INFECTION AND IMMUNITY, July 1971, p. 79-84

Vol. 4, No. 1
Printed in U.S.A.

# Cross-Protective Antigens of Group A Streptococci Types 3 and 31 and Types 46 and 51

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Received for publication 22 January 1971

Reciprocal cross-protective antigens have been demonstrated between types 3 and 31 cocci, and one-way cross-protective antigens have been demonstrated between types 46 and 51 cocci. The reciprocal cross-protective antigen of types 3 and 31 is distinct from the specific M protein of either type. In the one-way cross relationship, type 46 cocci contain both type 46 and type 51 M proteins and therefore stimulate protective antibodies against both types. Type 51 cultures contain only the homologous M antigen. These relationships were demonstrated by capillary precipitin tests, indirect bactericidal tests, and in agar-gel diffusion patterns. The practical significance of these relationships in the serological typing of group A streptococci is discussed along with their possible role in immunity to streptococcal infections in man.

The M protein of group A streptococci is responsible for the serological specificity of the 50 or more recognized serotypes of these organisms. This antigen also stimulates the production of protective antibodies in humans and animals after a natural infection or vaccination. At one time it was thought that each serotype contained a specific M protein; however, in recent years several investigators have reported the presence of protective antibodies in hyperimmune serum against one or more heterologous types after the vaccination of rabbits with one serotype. In addition, Fox and Wittner (2) reported cross-protective antibodies in serum from children with upper respiratory infections caused by either types 3 or 12 group A streptococci. Common protective antigens have been reported between the following established types: types 2 and 48 (9), 3 and 12 (2), 13 and 48 (6), 14 and 51 (11), and 33, 41, 43, 52, and 53 (12). In addition, Wiley and Bruno (12, 13) have described cross-protective antigens between established types and several uncertain types of group A streptococci.

Cross-protective antigens apparently are not uncommon among the group A streptococci. A knowledge of these relationships is important to investigators working in the area of immunity to streptococcal infections as well as to those involved in the preparation of specific anti-M sera used in epidemiological studies of streptococcal infections. Cross-protective antigens between types 3 and 31 and between types 46 and 51 are described. The former is a reciprocal cross, whereas the latter is a one-way cross.

## MATERIALS AND METHODS

**Cultures.** The group A cultures used in this study are listed in Table 1. Stock cultures were maintained in defibrinated rabbit blood at −60 C. Acid extracts were prepared from Todd-Hewitt broth cultures incubated at 37 C overnight.

**Serological tests.** The capillary precipitin tests were performed by the method of Swift, Wilson, and Lancefield (10) with crude hot-acid extracts and alcohol-precipitated M extracts (4). Agar-gel double-diffusion tests were done in 1% Ionagar in saline by using optimal dilutions of 10-fold concentrated alcohol-precipitated M extracts and either absorbed or unabsorbed antiserum. The indirect bactericidal tests were carried out as previously described (1, 6). Lancefield (6) has shown that the indirect bactericidal test reflects the same antigen-antibody reaction as the mouse protection test and can be used to identify either protective M antigen or its antibody.

**Preparation of antisera.** New Zealand white rabbits were inoculated intravenously with heat-killed vaccines (5). A vaccination series consisted of 3 inoculations per week for a total of 18 inoculations. When an adequate serological response was not obtained during the first series of inoculations, the rabbits were rested for 2 months and the inoculation series was repeated with a fresh vaccine. The vaccine strains used for each type are indicated by a footnote in Table 1. Grouping antibodies were removed from the antisera by absorptions with heterologous heat-killed cells at a ratio of one part wet-packed cells to three parts of antiserum. Absorption was carried out at 37 C for 50 min, and the absorbing cells were

