NONSPECIFIC COMPLEMENT ACTIVATION BY
STREPTOCOCCAL STRUCTURES
II. Properdin-Independent Initiation of the Alternate Pathway*

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The observation (1) that inhibition of HLA cytotoxicity by streptococcal antigens was, in reality, due to consumption of complement (C) by these antigens prompted us to further investigate the mechanism of this consumption. Several possibilities for this anticomplementary activity were considered. Since human sera often contain antibodies to a number of streptococcal antigens, a specific interaction between bacterial structures and their corresponding antibodies was conceivable. In general, antigen-antibody complexes trigger the classical pathway, but there have been recent reports (2) of activation of the alternate pathway by these complexes as well. The evidence against specific antibody as the main mechanism of C consumption was the observation that the preincubation of streptococcal membranes with rabbit C yielded the most significant loss of C activity. Although unlikely, the possibility that rabbit sera contain antistreptococcal antibodies could not be completely excluded.

Secondly, while we suspected that streptococcal cellular structures were responsible for the activation, one had to consider that other substances either exogenous or endogenous to the organism could be responsible for the effect. In the former category were agents such as endotoxin, known for its anticomplementary effect (3), the culture medium which contains yeast extract (perhaps related to zymosan (4)), or enzymes (5) used during the purification of the membranes (6). The endogenous source of contamination includes other cell constituents which might be present as contaminants of our membrane preparation. Among these possibilities one must consider ribonucleotides which are known to bind Clq (7). Other isolated fractions from our laboratory, such as M protein from the cell wall and antigens cross-reactive with sarcolemma, were also tested for anticomplementary activity.

Finally, the relative blocking efficiency of Group A streptococcal membranes was always consistently greater than that observed with Group D membranes. Was this effect due to differences in the amount of anticomplementary substance(s) present in each preparation, or were these discrepancies related to our assay system? In addition, was this anticomplementary activity present in gram-positive organisms other than Group A streptococci?

With these questions in mind, a number of experiments were designed to

* This work was carried out in partial fulfillment for the requirements of a Ph.D. (J. W. T.), supported by a Rockefeller University fellowship and private funding.
test each theoretical possibility. The present report demonstrates that streptococcal structures clearly activate the alternate pathway of C consumption. Neither antibody nor properdin is necessary for the activation process and a direct activation of factor D occurred. The particular pathway is clearly different from the mechanisms seen with other known activators such as endotoxin, immune, or nonimmune aggregates. The C activation appears to occur with both cell walls and membranes of Group A streptococci as well as with components of other gram-positive organisms. Using standard titration of whole C hemolytic activity, no significant differences between Group A and Group D streptococci could be found.

Materials and Methods

Bacterial Strains and Growth. In addition to the originally used strains, S 43/192/2 and A932, [see (1)], the following streptococci were obtained from the collection of Dr. R. C. Lancefield, The Rockefeller University, New York.

S43 GLOSSY. This is a Group A type 6 mutant strain which is an M-protein variant of the S43 strain, originally isolated from a patient.

A909. Group A type 12 obtained from the throat of a patient in 1966.

T12/126/6. Group A type 12 obtained from the urine culture of a patient in 1924.

S25 HUBBANK. An M-protein mutant of a Group A type 14 isolated from a patient with lobar pneumonia in 1918.

C74. Group C, not typed, from a human throat culture in 1941.

DleeB. Group G, not typed, from a skin lesion of a child with epidermolysis bullosa, 1939, (Presbyterian Medical Center, New York).

Dr. E. C. Gotschlich, The Rockefeller University, New York, kindly provided the following two gram-positive microorganisms:

Pneumococcus CW-1. Pneumococcus CW-1 is a mutant which does not produce pneumococcal autolyzing enzymes, thus permitting the isolation of its cell wall (8).

Bacillus pumilis SH-17. Bacillus pumilis SH-17 is a mutant strain which produces teichoic acid cross-reactive with Group A meningococcal polysaccharide (9).

