A REVIEW OF PAST ATTEMPTS AND PRESENT CONCEPTS OF PRODUCING STREPTOCOCCAL IMMUNITY IN HUMANS[1]

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Introduction

THE prevalence of streptococcal infection and the morbidity and mortality attending the suppurative and non-suppurative complications which follow such infection are imperative reasons for investigation of methods which would prevent the spread of this ubiquitous organism. The antibiotic era has provided potent weapons with which to effect such control, and chemoprophylaxis has been established as recommended treatment during and following an attack of acute rheumatic fever (1-5). Most modern work has been directed at the prevention of recurrences of rheumatic fever or the control of streptococcal infection in closed groups such as military instillations.

The incidence of streptococcal infection has been estimated as seven million per year in the United States (4). Study of epidemics in the military has established the attack rate of rheumatic fever as 3% following Group A streptococcus infection (6), and controlled studies in civilian populations indicate similar results (7). If all these infections were unrecognized and untreated, a significant number of an unprotected population would thus acquire first attacks of rheumatic fever.

Much effort has been directed toward the prompt recognition of streptococcal infection (5), and studies of upper respiratory infections now under way (7) can provide a sound basis for instituting antibiotic therapy. It is generally assumed that continuous prophylaxis will prevent repeated attacks of rheumatic fever in the highly susceptible rheumatic population, which has an attack rate of 50% following Group A streptococcal infection (8), and prompt treatment of Beta-hemolytic streptococcal pharyngitis will reduce the incidence of first attacks in the general population. However, streptococcal infections that occur as a breakthrough of prophylaxis in the closely watched rheumatic group may be followed by a 15 to 20% recurrence rate (9) and asymptomatic, subclinical infections accounting for 50 to 65% of observed streptococcal infections (7) place limitations on the effectiveness of chemoprophylaxis

Assuming that rheumatic fever represents an allergic phenomenon following Group A streptococcal infection, it is obvious that such prevention has been centered upon avoidance of the suspected antigen in rheumatic populations and prompt elimination of it, when recognized, in non-rheumatic individuals. Attempts at primary immunization against this antigen have been remarkably few. The elucidation of a rational approach to a biological method of preventing streptococcal infection has only been accomplished recently; in fact, our knowledge concerning the streptococcus and rheumatic fever is only some fifty years old (10).

A review of all past attempts at immunization against streptococcal disease requires a simultaneous survey of the advances in basic science, clinical observation and epidemiologic analysis which stimulated them. These "stimuli" comprise a vast amount of literature which

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we have only attempted to highlight. They seemed to fall spontaneously into three categories (investigations centered around scarlet fever, rheumatic fever and the antigenic complex of streptococci) which we have followed in presenting the information.

Scarlet Fever

The streptococcus was probably first recognized by Pasteur in 1879 in his discussion of puerperal sepsis (11), and described as a chain-forming coccus and named *Streptococcus pyogenes* in 1884 (12). Only one year later these bacteria were reported in the blood of patients dying of scarlet fever (13). Investigation of the relationship between the streptococcus and this disease which followed provided the stimulus for numerous "anti-scarlatinal vaccinations." These represent the first attempts at anti-streptococcal immunization, though investigators at the time were using these methods as one tool for determining the etiology of scarlet fever.

In 1899 Stickler inoculated 10 patients with material obtained from the throat and mouth of a patient who had a mild attack of scarlet fever (14). He was searching for a virus in the inoculated mucus but, following the development of systemic reactions and "nephritis," he abandonded his attempts. A known organism was first used by Gabritschewsky when he injected killed scarlatinal streptococci, together with the broth in which they were grown, into humans (15). Local and systemic reactions followed, and some cases developed scarlet fever. This latter phenomenon led Gabritschewsky to believe that the organism in his vaccine was really the specific agent of scarlatina, an observation preceding those of other investigators by 20 years. He reported favorable results, however, and the use of killed streptococci in their growth media as a vaccine was attempted on a large scale in the following years as a clinical method of preventing scarlet fever. Numerous European workers reported a marked decrease in the incidence of scarlet fever in vaccinated children compared to non-vaccinated controls (16), but correct interpretation of these results awaited further knowledge of the pathogenesis of the rash which labelled scarlet fever to these clinicians.

