY

An alternative pathway which activates the complement system bypassing C1, C4 and C2, was revived in 1968 in studies of the reaction of endotoxic lipopolysaccharide (LPS) with normal guinea pig serum. It was then shown that, like in the inactivation of C3 by cobra venom *(Naja naja)* and by zimosan (a mannanpeptide of the yeast cell wall), studied more than 30 years ago, an unusual C-fixing behavior was exhibited, in that C3-C9 were destroyed, while C1, C4, and C2 were not significantly affected.

Like properdin (P), in the classical work of Pillemer (1954), LPS does not react directly with C3, but through an intermediate, LPS-X, which is practically free of C4 and C2, but reacts exactly as the C3 convertase $\overline{C4b,2a}$ (11).

In the case of cobra venom, a purified factor, CVF, was isolated (12) which, according to recent work, may well correspond to C3b (13). This factor, a glycoprotein of 144,000 m.w., reacts with a thermolabile beta-pseudoglobulin of 80,000 daltons called C3 Proactivator (C3PA), in the presence of the corresponding enzyme C3PAse (20,000 daltons) and of C3, to build up an enzyme which, like $\overline{C4b,2a}$ converts C3 into C3a + C3b (cf. table 2 and figure 4).

Pillemer's original interpretation has been revised in the light of these findings and serum factors A and B of the properdin system were respectively identified to C3 and to C3PA. In the assembly of the alternative convertase $\overline{C3b,Bb}$, B is the counterpart of C2 in the classical C3 convertase (cf. ref. 2).

The alternative pathway is supposed to be of relevant importance in the pre-antibody phase of

resistance to bacterial infection, as exemplified by studies on the phagocytosis of encapsulated pneumococci in the absence of anticapsular antibody, by way of the heat labile opsonin (HLO) system of the pneumococcus. The activator agent seems to be a guinea pig alpha-2 immunoglobulin which combines with the microorganism. Then, C3PA(B) is taken up, C3 is cleaved and C3b is incorporated to the complex that becomes opsonized (14,15). We shall return to this subject when discussing the role of C in phagocytosis. Before closing this section it may be pertinent to indicate a number of tools which enable us to differentiate the alternative from the classical C4,2 pathway: (i) the alternative pathway is not inhibited by anti-C2; (ii) it takes place in the presence of EGTA (ethyleneglycol tetracetate), which chelates Ca^{++}, but not Mg^{++} (EDTA will prevent both pathways); it occurs with the sera of C4 deficient guinea pigs.

TABLE 1. *The "complement cascade" and some immunobiological properties of fragments and intermediate complexes*

$$S + A + C1 \xrightarrow{Ca^{++}} S\overline{AC1} + C4 = C4a + C4b + C2 = C2a + C2b$$

$$S' + C4b \longrightarrow S'C4b + C2a \xrightarrow{Mg^{++}} S'\overline{C4b,2a} \text{ (C3 convertase)} + C3 = C3b + C3a \text{ (Analphylatoxin)}$$

$$S'\overline{C4b,2a} + C3b \longrightarrow S'\overline{C4b,2a,3b} \text{ or C5 convertase. Immune Adherence. Phagocytosis.}$$

$$S'' + C3b \longrightarrow S''C3b. \text{ Immune adherence.}$$

$$S''' + C5b \longrightarrow S'''C5b \text{ (Unstable)} + C6 \longrightarrow S'''C5b,6 \text{ (Relatively stable)} + C7 \longrightarrow S'''C5b,6,7 \text{ (Stable). Chemotaxis.}$$

$$S'''C5b\text{–}7 + C8 \longrightarrow S'''C5b\text{-}8 \text{ (Partial hole)} + C9 \longrightarrow S'''C5b\text{-}9 \longrightarrow S \text{ (Complete hole)}$$

$$S'\overline{C4b,2a,3b} + KAF \longrightarrow C3c + S'C4b,2a,3d + K = \text{Conglutination.}$$

S are Forssman sites.
S' are C4b sites or closely adjacent C3b sites.
S'' are sites for C3b distant from S'.
S''' are C5b sites.

