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Arthropathic Group A Streptococcal Cell Walls Require Specific Antibody for Activation of Human Complement by Both the Classical and Alternative Pathways

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The induction of acute arthritis in rats by a single intraperitoneal injection of group A streptococcal cell wall is associated with the activation of complement. We have therefore investigated the interaction of arthropathic peptidoglycan-polysaccharide complex of streptococcal cell walls and human complement. The incubation of cell wall in normal human serum results in the formation of complexes of cell wall and the C3 and C4 components of complement. Using agammaglobulinemic serum, we have further shown that the activation of complement and formation of complement-cell wall complexes absolutely requires the presence of a small quantity of specific antibody. This antibody is present in normal human immunoglobulin G and is effective as the Fab fragment (alternative pathway). Although antibodies specific for three cell wall epitopes were capable of inducing complement-cell wall complex formation by the classical complement pathway, only anti-A polysaccharide antibody (*N*-acetyl-D-glucosamine epitope) was effective by the alternative complement pathway. A complement consumption assay showed that anti-cell wall antibody was required not only for complement-cell wall complex formation, but also for activation of complement by streptococcal cell wall in human serum. These studies suggest that a minimal level of anti-cell wall antibody may be required for the induction of arthritis in rats by streptococcal cell wall.

Complement activation plays an important role in the pathogenesis of inflammatory arthritis induced by a single systemic injection of an aqueous suspension of group A streptococcal cell walls in rats. During day 1 after cell wall injection, there is an initial decline in serum complement, as measured by both the classical and alternative pathways (17). After 2 days, the serum complement recovers and in fact rises to levels more than twice as high as normal. The depletion of complement over the short term by multiple injections of cobra venom factor depresses the acute phase of the group A cell wall-induced arthritis (26). However, the complement suppression is short-lived, and the chronic inflammatory arthritis that develops in cobra venom-treated animals is indistinguishable from that in controls given only cell wall. In addition, cell wall-complement complexes are present in the tissues of arthritic rats (Eisenberg and Schwab, manuscript in preparation). Such complement-cell wall complexes have also been suggested by in vitro work showing that cell wall incubated in serum is able to bind to cellular complement receptors (22).

Because of the involvement of complement in group A cell wall-induced acute arthritis in rats, we have been interested in investigating the mechanisms of complement activation by the arthropathic cell wall. A variety of bacteria or bacterial cell walls, as well as the isolated peptidoglycan, have been shown to be activators of the alternative complement pathway (9, 11). In some cases, an enhancing role for antibody has been demonstrated in this activation (29). In other cases, antibody is absolutely required (6). Some nonbacterial activators of the alternative pathway have also been shown to have either enhanced activation in the presence of specific antibody (20) or an absolute requirement for antibody (19). In the current investigation, we have focused on the activation of human complement by streptococcal cell wall, since appropriate human reagents are readily available. We have found that the arthropathic peptidoglycan-polysaccharide complex of group A streptococcal cell wall absolutely requires antibody for activation of either the classical or the alternative complement pathway and the subsequent formation of cell wall-complement complexes. Although the specificity of the anti-cell wall antibody is critical, sufficient activating antibody is present in normal human serum or normal human immunoglobulin G (IgG).

MATERIALS AND METHODS

Cell wall. Group A type 3 strain D58 streptococci were processed as previously described (10) to obtain arthropathic cell wall fragments, which consisted essentially of peptidoglycan covalently bound to the group A polysaccharide (14). In some experiments, cell wall was prepared in similar fashion from group A variant streptococci, which lack most of the *N*-acetyl-D-glucosamine (NADG) determinant, which is the characteristic epitope of the group A polysaccharide (3). Peptidoglycan was also prepared from group A variant cell wall by extraction of the group A variant polysaccharide with hot formamide (15).

