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Antigenic Variation in Group A Streptococci: Types 11 and 9

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A strain of group A *Streptococcus* which was virulent but M-nontypable was isolated from patients in a hospital nursery during an epidemic. This strain, Boston 11, reacted in T-agglutination tests with antisera for types 9 and 11, an unusual combination. A comparison of this strain with Lancefield's M-11 strain (NCDC SS-721) and Alabama 11 (Provisional 61) revealed three serologically related but distinct strains. Antiserum produced with the Boston 11 strain exhibited similar reactivity with all three "11" strains as well as with M-9 (SS-501) as demonstrated in precipitin tests. Immunodiffusion studies indicated that the Boston 11 antigen was partially identical with the M-11 and M-9 strains and shared at least one antigen with the Alabama 11 strain. The Boston 11 antiserum could be made specific for precipitin tests, but bactericidal activity for the Alabama 11, M-11, and Boston 11 strains was essentially negative.

At one time bactericidal activity was thought to be specific for each M antigen. However, in 1957 Lancefield (8) described a cross-reaction in bactericidal tests between types 13 and 48. Type 13 antiserum inhibited multiplication of the type 48 strain as well as that of the homologous strain, but type 48 antiserum lacked bactericidal properties for the type 13 strain. In 1961, Wiley and Wilson (16) described inhibition of type 51 strains by M-type 14 antibodies. In early 1968, Fox and Wittner (3) reported a cross-protective relationship between M-types 3 and 12 in human convalescent sera. Later in 1968, Wiley and Bruno (15) demonstrated precipitin and bactericidal cross-reactions among M-types 33, 41, 43, 52, and Ross. Our studies were undertaken to produce M antiserum to a highly virulent but M-nontypable strain (Boston 11); the results disclosed another example of cross-precipitin and perhaps cross-bactericidal reactions. With the aid of capillary precipitin tests, immunodiffusion, immunoelectrophoresis, and bactericidal tests, we demonstrated that Boston 11 was closely related to Alabama 11, Lancefield's M-11, and Williams' M-9 strains.

MATERIALS AND METHODS

Cultures. In Tables 1 and 3, the source, M type, and T-agglutination pattern of the stock group A and diagnostic strains are listed. All strains were preserved by desiccation (4) or freezing (or both) in blood at −20 C. Cultures for routine work were transferred to 5 ml of Todd-Hewitt (TH) broth (Difco) containing 0.05 ml of defibrinated rabbit blood, and then incubated at 37 C for 16 to 18 hr and stored at 4 C.

Streptococcal extracts. Extracts of all strains were prepared, and precipitin tests were performed against all National Communicable Disease Center (NCDC) streptococcal M-typing antisera for types 1 to 58, Alabama 11, and Boston 11 (7, 12). Refined M extracts of the stock strains were prepared by alcohol-sodium acetate precipitation of the crude extracts, by the method of Lancefield (7).

Suspensions for T-agglutination tests were prepared and typed against NCDC stock T-typing antisera (9). The supernatant fluid of the trypsin-digested suspension was used in immunoelectrophoresis and immunodiffusion tests.

Antisera. Using Becker's method (1) for enhancement of M-protein production, we rotated the Boston 11 culture with human blood and type 11 antiserum for a total of 6 hr. Subcultures revealed matt colonies which were picked to establish the vaccine strains. The vaccine and antiserum were prepared by a modification of Lancefield's technique, as described by Moody et al. (9). Cells were grown for 16 to 18 hr at 37 C in 400 ml of TH broth, centrifuged, heated at 56 C for 30 min, and resuspended in saline one-fourth the original volume of TH broth. Rabbits were immunized as prescribed by Lancefield (8).

To absorb Boston 11 antiserum, we used a slight modification of the method described by Harrell and Ashworth (6). The antiserum was absorbed at a ratio of 1 part broken type 6 cells and glass beads to 13 parts of Boston 11 antiserum. After absorption at 37 C for 1 hr, then at 4 C for 72 hr, the antiserum was absorbed at a 1:5 ratio with type 9 (SS-501) cells for 15 min at 37 C. The serum was chloroformed (14) and then checked for cross-precipitin reactions

TABLE 1. *Source and designation of strains*

M type	T type	NCDC no.	Source	Original designation
9	9	SS-501	R.E.O. Williams	R.51 Holmes
11	11	SS-551	Lancefield	T11/81/1
11	11	SS-721	Ashworth BRS-CDC	0011/B3
Ala 11	9/11	SS-875	Dillon	2998T
Boston 11	9/11	DS-3527-67	Hospital nursery	3814 Desch

against all NCDC stock M extracts. Included in this check were extracts of types 28, 34, 44 (SS-510), 48, Provisional 59, 60, and 61 (Alabama 11).

