CORRELATED ANTIGENIC AND BIOCHEMICAL PROPERTIES OF STAPHYLOCOCCI

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The general differentiation of pathogenic[1] from non-pathogenic staphylococci on the basis of pigment production and general fermentative activity has been considered by a long series of workers. [Rosenbach (1884), Welch (1891), Gordon (1903–4 and 1904–5), Dudgeon (1908), Winslow and Winslow (1908), Kligler (1913), Winslow, Rothberg and Parsons (1920).] A distinction on an antigenic basis was first made in 1902 by Kolle and Otto (1902) who found that staphylococci from purulent lesions were all agglutinated by immune serums prepared against one of them while strains from other sources were not agglutinated. The majority of the agglutinable strains were orange and were pathogenic for mice. In view of the very large number of confirmatory publications in the fifteen years following Kolle and Otto's paper [Otto (1903), Proescher (1903), Veiel (1904), Kutscher and Konrich (1904), Fraenkel and Bauman (1905), Noguchi (1911), Dreyer and Nothman (1912), Geisse (1913), Walker and Adkinson (1917)] it is curious that workers in more recent years have not been able to differentiate the staphylococci satisfactorily by means of direct agglutination. Hine (1922) found it necessary to use agglutinin absorption but by this technic did prove that

[1] The term "pathogenic" is very loosely applied to staphylococci, usually implying known pathogenicity for human beings. In the present stage of knowledge such an assumption for any particular strain is not warranted. For convenience, however, this term has been used in this paper to indicate strains from human lesions or strains proven to be pathogenic for animals, and the term "non-pathogenic" to indicate strains from other sources or non-pathogenic for animals.

the majority of strains from disease processes were distinct from those from other sources. Julianelle (1922) found no correlation of antigens with source or experimental pathogenicity in his three antigenic groups determined by agglutinin absorption. Hopkins and Barrie (1928) by absorption also established three groups, only one of which was found in disease processes. Dudgeon and Simpson (1927) resorted to a precipitin technic and, although they used only strains from infectious processes, found that pathogenicity for rabbits was correlated with the presence of a specific precipitinogen and, to a less extent, with orange pigment production. Julianelle and Weighard (1935) found that staphylococci from lesions contained a specific precipitable polysaccharide (A) which could be differentiated chemically as well as antigenically from a polysaccharide, (B), found in strains from other sources.

There is considerable disagreement in the literature as to the degree of correlation of hemotoxin production by staphylococci with pathogenicity or antigenicity. This is understandable considering the various technics and types of erythrocytes used. The majority of workers using broth cultures, or their filtrates, with rabbit erythrocytes have reported a considerable, although seldom absolute, correlation. [Neisser and Wechsberg (1901), Otto (1903), van Durme (1903); Veiel (1904), Kutscher and Konrich (1904), Fraenkel and Bauman (1905), Koch (1908), Noguchi (1911).]

Gordon (1903–4) found that the fermentation of mannitol was one property which differentiated staphylococcal strains from lesions, from those from other sources. Dudgeon (1908), Hine (1922), and Dudgeon and Simpson (1927), confirmed and extended his findings.

The ability of certain strains of staphylococci to coagulate oxalated or citrated plasma observed by Loeb (1903) and by Much (1908) was found by Daranyi (1926) to be associated with pathogenicity and with a strong hemotoxic ability. Gross (1931) and Chapman *et al.* (1934) also found a correlation of coagulase production with pathogenicity, hemotoxic activity and orange pigment production.

We are reporting here the results obtained in testing 286 strains of staphylococci isolated from various sources in respect to the various properties discussed above.

METHODS

The organisms were isolated on rabbits' or sheeps' blood agar plates. Single colonies were picked from two consecutive platings to establish presumptive purity. Inoculations were made into the various test media from 18-hour broth cultures. During the time of the experiments all the strains were preserved in the refrigerator on plain or blood-agar slants. Transfers were made monthly.

Immunization of rabbits

Rabbits were immunized during a four weeks' period by a series of intravenous injections of saline suspensions of 18-hour agar slant cultures. Heat-killed organisms were given during the first three weeks and living organisms during the final week. The animals were bled ten days after the last injection.

