THE IMMUNOLOGICAL SPECIFICITY OF STAPHYLOCOCCI

I. The Occurrence of Serological Types*

By L. A. JULIANELLE, Ph.D., and C. W. WIEGHARD

(From the Oscar Johnson Institute, Washington University School of Medicine, St. Louis)

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During the course of studies on the occurrence of heightened skin sensitivity to bacterial derivatives in patients with trachoma (1), reactions were observed that were reconcilable only on the predication that staphylococci are composed of immunologically different strains. Since the available information on this subject failed to furnish conclusive evidence that such a condition exists, it was decided to investigate the possible classification of these organisms into serologically distinct types. While a preliminary report (2) has already been made of the more salient aspects of this study, it is desirable to submit at the present time, the more detailed data and experiments.

Methods

Strains of Staphylococci.—The organisms studied in this investigation were isolated from a variety of sources during the past 2 to 3 years. The majority of the strains were cultivated from patients in Barnes Hospital (St. Louis) who were suffering from septicemia, osteomyelitis, furunculosis, acute conjunctivitis and, in one instance, pneumonia. These were considered as pathogenic, virulent strains. Other cultures were isolated from the normal conjunctiva and skin, from monkeys as bacteria fortuitously present in other infections, from the air, etc. Three cultures, Ha, Fs and D_1, were obtained for purposes of comparison.[1] The first two of these strains are pathogenic and toxigenic, while the last is both avirulent and non-toxigenic (3).

Immunization.—Only normal rabbits not possessing normal agglutinins for Staphylococcus were used for immunization. Broth cultures 15 to 18 hours old were centrifugated and the sediment was resuspended in sufficient saline to equal one-tenth the original volume. The bacteria were then killed by heating for 1 hour

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[1] These cultures were kindly sent by Dr. E. L. Burky of Johns Hopkins Hospital.

at 56–60°C. In making injections the suspensions were diluted to original volume. The animals were given four courses of intravenous injections consisting of inoculations on 3 successive days and a rest of 4 days. The dosage for the first course was 0.5 cc., and this was increased 0.5 cc. each course, until 2.0 cc. were injected. The animals were then bled 10 days after the last inoculation.

Agglutination.—Agglutinations were conducted with either living or heat-killed broth cultures. The usual technique was employed of incubating the tests in the water bath at 37°C. for 2 hours and storing in the ice chest overnight. Final readings were made on the following morning.

Precipitation.—Precipitation tests were conducted in the usual manner using 0.5 cc. of diluted antigen and 0.5 cc. of serum diluted 2:3.

Differentiation of Types by Precipitation

Rabbits were immunized as described to eight different strains of Staphylococcus. Eventually all the antisera were tested for agglutination with 30 odd strains. For purposes of illustration, however, typical agglutination of 12 strains in four antisera are presented in Table I. Examination of this protocol reveals that none of the cultures were agglutinated in sera pooled from six normal rabbits. It is important in this connection to point out that agglutination of Staphylococcus is not uncommon in normal rabbit serum. Further observation of Table I indicates that with some of the more recently isolated strains (13, C, 158) there appears to be a distinct tendency to separate into different immunological entities. On the other hand, in the case of some other strains (as B_2A, and D_1) a difference in serological reactivity is suggestive only, while in the remaining agglutinations (*e.g.* K, P, 161, Mx3), there is no indication of the existence of different types. In fact an analysis of all the agglutination reactions observed in this study offers at best only a suggestion of type differentiation, and the conclusion is therefore unavoidable that agglutination presents an uncertain and inconclusive method for distinguishing immunological types among the staphylococci.

