Journal of Pharmaceutical Sciences

OCTOBER 1977
VOLUME 66 NUMBER 10

REVIEW ARTICLE

Complement: A Host Defense Mechanism Ready for Pharmacological Manipulation?

BRIAN J. JOHNSON

Keyphrases □ Complement—mechanism of activation, associated biological effects, role in human diseases, inhibitory drugs, review □ Immunological systems—complement, mechanism of activation, associated biological effects, role in human diseases, inhibitory drugs, review □ Anticomplement drugs—complement inhibitors, review

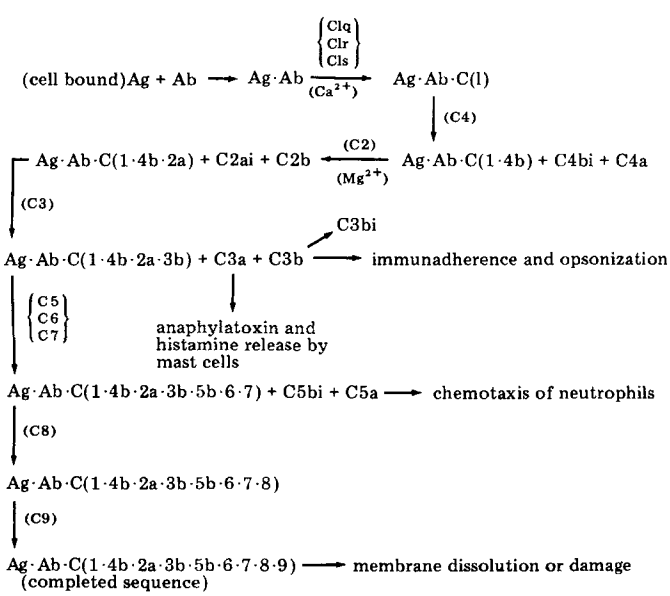
Complement is a group of 11 serum proteins that compose the nine different complement components. These components[1] are, in the order activated by the classical pathway, C1, C4, C2, C3, C5, C6, C7, C8, and C9. Complement is an important host defense mechanism against bacteria, viruses, and other injurious stimuli. Activation can be effected by antigen–antibody complexes, bacterial and plant polysaccharides, microbial and tissue enzymes, synthetic peptides and polypeptides, and cobra venom factor. Following activation, complement releases biologically active products that can effect increased vascular permeability, chemotaxis of polymorphonuclear leukocytes, enhancement of phagocytosis, and retention of leukocytes by immune adherence.

The past quarter of a century has seen great strides in the purification of the various complement components and their mechanistic role in the lysis of cellular material. All components of the classical pathway were obtained functionally pure from human serum (1). The purification of the components (C1q, C1r, and C1s) of the first complement protein C1 was described (2–4). The remaining components also were purified (5–11). Physicochemical characterization was achieved to varying extents (12). The serum concentrations of the various complement components range from traces of C8 and C9 to 1.3 mg/ml for C3. The molecular sizes of these components range from 100,000 to 200,000 daltons, with the exception of C1q (400,000 daltons) and C9 (79,000 daltons).

[1] This nomenclature follows the WHO recommendations: *Bull. WHO*, **39**, 939 (1968).

MECHANISM OF COMPLEMENT ACTIVATION

Activation of the complement system can follow two pathways. The first, the classical pathway, is probably the more studied of the two routes. This pathway (Scheme I) is activated by the binding of C1 through its C1q subunit to cell-bound antibody and consists of several steps resulting in the generation of the C1s subunit. The presence of Ca^{2+} ions is a prerequisite for C1 activation.



Scheme I

Usually, only the naturally occurring classes of immunoglobulins, IgG1, IgG2, IgG3, and IgM, are capable of binding C1q. Binding of C1 to monomeric IgG and IgM can be demonstrated *in vitro* under certain experimental conditions (13). However, a certain amount of aggregation of the immunoglobulins is necessary to bind C1q tightly. In fact, several studies showed no C1 binding until a critical number of IgG molecules had aggregated (14, 15). Specifically for immune hemolysis, at least two IgG molecules in close proximity are necessary for complement activation (16).

The next step consists of Ag·Ab·C(1)[2] enzymatically attacking and cleaving C4 into the large C4b fragment and a small C4a (15,500 daltons) fragment (17, 18). Failure of the C4b fragment to bind to the receptor site on the cell membrane results in the loss of the receptor site, and the C4b fragment becomes lytically inactive C4bi. The next occurrence is the absorption of C2 onto the cell-bound C4b. This reaction is reversible and requires Mg^{2+} (19). Component C2 is then cleaved into two fragments, C2a (81,000 daltons) and C2b (20, 21). Component C2b is released, and the C2a is bound to C4b to form Ag·Ab·C(1·4b·2a). Once formed, C(4b·2a) does not require the presence of Mg^{2+} or C(1) for its action.

The next sequence concerns the cleavage of C3 by C(4b·2a), generating two fragments, C3b and C3a (7200 daltons) (22, 23). Fragment C3a, which has anaphylatoxin and chemotactic factor activity, is released into the fluid phase. The C3b fragment can become attached to various parts of the cell membrane but most probably not to the C(4b·2a) site. Thus, the C(4b·2a) enzyme can produce a large number of C3b fragments, essentially by an amplification mechanism. Any C3b that does not become cell bound has a short half-life and becomes inactivated. When a C3b fragment binds to the immediate vicinity of the C(4b·2a) complex, a new enzyme C(4b·2a·3b) is created which can cleave C5. This cleavage produces two products, C5b and C5a (15,000 daltons) (24, 25). Product C5a has anaphylatoxin and chemotactic activities (26). The C5b fragment binds reversibly to the Ag·Ab·C(1·4b·2a·3b) to give Ag·Ab·C(1·4b·2a·3b·5b). This reaction is very inefficient, resulting in a large amount of C5bi (inactive).

The lytic activity of C5 is stabilized by the binding of C7 to form the trimolecular complex C(5b·6·7). Once bound, C(5b·6·7) forms a relatively stable site and lysis occurs upon reaction with C8 and C9 (27). The C8 component is physically absorbed onto the cell-bound C(5b·6·7) complex. The cell-bound C8 offers a binding site for C9, which completes the formation of a permanent membrane lesion on the cell membrane, leading to rapid lysis.

This discussion illustrated the activation of the complement system by a cellular antibody–antigen complex. However, immune complexes are not the only substances that can activate complement *via* the classical pathway. Other activating substances include protamine–heparin complexes (28), synthetic polynucleotides (29), single-stranded DNA–lysozyme complexes (30), and complexes of the acute phase C-reactive protein (CRP) with pneumococcal C-polysaccharide, lecithin, sphingomyelin, and protamine (31–33). Also, complexes of staphylococcal protein A or of reduced insulin with nonantibody IgG can activate the classical pathway (34–36). The evidence for the conclusions regarding the abilities of these substances to activate the classical pathway rests on their abilities to consume *in vitro* the total serum complement, including the early- as well as the late-acting components.

The second alternative pathway by which the complement system is activated was recognized in the study of the reaction of complement with naturally occurring materials. Only the late-acting components of complement, C3–C9, were consumed without the concomitant participation of C1, C4, and C2. The materials that accomplish this activation are bacterial and plant polysaccharides, bacterial lipopolysaccharides, cobra venom, and certain classes of immunoglobulins.

The mechanism of activation is through the generation of a C3 and a C5 cleaving enzyme rather than the respective classical pathway's C(4b·2a) and C(4b·2a·3b) enzymes. Once C5 is cleaved, activation of the remaining component, C6–C9, most probably proceeds *via* the classical pathway. The initiation of this alternative pathway apparently requires the participation of at least three proteins: properdin (P), factor B, and factor D. The presence of C3 is obviously needed. Not all of these factors may be required for activation by all substances. For example, properdin is thought to be necessary for activation of the complement system by polysaccharides but not by cobra venom factor (37).

A large amount of work was directed to understanding the mechanism of activation of complement *via* the alternative pathway. The activating mechanism initiated by zymosan was investigated (38, 39). Other bacterial polysaccharides and lipopolysaccharides from Gram-negative bacteria possess the property similar to that of zymosan of binding properdin and inactivating C3–C9 in whole serum (40). Properdin was obtained pure (41), factor B was identified as a C3 proactivator (42, 43), and factor A was identified as C3 (44, 45). Complement activation by bacterial lipopolysaccharides caused consumption of C3–C9 without consumption of C1, C4, and C2 (46, 47). Recently, the C3 and C5 cleaving enzymes on the surface of zymosan particles incubated with whole serum were studied (48–51).

Other mechanisms for activation of the alternative pathway can involve certain classes of immunoglobulins complexed to their homologous antigens. For example, guinea pig $\gamma 1$ immunoglobulin complexed with specific antigens consumed only the late-acting complement components C3–C9 without affecting C1, C4, and C2 (52). In contrast, guinea pig $\gamma 2$ consumed C1, C4, and C2 as well as C3–C9, indicating activation of the classical pathway (53, 54). Apparently, the site for the alternative pathway activation is in the $(Fab)_2$ portion of the immunoglobulin (55, 56), whereas the classical pathway activation locus is in the Fc portion (57, 58). IgA also appears capable of alternative pathway activation (59).