Preparation of antigens

Flasks containing 50 cc. of meat-infusion broth were inoculated with 18-hour cultures of the organisms and incubated for 4 days. The whole cultures were precipitated over night in the ice box with 4 volumes of 95 per cent alcohol plus 3 drops of glacial acetic acid. The precipitate and organisms were removed by centrifuging and were extracted with 2.5 cc. of N/16 HCl in a water bath at 100°C. for 15 minutes. The mixtures were then neutralized to litmus and centrifuged. The clear supernatants were used as antigens for the precipitin tests.

Precipitin tests

The precipitin tests were done by means of a capillary technic described previously (Thompson *et al.*, 1936). Equal quantities of serum and antigen were drawn into graded capillary tubes. The tubes were then supported upright by plasticine on microscope slides. Readings of the amount of precipitate were made after 2 hours at room temperature and 18 hours in the refrigerator.

Hemotoxin tests

The organisms were grown in beef-infusion broth, (pH 6.5) to which was added 0.03 per cent $MgSO_4$. The cultures were kept at

37°C. in an atmosphere of 20 per cent CO_2. After 10 days' growth they were filtered through Mandler filters and various dilutions of the filtrates were titrated for hemotoxin content against equal quantities of 1 per cent washed rabbit erythrocytes. The mixtures were incubated at 37°C. for 1 hour and kept in the refrigerator for 18 hours. Any definite trace of hemolysis was considered as evidence of hemotoxin production.

Mannitol fermentation

Cultures were inoculated into tubes of 1 per cent Difco mannitol in beef infusion broth. After 3 days' incubation the presence of acid was tested for by adding 2 drops of bromcresol purple.

Coagulase

Oxalated plasma was obtained by placing 10 cc. of fresh human blood into a centrifuge tube containing 0.02 gram of potassium oxalate, thoroughly mixing, and then centrifuging out the cells. 0.3 cc. of the plasma was mixed with 0.2 cc. of an 18–24 hour broth culture of the organism to be tested. Partial or complete clotting after 2 hours' incubation at 37°C. indicated the production of coagulase.

Pigment

The pigment was classified as orange, lemon or white by the appearance of the massed growth from a 72-hour agar plate placed upon white filter paper and pressed between two microscopic slides. No attempt was made to classify the various shades between orange and lemon. When any trace of orange color was present the strain was designated as orange.

RESULTS

Antigens were prepared from all 286 strains and tested for precipitation with serums obtained by immunization against 9 of the strains. In table 1 examples are given of the types of reactions obtained.

Ninety-three strains gave reactions identical with those given by Julianelle's strain 13 and have therefore been designated as group A. Twenty-nine strains were similar to Julianelle's MX and have been designated as group B in conformity with his terminology. Sixty-four strains gave moderate amounts of

precipitate with serum 32 but little or none with the other serums. Since these strains obviously contain an antigen distinct from Julianelle's A and B, this group has been designated as group C. Sixteen strains giving precipitates with both MX and 32 serums have been tentatively designated as group BC. Eighty-six

TABLE I

Typical precipitin reactions

ANTISERUM AGAINST STRAIN NUMBER	AMOUNT OF PRECIPITATE WITH ANTIGEN FROM STRAIN NUMBER														
	13*	6	I	MX*	Co	Cg	Be	O	32	G	Hn	D	Con	Bsh	Bd
13*	++++	++++	++++	±	±	0	0	0	0	±	±	0	±	0	0
8	++++	++++	++++	0	0	0	0	0	±	0	0	±	0	±	0
I	++++	++++	++++	0	±	±	±	0	0	0	±	0	0	0	0
MX*	±	±	±	++	+	++	±	0	±	++	++	+	0	±	0
32	0	±	0	0	0	±	+	++	+++	++	+	++	±	0	0
Designation of group.....		A			B			C			BC			O	
Number of strains giving each type of reaction.....		93			29			64			16			86	

* Strains 13 and MX were kindly sent to us by Dr. L. A. Julianelle as typical of his A and B strains respectively.

