Antipeptidoglycan in Rheumatic Fever: Agreement with Carditis (37705)

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Streptococcal peptidoglycan causes toxic effects in experimental animals, including changes in the skin of rabbits (1–3), rheumatic-like changes in the hearts of mice (4, 5), and persistent extensive carditis in rabbits (3). No one has yet determined whether or not streptococcal peptidoglycan plays a role in rheumatic carditis in man. In order to investigate this possibility, we attempted to detect the presence of antipeptidoglycan in the sera of the rheumatic fever patients with and without carditis.

For the detection of antipeptidoglycan, we used a heterologous system involving a test antigen derived from *Micrococcus lysodeikticus* and inhibitors obtained from *Staphylococcus aureus*. The basis of reactivity of peptidoglycan with the antibody in this system is the presence of two determinants: glycan with an immunodominant β-*N*-acetylglucosamine (6, 7) and peptide with an immunodominant *C*-terminal D-alanyl-D-alanine (8, 9), presented in Table I. In the peptide determinant, L-alanyl-γ-(α-D-glutamyl)-L-lysyl-D-alanyl-D-alanine, the α-carboxyl group of D-glutamic acid is substituted by glycine in *M. lysodeikticus,* and by an amide in *Streptococcus pyogenes* and *S. aureus*. The glycan determinant in all three bacterial species of the test system is composed of alternating units of *N*-acetylglucosamine and *N*-acetylmuramic acid linked β-1,4.

Materials and Methods. The human sera studied for the presence of antipeptidoglycan were obtained from patients with acute rheumatic fever, patients with streptococcal impetigo, and healthy individuals. Fifteen rheumatic fever patients, seven with carditis and eight without, were under treatment at The House of The Good Samaritan. All 15 patients met the revised Jones criteria for rheumatic fever (10). Rheumatic fever patients with definitely significant murmurs were considered to have carditis; those without significant murmurs were considered not to have carditis (11). In order to provide an adequate amount of serum for the tests, it was necessary to pool several serum specimens from each patient. For both the patients with carditis and those without carditis, the serum specimens were obtained within 1–3 months after admission to the hospital. The initial specimens used in each pool were collected within 1 month after the onset of rheumatic fever. As we have reported previously (11), there was no correlation between presence or absence of carditis and antistreptolysin O (ASLO) titer. For patients with carditis, the mean ASLO titer of pooled serum specimens varied from 230 to 920 units and averaged 479 units. The titer of pooled serum specimens of patients without carditis varied from 317 to 1300 units and averaged 661 units. The ages of the patients with carditis varied from 4 to 15 years; the ages of the patients without carditis varied from 5 to 14 years. Among those with carditis were two females, five males, three black children, and four white children. Among those without carditis were two females, six males, one black child, and seven white children. The sera from ten patients with streptococcal impetigo and 17 healthy individuals served as controls. The impetigo patients were under treatment at Boston City Hospital. Their age varied from 14 to 41 years. They included six females, four males, seven black individuals, and three white individuals. The sera of

healthy individuals were collected at Tufts School of Medicine and New England Center Hospitals. These healthy individuals were white males between the ages of 22 and 27 years.

The test antigen was obtained as previously described (6) from a culture of *M. lysodeikticus* ATCC 2665 grown in a medium containing ^{14}C lysine, preferentially incorporated into the cell walls. After harvesting, the cells were broken, and the cell walls were separated by differential centrifugation. Then the cell walls were solubilized by digestion with lysozyme. The nondialysable fraction labeled with ^{14}C was used as antigen.

A modified peptidoglycan from *S. aureus* H, obtained by treatment of the cell walls with 1 *N* NaOH and subsequent digestion with lysozyme, was designated Inhibitor I. Details of the procedure have been described previously (6).

S. aureus H cells treated with penicillin by the method of Park (13) yielded Inhibitor II, binding the antibody cross-reacting with the peptide determinant. The nature of the test antigen and both inhibitors is presented in Table I.