Another material that activates complement *via* the alternative pathway is cobra venom factor. The anticomplementary properties of cobra venom factor were first reported by Flexner and Noguchi (60). Cobra venom factor inactivates only the late-acting complement components without affecting C1, C4, and C2 (61, 62).

Cobra venom factor has been purified using ion-ex-

[2] In this review, a modification of the old nomenclature is used; the active complex is denoted by enclosure in parentheses rather than by a bar over the complex.

change and gel filtration chromatography and electrophoresis (63–65). It does not act on C3 directly but requires a normal serum cofactor. After incubation of cobra venom factor with whole serum, C3, C5, C6, C7, C8, and C9 were consumed with a concomitant generation of chemotactic activity (66). Cobra venom factor lysed unsensitized red blood cells, probably indicating the generation of hemolytically active C(5·6·7) complexes (64, 67). The generation of fluid phase C(5·6·7) complexes may contribute to the cobra venom factor-induced lysis of erythrocytes (68). Furthermore, the hemolytic potential of fluid phase C(5·6·7) generated during alternative pathway activation by cobra venom factor is regulated by C5·6·7 inhibitor (68).

In the first step, cobra venom factor forms a reversible complex with factor B (69). Factor B was described under various names including properdin factor B (70), β_2-glycoprotein type II (71), glycine-rich β-glycoprotein (GBG) (72), and C3-proactivator (C3PA) (73). The cobra venom factor complex with factor B is then cleaved in the presence of Mg^{2+} ions by the activated factor (D) into Ba (20,000 daltons) and Bb (60,000 daltons) (73). Fragment Bb remains bound to the cobra venom factor to form CVF·Bb (74). This complex has the ability to cleave C3 into C3a and C3b. The active site of this complex, CVF·Bb, resides on the Bb portion (75).

The activated (D) factor is a serine esterase (76). The precursor form of D can also be activated by trypsin. Other names for this activated (D) factor include C3PA convertase (44) and GBG-ase (77). A portion of factor D may exist in an active state in plasma (76). The generation of the C5 cleaving enzyme by cobra venom factor is not well understood (78). Obviously, cobra venom factor, unless administered to somebody, does not play a biological role *in vivo*. However, a cobra venom factor analog perhaps exists in human serum (79).

Component C3b, the larger fragment obtained upon cleavage of C3, might be considered as a cobra venom factor functional analog. Thus, incubation of C3b with factors B and D in the presence of Mg^{2+} ions results in the cleavage of B into Bb, with concomitant generation of C3 cleaving activity (44). Further evidence for this supposition was reported (80) when mixtures of highly purified cobra venom factor, factor B, and factor D in the presence of Mg^{2+} were chemotactic for human neutrophilic polymorphonuclear leukocytes. Thus, cleavage of C3 by either the classical or alternative pathway, *i.e.*, by C(1·4b·2a) or CVF·Bb, produces C3b. This C3b can then react with further quantities of factors B and D to form additional C3 convertase, probably C3b·Bb, leading to a further C3 cleavage. This reaction series is an amplification process. Furthermore, cell-bound C3b can react with factors B and D, giving rise to cell-bound C3-convertase which, upon reaction with C3, allows the cascade C3–C9 to occur, resulting in cell lysis (81). This cell-bound C3-convertase is very efficient in cleaving C3.

Evidence for the similarity between cobra venom factor and human C3 was reported (82). Rabbit anti-cobra venom factor cross reacts with human C3. However, as shown by electrophoresis, there was no change in the mobility of the cobra venom factor when incubated with human C3b inactivator. Also, there was no reduction in the ability of the cobra venom factor to inactivate C3 in normal human serum in the presence of this inactivator. However, incubation of cobra venom factor with cobra serum destroyed the ability of the cobra venom factor to induce C3 conversion in human serum. Possibly, cobra venom factor is altered cobra C3 that has the capacity, like human C3b, to activate the alternative pathway. The potent C3-inactivating activity of cobra venom factor probably derives from the fact that the cobra venom factor is not inactivated by mammalian C3b inactivator.

The complement system can also be activated by numerous enzymes. For example, plasmin activates C1s to the active form C(1s) (83). Lysosomal enzymes derived from human leukocytes activate C1s (84). Trypsin activates C1r to C(1r) (85), and this enzyme can replace C(1s) in the formation of C3-convertase from Ag·Ab·C(1·4b) and C2 (86). Trypsin can also replace factor (D) in the formation of CVF·Bb (74). The enzymes trypsin, plasmin, and pronase substitute for the factor (D) in the formation of C3-convertase by zymosan (51). Plasmin can directly cleave C3 to produce a fragment with chemotactic activity (87). Furthermore, trypsin, lysosomal enzymes from human leukocytes, and bacterial and tissue proteases can also cleave C3 and/or C5 directly to produce chemotactic factors and anaphylatoxins (88–91).

BIOLOGICAL ACTIVITIES ASSOCIATED WITH COMPLEMENT ACTIVATION

The biological activities generated by activation of the complement system are evident by acute inflammation and cell killing. These effects are manifest in increased vascular permeability, chemotaxis of leukocytes, enhanced phagocytosis, and membrane damage.

The release of a kinin and two anaphylatoxins results in increased vascular permeability. The kinin, which is released from C2 during its activation by C(1s) (92, 93), is distinguishable from bradykinin and, in contrast to the anaphylatoxins, has a direct effect on smooth muscle. It also is not histamine dependent and possibly plays a major role in the pathogenesis of hereditary angioedema (94).

Fragments C3a (7200 daltons) and C5a (15,000 daltons) are powerful anaphylatoxins (22, 26, 88, 92, 95, 96). Both of these materials induce *in vitro* contraction of guinea pig ileum and produce unresponsiveness after repeated applications. However, a smooth muscle made unresponsive to C3a is still reactive to C5a and vice versa (89). These anaphylatoxins also enhance vascular permeability and cause degranulation of mast cells. Unlike the kinin released from C2, these anaphylatoxins are mediated through release of histamine from the granules of mast cells (88, 89).

Both anaphylatoxins are chemotactic for leukocytes (97–101). The trimolecular complex C(5b·6·7) (27, 102) is also a potent chemotactic factor. Enhanced phagocytosis is another manifestation of the biological activity of complement. Phagocytosis is enhanced by the coating of cells with C3b, an opsonizing agent. Cells carrying C3b receptors include primate red cells, polymorphonuclear leukocytes, monocytes, and B-lymphocytes (103). This opsonizing effect probably is closely related to the capacity of the bound C3b to induce immune adherence (104, 105).

ROLE OF COMPLEMENT IN HUMAN DISEASES

From the discussion of the biological activities of the

complement system, it is seen that complement is an important *in vivo* effector system. Thus, *via* the anaphylatoxins, histamine may be released, causing smooth muscle contraction, peripheral vasodilation, increased vascular permeability, and edema. Through the action of its various chemotactic factors, polymorphonuclear leukocytes migrate into the area of complement activation. By providing immune adherence sites on surfaces such as the outer membranes of cells, the inner surface of blood vessels and capillaries, and deposits of immune precipitates, complement causes localization of chemotactically attracted or randomly moving leukocytes. In addition, through its opsonization function, it facilitates phagocytosis. Thus, complement is a very important host defense mechanism.

However, these advantageous biological activities of complement may become detrimental. For example, in acute glomerulonephritis, a deposition of an immune complex of γ-globulin and C3 along the glomerular capillary walls and in the mesangium (106–108) is seen. Serum complement component determinations indicate an acute reduction in circulating C1, C2, C3, C4, and C5 (109–113). The presence of bound complement in association with bound antibody in the kidneys strongly suggests that complement plays a role in the production of tissue damage in this disease.

In systemic lupus erythematosus, there is evidence that complement participates in the production of generalized tissue damage. There is usually a severe lowering of the serum level of multiple complement components (110, 112). The most probable cause of low serum hemolytic activity and increased complement catabolism is consumption of complement by circulating and localized immune complexes. The pattern of deposition of immunoglobulins and complement seen in blood vessels in numerous organs and the glomeruli is similar to that found in immune complex deposition diseases such as serum sickness (108, 114, 115). Bound complement has also been found in the skin in areas of the lupus rash but not in adjoining clinically normal skin (116).

Eluates of diseased kidneys reveal the presence of DNA (117) and anti-DNA (117, 118) in addition to antibody to various other antigens (117, 118). DNA–anti-DNA (119) and other complexes are complement fixing (116, 117). Lupus probably is caused by circulating complement-fixing immune complexes that become trapped and localized in various organs. Therefore, these complexes can trigger the complement system and cause tissue damage and inflammation.

Other diseases involving complement are the autoimmune hemolytic anemias. In many cases, the red cells are coated with complement proteins in the absence of detectable antibody (120). Possibly, the sensitivity of the detection systems used is too low for visualizing the antibody. Another possible explanation for this coating is a disease-related change in the red cell membrane that permits binding of complement without participation of antibody.

Rheumatoid arthritis is another disease that involves the complement system. Its cause is unknown, and the reason for joint predilection with the events leading to a generalized disease are poorly understood. However, there is no doubt that complement activation, with its potential for tissue damage, occurs within the joint space and probably explains the inflammatory symptoms. Thus, in the synovial joint fluid of patients with rheumatoid arthritis, whole complement (121, 122), C1 (123), C2 (121), and C4 (121, 123) are markedly depressed. Also γ-globulin, C3, and C4 are regularly deposited in the synovium of patients with active disease (124, 125). Rheumatoid joint fluids often contain leukocytes with inclusion bodies, and some of these inclusions contain γ-globulin. These findings indicate extensive activation of the complement system within the joint space.

