CLINICAL PROGRESS

Antibody Titers in Acute Rheumatic Fever

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The indistinct boundaries of the clinical picture of rheumatic fever and the lack of a specific diagnostic test have led to the formulation of an arbitrary list of criteria for the diagnosis of the acute disease and to the use of laboratory aids that are nonspecific but nevertheless informative.

The laboratory tests used in rheumatic fever fall into 2 general groups, (1) the acute phase reactants and (2) bacteriologic and immunologic studies confirmatory of a preceding streptococcal infection. Each of these groups yields information relevant to a different facet of the disease process. None of the tests in either group is diagnostic of rheumatic fever.

The acute phase reactants, which include the erythrocyte sedimentation rate, the C-reactive protein, and the serum mucoprotein determinations, are positive in a wide variety of other inflammatory diseases as well as acute rheumatic fever. These tests are of no differential diagnostic value, but in patients with an established diagnosis of rheumatic fever, they roughly parallel and are useful indicators of the presence or absence of disease activity. As such, they are indispensable guides for following the clinical course of the disease.

The laboratory tests in the second group do not reflect disease activity. In supplying information relative to streptococcal infection, they indicate whether the stage has been set for the development of rheumatic fever. These aids include a throat culture for the presence of group A beta-hemolytic streptococci and the various streptococcal antibody tests (e.g., the ASO or antistreptolysin-O titer). These tests are nonspecific in the sense that they do not differentiate in any clinically useful or consistent way between those patients who do and those patients who do not develop rheumatic fever following streptococcal infections.

Nevertheless, when used with the clinical findings, these bacteriologic and immunologic tests help in determining whether the disease process is properly classified as acute rheumatic fever.

In patients with acute rheumatic fever streptococcal antibody tests are in general a more reliable indicator of recent streptococcal infection than throat cultures. At the time rheumatic fever is suspected, a routine throat culture is of rather limited value. By this time, the group A beta-hemolytic streptococci may have been eradicated by antibiotic therapy or, even without antibiotics, may be so reduced in number as to evade detection in a single routine culture. Therefore, a negative culture is of no significance. Repeated throat cultures and cultures by special technics result in the demonstration of the infecting organism in a higher percentage of patients.

The great variety of antigenic products of group A streptococci provides a rather wide selection of antibodies that can be used as
Indicators of recent streptococcal infection. Those which have been studied most thoroughly following natural streptococcal infection in man include antibody responses to streptolysin O, erythrogenic toxin, type-specific M protein, streptokinase, hyaluronidase, the desoxyribonuclease, and diphosphopyridine nucleotidase. With the exception of M protein, which is a surface component of the cell, these substances are extracellular products of the streptococcus, i.e., products released into the surrounding medium (fig. 1). In addition to their antigenicity they are all biologically active. Indeed, it is largely by their biologic activity that they are known, since chemically pure preparations have not been obtained. Furthermore, specific antibody for each product is determined by neutralization of its specific biologic activity. In studying human infections antibodies to streptococcal substances other than those named have not proved satisfactory either because they are nonantigenic (streptolysin S, hyaluronic acid), poorly antigenic (proteinase, group-specific carbohydrate), nonspecific for beta-hemolytic streptococci (nucleoprotein fraction), or poorly differentiated in unabsorbed sera (T protein, R protein, group-specific carbohydrate). Naturally occurring antibodies to other substances (ribonuclease, amylase, beta glucuronidase, lipoproteinase, and polyglycerophosphate) have not been extensively studied in human beings. Antibody to polyglycerophosphate is not likely to be useful in studying streptococcal infections in man because the antigen is present in a number of other gram-positive bacteria.

Earliest interest in streptococcal immunology quite naturally focused on those antibodies that neutralize streptococcal substances associated with the most striking clinical and laboratory manifestations of infection. Attention was drawn to antibody neutralizing the erythrogenic toxin of scarlet fever (antitoxin), to antibodies responsible for protection in experimental infections (antibodies to the M proteins), and to antibodies inhibiting hemolytic substances produced by streptococci (streptolysins).

Figure 1
Diagrammatic representation of known cellular components and extracellular products of group A streptococci.
Antibodies to M Proteins (Type-Specific, Bactericidal, or Bacteriostatic Antibodies)

For some years immunity to streptococcal infections was in a state of confusion, confounded by a lack of knowledge of the antigenic composition of these organisms and by a preoccupation of many clinical investigators with scarlet fever and its antitoxin, which was subsequently shown to control the rash of scarlet fever but to afford no protection against invasion by streptococci.

The fundamental studies of Griffith\(^7\) and of Lancefield\(^8\) pointed to the antigenic diversity of these organisms and afforded a firm basis for their serologic classification. Griffith classified hemolytic streptococci into a number of types according to their T proteins (identified by agglutination reactions). A more natural classification arose from the studies of Lancefield. In the first place, she showed that hemolytic streptococci were divided into groups A through O according to the serologic specificity of the carbohydrate component of the cell wall (referred to as group-specific carbohydrate or "C" substance). Most infections in man, and all those resulting in acute rheumatic fever, are caused by group A hemolytic streptococci,\(^8\) which have in their cell walls a common group-specific substance, known as group A carbohydrate. Lancefield has also shown that group A streptococci can be further subdivided, according to their specific M-proteins, into more than 40 different serologic types, which are designated numerically. Like T proteins, the M proteins are surface components of the streptococcal cell but their greater importance stems from their implication in the virulence of the organism and from the demonstration that immunity to streptococcal infection in animals and in man is dependent upon antibody to the specific M protein.\(^8,\)\(^9\)

Immunity is type-specific. Antibody to the M protein of one type does not protect against infection with a streptococcus producing M protein of a different type. This explains why multiple streptococcal infections occur.