Differentiation of Types by Precipitation

The original observation by Avery and Heidelberger (4) that the type specificity of pneumococci is determined by chemically different carbohydrates or soluble specific substances has been confirmed and enlarged upon by studies of other bacteria as well as Pneumococcus. The results of these combined studies indicate that in the present

TABLE I

Representative Cross-Agglutination Reactions with Different Strains of Staphylococcus

Strain	Antisera and dilutions																									
	Anti-13					Anti-B_2A					Anti-M_{11}					Anti-Mx3					Pooled normal serum				
	1:20	80	320	1280	5120	1:20	80	320	1280	5120	1:20	80	320	1280	5120	1:20	80	320	1280	5120	1:20	80	320	1280	5120
13	++++	++++	++	+	−	++++	++++	+++	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B_2A	++++	++++	++	±	−	++++	++++	++++	++	−	+++	++	−	−	−	++	+	−	−	−	−	−	−	−	−
K	++++	++++	+++	++	+	++++	++++	+++	++	±	+++	++	+	±	−	++++	+++	++	+	±	+	+	±	−	−
C	++++	++++	++	±	−	++++	++++	++++	++	−	++	±	−	−	−	+	−	−	−	−	−	−	−	−	−
P	++++	+++	++	+	−	++++	++++	++++	++	−	++++	++	+	−	−	++++	++	+	−	−	−	−	−	−	−
Ha	++++	++++	++++	+++	±	++++	++++	++++	+++	+	++++	++++	+++	++	−	+	±	−	−	−	−	−	−	−	−
157	−	−	−	−	−	++++	+	−	−	−	++	+	−	−	−	++++	++	+	−	−	−	−	−	−	−
158	−	−	−	−	−	±	−	−	−	−	+++	++	+	−	−	++++	++	±	−	−	−	−	−	−	−
161	++++	++++	+++	±	−	++++	++++	++++	++	+	++++	+++	++	±	−	++++	++	+	±	−	−	−	−	−	−
D_1	++	+	−	−	−	++	+	−	−	−	+++	++	−	−	−	++++	++++	++	+	−	+	−	−	−	−
Mx3	++++	++++	++++	++	+	++++	++++	+++	++	+	++++	+++	+++	+	−	++++	+++	++	+	−	−	−	−	−	−
M_{11}	+++	+++	++	−	−	++++	++++	++	+	−	++++	+++	+++	++	±	+++	+++	+	−	−	−	−	−	−	−

state of knowledge immunological specificity of bacteria is most frequently a property associated with the polysaccharide fraction of the cell. Since, as pointed out above, strains of staphylococci occasionally exhibit a definite tendency to type specificity it seemed wise to investigate the occurrence and behavior of carbohydrates derivable from these organisms. Ultimately a satisfactory method for extracting polysaccharides from staphylococci was devised. This method together with the chemical properties and characteristics of the soluble specific substances will be the subject of a subsequent report.

Preliminary precipitation tests with the soluble specific substances of different strains indicated in certain instances a serological reactivity in dilutions up to 1 to 6 to 8 million. It was soon discovered, however, that while all antibacterial sera contain agglutinins in high titre, anticarbohydrate antibody is frequently lacking, or present in titres too low for utilization in a study of differential precipitation. Since the sera of animals receiving the same suspensions of bacteria vary in the presence of precipitins, it must be assumed that the absence of antibody formation is dictated by the individual rabbit rather than the antigenic carbohydrate complex. From the data already on hand, it appears that only one of three or four antisera contain precipitins for the homologous polysaccharide. This figure, however, must be accepted as tentative, since the number of sera studied is not sufficiently extensive to allow accurate generalization.

Precipitation tests have been made with a number of purified carbohydrates derived from different strains of Staphylococcus. In Table II typical reactions of the polysaccharides from 12 different strains are presented. The results of these reactions show that on the basis of precipitation of the purified carbohydrates in antibacterial sera, staphylococci are sharply separable into two different and distinct types. As a matter of fact, a total of 16 strains have been studied by this method, and they all fall into one or the other of the two types. Of the cultures studied, nine isolated from different human infections and therefore considered virulent have fallen into one type, while the remaining, all isolated from non-pyogenic sources and consequently regarded as avirulent, have fallen into the second type. It is proposed, therefore, to designate the pathogenic strains as Type A and the non-pathogenic as Type B.

