CROSS-REACTIONS AMONG GROUP A STREPTOCOCCI

I. Precipitin and Bactericidal Cross-Reactions Among Types 33, 41, 43, 52, and Ross

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The precipitin classification of Group A streptococci depends upon the M antigens which are considered serologically specific for each of the many types. These protein antigens are located in the cell wall of the cocci where they function as virulence factors, along with the hyaluronate capsule to some extent, by enabling the cocci to resist phagocytosis and intracellular destruction. As a consequence, the cocci are able to multiply freely in the extracellular fluid of tissues. M antigens can be recognized in precipitin tests with specific M antisera and extracts of the cocci (1), by the long chain reaction (2), or in mouse protection or bactericidal tests (3, 4), in which M antibodies inhibit multiplication of cocci by promoting their phagocytosis. It is on such interaction of M antigen and its antibody that type-specific protection from infection by these organisms depends.

Accumulated evidence, however, indicates that some cross-protection exists among various types. For example, Lancefield described a cross-reaction in bactericidal tests between Types 13 and 48 in which Type 13 antiserum inhibited multiplication of Type 48 cocci as well as that of the homologous organisms, but Type 48 antiserum lacked bactericidal properties for Type 13 cocci (3). She also reported reciprocal reactions in mouse protection tests between Types 2 and 48 (5). Recently, Fox reported a cross-protective relationship in human convalescent sera between Types 3 and 12 (6). Experiments in this laboratory showed that Type 14 cocci, in addition to the homologous Type 14 M antigen, carried another whose antibodies inhibited multiplication of cocci subsequently assigned to Type 51 (7). A recent report from this laboratory also described cross-protective relationships among Type 33 cocci and certain nontypable strains (8). Continued work with these strains and others shows that such cross-protection, as measured in bactericidal tests, is more common than is generally recognized, and, if a corresponding situation exists in vivo, is widespread enough to be a factor in protection against streptococcal infection. The studies that are the subject of this paper include capillary precipitin, agar gel diffusion, and bactericidal tests with strains of Types 33,

41, 43, 52, and certain nontypable strains. Besides strong homologous reactions, the experiments disclosed a complex pattern of cross-reactions in precipitin and bactericidal tests, the strength of which ranged in both tests from slightly less than the stronger homologous reactions to some which were questionable. In agar gel, the precipitin cross-reactions, which were strong enough to test in this way, formed bands which joined with spur formation on the side of the homologous reaction.

Materials and Methods

Strains and Cultures.—The source, type, and T-agglutination reactions of the Group A strains tested in this work are listed in Table I. Long-term preservation of the strains was either in the frozen and dried state or at −60°C in blood broth. Cultures for routine work were inoculated into blood broth, incubated at 37°C overnight, and stored at 4–6°C. At intervals, these blood broth cultures were streaked on tryptose infusion blood agar with hyaluronidase (9) and examined for the development of glossy colonies among the matt colonies. Glossy colonies often indicate M antigen–deficient cocci which do not resist phagocytosis and consequently render the culture unable to multiply vigorously in normal human blood (3). Such mixed cultures are therefore unsuitable for bactericidal tests. When glossy colonies were found, a matt colony was picked to blood broth to provide a stock, working culture.

TABLE I

Group A Streptococcal Strains

Strain	Source		Slide agglutination (anti-T sera)	M type
C107/24*	Type representative	Dr. Lancefield	3, 13, B3264, 33	33
C101/21	" "	"	No agglutination	41
C126/21	" "	"	3	43
A871/14	" "	"	B3264	52
Ross	Impetigo	Dr. Kuttner	3, 13, B3264, 14	?
AD1265	Throat	A. I. du Pont Inst.	3, B3264, 14	?

* The numeral following the diagonal indicates the number of mouse passages.

Streptococcal Extracts.—The capillary precipitin tests were done with M extracts prepared by alcohol precipitation of crude extracts according to the method of Lancefield (1). Concentrated M extracts for the agar gel tests were made by taking up the final alcohol precipitate of M extracts in 0.1 the usual volume of saline.

Antisera and Capillary Precipitin Tests.—Vaccines were prepared by suspending heat-killed cocci (56°C for 30 min) in one-fourth the amount of normal saline as the original volume of culture in Difco Todd-Hewitt broth. Lancefield's immunization schedule was followed in which rabbits received intravenous inoculations the first 3 days of the week and were rested the following 4 days: 0.5 ml/day the 1st wk and 1 ml subsequently. When test bleedings revealed suitable antibody titer, the animals were major bled (40 ml) from the marginal ear vein for not more than three weekly bleedings. After 7 wk of inoculation or three major bleedings, they were rested for 6–8 wk and the immunization schedule repeated. The capillary precipitin tests were performed by the technique of Swift et al. (10) using M extracts and antisera ab-

sorbed free of anti-C, anti-E4 (11), and anti-PGP (12) with heat-killed cocci of Type 1 in the proportion of one part of packed cells to three of serum.