Transplantation of tissue from genetically dissimilar patients obviously introduces new antigenic sites in the recipient. Even with extensive tissue typing of such histocompatibility antigens, the possible introduction of foreign immunogens always remains.

In studies on renal allograft rejection, two events appear to be necessary to sensitize the host. Immediately after transplantation, antigen from the donor organ is shed into the systemic circulation of the recipient by way of the renal vein. Processing of this antigen by macrophages may be necessary to transfer antigens to a form that causes sensitization of lymphocytes. Possibly, the RNA of the macrophage is linked to some part of the antigen that reaches a regional lymph node. This event initiates conversion of lymphocytes to lymphoblasts, which, in turn, divide to produce lymphocytes specifically sensitized to the graft.

Immune globulin produced by the sensitized lymphocyte (or plasma cells) may also be released as circulating antibody against the graft. Circulating lymphocytes are capable of being sensitized during their passage through the graft, perhaps by direct contact with antigens contained on the vascular endothelium of the graft. These sensitized lymphocytes homing to a lymph node may represent a population of sensitized lymphocytes that produce immune globulin specific for the graft. Both sensitized lymphoid cells (126) and humoral antibody from patients sensitized by previous kidney grafts (127) have been shown to be responsible for destruction of kidney cells cultured *in vitro* or utilized as a monolayer substrate. The destruction of the graft probably is mediated by both the immune cellular and humoral responses. This combination results in the release of macrophage migration inhibitory factor (MIF), which causes the clustering of monocytes at the vascular endothelium. Fixation of antibody activates the complement system with the release of chemotactic factor, leading to the accumulation of polymorphonuclear leukocytes.

Prolonged renal allograft rejection reactions in humans are characterized by reduced levels of C2 (128) and C3 (129), although in early rejection episodes the complement levels are variable and may be normal, elevated, or depressed (130). Three turnover studies with radiolabeled C3 and C4 were reported (131). Two of the C4 recipients and all of the C3 recipients showed increased rates of catabolism of the protein during rejection episodes. Furthermore, C1 and C3 were found in bound form in rejected renal allografts (132, 133). However, the role of complement in allograft rejection is far from clear. Thus, in dogs and mice, rejection episodes did not decrease complement levels, and complement suppression had no influence on rejection (134). C5-Deficient mice reject skin grafts normally (135), and studies with C6-deficient rabbits indi-

cated a significant prolongation of skin grafts in about half of the recipients (136). These data suggest that there may be two distinct pathways for rejection, only one of which is complement dependent.

The difference in tissue antigens between donor and recipient and the strength of these antigens may modify the histological and clinical phenomena accompanying the rejection. Thus, grafts between species are apt to be rejected violently, whereas those between identical twins undergo no rejection. However, this latter case is very uncommon in practice. Thus, the donor and recipient can be matched closely with such techniques as mixed lymphocyte culture and direct cross-matching using recipient serum and donor cells. However, even after these precautions, immunosuppressive drugs such as azathioprine, corticosteroids, and antilymphocyte globulin are still required.

These drugs can act at various levels of the immune response, including (*a*) interference with the initial sensitization or recognition of an antigen, (*b*) depression of the cellular proliferative response of either the cellular or humoral type, (*c*) interference with the function of the complement-sensitized cells, or (*d*) interference with the function of the secondary mediators that produce damage following interaction of antibody and tissue antigens. These drugs have serious side effects such as alopecia, anemia, leukopenia, hepatitis, bone marrow depression, nausea, vomiting, stomatitis, hyperglycemia, peptic ulceration, GI bleeding, and aseptic bond necrosis. The antilymphocyte globulin treatment has been reported to cause pain at the injection site, fever, and, in rare cases, anaphylaxis and thrombocytopenia.

SEARCH FOR ANTICOMPLEMENT DRUGS

One approach to immunosuppression would be to affect the complement system. Possibly by inhibiting or lowering the complement levels in the host by nontoxic anticomplement drugs, a useful therapeutic tool would become available. Such drugs would be useful not only for treating transplantation rejection but also for alleviating the distress caused by autoimmune diseases and those diseases where deposition of immune complexes can activate the complement system, *e.g.*, acute glomerulonephritis.

The idea of inhibiting or controlling the complement system is not new. Indeed, a natural control mechanism is built into the complement system itself, namely, the instability of the activated components such as C(4b·2a) and C(4b·2a·3b·5b). The rapid decay of these intermediates serves as a self-limiting mechanism. Moreover, there are naturally occurring inhibitors such as the C1 inhibitor (C1INH) and the C3b inactivator (C3bINA), also known as conglutinogen activating factor (KAF). The C1 inhibitor combines stoichiometrically with C(1s) to inhibit its hemolytic and esterolytic activities (137, 138). This inhibitor also inhibits the enzymatic activity of C(1r) (139), and it can block the action of other enzymes such as plasmin (140), kallikrein (141), and Hageman factor (142).

The inactivator C3bINA inhibits the hemolytic and immune adherence activities of bound and fluid phase C3b by cleaving it into two smaller fragments, C3d and C3c (143–147). The biological significance of these inhibitors of C1 and C3b is illustrated in patients deficient in them. Patients deficient in C1 inhibitor illustrate hereditary angioneurotic edema characterized by recurrent episodes of circumscribed edema of the skin, larynx, and intestinal mucosa (148). This edema is most probably due to uncontrolled activation of C1. The genetic deficiency of the C3b inhibitor increases the susceptibility of patients to pyogenic bacterial infections (149–151). Inactivators to C6 (143) and of C4b (152) also have been described. Recently, the C(5·6·7) inhibitor was discussed (153, 154); it acts upon C(5·6·7) reversibly in the fluid phase to prevent its attachment to bystanding erythrocytes during the short time the binding site of C(5·6·7) is active.

Synthetic inhibitors of the complement system have been used and reviewed (155). Ideally, it should be possible to obtain a series of inhibitors that inhibit specifically each complement component. However, this has not been achieved in practice. Indeed, one can argue that an inhibitor to be used as a therapeutic agent in humans need not have a stringent specificity of action for a particular complement component. As long as the action of the inhibitor on noncomplement systems does not lead to toxic effects, a certain degree of nonspecificity might even be desirable.

For example, if an inhibitor only affected C8 or C9, then the lytic properties of complement would be affected but not the production of the kinins, anaphylatoxins, and immune adherence fragments. Similarly, inhibition of the early-acting components might prevent activation of complement by the classical pathway, C1, C4, and C2, but not by the alternative pathway triggered at C3. Such a situation would be advantageous if a given immune complex only caused complement activation through the classical pathway. Thus, in designing an inhibitor, there are advantages in trying to obtain a material that inhibits either all of the complement components or just C3. This latter approach seems easier.

In the complement cascade, activation of one component then causes activation of the next in the sequence. Thus, an inhibitor, if it is to be effective, must be able to react with the active site of the particular complement enzyme in the short time interval between activation of the complement enzyme and reaction of the activated enzyme with the next component. An effective inhibitor must not only be very active in its action, but it should be present in high concentration. Many complement components not only have enzyme active sites but also have combining sites. Thus, inhibitors can be designed for blocking such binding sites, *e.g.*, C3b to the cell membrane. However, the requirements of high activity and high concentration of inhibitor are necessary.

Complement inhibitors have been used for a number of distinct purposes. One use has been to study the nature of the complement components and the mechanisms of their action, as in the studies with organophosphorus inhibitors such as isoflurophate (diisopropyl phosphorofluoridate) and phosphonate esters. Another use for complement inhibitors is to obtain evidence that a certain biological activity requires, or does not require, complement. Thus, for *in vivo* reactions, it is necessary to have an inhibitor that is highly active and highly specific. This latter requirement is difficult to obtain; cobra venom factor, a naturally occurring activator of complement, probably comes closest to the ideal. When inhibitors are used to determine com-

plement dependency, specificity is crucial. This inability to obtain synthetic materials possessing high specificity in their actions has led to the development of animals that are genetically deficient in a particular complement component.

Isoflurophate inhibits the activity of whole complement (156); it acts upon C(1) (157) by inhibiting C(1s) (158). This result is not surprising, since isoflurophate inactivates serine esterases. Baker and coworkers (159–161) synthesized a series of sulfonyl fluorides, many of which were irreversible inhibitors of C(1), and suggested that C(1) contained an anionic site in its active center. This conclusion was based on the ability of tosyl-L-arginine methyl ester to be a substrate for C(1). Indeed, this ester competitively interferes with the hemolytic activity of C(1) (162).