TABLE II

Precipitation of Purified Carbohydrates Derived from Staphylococcus in Homologous and Heterologous Antibacterial Sera

S.S.S. from strain	Antisera and dilutions of carbohydrates expressed in thousands																							
	Anti-13						Anti-B_2A						Anti-148						Anti-Mx3					
	1:20	50	100	200	500	1000	1:20	50	100	200	500	1000	1:20	50	100	200	500	1000	1:20	50	100	200	500	1000
13	++++	++++	++++	++++	+++	++	++++	++++	++++	+++	++	+	−	−	−	−	−	−	−	−	−	−	−	−
B_2A	++++	++++	++++	+++	+	±	++++	++++	++++	+++	++	±	−	−	−	−	−	−	−	−	−	−	−	−
K	++++	++++	++	+	±	−	++++	+++	+++	++	+	−	−	−	−	−	−	−	−	−	−	−	−	−
C	++++	++++	+++	++	+	−	++++	++++	+++	++	±	−	−	−	−	−	−	−	−	−	−	−	−	−
P	++++	++++	++++	++++	++	+	++++	++++	++++	++	+	±	−	−	−	−	−	−	−	−	−	−	−	−
Ha	++++	++++	+++	++±	++	+	++++	++++	++++	+++	++	±	−	−	−	−	−	−	−	−	−	−	−	−
148	−	−	−	−	−	−	−	−	−	−	−	−	++++	++++	+++	++	+	±	++++	++++	++++	++++	+++	++±
Mx3	−	−	−	−	−	−	−	−	−	−	−	−	++++	++++	+++	+	±	−	++++	++++	+++	+++	++	+
Mx2	−	−	−	−	−	−	−	−	−	−	−	−	++++	++++	+++	++	+	−	++++	++++	+++	+++	+	−
M31	−	−	−	−	−	−	−	−	−	−	−	−	++++	++++	++++	+++	+	±	++++	++++	++++	++++	+++	++
M_{11}	−	−	−	−	−	−	−	−	−	−	−	−	++++	++++	+++	++	+	−	++++	++++	+++	++	+	±
D_1	−	−	−	−	−	−	−	−	−	−	−	−	++++	++++	+++	++	+	−	++++	++++	++++	++	++	+

++++, heavy, compact, disk precipitate; +++, marked disk precipitate; ++, thin, film-like scale; +, ground glass turbidity; ±, slight turbidity.

All the carbohydrates indicated above were tested in the same dilutions in the pooled sera of six normal rabbits. Precipitations occurred in no instance.

That the carbohydrates are characteristic of Staphylococcus was later demonstrated by testing their reactivity in antibacterial sera of different species. The results of this experiment are given in Table III. Examination of this protocol discloses that while the carbohydrates of Type A and Type B Staphylococcus are precipitated in homologous immune sera, they do not precipitate in antipneumococcus, Type I, II or III sera, or anti-Friedländer, Type A, B or C sera, or in antityphoid serum. It is obvious, therefore, that the carbohydrates are genuinely specific of Staphylococcus.

The method of obtaining a relatively purified carbohydrate from Staphylococcus makes a rapid typing difficult. It was proposed, therefore, to determine the possibility of employing crude extracts of the bacteria for typing purposes. Accordingly, cultures were seeded into flasks containing 50 cc. of broth and they were incubated for 20 hours. The broth cultures were then centrifugated, and the clear supernatant fluid was saved, while the sedimented bacteria were extracted with acid. 10 cc. of 16/N HCl was added to the bacteria and the suspension was boiled in a water bath for 15 minutes. It was then cooled and made neutral to litmus by adding 16/N NaOH. This was then brought to original volume with saline and centrifugated. The clear supernatant was used in the precipitation test. The results of this experiment are recorded in Table IV. It will be seen that type specificity may be detected by precipitation tests performed with the supernatant of broth cultures. This is particularly true of Type A strains which apparently elaborate greater quantities of soluble specific substance. The reactions are even more striking when acid extracts are used for precipitation. In some instances it will be noted that there is a certain degree of cross-precipitation (*e.g.* Mx3) but when the reaction is specific the precipitate forms as a firm compact disk which is disrupted with difficulty, while the non-specific precipitate is granular and is disturbed readily on agitation. It appears, therefore, that with certain strains sufficient carbohydrate may be obtained for precipitation tests either by centrifugation of young broth cultures or by acid extraction of the sedimented bacteria.