Prior to the discovery of erythrogenic toxin (see below) the etiology of scarlet fever was clouded in confusion. The causative agent had been rapidly established as being present in the throat secretions of patients with the disease and the first step toward classifying streptococci had been proposed by Schöttmuller, with his classic demonstration of the varying ability of streptococci to hemolyze red blood cells on a blood agar plate (17). Thus attention had been focused on the hemolytic streptococcus, but two major obstacles prevented unanimous acceptance of this organism as the definitive etiological agent. One difficulty was an inability to prove that the streptococci associated with scarlet fever differed specifically from hemolytic streptococci causing other septic processes. The other concerned the difference in the duration of immunity to scarlet fever compared to other streptococcal infections.

Classification of streptococci in the early 1920's depended on hemolytic reactions and biochemical and cultural characteristics (13). Brown further clarified the hemolytic reactions of the organism when he recommended alpha, beta and gamma terminology in 1919 (18), but the study of biochemical and cultural characteristics could not be correlated with pathogenicity (19). Such studies only served to confuse the issue and could not provide criteria for separating the organisms causing scarlet fever from other hemolytic streptococci.

Dochez and his coworkers began an immunological classification in 1919 (20). With agglutination and animal protection experiments, they studied a great number of strains and succeeded in identifying many different antigenic types among hemolytic streptococci isolated from human infections. These methods indicated some degree of serologic specificity but did not provide conclusive answers.

In early attempts to establish type specificity of the scarlatinal streptococcus, Moser and Pirquet had prepared serum from horses injected with streptococcus scarlatina as an antigen (21). Studies with this serum demonstrated its ability to agglutinate some strains of scarlatinal

streptococci in higher titres than hemolytic streptococci from other sources, prevent the development of local and systemic reactions if administered to individuals prior to injection with Gabritschewsky's vaccine (see above), and have some therapeutic value in decreasing the symptoms and alleviating the course of acute scarlet fever (13).

Analogies were made between Moser's antiserum and diptheria antitoxin (13), but the outstanding objection to the acceptance of streptococci as the cause of scarlet fever remained the impossibility of differentiating that organism from hemolytic streptococci associated with other septic conditions. Other etiologic agents were searched for, Moser's serum dropped into disuse and streptococcal vaccine was no longer used in the prophylaxis of scarlet fever.

The second major attempt at immunization against streptococal disease involved extra-cellular products of the organism. The stimulus here began in 1923 when Dick experimentally produced scarlet fever in humans with the hemolytic streptococcus (22). It was during these, and animal, experiments by Dochez and Sherman (23) that the idea took shape of a toxic substance elaborated by streptococci as being the causative factor in producing the clinical picture of scarlet fever. Schultz and Charlton (24) and Mair (25) had established that serum from persons who have had scarlet fever could blanch the rash of the disease. Dochez was also able to produce this "extinction phenomenon" by using horse antiserum prepared against S. scarlitinae (26). Antisera prepared from other hemolytic streptococci did not induce blanching of the rash.

The presence of a toxic substance in the blood broth cultures of *S. scarlatinae* was demonstrated by Dick and Dick in 1924 (27). They described a skin test analogous to the Schick test, were able to neutralize the toxic filtrate *in vitro* and *in vivo* by the use of convalescent serum, and showed that susceptible individuals could be immunized by repeated doses of toxin until skin reactivity became negative.

The elucidation of an etiologic agent, a toxin which causes clinical symptoms and a means of testing immunity gave workers the tools with which again to attempt active immunization, and in the 10 years following 1924, countless studies were performed in an attempt to produce immunity against scarlet fever (16). Most of these involved graded toxin injections into susceptible individuals followed by reversal of the Dick test, and thus explained the favorable results sporadically achieved by earlier work with killed streptococci and growth media (see above), erythrogenic toxin being the antigen responsible for producing "anti-scarlatinal immunity."

It was during those years that the relationship between rheumatic fever and the streptococcus was first well indicated, the studies of scarlet fever bringing it into sharper focus.

Rheumatic Fever

Clinical observations had related sore throat to rheumatic fever as far back as the late 19th century (28), but now epidemiologic studies drew attention to the similar geographic distribution of scarlet fever and rheumatic fever (29-31) and clinical studies (32) and immunologic surveys of Dick-positive individuals in rheumatic populations (33-36) indicated a low incidence of scarlet fever occurring in rheumatic populations.

