

RAPID TESTING OF BIOMATERIALS

For Complement Activation Using In Vitro Complement Immunoassays

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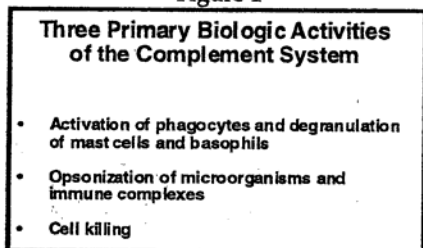
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1. The Complement System.

The complement system is part of the human immune system. One of its primary functions is to resist microbial infections by promoting an inflammatory response. Three important biological activities of the system are shown in Figure 1. When biomaterials activate complement, the ensuing inflammatory response, normally directed against microorganisms, can lead to harmful acute effects and potentially to less well-understood chronic effects (eg., immunosuppression).

Figure 1



The complement system consists of 20 different plasma proteins. It has two activation pathways: The classical pathway, shown on the left in Figure 2, and the alternative pathway, shown on the right.

The primary goal of each pathway is to form a proteolytic enzyme which can cleave C3. C3 is the most abundant complement protein in our blood (approx. 1000 µg/mL). It is the key protein associated with the major biologic activities of complement.

Upon C3 cleavage, two fragments are produced, C3a and C3b; C3b can participate in the formation of either of two enzymes which cleave C5. Cleavage of C5 triggers activation of the common terminal pathway, producing the most potent anaphylatoxin, C5a, and leading to the most dramatic effect of complement activation – cell lysis or killing.

In Figure 2, the names of four different proteins are indicated: C4; Factor B; C3; and C5. As shown in subsequent pages, monitoring these proteins forms the basis of our ability to detect the activation of the different complement pathways and the C3 protein by biomaterials.

Figure 2

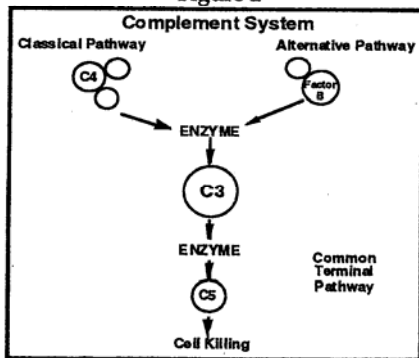
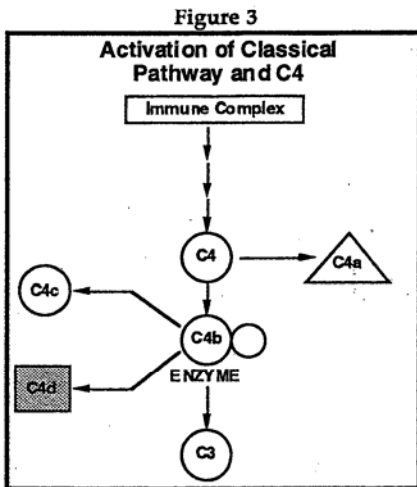


Figure 1. For simplicity, only four of the complement proteins are indicated by name in this figure. For a thorough discussion of the interactants, see reference 1.

2. Classical Pathway.

The classical pathway, shown in Figure 3, is usually activated by immune complexes, i.e., antigen-antibody complexes. The primary goal of activation is to make an enzyme for cleaving C3; however, in the steps leading to the assembly of this enzyme, complement protein C4 is cleaved giving two initial activation fragments: C4b and C4a — an anaphylatoxin (shown in the triangle).



C4b combines with a fragment of C2 to form an enzyme, the classical pathway C3 convertase. Most enzymes in the complement system are under stringent regulation by complement control proteins. C4b is the target of some of these control proteins (C4 binding protein and Factor I) and readily converted to C4d and C4c. The C4d fragment, as well as C4c, are devoid of

the ability to participate in the formation of any new enzyme. In this manner, the classical pathway C3 convertase (and C5 convertase) are negatively regulated.