Bing and coworkers (163–165) showed that various aromatic compounds competitively inhibited the esterase activity of C(1s) and suggested that C(1s) possessed not only an anionic but also a hydrophobic site in its active center. ϵ-Aminocaproic acid in high concentrations prevents the activation of C1 to C(1) in its inhibition of the hemolytic activity of whole complement (166). Selective inhibition of C1 without effect on C4, C2, C3, and C9 levels in whole serum recently was reported (167). Furthermore, the effect was reversible by dialysis. In another study (168), high levels of ϵ-aminocaproic acid enhanced the cleavage of C3 with the accompanying appearance of the biologically active C3a. However, low levels of ϵ-aminocaproic acid inhibited, rather than enhanced, C3 cleavage in serum. The inhibitory activity of ϵ-aminocaproic acid was attributed to its interaction with the properdin factors. The enhancing effect of this ϵ-aminocaproic acid is thought to be due to its inhibitory effects on the C3b inactivator (168).

These inhibitors of C1 afford insights into the mechanism and nature of the active site of C(1). However, it is doubtful if these materials would be too useful for *in vivo* suppression of complement levels, since the biological activities of the complement fragments could still arise through activation of complement by the alternative pathway.

The aromatic amino acid derivatives or inhibitors of complement suggest that one component of complement might be a proteolytic enzyme with enhanced activity toward such moieties (169). The aromatic derivatives competitively inhibited the action of Ag·Ab·C(1·4b·2a·3b) in forming the heat-stable intermediate Ag·Ab·C(1·4b·2a·3b·5b·6·7) (170). This result then leads to the direct demonstration that C(1·4b·2a·3b) has peptidase activity for aromatic amino acid derivatives such as glycyl-L-tyrosine (171, 172).

Inhibitors of the binding sites of the various complement components have been investigated. Benzoxycarbonyl-L-glutamyl-L-tyrosine (173) and maleopimaric and maleofumaric acids presumably dissociate the activated C(5·6·7) complex (174, 175). Several diamines dissociate the activated C1, C(1), from the antigen–antibody complex, probably by chelating the Ca^{2+} ions necessary for the binding between C1q and the other C1 subcomponents (169).

Maleopimaric and maleofumaric acids are potent inhibitors of complement *in vitro*. Fumaropimaric acid given repeatedly over 3–4 days reduced complement levels (176), but its mode of action is not known.

Copper–chlorophyllin inhibited anaphylaxis (177), and this effect was related to inhibition of complement. This conclusion was confirmed (178); the inhibition of complement occurs at the reaction of Ag·Ab·C(1·4b·2a·3b) with C5.

Heparin has been long known to possess anticomplementary activity. Its major activity is reported to be with C1 (179). Interactions of heparin and protamine in fresh human serum, in amounts far below those required for complement depletion by either agent alone, induced virtually complete removal of total hemolytic complement activity (180). The predominant complement component depleted was C1. It has been suggested (180) that the reaction between heparin and protamine, like the interaction between antibody and antigen, markedly enhances the ability of heparin to interact with the first component of complement and that this activation goes through the classical pathway. It was also suggested (180) that interactions between certain polycations and polyanions, similar to those between antibodies and antigens, may have a role in the initiation of the inflammatory response.

However, in another study using multiple polyanions and polycations, a test for their ability to influence formation of E·C(5·6·7) from C(5·6), C7, and sheep erythrocytes (E) was performed (181). Six of the 11 polyanions tested, including polyanethol sulfonate, heparin, and dextran sulfate, inhibited this reaction. By contrast, five of seven polycations, including polybrene, protamine, and polyornithine, potentiated formation of E·C(5·6·7). This inhibition seemed to mimic the C(5·6·7)INH, while the potentiation seemed to involve the neutralization of this inhibitor (180).

Various polyanions alone react with the first component of complement (139, 182–187). The effect of eight different polyanions on the fluid phase and on bound C(1) as well as on purified C4 and C2 was tested (188). The polyanions were dextran sulfate, polyvinyl sulfate, polyanethol sulfonate, heparin, polyionsinic acid, chrondroitin sulfate, germinin, and pentosan-polysulfoester. These polyanions had no effect on purified C4 and C2, but they showed a strong inhibition of C(1) activity. Further investigation of the mode of interaction of some of these polyanions (189) indicated that they prevented the uptake of C2 by EA·C4b, where EA represents sensitized sheep erythrocytes. In contrast, treatment of the complex EA·C4b·2 with polyanions led to the dissociation into EA·C4b and C2.

Some synthetic polyanionic polypeptides were screened in this laboratory for their anticomplementary activities. The assay involved incubating the synthetic polypeptide with human serum and then, after dilution, performing a CH_{50} determination (190). Comparison of the number of CH_{50} units in the control with the number of CH_{50} units remaining in the test sample gave the amount of complement consumed (Table I).

Materials possessing aromatic moieties as well as the anionic groups consume complement. This criterion covers such diverse compounds as I and XXII (Table I). Compound I has a polypeptide backbone, and the aromatic, tyrosyl group is adjacent to the anionic glutamyl residue; XXII has a carbon backbone, and the aromatic and anionic moieties are randomly arrayed. This requirement of an

Table I—Complement-Consuming Abilities of Polyanions

Assigned Number	Compound[a]	Moles of Amino Acid Bound per Gram of Polymer	Percentage of CH_{50} Removed[b]	Number of CH_{50} Units Removed per Milligram of Material
—	Human IgG1	—	—	30.7
I	$(Tyr\text{-}Glu\text{-}Ala\text{-}Gly)_n Gly$	Equimolar	100	15.4
II	$(Phe\text{-}Glu\text{-}Ala\text{-}Gly)_n Gly$	Equimolar	0	—
III	$(Trp\text{-}Glu\text{-}Ala\text{-}Gly)_n Gly$	Equimolar	25	—
IV	$(Tyr\text{-}\gamma\text{-}Glu\text{-}Ala\text{-}Gly)_n Gly$	Equimolar	32	—
V	$(Tyr\text{-}Glu\text{-}Gly\text{-}Gly)_n Gly$	Equimolar	30	—
VI	$(Tyr\text{-}Glu\text{-}Ala\text{-}\beta\text{-}Ala)_n Gly$	Equimolar	100	—
VII	$(Ala\text{-}Glu\text{-}Ala\text{-}Gly)_n Gly$	Equimolar	0	—
VIII	$(Glu)_n$	Equimolar	0	—
IX	$(Glu\text{-}Ala)_n$	Equimolar	0	—
X	$(Glu\text{-}Ala\text{-}Tyr_4)_n$	$(3.6:6.7:0.25) \times 10^{-3}$	0	—
XI	$(Glu\text{-}Ala\text{-}Tyr_{10})_n$	$(3.4:6.3:0.61) \times 10^{-3}$	0	—
XII	(Maleic acid–vinyl methyl ether)$_n$	0	0	—
XIII	(Maleic acid–Tyr–methyl ether)$_n$	1.44×10^{-3}	100	58.9
XIV	(Maleic acid–Phe–vinyl methyl ether)$_n$	1.40×10^{-3}	100	43.5
XV	(Maleic acid–His–vinyl methyl ether)$_n$	1.10×10^{-3}	0	—
XVI	(Maleic acid–Leu–vinyl methyl ether)$_n$	1.20×10^{-3}	0	—
XVII	(Maleic acid–Pro–vinyl methyl ether)$_n$	1.77×10^{-3}	0	—
XVIII	(Maleic acid–Gly–vinyl methyl ether)$_n$	1.70×10^{-3}	0	—
XIX	(Maleic acid–Lys–vinyl methyl ether)$_n$	1.67×10^{-3}	0	—
XX	(Maleic acid–Trp–Gly–vinyl methyl ether)$_n$	1.35×10^{-3}	100	—
XXI	(Maleic acid–styrene)$_n$	0	100	87.0
XXII	(Maleic acid–styrene–Tyr)$_n$	0.75×10^{-3}	100	105.3

[a] Molecular weight greater than 30,000 daltons. [b] By 2000 μg.

aromatic moiety is illustrated by comparison of I and VII. Replacement of the tyrosyl residue in I by an alanyl moiety as in polymer VII produces a complete loss of complement-consuming ability.

Further evidence for the necessity of an aromatic residue is evident by comparison of XII, XIII, XIV, and XX. These materials are identical, except that polymers XIII, XIV, and XX have small amounts of aromatic residues attached to them. These residues are necessarily next to the anionic carboxyl residue, since these polymers were synthesized from the precursor polymer poly(maleic anhydride–vinyl methyl ether). Thus, XIII could be thought of as having repeating tyrosyl and carboxyl moieties along a carbon chain, somewhat analogous to I. Although II, X, and XI all have aromatic and anionic moieties, none consume complement. Compounds X and XI are random copolymers. It is possible that insufficient tyrosine is incorporated in them to satisfy the aromatic requirement or that the tyrosyl residues are not adjacent to the anionic carboxyl group of glutamic acid.

A weight basis comparison of the amounts of each polymer and the human immunoglobulin IgG1 to remove half of the available CH_{50} units per milliliter of human serum is given in Table I. Polymer I has half of the capability of the human immunoglobulin IgG1 to consume complement, whereas the other polymers are somewhat better. Comparison of the tyrosyl-substituted XXII with the unsubstituted XXI further indicates the role of the tyrosyl residue. The complement-consuming ability of the polymer is increased by 20% when this residue is incorporated. The potent anticomplementary activity of poly(maleic acid–styrene) indicates that the necessary structural requirement for anticomplementary activity in this series occurs when a carboxyl group is in close proximity to a side-chain carboxyl group. This requirement may possibly explain the lack of anticomplementary activity of the random copolymers X and XI, which possess small amounts of tyrosine. Since their sequences are random, there is the possibility that the tyrosyl residues are not situated in the correct sequence for anticomplementary activity.