Sera. Serum from three normal donors was used. In addition, samples were obtained from a 42-year-old female patient with severe agammaglobulinemia secondary to common variable immunodeficiency. The serum immunoglobulin levels of this patient were as follows: IgG, 20 μg/ml; IgA, 70 μg/ml; IgM, 30 μg/ml; and IgE, less than 1 U/ml. Serum and heparinized plasma were used interchangeably with entirely comparable results. In experiments in which the agammaglobulinemic serum was utilized, serum from the patient's husband, which had been obtained at the same time and processed in parallel, was used as a control.

Antibodies. Polyclonal human IgG was a DEAE-purified

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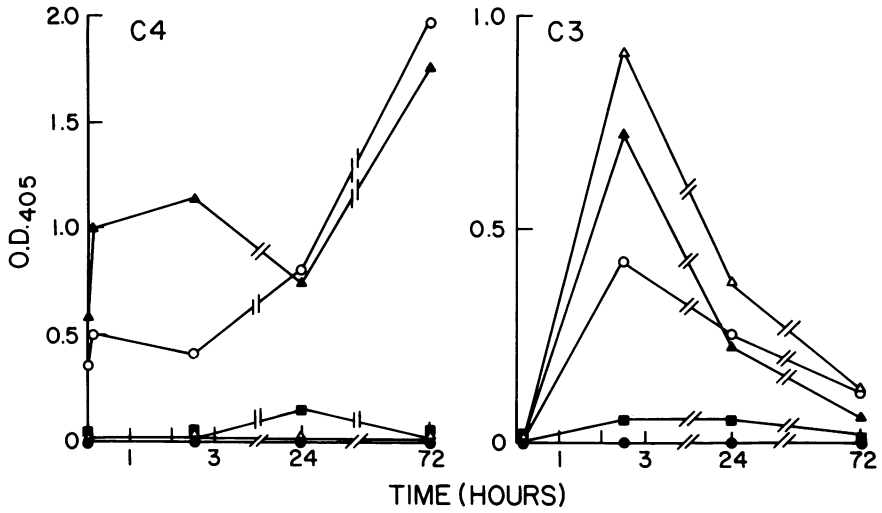

FIG. 1. Formation of complement-cell wall complexes in normal human serum. Group A streptococcal cell wall (100 μg) was mixed with human serum and incubated for the indicated periods of time. C4- and C3-cell wall complexes were measured by ELISA as described in the text. ○, Normal human serum; ●, normal human serum plus 0.01 M EDTA; △, normal human serum plus 0.01 M EGTA and 0.005 M Mg^{2+}; ▲, normal human serum heated to 50°C for 20 min; ■, normal human serum heated to 50°C for 20 min plus 0.01 M EGTA and 0.005 M Mg^{2+}. $O.D._{405}$, Optical density at 405 nm.

preparation made from commercial Cohn FII. In one experiment, the Fab fragments prepared by papain digestion of the DEAE-purified IgG from a single individual were utilized. Specific antibodies to the group A polysaccharide (NADG), the group A variant polysaccharide (rhamnose oligosaccharide), and peptidoglycan (D-Ala-D-Ala) were affinity purified from rabbit sera as previously described (8). Human complement components were detected by monoclonal antibody to C4 (the kind gift of J. Donald Capra, Dallas, Tex.) or with a polyclonal affinity-purified rabbit anti-human C3.

Formation of cell wall-complement complexes. Group A cell wall fragments were added to human serum samples at a concentration of 100 μg/ml on ice. The serum was then placed at 37°C for 1 h, except in those studies in which multiple times of incubation were tested. At the end of the incubation time, sufficient 0.1 M EDTA was added to yield a final concentration of 0.01 M EDTA, and the tubes were held on ice until they were ready for testing. Sera were also incubated with cell wall after the following manipulations: the addition of 0.01 M ethylene glycol-bis(aminoethyl ether)-*N,N,N',N'*)tetraacetic acid (EGTA)–0.005 M Mg^{2+} to test only the alternative pathway; the addition of 0.01 M EDTA as a negative control; heating to 50°C for 20 min to test only the classical complement pathway; heating to 50°C for 20 min and the addition of 0.01 M EGTA–0.005 M Mg^{2+} as a negative control; and heating at 56°C for 30 min as a negative control. At least one form of negative control was included in every experiment.