The technique of inhibition of radioimmunoprecipitation involved measuring the amount of radioactive antigen precipitated by the serum under study in the presence and absence of nonradioactive inhibitor. The serum (100 μl) was preincubated for 30 min at 37° with 100 μl of nonradioactive inhibitor and for an additional 30 min at 37° with 100 μl of the test antigen. Both the inhibitor and the antigen were dissolved in phosphate-buffered saline, pH 7.2. An identical test without inhibitor was also performed. Controls included serum without either antigen or inhibitor, serum with inhibitor, and antigen without serum or inhibitor. All determinations were run in duplicate. The tubes were incubated at 4° overnight and centrifuged at 27,000*g* for 10 min. The supernatants were decanted, and the precipitates were washed twice with 2 ml cold phosphate-buffered saline, and freeze-dried. Each precipitate was dissolved in 0.2 ml of 0.1 *N* NaOH. This dissolved precipitate was added to 10 ml of scintillation fluid and counted in a liquid scintillation counter.

Identification of antipeptidoglycan in human sera used in this report is based on the following experimental model (6): serum of rabbits hyperimmunized with *S. pyogenes* group A variant precipitated the radioactive test antigen. This reaction was inhibited by pretreating the sera with Inhibitor I, and in another series of experiments with Inhibitor II. It was found that the degree of inhibition was always higher with Inhibitor I (67–80%) than with Inhibitor II (30–49%). When this technique of inhibition of radioimmunoprecipitation was applied to human sera, it was designated double inhibition. A serum was considered positive for antipeptidoglycan when the precipitation was inhibited by both inhibitors, but to a higher degree by Inhibitor I than by Inhibitor II. For instance, a positive serum from one of the patients with impetigo precipitated 2.3 μg antigen/ml; the precipitation was inhibited 57% with Inhibitor I and 27% with Inhibi-

TABLE I. The Nature of the Test Antigen and the Inhibitors.

Substance and source	Antigen	Determinant	Immunodominant group
Test antigen *M. lysodeikticus*	peptidoglycan	glycan[a]	β-*N*-acetylglucosamine
		peptide[b]	N^{α}-D-alanyl-D-alanine
	teichuronic acid		not defined
Inhibitor I *S. aureus*	peptidoglycan	glycan[a]	β-*N*-acetylglucosamine
		peptide[c]	N^{α}-D-alanyl-D-alanine
Inhibitor II *S. aureus*	peptidoglycan	peptide[c]	N^{α}-D-alanyl-D-alanine

[a] Built of repeating units of β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl-.

[b] N^{α}-L-Alanyl-γ-(α-D-glutamyl-Gly)-L-lysyl-D-alanyl-D-alanine.

[c] N^{α}-L-Alanyl-γ-(α-D-glutamyl-NH_2)-L-lysyl-D-alanyl-D-alanine.

TABLE II. Antipeptidoglycan in Fifteen Rheumatic Fever Patients With and Without Carditis.

Sera	Carditis		Agreement of carditis[a] and antipeptidoglycan
	Present	Absent	
Antipeptidoglycan positive	7	3	7
Antipeptidoglycan negative	0	5	5
Total number	7	8	12

[a] Chi-square test: χ^2 (1) = 4.257, $p < 0.05$.

tor II. A negative serum from one of the patients with rheumatic fever precipitated 3.7 μg of antigen/ml; the precipitation was inhibited 49% by Inhibitor I but not inhibited by Inhibitor II.

Results. Table II shows the presence or absence of antipeptidoglycan in pooled serum specimens of the 15 patients with rheumatic fever. It also shows the presence or absence of carditis in the 15 patients and the agreement between antipeptidoglycan and carditis. All seven patients with carditis had antipeptidoglycan; of eight patients without carditis, five did not have these antibodies. Statistical evaluation showed a significant difference between the relative agreement and nonagreement of antipeptidoglycan and carditis.

Table III shows the distribution of antipeptidoglycan in the patients with rheumatic fever and controls. Of the 15 sera of rheumatic fever patients, ten had antipeptidoglycan. Of the ten control patients with impetigo, all but one were positive, but of the 17 control healthy individuals, only six were positive. The Chi-square test showed a significant difference between the distribution of antipeptidoglycan in the three groups.