Another area being pursued vigorously in this laboratory is the synthesis of peptides based on the knowledge of the complement-fixing sites of immunoglobulins. The modification of a few tryptophan residues in rabbit IgG and Fc with 2-hydroxy-5-nitrobenzyl bromide eliminated the anticomplementary activity of these protein molecules in the presence of whole guinea pig complement, but it did not significantly alter their C1q-binding capacity (191, 192). Furthermore, a particular tryptophan residue of V, a cleavage peptide of rabbit Fc, was most probably responsible for this change in anticomplementary activity. According to studies on the amino acid sequences of rabbit heavy chains (193), tryptophan residue No. 277 at the *N*-terminal side of the carbohydrate group and within the first intrachain disulfide bridge of the Fc appeared to be involved in complement fixation. Other reports (57, 58, 194–197) also indicated that the anticomplementary activity of IgG was associated with the Cγ2 domain of the immunoglobulin.

To define more closely the residues participating in the complement-binding and/or complement-fixing site of a human IgG1, human IgG1 was modified by dansylation (198–200), nitration (201), and alkylation with 2-hydroxy-5-nitrobenzyl bromide (191) and 2,3-butanedione reagent (202). The alkylation of human IgG1 with 2-hydroxy-5-nitrobenzyl bromide reduced its ability to consume complement. This result agrees with that already reported for rabbit IgG (191). Similarly, nitration, dansylation, and arginine modification with 2,3-butanedione each lowered the anticomplementary abilities of human IgG1. These results imply that the amino acid residues, tryptophan, lysine, arginine, and tyrosine (203), constitute part of the active site of the complement-fixing locus and/or part of the C1 binding site of the immunoglobulin.

Comparison of some amino acid sequences of the complement-fixing immunoglobulins of human IgG1 (204),

Table II—Sequence Comparisons of Various Immunoglobulins Possessing the Tryptophan Residue Implicated in Complement Fixation

Immunoglobulin C_H2 Domain	Amino Acid Residues	Sequence												
Human IgG1	271–283	Pro	Gln	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Gln
Guinea pig IgG2	271–283	Pro	Glu	Val	Gln	Phe	Thr	Trp	Phe	Val	Asp	Asn	Lys	Pro
Rabbit IgG	271–283	Pro	Glu	Val	Glx	Phe	Thr	Trp	Tyr	Ile	Asx	Glu	Gln	Val
Murine IgG2a	271–283	Pro	Asp	Val	Gln	Ile	Ser	Trp	Phe	Val	Asp	Asn	Val	Glu
Human IgM[a]	482–494	Ala	Asp	Val	Phe	Val	Glu	Trp	Met	Gln	Arg	Gly	Glu	Pro
Human IgM	264–276	Phe	Ser	Pro	Arg	Gln	Val	Trp	Ser	Leu	Arg	Glu	Gly	Lys

[a] $C_\mu4$ domain.

guinea pig IgG2 (205), mouse IgG2 (197), rabbit IgG (206), and human IgM (207) is given in Table II. Several peptides based on these sequences were synthesized; those peptides that mimicked the sequences around the tryptophan residue at position 277 of human IgG1 fixed complement (208). These results confirm the hypothesis (183) that this tryptophan moiety is part of the complement-fixing site (209). These sequences surrounding this aromatic amino acid are hydrophobic and also contain anionic side-chain amino acid residues. These observations correlate quite well with previous work.

At the moment, neither *in vivo* studies nor the mechanisms of action of these materials have been investigated. However, due to their low molecular weights, these materials are not expected to be antigenic; should they lower complement levels *in vivo*, they should be capable of pharmacological manipulation.

CONCLUSIONS

Under normal circumstances, the complement system is a potent natural defense mechanism of the host. However, in certain situations, this mechanism can be disadvantageous, *i.e.*, in transplantation rejection, autoimmune diseases, and other diseases where deposition of immune complexes can activate the complement system such as acute glomerulonephritis. The time has come for the development of anticomplement drugs to alleviate this problem. Such drugs would ideally be specific for a particular complement component and also have a low toxicity and antigenicity.

The most promising area to exploit would be drugs that affect the third complement component, since activation of the complement system by either the classical or the alternative pathway involves this component. Indeed, lowering of the level of C3, or its temporary elimination, in the patient would reduce production of the anaphylatoxin C3a and its concomitant detrimental biological activity. At the moment, small peptides based on the sequences of known anticomplement materials such as the immunoglobulins appear promising. However, further investigations on the complement active sites of other anticomplement materials such as cobra venom factor are required. Such information will be of value in designing materials that specifically bind or activate particular complement components.

Another avenue of approach to this problem is the use of high molecular weight polyions. Even though some of these materials are antigenic (210), they may have therapeutic value if used in *in vitro* situations. For example, several investigators (211–213) suggested that complement plays an immunopathological role in the inflammatory periodontal disease process. The gingival crevicular fluid from normal patients possesses the complement components C3, C4, and C5; that from patients with inflamed gingiva contains properdin factor B and electrophoretically converted C3 (214). In addition, the functional nature of complement components in the crevicular fluid from periodontal patients was demonstrated (215).

If the levels of complement could be lowered in the gingival crevicular fluid, then tissue damage and inflammation due to complement activation could be controlled. Gingival crevicular fluid possesses some similarity to serum (216, 217); since some of the polyanionic polymers are good serum complement-removing materials, these polymers may find therapeutic use for patients with periodontal disease.

REFERENCES

(1) D. H. Vroon, D. R. Schutz, and R. M. Zarco, *Immunochemistry*, **7**, 43 (1970).

(2) K. Yonemasu and R. M. Stroud, *J. Immunol.*, **106**, 304 (1971).

(3) M. M. E. deBracco and R. M. Stroud, *J. Clin. Invest.*, **50**, 838 (1971).

(4) K. Nagaki and R. M. Stroud, *J. Immunol.*, **102**, 421 (1969).

(5) H. J. Müller-Eberhard and C. E. Biro, *J. Exp. Med.*, **118**, 447 (1963).

(6) M. J. Polley and H. J. Müller-Eberhard, *ibid.*, **128**, 533 (1968).

(7) U. R. Nillson and H. J. Müller-Eberhard, *ibid.*, **122**, 277 (1965).

(8) C. M. Arroyave and H. J. Müller-Eberhard, *Immunochemistry*, **8**, 995 (1971).

(9) C. M. Arroyave and H. J. Müller-Eberhard, *J. Immunol.*, **111**, 302 (1973).

(10) J. A. Manni and H. J. Müller-Eberhard, *J. Exp. Med.*, **130**, 1145 (1969).

(11) U. Hadding and H. J. Müller-Eberhard, *Immunology*, **16**, 719 (1969).

(12) J. E. Volanakis, *J. Oral Pathol.*, **4**, 3 (1975).

(13) W. Augener, H. W. Grey, N. R. Cooper, and H. J. Müller-Eberhard, *Immunochemistry*, **8**, 1011 (1971).

(14) K. Ishizaka, T. Ishizaka, and J. Banovitz, *J. Immunol.*, **95**, 824 (1965).

(15) N. E. Hyslop, R. R. Dourmashkin, N. M. Green, and R. R. Porter, *J. Exp. Med.*, **131**, 783 (1970).

(16) T. Borsos, H. J. Rapp, and U. L. Walz, *J. Immunol.*, **92**, 108 (1964).

(17) R. A. Patrick, S. B. Taubman, and I. H. Lepow, *Immunochemistry*, **7**, 217 (1970).

(18) D. B. Budzko and H. J. Müller-Eberhard, *ibid.*, **7**, 227 (1970).

(19) G. Sitomer, R. M. Stroud, and M. M. Mayer, *ibid.*, **3**, 59 (1966).

(20) R. M. Stroud, K. F. Austen, and M. M. Mayer, *ibid.*, **2**, 219 (1965).

(21) R. M. Stroud, M. M. Mayer, J. A. Miller, and A. T. McKenzie, *ibid.*, **3**, 163 (1966).

(22) D. B. Budzko, V. A. Bokisch, and H. J. Müller-Eberhard, *Biochemistry*, **10**, 1166 (1971).

(23) H. J. Müller-Eberhard, in "Progress in Immunology II," vol. 1, L. Brent and J. Holborow, Eds., North-Holland, New York, N.Y., 1974, pp. 173–182.