The following bacteria were isolated from skin swabs:

Staphylococcus epidermidis. Staphylococcus epidermidis is a strain which does not produce protein A and thus avoids the possibility of nonspecific binding of antibody (10).

All these microorganisms were resuspended from lyophilized form. After several subcultures, the final harvest was carried out in 0.25-3.0 liters of medium (see reference 1).

Isolation of Walls and Membranes. All strains (except S43/192/2, see Table I) were identically treated by mechanical disruption with glass beads for 40-60 min (11) until more than 90% of the organisms became gram negative. Differential centrifugation was followed by a brief treatment with RNase, including a saline wash before resuspension in distilled H2O and lyophilization (1).

Lipid Extraction and Fractionation. 500 mg lyophilized membranes of S43/192/2 were resuspended in 10 ml Foch reagent (chloroform to methylalcohol, 2:1) (12) in the cold with continuous sonication in a water bath (see reference 1) for 2 h. After paper filtration and vigorous shaking with 2 ml 0.04% CaCl2 for 2 min, centrifugation for 10 min at low speed resulted in the separation of two phases. Thus, three fractions resulted from this procedure:

\[
\begin{align*}
\text{Filter residue} & = \text{mainly proteins} \\
\text{Top phase } (H_2O) & = \text{polar lipids} \\
\text{Bottom phase } (CHCl_3) & = \text{nonpolar lipids}
\end{align*}
\]

The last was further fractionated into simple and composite lipids by column (0.8 x 16 cm) chromatography with silicic acid (Lipid Chromatograph grade, -325 mesh; Sigma Chemical Co., St. Louis, Mo.) (12).

The simple lipids, eluted with 10 ml CHCl3, include fatty acid triglycerides, glycerol, and steroids. The composite lipids, eluted with MeOH, mainly consist of phospholipids clearly visible
initially as a yellow band 1 cm underneath the top of the column. The three lipid fractions were blown to dryness under nitrogen and the filter residue was lyophilized. No attempt was made to further quantify the yield. Phospholipids make up approximately 50% of the total lipids, the latter varying between 25–30% of the membranes (13).

The residue remaining after lipid extraction was treated in several different ways. A sample was treated with 3 M KCl in acetate buffer, pH 5.0, for 48 h at room temperature, followed by high speed centrifugation at 100,000 g. The supernatant fluid was subjected to molecular-sieve chromatography on a Sephadex G100 column equilibrated in acetate buffer, pH 5.0. A second sample was mixed with 1% Triton-X in 0.01 M Tris buffer, pH 8.3, and continuously sonicated overnight at 45°C. This was followed by high speed centrifugation (see above) and ion-exchange chromatography using DEAE-cellulose and a linear gradient from 0 to 3 M NaCl. A third sample was placed in 5% Emulphogen (GAF Corp., New York) in 0.01 M Tris buffer, pH 8.3, and sonicated overnight and centrifuged as outlined. The supernate was placed on an isoelectric-focusing column and run for 48 h, using a linear pH gradient from 5 to 8 in 50% sucrose. These pooled lyophilized fractions were stored at 4°C until further use. The protein patterns of these fractions were analyzed in sodium dodecyl sulfate polyacrylamide slab electrophoresis (9).

Enzyme Studies. Whole membranes from strain S43/192/2, as well as the residue after lipid extraction, were digested with 1 mg/ml trypsin (Worthington Biochemical Corp., Freehold, N. J.), 180 U/mg in 0.046 M Tris, pH 8.1 with 0.0115 M CaCl₂ and/or with 10 mg/ml phospholipase LP 5550 from wheat germ (1.5 U/mg) (kindly supplied by Dr. M. W. Chase, The Rockefeller University, New York) in 0.15 M Na acetate, pH 5.0, for various periods of time at 37°C. The trypsin reaction was stopped by adding a twofold excess of Soybean trypsin inhibitor (ICN Nutritional Biochemical Div., International Chemical & Nuclear Corp., Cleveland, Ohio). This was followed by high speed centrifugation at 150,000 g for 3 h. Both pellet and supernate were tested as indicated below.