Antiserum to Alabama 11 was supplied by Hugh Dillon.

Indirect bactericidal tests. Indirect bactericidal tests were performed as described by Lancefield (8). A 10^{-5} dilution of a 2-hr TH broth culture of the strain to be tested was made in TH broth and further diluted 1:4, 1:16, and 1:64 in TH broth. A 0.1-ml amount of each dilution was plated to determine the inoculum size. A 0.1-ml amount of each of the three dilutions, 0.05 ml of antiserum, or normal rabbit serum (NRS), and 0.3 ml of lightly heparinized, freshly collected whole blood were placed in Siliclad (Clay-Adams) tubes (12 by 75 mm). The rubber-stoppered tubes were then rotated for 3 hr at 7 rev/min at 37 C. After rotation, melted blood-agar was inoculated with 0.1 ml and poured into plates. Colonies in the poured plates were counted after incubation overnight at 37 C.

Immunoelectrophoresis. Crude and refined Lancefield extracts and the supernatant fluid from T-agglutination suspensions were electrophoresed by using a modification of Peetoom's technique (11). Glass slides [1 by 4.5 inches (2.54 by 11.43 cm)] were precoated with sterile 3% Noble agar in distilled water and then dried at 37 C. The slides were layered with 4 ml of 0.6% agarose (Fisher) in 75 ml of 0.05 M sodium barbital buffer, *p*H 8.6, and 25 ml of distilled water. Pelon wicks were soaked in buffer for 20 to 30 min and then placed on the agarose slide. Electrophoresis was carried out at room temperature at 12.5 ma/slide for 30 min. An antiserum trough was cut and filled, and immunodiffusion was developed at 4 C for up to 72 hr.

Agarose double diffusion. Slides [2 by 3 inches (5 by 7.6 cm)] were prepared for double-diffusion tests, and the same agarose support used in immunoelectrophoresis was employed. Slides were developed at 4 C for up to 72 hr.

RESULTS

Capillary precipitin typing of SS-501, SS-721, and SS-875 against NCDC and Alabama 11 antisera yielded only strong homologous reactions. The Boston 11 extracts, however, crossed 1–2+ with the Alabama 11 antiserum.

In T-agglutination tests, the Boston 11 strain reacted strongly with Z and W pools and with types 9 and 11 in the respective pools. Single-colony isolates reacted in the same manner. The strength of reactions with monospecific antisera was not affected by testing antigens subjected to different incubation temperatures (10) or by heating at 50 C for 15-min intervals up to 1 hr. This 9/11 T-pattern was also demonstrated with the Alabama 11 strain

Two attempts were made to produce specific bactericidal and precipitin Boston 11 antiserum; three rabbits were used each time. Rabbits were bled in the 5th, 7th, 9th, and 25th weeks of the immunization schedule. Immunization with the Boston 11 vaccine yielded antiserum which precipitated equally well with alcohol-precipitated extracts of SS-721, SS-501, SS-875, and with Boston 11. The serum from each bleeding was trial-absorbed by using various cells, cells-to-sera ratios, times, and temperatures. Absorption with SS-721 or SS-875 removed the homologous as well as the cross-precipitin reactions. Of the six rabbits used, only one rabbit produced antiserum which showed any indication of bactericidal activity and which could be absorbed without loss of the homologous Boston 11 reaction. This absorbed antiserum, when checked against NCDC stock extracts, was found to be specific for Boston 11.

Even though the antiserum could be made specific as demonstrated by precipitin tests, the question of specific bactericidal activity remained. The bactericidal activity of absorbed homologous antisera and absorbed Boston 11 antiserum against strains SS-501, SS-721, SS-875, and Boston 11 is shown in Table 2. If Potter's bactericidal index (*personal communication*) is used as an indicator of bactericidal activity, then only strain SS-875 against Alabama 11 antiserum with an index of 69 would have any bactericidal activity. The bactericidal index is as follows: (number of organisms inoculated/number of organisms after incubation with test serum) ÷ (number of organisms inoculated/number of organisms after incubation with NRS). An index of 50 indicates bactericidal activity; below 50, questionable or no bactericidal activity; above 50, moderate to strong activity.