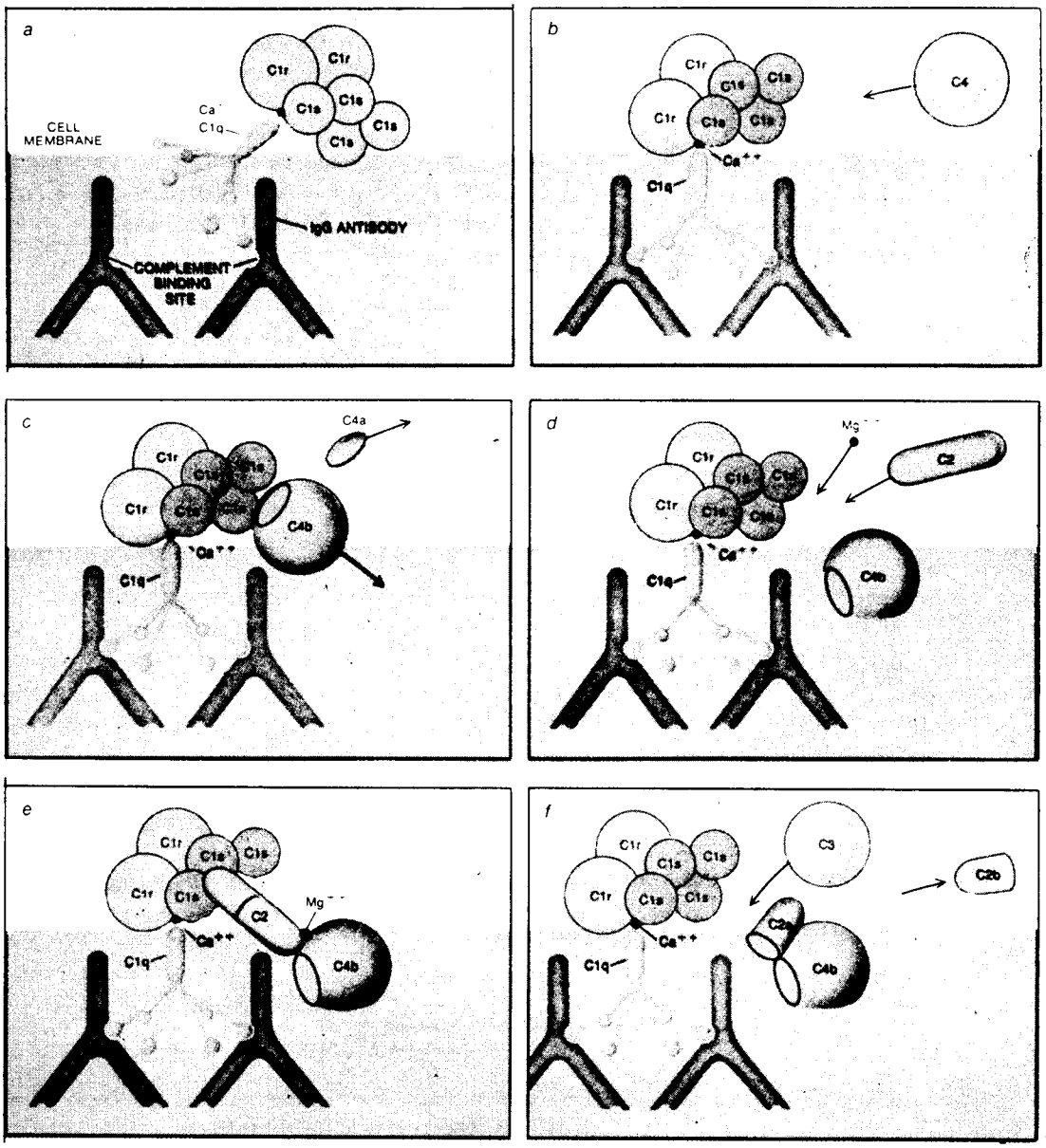