C Assays. C activation was determined subsequent to incubation with the various test fractions for 1 h at 37°C, measured by standard hemolytic titration (CH₅₀) and/or direct immuno-electrophoretic determination of conversion of factor B to activated factor B (B⁺) and C3 to C3i utilizing specific antisera (14). In order to differentiate between the two different pathways of C activation, 1/10 volume of the following buffers were added to C before incubation:

- 0.1 M ETHYLENEGLYCOL-BIS-(BETA-AMINO-ETHYL ETHER) N,N'TETRA-ACETIC ACID (EGTA) (Sigma Chemical Co.) with 0.05 M MgCl₂ in normal saline, pH 7.4, known to selectively block the classical pathway by chelation of Ca²⁺ (15); the concentration used was shown to completely prevent hemolysis by the classical pathway.
- 0.1 M ETHYLENEDIAMINETETRA-ACETIC ACID (EDTA) (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.) in normal saline, pH 7.4, which by chelating both Ca²⁺ and Mg²⁺ blocked both pathways, the classical and the alternate.

NORMAL SALINE. The residual C was calculated as the percentage of the control incubated simultaneously with the corresponding buffer alone. The amount of C consumed by the test substances was then achieved by subtracting the results obtained from 100%.

A semiquantitative microassay of C (16) was modified as follows: Doubling dilutions of test materials were incubated with equal volumes of diluted C in microculture plates (catalogue no. 13-7985-03; Ace Scientific Supply Co., Inc., Linden, N. J.) at 37°C for 30 min. Sheep red cells sensitized with rabbit antisheep red cell antibody (EA) (17) were added to the microplates and incubated for an additional 60 min. The degree of hemolysis of EA obtained was a measure of the residual C.

C Sources. Whole human serum obtained from normal, healthy individuals was stored in aliquots at −70°C until use. For the study of the alternate pathway, individual serum proteins were removed as follows:

- PROPERDIN. Fresh human serum was incubated with 2 mg/ml zymosan (catalogue no. Z-4250 from S. cerevisiae yeast; Sigma Chemical Co.) at 17°C for 1 h, centrifuged in the cold at 2,000 g for 30 min and the supernate stored at −70°C (18).
- FACTOR D. 2 ml of fresh human serum were subjected to molecular-sieve chromatography on a Sephadex G75 column (1.5 × 90 cm, equilibrated with 0.15 M NaCl and 0.002 M EDTA, pH 7.3). The bulk of the serum proteins was eluted in two peaks, pooled, and concentrated to the original volume with a PM10 Amicon filter (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) at 2–
4°C. Factor D, because of its low molecular weight, eluted later from the column (19) and was discarded.

Factor B. Whole human serum was incubated at 50°C for 30 min (20).

Component C3. Whole human serum was incubated at 37°C for 60 min with 0.015 M hydrazine hydrate (21).

Component C2. Whole human serum from a patient homozygous for C2 deficiency (22) was kindly provided by Dr. S. M. Fu and Dr. H. G. Kunkel, both from The Rockefeller University, New York.

Immunglobulins. Whole human serum from a patient with severe hypo-gammaglobulinemia was kindly provided by Dr. T. Hoffman, The Rockefeller University, New York, and Dr. H. G. Kunkel. This serum was known to have normal C levels and the alternate pathway was shown to be intact. IgA and IgM were not detectable while IgG was 140 mg/ml (T. Hoffman, personal communication).

Endotoxin Assays. The Limulus gelating test (23), as well as the chicken embryo test (24), were carried out according to standard procedures.

Biochemical assay.

Glucose. Glucose was determined enzymatically utilizing Glucostat (Worthington Biochemical Corp.) in the semimicro method.

Rhamnose. Rhamnose was determined as methyl pentose according to the methods of Dische and Shettes (25).