Since C4 is unique to the classical pathway, i.e., it is not consumed in the alternative nor in the common terminal pathway, **detection of C4d in a test sample is proof of C4 activation and, therefore, classical pathway activation.**

3. Alternative Pathway.

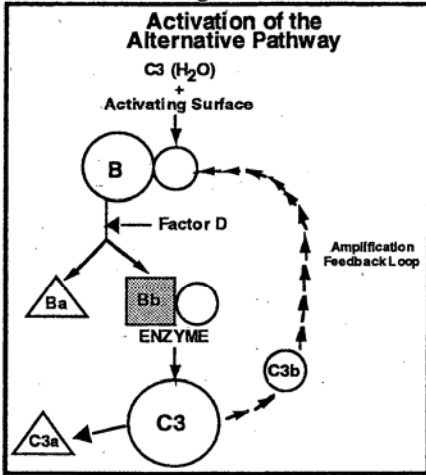
The alternative pathway can be activated by water-reacted C3 (which is constantly being formed *in vivo* at a low level) plus an appropriately receptive surface — for example, a surface like those seen in cellulosic hemodialyzers or the surface of a bacterium which has accessible hydroxyl or amino groups.

Like the classical pathway, the primary goal of the alternative pathway is to generate a C3-cleaving enzyme, in this case the alternative pathway C3 convertase. In the process of assembling such an enzyme, Factor B is cleaved by Factor D, shown in Figure 4, giving two activation fragments called Bb and Ba. The Bb fragment contains the proteolytically active site of the C3-cleaving enzyme of the alternative pathway.

Since Factor B is unique to the alternative pathway, detection of Bb in a test sample is proof of activation of Factor B and of its corresponding alternative pathway.

A special feature of this pathway is that C3b, produced by C3-cleavage, can actually feedback and promote the generation of more alternative pathway C3 convertases. This feedback loop greatly amplifies the amount of C3 which will be cleaved as a result of complement activation.

Figure 4



Most of the complement bioincompatibility associated with biomaterials is due to activation of Factor B, the alternative pathway, and its amplification feedback loop.

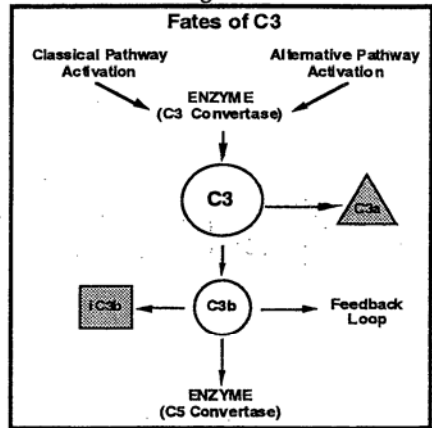
Figure 5 shows some of the fates of C3. Once either activation pathway is triggered, a C3-cleaving enzyme will be formed. This enzyme, whether from the classical or alternative pathway, cleaves C3 into two fragments: C3b and C3a. C3a, in the triangle, is a potent anaphylatoxin. C3b is the activated form

of C3 which is responsible for many of the biological activities of complement.

Because C3b is so biologically active, it is stringently controlled *in vivo*. This is accomplished by cleavage of C3b to iC3b shown in the box ("i" means inactivated).

Because C3 can be activated by either the classical or alternative pathway, the detection of either iC3b or C3a in a test specimen is proof of complement activation by one or both pathways.

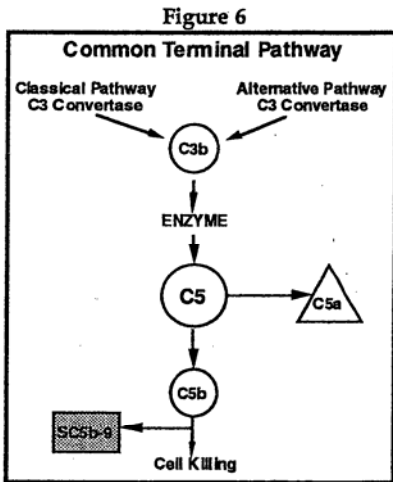
Figure 5



Therefore, detection of an increase of either fragment, C3a or iC3b, as a result of exposure of a biomaterial to human serum -- in a properly controlled experiment -- is proof that the biomaterial can activate complement.

4. Common Terminal Pathway.

As shown in Figure 6, the C3b which is generated as a result of C3 cleavage can also participate in the formation of new enzymes which cleave C5 to yield two fragments: C5a, the most potent anaphylatoxin, and C5b, which possesses the capacity to drive the rest of the common terminal pathway of complement.