Antibody to the M protein of the infecting strain (type-specific antibody) develops regularly following untreated streptococcal infections. It is relatively slow in appearing, sometimes requiring several months before it can be detected.\(^10,\)\(^11\) This antibody is sharply suppressed by the administration of antibiotics, particularly penicillin.\(^11,\)\(^12\) It often persists for many years, furnishing rather permanent protection against reinfection with strains of this specific type.\(^13\)

Antibodies to the specific M proteins can be demonstrated by passive protection tests in experimentally infected animals. A more convenient test for type-specific or M antibody is the bactericidal or bacteriostatic test.\(^14\) In this test, the M antibody is detected by its ability to facilitate the phagocytosis of streptococci of this type. Human blood lacking M antibody for the type being tested is used as a source of leukocytes. To this are added known quantities of streptococci, producing M protein of the type being tested, plus known quantities of the serum being tested for M antibody. After a suitable period of incubation and rotation to facilitate surface phagocytosis, the number of surviving chains of streptococci is estimated by counting the colonies produced by an aliquot seeded into a blood agar plate.\(^13\) The reduction in colony count as compared with suitable controls indicates in a roughly quantitative fashion the amount of type-specific antibody present. This is a complex biologic test system, subject to a number of variables. More recently it has been shown that type-specific antibody can also be identified by its ability to promote the formation of long chains by streptococci of homologous type growing in broth culture.\(^15\) This test is simpler to perform than the other 2 but is probably less quantitative and requires special strain variants, which will readily form long chains in the presence of type-specific antibody.

Because of their type-specific nature and of their persistence for long periods of time, antibodies to the M proteins are of no value as a general indicator of the probability of recent streptococcal infection. Moreover, the complexity of the tests involved and the multiplicity of different M antigens generally restrict their usage to special research studies.
ANTIBODIES IN ACUTE RHEUMATIC FEVER

The demonstration, however, that infection by certain specific types of streptococci is associated with the development of acute nephritis has stimulated new and widespread interest in these antibodies as a source of information regarding the infecting type in patients from whom the organism can no longer be isolated.

Antitoxin (Antibody to Erythrogenic Toxin)
Antitoxin titers have proved to be rather unsatisfactory measurements of streptococcal antibody not only because of the existence of more than one erythrogenic toxin, but more seriously because erythrogenic toxins and their antitoxins can only be measured in terms of a skin reaction in susceptible human subjects or animals. This results in gross limitations in precise standardization and accurate quantitation of antibody levels.

Antistreptolysin O
Within the past several decades, antibody to streptolysin O (antistreptolysin O, sometimes designated simply as antistreptolysin or commonly abbreviated ASO or ASL) has replaced antibody to erythrogenic toxin as the classic antibody for studying the response to streptococcal infections in man. This antibody is easily quantitated and standardized, and has been extensively studied and widely used. Since antistreptolysin O is typical of the general pattern of antibody response following streptococcal infection, it will be considered in some detail.

Confusion concerning the hemolytic properties of streptococci was clarified by the work of Todd, who demonstrated that streptococci produce 2 kinds of hemolysin, streptolysin S and streptolysin O. Streptolysin S is oxygen-stable and is responsible for the clear (beta) hemolysis surrounding colonies of Streptococcus pyogenes grown on the surface of aerobically incubated blood agar plates. The letter S refers to the fact that serum greatly enhances the formation of this hemolysin. Streptolysin S is inhibited by labile lipoprotein complexes in normal human serum, but neutralizing antibody for streptolysin S does not develop following natural infection in man nor following injection of this antigen into laboratory animals. In contrast, streptolysin O is antigenic upon injection and antibodies are readily demonstrated in the sera of patients following streptococcal infections. Streptolysin O is so named because it is oxygen-labile, existing in 2 forms: the inactive (oxygenated) form which can be reversibly reactivated to the active (reduced) form. Both streptolysin O and S may contribute to the hemolysis of subsurface colonies of Streptococcus pyogenes. Streptolysin O is inhibited by specific antibody and by cholesterol and certain other lipids.

Streptolysin O may be stored in its active (reduced) state or may be reactivated just prior to use by the addition of a reducing agent (e.g., cysteine). The activity of streptolysin O is measured by its ability to hemolyze red cells. In tests for antistreptolysin O, varying dilutions of serum are incubated with constant standard amounts of active hemolysin. These mixtures are tested for residual (unneutralized) hemolytic activity by incubation with a standard amount of rabbit red cells. The antistreptolysin-O titer is that dilution of serum which will completely neutralize hemolytic activity under these standard conditions.