(24) H. S. Shin, R. J. Pickering, M. M. Mayer, and C. T. Cook, *J. Immunol.*, **101,** 813 (1968).
(25) U. Nillson and J. Mapes, *ibid.*, **111,** 293 (1973).
(26) J. A. Jensen, *Science,* **155,** 1122 (1967).
(27) P. J. Lachmann and R. A. Thompson, *J. Exp. Med.*, **131,** 643 (1970).
(28) R. Rent, N. Ertel, R. Eisenstein, and H. Gewurz, *Immunology,* **114,** 120 (1975).
(29) S. Yachnin, D. Rosenblum, and D. Chatman, *J. Immunol.*, **93,** 549 (1964).
(30) W. F. Willoughby, R. T. Ford, and H. S. Shin, *ibid.*, **111,** 296 (1973).
(31) M. H. Kaplan and J. E. Volanakis, *ibid.*, **112,** 2135 (1974).
(32) J. E. Volanakis and M. H. Kaplan, *ibid.*, **113,** 9 (1974).
(33) J. Siegel, R. Rent, and H. Gewurz, *J. Exp. Med.*, **140,** 631 (1974).
(34) J. Sjöquist and G. Stalenheim, *J. Immunol.*, **103,** 467 (1969).
(35) G. Kronvall and H. Gewurz, *Clin. Exp. Immunol.*, **7,** 211 (1970).
(36) J. W. Cantrell, R. M. Stroud, and K. M. Pruitt, *Diabetes,* **21,** 872 (1972).
(37) A. G. Osler and A. L. Sandberg, *Prog. Allergy,* **17,** 51 (1973).
(38) L. Pillemer, L. Blum, I. H. Lepow, O. A. Ross, E. W. Todd, and A. C. Wardlaw, *Science,* **120,** 279 (1954).
(39) L. Pillemer, L. Blum, I. H. Lepow, L. Wurz, and E. W. Todd, *J. Exp. Med.*, **103,** 1 (1956).
(40) L. Pillemer, M. D. Schoenberg, L. Blum, and L. Wurz, *Science,* **122,** 545 (1955).
(41) J. Pensky, C. F. Hinz, E. W. Todd, R. J. Wedgwood, J. T. Boyer, and I. H. Lepow, *J. Immunol.*, **100,** 42 (1968).
(42) I. Goodkofsky and I. H. Lepow, *ibid.*, **107,** 1200 (1971).
(43) C. A. Alper, I. Goodkofsky, and I. H. Lepow, *J. Exp. Med.*, **137,** 424 (1973).
(44) H. J. Müller-Eberhard and O. Götze, *ibid.*, **135,** 1003 (1972).
(45) I. Goodkofsky, A. H. Stewart, and I. H. Lepow, *J. Immunol.*, **111,** 287 (1973).
(46) H. A. Bladen, H. Gewurz, and S. E. Mergenhagen, *J. Exp. Med.*, **125,** 767 (1967).
(47) H. Gewurz, H. S. Shin, and S. E. Mergenhagen, *ibid.*, **128,** 1049 (1968).
(48) V. Brade, G. D. Lee, A. Nicholson, H. S. Shin, and M. M. Mayer, *J. Immunol.*, **111,** 1389 (1973).
(49) A. Nicholson, V. Brade, C. D. Lee, H. S. Shin, and M. M. Mayer, *ibid.*, **112,** 1115 (1974).
(50) V. Brade, A. Nicholson, G. D. Lee, and M. M. Mayer, *ibid.*, **112,** 1845 (1974).
(51) V. Brade, A. Nicholson, D. Bitter-Suermann, and U. Hadding, *ibid.*, **113,** 1735 (1974).
(52) A. G. Osler, B. Oliveira, H. S. Shin, and A. L. Sandberg, *ibid.*, **102,** 269 (1969).
(53) A. L. Sandberg, A. G. Osler, H. S. Shin, and B. Oliveira, *ibid.*, **104,** 329 (1970).
(54) A. L. Sandberg and A. G. Osler, *ibid.*, **107,** 1268 (1971).
(55) A. L. Sandberg, O. Götze, H. J. Müller-Eberhard, and A. G. Osler, *ibid.*, **107,** 920 (1971).
(56) K. B. M. Reid, *Immunology,* **20,** 649 (1971).
(57) J. M. Kehoe and M. Fougereau, *Nature,* **224,** 1212 (1969).
(58) J. R. Ellerson, D. Yasmeen, R. M. Painter, and K. J. Dorrington, *FEBS Lett.*, **24,** 318 (1972).
(59) H. L. Spiegelberg and O. Bötze, *Fed. Proc.*, **31,** 2478 (1972).
(60) S. Flexner and H. J. Noguchi, *J. Exp. Med.*, **6,** 277 (1903).
(61) H. Ritz, *Z. Immunol. Exp. Ther.*, **13,** 62 (1912).
(62) P. G. Klein and H. J. Wellensiek, *Immunology,* **8,** 590 (1965).
(63) R. A. Nelson, *Surv. Ophthalmol.*, **11,** 498 (1966).
(64) M. Ballow and C. G. Cochrane, *J. Immunol.*, **103,** 944 (1969).
(65) H. J. Müller-Eberhard and K. Fjellström, *ibid.*, **107,** 1666 (1971).
(66) H. S. Shin, H. Gewurz, and R. Snyderman, *Proc. Soc. Exp. Biol. Med.*, **131,** 203 (1969).
(67) R. J. Pickering, M. R. Wolfson, R. A. Good, and H. Gewurz, *Proc. Natl. Acad. Sci. USA,* **62,** 521 (1969).
(68) B. McLeod, R. F. Lint, P. Baker, C. Behrends, and H. Gewurz, *Immunology,* **28,** 741 (1975).
(69) N. R. Cooper, *J. Exp. Med.*, **137,** 451 (1973).
(70) L. Blum, L. Pillemer, and I. H. Lepow, *Z. Immunol. Exp. Ther.*, **118,** 349 (1959).
(71) H. Haupt and K. Heide, *Clin. Chim. Acta,* **12,** 419 (1965).
(72) T. Boenisch and C. A. Alper, *Biochim. Biophys. Acta,* **221,** 529 (1970).
(73) O. Götze and H. J. Müller-Eberhard, *J. Exp. Med.*, **134,** 905 (1971).
(74) W. Vogt, L. Dieminger, R. Lymen, and G. Schmidt, *Hoppe-Seyler's Z. Physiol. Chem.*, **355,** 171 (1974).
(75) N. R. Cooper, in "Contemporary Topics in Molecular Immunology," vol. 2, R. A. Reisfeld and W. J. Mandy, Eds., Plenum, New York, N.Y., 1973, p. 155.
(76) D. T. Fearon, K. F. Austen, and S. Ruddy, *J. Exp. Med.*, **139,** 355 (1974).
(77) C. A. Alper and F. S. Rosen, *Adv. Immunol.*, **14,** 251 (1971).
(78) L. G. Hunsicker, S. Ruddy, and K. F. Austen, *J. Immunol.*, **110,** 128 (1973).
(79) H. J. Müller-Eberhard, U. R. Nillson, A. P. Dalmasso, M. J. Polley, and M. A. Calcott, *Arch. Pathol.*, **82,** 205 (1966).
(80) S. Ruddy, K. F. Austen, and E. J. Goetzl, *J. Clin. Invest.*, **55,** 587 (1975).
(81) D. T. Fearon, K. F. Austen, and S. Ruddy, *J. Exp. Med.*, **138,** 1305 (1973).
(82) C. A. Alper and D. Balavitch, *Science,* **191,** 1275 (1976).
(83) O. D. Ratnoff and G. B. Naff, *J. Exp. Med.*, **125,** 377 (1967).
(84) S. B. Taubman, P. R. Goldschmidt, and I. H. Lepow, *Fed. Proc.*, **29,** 1065 (1970).
(85) G. Valet and N. R. Cooper, *J. Immunol.*, **112,** 1667 (1974).
(86) M. Loos, T. Borsos, and H. J. Rapp, *ibid.*, **109,** 434 (1972).
(87) P. A. Ward, *J. Exp. Med.*, **128,** 189 (1967).
(88) W. Dias da Silva and I. H. Lepow, *ibid.*, **125,** 921 (1967).
(89) C. G. Cochrane and H. J. Müller-Eberhard, *ibid.*, **127,** 371 (1968).
(90) J. H. Hill and P. A. Ward, *ibid.*, **130,** 505 (1969).
(91) I. H. Lepow, in "Progress in Immunology," vol. I, B. Amos, Ed., Academic, New York, N.Y., 1971, p. 567.
(92) M. R. Klemperer, F. S. Rosen, and V. H. Donaldson, *J. Clin. Invest.*, **48,** 44a (1969).
(93) I. H. Lepow, in "Biochemistry of the Acute Allergic Reactions," K. F. Austin and E. L. Becker, Eds., Blackwell, Oxford, England, 1971.
(94) V. H. Donaldson, D. D. Ratnoff, W. Dias da Silva, and F. S. Rosen, *J. Clin. Invest.*, **48,** 642 (1969).
(95) W. Dias da Silva, J. W. Eisle, and I. H. Lepow, *J. Exp. Med.*, **126,** 1027 (1967).
(96) H. S. Shin, R. Snyderman, E. Friedman, A. Mellors, and M. M. Mayer, *Science,* **162,** 361 (1968).
(97) R. Snyderman, H. Gewurz, and S. E. Mergenhagen, *J. Exp. Med.*, **128,** 259 (1968).
(98) V. A. Bokisch, H. J. Müller-Eberhard, and C. G. Cochrane, *ibid.*, **129,** 1109 (1969).
(99) P. A. Ward, in "Cellular and Humoral Mechanisms in Anaphylaxis and Allergy," H. Z. Movat, Ed., S. Karger, Basel, Switzerland, 1969, p. 279.
(100) J. A. Jensen, R. Snyderman, and S. E. Mergenhagen, in *ibid.*, p. 265.
(101) H. S. Shin, H. Gewurz, and R. Snyderman, *Proc. Soc. Exp. Biol. Med.*, **131,** 203 (1969).
(102) P. A. Ward, C. G. Cochrane, and H. J. Müller-Eberhard, *J. Exp. Med.*, **122,** 327 (1965).
(103) C. Bianco, R. Patrick, and V. Nussenzweig, *ibid.*, **132,** 702 (1970).
(104) I. Gigli and R. A. Nelson, *Exp. Cell. Res.*, **51,** 45 (1968).
(105) K. Nishioka and W. D. Linscott, *J. Exp. Med.*, **118,** 767 (1963).
(106) J. D. Feldman, M. R. Mardiney, and S. E. Shuler, *Lab. Invest.*, **15,** 283 (1966).
(107) D. Koffler and F. Paronetto, *J. Clin. Invest.*, **44,** 1665 (1965).
(108) P. J. Lachmann, H. J. Müller-Eberhard, H. G. Kunkel, and F. Paronetto, *J. Exp. Med.*, **115,** 63 (1962).
(109) C. A. Alper and F. S. Rosen, *J. Clin. Invest.*, **46,** 2021 (1967).
(110) H. Gewurz, R. J. Pickering, D. S. Clark, A. R. Page, J. Finstad, and R. A. Good, in "Immunologic Deficiency Diseases in Man," D. Bergsma, Ed., National Foundation, New York, N.Y., 1968.
(111) P. F. Kohler and H. J. Müller-Eberhard, *Clin. Res.*, **15,** 296 (1967).
(112) P. H. Schur and K. F. Austen, *Ann. Rev. Med.*, **19,** 1 (1968).
(113) C. D. West, J. D. Northway, and N. C. Davis, *J. Clin. Invest.*, **43,** 1507 (1964).
(114) F. Paronetto and D. Koffler, *ibid.*, **44,** 1657 (1965).