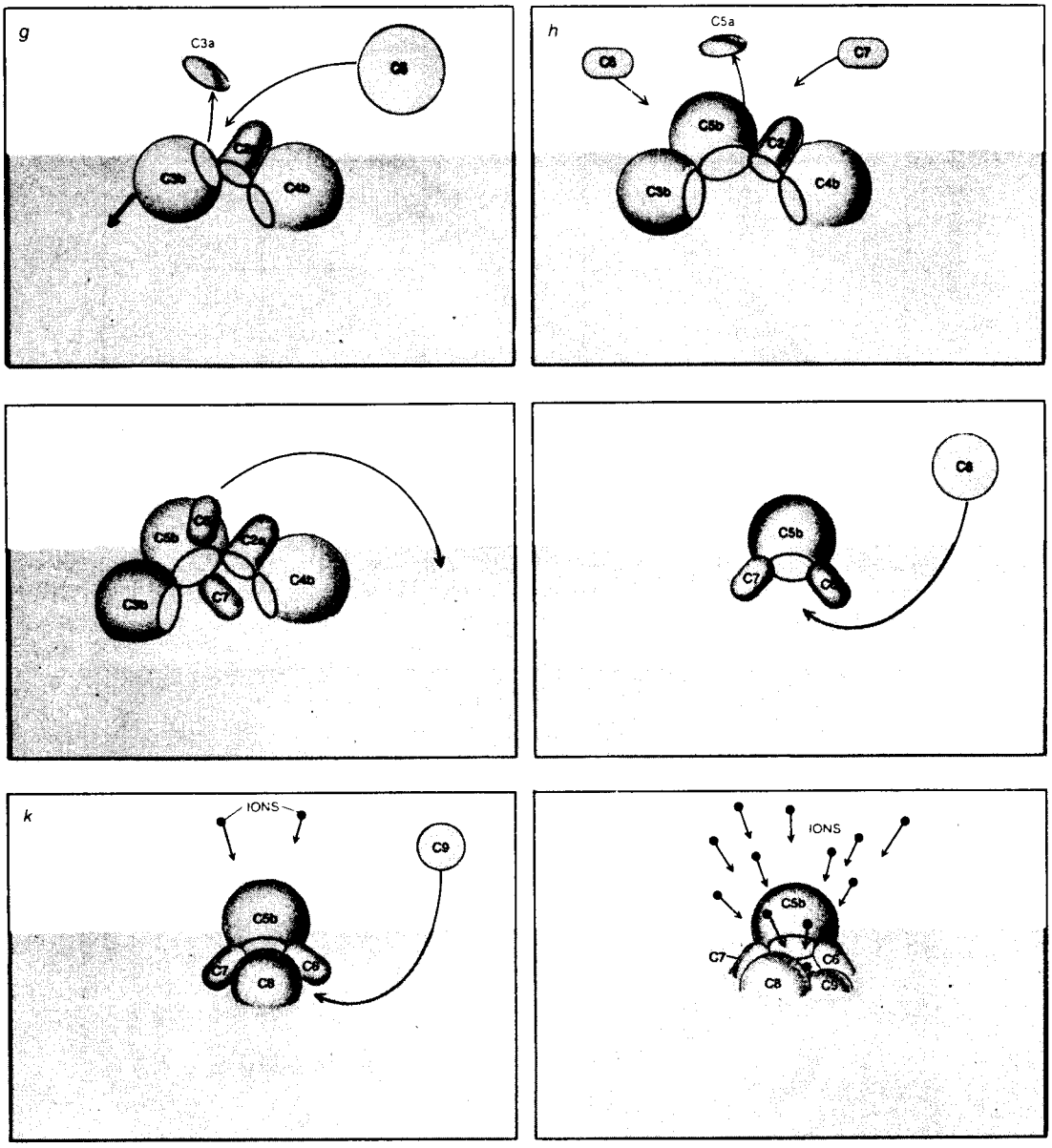