In the early 1930's Coburn and Pauli correlated epidemiologic, immunologic and bacteriologic studies and pointed to the hemolytic streptococcus as initiating the infectious process preceding most cases of rheumatic fever (37-40).

Workers were aware that the high incidence of a Dick negative reaction among rheumatic children who have not had scarlet fever indicates that these children probably have had frequent infections with erythrogenic organisms producing antitoxin which would prevent the rash of scarlet fever (40).

Similarity was noted between the types of hemolytic streptococci associated with the two diseases. Organisms were recovered from scarlet fever patients which were serologically identical with strains known to initiate recurrences of rheumatic fever (41,42); both were found to elaborate soluble toxin (41) and streptolysin (43); and both stimulated antibody production in high titre by the ASO test (44).

These data suggested that erythrogenic toxin played a role in the pathogenesis of rheumatic fever, and Coburn and Pauli investigated the possibility of active and passive immunization against the toxin as a means of protecting the rheumatic subject (40), the third major attempt at producing immunity to the streptococcus or its products. Because of the effectiveness of antitoxin in reducing the incidence of scarlet fever, many investigators considered active immunization with erythrogenic toxin beneficial in lowering the incidence of throat infections and their complications such as rheumatic fever (16), as well as a good method of prophylaxis in rheumatic children (45). This view was prevalent until Coburn and Pauli's work showed that active and passive immunization with Scarlatinal NY5 toxin and antitoxin produced reversal of skin sensitivity but did not decrease the incidence of streptococcal infection and the development of rheumatic disease in the studied group as compared to suitable controls (40). A decade later Jackson verified this work by again showing that immunization with Dick toxin protects against the rash of scarlet fever but not against invasion by streptococci (46).

Other investigators had attempted to produce rheumatic lesions in laboratory animals by repeated exposure to streptococci (47). The concept of streptococcal hypersensitivity as being responsible for the acute rheumatic state gave impetus to intravenous vaccination with streptococci as a means of desensitization and prevention of relapses (48-50). Swift used heat-killed hemolytic streptococci, nucleoprotein and a mixture of pulverized organisms as three different intravenous vaccines (49). Working without knowledge of type-specificity, he realized that the failure of many patients to react favorably to immunization with a single strain suggested that the immunizing strain is too far removed antigenically from the sensitizing strain which presumably caused the disease state, and resolved the problem into the need for "a more accurate determination of the strain or strains responsible for maintenance of the hypersensitive state" (49).

This need was met by the concept of streptococcal type-specificity which was then emerging from basic work in the bacteriology and immunology of the organism.

The Antigenic Complex and Type-Specificity

The serological classification of streptococci, dependent on intracellular components of the bacterial cell was the major advance on which the concept of type-specificity has been built, and marks the beginning of our current approach to the problem of producing active streptococcal immunity.

Specific serologic types of hemolytic streptococci, pathogenic for man, were first demonstrated from study of epidemic strains in army camps in 1918 by agglutination reactions (20). Group-specific polysaccharide, however, was first fully studied after its demonstration in the 1920's (51), and most groups of hemolytic streptococci were characterized according to their source in nature and pathogenecity in man and animals. From this work the Group A streptococcus rapidly emerged as the significant human pathogen (52).

Griffeth, using a slide agglutination technique, first established a classification of Group A streptococci according to types (53). His method revealed the dominant antigen which determined type-specificity but analysis of the whole antigenic structure of the organism was begun by Rebecca Lancefield in 1928 (54). With the precipitin and mouse protection tests she demonstrated four distinct substances in extracts of the hemolytic streptococcus and designated them as "C", "T", "P" and "M" antigens, showing that the intact bacterial cell induced antibody formation against all of them.

"C" substance was found to be chemically similar to the specific carbohydrate of pneumococci and responsible for the serological grouping of streptococci. It was not type specific and could not stimulate antibody production when separated from the cell (54c).

"T" substance represented the antigen primarily responsible for antibodies involved in the agglutination reaction but

unable to confer protective action when present in sera (54b).

"P" substance, or nucleoprotein, extracted from the streptococci easily immunized rabbits, but the antisera thus produced was precipitated equally well by nucleoproteins from all strains, as well as from non-hemolytic streptococci and pneumococci. This, however, was the first fraction of hemolytic streptococcus extracts which was truly antigenic after separation from the bacterial cell (54b).