Table III also shows the difference in the mean amounts of antigen precipitated by the sera of patients with rheumatic fever, impetigo, and healthy individuals. The dispersion of the amounts of antigen precipitated within each group is measured by standard deviation. The amounts of antigen were smallest in the rheumatic sera and greatest in the sera of healthy individuals. The analysis of variance showed a significant F ratio. The specific locus of significant difference was then determined by the Student's t tests. The difference between the mean amounts of antigen precipitated by the sera of the three groups, but not between the amounts precipitated by the rheumatic and impetigo sera, were significant.

Discussion. M. lysodeikticus cell wall was selected as the test antigen in the present study because its chemical composition is relatively simple. It is composed of peptidoglycan (approximately 80%) and teichuronic

TABLE III. Distribution of Antipeptidoglycan[a] and Precipitation of the Test Antigen[b] in Rheumatic and Control Sera.

Sera		A Rheumatic fever patients	B Impetigo patients	C Healthy individuals
Number of antipeptidoglycan positive		10	9	6
Total number		15	10	17
Antigen precipitated/ml serum (μg)	Mean	1.92	4.43	7.71
	Standard deviation	1.38	2.19	4.99

[a] Chi-square test: χ^2 (2) = 8.189, $p < 0.02$.

[b] Analysis of variance: F (2, 39) = 13.38, $p < 0.001$; A/B: $t = 1.92$, $p \simeq 0.07$. Student's t tests for C/B: $t = 2.39$, $p < 0.05$; C/A: $t = 5.06$, $p < 0.001$.

acid built of equimolar proportions of 2-acetamido-2-deoxymannuronic acid and glucose (14). Furthermore, the peptidoglycan cross-reacts with peptidoglycan of *S. pyogenes* (6). However, peptidoglycan cross-reacts with other antigens, such as C polysaccharide of *S. pyogenes* group A (7). Furthermore, when *M. lysodeikticus* cell wall was used as a test antigen with a variety of human sera, we found precipitation in nearly all instances. Thus, in 9 of 17 serum specimens from healthy individuals in which there was no inhibition with either inhibitor, the amounts of precipitated antigen were generally large. The precipitation of the test antigen by human sera suggests that they contain other antibodies cross-reacting with *M. lysodeikticus* antigens, or that the precipitation is nonspecific. In an attempt to explain the precipitation, serum from a healthy individual was subjected to extensive additional studies. This serum was selected because it precipitated a relatively large amount (15 μg) of test antigen, but the inhibition tests were negative both with Inhibitors I and II. The additional studies included a series of inhibition tests with substances obtained from *M. lysodeikticus* cell wall by a variety of treatments. All substances which contained teichuronic acid inhibited the precipitation, whereas purified peptidoglycan did not. These observations suggest that the precipitation of the test antigen by human sera is due, at least in part, to cross-reactivity with teichuronic acid. It is unclear, however, why the amounts of precipitated antigen were relatively small with impetigo sera of which all but one contained antipeptidoglycan and in rheumatic fever sera irrespective of presence or absence of antipeptidoglycan. In this connection it is to be noted that the rheumatic fever patients and the two control groups were not comparable with regard to age, sex, and race. However, there was no significant difference between the rheumatic patients with carditis and those without carditis with regard to the same three variables.

The results of the test for antipeptidoglycan in the sera of the 15 patients with rheumatic fever showed a significant difference between relative agreement and nonagreement of antipeptidoglycan and carditis. If this observation were to be confirmed in studies of a larger number of rheumatic fever patients, it would suggest that peptidoglycan plays a role in the pathogenesis of rheumatic carditis. Any hypothesis implicating peptidoglycan in the pathogenesis of rheumatic carditis must take into consideration the fact that patients with impetigo having antipeptidoglycan in their sera do not develop carditis (15). It is likely, therefore, that if peptidoglycan plays a role in the development of rheumatic carditis, there must also be one or more additional factors which influence the localization of peptidoglycan in the heart in some cases of group A streptococcal pharyngitis. These factors must be absent in impetigo.

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