FIGURE 1. CLASSICAL WAY OF ACTIVATION OF COMPLEMENT IN IMMUNOHEMOLYSIS (repr. from Mayer, M.M., ref. 1). Two molecules of IgG bound to contiguous Forssman sites of the red cell membrane (a) provide in their Fc regions the combining sites for C1q(b), which is then activated to C1s, a proesterase able to cleave C4 and C2(c,d). C4b fixed to nearby sites provides the receptor for C2a and an enzyme is formed,



$\overline{C4b,2a}$ (C3 convertase) which is able to split C3 into C3a + C3b(e,f,g), the latter being incorporated to the complex to build up $\overline{C4b,2a,3b}$ (C5 convertase), an enzyme which splits C5 into C5a + C5b (h). The reaction proceeds by stabilization of bound C5b by the succesive binding of C6 and C7 (i,j). Finally, 1 molecule of C8 and 6 of C9 (only 1 is shown in the figure) are incorporated to form the cytolytic attack element (k,l).

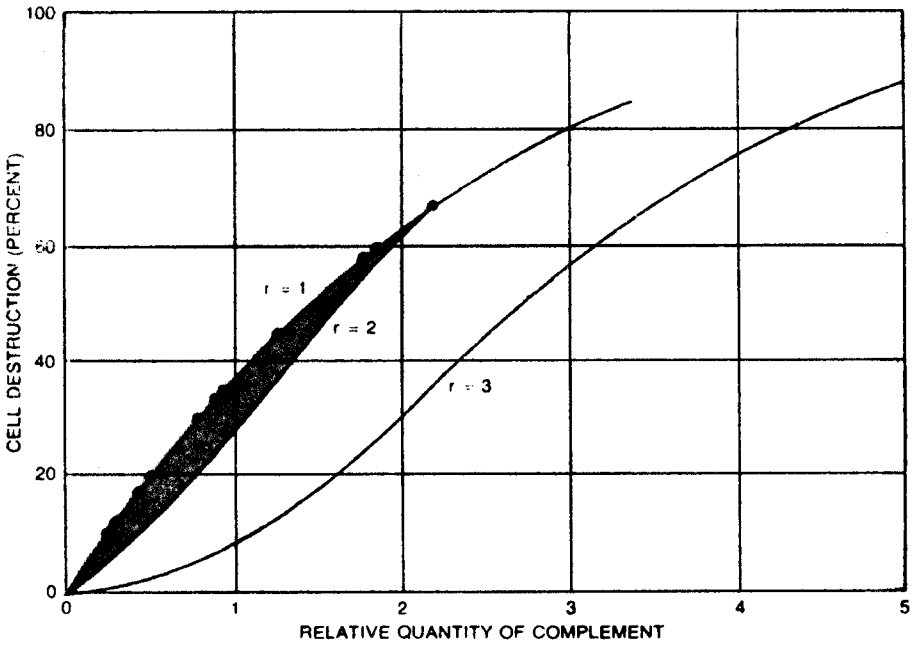


FIGURE 2. DEMONSTRATION OF THE ONE-HIT THEORY (repr. from Mayer, M.M. ref. 1). Theoretical curves were calculated from the binomial probability distribution for r = 1, r = 2, and r = 3. The number of lysed cells from a fixed amount of E and variable amounts of C fits the one-hit curve.