The "M" extract was first characterized as an alcohol-soluble protein, resistant to heating at low pH and highly susceptible to the action of proteolytic enzymes. Early work in its immunological properties classified this protein in the acid extracts as an haptene since it did not give rise to antibodies when injected intravenously into rabbits but did react in the precipitin test with antibody prepared against whole cells (54b). Hirst and Lancefield found that under better conditions of extraction the "M" substance was antigenic and, in fact, the originally prepared hot acid extract was antigenic if large enough doses were employed (55). Nevertheless the substance isolated from the cells was a much poorer antigen than the intact cells from which it was derived (56-58).

M protein was shown to be involved with the virulence of a given strain, initially by relating its presence or absence to colony form. Immunization with virulent or avirulent matt forms induced the formation of anti-M precipitins and protective antibodies, while immunization with the avirulent glossy forms failed to induce "either" of these (59,60).

Analogy was made between the M protein of streptococci and the type specific capsular polysaccharide of pneumococci by virtue of the type-specificity common to both, the type-specific protection endowed on animals given anti-M and agglutination and precipitin reactions (52).

The action of trypsin on M protein elucidated further knowledge of the antigen. Originally streptococci were treated with trypsin in order to transform spontaneously agglutinating cultures into stable diffuse suspensions suitable for slide agglutination (61). It was observed that this treatment allowed the organisms to agglutinate with heterologous agglutinins (62) and analogy was made to the change from matt to glossy forms by the loss of type-specific M protein (59,60). M substance could not be isolated from matt colonies grown in the presence of, or subjected to, trypsin. Since the bacteria were not killed by digestion, and subsequent generations of the trypsin-treated streptococci were mouse virulent, it was concluded that M substance in living bacteria is readily accessible to the action of proteolytic enzymes without injury occurring to other vital functions of the living cell, and, therefore, is probably located near the outer surface of the streptococcus (52).

In 1942, Zittle and coworkers were able to extract M protein from the residue of sonically treated organisms (63), suggesting that M protein is part of a surface which is disrupted but not brought into solution by the sonic treatment. They chemically separated the protein from group specific polysaccharide and, in later work (64), described the weight and shape of the molecule.

It had been shown that the yield of M protein after sonic treatment contained nucleic acid (63), and further purification by Lancefield and Perlmann was carried out in 1952 (65). They prepared M protein free of ribonucleic acid by using ribonuclease and were able to produce antibodies in rabbits by injecting a total dose of 45 mg intravenously, though cross reactions in these antisera indicated remaining impurities in the "purified antigen."

The implications of a substance found near the streptococcal cell surface which is antigenic and produces protective antibodies in animal sera as a means of producing immunity are now obvious, but a great deal of work was necessary to prove its significance in human streptococcal infection.

Human Type-Specific Antibody

The demonstration of type-specific anti-M antibody in humans was first attempted with the same methods which had originally detected M-antigen, the slide agglutination test, the precipitin test and mouse protection (66-68). The

complexity of the antigenic structure of the hemolytic streptococcus made the interpretation of slide agglutination results very difficult (54a), and passive protection in mice was unsatisfactory because of the problems of obtaining mouse virulent strains and satisfactory protective antisera for controls (69,70). Some work was accomplished with the precipitin test (68), but it was not until the bactericidal test was perfected that incisive studies appeared.

Todd first described the bactericidal action of human blood in 1927 (71). In 1934 Fothergill and Lium used the blood of convalescent scarlet fever patients in the test and demonstrated an increased bactericidal activity in those with purulent complications (72). Puerperal sepsis patients were shown to develop "bactericidal antibodies" in 1935 (73), erysipelas patients in 1936 (74), and rheumatic children in 1943 (75).

Kuttner and Lernert studied the bacteriostatic properties of blood from patients recovering from streptococcal pharyngitis, comparing it to agglutinin and precipitin tests (76). They found non-specific results with the agglutination method and completely negative results with the precipitin test. The bacteriostatic test, however, showed a well marked difference between the blood of children who had been infected with the type of streptococcus being studied and those who had not been infected. The bacteriostatic activity persisted for many months, demonstrable up to a year, and was not correlated with the development of symptoms referable to the pharyngitis or with the development of rheumatic fever.