A rise in antistreptolysin-O titer can be interpreted as reliable evidence of a streptococcal infection. Because hemolytic streptococci of groups C and G also produce streptolysin O, an increase in antistreptolysin-O titer may follow infection with these groups as well as with group A streptococci. Other species of bacteria, such as the pneumococci and the clostridia, produce hemolysins that are immunologically related to those of group A streptococci, but the degree of cross reactivity does not appear to be sufficient to result in practical difficulties. Immunization procedures and infectious diseases of nonstreptococcal etiology do not result in an increase in antistreptolysin-O titer. Although cholesterol is known to be a potent inhibitor of streptolysin O, the cholesterol of normal serum does not inhibit streptolysin O, as it is apparently in a state in which it does not combine with strep-
tolysin O. On the other hand, the presence of nonspecific lipoprotein inhibitors in the sera of patients with hepatitis and in sera that have become contaminated may result in spuriously high titers that do not reflect true antibody inhibition. Technical difficulties, other than contaminated sera, which may lead to false titers include the use of streptolysin-O preparations which are incompletely activated. Commercial preparations of streptolysin O should be used as soon as reconstituted. On standing, a portion or all of the preparation may convert to a nonhemolytic state in which it will nevertheless still combine with antibody. Even when freshly prepared hemolysin is used, it is hazardous to assume that the potency of the preparation has not changed since standardization by the manufacturer. One, or preferably several, standard antisera should be titrated as controls in each set of antistreptolysin O determinations.

In patients with well-documented streptococcal infections, a rise in antistreptolysin-O titer can often be demonstrated after 1 week, but titers do not reach maximal levels until 3 to 5 weeks after infection. There is considerable variation in the rapidity with which titers return to preinfection levels. The cause for this individual variation is largely unknown although a number of factors may affect the rate of decline. For example, steroid therapy accelerates the rate of decline. Other factors, which may influence the rate of fall in titer, include persistence of the organism, and perhaps indirectly the presence or absence of tonsils and the administration of antibiotics. Reinfecion may result in a sustained or continuously rising titer. Generally, in the absence of reinfection, titers tend to decline gradually and to approach preinfection levels by 6 to 12 months.

The magnitude of the antibody response also shows considerable variation. Factors such as the nature or the severity of the preceding infection or the serologic type of the infecting group A streptococcus may influence the magnitude of the antistreptolysin-O response. Of considerable attraction is the observation that patients who develop rheumatic fever are in general more likely to show a higher antibody response than those whose infection is not followed by this late complication. This suggests that rheumatic individuals are "immunological hyper-responders." An alternative interpretation is that individuals developing rheumatic fever have in some way been subjected to a greater antigenic challenge. More definitive information as to which interpretation is correct can be obtained by studying the antibody response of rheumatic subjects to injected antigens where the dose of antigen administered can be easily quantitated and controlled. Studies of the antibody response of rheumatic children to intramuscular injection of a concentrate containing streptolysin O suggest that, as a group, these children show greater antistreptolysin-O responses than nonrheumatic children. Interpretation of this study, however, is complicated by the varying doses injected and by the different average initial titers in the rheumatic and nonrheumatic groups. If rheumatic individuals are "hyper-responders," it appears that this immunologic hyper-responsiveness is specific for streptococcal antigens. Most studies have indicated no unusual antibody response in rheumatic subjects following the injection of nonstreptococcal antigens. Moreover, the difference in response to streptococcal antigens is by no means constant. Of the 20 rheumatic children injected with streptolysin O, 5 showed no detectable antibody response. Also the difference in antibody response following natural infection between those who do and do not develop rheumatic fever is based on an average figure, and there is a wide area of overlap of individual responses in the 2 groups of patients. Indeed, some patients who develop rheumatic fever show no detectable rise in antistreptolysin-O titer.

Occasional differences in the antibody response may be related to differences in the production of streptolysin O by strains of group A streptococci. Although it is possible to quantitate production under in vitro conditions, it is not possible by present techniques to estimate in vivo production. Experience
with injected antigens, however, where the amount of antigen can be precisely controlled, has indicated considerable individual variation in antibody response due to unknown host factors. Two factors that appear to influence both the magnitude and the frequency of the antistreptolysin-O response are previous exposure to the streptococcal antigen and, perhaps indirectly, the age of the patient. It is well known from experimental studies with a variety of antigens that antibody responses are less impressive in patients or animals who are experiencing their first exposure to an antigen (primary response) than in those who have been previously exposed (secondary response). Streptococcal infections are not only fairly common but repeated infection occurs with reexposure to antigens encountered previously. In most clinical situations, we are not dealing with the patient’s first experience with streptococcal antigens. Therefore, the frequency and the degree of antibody response will depend upon previous experience with the streptococci. Such factors as the number of past streptococcal infections, the length of time since the last infection, and the height of the residual antibody level at the time of reinfection may be involved.

It is generally agreed that antistreptolysin-O responses are usually poorer in infants than in older children or adults. Except in the first few months of life, it would seem unlikely that poor responses can be attributed to an immaturity of immunologic mechanisms. It seems more likely that responses tend to be poorer because this is the infant’s or child’s first encounter with streptococcal antigens (primary response).

The natural course of streptolysin-O response can be modified by the administration of drugs. Cortisone may delay antibody development. Penicillin may modify the antistreptolysin-O response even more strikingly, by reducing the over-all number of responders and by influencing the magnitude of the antibody response. The effect of penicillin is related to the time therapy is started, to the dosage employed, and to the length of time given. Bactericidal drugs such as penicillin are more likely to influence the antibody response than bacteriostatic drugs such as sulfonamides and tetracyclines.