ELISA. The streptococcal cell wall-complement complexes were detected by an enzyme-linked immunosorbent assay (ELISA) adapted from the radioimmune assay for cell wall previously described (8). Polyvinyl flat-bottom microtiter plates (96 well; Dynatech Corp., Alexandria, Va.) were coated with a 3-μg/ml solution of anti-C4 or anti-C3 or with anti-group A cell wall in borate-buffered saline (1 μg/ml; 0.2 M borate, 0.075 M NaCl [pH 8.4]) for 5 h at 4°C. Nonspecific binding sites were then blocked by the addition of 200 μl of borate-buffered saline plus 0.5% human serum albumin, 0.4% Tween 80, and 0.01 M EDTA (BHTE) per well. Then samples were added in 100 μl at a dilution of 1:1,000 in BHTE. After overnight incubation, affinity-purified biotinylated antibodies to group A cell wall were added for 3 h at 4°C. Avidin-alkaline phosphatase conjugate (100 μl) was then added for a further incubation of 3 h at 4°C. Finally, 100 μl of *para*-nitrophenyl phosphate at 1 mg/ml in 0.01 M diethanolamine (pH 9.8) was added. The development of color was monitored by an automated microELISA reader (Dynatech model MR 580). All results shown are the net optical density, calculated by subtracting the appropriate assay background in each case. Control assays confirmed the requirement for cell wall, complement, and specific antibodies in these assays.

Erythrocyte lysis assay. Consumption of hemolytic complement via the alternative pathway was determined by rabbit erythrocyte lysis (5). Agammaglobulinemic serum plus EGTA-Mg^{2+} was incubated alone, with 100 μg of group A streptococcal cell wall per ml, with 40 μg of rabbit anti-A polysaccharide affinity-purified antibody per ml, or with both cell wall and antibody for 180 min at 37°C. After incubation, the samples were tested for residual hemolytic complement by making serial 1.5-fold dilutions in EGTA-Mg^{2+} and then adding an equal volume of 1% washed normal rabbit erythrocytes in Veronal-buffered saline–EGTA–Mg^{2+}. After incubation at 37°C for 30 min, the plates were centrifuged at 2,500 rpm (J6B centrifuge; Beckman Instruments, Inc., Palo Alto, Calif.) for 5 min, and 15 μl of the supernatants was transferred to clean, flat-bottom microtiter wells for determination of released hemoglobin by the microELISA reader.

RESULTS

Formation of complement-cell wall complexes in normal human serum. The incubation of group A streptococcal cell wall in human serum resulted in the formation of C4-cell wall complexes by the classical complement pathway and C3-cell wall complexes by both the classical and alternative complement pathways. The C4 complexes were formed within minutes in serum with an intact classical pathway and did not decrease appreciably thereafter (Fig. 1). The C3 complexes were also rapidly formed, but decreased over a period of 1 to 3 days.