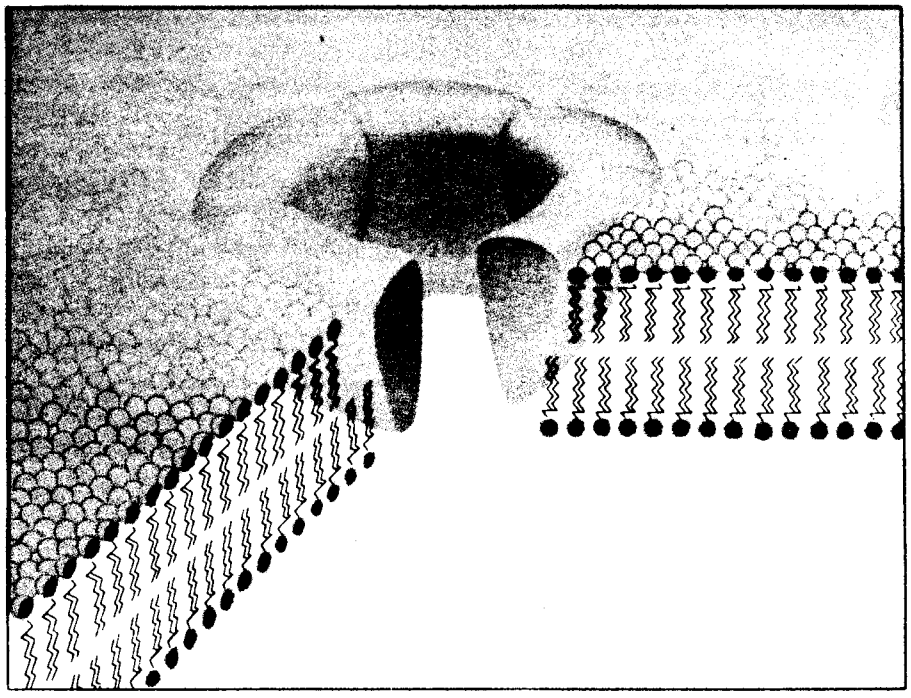


FIGURE 3. THE "DOUGHNUT HYPOTHESIS" (repr. from Mayer, M.M., ref. 1). The holes produced by C5b-9 would have a doughnut-shaped structure. Electron micrographs show such holes with a diameter of 10-11 nanometers for the human red blood cell.

TABLE 2. *The alternative pathway of complement activation*

1. Original observations of Pillemer (1954) on the properdin system

1.1 $Z + P + A + B \xrightarrow[15°]{Mg^{++}} PZ\ (Sediment) + RP\ (Supernate)$

1.2 $PZ + RP + C3 \xrightarrow[37°]{Mg^{++}} Destruction\ of\ C3$

The unit of properdin was defined as the amount required to inactivate all the C3 from a solution of RP containing 120 units of C3/ml, in the presence of a standardized amount of Z.

2. Present interpretation of the alternative pathway

2.1 $P + Z \xrightarrow{Mg^{++}} \overline{P}$

$P + \overline{IF} \longrightarrow \overline{P} \longrightarrow D$

2.2 $\overline{P} + A(C3) + B + \overline{D} \longrightarrow \overline{C3b.Bb}$

$CVF(C3b) + C3PA + C3PAse \longrightarrow$

2.3 $\overline{C3b,Bb} + C3 \longrightarrow C3a + C3b$

2.4 $C3b + KAF(C3INA) \longrightarrow C3c + C3d$

NOTES:

a) Other activating agents besides Z and $\overline{IF}$ are LPS, the HLO system of pneumococcus, the envelope of cercariae of *S. mansoni* or of epidemastigote forms of *T. cruzi*, etc.

b) Pillemer's A has been identified to C3, this showing the autocatalytic nature (feedback action of C3b) of the alternative pathway (cf. steps 2.2 and 2.3). The alternative C3 convertase includes Bb, a fragment of B, as the counterpart of C2a in the classical C3 convertase includes Bd, a fragment of B, as the counterpart of C2a in the classical C3 convertase.

c) KAF acts as a regulator and modulator, as shown in step 2.4.

BIOLOGICAL ACTIVITIES

Complement as a mediator of opsonization

Although the enhancing effect of C on the phagocytosis of bacteria treated with specific antibodies contained in normal serum (opsonins) was clearly demonstrated in the beginning of the century and a similar effect was observed with antibodies of hyperimmune sera (immuneopsonins or bacteriotropins) some 45 years ago, the manner in wich C mediated opsonization was entirely obscure until the 1950's when the phenomenon of immune adherence (IA) was discovered (16). The adherence of sensitized bacteria onto the surface of primate erythrocytes would enhance phagocytosis by a mechanism similar to that operating in surface phagocytosis. By the 1960's it was shown