TABLE 2

Biochemical properties of antigenic groups

ANTIGENIC GROUP	NUMBER OF STRAINS	NUMBER OF STRAINS IN EACH ANTIGENIC GROUP SHOWING											
		Orange pigment Mannitol + Coagulase + Hemotoxin +		Orange pigment		Mannitol fermentation		Coagulase production		Hemotoxin production		Not orange Mannitol neg. Coagulase neg. Hemotoxin neg.	
(A)	93	68	73%	84	90%	75	80%	85	91%	86	92%	2	
(B)	29	1		1		1		1		2		26	89%
(C)	64	0		2		4		2		2		56	91%
(BC)	16	0		0		0		0		0		16	100%
(O)	86	5		18	20%	21	24%	6		13	15%	54	63%

strains showed no definite precipitate with any serum and have been called group O.

Group A is clearly set apart from the other groups, not only by the specificity of its antigen but also by the much greater amount of precipitate produced.

In table 2 are shown the results of the biochemical tests arranged according to antigenic groups.

The majority of strains of group A gave positive reactions in all or most of the biochemical tests in contrast to the strains of groups B, C, and BC, of which 90 to 100 per cent were entirely negative in all tests.

TABLE 3

Hemotoxin production on rabbits' blood plates and in CO_2 toxin broth

ANTIGENIC GROUP	NUMBER OF STRAINS COMPARED	PER CENT SHOWING HEMOLYSIS ON PLATES	PER CENT OF THESE PRODUCING NO HEMOTOXIN IN TOXIN BROTH
A	78	96	2.6
B	29	65	89
C	64	64	95
BC	15	46	100
O	85	61	77

TABLE 4

Antigenic groupings of strains from different sources

SOURCES OF CULTURES	NUMBER OF STRAINS	ANTIGENIC GROUP									
		A		B		C		BC		O	
Infections	59	46	78%	1		3		1		8	16%
Normal Conjunctiva Nose Throat	191*	41	21%	24	12%	59	29%	15	9%	54	28%
Air	24	2		1		4		0		17	73%

* 178 strains from normal conjunctivae.

A certain degree of correlation with antigenic group was also found for two other properties not included in the table. Forty to sixty per cent of the colonies of groups B, C, and BC strains were definitely viscid but only 9 per cent of group A strains produced viscid colonies. A number of strains in all groups were tested for their power to reduce methylene blue.[2] The speed of

[2] 1 drop of 1 per cent aqueous methylene blue was added to 24-hour broth cultures and the time required for complete reduction at 37°C. was determined.

reduction by the same strains varied from time to time so that no absolute standard could be set. However, when a number of cultures from different groups was tested at the same time, most rapid reduction was always produced by the group A strains.

When comparisons were made of the ability of any of the strains of staphylococci to produce hemolysis of rabbits' blood plates with production of hemotoxin by the method described above, many strains were found which were able to produce marked hemolysis of rabbits' blood plates although no hemotoxin could be detected in the broth filtrates. The results of these comparisons are summarized in table 3.

In table 4 the relationship of antigenic grouping to the sources of the cultures is shown.

Seventy-eight per cent of strains from infectious sources were in antigenic group A; only 8 per cent of these "infection" strains were in groups B, C, and BC.

Fifty per cent of the strains from mucous membranes were members of groups B, C, or BC; 21 per cent were of group A.

Seventy-three per cent of the strains[3] isolated from the air were in group O, giving no precipitates with any of the serums.

DISCUSSION

The very clear-cut antigenic differentiation of group A is further supported by the great degree of its correlation with the other biochemical properties and with the origin. This is in harmony with the conception that staphylococci from disease sources tend to fall into a fairly uniform group possessing certain biochemical and antigenic properties differentiating them from "non-pathogenic" staphylococci. In particular it is in agreement with the work of Dudgeon and Simpson (1927) who found that the presence of a specific precipitinogen in staphylococci was correlated with pathogenicity, and with the recent work of Julianelle and

[3] All strains were morphologically Gram-positive cocci in typical irregular clusters on solid medium and produced typical staphylococcus-like colonies. Many did not liquefy gelatin within the three weeks' period allowed.

The majority of the air strains were kindly given to us by Dr. L. Buchbinder of the Bacteriological Division of the New York City Air Pollution Survey.