Results

Differentiation between Classical and Alternate Pathway of C Consumption. Considering the possibility that human sera might contain antibodies to streptococcal antigens, C consumption experiments were first carried out in the presence and absence of chelating agents. Fig. 1 shows that EGTA (hatched bars) completely fails to prevent C consumption in the presence of 0.5 mg/ml of streptococcal membranes. This indicates that the C activation was all accomplished via the alternate pathway. At other concentrations of streptococcal membranes, however, there is a consistent difference between C consumption in the presence of EGTA (hatched bars) and the controls without chelating agents (white bars). This small difference suggests that a small amount of C is consumed via the classical pathway possibly due to the presence of specific anti-streptococcal antibody in the human sera used. These observations were confirmed on the same samples by a different assay system in which activation of C was measured indirectly by immunoelectrophoretic conversion of factor B to B (Fig. 2) and C3 to C3i (not shown). Both these factors were converted when whole serum was incubated with streptococcal membranes. This effect was only slightly diminished by the presence of EGTA. However, this difference (compare also Fig. 1), although small, perhaps indicates antibody-mediated activation of C via the classical pathway. EDTA completely inhibited consumption of C (data not shown).

Localization of Bacterial Factors Responsible for the Effect. In the same pilot study an attempt was made to determine on a weight basis which of the two major streptococcal cellular structures (membranes or cell walls) contained the greatest amount of the activating substances. Since previous work (12) has demonstrated that cell walls are usually contaminated by membranes, a cell wall preparation was selected which contained the least amount of membrane contamination (Table I). Similarly the purest "wall-free" membrane preparation was selected for these experiments. As can be seen in Fig. 1, membranes at two different concentrations contained more of the material capable of consuming C
FIG. 1. The difference in the percentage of C consumption by streptococcal plasma membranes and cell walls, respectively. The white bars refer to the percentage of C consumed in the absence of chelators. The hatched bars refer to consumption in the presence of chelating agent EGTA, which permits activation of the alternate pathway only.

FIG. 2. The conversion of B to B. Native B shows migration to the anode (upper pair), while active B migrates to the cathode (lower pair). Activation was achieved with 0.4 mg/ml Group A streptococcal (Strep) membranes from 543/192/2. The strength of the precipitates can be estimated, and thus quantitative information is available. The lower wells of each pair show the conversion after incubation with Mg/EGTA. The 10% remaining native B (last well) correlates to the amount of C activated via classical pathway found by hemolytic titrations (Fig. 1).

via both (white bars), as well as via the alternate pathway only (hatched bars) than did wall preparations.

Determination of Essential Serum Factors. Since the above experiments indicated that the primary activation of C occurred via the alternate pathway, the next step taken was to attempt to determine at what point in the alternate pathway did this activation occur. For the sake of clarity, the major components of this pathway have been schematically outlined in Fig. 3. The different
**Table I**

*The "Purest" Bacterial Preparations Used*

| Microorganism | Strain | Group | Type | Rhamnose | Contamination with wall* | Dry weight | Glucose | Contamination with membrane† |
|---------------|--------|-------|------|----------|--------------------------|------------|--------|-------------------------------|
|               | S43/192/2§ | A     | 6    | 0.5      | 1.9                      | 300.011    | 0.3    | 14.3                          |
|               | T 12/126/6 | A     | 12   | -        | -                        | -          | -      | -                            |

* On the basis of 27% rhamnose in walls.
† On the basis of 1.9% glucose in membranes.
§ This strain was treated with lysin to achieve the separation of wall and membranes.
∥ Kindly provided by Geraldine Siviglia, The Rockefeller University, New York.