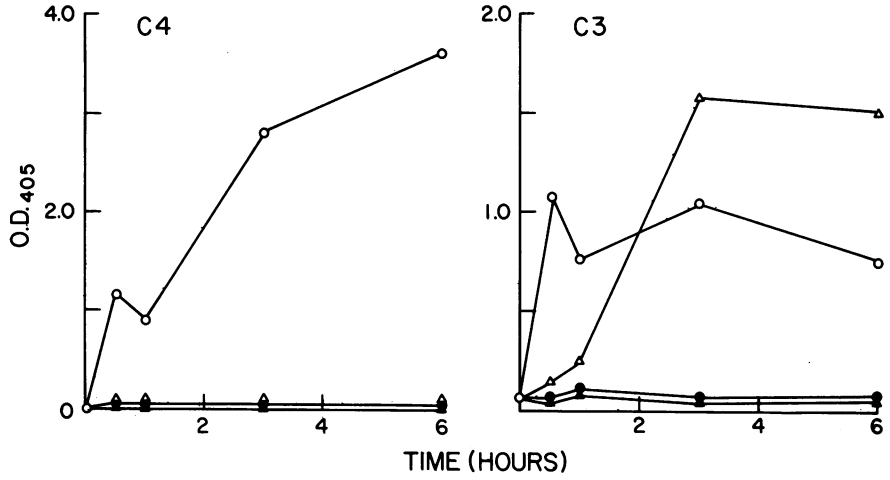

FIG. 2. IgG requirement for formation of complement-cell wall complexes in agammaglobulinemic human plasma. Cell wall was incubated in agammaglobulinemic plasma treated to permit only classical complement pathway activation (50°C for 20 min [○, ●]) or only alternative complement pathway activation (EGTA-Mg^{2+} [△, ▲]). ○ and △, Addition of 8 mg of normal human IgG per ml; ● and ▲, addition of buffer. C4- and C3-cell wall complexes were measured by ELISA at the indicated times. $O.D._{405}$, Optical density at 405 nm.

Requirement for immunoglobulin for cell wall-complement complex formation. The availability of serum and plasma from a patient with unusually low levels of serum immunoglobulin permitted us to investigate the role of antibody in the formation of cell wall-complement complexes. Initial observations showed that the agammaglobulinemic serum failed completely to form cell wall-complement complexes with C3 and C4 by either the classical or alternative complement pathway. However, by adding normal human IgG to a concentration of 8 mg/ml, the complement-activating capacity of the agammaglobulinemic serum was restored (Fig. 2). The agammaglobulinemic serum plus IgG formed C4-cell wall complexes in the presence of the classical complement pathway and formed C3-complement complexes in the presence of either the classical or the alternative complement pathway. In the absence of additional immunoglobulin, no complement-cell wall complexes were formed for the entire 6 h of the experiment. In addition, C4-complement complexes were never formed in the presence of the alternative complement pathway. Fab fragments of serum IgG also permitted the formation of C3-cell wall complexes (but not C4 complexes) in unmanipulated plasma or in EGTA-Mg^{2+} plasma (data not shown).

Specific antibody required for complement activation. The addition of normal IgG to agammaglobulinemic serum to restore the ability of the serum to form cell wall-complement complexes via alternative pathway activation suggested that anti-cell wall antibody might be required. This interpretation was further supported by the fact that preabsorption of normal human IgG with cell wall specifically removed its complement-activating ability by both the classical and alternative pathways (data not shown). The specificity of this antibody requirement was investigated by adding graded concentrations of three preparations of affinity-purified rabbit anti-cell wall antibody to agammaglobulinemic plasma and testing for the formation of C4- and C3-cell wall complexes by either the alternative or classical complement pathway. Antibodies against the A polysaccharide (NADG), the A variant polysaccharide (rhamnose oligosaccharide), or the D-Ala-D-Ala determinant of the peptidoglycan all were capable of the induction of complement activation via the classical pathway and the subsequent formation of C4- and C3-cell wall complexes (Fig. 3). On the other hand, only the anti-A polysaccharide antibody was efficient at inducing activation via the alternative complement pathway. The anti-A variant antibody induced some activation via the alternative complement pathway at the highest concentration, whereas the anti-D-Ala-D-Ala antibody was completely ineffective. The loss of complex detection at the highest concentration of the anti-A antibody was presumably due to competition of the added antibody with the biotinylated anti-A antibody used to develop the ELISA, since an anti-cell wall assay on this material was similarly inhibited (data not shown).