Rothbard, in 1945, explained the mechanism of the bactericidal test by showing that antisera containing M antibody enhance the phagocytic activity of human leukocytes and thus prevent the growth of streptococci in human blood (77). He also found that type-specific antibody occurs 3 to 5 weeks following infection and persists for at least 37 weeks. With coworkers one year later, he studied immunity to induced streptococcal, nasopharyngeal infections in monkeys as the closest model to man for demonstrating the protective action of type-specific antibody (78). In the paper they indicate that there is "no known instance in which a patient (human) has suffered more than one nasopharyngeal infection with the same type of streptococcus" and demonstrate conclusively that following infection with a known type of streptococcus, monkeys are resistant to reimplantation with an homologous type and readily infected with heterologous types. Resistance was correlated with the development of anti-M antibody as measured by the bacteriostatic test, and the relationship of M antigen to virulence of strains (see above) was further documented by the fact that glossy variants failed to implant; cause-antibody rises, or protects against subsequent implantation with matt strains. In later work the bacteriostatic test for M antibody was found to be more consistently type-specific than precipitin reactions, and M antibody was found 2 to 10 weeks following infection, distinctly later than the rise in ASO titre (79).

More recently, type-specific antibody has been shown to develop in the majority of individuals following streptococcal respiratory infections though usually more slowly and with more variation in time of development than anti-streptolysin 0 (80). Antibiotic treatment has been demonstrated to inhibit anti-M formation (81), the degree of inhibition related to the successful elimination of the organism by therapy (80).

The persistence of type-specific antibody for as long as 32 years has been demonstrated by Lancefield with the bactericidal test, in a study of individuals who were infected with known types of streptococci before antibiotic therapy was available (82). In some cases mouse-protection tests demonstrated that these persistent, type-specific antibodies are protective against homologous infection in mice and probably "a reliable indication of immunity."

A new method of detecting type-specific antibody in human sera was indicated in 1957 when Stollerman and Ekstedt reported the growth of streptococci in significantly longer chains on incubation with sera containing homologous antibody compared to short chain formation in sera containing heterologous antibody or in growth media without

antibody (83). A study of the development of type-specific antibody in children infected with known types of streptococci, comparing the long chain phenomenon to the bactericidal test, established the former as an almost equally sensitive and reliable method of detecting anti-M antibody (84).

A complement-fixation test has also been reported as a method of detecting the antibody against two types of streptococci (85). The data presented indicates a high degree of type-specificity, though more types would have to be studied for this method to become generally applicable.

The development of type-specific antibody in humans, which is present for many years and apparently confers protection against subsequent infection with homologous types, and the emergence of techniques for detecting this substance provide the current stimulus for attempting primary immunization against the Group A streptococcus.

Presenting the antibody-forming sites of the body with sufficiently antigenic M protein has been the major obstacle to producing type-specific immunity experimentally, while the problems of protecting a given individual against at least 45 specific types raise serious doubt in regard to its clinical application.

In 1946 a naval epidemiological unit reported attempts at immunization by using heat and ultraviolet killed organisms as the antigen (86). Small amounts of vaccine were used, and results were evaluated only on the basis of natural infection, no attempt being made to measure antibodies. No reduction in total respiratory or streptococcal illness was noted in vaccinated groups as compared to controls, and injections with organisms homologous to the type used in the vaccine occurred with similar frequency in both groups. The lack of response reflects two aspects of the problem: insufficient total dosage and exposure to the antigen for a short period of time, since these investigators used 1.5×10^9 organisms as a total dose administered in three divided doses in one week. Reactions to the infection of whole organisms included severe general reactions.

Rantz and coworkers studied the problem of immunizing with whole organisms more intensely, measuring the production of type- and group-specific antibodies (87). They noted wide variation in the tolerance of adult human beings for subcutaneously injected killed streptococci. Toxic phenomena occurred in some subjects with as little as 8 micrograms of nitrogen while others could tolerate 600 to 800 micrograms without difficulty. Some individuals developed increasing sensitivity to the vaccine and showed marked reactions to doses which initially were tolerated without toxicity. Antibodies, measured by the bactericidal technique, developed only after injection of 370 micrograms or more of nitrogen in nine injections at weekly intervals, shorter courses or smaller amounts failing to induce type-specific antibody. Because of the common and severe reactions and the poor and unpredictable antibody response, the authors conclude that the large amounts of whole cell vaccine required to produce a response could be administered to few human beings without initial high toxicity. The fact that sensitization apparently occurred in some individuals adds a further pessimistic note. As is pointed out, however, the reactions may have been due to erythrogenic toxin incompletely removed from the cells, since none of the subjects were Dick tested, and the development of hypersensitivity could not be directly attributed to M protein since the entire antigenic complex of the organism was injected.