Aside from biologic factors and the influence of drugs, certain differences in reports of the frequency and magnitude of antistreptolysin-O responses are inherent in the schemes of dilution increments used in measuring antibody. In table 1 are listed a variety of dilution schemes that have been used for measuring antistreptolysin O or other streptococcal antibodies. Most of these are devised according to a plan that makes it technically easy to make dilutions in a serial fashion. Some are constructed in a logical mathematical pattern, showing increments that are logarithmic. These result in a straight-line progression, as shown in figure 2. The slopes of the 3 lines representing these dilution methods vary considerably. The 2-fold system progresses to relatively high titers within a few dilutions,

<table>
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<tr>
<th>Logs</th>
<th>2-fold</th>
<th>1/2-fold</th>
<th>1/4-fold</th>
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<th>Rantz</th>
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<td>2100</td>
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Table 1
Dilution Schemes Commonly Used in Streptococcal Antibody Titrations
whereas the 1 1/2-fold (Kaplan) method\textsuperscript{33} progresses more slowly and the 1 1/4-fold or 0.1 log (CARD)\textsuperscript{21} system shows even smaller increments. The design of the 2 other schemes results in titers that follow no constant mathematical sequence. The method of Hodge and Swift\textsuperscript{34} is by arithmetical progression, which changes at intervals. This method produces an irregular curve, but has the advantage of resulting in numerical titers that are easy to remember. Although logarithmic in trend, the method of Rantz and Randall\textsuperscript{35} is irregular in progression and results in titers that are more difficult to remember. Despite these disadvantages, this latter method is becoming more and more widely used because it is often the scheme which accompanies the commercially available reagents.

Obviously, the spacing of increments in the particular scheme being used will influence to some extent both the percentage of patients showing an "antibody response" and the "magnitude" of these responses. For example, within the limits of reproducibility, an antibody rise will be more frequently demonstrated by schemes with smaller increments. Moreover, within any given scheme, the percentage of patients exhibiting an antibody rise will vary, depending upon the level of the initial antibody titer. Although some of the schemes are irregular, in general the arithmetic increments become progressively larger as one moves from the lower to the higher dilutions. Consequently, an antibody response is more likely to be demonstrated in patients with low initial titers than in patients with high initial titers. To illustrate, with the 0.1-log increment system (CARD), a 100-unit rise in a patient whose initial titer is 63 units will result in a 4-tube increase, whereas an identical rise in a patient whose initial titer is 500 would not be detected (table 1).

With careful technics, increments as close as 0.1 log can be accurately and reproducibly measured, and increases of 0.2 log (2 tubes) can be accepted as evidence of a rise in titer. Following well-documented untreated streptococcal infections, antistreptolysin-O responses can be demonstrated in about 80 to 85 per cent of patients.\textsuperscript{21, 23} In some series the percentage is lower (60 to 80 per cent).\textsuperscript{3, 36-39} This may be due to a number of factors, including antibiotic therapy, age of the patient, high-initial antibody titers, infection with strains that are poor streptolysin-O producers, and dilution of the series with patients who by clinical or bacteriologic criteria appear to be infected with streptococci but whose current illness is indeed due to some other agent.

In the usual clinical situation, an antistreptolysin-O titer is not obtained until rheumatic fever or some other complication of streptococcal infection is suspected. By this time the antibody titer may be at or near its peak and it may be impossible to demonstrate an antibody rise. Without a baseline titer for comparison, it is considerably more difficult to interpret the meaning of a titer taken at the time rheumatic fever is suspected. One must resort to a comparison of this single antibody determination with an average level in the population. Some titers that are low in comparison with the average population may represent a definite increase over the baseline titer for this individual and may, therefore, represent streptococcal infection. Other titers may be high but may represent a residual from a streptococcal infection too distant to be directly related to the onset of rheumatic fever. The difficulty is compounded by the fact that average antistreptolysin-O levels may vary for different populations by age, economic status, geographic area, season, year, and other factors related to the frequency of streptococcal infections.\textsuperscript{28}

Because of the ubiquity of streptococcal infections and of the commonness of streptolysin O as an antigen of group A streptococci, sera from normal individuals generally show some antibody to streptolysin O, reflecting contact with the streptococci at some time in the distant past. These low titers (< 250 Todd units) are commonly found in school-age children and young adults who have not had a recent streptococcal infection.\textsuperscript{28} The antibody is passively transferred to the fetus via the placental circulation, so that levels in cord bloods and in newborns are equal to or slightly

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in excess of those of the mother. This passively acquired antibody is lost during the first half year. Consequently, between 6 months and 2 years of age antistreptolysin-O titers are usually very low or not detectable. After infancy and particularly when the child reaches school age, the chances of acquiring a streptococcal infection increase, so that average antistreptolysin levels tend to increase during this period. Highest normal levels are found in the school-age child. These tend to stabilize or decline slightly in the young adult and to decrease in later years as the frequency of streptococcal infection decreases.

In normal school-age populations about 80 per cent of individuals will have a titer of 333 units or less (table 2). In young adults a titer of greater than 200 Todd units may be out of line with the general population. In older adults, except those in situations where exposure to streptococcal infection is high, titers greater than 100 to 150 units may be considered uncommon. Adults who may be unusually exposed to streptococcal infection include those in the military services and mothers of school-age children.