(115) K. H. Svec, J. D. Blair, and M. H. Kaplan, *ibid.*, **46,** 558 (1967).
(116) E. M. Tan and H. G. Kunkel, *Arthritis Rheum.*, **9,** 39 (1966).
(117) D. Koffler, P. Schur, and H. G. Kunkel, *J. Exp. Med.*, **126,** 607 (1967).
(118) C. Krishnam and M. H. Kaplan, *J. Clin. Invest.*, **46,** 569 (1967).
(119) E. Wasserman and L. Levine, *J. Immunol.*, **87,** 290 (1961).
(120) J. P. Leddy, *Semin. Hematol.*, **3,** 48 (1966).
(121) G. Fostiropoulos, K. F. Austen, and K. J. Block, *Arthritis Rheum.*, **8,** 219 (1965).
(122) H. Hedberg, *Acta Rheum. Scand.*, **9,** 165 (1963).
(123) N. J. Zvaifler and T. Pekin, *Clin. Res.*, **11,** 180 (1963).
(124) A. J. Fish, A. F. Michael, H. Gewurz, and R. A. Good, *Arthritis Rheum.*, **9,** 267 (1966).
(125) W. S. Rodman, R. C. Williams, R. J. Bilka, and H. J. Müller-Eberhard, *J. Lab. Clin. Med.*, **69,** 141 (1967).
(126) A. Covaerts, *J. Immunol.*, **129,** 724 (1960).
(127) F. Milgrom, B. I. Litvak, K. Kano, and E. Witebsky, *J. Am. Med. Assoc.*, **198,** 226 (1966).
(128) K. F. Austen and P. S. Russell, *Ann. N.Y. Acad. Sci.*, **129,** 657 (1966).
(129) C. B. Carpenter, T. J. McGill, J. P. Merrill, and G. S. Dammin, *Am. J. Med.*, **43,** 854 (1967).
(130) C. B. Carpenter, S. Ruddy, I. H. Shehadeh, J. P. Merrill, K. F. Austen, and H. J. Müller-Eberhard, *Transplant. Proc.*, **1,** 279 (1969).
(131) C. B. Carpenter, S. Ruddy, I. H. Shehadeh, H. J. Müller-Eberhard, J. P. Merrill, and K. F. Austen, *J. Clin. Invest.*, **48,** 1495 (1969).
(132) R. R. Linquist, R. P. Guttman, J. P. Merrill, and G. J. Dammin, *Am. J. Pathol.*, **53,** 851 (1968).
(133) K. A. Porter, G. A. Andres, M. W. Calder, J. B. Dossetor, K. C. Hsu, J. W. Rendall, B. C. Seegal, and T. E. Starzl, *Lab. Invest.*, **18,** 159 (1968).
(134) H. Gewurz, D. S. Clark, J. Finstad, W. D. Kelly, R. L. Varco, R. A. Good, and A. E. Gabrielson, *Ann. N.Y. Acad. Sci.*, **129,** 673 (1966).
(135) L. D. Caren and L. T. Rosenberg, *Immunology*, **9,** 359 (1965).
(136) U. Rother, D. L. Ballantyne, C. Cohen, and K. Rother, *J. Exp. Med.*, **126,** 565 (1967).
(137) O. D. Ratnoff and I. H. Lepow, *ibid.*, **106,** 327 (1957).
(138) L. R. Levy and I. H. Lepow, *Proc. Soc. Exp. Biol. Med.*, **101,** 608 (1959).
(139) G. B. Naff and O. D. Ratnoff, *J. Exp. Med.*, **128,** 571 (1968).
(140) O. D. Ratnoff, J. Pensky, D. Ogston, and G. B. Naff, *ibid.*, **129,** 315 (1969).
(141) I. Gigli, J. W. Mason, R. W. Coleman, and K. F. Austen, *J. Immunol.*, **104,** 574 (1970).
(142) A. D. Schreiber, A. P. Kaplan, and K. F. Austen, *J. Clin. Invest.*, **52,** 1402 (1973).
(143) N. Tamura and R. A. Nelson, *J. Immunol.*, **99,** 582 (1967).
(144) P. J. Lachmann and H. J. Müller-Eberhard, *ibid.*, **100,** 691 (1968).
(145) S. Ruddy and K. F. Austen, *ibid.*, **102,** 533 (1969).
(146) *Ibid.*, **107,** 742 (1971).
(147) J. D. Gitlin, F. S. Rosen, and P. J. Lachmann, *J. Exp. Med.*, **141,** 1221 (1975).
(148) V. H. Donaldson and R. R. Evans, *Am. J. Med.*, **35,** 37 (1963).
(149) C. A. Alper, N. Abramson, R. B. Johnston, J. H. Jandle, and F. S. Rosen, *N. Engl. J. Med.*, **282,** 349 (1970).
(150) N. Abramson, C. A. Alper, P. J. Lachmann, F. S. Rosen, and J. H. Jandle, *J. Immunol.*, **107,** 19 (1971).
(151) C. A. Alper and F. S. Rosen, *Adv. Immunol.*, **14,** 251 (1971).
(152) N. R. Cooper, *J. Exp. Med.*, **141,** 890 (1975).
(153) B. C. McLeod, P. Baker, and H. Gewurz, *Immunology*, **26,** 1145 (1974).
(154) B. C. McLeod, P. Baker, and H. Gewurz, *Int. Arch. Allergy Appl. Immunol.*, **47,** 623 (1974).
(155) E. L. Becker, in "Inflammation Mechanisms and Control," I. H. Lepow and P. A. Ward, Eds., Academic, new York, N.Y., 1972.
(156) L. Levine, *Biochim. Biophys. Acta*, **18,** 283 (1955).
(157) E. L. Becker, *J. Immunol.*, **77,** 462 (1956).
(158) A. L. Haines and I. H. Lepow, *ibid.*, **92,** 468 (1964).
(159) B. R. Baker and J. A. Hurlbut, *J. Med. Chem.*, **12,** 415 (1969).
(160) *Ibid.*, **12,** 902 (1969).
(161) B. R. Baker and M. Cory, *J. Med. Chem.*, **14,** 119 (1971).
(162) R. M. Stroud, K. F. Austen, and M. M. Mayer, *Immunochemistry*, **2,** 219 (1965).
(163) D. H. Bing, *Biochemistry*, **8,** 4503 (1969).
(164) D. H. Bing, J. L. Mernitz, and S. Spurlock, *ibid.*, **11,** 4263 (1972).
(165) D. H. Bing, M. Cory, and M. Doll, *J. Immunol.*, **113,** 584 (1974).
(166) F. B. Taylor and H. Fudenberg, *Immunology*, **7,** 319 (1964).
(167) N. A. Sorter, K. F. Austen, and I. Gigli, *J. Immunol.*, **114,** 928 (1975).
(168) W. Vogt, G. Schmidt, R. Lynen, and L. Dieminger, *ibid.*, **114,** 671 (1975).
(169) W. Cushman, E. L. Becker, and G. Wirtz, *ibid.*, **79,** 89 (1957).
(170) R. S. Basch, *ibid.*, **94,** 629 (1965).
(171) N. R. Cooper and E. L. Becker, *ibid.*, **98,** 119 (1967).
(172) N. R. Cooper and H. J. Müller-Eberhard, *Fed. Proc.*, **26,** 361 (1967).
(173) P. A. Ward, C. G. Cochrane, and H. J. Müller-Eberhard, *Immunology*, **11,** 141 (1966).
(174) M. M. Glovsky, E. L. Becker, and N. J. Halbrook, *J. Immunol.*, **100,** 979 (1968).
(175) M. M. Glovsky, P. A. Ward, E. L. Becker, and N. J. Halbrook, *ibid.*, **102,** 1 (1969).
(176) N. N. Azar and R. A. Good, *Proc. Soc. Exp. Biol. Med.*, **137,** 429 (1971).
(177) K. H. Busing, *Allerg. Asthma*, **3,** 15 (1957).
(178) D. Lorenz and H. Uebel, *Arzneim.-Forsch.*, **7,** 357 (1957).
(179) K. Lauenstein, H. G. Siedeutopf, and H. Fischer, *Z. Naturforsch.*, **206,** 575 (1965).
(180) R. Rent, N. Ertel, R. Eisenstein, and H. Gewurz, *J. Immunol.*, **114,** 120 (1975).
(181) P. J. Baker, T. F. Lint, B. C. McLeod, C. L. Behrends, and H. Gewurz, *ibid.*, **114,** 554 (1975).
(182) V. Agnello, R. I. Carr, D. Koffler, and H. G. Kunkel, *Fed. Proc.*, **28,** 2447 (1969).
(183) T. Borsos, H. J. Rapp, and C. Crisler, *J. Immunol.*, **94,** 662 (1965).
(184) G. E. Davies, *Immunology*, **6,** 561 (1963).
(185) M. Loos, T. Borsos, and H. J. Rapp, *J. Immunol.*, **108,** 683 (1972).
(186) S. Yachnin, *J. Clin. Invest.*, **42,** 1947 (1963).
(187) S. Yachnin, D. Rosenblum, and D. Chatman, *J. Immunol.*, **93,** 540 (1964).
(188) E. Raepple, H. U. Hill, and M. Loos, *Immunochemistry*, **13,** 251 (1976).
(189) M. Loos, J. E. Volanakis, and R. M. Stroud, *ibid.*, **13,** 257 (1976).
(190) E. A. Kabat and M. M. Mayer, in "Experimental Immunochemistry," 2nd ed., Charles C Thomas, Springfield, Ill., 1967, p. 133.
(191) R. Allan and H. Isliker, *Immunochemistry*, **11,** 175 (1974).
(192) *Ibid.*, **11,** 243 (1974).
(193) E. Appella, A. Chersi, R. G. Mage, and S. Dubiski, *Proc. Natl. Acad. Sci. USA*, **68,** 1341 (1971).
(194) S. Utsumi, *Biochem. J.*, **112,** 343 (1969).
(195) Z. Ovary, P. H. Saluk, L. Quijada, and M. E. Lamm, *J. Immunol.*, **116,** 1265 (1976).
(196) G. E. Connell and R. R. Porter, *Biochem. J.*, **124,** 53 (1971).
(197) J. M. Kehoe, J. A. Bourgois, J. D. Capra, and M. Forgereau, *Biochemistry*, **13,** 2499 (1974).
(198) G. Weber, *Biochem. J.*, **51,** 145 (1952).
(199) P. Wohl and G. Weber, *J. Mol. Biol.*, **30,** 371 (1967).
(200) J. K. Weltman and G. M. Edelman, *Biochemistry*, **6,** 1437 (1967).
(201) M. Z. Atassi and A. F. S. A. Habeeb, *ibid.*, **8,** 1385 (1969).
(202) A. L. Grossberg and D. Pressman, *ibid.*, **7,** 272 (1968).
(203) B. J. Johnson and K. E. Thames, *J. Pharm. Sci.*, **66,** 427 (1977).
(204) G. M. Edelman, B. A. Cunningham, W. E. Gall, P. D. Gottlieb, V. Rutishauer, and M. J. Waxdal, *Proc. Natl. Acad. Sci. USA*, **63,** 78 (1969).
(205) D. E. Tracey and J. J. Cebra, *Biochemistry*, **13,** 4796 (1974).
(206) R. L. Hill, H. E. Lebovitz, R. E. Fellows, and R. Delaney, in "Gamma Globulins, Proceedings of the 3rd Nobel Symposium," 1967, p. 109.
(207) F. W. Putman, G. Florent, C. Paul, T. Shinoda, and A. Shimizu, *Science*, **182,** 287 (1973).
(208) B. J. Johnson and K. E. Thames, *Fed. Proc.*, **35,** 494 (1976).
(209) B. J. Johnson and K. E. Thames, *J. Immunol.*, **117,** 1491 (1976).
(210) B. J. Johnson, *J. Pharm. Sci.*, **63,** 313 (1974).