TABLE 1. *Source and designation of strains*

| M type | Strain no. | Source |
|---|---|---|
| 3 | SS265[a] | CDC[b] diagnostic culture, 1950 |
| 3 | B930/24 | R. Lancefield |
| 3 | DS-3590-70 | CDC diagnostic culture, 1970 |
| 3 | DS-3161-70 | CDC diagnostic culture, 1970 |
| 31 | J137/34[a] | R. Lancefield |
| 31 | J137/69 | R. Lancefield |
| 31 | DS-1243-70 | CDC diagnostic cultures, 1970 |
| 31 | DS-3544-70 | CDC diagnostic culture, 1970 |
| | D58X | R. Lancefield, M negative, contains 3R antigen |
| 46 | R52.678 | Colindale |
| 46 | C105[a] | R. Lancefield |
| 51 | AD309/60[a] | Grove G. Wiley |
| 14-51 | R47.471 | Colindale |
| 14-51 | 14/25 | R. Lancefield |
| 14 | Co8199 | Grove G. Wiley |

[a] Strains used as vaccines.
[b] Center for Disease Control.

removed by centrifugation. Reciprocal absorptions were carried out in the same manner with the appropriate cells.

## RESULTS

**Reciprocal protective antigens between types 3 and 31.** Unabsorbed antisera collected from 14 rabbits given one series of inoculations with type 3 cells were bactericidal for types 3 and 31 cocci. After the grouping antibodies were removed, these antisera gave strong precipitin reactions with acid extracts of types 3 and 31 cells as well as with extracts of types 12, 39, 52, and 55 cells. Bactericidal tests were negative for the latter four types. Absorption of type 3 antiserum with type 31 cells removed the precipitin and bactericidal activity for type 31 cocci but did not affect these reactions for the homologous cocci (Table 2). Cross-precipitin reactions against the other serotypes were removed by absorption with strain B3264 which contains the B antigen described by Hambly (3).

In the reciprocal relationship, unabsorbed antisera collected from nine rabbits given one series of inoculations with type 31 cells were bactericidal for types 3 and 31 cocci. After the grouping antibodies were removed, these antisera gave strong precipitin reactions with acid extracts of types 3, 31, and 12 cells. These antisera were not bactericidal for type 12 cocci. Absorption of the type 31 antisera with type 3 cells removed the precipitin and bactericidal activity for type 3 cocci, as well as the type 12 precipitin reaction, without affecting the homologous reactions (Table 2).

Three consecutive absorptions of type 3 antiserum with type 31 cells and vice versa (Table 2) did not significantly reduce the homologous precipitin or bactericidal activity of either antisera. This finding indicates that the common protective antigen is distinct from the specific M antigen of either type.

Results of agar-gel diffusion studies on unabsorbed types 3 and 31 antisera are shown in Fig. 1. Types 3 and 31 antisera were placed in wells 1 and 2, respectively, and concentrated extracts of types 31, 3, and 31 were placed in wells 3, 4, and 5, respectively. The precipitin lines between the two antisera and type 3 antigen appear to be lines of partial identity. However, since multiple absorptions of either antisera with heterologous cells did not significantly reduce the homologous reactions, the heavy precipitin line between type 3 antiserum and antigen probably represents both the type 3 M antigen plus the common protective antigen. Additional evidence for this may be seen (Fig. 1) when each antiserum is reacted with both antigens. For example, lines of nonidentity are formed when type 31 antiserum (well 2) is reacted against extracts of types 3 and 31 cells (wells 4 and 5, respectively). Attempts to separate these lines by adjusting the concentration of reactants were unsuccessful because the heterologous reactions were lost when either the antiserum or antigens were diluted.

Figure 2 shows the agar-gel patterns obtained when absorbed and unabsorbed type 3 antisera (wells 1 and 2, respectively) are reacted against extracts of types 31, 3, and 31 cells (wells 3, 4, and 5, respectively). Lines of identity are formed between the two antisera and type 3 antigen. A precipitin line can also be seen between the unabsorbed serum and type 31 extract (wells 2 and 5, respectively). The line is not present between the absorbed serum and type 31 extract (wells 1 and 3, respectively). The precipitin lines between absorbed and unabsorbed antisera (wells 1 and 2) are apparently due to soluble type 31 antigens that remain in the absorbed antiserum after absorption with type 31 cells. Precipitin lines similar to those in Fig. 2 are obtained when absorbed and unabsorbed type 31 antisera are reacted against types 31 and 3 antigens.