Fig. 3. A schematic representation of the various components of the alternate C pathway (for synonyms see Table II).

nomenclatures for each component of the system are given in Table II. For this purpose, the immuno-electrophoretic conversion system was used (14) with sera selectively deprived of particular C proteins. The results are given in Table III. As expected, whole human serum (WHS) exhibited almost total electrophoretic conversion of factor B to factor B and C3 to C3i. C2-deficient serum (C2) gave essentially identical results to those obtained with WHS. Surprisingly, a similar degree of conversion was obtained in serum lacking properdin (P) suggesting that the activation had occurred in the absence of P. No conversion was obtained in the serum lacking factor D, thus indicating that this factor was essential for the reaction. Removal of C3 also led to some reduction in degree of conversion of factor B. However, this may be due simply to the interruption of the "amplification loop" via C3b (26). Finally, serum lacking factor B activity failed to activate C3, an observation which thereby excluded a direct effect of the agents on C3 (5).
PROPERDIN-INDEPENDENT INITIATION OF ALTERNATE PATHWAY

TABLE II
Terminology of the Alternate Pathway

| Symbol | Synonyms | mol wt |
|--------|----------|-------|
| IF     | Initiating factor or nephritogenic factor (C3 NeF) | 150,000 |
| P      | Activated P | 184,000 |
| D      | Native factor D or C3 proactivator convertase (C3 PA convertase, C3 PAase GBGase) | 25,000 |
| D      | Activated factor D | 235,000 |
| B      | Native factor B or C3 proactivator (C3 PA) or glycin-rich beta glycoprotein (GBG) | 80,000 |
| B      | Active factor B or C3 activator (C3 A) or glycin-rich gamma glycoprotein (GGG) | 60,000 |
| —      | Glycin-rich alpha glycoprotein (GAG) | 20,000 |
| C3     | Third component or former factor A | 180,000 |
| CoVF   | Cobra venom factor | 140,000 |

TABLE III
Alternate Pathway Activation by Streptococcal Membrane or Inulin in Sera Deprived of Individual Factors

| Missing serum factor | C3* | B‡ |
|----------------------|-----|----|
| —                   | 90§ | 80 |
|                     | 45|| | 20 |
| P                   | 85  | 80 |
|                     | 10  | 5  |
| C3                  | 25  | 0  |
| D                   | 0   | 0  |
| B                   | 5   | 10 |
|                     | 0   | 0  |
|                     | 0   | 0  |

* % conversion of C3 to C3i after subtraction of parallel control, utilizing saline alone.
‡ % conversion of B to B after subtraction of parallel control, utilizing saline alone.
§ % conversion after incubation with 0.2 mg/ml membranes from streptococcus A/6, strain S43/192/2 (top line in each pair of entries).
|| % conversion after incubation with 10 mg/ml inulin (bottom line in each pair of entries).

To elucidate the difference in the point of activation between streptococcal membranes and the plant polysaccharide inulin (a known activator of the alternate pathway), both compounds were simultaneously tested. As shown in Table III, in the absence of P, 10 mg/ml of inulin led to only minimal conversion of factor B, a degree comparable to that of the saline control. In contrast, 0.2 mg/ml of streptococcal membranes produced approximately 80% conversion of factor B.

In an attempt to determine what role, if any, antibody to streptococcal antigens played in the conversion of factor B, a hypogammaglobulinemic serum
Table IV

Alternate Pathway Activation by 0.1 mg/ml Streptococcal Membranes in Hypogammaglobulinemic Serum* Deprived of P

| Serum reconstituted with: | C3‡ | B‡ |
|--------------------------|-----|----|
| —                        | 50  | 35 |
| Antistreptococcal antibody§ | 55  | 45 |

* All incubations carried out in the presence of Mg/EGTA, thus blocking the classical pathway of C activation.
‡ See Table III.
§ Addition of 1/10 volume heat-inactivated serum of a rheumatic patient with a high titer of heart-reactive antibody.

was used instead of the normal serum. Table IV demonstrates that this serum, even though deficient in immunoglobulins, showed approximately 35% conversion of factor B to B. Addition of a serum known to contain antistreptococcal antibodies increased the conversion by only 10%. These results suggested that immunoglobulins play only a minor role in the activation mechanism under investigation.