In an additional set of experiments, different cell wall

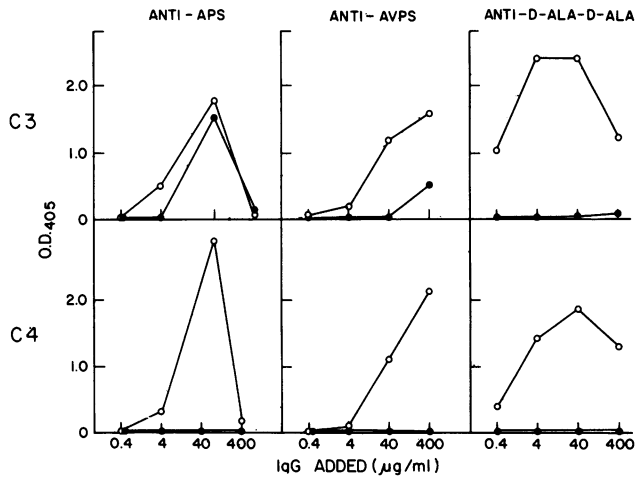

FIG. 3. The role of specific antibody in the formation of complement-cell wall complexes in agammaglobulinemic plasma. The indicated quantities of affinity-purified rabbit antibody specific for group A polysaccharide (anti-APS), A variant polysaccharide (anti-AVPS), or D-Ala-D-Ala terminus of the pentapeptide of peptidoglycan (anti-D-Ala-D-Ala) were incubated with agammaglobulinemic plasma and 100 μg of group A streptococcal cell wall per ml for 1 h at 37°C. C3- and C4-cell wall complexes were measured by ELISA. ○, Plasma heated to 50°C for 20 min; ●, plasma plus EGTA-Mg^{2+}. $O.D._{405}$, Optical density at 405 nm.