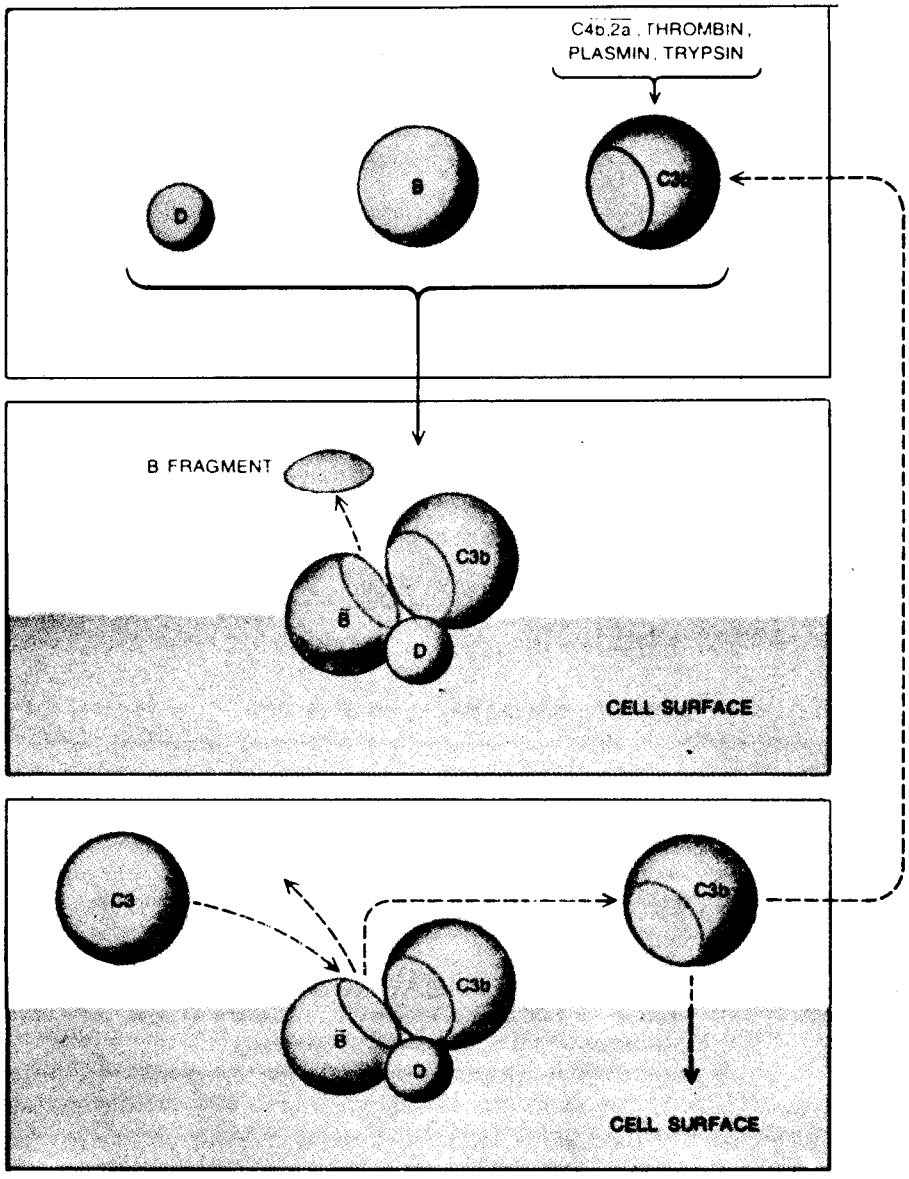


FIGURE 4. COMPLEMENT ACTIVATION BY THE ALTERNATIVE PATHWAY (repr. from Mayer, M.M., ref. 1). Activation by the alternative pathway requires at least 3 serum factors: C3b, B and $\overline{D}$. B is cleaved into Ba + Bb (not shown in the figure) and an enzymatic complex is formed C3b,$\overline{B}$,D (the tendency today is to ascribe to this complex the composition $\overline{C3b,Bb}$, Bb being the counterpart of C2a in the classical C3 convertase), which splits C3 into C3a + C3b.

that EAC1,4b,2a,3b was markedly susceptible to phagocytosis and that no increase resulted following the addition of C5-9 (17).