In spite of the relative paucity of data on antistreptolysin-O titers in “normal” populations and in spite of some degree of overlapping of titers in “normal” and “rheumatic” populations (table 2), the occurrence of an isolated, single titer that is quite high (500 units or more) is reasonable evidence of a recent streptococcal infection. Furthermore, the infection is probably recent enough to be causally related to the present illness suspected of being acute rheumatic fever. In about 80 per cent of patients with acute rheu-
rheumatic fever, the antistreptolysin-O titer during the first 2 months of illness is 250 units or higher.\textsuperscript{22} If the admission titer is low or borderline, further specimens taken at weekly intervals sometimes reveal an elevated titer, since the level of antibody may continue to rise for some time after the onset of acute rheumatic fever. In patients with subsiding, inactive, or chronic rheumatic fever the antibody titer has frequently declined to normal levels.\textsuperscript{22} Because of the prolonged latent period of several to many months frequently observed between the streptococcal infection and the onset of acute chorea, patients presenting with this rheumatic manifestation often show antistreptolysin titers that have returned to normal levels.\textsuperscript{47}

The determination of antistreptolysin O is probably the best routine streptococcal antibody test available, although the extent of overlapping values between normal and acute rheumatic fever populations does not make it an ideal differential test. Since it was one of the first such tests studied, it has been widely used in investigative work and, with the commercial availability of the necessary reagents, has become a routine test in many hospital laboratories. The units of antibody measured are fairly well standardized in terms of the Todd unit of the original procedure. Therefore, in the various modifications of this method currently in use, one can be reasonably sure, with careful attention to technic and standardization, that the antibody titers obtained in one laboratory are comparable to those obtained in others. Moreover, the percentage of patients with streptococcal infection who subsequently show an antibody response is as high as or higher than that with any other single streptococcal antigen. Nevertheless, about 20 per cent of patients fail to show an antibody response to streptolysin O, and a similar percentage of patients with acute rheumatic fever present with low or borderline antistreptolysin-O titers (250 units or less).\textsuperscript{3, 23} Consequently, a diagnosis of acute rheumatic fever can never be excluded on the basis of the antistreptolysin-O titer alone.

The failure of certain patients with strepto-

coccal infection to show an antistreptolysin-O response and the consequent finding of low or borderline titers in some patients with acute rheumatic fever has stimulated interest in other streptococcal antibodies that may be used as secondary tests. Determination of antibody to one or more additional streptococcal antigens is of value because titers of antibody to one streptococcal antigen sometimes do not parallel those of another. For example, a patient may have a high antistreptolysin-O titer and a low antihyaluronidase titer or vice versa. Therefore, if a secondary test is performed, the percentage of patients showing an elevated titer is increased and if multiple antibody tests are done, this will approach 100 per cent.\textsuperscript{3, 22} A number of secondary tests are available: antistreptokinase, antihyaluronidase, antidiphosphopyridine-nucleotidase (anti-DPNase), and antidesoxyribonuclease B (anti-DNase B).

**Antistreptokinase (“Antifibrinolysin”)**

The phenomenon of liquefaction of human fibrin clots by broth cultures or culture filtrates of beta-hemolytic streptococci as reported by Tillett and Garner in 1933,\textsuperscript{48} was ascribed first to “fibrinolysin.” Further studies indicated that this streptococcal substance does not lyse fibrin directly but results in the conversion of a normal serum component (plasminogen) to an active proteolytic enzyme (plasmin). Plasmin digests not only fibrin but also other protein substrates. Thus, the term fibrinolysin is a misnomer in several respects, and it has been generally replaced by streptokinase when referring to the streptococcal substance that activates the plasminogen-plasmin system. This system is a complicated one, involving not only the factors already named but also inhibitors and other activators.

With the establishment of the antigenicity of streptokinase in man,\textsuperscript{49} it became apparent that the estimation of the corresponding antibody levels in human sera might serve as an indication of streptococcal infection. A quantitative method for assaying the antistreptokinase levels of human sera was introduced by

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Kaplan in 1946. This method involved a neutralization test in which a constant, standardized amount of streptokinase was incubated with serial dilutions of the serum to be tested, following which an indicator system consisting of fibrinogen, plasminogen, and thrombin was added. The end-point of the test was defined as the reciprocal of the highest dilution of serum that completely prevented lysis of the clot during a second period of incubation.

Information on antistreptokinase levels is much less extensive than that for antistreptolysin O, particularly in normal populations (table 2). The rise in antistreptokinase titer following a streptococcal infection with and without complications follows the same general pattern as the antistreptolysin-O titer, although several studies indicate that antibody responses occur less frequently to streptokinase. The relative infrequency of response to streptokinase may be explained by marked differences in production of this substance by infecting strains. Another possible explanation would be the production of several immunologically distinct streptokinases by different strains of streptococci, but the evidence on this point is at present conflicting.

Apart from the relative infrequency of antibody response reported in these studies, antistreptokinase determinations have not been altogether satisfactory because of difficulties in standardization. The various components of the test system are not available in a pure state. Reproducible results can be obtained in one laboratory with the use of a single lot of substrate and of other components, but these results may not compare with those of other laboratories. Moreover, the sera being tested may contribute varying amounts of nonantibody substances (e.g., nonspecific inhibitors, plasminogen) that may affect the test system. Attempts to overcome these difficulties have been made by modifying the antisera in various ways prior to testing. For example, heating will inactivate the plasminogen in antisera but the test results may still be influenced by the fact that heat-inactivated plasminogen binds streptokinase. Another method consists of separating antibody globulin from the antisera prior to testing. This appears to offer a more promising and reliable approach to the measurement of antistreptokinase but makes the routine determination of this antibody somewhat complicated. As a consequence of the above considerations, the

Table 2
Usual Limits of Streptococcal Antibody Titers in Normal Individuals and in Patients with Early Acute Rheumatic Fever

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<th>Antibody</th>
<th>Upper limits in normal population*</th>
<th>Lower limits in early acute rheumatic fever†</th>
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<tr>
<td></td>
<td>5 - 12 years‡</td>
<td>Children &amp; adults‡</td>
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<td>28, 39</td>
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<td></td>
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<td>(160-250)</td>
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<td>Antihyaluronidase</td>
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<td>(100-128)</td>
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<td>Anti - DNAse B</td>
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*Approximately 80 per cent at this level or lower.
†Approximately 80 per cent at this level or higher.
‡Where several different values were obtained from the references cited, the range is indicated in parenthesis and an approximate median value is represented by the figure in bold face.
§Includes some children as well as young adults.