One consequence of C5b generation, after sequential interaction with four other proteins, is the formation of the membrane attack complex (MAC) in cell membranes and target cell killing. With *in vitro* test samples, however, and to a measurable extent *in vivo*, much of the C5b generated does not result in the formation of MAC, but is diverted by control proteins (S protein) to form a soluble, lytically inert complex called SC5b-9, shown in the box in Figure 6. Recently another serum protein called

clusterin (also known as Apo J and SP40,40) was shown to bind C5b67 and prevent the formation of MAC in a manner analogous to that of the S protein.

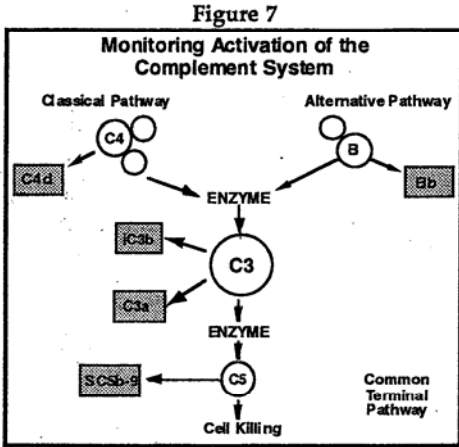
Because C5 is unique to the common terminal pathway of complement, detection of C5a or SC5b-9 in a test sample is absolute proof of activation of the terminal pathway of complement in the specimen.

An important point to remember is that for each mole of SC5b-9 detected, a mole of C5a is also generated. C5a is a small, sticky, and elusive molecule. It has a very brief *in vivo* half-life (less than one minute), since it is rapidly captured by cells with C5a receptors. SC5b-9, on the other hand, is an extremely stable, soluble macromolecular complex, and can be used legitimately as a marker for C5 cleavage, and, therefore, C5a anaphylatoxin generation.

5. Monitoring Complement Activation.

To summarize, by measuring activation fragments of C4, Factor B, C3 or C5 in a test sample, it is possible to determine whether complement has been activated as a result of exposure of serum to a test material, and, furthermore, which pathway or pathways were involved.

Figure 7 depicts the complement system and, in boxes, shows the complement fragments which can be used as markers to assess the activation status of different pathways or proteins in the system.



6. Anaphylatoxins.

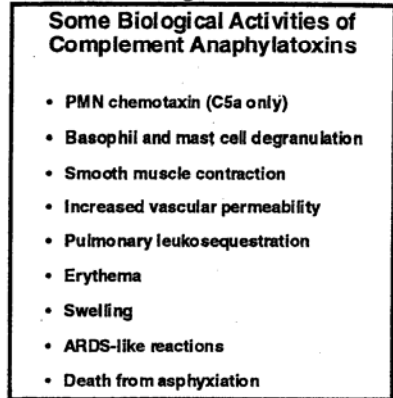
As mentioned above, complement has many important biological activities. For purposes of this monograph, we want to emphasize those associated with the anaphylatoxins. Generation of excessive quantities in a patient as a result of exposure to a complement activating material can lead to a number of biologic responses, shown in Figure 8.

This is one reason why assessing the complement activation potential of new biomaterials is important.

Notably, some of the other biological consequences of complement activation can also harm a patient especially over

the long term — but those induced by anaphylatoxins are the most dramatic and the ones which should be avoided absolutely.

Figure 8



7. Assays for Monitoring Complement Activation.

QUIDEL has developed five assays to detect and quantify many of the complement activation fragments in human serum. These are depicted in Figure 9; the analytes are shown also in Figure 7.

If exposure of a material to human serum *in vitro* produces one or more of these fragments, then the material is a complement activator. If, in properly designed experiments, exposure *in vivo* produces elevated complement fragments, then either the biomaterial is an activator and/or the mechanical or indirect effects of the device incorporating the biomaterial results in complement activation.

Using one or more of these assays in appropriately designed experiments, it is now possible to determine whether a test material is complement activating and, if so, by which pathway.

Figure 9

Commercial Enzyme Immunoassays Available from QUIDEL	
<u>Activation Fragment Detected</u>	<u>Proof of Complement Pathway Activation</u>
C4d	Classical Pathway
iC3b	Classical and/or Alternative Pathway
C3a	Classical and/or Alternative Pathway
Bb	Alternative Pathway
SC5b-9	Common Terminal Pathway

Figure 10 shows some of the features of these assays. They are standard microassay plate enzyme immunoassays and are accordingly easy to use. They are familiar to most laboratory technicians who conduct immunoassays. They require no radioactivity and the expense

Figure 10

Important Features of the Complement Activation Assays
• Enzyme Immunoassays
• Breakaway 96-well microassay plates
• No radioactivity
• No special processing of test sample
• Include standards and controls
• Require only 3 hours (including incubation)
• Have several months shelf-life
• Automatable
• Constitute a comprehensive panel
• Available now

and safety hazards associated with radioactivity. Each kit includes standards and controls for quality control and quantitation. Each kit has several months shelf-life. Standard laboratory equipment can be used to automate most of the procedural steps. And, if needed, a comprehensive complement activation test panel covering all the complement activation pathways can be assembled.