Characterization of C-Activating Principles. While the experiments described in Fig. 1 indicated that streptococcal membranes contained more of the C-activating factor than did the cell walls, it was not clear which particular portion of the membrane contained the active principle. Accordingly, the membrane preparation with the least amount of cell wall contamination (see Table I) was further divided into a lipid fraction and a protein residue, respectively. An undissociated membrane preparation obtained from the same batch of membranes served as a control. Fig. 4 demonstrates that the majority of the anticomplementary activity of streptococcal membranes resided in the protein residue portions of the membrane after lipid extraction. While the composite lipid portion of the membrane was also anticomplementary, the use of selective chelating agents indicated that C activation by the lipid components of the streptococcal membrane was exclusively confined to the activation of C by the classical pathway.

Further experiments revealed a number of biological characteristics of this C-activating factor. The material was heat sensitive and lost 75% of its activity after 2 h incubation at 70°C. It was resistant to the action of both trypsin and phospholipase enzymes. The material contained little or no endotoxin as evidenced by the lack of activity detected in the chicken embryo test, and less than 0.04% contamination was noted in the Limulus test. Column chromatography of the solubilized fractions of the protein residue (see Lipid Extraction and Fractionation in the Materials and Methods) revealed that protein fractions less than 60,000 daltons resulted in loss of activity. In addition, partially purified immunologically active M protein (kindly provided by Dr. V. A. Fischetti, The Rockefeller University, New York) preparation (~40,000 daltons) and streptococcal antigens (kindly provided by Dr. I. van der Rijn, The Rockefeller University, New York) cross-reactive with human sarcolemma (~30,000 daltons) failed to activate the C system. As a final control, both the culture medium used to grow the streptococci as well as the phage lysoin used for the isolation of the
streplococcal membranes were tested for their anticomplementary activity and were found to be nonreactive.

**C Consumption by Other Hemolytic Streptococci.** Since the majority of the experiments had been carried out with purified membranes obtained from a single Group A streptococcal strain, it was of interest to determine whether other hemolytic streptococci contained the active principle. For those purposes, mechanically disrupted streptococci were employed in order to achieve more uniform preparations of streptococcal cell walls and membranes. Also included in this series of experiments were strains from Groups C, D, and G streptococci. Tests were employed to measure consumption of hemolytic C and as in previous experiments each preparation was tested in the presence or absence of selective chelating agents in order to determine the relative degree of C activation by each pathway. As shown in Figs. 5 a and 5 b, the activating principle was present in all strains tested. Both cell walls and membranes contained the activating factor. By utilizing the chelating agents, it was possible to show that cell walls activated the classical pathway to a greater degree than did streptococcal membranes. This is perhaps not surprising in view of the many antigens present in streptococcal cell walls capable of inducing the formation of streptococcal antibodies in human sera (27). It must be emphasized however that the method of preparation of these structures makes it difficult to decide which fraction of the streptococcal cell contained the greatest amount of C activation. A perusal of Table V indicates that all cellular components tested contained significant amounts of mutual contamination of cell walls with membranes and vice versa.

With respect to membrane preparations, the method of isolation did not appear to influence the results. Fig. 6 demonstrates that the degree of C consumption was the same irrespective of whether membranes were prepared by lysis extraction or were mechanically disrupted.

**Consumption by Other Gram-Positive Organisms.** Experiments were designed to determine whether gram-positive organisms other than hemolytic streptococci were capable of activating the system. Several gram-positive strains were selected. In each case (see Fig. 7 a and 7 b) both cellular structures (membranes and walls) were capable of activating the C pathways. In the
FIG. 5. (a) Anticomplementary activity of membranes from various streptococcal (Strep.) strains. (b) Anticomplementary activity of cell wall preparations from various streptococcal strains.