To ensure that these results were not due to mixed cultures, 24 isolated colonies each of type 3 and type 31 strains were transferred to Todd-Hewitt broth, and acid extracts were prepared from overnight cultures. When tested in the

TABLE 2. *Precipitin and bactericidal tests with types 3 and 31 antisera*

| Antisera | Precipitin tests[a] | | Bactericidal tests[b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | B930/24 type 3 | | | | J137/69 type 31 | | | |
| | B930/24 type 3 | J137/64 type 31 | TM[c] | 122 | 28 | 3 | 232 | 40 | 11 | 4 |
| Normal rabbit | | | L | L | L | L | L | L | L | L |
| Unabsorbed type 3 | 4+ | 4+ | 55[d] | 35 | 0 | 0 | 8 | 3 | 0 | 0 |
| Absorbed once with 31 cocci | 4+ | – | 0 | 0 | 0 | 0 | L | L | PL | 615 |
| Absorbed three times with 31 cocci | 4+ | – | 8 | 0 | 0 | 0 | L | L | L | PL |
| | | | 396[c] | 148 | 35 | 12 | TM | 373 | 59 | 18 |
| Normal rabbit | | | L | L | PL | PL | L | L | PL | 595 |
| Unabsorbed type 31 | 3+ | 4+ | 40 | 0 | 0 | 0 | 25 | 10 | 3 | 0 |
| Absorbed once with 3 cocci | – | 4+ | L | L | L | PL | 8 | 0 | 0 | 0 |
| Absorbed three time with 3 cocci | – | 4+ | L | L | L | PL | 60 | 32 | 0 | 0 |

[a] Precipitin reactions graded from – to 4+.
[b] L, PL, TM, stand for laked, partially laked, and too many to count, respectively.
[c] Inoculum.
[d] Numbers are colony counts from 0.1 ml multiplied by 4 (total volume in each tube).

capillary precipitin tests, each of these extracts gave the same cross-reactions with unabsorbed antisera as those described above. The common protective antigen may represent a unique situation in the particular strains of types 3 and 31 used for this study. Two strains each of types 3 and 31 isolated in 1970 from different parts of the United States were obtained from the Streptococcal Laboratory of the Center for Disease Control. Acid extracts of these four isolates gave a strong precipitin reaction with unabsorbed type 3 and 31 antisera. In the reciprocal bactericidal test, one isolate of each type was phagocytized by both of the unabsorbed antisera. Thus, this cross-protective relationship is probably not the result of mixed cultures or of an unusual antigen present in the two vaccine strains.

Absorbed and unabsorbed types 3 and 31 antisera were also reacted with acid-extracts of type 3 strain D58X. This strain contains the 3R antigen but no type 3 M antigen (7). No precipitin reaction was observed with this extract. This lack of reaction indicates that these antisera do not contain antibodies to the 3R antigen.

**One-way protective antigen between types 46 and 51.** Unabsorbed antisera collected from rabbits that received two complete series of inoculations

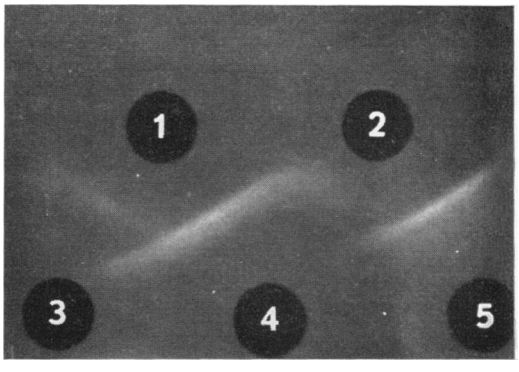


FIG. 1. *Agar-gel tests showing cross-precipitin reactions between types 3 and 31 unabsorbed antisera. Well 1, type 3 serum; well 2, type 31 serum; wells 3 and 5, type 31 acid extract; well 4, type 3 acid extract.*