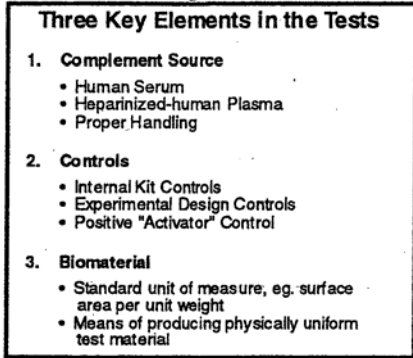
8. Experimental Design.

Key-Elements. In testing biomaterials for their complement activation potential, three elements in particular should be emphasized (Figure 11).

First, the Complement Source. It must be human serum or heparin plasma. ACD and EDTA plasma will not work (the anticoagulants inhibit complement). Serum is the preferred complement source, since heparin also can modulate complement activity. Proper handling of the complement source is critical, since mishandling can lead to artifactual complement activation.

Second, the Controls. The kits contain internal controls and standards. Also, the experimental design must account for complement activation which occurs spontaneously, independently of interaction of the biomaterial with serum. This control will be explained in more detail later.

Figure 11



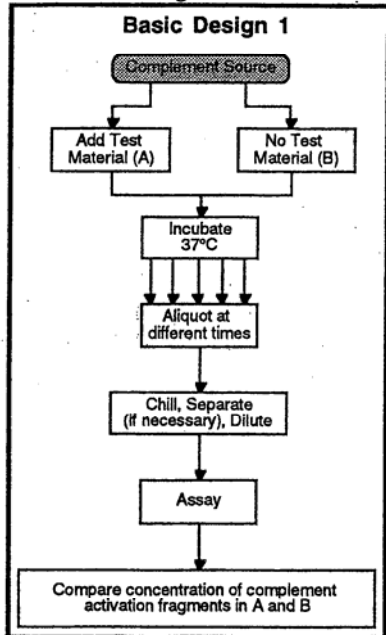
And third, the **Biomaterial**. Complement activation induced by biomaterials occurs (usually) at the interface of the biomaterial surface and the fluid phase, complement source. A standard unit of measure such as surface area per unit weight is useful to allow calculation of the complement activating potential of the biomaterial. Given this fact, a means of producing physically uniform test material, for example, a powder of known, reproducible particle size, would be helpful.

There are two basic experimental designs.

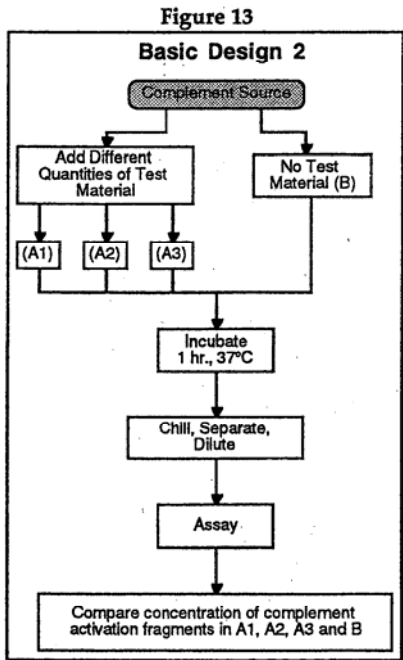
Experimental Design I. In Design 1, shown in Figure 12, a constant amount of test material is added to the complement source, giving reactant mixture A. In parallel, the same complement source without added test material is also prepared, giving B. Complement activation is allowed to proceed by incubation at 37°C. Aliquots are taken at different time points from A and B. If activation is occurring, then there should be a steady increase in the

concentration of the activation fragment over time. By comparing the concentrations of complement activation fragments in A to those in B, one can conclude if activation, as a result of exposure to the test material, has occurred.