Weighard (1935) who reported that pathogenic staphylococci possess a specific carbohydrate antigen distinct from a similar carbohydrate present in "non-pathogenic" strains. The antigenic subdivision of the remaining strains is less clear-cut and not correlated with other factors to any great extent so that the groupings are tentative and subject to confirmation. Our results indicate, however, that at least one antigen, in addition to Julianelle's "B" carbohydrate, is present in the staphylococci from mucous membranes, and that these strains may be further subdivided on this basis. This is in agreement with Hine (1922) and with Hopkins and Barrie (1925) both of whom, by agglutinin absorption, divided their "non-pathogenic" strains into two groups.

The majority of the staphylococci from the air may be separated from the "pathogenic" group and from the "mucous membrane" groups in that they possess no acid-soluble, heat-stable antigen in common with these groups.

The discrepancies between the results of the estimation of hemotoxin production in broth cultures and on blood plates are worthy of note. Neisser (1925) described similar differences and suggested that two different mechanisms are concerned.

We believe that the discrepancies are not due to different mechanisms being concerned but to the fact that the 2 per cent blood agar is a more favorable medium for the production of hemotoxin, even without CO_2, than is the broth which we used. The hemotoxins produced by the B and C strains on agar plates are soluble; are neutralizable by antitoxin against A strains; and are increased by growth in 20 per cent CO_2. All these properties indicate their essential identity with A type toxin.

SUMMARY

Heat-stable, acid-soluble antigens prepared from 286 strains of staphylococci from various sources were tested for precipitation with immune serums against 9 of the strains. The organisms were found to fall into 5 groups: Group A, 93 strains including Julianelle's Type A; group B, 29 strains including Julianelle's

Type B; group C, 64 strains; group BC, 16 strains precipitated by both B and C serums; group O, 86 strains not precipitated by any serum.

When the cultures were tested for orange pigment production, mannitol fermentation, coagulase production and hemotoxin production (in broth under 20 per cent CO_2) a marked correlation of these properties with the group A antigen was found. The correlation of hemotoxin production was less marked when hemolysis of rabbits' blood plates was used as a criterion; considerable percentages of groups other than A producing visible hemolysis of the plates. The evidence indicates that the hemotoxins produced by the B and C strains are identical with those produced by the A strains.

A definite correlation of antigenic grouping with the sources of the cultures was evident. Seventy-eight per cent of strains from infections were in group A. Fifty per cent of strains from mucous membranes were in groups B, C, and BC, with 21 per cent and 28 per cent in A and O respectively. Seventy-three per cent of the strains isolated from the air fell into group O.

REFERENCES

CHAPMAN, G. H., BERENS, C., PETERS, A., AND CURCIO, L. 1934 Coagulase and hemolysin tests as measures of the pathogenicity of staphylococci. J. Bact., **28**, 343–363.

DARANJI, J. 1926 Pathogenität und Einteilung der Staphylokokken. Zent. Bakt. I. Orig., **99**, 74–79.

DREYER, L., AND NOTHMANN, F. 1912 Zahl, Art und Virulenz der aus aseptischen operations Wunden beim heutigen Desinfektions verfahren züchtbaren Bakterien. Beitr. z. Klin. Chirurg., **80**, 100–120.

DUDGEON, L. S. 1908 Differentiation of staphylococci. J. Path. and Bact., **12**, 242–257.

DUDGEON, L. S., AND SIMPSON, J. W. H.: 1927–8 On the differentiation of the staphylococci with special reference to the precipitin reactions. J. Hyg., **27**, 160–173.

FRAENKEL, C., AND BAUMANN. 1905 Ueber Hämolysinbildung und Agglutination der Staphylokokken. Münch. Med. Wschrft., **52**, 937–939.

GEISSE, A. 1913 Die Differenzierung Pathogener und Saprophytischer Staphylokokken. Zeitschrift f. Hyg., **76**, 282–306.

GORDON, M. H. 1903–4 Reports of some characters by which various streptococci and staphylococci may be differentiated and identified. Local British Government Board, Report of Medical Officer, **33**, 388–430.