with alkaline-treated type 46 vaccine (1) were bactericidal for types 46 and 51 cocci but not for type 14–51 cocci (Table 3). After absorption with type 6 cells to remove grouping antibodies, these antisera gave a 2+ to 4+ capillary precipitin reaction with acid extracts to types 46, 14–51, and 51 cells. Absorption of the antisera with either type 51 cells or type 14–51 cells removed the

precipitin and bactericidal activity for type 51 cocci as well as the precipitin reaction with type 14–15 extracts but did not affect the homologous reactions (Table 3).

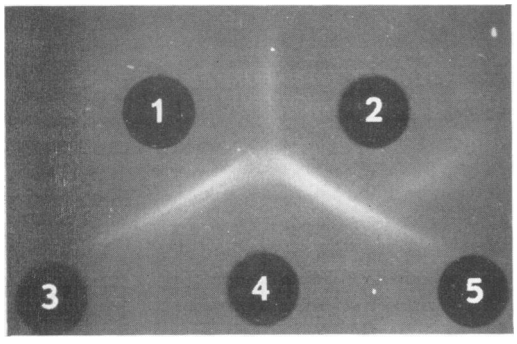


FIG. 2. *Agar-gel tests showing precipitin reaction with absorbed and unabsorbed (wells 1 and 2, respectively) type 3 antisera. Wells 3 and 5, type 31 acid extracts; well 4, type 3 acid extract.*

Unabsorbed antisera collected from rabbits vaccinated with heat-killed type 51 vaccine were bactericidal for type 51 cocci only (Table 3). After absorption with type 18 cells, these antisera gave a 2+ precipitin reaction with types 46 and 14–51 acid extracts and a 4+ precipitin reaction with type 51 extracts.

Agar-gel diffusion studies on unabsorbed type 46 antiserum are shown in Fig. 3. A heavy precipitin line is present between the antiserum (well 1) and concentrated extracts of type 46 cells (wells 2 and 5). The two additional precipitin lines present in Fig. 3 are lines of identity formed when the antiserum is reacted against concentrated extracts of types 46, 51, 14–51, and 46 cells (wells 2, 3, 4, and 5, respectively). The latter lines indicate the presence of common antigens in acid extract of cells of all three types. Absorption of this antiserum with either type 14–51 or type 51 cells leaves only the heavy precipitin line between the type 46 antiserum and type 46 extracts. These results plus the bactericidal data (Table 3) show

TABLE 3. *Precipitin and bactericidal tests with types 46 and 51 antisera*

| Culture | Antisera | Precipitin tests[a] | Bactericidal tests | | | |
|---|---|---|---|---|---|---|
| | | | 393[b] | 103 | 21 | 1 |
| Type 46 (R52.678) | Normal rabbit | | L[c] | L | L | 876[d] |
| | Type 51 unabsorbed | 2+ | L | L | PL | 776 |
| | Type 46 unabsorbed | 4+ | 8 | 8 | 0 | 0 |
| | Type 46 absorbed with 51 cocci | 4+ | 4 | 0 | 0 | 0 |
| | Type 46 absorbed with 14–51 cocci | 4+ | ND | | | |
| | | | TM[b] | 164 | 37 | 10 |
| Type 51 (AD309/60) | Normal rabbit | | L | L | L | L |
| | Type 46 unabsorbed | 4+ | 24 | 12 | 0 | 0 |
| | Type 46 absorbed with 51 cocci | – | L | L | L | TM |
| | Type 46 absorbed with 14–51 cocci | – | L | L | L | L |
| | Type 51 unabsorbed | 4+ | 4 | 0 | 0 | 0 |
| | | | TM[b] | 161 | 43 | 10 |
| Type 14–51 (R47.471) | Normal rabbit | | L | L | TM | 516 |
| | Type 46 unabsorbed | 2+ | L | L | PL | PL |
| | Type 51 unabsorbed | 2+ | L | L | TM | 448 |
| | Type 14 | 4+ | 0 | 0 | 0 | 0 |