The obvious answer to these problems would be the injection of pure M protein as an antigen. Schmidt, using Lancefield's partially purified antigen (see above), found minimal reaction to this product if injected subcutaneously, and was able to detect a primary antibody response in 2 of 7 adults receiving from 300 to 700 micrograms of the material (88). Using aluminum phosphate as an adjuvant he studied two groups of children on penicillin prophylaxis, administering 300 to 350 micrograms of protein over a 12-week period. These injections were well tolerated and homologous antibody detectable by the bactericidal test was found in two of twenty-two sera, a smaller percentage of children developing type-specific antibody after M protein plus adjuvant than adults given the same

antigen in saline. He summarized the work with the statement, "Further immunization studies with M antigen should probably be deferred until more native preparations of greater antigenic potential become available."

It appears from the above work that whole cells in large enough doses to produce a response are too toxic, and the purest form of M protein available, either alone or with an adjuvant, is not an adequate antigen. One would suspect that between the extremes of the whole streptococcal cell and chemically purified M protein, a material could be found which is not toxic yet is antigenically potent. Such a substance may prove to be the streptococcal cell wall.

Cell Walls

Cell wall preparations of Group A streptococci have been obtained by two methods, sonic oscillation (89) and Mickle disintegration (90). Both are procedures which physically disrupt the organism, allowing the cytoplasm to escape into solution and the insoluble cell walls to be collected by centrifugation, probably by a mechanism of rupturing the cell wall (91, 92). In the former, high frequency sound waves accomplish this, while in the latter, agitation of the whole cells with tiny glass beads produces the same result.

M protein was isolated from the residue of sonically treated organisms in 1942 (63), and in 1952 Smolens and Warner reported studies of the immunologic properties of such preparations (89).

They worked with ten types of Group A streptococci, on the basis of epidemiologic surveys of army camps which showed that these accounted for 70% of streptococci found in scarlet fever patients, over 70% of all upper respiratory infections, and over 90% of the types found in cases of rheumatic fever.

The washed, insoluble fractions of these sonically treated organisms, comprising 25 to 35% of the weight of the original bacteria, were used as antigens. Each of the ten types was able to produce a variable degree of active mouse protection after three immunizing doses of 100 micrograms each. Type 3 cell walls were used to prepare rabbit antisera which conferred passive mouse protection against the homologous organism.

One-hundredth microgram of cell walls was used in human skin tests. Laboratory personnel "exposed" to streptococci gave local reactions while those without exposure did not react. Positive reactions, however, were all local and disappeared in 4 days. Subcutaneous immunization of normal medical students with approximately 200 micrograms in three graded, weekly doses was accomplished with little or no sensitivity, and the data suggests that anti-M antibody as measured by the passive mouse protection test was produced in some individuals.

Although this work is not conclusive as regards the production of human immunity, it represents two important observations which are partial answers to the difficulties of multiplicity of types and a non-toxic antigen discussed above. It is feasible that yearly surveys of the frequency with which certain types of Group A streptococci are involved in human infection in a given region could provide data on which a polyvalent vaccine offering a high percentage of protection could be based. Cell walls are not as toxic to humans as are whole cells, are antigenic enough to produce homologous antibodies in mice and rabbits after relatively small dosages, and may do the same in man.

Slade and coworkers have further investigated the action of sonic oscillation on streptococcal cells (93). Following the process of continued oscillation with electron microscopy and chemical and immunological analysis of the extracts, they found that the insoluble residue gives strong precipitin reactions with type-specific homologous antisera, clearly demonstrating the presence of M protein in this fraction. Longer periods of sonic oscillation, however, produced soluble fractions which also gave strong type-specific reactions, suggesting that the cell wall is composed of layers around a "hard core" of insoluble material which consistently gives positive precipitin tests for M protein. They postulate that the energy of sonic oscillation first solubilizes the cytoplasm of the cell and then solubilizes part of the cell wall, thus ac-

counting for the presence of M protein in the soluble fractions.