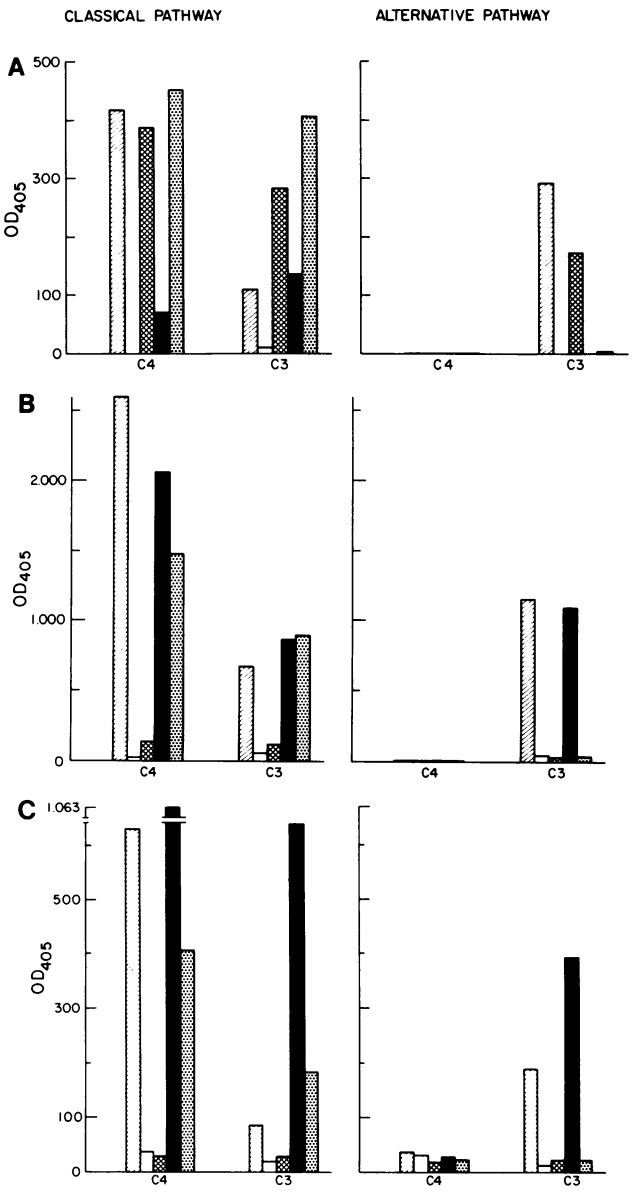

FIG. 4. Specificity of antibody required for the formation of complement-cell wall complexes with different cell wall preparations. (A) Group A streptococcal cell wall; (B) group A variant streptococcal cell wall; (C) group A variant peptidoglycan. Cell wall was incubated with indicated specific antibodies at 40 μg/ml and agammaglobulinemic serum for 1 h at 37°C and then tested for C4- and C3-cell wall complexes. Classical pathway activation was determined in serum that had been heated to 50°C for 20 min, whereas alternative pathway activation was determined in serum to which had been added EGTA-Mg^{2+}. ▨, Normal human serum; □, agammaglobulinemic serum plus normal rabbit IgG; ▩, agammaglobulinemic serum plus anti-A polysaccharide antibody; ■, agammaglobulinemic serum plus anti-A variant polysaccharide antibody; ▩, agammaglobulinemic serum plus anti-D-Ala-D-Ala antibody. OD_{405}, Optical density at 405 nm.

preparations were combined with the affinity-purified rabbit anti-cell wall antibodies in the presence of agammaglobulinemic serum. The antibodies were all used at a concentration of 40 μg/ml. Figure 4A shows the results with group A streptococcal cell wall. As previously indicated (Fig. 3), activation via the classical complement pathway could be obtained with antibodies to the A polysaccharide, A variant polysaccharide, or peptidoglycan. However, only antibodies to A polysaccharide were able to induce the formation of C3-cell wall complexes by the alternative pathway. This restriction was true even though the anti-peptidoglycan antibodies were as good inducers of the classical pathway as the anti-A polysaccharide antibodies. With group A-variant cell wall, the results were somewhat different (Fig. 4B). This material is not well recognized by the anti-A polysaccharide antibody, and therefore this antibody was unable to activate complement by either pathway. Group A variant cell wall is recognized by both the anti-A variant polysaccharide antibodies and the anti-peptidoglycan antibodies, and both of these preparations produced efficient C4- and C3-cell wall complex formation by the classical complement pathway. However, only the anti-A variant polysaccharide antibody was able to induce the formation of C3-cell wall complexes by the alternative complement pathway. The results for A variant peptidoglycan (Fig. 4C) are qualitatively similar to those for the A variant cell wall, presumably because, as we have previously found, the A variant peptidoglycan preparation is contaminated with A variant polysaccharide and therefore is recognized by both the anti-A variant polysaccharide antibody and the anti-peptidoglycan antibody (unpublished results).

Consumption of hemolytic complement by the alternative and classical complement pathways. Up to this point, we have been using the concepts of complement activation and formation of C4- and C3-cell wall complexes interchangeably. This usage was based on the assumption that if the streptococcal cell wall was able to activate complement in serum, it would lead to the formation of such complexes. In a further set of experiments, we tested this assumption by measuring complement activation (i.e., consumption) directly in agammaglobulinemic serum via the alternative complement pathway. Agammaglobulinemic serum was incubated in the presence of EGTA-Mg^{2+} for 3 h at 37°C. Serum samples were premixed with group A streptococcal cell wall alone, cell wall plus anti-A antibody, anti-A antibody alone, or neither cell wall nor antibody. These samples were compared with agammaglobulinemic serum which was held on ice during the 3 h. Serial dilutions of each sample were tested for the ability to lyse rabbit erythrocytes in the presence of EGTA-Mg^{2+}, as a measure of hemolytic complement initiating through the alternative complement pathway. There was a slight loss in hemolytic complement in the serum samples that had been incubated at 37°C, but it made no difference whether there had been antibody present, cell wall present, or neither (Fig. 5). Only that serum sample which had both antibody and cell wall showed marked complement consumption. This experiment supports our assumption that the failure to form cell wall-complement complexes was indicative of a failure to activate complement.