[a] Precipitin reactions graded from – to 4+.
[b] Inoculum.
[c] L, PL, TM, ND, stand for laked, partially laked, too many to count, and not done, respectively.
[d] Numbers are colony counts from 0.1 ml multiplied by 4 (the total volume in each tube).

the presence of types 46 and 51 antibodies in antiserum prepared with type 46 vaccines. This finding indicates that type 46 cells contained both type 46 and type 51 M antigens. Further evidence for this conclusion is seen when absorbed type 51 antiserum is reacted against concentrated extracts of types 51, 14–51, and 46 cells in agar-gel diffusion tests. Precipitin lines of identity are formed with all of the extracts (Fig. 4).

Not all extracts of type 46 cells react in the capillary precipitin test with type 51 antiserum. In retrospect, we noted that most of the extracts prepared several years ago reacted with absorbed type 51 antiserum, but recently prepared extracts did not. This observation was also made by Wiley and Bruno (*personal communication*), who found that the presence of this cross-reaction was dependent upon the particular lot of Todd-Hewitt broth used for growing the cells. Thus, acid-extracts of fresh isolates of type 46 strains may or may not react with type 51 antiserum.

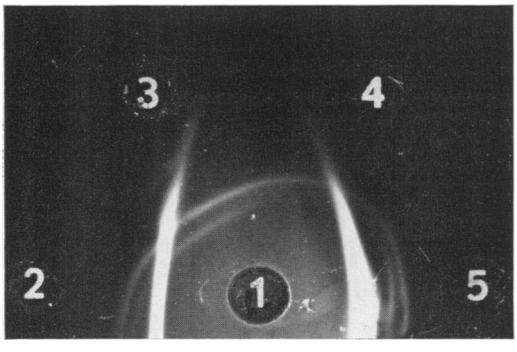


FIG. 3. *Agar-gel tests with type 46 unabsorbed antiserum. Well 1, type 46 antiserum; wells 2 and 5, type 46 acid extracts; well 3, type 51 acid extract; well 4, type 14–51 acid extract.*

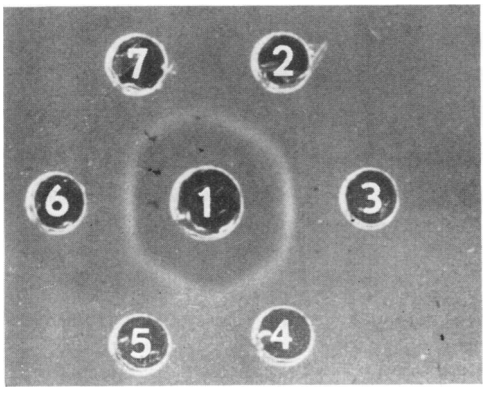


FIG. 4. *Agar-gel tests showing precipitin lines of identity when absorbed type 51 antiserum (well 1) is reacted against acid extracts of type 51 (wells 2 and 5), type 14–51 (wells 3 and 6), and type 46 (wells 4 and 7).*

## DISCUSSION

The presence of reciprocal and one-way protective antigens in group A streptococci has been reported in several serotypes other than those cited in this paper. The reciprocal relationship was observed by Lancefield in certain strains of types 2 and 48 (9) and by Wiley and Bruno in types 43 and 52 (12) and in type 41 and a strain of uncertain type labeled G1 (13). Cross-absorption of antisera against each of these types removed the heterologous bactericidal and precipitin antibodies without significantly affecting these properties for the homologous antigens. In this paper, we have reported this same reciprocal relationship in types 3 and 31 cocci. The results of cross-absorption experiments clearly show that the cross-protective antibodies are stimulated by an antigen that is distinct from the specific M antigen of either type. Since one of the major characteristics of an M protein is the ability to stimulate protective antibodies, it seems reasonable to assume that these cross-protective antigens are also M proteins.