(211) S. E. Mergenhagen, T. P. Tempel, and R. Snyderman, *J. Dent. Res.*, **49**, 256 (1970).
(212) R. Snyderman, *J. Am. Dent. Assoc.*, **87**, 1020 (1973).
(213) R. J. Genco, P. A Mashimo, G. Krygier, and S. A. Ellison, *J. Periodont.*, **45**, 330 (1974).
(214) R. Attström, A. Laurel, U. Lahsson, and A. Sjöholm, *J. Periodont. Res.*, **10**, 19 (1975).
(215) F. J. Courts, R. J. Boakle, H. H. Fudenberg, and M. S. Silverman, *J. Dent. Res.*, **56**, 327 (1977).
(216) P. Brandtzaeg, *Arch. Oral Biol.*, **10**, 795 (1965).
(217) E. J. Shillitoe and T. Lehner, *ibid.*, **17**, 241 (1972).

ACKNOWLEDGMENTS AND ADDRESSES

Received from the *Department of Microbiology, University of Alabama Medical School, Birmingham, AL 35294.*

Some work reported in this article was supported by Grant AI 11970 from the National Institutes of Health.

RESEARCH ARTICLES

Mechanism of Surface Lubrication: Influence of Duration of Lubricant–Excipient Mixing on Processing Characteristics of Powders and Properties of Compressed Tablets

A. C. SHAH ˣ and A. R. MLODOZENIEC

Abstract □ A mathematical expression for tablet hardness was related to lubricant mixing by considering increases in the surface coverage with prolonged mixing time. The duration of lubricant mixing significantly changed the apparent bulk volume of the mix, ejection force during tableting, hardness, and disintegration and dissolution properties of tablets. These findings may provide some rationale for the changes in processing characteristics and properties of finished drug products often encountered in the scale-up of solid dosage formulations. Several lubrication mechanisms are discussed in connection with the duration of mixing effects and scanning microscopy studies.

Keyphrases □ Tablets, compressed—effect of duration of lubricant–excipient mixing on physical properties □ Powders—effect of duration of lubricant–excipient mixing on processing characteristics □ Lubrication, surface—effect of duration of lubricant–excipient mixing on physical properties of tablets and processing characteristics of powders □ Dosage forms—tablets compressed, effect of duration of lubricant–excipient mixing on physical properties

The formulation of a solid dosage form often requires precise processing control of the powder mixture to ensure a volumetric delivery of a homogeneous aliquot. Thus, various adjuvants are gravimetrically added to form the bulk mix to achieve uniform mixing and flow of the powders as in capsule or tablet die filling.

In tablet formulation, a lubricant usually permits resolution of several production problems related to compression. As an essential unit operation in the production of a compressed tablet, lubrication facilitates glidancy of the powders during material flow, eliminates binding of the compact to the die, and minimizes sticking and picking by the punch face surfaces in contact with the compressed tablet. Lubrication, in general, involves adding small quantities of an antifriction agent to powders or granules and mixing them for a specified time.

BACKGROUND

Many studies evaluated different types of lubricants (1–5), the influence of lubricant concentration (6, 7), the relationship between lubricant effects and tablet properties (hardness and disintegration) (8, 9), and changes in physical properties of powder mixes as a function of lubrication (10–15). Few of these studies, however, concerned the influence of mixing time on the processing characteristics of the powder and, especially, the performance properties of the compressed tablet.

Properties of the compact critical to its performance include the ejection force, tablet hardness, disintegration, and dissolution. It is generally recognized that a lubricant modifies these properties. However, the duration of mixing in the lubricant component may not only affect the properties of the compact but also the properties of the blended mixture by altering the apparent bulk volume, the compression force required to make a prescribed compact, and the hydrophobic character of the mixture. The research studies described in this report concerned the interdependency of these physical properties for several model systems designed to test the effects of mixing time.

Previous mixing studies dealt with the homogeneity of the mixture (16–20), evaluations of mixing equipment (21–23), and segregation kinetics associated with model systems (24–27). This work emphasized the importance of solid–solid mixing related to drug distribution and homogeneity of the mix. Since content uniformity of the active ingredient is a primary control for accurately dosing a patient with a unit dosage form, emphasis is always given to the final composition of the assayed tablet or other dose form. Nonetheless, the release characteristics and performance criteria (such as physical integrity and stability) also rely on the nature and extent of distribution of inert excipients as well as the active ingredient. Of significance is the lubricant–excipient interaction and the manner in which these materials are affected by mixing. Possibly, mixing times can modify the intended role of these adjuvants, in some cases altering or diminishing their primary function.

The present work investigated the mixing of direct compaction excipients such as lactose and microcrystalline cellulose with various commonly used lubricants. Furthermore, the occasional nonpredicted increased disintegration time of a compressed tablet associated with a decreased hardness or crushing force requirement prompted an investigation of the effects of lubricants (*e.g.*, stearates) and mixing duration on the physical properties of a blended mixture and compact.