TABLE 2. *Bactericidal activity of antisera produced with various M-11 group A streptococci*

Strain	Determination	Plate counts resulting from various serum dilutions[a]		
		10^{-5}/1:4	10^{-5}/1:16	10^{-5}/1:64
SS-721 (Stock 11)	No. of chains inoculated	130	32	8
	No. of chains at end of test with normal human blood and			
	(a) Boston 11 antiserum	1,200	300	70
	(b) Normal rabbit serum	L	PL	300
SS-875 (Alabama 11)	No. of chains inoculated	340	120	30
	No. of chains at end of test with normal human blood and			
	(a) Boston 11 antiserum	1,020	250	40
	(b) NCDC 11 antiserum lot 4	1,600	400	90
	(c) Ala-11 antiserum	100	20	4
	(d) Normal rabbit serum	L	1,900	400
Boston 11	No. of chains inoculated	190	50	20
	No. of chains at end of test with normal human blood and			
	(a) Boston 11 antiserum	300	100	30
	(b) NCDC 11 antiserum lot 4	400	250	10
	(c) Ala-11 antiserum	600	200	60
	(d) 9 Antiserum-experimental	1,200	500	100
	(e) Normal rabbit serum	1,200	600	80
SS-501 (Stock 9)	No. of chains inoculated	320	80	20
	No. of chains at end of test with normal human blood and			
	(a) Boston 11 antiserum	PL	2,000	500
	(b) Normal rabbit serum	L	PL	520

[a] L, laked; PL, partially laked.

Type 9 antiserum had no bactericidal activity against the Boston 11 strain, although the type 9 antiserum had previously been found to be bactericidal for homologous type 9 strains. When the Boston 11 strain was tested against Boston 11 antiserum, NCDC stock 11 antiserum, and Alabama 11 antiserum, the bactericidal indices were from 1.3 to 8, indicating little if any bactericidal activity. Even though the indications of bactericidal cross-reactivity were slight, the results could be repeated.

When bactericidal tests between the Boston 11 strain and all NCDC M-typing antisera were set up, no cross-bactericidal activity could be demonstrated.

Agarose double-diffusion tests of unabsorbed Boston 11 antiserum against all NCDC stock M extracts indicated that only the crosses of SS-501, SS-551, SS-721, and SS-875 existed. This included checks against strains known to contain a large amount of R antigen (1) and those which contain a large amount of B antigen as described by Hambly (5). The diagrammatic sketch (Fig. 1) shows the precipitin reactions in agarose gel. Bands of reactivity formed between Boston 11 extract and M-9 extract against Boston 11 antiserum, and between Boston 11 extract and M-11 extract against Boston 11 antiserum. Because the Boston 11 antigen is related both to M-9 and M-11 antigens, and since the Boston 11 antiserum contains antibodies to M-9, M-11, Boston 11, and

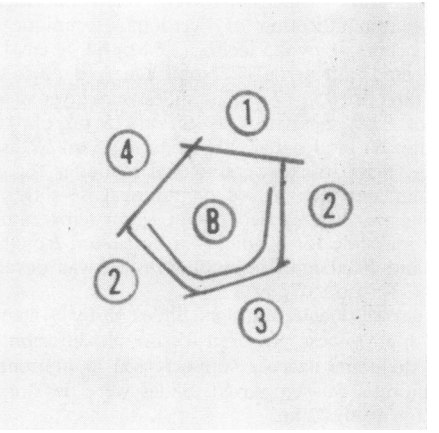

FIG. 1. *(1) SS-501 antigen (M9, T9); (2) Boston 11 antigen; (3) Ala-11 antigen; (4) SS-721 antigen (M11, T11); (B) Boston 11 antiserum, center well.*

Alabama 11, precipitin spurs of partial intersection or partial identity were resolved in the above reaction. As expected, a pattern of intersection or nonidentity was formed when extracts of M-9 and M-11 were reacted with Boston 11 antiserum, since these antigens are not related to each other, but both are related to the Boston 11 antigen used to prepare the Boston 11 antiserum. Between the Alabama 11 extract and Boston 11 extract, two bands of homology formed, indicating that these strains are closely related and perhaps identical.