Antigens that stimulate cross-productive antibodies which act in only one direction have been reported in types 13 and 48 (6), types 14 and 51 (11), types 43 and 41 (12), and, in this paper, in types 46 and 51. The presence of bactericidal antibodies for type 46 and type 51 cocci in unabsorbed type 46 antiserum and the absence of reciprocal bactericidal antibodies in type 51 antiserum are analogous to the one-way cross reported by Wiley and Wilson for types 14 and 51 (11). Thus, although one can conclude that both types 14 and 46 cocci may contain type 51 M protein in addition to their homologous M protein, its presence in the two types appears to be dependent upon different factors. In a study of 42 strains previously identified as type 14, Wiley and Wilson (11) found that 37 of the strains possessed both type 14 and type 51 M protein, 4 strains had only type 51 M protein, and 1 strain had only type 14 M protein. Representive strains of each type, that is, types 14, 14–51, and 51, were isolated from patients with streptococcal infections. These isolations demonstrated the presence of these types in nature. The 46–51 relationship, on the other hand, may be dependent on environmental factors, since some acid extracts of type 46 cells contain both types of M protein, whereas other extracts of the same strain contain only type 46 M protein. As noted earlier, Wiley and Bruno (*personal communications*) found that the

presence or absence of type 51 M protein in type 46 cells is dependent upon the particular lot of Todd-Hewitt broth used. The practical significance of this observation in the serological typing of diagnostic cultures is not known. However, acid extracts of cultures of unknown type that give positive precipitin reactions with type 51 antiserum should be checked with specific types 14 and 46 antisera to ensure the correct serological type of the culture.

The evidence for cross-protective antibodies in sera obtained from humans after streptococcal infections is equivocal at this time. Lancefield (8) investigated the bactericidal activity of approximately 40 human sera from individuals who had known streptococcal infections as long as 32 years prior to her study. Two of the individuals in that study had infections with either type 2 or type 13 organisms, types which were known to stimulate cross-protective antibodies against type 48 in animals (6, 9). The serum from the individual with a type 13 infection had some bactericidal activity against type 48 cocci, but the other serum was negative for these organisms. However, in both instances, the known infections had occurred 22 years before Lancefield's (8) study; therefore, it is difficult to interpret the significance of the presence or absence of cross-protective antibodies in these sera. Fox and Wittner (2) studied the sera from 11 children with types 3 or 12 streptococcal upper respiratory infections for cross-protective antibodies against these two types. One of the sera from a child with type 12 infection was bactericidal for type 3 cocci but negative for type 12 cocci. Sera from two children with type 3 infections were bactericidal for type 12 cocci, but only one of these was bactericidal for the homologous infecting type. Again, interpreting the significance of these observations is difficult, since Lancefield (8) has shown that type-specific bactericidal antibodies may persist for at least 32 years after an infection and that bactericidal antibodies for a particular type may be present in sera from individuals with no previous history of an infection with that type.

We have examined two sera from one individual, taken approximately 1 year apart, that were strongly bactericidal for type 3 cocci but negative for types 12 and 31 cocci. During a 4-year study, Wilson and Zimmerman (*personal communication*) had isolated type 1 cultures from this individual several times. They had also demonstrated type 1 bactericidal antibodies in his sera but had not tested for type 3 antibodies, since type 3 cultures had not been isolated from him.

It is apparent that sufficient data are not available to determine what role, if any, cross-protective antibodies play in streptococcal immunity in man. Information in this area is desirable, however, for a better understanding of immunity to streptococcal infections and for development of vaccines for immunization of humans. Such data are difficult to obtain because the bactericidal test is cumbersome to run. It is difficult, therefore, to check sera against a large number of cultures of known serotype. A knowledge of those serotypes that stimulate cross-protective antibodies in hyperimmune rabbit sera should simplify investigations of this nature.

ACKNOWLEDGMENTS

The authors wish to thank R. A. Zimmerman and Elizabeth Wilson, Streptococcal Disease Section, CDC, Fort Collins, Colorado, for the human sera used in this study and for supplying data they had previously obtained on this patient.

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