Figure 12

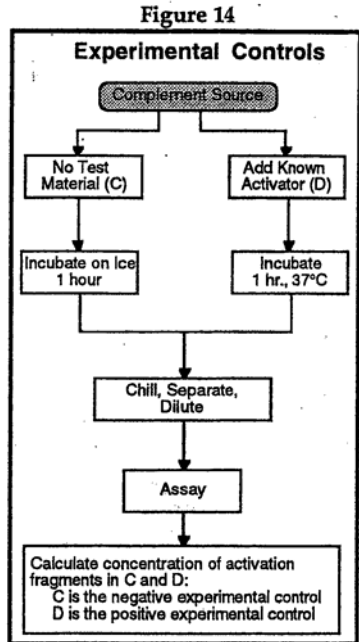


Experimental Design II. In Design 2, shown in Figure 13, the complement source is incubated with increasing amounts of the test material, giving A1, A2, etc. The complement source control, B, is also included. After a standard incubation, eg. 1 hour at 37°C, the concentrations of complement activation fragments in reaction mixtures, As, are compared to those in B, which was not exposed to the test material.



Two experimental controls can be included with either design 1 or 2 (see Figure 14). Control C should always be conducted, whereas D is optional, but recommended.

If the complement source is incubated at 0°C, no complement activation should occur (Control C). Control C gives the actual baseline value in the complement source. At 37°C, even in the absence of Test material, some activation will occur spontaneously (Control B).



By adding a known activator to the complement source, one can monitor the performance of the entire test system, including the quality of the complement source and the technical performance of the experiment and the subsequent assay. D is a positive control.

Depending upon the needs of the investigator, the known activator can be synthetic material, eg., Sepharose®, cellulose, nylon, etc. or a chemical activator such as inulin, zymosan, heat-aggregated gamma globulin, or cobra venom factor. The activation potential of an unknown test material can be compared to one of these known activators and expressed, eg., as a percentage of complement activation achieved with one of these known activators under standard conditions.

9. Activation of C3 by Sepharose®.

Figure 15 shows the activation of C3 by Sepharose 4B and by cobra venom factor (CVF), an extremely potent activator of complement used *in vitro* and *in vivo* experimentally to deplete animals. On the y-axis is shown the concentration of iC3b, one of the activation fragments of C3. On the x-axis is shown time in minutes. Aliquots were taken at each time point as described earlier in Design 1 (Figure 12) and tested for iC3b in the microplate enzyme immunoassay.

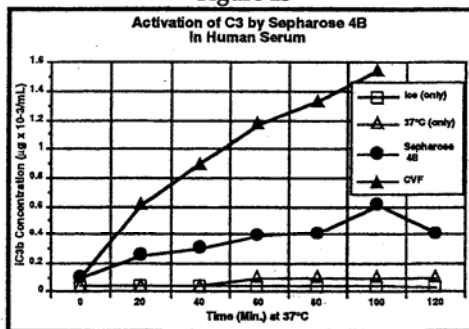
As shown, both Sepharose 4B (closed circles) and CVF (closed triangles) activate C3. Also, the amount of C3 activation and, hence iC3b, increases over time. A maximum level of activation by Sepharose 4B is achieved at about 90 minutes under these conditions.

The amount of activation by Sepharose B is less than that seen with cobra venom factor because:

- (1) the number of chemically receptive sites on Sepharose 4B for activated C3 are limited; and
- (2) activation on the Sepharose surface is subject to control by complement control proteins — whereas activation by CVF is not subject to control.

Note that no change in the level of the analyte, iC3b, was observed when the complement source was incubated on ice for 2 hours (Control C). The concentration of iC3b did increase in the 37°C control (without Test Material) to about twice the level in the starting material (Control B).

Figure 15

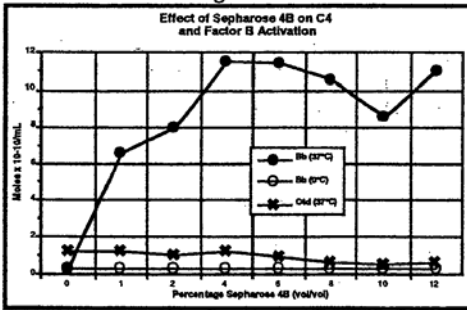


Nevertheless, it is clear that Sepharose 4B is an activator of human complement.

But what pathway, classical or alternative, is being activated?