Cell wall preparations of many bacteria were obtained after agitation with glass beads in a Mickle disintegrator in 1951 (92). Salton demonstrated the presence of M protein in such preparations of Group A streptococci with the precipitin tests in 1953 (94).

Barkulis and coworkers used this method to prepare cell walls from Group A, Type 14 streptococci (90). The cell walls comprised 23 to 24% of the dry weight of the original organism, similar to the range reported after sonic oscillation (above). The structure of the cell walls was chemically analyzed and 15% consisted of D-rhamnose and hexosamine, the principal constituents of group-specific C substance. The remainder was a protein which was readily removed by trypsin and accounted for the type-specificity of the preparation. Typable M protein was extracted from these cell walls in quantities similar to that obtained from whole cells, establishing that M protein is "localized principally, if not completely, to the cell wall." Since two acid-heat extractions removed most of the serologically reactive M protein, consisting of less than half of the total protein found on the cell wall, it was concluded that the widely used acid heat extraction procedure liberates only part of the outer protein surface of the cell wall, and though this molecule reacts with type specific antibodies, "antigenicity may be a function of a larger protein moiety, a portion of which is still retained on the cell surface following extraction."

The immunology of these cell wall preparations was studied in rabbits (95). After immunization with small doses, antibody measurable by passive mouse protection, bacteriostatic and quantitative precipitin tests was demonstrated, titres determined by the third method showing that cell walls produced less antibody than a corresponding number of whole cells. From these data the authors conclude that a cytoplasmic component of the organism probably adheres to the cell after lysis in the host and in some way potentiates the antigenic stimulus of the M protein.

The only attempt at producing immunity in humans with cell wall preparations obtained after Mickle disintegration has resulted in the report that "cell walls in saline are of doubtful value in producing type specific antibody." (96) The study was performed on adults who were being followed as outpatients for chronic lung disease. Prior to immunization they were skin tested with 3 micrograms of the material, and if negative, they received three intra-muscular injections of cell wall suspension one month apart. Sera were tested before and after immunization with the bacteriostatic test.

Seventy-two patients were skin tested and eleven of these showed significantly positive reactions to preclude immunization, though only one had demonstrable homologous antibody prior to injection. Of 40 patients immunized with doses of 30 micrograms to 16 mg, 2 showed a primary antibody response, one of these receiving the smallest dose of 30 micrograms. Seven patients who were weakly positive in the bacteriostatic test before immunization showed an apparent elevation of antibody titre or secondary response. No correlation was found between the presence of circulating antibody and skin reaction to the cell walls.

Though only 2 of 40 patients showed a primary antibody response, the relative ease with which cell walls are tolerated by humans was demonstrated by this study, and the suggestion of antigenicity warrants further investigation of such preparations as they are the least toxic and most antigenic preparations presently available.

An attempt is being made to produce a secondary or anamnestic antibody response with these preparations in a group of children who have been infected with a known type of Group A streptococcus; whose type-specific antibody level has been followed and observed to fall since its initial rise (97). Skin sensitivity to cell walls of the homologous organism has not been significant in the small number of children thus far tested and systemic reactions have not occurred. Measurements of antibody response with the bactericidal and long chain methods currently being carried on

TABLE I

ATTEMPTS AT IMMUNIZATION AGAINST THE STREPTOCOCCUS

	Date	Antigen	Toxicity	Clinical Immunity	Stimulus	References
I	1899	pharyngeal exudate of SF patients (14)	severe	not studied	search for etiology of SF	(11-13)
	1900–1920	killed S. scarlatina plus growth media (15, 16)	mild	decreased incidence of SF	?? etiology of SF	(15-21)
II	1924–1930	erythrogenic toxin (27)	mild	Dick test reversal and prevention of SF	S. Hemolyticus produces toxin which causes SF	(22-26)
III	1930–1940	erythrogenic toxin (40, 45, 46)	mild	no effect on streptococcal infection or incidence of RF	Strep. related to RF by study of relationship between SF and RF	(28-44)
IV		heat-killed strep. and nucleoprotein (48-50)	mild-severe	failure to reduce symptoms of acute RF or effectively reduce recurrences	RF represents "hypersensitivity" to strep. infect.	(47-50)
V	1940–1960	heat and UV killed strep. of known type (86)	mild	no decreased infection by homologous strain	antigenic classification of Gr. A strep., M protein and type-specific immunity, methods for measuring human anti-M antibody	(51-85)
VI		heat-killed strep. of known type (87)	severe	? clinical immunity but Anti-M demonstrated		
VII		"Purified" M protein (88)	mild	?? clinical immunity, ? anti-M		
VIII		cell walls (89, 96, 97)	mild	? immunity, anti-M probably produced	M protein on strep. cell wall, methods for obtaining antigenic suspension of cell walls	(89-97)