DISCUSSION

We have found that group A streptococcal cell walls, which are capable of inducing a chronic inflammatory destructive arthritis when injected into rats (4), can activate complement in human serum by both the classical and alternative pathways and can form complexes containing cell wall and the C4 and C3 complement components. In serum, the C4 component of the complex is relatively stable over 3 days at 37°C, whereas the C3 determinants are gradually lost. This catabolism of C3 probably represents, at least in

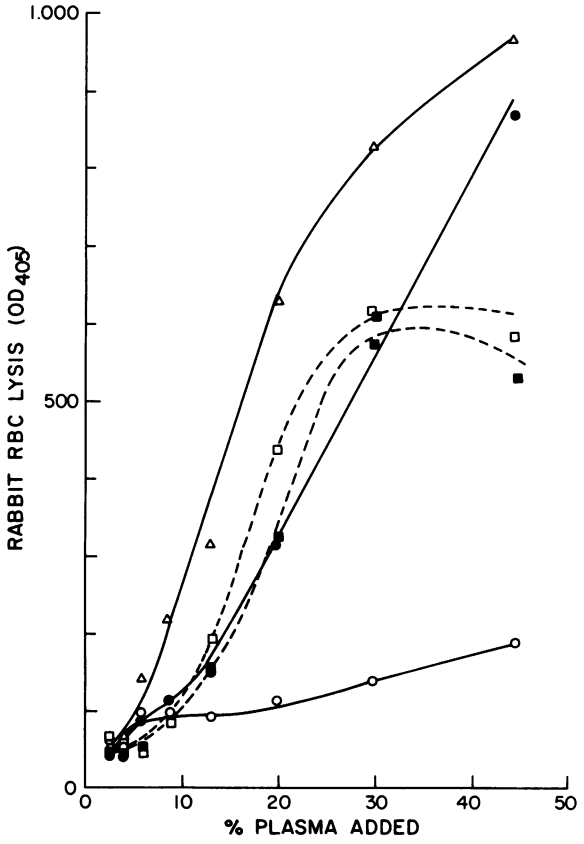

FIG. 5. Consumption of complement by the alternative pathway by streptococcal cell wall requires antibody. Agammaglobulinemic plasma with EGTA-Mg^{2+} was incubated for 3 h at 37°C, and then residual lytic complement activity via the alternative pathway was measured by testing serial 1.5-fold dilutions for lysis of rabbit erythrocytes (RBC) in the presence of EGTA-Mg^{2+}. ○, Plasma plus 40 μg of anti-A polysaccharide antibody per ml and 100 μg of cell wall per ml; ●, plasma plus cell wall alone; □, plasma plus anti-A antibody alone; ■, plasma plus neither cell wall nor antibody; △, plasma held at 4°C before being tested. OD_{405}, Optical density at 405 nm.

part, the action of factors H and I, as C3c is lost preferentially to C3dg, particularly in the presence of erythrocytes (data not shown; 16, 18, 25). We are confident that our studies were able to separate reliably the classical and alternative complement pathways since (i) EGTA-treated serum never permitted C4 complex formation; (ii) serum heated at 50°C for 20 min permitted C4 complex formation, but did not permit C3 complex formation in the presence of Fab fragments; and (iii) the combination of EGTA-Mg^{2+} and 50°C for 20-min treatments resulted in inactivation of both pathways.

We have also shown that specific anti-cell wall antibody was absolutely required for activation of the alternative complement pathway by the streptococcal cell wall. No alternative pathway activation or formation of C3-cell wall complexes occurred in agammaglobulinemic serum. Using affinity-purified rabbit antibodies specific for three immunodeterminants of the group A streptococcal cell wall, we found that only anti-NADG antibodies could activate the alternative pathway efficiently. Anti-A variant antibodies (which recognize the polyrhamnose backbone of the polysaccharide) and anti-peptidoglycan antibodies (which recognize the D-Ala-D-Ala determinant of the pentapeptide side chain) showed little or no alternative complement pathway activation with group A streptococcal cell walls, although both of these antibodies were good activators of the classical complement pathway. On the other hand, the anti-A variant antibody provided excellent alternative complement pathway activation with A variant cell walls. Therefore, the relative activities of these antibodies vis-a-vis the alternative complement pathway are a function of both their binding specificity (21) and the molecular structure of the substrate with which they interact.