Two pathways can result in activation of C3 (see Figures 2 through 6). It may be important for the investigator to know whether one or both are involved. This can be determined by testing the serum exposed to the test material for increases in C4d, Bb or both. Figure 16 shows that Factor B is activated by exposure of serum to Sepharose 4B (closed circles). The amount of activation occurring within 60 minutes increases with increasing quantities of Sepharose from 0 to 4% by volume. Factor B is not activated by incubating the complement source alone at 37°C (open circles). The X's show that C4 is not activated by Sepharose 4B. This experiment employed Design 2 shown in Figure 16.

Figure 16

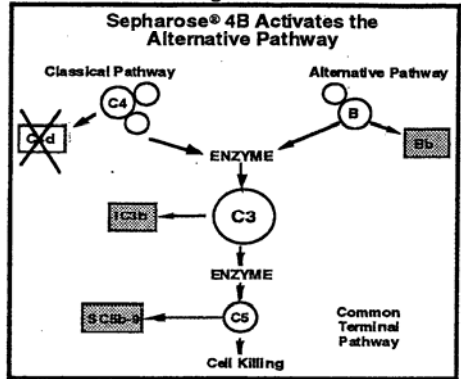


Since Sepharose 4B activates Factor B and C3, but not C4 — depicted in Figure 17 — then Sepharose activates the alternative pathway of complement and not the classical pathway.

By measuring SC5b-9 in similar experiments, we have shown that

Sepharose 4B also triggers the common terminal pathway causing C5 cleavage and the production of SC5b-9 and C5a.

Figure 17



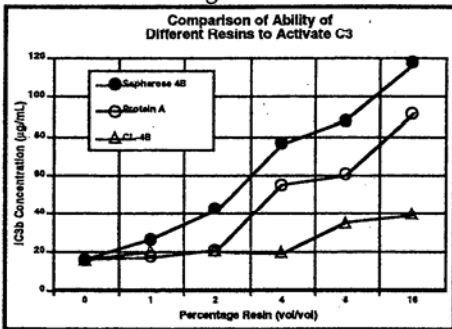
Therefore, one activation experiment and 3 EIAs allow the demonstration that:

- (1) Sepharose activates complement, since iC3b is generated;
- (2) that the classical pathway is not activated, since C4d is not generated;
- (3) that the alternative pathway is activated, since Bb is produced; and
- (4) that the terminal pathway is activated, since SC5b-9 is produced.

10. Comparison of Activation of Complement by Sepharose, Protein A Sepharose, Cross-Linked Sepharose.

In this experiment, Figure 18, the ability of Sepharose 4B alone, protein A Sepharose 4B, and chemically cross-linked Sepharose 4B to activate C3 were compared. Different amounts of the resins were incubated with human serum for 1 hour at 37°C and the iC3b concentration in each reaction mixture was determined. As shown, Sepharose 4B (closed circle) was a more potent activator than Protein A Sepharose (open circle), which was more potent than cross-linked Sepharose (open triangle).

Figure 18



These differences are possibly attributable to decreasing numbers of chemical binding sites available to C3b on the derivatized forms of Sepharose 4B and/or altered ability of control proteins to impact the function of the C3b on the different surfaces.

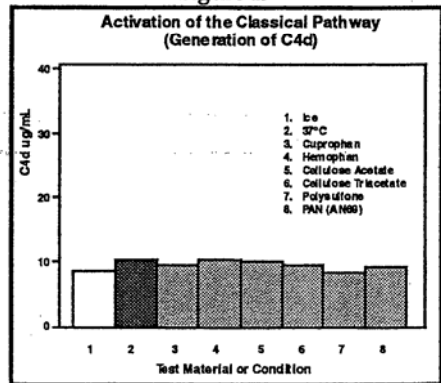
11. Hollow Fiber Membranes and Complement Activation.

Using the experimental approaches outlined above, we have compared the ability of different materials (used in hollow fiber membranes) to activate complement *in vitro*. The next four Figures show the results expressed as µg/mL of activation fragment generated upon exposure to the test material at 37°C for 1 hour.

The first bar in each Figure shows the level of the analyte present in the ice control (Control C). The solid black bar shows the level of analyte generated in the 37°C control (Control B). Comparison of all test values to this value is required to determine if the Test Material activates complement.