GORDON, M. H. 1904–5 Report of bacteriological test whereby particles shed from the skin may be detected in air. Local British Government Board, Report of Medical Officer, **34**, 387–413.

GROSS, H. 1931 Plasmagerinnungstoff der Staphylococci. Zent. Bakt., I. Orig., **122**, 354–361.

HINE, T. C. M. 1922 Serological classification of the staphylococci. Lancet, **2**, 1380–1382.

HOPKINS, J. G., AND BARRIE, N. 1928 Antigenic relationship of certain strains of *Staphylococcus aureus* as shown by agglutinin absorption. Proc. Soc. Exp. Biol. and Med., **25**, 701–702.

JULIANELLE, L. A. 1922 Studies of hemolytic staphylococci. J. Inf. Dis., **31**, 256–284.

JULIANELLE, L. A., AND WEIGHARD, C. W. 1935 The immunological specificity of staphylococci. J. Exp. Med., **62**, 11–37.

KLIGLER, I. J. 1913 A systematic study of the coccaceae in the collection of the Museum of Natural History. J. Inf. Dis., **12**, 432–452.

KOCH, J. 1908 Über das Vorkommen Pathogener Staphylokokken auf der Körperoberfläche des Mensches und seiner Ungebung. Ztschrft. f. Hyg., **58**, 287–326.

KOLLE, W., AND OTTO, R. 1902 Die Differenzierung der Staphylokokken mittelst der Agglutination. Zeitschrift f. Hyg., **41**, 369–379.

KUTSCHER AND KONRICH, F. 1904 Untersuchungen über die Beziehunger von Hämolysinbildung und Agglutinabilität der Staphylokokken. Zeitschrift f. Hyg., **48**, 249–268.

LOEB, L. 1903 The influence of certain bacteria on the coagulation of the blood. J. Med. Res., **10**, 407–409.

MUCH, H. 1908 Über eine Vorstufe des Fibrinfermentes in Kulturen von *Staphylokokkus aureus*. Biochem. Ztschrift., **14**, 143–155.

NEISSER, M. 1928 Handbuch der Mikroorganismen. Kolle, Kraus, Uhlenuth, Gustave Fischer, Jena. **4**, 437.

NEISSER, M., AND WECHSBERG, F. 1901 Über das Staphylotoxin. Zeitschrift. f. Hyg., **36**, 299–349.

NOGUCHI, Y. 1911 Ueber die Farbstoff Produktion und Pathogenität der Staphylokokken. Arch. f. Klin. Chirurg., **96**, 696–758.

OTTO, R. 1903 Weiter Beiträge zur Agglutination der Staphylokokken. Zent. f. Bakt., Orig., **34**, 44–48.

PROESCHER 1903 Ueber die Künstliche Immunität gegen Staphylokokken. Zent. f. Bakt., Orig., **34**, 437–445.

ROSENBACH 1884 Microörganisms of wound infections. Wiesbaden. Quoted by Fleming, A. in "A System of Bacteriology". Medical Research Council, **2**, 11–28, 1929.

THOMPSON, RICHARD, GALLARDO, E., AND KHORAZO, D. 1936 Precipitins in the ocular tissues of rabbits generally and locally immunized with crystalline egg albumin. Am. J. Ophth., **19**, 852–858.

VAN DURME, P. 1903 Über Staphylokokken und Staphylolysin. Hygienische Rundschau, **13**, 66–68.

VEIEL, F. 1904 Die Staphylokokken des Chronischen Ekzems. Münch. Med. Wchnschrift., **51**, 13–16.

WALKER, I. C., AND ADKINSON, J. 1917 Studies on *Staphylococcus aureus*, *albus* and *citreus* and on *Micrococcus tetragenus* and *M. catarrhalis*. J. Med. Res., **35**, 373–390.

WELCH, W. H. 1891 Conditions underlying infection of wounds. Am. J. Med. Sciences, **102**, 439–465.

WINSLOW, C.-E. A., ROTHBERG, W., AND PARSONS, E. I. 1920 Notes on the classification of the white and orange staphylococci. J. Bact., **5**, 145–167.

WINSLOW, C.-E. A., AND WINSLOW, A. R. 1908 Systematic relationships of the coccaceae. Wiley and Sons, New York.