Studies with several normal sera, as well as with preparations of human IgG from individuals not specifically immunized against streptococcal cell wall antigens, indicate that the antibodies required for alternative complement pathway activation were present as part of background serum immunoglobulins. The actual level of specific antibody necessary for alternative pathway activation was approximately 40 μg/ml, although much lower amounts of anti-peptidoglycan antibody were effective for classical pathway activation. The absorption experiments with normal human IgG indicated that the active principle was indeed specific antibody. Finally, the finding that Fab fragments of antibody were also effective in alternative complement pathway (but not classical complement pathway) complex formation was in agreement with previous systems in which antibody has been shown to play a facilitating role for the alternative complement pathway (23).

Most activators of the alternative pathway are believed to proceed by an antibody-independent route. However, several bacterial substrates have been found to require antibody for alternative complement pathway activation (1, 2, 6, 7, 13, 23, 24, 27, 28). The killing of *Escherichia coli* in bovine serum via the alternative complement pathway was shown to require a factor specifically absorbable at 0°C with homologous bacteria (13), whereas in similar experiments with *Shigella* spp. in human serum, it was possible to restore the opsonic capacity of absorbed serum with IgM antibody (24). In studies with *Haemophilus influenzae*, it was shown that the alternative complement pathway was activated by encapsulated strains in C4-deficient guinea pig serum, but killing of the bacteria occurred only in the presence of anti-polysaccharide antibody (28). More recent work with *H. influenzae* type B in human serum showed that activation and killing by the alternative complement pathway were absolutely antibody dependent (27). In addition, this last study found that the alternative complement pathway required anti-capsular antibodies specifically, while classical pathway-mediated killing could proceed with either anti-capsular antibodies or other antibodies that reacted with the bacterial organism. These results are entirely parallel to our findings with group A streptococcal cell wall. Another study measured the role of the alternative complement pathway in the opsonophagocytosis of type III group B streptococcus (6, 7). This investigation found that in organisms with intact sialic acid residues in the capsular polysaccharide, anti-type-specific antibody was required for alternative complement pathway activation. However, if these sialic acid residues were removed, the bacteria could be opsonized by the alternative complement pathway in the absence of antibody. Both this study and the study of *H. influenzae* (27) showed that the quantity of antibody necessary for alternative complement pathway activation was greater than that needed for the classical complement pathway. This result again is in agreement with our results with the streptococcal cell wall (Fig. 4).

We have recently found that streptococcal cell wall in rat

serum similarly forms complexes with the C3 complement component by both the alternative and classical pathways. In addition, such complexes are present in the tissues, particularly the joints, of arthritic rats (Eisenberg and Schwab, in preparation). Although we do not have the appropriate reagents to prove directly a requirement for antibody in alternative complement pathway activation in rat serum, we feel this requirement is a reasonable assumption. This situation then sheds a new light on the potential role of antibody in the production of chronic inflammatory arthritis in cell wall-injected rats. Our previous studies have shown no obvious positive correlation between overall antibody titers and the development of disease (12). On the other hand, IgM anti-peptidoglycan antibody levels in the chronic phase of joint disease appear to have a suppressive effect on the further development of chronic arthritis (R. E. Esser, S. K. Anderle, R. A. Eisenberg, and J. H. Schwab, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, B22, p. 21). Our present results suggest that a minimal antibody level is required for activation of complement by either the classical or the alternative pathway. Therefore, as complement plays a role in the production of disease (26), it is likely that natural antibody is also essential. Since the levels of antibody necessary are quite small (e.g., less than 1 μg of anti-peptidoglycan per ml for the classical pathway activation) and since such antibodies are presumably formed in the course of exposure to environmental agents, it would probably be difficult to demonstrate directly such a requirement in vivo.

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