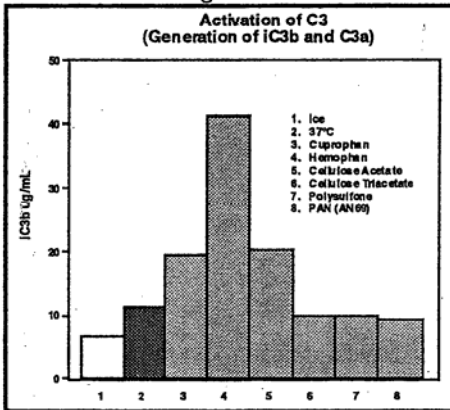
As shown by assaying for analyte C4d, none of the test materials activated C4 of the classical pathway (see Figure 19).

Figure 19



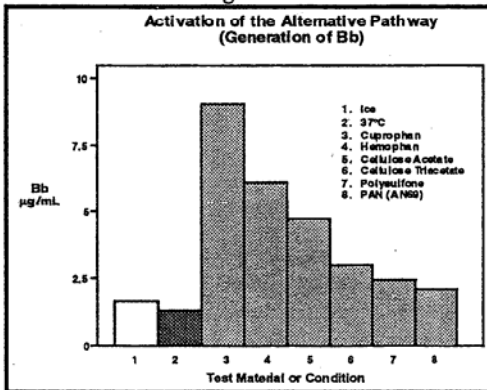
As shown in Figure 20 for analyte iC3b, several hollow fibers, especially those numbered 3, 4, and 5 (Cuprophan, Hemophan and Cellulose Acetate) activated C3.

Figure 20



As shown in Figure 21 for analyte Bb, five materials activated Factor B of the

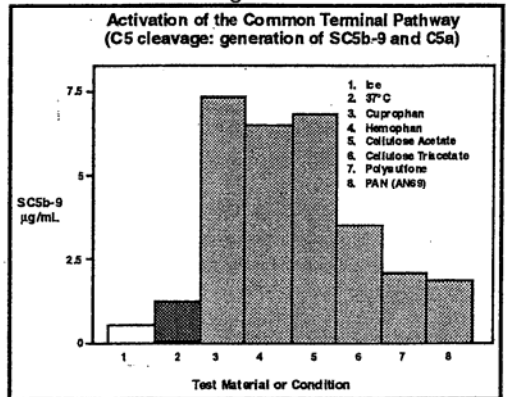
Figure 21



alternative pathway, especially those numbered 3 through 6.

And, as shown in Figure 22, several activated the common terminal pathway as shown by SC5b-9 production.

Figure 22

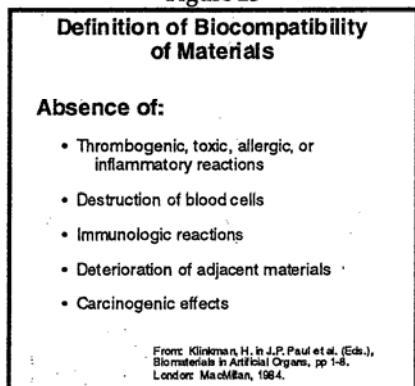


Therefore, this study allows ranking the various materials examined by their apparent complement activating potential with Cuprophan ≥ Hemophan > Cellulose Acetate > Cellulose Triacetate > Polysulfone > PAN, where Cuprophan is a good activator and PAN is a non activator.

12. Biocompatibility.

Figure 23 shows five key properties of biocompatible materials as described by Klinkman. To the best of our knowledge, with the exception of "Absence of carcinogenic effects," complement activation can cause all four of the other unwanted effects.

Figure 23



The effects of long term, chronic, moderate activation of complement are not known.

13. Conclusion.

In summary, activation of complement can result in acute inflammatory reactions which are life-threatening. Use of materials which promote significant activation of complement should be avoided when developing devices which use biomaterials which will come in contact with human blood. The long-

term effects of lower-level chronic activation of complement on the patient or on the function of the device incorporating the biomaterial are not well-understood.

QUIDEL has developed simple enzyme immunoassays which are available commercially. We have shown that they can be used readily to assess the complement-activating potential of different materials.

There is still a lot of work to be accomplished. Effort is required to develop standard methods for preparation of the biomaterials for testing and refinements are needed in the quantitative methods used to express the complement activation potential of biomaterials.

14. References.

1. The Complement System. M.K. Liszewski and J.P. Atkinson. In: *Fundamental Immunology*; Third Edition; ed. W.E. Paul; Raven Press, Ltd., New York. pp 919-939 (1993).