TABLE V

Biochemical Composition of Mechanically Disrupted Streptococcal Strains

| Streptococcal strains | Group | Membranes | Cell walls |
|-----------------------|-------|-----------|------------|
|                       |       | Rhamnose  | Contamination* | Glucose | Contamination* |
|                       |       | %         | %            | %       | %            |
| S43 Glossy (Type 6)   | A     | 4.1       | 15.2         | .41     | 20.4         |
| S23 Burbank (Type 14) | A     | 5.3       | 19.6         | .52     | 26.0         |
| C74                   | C     | 4.1       | 15.2         | .58     | 29.1         |
| D166B                 | G     | 5.9       | 21.9         | .37     | 18.6         |

* See Table I.

FIG. 6. The anticomplementary activity of mechanically prepared membranes as compared to lysin-extracted material. No significant difference in C consumption was seen between the two preparations. Strep., streptococcal.
Discussion

Our finding that streptococcal antigens as well as constituents from certain other gram-positive microorganisms may activate the alternate C pathway at the particular level of factor D is rather unexpected. Due to the availability of the two deficient sera, one lacking C2 and the other hypogammaglobulinemic, it has been possible to confirm the initial indications that certain gram-positive microorganisms contain factors which can activate the alternate pathway by a mechanism not requiring immunoglobulins. Our studies further indicate that P is not required for this activation. Although we know that certain cell wall constituents from gram-negative organisms such as endotoxin (3) also activate the alternate pathway, this mechanism is P dependent and thus differs from our findings. On the other hand, certain bacterial enzymes act directly on C3 (5). That this is not the case in our experiments was clearly demonstrated by the observation that no conversion was seen with a serum which was deficient in factor D.

Our attempts to localize the active principles led to the conclusion that the necessary factors are certainly present in membrane preparations which were shown to be almost free of cell walls. Nevertheless, these factors also appear to be present in cell wall fractions. It must be emphasized however that our presence of EGTA the differences in C consumption between strains as well as the differences between the various cellular components were minimal. However, it must be emphasized in these experiments that the methods used for the preparation of pure cell walls and membranes are crude and only reflect a general enrichment of the particular cellular components.
methods for isolating cell walls are crude and all cell wall preparations contained a significant amount of membrane material. In addition, the conformation of these membranes may be important. For example, contaminating membranes are attached in a more rigid flat fashion in cell wall preparations, while they tend to fold into spheres when isolated as ruptured protoplasts (28). This conformational shape of the membranes could hinder the exposure of their whole surface and thus explain different quantitative results. The sonication technique used before incubation of the preparations with C is a rather mild procedure and may not dissociate aggregated protoplast membranes.

Studies of the localization of the activity within the membrane shows that the active principle is in the residual material after lipid extraction. Further studies are planned to identify and isolate the responsible moiety within the residue fraction. The active principle appears to be a high molecular weight fraction since, in preliminary experiments, all fractions having a mol wt below 40,000 showed no consumption of C.

How do these findings fit with the data reported by Hirata and Terasaki (29) and Rapaport et al. (30) who did not find substantial anticomplementary activity in their streptococcal extracts? With respect to the former authors, they stated that only M-protein preparations prepared from type 1 streptococci were active while other M-protein extractions were noninhibitory. Since we did not use type 1 membranes, it is conceivable that the HL-A cross-reactivity is specifically related to a given M protein from a particular strain. However, the possibility exists that their preparations did in fact exhibit an anticomplementary effect but the conditions of their experiment precluded the detection of the anticomplementary effect. For example, no inhibition of cytotoxicity was observed when the HLA antiserum was concentrated enough to kill 80% of the cells. This finding is similar to results obtained by us. Yet, if the serum is diluted to a 1:16 concentration, the C effect is clearly seen (1). As we have pointed out, both the dilution of the antiserum and the concentration of the antigens, and the mixture of the various components of the cytotoxicity assay system are crucial for detection of the activation of the alternate pathway of C.