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antistreptokinase test has been generally less acceptable and less widely used than other streptococcal antibody tests.

**Antihyaluronidase**

Because of possible implications in the pathogenesis of rheumatic fever, a considerable flurry of interest followed the demonstration that group A streptococci produce an enzyme that depolymerizes hyaluronic acid, a constituent of synovial fluid and other connective tissues. This streptococcal enzyme is adaptively produced, i.e., production is stimulated by the addition of substrate to the culture medium. Only group A streptococci of types 4 and 22 produce appreciable amounts of the enzyme under in vitro conditions. The hyaluronidase of group A streptococci is immunologically distinct from the hyaluronidases from other sources (other groups of streptococci, other bacteria, mammalian tissues).

This enzyme is inhibited by 2 factors present in human sera. The first, a nonspecific inhibitor (NSI) inhibits hyaluronidase-activity, regardless of the source of enzyme. It is heat-labile and requires magnesium ions for its action. This nonspecific inhibitor is an acute-phase reactant rather than an antibody; as such it develops early in infection, subsiding during convalescence unless suppurative or nonsuppurative complications develop. The specific inhibitor (SI) is a true antibody, which develops several weeks after the onset of streptococcal infection, persists after the subsidence of inflammation, appears in the gamma-globulin fraction of serum, and neutralizes the hyaluronidase of group A streptococci specifically. It is heat-stable and does not require magnesium ions for its activation.

The in vitro determination of antihyaluronidase in human sera is based on the specific inhibition of the capacity of streptococcal hyaluronidase to digest hyaluronic acid. Non-specific inhibitor is inactivated by heating the sera at 56°C. for 30 minutes. Two commonly used methods in the determination of hyaluronidase activity are the turbidimetric and mucin clot prevention (MCP) methods. Both methods depend upon a property of hyaluronic acid that results in binding with protein in an acid medium. Under the conditions of the first test the hyaluronic acid-protein complex appears as a turbid suspension, whereas in the second test the complex forms a gelatinous mucin clot. Both tests may be adapted for the estimation of antibody. Though the turbidimetric method is more quantitative, the MCP test is the more widely used because of its adaptability in the assay of large numbers of sera. A modification of the latter test has been reported by DiCaprio et al. A reliable substrate for this test is somewhat difficult to prepare, and commercial preparations are not always sufficiently highly polymerized to be suitable.

Standardization of antihyaluronidase titers has proved difficult, and the titers obtained in various laboratories vary quite markedly (table 2). This is apparently due to a number of factors including the variability encountered in lots of substrate, minor variations in the test procedure (which is greatly susceptible to critical factors such as salt concentration and pH), and difficulty in reading the end points. Because of this latter difficulty one cannot use serum increments as close as those that may be used with antistreptolysin O. Dilutions at closer intervals than 2-fold have usually proved unsatisfactory, and some investigators have used 4- or 5-fold dilutions.

The general pattern of antibody response to hyaluronidase is similar to that of streptolysin O, although considerable variation is noted in the levels reported by various authors for normal subjects and for patients with acute rheumatic fever (table 2). Several investigators have been impressed with the higher titers found in patients with acute rheumatic fever when compared with convalescent uncomplicated streptococcal infections, suggesting that the differential rise with this antibody might be greater than that with other antibodies such as antistreptolysin O. The percentage of patients developing an antihyaluronidase response following...
streptococcal infection is somewhat lower than the percentage developing an antistreptolysin-O response.\textsuperscript{37, 45, 63} This may be due in part to the technical difficulties in reliably demonstrating small increments of antihyaluronidase. It is indeed rather curious that so many patients do show an increase in antihyaluronidase titer following streptococcal infection, since only a few strains (types 4 and 22) produce appreciable amounts of this enzyme in vitro. Possible explanations for this discrepancy are that in vivo environments may be more favorable for production of this enzyme\textsuperscript{65} or that it may be present as a proenzyme.

The wide differences in titers between normal individuals and patients with rheumatic fever (table 2) suggest that this might be one of the better differential serologic tests in acute rheumatic fever; but because of difficulties in standardization, extreme differences in titers reported in the literature, and certain technical difficulties, the antihyaluronidase determination has not been altogether satisfactory as an antibody test. Nevertheless, until recently this has probably been the best available and most widely used streptococcal antibody test other than the antistreptolysin-O determination. Two new antibody tests, anti-DPNase and anti-DNase B have recently been described that appear to have certain advantages as secondary antibody tests.