Double Diffusion Tests.—These tests were done as described in a previous publication from this laboratory (11), using optimal dilutions of 10× M extracts and absorbed antisera.

Indirect Bactericidal Tests.—The details of this test are given in another publication from this laboratory (7). Briefly, it consists of rotating mixtures of dilutions of streptococci grown in filtered beef heart infusion broth (0.1 ml), normal rabbit serum or antiserum (0.05 ml), and heparinized normal human blood (0.25 ml) in silicone-stoppered, 7 × 10 mm glass test tubes for 3 hr at 37°C. 0.1 ml of this mixture was then plated in blood agar. After overnight incubation, the amount of growth was reported as L, PL, or TM, depending upon whether the blood was laked, partially laked (confluent hemolysis), or there were too many colonies to count,

TABLE II

Capillary Precipitin Tests

Antisera absorbed		M extracts					
		C107 Type 33	C101 Type 41	C126 Type 43	A871 Type 52	Ross* Type Ross	AD1265 Type Ross
C107	Type 33	++++	++	++	+++	++	++
C101	Type 41	++	++++	+	+	++	++
C126	Type 43	+++	—	++++	++	++	+++
A871	Type 52	—	—	++	++++	++	+++
Ross	Type Ross	+++	++	+++	++	++++	++++

The overnight readings are recorded on a scale of 1–4+.

* This strain carried a serologically distinct M antigen which was identical with strain AD1265. Therefore, these strains are referred to as Type Ross.

respectively. When possible, the colonies were counted and their number recorded. The total number of chains present after rotation may be calculated by multiplying the figure in the tables by four.

Slide Agglutination Tests.—These tests were done using a slight modification (substituting Difco Todd-Hewitt for Hartley broth) of the procedure recommended by the Streptococcal Reference Laboratory, Public Health Laboratory Service, London, England. The sediment from a 48-hr culture grown at room temperature was digested with pancreatic extract (Cole and Onslow) and tested for agglutination after approximately 4 hr and again after digesting overnight.

The T-agglutinating sera were generously supplied by Dr. M. T. Parker and Mr. W. R. Maxted of the Public Health Laboratory Serivce.

Dr. A. A. Ferris, Fairfield Hospital, Melbourne, Australia supplied the Type 33 agglutinating serum.

EXPERIMENTAL

Selection of Antisera.—Different antisera of the same type varied in their capacity to cross-react with one or another of the heterologous strains. Therefore, since only the strongest reactions were included in the data, more than one antiserum may represent the type in Tables II and III. However, the precipitin and corresponding bactericidal test were performed with the same antiserum.

Capillary Precipitin Tests.—The data in Table II, which represent overnight readings, show reciprocal cross-reactions among all of the types represented in the table except for Type 43 serum which failed to react with Type 41 extract and Type 52 serum which did not react with extracts of either Type 33 or Type 41, although the reciprocal reactions for each of these exceptions were positive.

In all cases, the homologous was stronger than the cross-reaction. However, the difference was sometimes small, as in the case of Type 33 serum which gave a 4+ homologous reaction compared with a 3+ cross-reaction with Type 52 extract. Sera which cross-reacted as strongly as this were difficult to absorb

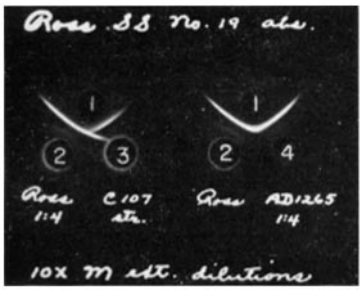

FIG. 1. The test with strains Ross (Type Ross) and C107 (Type 33) is an example of the kind of agar gel precipitin reaction that occurred with many of the cross-reacting strains. For comparison AD1265 (Type Ross) was included to illustrate a reaction of identity with the same experimental arrangement. The upper wells contained absorbed Ross antiserum, the lower wells dilutions of concentrated M extracts of the indicated strains. The homologous, Type Ross, reaction formed a spur with the C107, Type 33, cross-reaction in the first test and joined the AD1265 band in a reaction of identity in the second test. The antiserum contained bactericidal antibodies for all three strains.

free of cross-reacting antibodies without removing the homologous reaction as well. This difficulty was of practical importance in preparing type specific antisera and was most easily circumvented by selecting bleedings as early as possible in the immunization schedule, before the appearance of strong cross-reactions which often developed later than the homologous reactions.