Results of immunoelectrophoresis of SS-501, SS-721, and Boston 11 extracts are shown in Fig. 2. The extracts involved were reacted against unabsorbed Boston 11 antiserum. Slide 1 (Fig. 2) indicates that the migration rate of SS-721 and Boston 11 were identical and are probably closely related. However, there is some hazard in basing data on immunoelectrophoretic mobilities (2). Two bands formed when SS-721 was reacted with Boston 11 antiserum, whereas three bands formed for Boston 11. The bands at the origin are the previously determined group A antigen-antibody complex. Slide 2 (Fig. 2) is that of the T-suspension supernatant fluid of Boston 11 and the alcohol-precipitated extract of Boston 11. The T antigen appears to be a single separate band. Apparently, the M protein was not completely digested by trypsin, since three bands which appear to be the M-antigen-antibody complex were formed. There was a slight difference in the migration rate of SS-501 and Boston 11 as shown in slide 3 (Fig. 2); but, there again, the importance of the migration rate is not known.

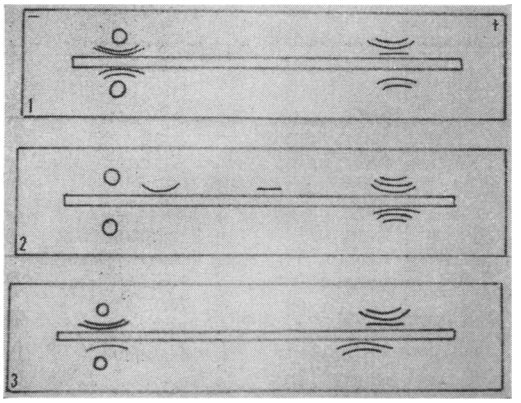

FIG. 2. *(1) Top well: Boston 11 extract; bottom well: SS-721 NCDC 11 extract; trough: Boston 11 antiserum. (2) Top well: Boston 11 T-suspension; bottom well: refined Boston 11 extract; trough: Boston 11 antiserum. (3) Top well: Boston 11 extract; bottom well: SS-501 NCDC 9 extract; trough: Boston 11 antiserum.*

In Table 3 are shown the results of the capillary precipitin tests performed with extracts of diagnostic strains with 9/11/12 T-patterns and combinations thereof and antisera for Boston 11, Alabama 11, and M-11. The Alabama 11 antiserum precipitated slightly (1–2+) with the Boston 11 stock strain. Of the 54 strains tested, 20 reacted strongly (3–4+) with Boston 11 antiserum, 8 strains gave at least 2+ reactions with Alabama 11 antiserum, and 2 strains gave strong (4+) reactions with NCDC M-11 antiserum. There was no correlation between clinical or geographical source with the appearance of either Boston 11 or Alabama 11 strains in this series. When 42 isolates from the original hospital outbreak were typed with Boston 11 antiserum, all reacted moderately with no cross-reactions with Alabama 11 or M-11 antiserum. Another nursery epidemic occurred recently; strains T-9/11, which were non-M-typable, were isolated. These cultures were not precipitin typed with either Boston 11 or Alabama 11 antiserum, since both antisera have a relatively short shelf life and are difficult to produce.

DISCUSSION

Since M protein, along with the hyaluronate capsule, functions as a virulence factor by enabling streptococci to resist phagocytosis, the Boston 11 strain which caused several deaths would have been expected to be highly antigenic. But it was extremely difficult to produce and absorb antiserum that was specific for the Boston 11 strain because of variations in the response of individual rabbits and the close antigenic relationship between Boston 11, M-11, and Alabama 11 strains. If a strain exceeds the number of chains inoculated by a factor of at least 40 after 3 hr of rotation at 37 C in normal human blood, the strain can be expected to contain more than a moderate amount of M antigen (8, 13). The Boston 11 strain did exceed this number in Becker's M-protein-enhancement procedure; therefore, M protein or some undefined antigen is present in the Boston 11 strain. On the other hand, the lack of definite bactericidal activity, as described by Potter, would indicate that either the M antibody and bactericidal antibody are not the same or that the M-antigen-antibody complex, in this specific case, is not responsible for the precipitin reaction. Perhaps the Boston 11 strain is an intermediate lying somewhere between Alabama 11 and the long-established M-11. These strains can be distinguished from one another by immunodiffusion, but cross-absorption of Boston 11 antiserum with either Alabama 11 or M-11 cells also removes the Boston 11 precipitin reaction. Strains such as the Boston 11 pose a difficult