Representative strains of both Types A and B were tested in rabbits for virulence and for toxicity of broth culture filtrates. The strains studied were 13, Ha and F_s (Type A) and Mx3 and D_1 (Type

TABLE III

Precipitation of Purified Carbohydrates Derived from Staphylococcus in Antisera of Different Bacteria

Antiserum	Dilution of carbohydrate											
	Type A (13)						Type B (Mx3)					
	1:20	50	100	200	500	1000	1:20	50	100	200	500	1000
Staphylococcus, Type A.....	++++	++++	++++	++++	+++	++	–	–	–	–	–	–
" " B.....	–	–	–	–	–	–	++++	++++	++++	+++	++	+
Pneumococcus " I.....	–	–	–	–	–	–	–	–	–	–	–	–
" " II	–	–	–	–	–	–	–	–	–	–	–	–
" " III....	–	–	–	–	–	–	–	–	–	–	–	–
Friedländer " A.....	–	–	–	–	–	–	–	–	–	–	–	–
" " B.....	–	–	–	–	–	–	–	–	–	–	–	–
" " C.....	–	–	–	–	–	–	–	–	–	–	–	–
Typhoid.....	–	–	–	–	–	–	–	–	–	–	–	–

TABLE IV

Precipitation of Crude Preparations of Carbohydrates in Homologous and Heterologous Antisera

Antigen		Dilution of antigen in									
		Type A serum					Type B serum				
		1:1	5	20	100	200	1:1	5	20	100	200
Supernatant 20 hr. broth culture	13 (Type A)	++++[s]	++++	++++	+++	++	−	−	−	−	−
	B_2A " "	++++[s]	++++	++	+	−	±	−	−	−	−
	Ha " "	++++[s]	++++	++	+	−	−	−	−	−	−
	F_8 " "	+++[s]	+++	+	−	−	+	−	−	−	−
	Mx3 (Type B)	++[g]	+	±	−	−	++++[s]	+++	+	±	−
	D_1 " "	−	−	−	−	−	++[s]	±	−	−	−
	148	+[g]	±	−	−	−	+++[s]	++	+	−	−
Neutralized acid extract	13 (Type A)	++++[s]	++++	++++	++++	+++	+	±	−	−	−
	B_2A " "	+++[s]	+++	++	++	+	−	−	−	−	−
	Ha " "	+++[s]	++	+	+	−	++	±	−	−	−
	F_8 " "	+++[s]	+++	+++	++	+	++	±	−	−	−
	Mx3 (Type B)	++[g]	+	±	−	−	+++[s]	+++	++	±	−
	D_1 " "	−	−	−	−	−	−	−	−	−	−
	148	+	−	−	−	−	++	++	±	−	−

[s] indicates precipitate was of the compact disk variety; [g], of the granular type readily disrupted on agitation.

B), and filtrates of these organisms were prepared according to the technique described by Burky (3). Strains 13 and Ha killed rabbits regularly, while F_S was irregular. Neither Mx3 nor D_1, however, when injected in similar quantities were virulent for rabbits. In testing the toxigenic properties of the same strains by the injection of broth filtrates in rabbits, it was found that filtrates of 13 and Ha were toxic while that of F_S was only slightly so. Neither filtrate of the Type B cultures studied caused any reaction in rabbits. It may be concluded, therefore, that Type A strains may be both virulent and toxigenic for rabbits but that Type B strains exhibit neither property.