The strength of precipitin cross-reactions did not correspond necessarily with the results of the bactericidal cross-reaction, a discrepancy which will be discussed with the bactericidal tests.

Agar Gel Diffusion Tests.—The precipitin reactions in agar gel revealed a reaction of identity with homologous strains, as would be expected. With the cross-reacting strains, however, the precipitin bands joined with formation of a spur on the side of the homologous reaction. Examples are given in Fig. 1. In both tests, the upper wells contained Ross antiserum. In the first test, the left and right lower wells contained Type Ross and Type 33 extracts, respectively.

The cross-reacting Type 33 precipitin band joined the stronger homologous Ross band with formation of a spur on the side of the homologous reaction. Most of the precipitin cross-reactions which were strong enough to test in agar gel reacted in this way. The second test in Fig. 1 was included as an example of a reaction of identity with this particular experimental arrangement. The precipitin bands with strains Ross and AD1265, considered together as Type Ross, joined in a reaction of identity.

Bactericidal Tests.—Results of these tests are summarized in Table III from which the controls for each test have been omitted in order to simplify presentation of the data. These controls included a tube containing normal rabbit serum and one with heterologous antiserum, usually of Types 3, 6, 14, or 49. In these tubes, inocula as small as five chains multiplied so extensively during 3 hr rotation that blood agar plates inoculated with 0.1 ml of the test mixture often contained too many colonies to count. In contrast, suppression of multiplication in tubes containing cross-reacting antisera ranged from approximately that which occurred with the homologous antisera, sometimes sterilization, to no detectable effect.

Marked cross-protection in these tests was either reciprocal or operated in one direction only. For example, reciprocal protection existed between such combinations as Types 43 and 52 but the bactericidal relationship between others, such as Types 43 and 41, was in one direction only. Type 41 serum strongly suppressed multiplication of Type 43 cocci but the bactericidal effect of six Type 43 antisera tested with Type 41 cocci was feeble or frankly negative.

It should be pointed out that Type 43 bactericidal antigenic determinants were shared by all of the strains since antisera prepared against each of them strongly suppressed multiplication of Type 43 cocci. The same can also be said of Ross cocci whose antisera were bactericidal for all of the strains, although no heterologous sera were markedly bactericidal for Ross cocci in these tests.

In three instances there were discrepancies between the precipitin and bactericidal tests. Positive reciprocal precipitin tests between Types 33 and 41 were associated with questionable or negative bactericidal tests. Also a Type 41 serum which gave a positive precipitin test with Ross cocci lacked bactericidal antibodies for these cocci. The antigens responsible for these positive precipitin tests have not been studied but may represent nonprotective, R antigens of the kind described by Lancefield (5), (13).

Effect of Rabbit Variation and Amount of Immunization on the Development of Cross-Reactive Antibodies.—Cross-reactive antibodies developed more readily in the sera of some rabbits than others and required for their production more immunization than homologous antibodies.

Variation in the immune response of rabbits is illustrated by rabbits 12-11 and 12-12 which were immunized with the same Type 41 vaccine during the same period of time for a total of 9–10 wk with a rest period after 7 wk of

TABLE III

Bactericidal Tests Showing Cross-Protection Among Strains of Types 33, 41, 43, 52, and Ross

Antisera unabsorbed		Culture dilutions of the indicated strains*																	
		C107 Type 33				C101 Type 41				C126 Type 43				A871 Type 52				Ross Type Ross			
C107	Type 33	13‡	3	0	0	PL	TM	126	58	4	2	0	0	TM	5	11	0	PL	96	45	0
C101	Type 41	L	L	PL	175	1	0	0	0	14	0	0	0	PL	PL	189	19	L	L	TM	168
C126	Type 43	TM	60	6	0	TM	TM	119	31	0	0	0	0	327	38	0	0	TM	204	62	4
A871	Type 52	PL	TM	159	22	L	L	PL	166	4	1	0	0	4	0	0	0	PL	TM	28	0
Ross	Type Ross	63	13	0	0	119	19	9	0	1	0	0	0	5	0	0	0	0	0	0	0
Typical growth in:																					
Normal rabbit serum		L	L	PL	TM	L	L	PL	TM	L	L	PL	TM	L	L	PL	TM	L	L	PL	TM
Heterologous serum of Types 3, 6, 14, 49		L	L	PL	TM	L	L	PL	TM	L	L	PL	TM	L	L	PL	TM	L	L	PL	TM

* Typical inocula for the four tubes were approximately 5000, 500, 50, 5 chains.

‡ Numbers indicate the actual number of colonies from 0.1 ml of rotated mixture. L, PL, TM, stand for Laked, Partially Laked, and Too Many colonies to count, respectively.