indicate that non-toxic doses of cell walls may indeed prove antigenic enough to stimulate the production of homologous antibodies, at least as a "booster" in individuals who already possess circulating anti-M antibody.

Summary and Discussion

Table I summarizes the evolution of the present concept of producing streptococcal immunity with particular reference to those advances which stimulated past attempts.

"M substance," a type-specific protein located on the cell wall of virulent Group A streptococci, has emerged as the antigen against which protective antibodies are formed and, therefore, the material with which immunization should be attempted.

Definite proof of the protective nature of these antibodies is lacking, but until one is brave enough to inoculate the pharyngeal mucosa of humans who have known type-specific circulating antibody with the homologous virulent organism,

we must assume it to be true on the basis of animal experiments and clinical observations. Similarly, the question of how high a titre of such antibody would protect humans is also unanswered, though this involves more than demonstrating protection, as methods for quantitating the amount of circulating anti-M in sera are either crude estimations with the bactericidal and long chain tests or involve complex methods of quantitative precipitation with purified M protein (98).

Even the qualitative detection of the antibody in human sera is a procedure that requires an active streptococcal laboratory, because virulent, live organisms of known type are needed for the bactericidal and long chain tests, as well as experience in setting up and interpreting the results.

Nevertheless, reliable results can be achieved in the detection of human type-specific antibody, and the major problem centers around the antigenicity and toxicity of various M protein preparations. Suspensions of cell walls appear to be more antigenic than purified M protein and less toxic than whole cells. A well controlled, comparative study of the antigenicity of whole cells, cell walls and purified M protein in rabbits, with evaluation of antibody formation by mouse protection, bactericidal and long chain tests, would hopefully provide more direct evidence for the relative antigenicity of these preparations; however, other possibilities for obtaining an antigenic agent exist.

Leukocytes which have phagocytized virulent streptococci may contain M protein which is non-toxic yet antigenic since this is conceivably the method by which M protein is brought to antibody-forming sites of the body during natural infection. Methods for detecting the presence of M protein in such leukocytes are being actively pursued (99) and may offer a new preparation of M protein with which to attempt the experimental production of antibodies.

It has been demonstrated that delayed allergy, or hypersensitivity to M protein and other streptococcal products, may be passively transferred by leukocytes similar to that of Tuberculin hypersensitivity (100). Since it is still not known whether bacterial hypersensitivity confers protection or how it relates to circulating antibody, this suggests another method by which streptococcal immunity and/or hypersensitivity may be studied. Intradermal inoculation of antigen-antibody complexes of other sytems has been shown to produce delayed allergy (101). The M-anti-M system seems to be a likely one for expanding such studies as well as offering another method of presenting the body with antigen.

The study of "L forms" or protoplasts of streptococci has shown that after digestion of the cell wall with phage-associated lysin, M protein is not present on the remaining cell membrane but is elaborated into the medium surrounding these organisms (102). This M protein, apparently in its native state except for being unbound to the cell wall, may be antigenically, if not chemically, different from the heat-extracted, purified M protein and represents still another potential preparation with which to attempt immunization.

While investigations along these lines might prove to be of useful significance to the bacteriologist and immunologist, a serious clinical objection to the effectiveness of producing streptococcal immunity remains the multiplicity of types of Group A organisms which cause serious disease. If a vaccine were developed, the possibility of protecting the general population against rheumatic fever by such means is certainly remote; however, epidemics in army camps and other closed institutions with one or two known types provide situations where a type-specific or polyvalent vaccine might be prophylactically effective. Finally, the production of type-specific streptococcal immunity would seem to have its greatest overt clinical significance in the prevention of post-streptococcal glomerulonephritis, a disease which apparently follows infection with only a few types of the Group A streptococcus (85).

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