The observation by Rapaport et al. that low molecular weight trypsin digest of membranes (30) is not anticomplementary has been confirmed in our hands. However, their preparation is a soluble substance and therefore does not strictly reflect the experimental conditions in cytotoxicity studies where one deals with solid-phase conditions due to the presence of cell surface membranes. Their negative control (as well as ours) after preincubation of this soluble material with C alone, i.e. omitting the solid phase provided by the later addition of target cells, remains therefore ambiguous. The anticomplementary activity of this soluble digest in the presence of a membrane surface, such as the lymphocyte membrane, cannot be excluded and remains a distinct possibility.

Finally, a word has to be said about the clinical implications of activation of the alternate pathway by streptococcal antigens. For example, in acute poststreptococcal glomerulonephritis C1, C4, and C2 components of C are depressed early in the disease, yet later, there is a selective depression of C3 while the early components return to normal. One may speculate that the reason some individuals contract the disease or progress to a more chronic form of glomerulo-
nephritis (31) may be related in part to an aberration within the alternate pathway. Even stronger evidence for this possibility may be found in some forms of chronic glomerulonephritis not directly related to acute streptococcal infections (32). Here C3 components are characteristically lowered while C1, C4, and C2, the early components, remain normal. Since many organisms were found to be capable of activating the alternate pathway, it is conceivable that these forms of glomerulonephritis are also due to an anomaly in the alternate pathway.

Quite apart from these practical viewpoints, our data may give some insights into the phylogeny of C. From the work on the starfish, we have learned that the factors of the alternate pathway may very well be the ancestors of the C system. This species lacks immunoglobulins as well as the corresponding early C components such as C1, C4, and C2. However, an interesting protein could be isolated from the hemolymph of such species which resembles in its enzymatic activity factor B; both lower vertebrates as well as invertebrates possess a lytic C system which can be activated independent of antibody, namely via a pathway initiated by cobra venom (33). This would suggest that parts of the C system are more primitive than the immune system itself. It is clear that these archaic C functions may represent the alternate pathway. This would mean that a primitive defense system existed already before the elaborate equipment for specific identification of targets was present. Accordingly, activators of the alternate pathway, rather than being unique, should be widely spread compounds which are shared by a variety of different microbes (as for example lipid A in various endotoxins of numerous gram-negative microbes). Our findings that not only several streptococcal strains but different species of other gram-positive bacteria also contain activating factor(s) are in agreement with the results from other laboratories (34, 35) and support the theory that the alternate pathway might have been designed as a broad, nonspecific defense system. The immune system came later to improve specific identification and efficient elimination of appropriate targets. Thus, antibody actually completes and enhances the effects of C rather than vice versa.

However, the questions remain: why did the factors of the alternate pathway persist up to the present day? Is it possible that the alternate pathway has to fill occasional loopholes of specific immunity? For example, certain groups of streptococci possess structures that closely resemble normal tissue antigens and thus present forbidden targets for specific immune reactions. In this situation, the body would have to rely on other defense mechanisms. In this context, the alternate pathway would gain an important meaning far beyond that of a simple evolutionary relic. Correspondingly, a specific defect in the alternate pathway system might represent a substantial handicap. It could, in the first place, lead to a protracted beleaguering of the host by corresponding pathogens. Secondly, after repeated challenges, the host's suppressive mechanisms which protect it from self-destruction might become exhausted and fail to prevent the final production of an autoantibody. These possibilities may have to be taken into consideration in a future analysis of streptococcal-related diseases.

Summary

Complement consumption by isolated membranes and walls from Group A streptococci and various other gram-positive microbes has been tested. These
microbial structures were found to activate the alternate complement pathway. However, unlike endotoxin, inulin, or other plant polysaccharides, activation of complement by our material was found to bypass properdin. The activating factor(s) also differs from cobra venom in its/their requirement for factor D. Preliminary experiments suggest this factor isolated from membranes to be a protein and to have a mol wt greater than 40–60,000 daltons. Our studies have led us to speculate that the phylogenetic role of the alternate complement pathway may be the primordial nonspecific defense system which has retained certain fundamental aspects up to the present time.

Linda Metakis cheerfully assisted with continuous technical advice.

Received for publication 3 February 1976.

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