\textbf{Anti-Diphosphopyridine-Nucleotidase (Anti-DPNase, ASDA)}

The identification and characterization of an enzyme in group A streptococcal culture supernates that splits DPN (coenzyme I, cozymase, diphosphopyridine nucleotide) was reported by Carlson et al. in 1957.\textsuperscript{66} There appears to be considerable strain variation in the in vitro production of streptococcal diphosphopyridine-nucleotidase (DPNase), with type 12 and other nephritogenic strains being among the better producers. Kellner et al.\textsuperscript{67} have presented evidence that this enzyme is antigenic in animals and in man. They referred to the antibodies directed against streptococcal DPNase as ASDA ("antistreptococcal diphosphopyridine-nucleotidase activity") although antistreptococcal DPNase or anti-DPNase are perhaps preferable abbreviations, which are less likely to be confused with antibody to the streptococcal desoxyribonucleases (streptodornases).

The assay of antibody to streptococcal DPNase is based on the ability of sera containing such antibody to neutralize the specific action of the streptococcal enzyme in splitting a standard DPN substrate. At the end of the reaction period, the enzyme activity is stopped by the addition of sodium cyanide and the undigested DPN is measured spectrophotometrically as a cyanide-substrate complex. A unit of DPNase activity has been arbitrarily defined as that amount which will destroy 0.01 micromoles of DPN under the conditions of the test. One unit of anti-DPNase is that amount of antibody which will neutralize 100 units of the enzyme. Kellner et al.,\textsuperscript{67} using the spectrophotometric assay procedure for DPNase described by Colowick et al.,\textsuperscript{68} reported reproducibility with a range of plus or minus 30 per cent. Bernhard and Stollerman, with a minor modification of the above methods, obtained results showing a standard deviation that was within 10 per cent of the mean titer in replicate determinations made on a pool of human sera.\textsuperscript{37}

The initial survey made on random sera by Kellner et al.\textsuperscript{67} showed a pattern of antibody titers similar to that for other streptococcal antibodies. The lowest titers were present in children between 6 months and 5 years, while infants up to the age of 6 months showed a mean titer similar to the adult. A definite rise in titer was observed in approximately 75 per cent of patients following streptococcal infection. These findings were confirmed by Bernhard and Stollerman,\textsuperscript{37} who reported a significant rise of the anti-DPNase titer in 63 per cent of children with pharyngitis associated with positive culture for group A streptococci as compared with a rise of antistreptolysin-O titer in 62 per cent of patients in this group. In patients with acute rheumatic fever, 87 per cent showed an elevated anti-DPNase titer, whereas an equal percentage showed an elevated antistreptolysin-O titer.

Although many strains of group A strepto-
cocci fail to produce DPNase in vitro,\textsuperscript{69} it appears that the majority of patients show an antibody response to this enzyme following streptococcal infection.\textsuperscript{37, 70} This paradoxical situation is similar to that previously noted in the case of antibody responses to hyaluronidase. Only a few studies\textsuperscript{37, 67, 70} have been made of levels of this antibody in normal and rheumatic populations, and some of these are not interpretable in terms of the categories in table 2. Therefore, the figures given must be considered preliminary.

Anti-DPNase is relatively easy to determine, although it requires the use of a spectrophotometer or a photocolorimeter. A 95 percent pure substrate can be purchased inexpensively. Since the test is based on a quantitative chemical determination of the undigested substrate, it is quite accurate and reproducible. Thus, the anti-DPNase test appears to be one of the most promising of the new antibody tests available.

**Antidesoxyribonuclease B (Anti-DNase B)**

The depolymerization of desoxyribonucleic acid (DNA) by culture supernates of group A streptococci has been independently reported by Tillett et al.\textsuperscript{71} and by McCarty.\textsuperscript{72} This activity was ascribed to an enzyme called streptococcal desoxyribonuclease (DNase) or streptodornase. In vitro studies have indicated that virtually all group A strains show DNase activity in relatively large amounts. It was, therefore, somewhat disappointing when it was originally found that comparatively few patients appeared to develop antibody following natural infection.\textsuperscript{73}

This paradox (of a reverse sort from that observed in the antibody response to hyaluronidase and to DPNase) was resolved when it was shown that group A streptococci can produce 3 distinct desoxyribonucleases, DNases A, B, and C.\textsuperscript{74} The 3 enzymes have been differentiated in a number of ways, but one of the most important differences is their serologic specificity. Antibody against one of the enzymes neutralizes the activity of that enzyme but not that of the other 2.

Since most strains of group A streptococci produce predominantly DNase B, it is not surprising that low titers of antibody to this enzyme are often found in normal individuals and that an antibody rise occurs regularly following streptococcal infection. The frequency of the anti-DNase B response compares favorably with that for antistreptolysin O. High titers are also frequent among patients with acute rheumatic fever (table 2).\textsuperscript{38} The titer limits in normal individuals and in patients with acute rheumatic fever, as given in table 2, must be considered tentative until additional and more extensive studies are available.

Anti-DNase B is measured by its ability to neutralize the specific DNase-B activity in a test that superficially resembles the mucin clot prevention (MCP) test.\textsuperscript{38, 73} Serum is heated to destroy circulating mammalian DNase activity, and dilutions are incubated with a constant amount of DNase B. The mixture is then tested for unneutralized enzyme activity by adding a standard amount of substrate (DNA). After further incubation, alcohol is added and undigested substrate forms a white clump. With care in standardization, the test can be read at serum dilutions similar to those employed in the antistreptolysin test (0.1 log or greater), although 2-fold dilutions are more satisfactory for general use. Suitable group A strains can be found that produce DNase B in great excess of the other 2 enzymes, so that the crude culture supernate can be used as a source of enzyme. The substrate can be prepared without too much difficulty or may be obtained commercially. Not all commercial preparations are satisfactory as a highly polymerized preparation is essential.