The opsonic effect of C3b started however to be considerably clarified by the works of Nussenzweig, Bianco and Rabinovich in the last ten years (18,19).

Briefly summarizing, these authors have definitely established that immunophagocytosis depends on the presence on phagocytes of receptors for Fc of IgG, as well as of receptors for C3b (cell receptor I) or for C3d (CR II). IgM by itself is not opsonizing, although very effective in promoting adhesion, as evidenced by rosette formation. With E IgM C3b a marked formation of rosettes with PMN-leukocytes was observed, but no phagocytosis (direct observation after lysis of adherent particles with water). If, however, IgG was added to the system, a massive phagocytosis ensued.

The experiments showed conclusively that adhesion alone does not provide an effective signal for ingestion and that the effect of bound C3b (or C3d) is perhaps ascribable to the approximation of phagocyte particles, bringing them outside repulsion forces. The addition of IgG would then have a zippering effect and promote englobement. Experiments by ultracentrifuging E + IgG seem to substantiate this hypothesis.

Complement and anaphylaxis

The role of C in anaphylaxis has never been satisfactorily demonstrated and, as a matter of fact, evidence accumulated in the last years indicate that complement does not participate in "true" anaphylactic reactions involving the production of IgE antibodies in actively sensitized animals.

"Decomplementation" experiments performed in rats with heterologous (rabbit) antibodies, using Ovary's PCA reaction, led to the finding that such local reactions were entirely abolished (20), but the final proof, the regeneration of sensitivity by "recomplementation" could never be provided. Furthermore, it is quite possible that anaphylatoxin and other inflammatory mediators may be produced locally in experiments of this kind. May as it be, it is quite interesting to note that 2 fragments of C components, as shown some ten years ago, are endowed with anaphylatoxin activity, namely C3a and C5a (21-23). In human serum, C3a has a molecular weight of 8,900 daltons and C5a 16,500. Both are peptides and activate a proesterase of PMN-leukocytes, this constituting an obligatory step in the chemotactic events elicited by them.

Using an immunofluorescent technique it was possible to show that C3a binds on the plasma membranes of mastocytes and the distribution of the dye suggest the presence of distinct receptors for this fragment. Uptake of I^{131} labeled C3a correlates with histamine release.

C3a is derived from the N-terminal end of the alpha-chain of C3 by cleavage between amino acids 77 and 78 by the classical or alternative C3 convertase. The presence of COOH-terminal arginine in C3a is a prerequisite for its biological activity.

Likewise, C5a derives from the alpha-chain of C5 by the action of C5 convertase of either pathway and the removal of its COOH-terminal arginine residue abrogates its biological activity. Thus, both C3a and C5a are controlled by carboxypeptidase A, also called anaphylatoxin inactivator, an alpha-globulin enzyme of m.w. 300,000 daltons (cf. ref. 2).

Other important differential characteristics of C3a and C5a are given in table 3.

Complement and immune complex diseases

While the participation of C in anaphylaxis induced by IgE antibodies is probably nil, converging lines of evidence derived particularly from experimental models indicate that activation of C is an essential component in the pathogenesis of immune complex disease.

The evidences referred to above include among others:

1. The prevention by "decomplementation" of passive Arthus reaction (24), as well as of nephrotoxic nephritis and of artery lesions in the acute immune complex disease of the rabbit (25).

2. The demonstration by immunofluorescence of both antigen-antibody complex and complement at the sites of tissue damage, such as the wall

TABLE 3. *Differential characteristics between C3a and C5a**

Fragment	Cleaving enzymes	Contraction of g.p. ileum	Contraction of rat uterus	Mastocyte lesions Rat	Mastocyte lesions G.P.	Histamine liberation from mastocytes Rat	Histamine liberation from mastocytes G.P.
C3a	$\overline{C4b,2a}$ or $\overline{C3b,Bb}$	+	—	+	+	+	+
	Trypsin	+	—	+	+	+	+
	Plasmin	+	—	+	+	+	+
	Complex CVA+C3PA	+	—	+	+	+	+
C5a	$\overline{C4b,2a}$ or $\overline{C3b,Bb}$	+	—	—	+	—	+
	Trypsin	+	—	—	+	—	+
	Agar	+	—	—	+	—	+

*(mod. from Dias da Silva, in Bier *et al.*, cf. p. 149, ref. 6).

of affected vessels in the immunological vasculitis and the basement membrane of kidney glomeruli (GBM) in experimental and clinical glomerulonephritis.