TABLE IV

Bactericidal Tests Illustrating Difference in Capacity of Rabbits to Develop Cross-Protective Antibody

Antisera obtained after 9–10 wk immunization with Type 41 cocci	C126/21 cocci Type 43			
	Inoculum (chains)			
	8000	800	80	8
	Results			
NRS	L	L	PL	TM
Rabbit 12-11*	14	0	0	0
Rabbit 12-12*	L	L	TM	41

* Both antisera gave strong homologous (Type 41) bactericidal reactions.

TABLE V

Bactericidal Tests Illustrating the Prompt Appearance of Homologous Protective Antibody During Immunization and the Delayed Appearance of Cross-Protective Antibody

Rabbit 42-3 immunized against C107 (Type 33)	C107 cocci Type 33				C126 Cocci Type 43			
	Inoculum (chains)							
	4440	444	44	4	9100	910	91	9
	Results				Results			
NRS	L	L	PL	TM	L	L	PL	TM
Antiserum after 6 wk	1	0	0	0	L	L	PL	TM
	Inoculum (chains)							
	9300	930	93	9	7100	710	71	7
	Results				Results			
NRS	L	L	PL	TM	L	L	PL	TM
Antiserum after 11 wk	2	0	0	0	226	35	5	0

After 7 wk of immunization, rabbit 42-3 was rested for 6 wk before immunization was resumed.

inoculation (Table IV). Both rabbits developed strong homologous bactericidal antibodies but only No. 12-11 developed cross-protective antibody for Type 43 cocci.

The influence of the amount of immunization is illustrated by the data in Table V. Rabbit 42-3, immunized for 6 wk with Type 33 cocci developed strong

homologous antibody but none for Type 43 cocci. After 6 wk of rest and an additional 5 wk of immunization, bactericidal antibodies for Type 43 cocci were present in the serum.

DISCUSSION

Positive results in bactericidal tests are usually considered evidence of serological identity among strains. It is evident from this work, however, that such is not always the case, and some reassessment of the significance of this property is required. The results may at times indicate a serologically close but not identical relationship.

Comparison of homologous and cross-reactions in these experiments revealed major differences which helped to distinguish between them. The latter were usually weaker than the homologous reactions both in bactericidal and precipitin tests. Sometimes, but not always, the weaker bactericidal cross-reactions were removed by heterologous absorption of the antiserum. This did not occur with the homologous reactions in these experiments. Also, the cross-reactions were present in some antisera with strong homologous reactions and absent from others against the same strain. Usually one or more of these differences existed to indicate the nature of the reaction but sometimes the agar gel test was needed for confirmation and this was especially true when there was reciprocal protection.

There is no way at present to assign nontypable strains, such as Ross and AD1265, to established types even though their M antigens are closely related to those of recognized types. The alternatives have been to assign new type numbers when enough identical strains were encountered to warrant recognition as a type or to classify them by slide agglutination with anti-T sera. The slide agglutination patterns given in Table I show that these strains which are closely related through their M antigens share agglutinating antigens as well. Three strains reacted with one or more sera in the 3, 13, B3264, 33 pattern, and two serologically identical strains, Ross and AD1265, reacted also with the Type 14 serum. One strain, C101/21, Type 41, was nonagglutinable, but four other Type 41 strains in this laboratory's collection reacted as 3, 13, B3264, 33 while one other was nonagglutinable. All of the strains, then, were related by reactions with at least one of five agglutinating sera, 3, 13, B3264, 33, or 14. Top et al. made a comprehensive study of strains with the 3, 13, B3264 agglutinating pattern and found among them, as with the strains in this work, cocci with different M antigens, including strains of Types 41, 52, and provisional new type 53 (14).

SUMMARY

Strains of four streptococcal types, 33, 41, 43, 52, and a nontypable strain, Ross, cross-reacted in precipitin and bactericidal tests. The homologous re-

actions, which determined the type, afforded the major protection and developed promptly and regularly in the serum of rabbits during immunization. The associated cross-reactions, on the other hand, appeared in the serum of certain rabbits only, were often not as strong as the associated homologous reactions, and required for their presence a longer period of immunization than the homologous reactions.

Agar gel analysis of the homologous precipitin reactions revealed, as would be expected, reactions of serological identity, while those cross-reactions which were strong enough to test in this way formed bands of precipitate which joined with spur formation on the side of the homologous reaction.

These experiments and others referred to in the text suggest that cross-protection, as demonstrated in bactericidal tests, is sufficiently widespread to be a factor in streptococcal immunity, if a corresponding protection occurs in vivo. Thus, streptococcal infection with one of the cross-reacting strains might confer, in addition to strong homologous protection, a certain amount of cross-protection.

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