TABLE 3. *Precipitin reactions of various M "11" group A streptococcal antisera*

NCDC no.	T type	Precipitin reactions with[a]			Source	Geographical location
		Boston 11	Alabama 11	Stock 11		
2915-69	11	—	—	—	Impetigo	Kansas
2921-69	9/11	4+	—	—	Wound	New Mexico
2929-69	9/11	3+	—	—	Cord	New Mexico
2986-69	9/11	3+	—	—	Cord	New Mexico
3069-69	9	—	—	—	Pharyngitis	Alabama
3086-69	9/11	4+	—	—	Skin	Tennessee
3089-69	9/11	2-3+	—	—	Skin	Tennessee
3091-69	9	—	—	—	Skin	Tennessee
3159-69	11	3+	—	—	Unknown	Illinois
3184-69	9	—	—	—	Skin	Trinidad
3235-69	9/11	3+	—	—	Pharyngitis	Alabama
3261-69	11	—	—	—	Throat	Connecticut
3262-69	9/11	—	—	—	Throat	Connecticut
3268-69	11	4+	—	—	Pharyngitis	Arkansas
3290-69	11/12	—	1-2+	—	Pharyngitis	Maryland
3296-69	9/11	4+	—	—	Psoriasis	California
3340-69	11/12	—	—	—	Wound	Oklahoma
3459-69	11	3+	—	—	Pharyngitis	Kansas
3465-69	11/12	3+	—	—	Wound	Massachusetts
3466-69	11/12	—	—	—	Wound	Massachusetts
3467-69	11/12	1-2+	1-2+	—	Wound	Massachusetts
3468-69	11/12	—	1-2+	—	Unknown	Massachusetts
3504-69	9/11	—	1-2+	1-2+	Acute rheumatic fever	Egypt
3540-69	9/11	—	2-3+	—	Impetigo	New Mexico
14-70	9/11	—	3+	—	Unknown	Louisiana
19-70	9/11	—	3+	—	Unknown	Louisiana
85-70	9/11	1-2+	—	—	Wound	Oklahoma
86-70	9/11	3+	—	—	Pharyngitis	Oklahoma
284-70	11	2-3+	—	—	Throat	Delaware
285-70	11	—	1-2+	—	Throat	Delaware
287-70	11	—	3+	—	Throat	Delaware
312-70	11	1-2+	1-2+	—	Pharyngitis	Kansas
324-70	9/11/12	—	—	4+	Wound	Oklahoma
390-70	11	—	3+	—	Pharyngitis	Alabama
422-70	11	—	1-2+	—	Pharyngitis	Kansas
432-70	11	2-3+	—	—	Blood	North Carolina
436-70	11	—	—	—	Sore	Alabama
474-70	9/11	—	2-3+	—	Pharyngitis	Alabama
479-70	9/11	1-2+	1-2+	—	Pharyngitis	Alabama
507-70	11	—	—	—	Pharyngitis	Georgia
545-70	9/11	—	1-2+	—	Pharyngitis	Kansas
549-70	9/11	—	—	4+	Impetigo	Kansas
579-70	9/11	4+	1-2+	—	Erysipelas	North Carolina
581-70	9/11	—	3+	—	Pharyngitis	North Carolina
673-70	9/11	1-2+	—	—	Throat	New Mexico
677-70	9/11	2-3+	—	—	Wound	New Mexico
686-70	9/11	—	2+	—	Throat	Kansas
689-70	9/11	1-2+	—	—	Pharyngitis	Kansas
692-70	11	4+	—	—	Unknown	New York
712-70	9/11/12	4+	—	—	Throat	Wisconsin
718-70	9/11	1-2+	1-2+	—	Cellulitis	North Carolina
730-70	9/11	3+	1-2+	—	Pharyngitis	Alabama
738-70	9/11	2-3+	2-3+	—	Pharyngitis	Alabama
776-70	9/11	3+	1-2+	—	Pharyngitis	North Carolina

[a] Scale: 1+, no settling of precipitate; 2+, settling of precipitate less than 0.5 mm; 3+, settling of precipitate 0.5 mm to 1.5 mm; 4+, settling of precipitate 1.5 mm or over.

question if one wishes to designate them as a single M type when definite bactericidal activity cannot be demonstrated in antisera produced with them. Until the difficulty in producing a bactericidal and M-specific antiserum for the Boston 11 strain can be overcome, these strains probably should be classified according to their T-agglutination pattern; their predominance in wound and umbilical cord infections should also be recognized.

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