3. In these situations the mere presence of immune complexes is not sufficient to produce tissue damage, as evidenced in "decomplemented" animals. The real nosogenic agent is complement, whose activation by either pathway induces chemotactic attraction of PMN-leukocytes mainly through the local release of C5b,6,7.

4. Only C-fixing antibodies are able to induce passive Arthus reactions. Guinea pigs antibodies of the IgG-1 subclass do not fix C and are able to induce anaphylaxis, but not the Arthus reaction. Vice-versa, g.p. antibodies of the IgG-2 subclass bind C and elicit the Arthus reaction, but not anaphylaxis.

5. In the experimental serum sickness of the rabbit produced by massive injection of heterologous protein, as detectable immune complexes appear in circulating blood, complement drops drastically and morphologic lesions appear in the heart, arteries, joints and kidneys.

6. Low C levels are found in the autologous phase of experimental nephrotoxic nephritis, as well as in immune complex nephritis, e.g., in Systemic Lupus Erythematosus. In the first case antibody is directed against GBM, while in lupus deposits of DNA-antiDNA are demonstrated in a lumpy pattern between GBM and the epithelium. Complement is activated, PMN-leukocytes are attracted by C5b,6,7 and proteolytic enzymes liberated by accumulated leukocytes "chew up" GBM. Identical mechanism seems to operate in the case of the Arthus reaction, a further element to be considered in the pathogenesis being the acceleration of blood coagulation.

Complement and blood coagulation

The relation of C to blood coagulation has long be suspected, but only recently it could be established on a solid basis with the availability of suitable experimental techniques.

At present there are two lines of evidence linking the blood coagulation and complement systems:

1. Hageman Factor (XII), when activated by glass surfaces or ellagic acid, converts plasminogen to plasmin, an enzyme shown to be an activator of C1 to $C\overline{1s}$. This conversion can be demonstrated with the plasma of patients with hereditary angioedema, which lack $C\overline{1s}$ inhibitor, but not with the plasma of normal subjects (26).

2. The addition of immune complexes to normal rabbit platelets in the presence of Mg^{++} leads to thrombocytolysis with the release of histamine and serotonin, as well as of a clot factor. Rabbits deficient in C6 have a prolonged clotting time and this stems from its inability to promote allergic platelet lysis. The addition of C6 corrects this abnormality of blood coagulation (27).

The activation of the complement system in these kinds of experiments proceeds apparently through the alternative pathway, since guinea pig IgG-1 antibodies and the F $(ab')^2$ of IgG2 which are unable to bind complement via C4,2 are as efficient in triggering immunological platelet injury as C-fixing "whole" IgG2 antibodies. Moreover, the reaction requires only Mg^{++}, being inhibited by EDTA, but not by EGTA (cf. ref. 5 , pp. 140-147).

Studies with human platelets treated with zymosan or anti-platelet antibody in the presence of complement result also in the appearance of a thrombin-like activity which may be related to the same clot factor involved in the allergic lysis of rabbit platelets referred to above.

SUMMARY AND PROSPECTUS

An attempt was made to analyse the role of complement in immunobiological phenomena, by restricting the compendium of its activities to four selected fields which have been better explored up to the present time. Many other biological activities could be listed, such as the role of complement in antibody systhesis (28, 29), in the rejection of allografts (31) or in tumor immunity, (cf. ref. 4, p. 77) but a rigid evaluation of the role of complement in these phenomena awaits additional clarifying data which will certainly become available with the elucidation of the precise nature of the various modes of interaction between complement molecules and target cell membranes.

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