The wide distribution of DNase B among strains of group A streptococci and the frequency of antibody response following streptococcal infection establish this as an antibody test that compares favorably with that for streptolysin O. In patients with rheumatic fever who present with low or borderline antistreptolysin-O tests, this antibody is frequently unquestionably elevated.\textsuperscript{38} These findings recommend it as one of the best available secondary tests.
Conclusions

Laboratory tests specifically indicating the presence of acute rheumatic fever are not presently available. Although antibody tests do not differentiate the patient with acute rheumatic fever from the patient who has recently recovered from a streptococcal infection without such a complication, they are useful as indicators of the probability of a recent streptococcal infection which could have triggered the development of acute rheumatic fever.

The antibody response to different streptococcal antigens varies independently following infection. No more than about 80 to 85 per cent of patients show a response to any single antigen, but if antibody responses to multiple antigens are looked for, this approaches 100 per cent.

Because the antistreptolysin-O (ASO) determination has been widely used and well standardized, it is probably the best antibody test available. However, some patients fail to show an antibody response to streptolysin O following streptococcal infection, and the values for normal populations and patients with acute rheumatic fever show appreciable overlapping. Therefore, some patients with acute rheumatic fever will present with low or borderline ASO titers. In such patients, the performance of one or more secondary antibody tests is desirable.

In the past the antihyaluronidase (ASH) test was probably the best secondary test available. Recently 2 new antibody tests have been described that seem to have certain advantages over the antihyaluronidase test. The anti-diphosphopyridine-nucleotidase (anti-DPNase or ASDA) test appears to offer some advantage in standardization in that the undigested substrate can be determined chemically. Although in vitro studies indicate that production of this enzyme is restricted to certain strains (mostly of the nephritogenic types), preliminary studies suggest that antibody responses are more broadly distributed. The second antibody test, antidesoxyribo nuclease B (anti-DNase B) measures neutralizing antibody for an enzyme that appears to be widely distributed among group A streptococci. Antibody responses to this enzyme occur regularly following streptococcal infection, and even those patients with acute rheumatic fever who show low titers of ASO often show distinctly elevated titers of anti-DNase B.

Although no problem arises in those patients suspected of acute rheumatic fever who show markedly elevated ASO titers, the use of one or more secondary antibody tests is particularly helpful in patients whose ASO titer does not conform with the clinical impression.

Summario in Interlingua

Tests laboratorial capaces a indicar specificamente le presentia de acute febre rheumatic non es disponibile a iste tempore. Ben que tests de anticorpo non differentia le patiente con acute febre rheumatic ab le patiente recentemente restabilisce ab un infection streptococcal sin le complication de febre rheumatic, tal tests es utile como indicatos del probabilitate de un recent infection streptococcal e assi del presentia de un causa al minus potential pro le precipitation del disvelloppamento de acute febre rheumatic.

Le responsa anticorpo a diferente antigenos streptococcal varia sin interdependencia post le occurrentia de un infection. Non plus que 80 a 85 pro cento del patientes responde positivamente a un antigeno individual, sed si le responsas a multiple antigenos es investigate, le proportion del casos positive approcha 100 pro cento.

Viste que determinationes a antistreptolysina-O (ASO) ha esse utase extensamente de manera que iste metodo es ben standardisate, illo es probablemente le melior del tests anticorpo nunc disponibile. Tamen, certe patientes non exhibi un response anticorpo a streptolysina-O post infectiones streptococcal, e le valores obtenite in le population normal e in gruppos de patientes con acute febre rheumatic monstra appreciabile areas de coincidentia. Per consequente, certe patientes con acute febre rheumatic manifesta basse o questionabile titros de ASO. In tal casos, le efectuation de un o plures secundari tests de anticorpo es desirabile.

In le passato, le melior del disponibile tests secundari eseva probablemente le test a antihyaluronidase (ASH). Recentemente 2 nove tests a anticorpo eseva describite que pare haber certe advantages in comparation con le test a antihyaluronidase. Le test a anti-diphosphopyridina-nucleotidase (anti-DPNase a ASDA) pare offerer certe advantages de standardisation in tanto que le nondigerite substrato pote esser determinate chimicamente. Ben que studies in vitro indica que le production de iste enzyma es restringe
a certe razas (predominantemente del tipo nephritogeno), studis preliminaris suggest que responsas anticrocorpe es distribuite plus largemente. Le secunde test a anticrocorpe, antidioxyribonuclease B (anti-DNase B) mesura le anticrocorpe neutralizatori pro un enzyme que par esse extensemente distribuite inter streptococces de gruppo A. Responsas anticrocorpe a iste enzyme occurre regularmente post infection streptocceal, e mesmo le patientes con acute febre rheumatic qui exhibi basse titros de ASO va frequentemente exhibit distintemente elevate titros de anti-DNase B.

Ben que nulle problema existe in patientes suscept de acute febre rheumatic quando illes exhibit marente elevate titros de ASO, le uso de un o plure tests secundari a anticrocorpe es specialmente utile in patientes in qui le titro de ASO non congrue con le impression clinici.

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Antibody Titers in Acute Rheumatic Fever
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