An attempt was made later to differentiate the two types of Staphylococcus on the basis of lysis by bacteriophage. Three strains of staphylococcus phage were obtained from Dr. J. J. Bronfenbrenner. The activity of the phage was then tested against representative strains of each type in the usually prescribed manner. The results of this experiment were definite in demonstrating that the function of lysis of Staphylococcus by bacteriophage is not related to the type of a given strain.

The small number of antisera containing type specific precipitins following intravenous immunization made it desirable to determine the effect of different methods of immunization on the formation of precipitins. Rabbits given repeated injections of heat-killed staphylococci intracutaneously showed high titres of agglutinins in their sera but in no instance antibodies reactive with the carbohydrates. In other rabbits, living organisms were injected in agar foci also intracutaneously, and while the agglutinin titre following this immunization was high, the sera was completely devoid of type specific precipitins. It may be concluded, therefore, that of the three methods studied, intravenous immunization is the most effective for stimulating the anticarbohydrate antibody.

DISCUSSION

That staphylococci are separable into different immunological types has been suggested by Julianelle (5) and Hine (6), on the basis of the agglutination reaction. So, also, Hopkins and Barrie (7) indicate a possible antigenic differentiation of this species as brought out by a modified agglutinin adsorption test. In each instance, however, the

types were not strictly specific nor did they show a sharp demarcation from each other. Similarly it has been observed in the present investigation that the agglutination reaction does not suffice in detecting types decisively. While more recently isolated strains have a distinct tendency to divide into different types, in many instances there is no indication even of type specificity. It was for this reason that a study was made to determine the specificity of carbohydrates derived from Staphylococcus.

The results of the experiments on the specificity of the carbohydrates reveal that at least two immunological types exist among the staphylococci. The indications are that pathogenic strains fall into one type, A, and the non-pathogenic strains comprise a second type, B. It is interesting to point out that both virulent and avirulent strains are capable of elaborating type specific carbohydrates and that virulence is therefore associated not with the presence of soluble specific substance, but rather with the particular type of polysaccharide. This it will be remembered is in contrast with Pneumococcus (8) and Friedländer's bacillus (9) where virulence accompanies elaboration of the type specific carbohydrate.

The differentiation of staphylococcus types may be demonstrated even with crude preparations of carbohydrates. Supernatant fluid of centrifugated broth cultures and acid extracts of the sedimented bacteria may also precipitate in homologous immune sera. The evidence indicates that the carbohydrates are apparently contained within the cell in striking contrast to the ectoplasmic distribution of the type specific polysaccharides of encapsulated bacteria. Attempts to distinguish type specificity on the basis of lysis by bacteriophage were unsuccessful.

The presence of type specific antibodies in the sera of immunized rabbits varies in individual animals despite a constantly high agglutinin titre in all antisera. The effect of different methods of immunization on precipitin formation was studied, and it was found that type specific precipitins are stimulated only following intravenous injections of the bacteria.

SUMMARY AND CONCLUSIONS

1. Agglutination is not a precise method for the demonstration of serological types among staphylococci.

2. Precipitation of soluble specific substance derived from these organisms demonstrates the existence of at least two immunologically distinct types.

3. The one type, designated A, is composed of apparently virulent strains, while the other, Type B, contains the avirulent strains.

4. Precipitation tests performed with centrifugates of young broth cultures or with acid extracts of sedimented bacteria may also demonstrate type specificity.

5. Lysis by bacteriophage fails to detect the specific types of Staphylococcus.

6. Immunization by intravenous methods stimulates agglutinin formation in all rabbits and precipitin formation in only one of three or four animals.

7. Immunization by repeated intracutaneous injections of dead staphylococci or living organisms in an agar focus also stimulates agglutinin formation but fails to incite the formation of type specific precipitins.

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