LOSS OF THE PROPERTIES OF HEMOLYSIN AND PIGMENT FORMATION WITHOUT CHANGE IN IMMUNOLOGICAL SPECIFICITY IN A STRAIN OF STREPTOCOCCUS HAEMOLYTICUS

By REBECCA C. LANCEFIELD, Ph.D.

(*From the Hospital of The Rockefeller Institute for Medical Research*)

The evidence presented in this paper shows that a strain of hemolytic streptococcus lost its functions of producing hemolysin and pigment but retained its serological specificity.

Valentine and Krumwiede (1) reported alterations in a culture of hemolytic streptococcus of human origin which resulted in a suppression of the power to elaborate hemolysin. Among numerous strains of hemolytic streptococci investigated by them one gave rise, after repeated subcultures, to about 10 per cent of non-hemolytic colonies which were surrounded by a green zone, while the remaining colonies were normally hemolytic. Subcultures from individual colonies of both forms remained constant in their effect on blood, but the organisms were, nevertheless, immunologically identical in so far as could be determined by immune sera prepared against pure cultures of each form. The immunological identity of the two strains was established by the reactions of agglutination and agglutinin absorption. The hemolytic form was slightly more virulent than the non-hemolytic, although in both instances the virulence was so low as to be of doubtful significance. The fact that mouse passage enhanced the virulence of the non-hemolytic form without restoring its hemolytic power indicated that the two functions were probably unrelated.

Todd (2) observed that the property of producing hemolysin disappeared in two strains of *Streptococcus haemolyticus* as the virulence of the organisms was increased by mouse passage. The formation of hemolysin by these strains was greatly influenced by growth under reduced oxygen tension. The virulent form, which was non-hemolytic in the presence of oxygen, was, when grown under anaerobic conditions, almost as hemolytic as the original avirulent organism. After many transfers on artificial media, the streptococci regained their hemolytic property and lost progressively their virulence for mice. This reversion, together with the fact that the cultures were always hemolytic in the absence of oxygen, was considered sufficient identification of the organisms as modified forms of *Streptococcus haemolyticus*. In a subsequent study of one of these strains, the immunological

459

identity of the two forms was established by type-specific (M) precipitin reactions and mouse protection tests (3).

A somewhat similar change to that observed by Todd as occurring during mouse passage was reported recently by Fry (4) as probably occurring in the human subject. In six cases of puerperal sepsis both hemolytic and green-forming streptococci were isolated in aerobic cultures from different foci of infection in the same patient. In four other cases only green-producing streptococci were obtained, while in one additional case a mixture of green-producing and hemolytic forms was found in several successive blood cultures from this patient. In addition, at least two cultures which were slightly hemolytic and one which was strongly hemolytic when first isolated, lost their hemolytic capacity and became green-forming after several subcultures. All of these strains produced hemolysin when grown anaerobically or when grown in 10 per cent horse serum broth. Serological studies, made to test the identity of the strains obtained from a single patient, were completed in two cases. In both instances, reciprocal agglutinin absorption experiments were strongly suggestive of the serological identity of the hemolytic and green-producing strains isolated from the same individual.

The loss of certain functions, recorded in this paper, occurred in *Streptococcus haemolyticus* O 90 during a serological study of this organism. As previously noted (5, 6), this strain of streptococcus was classified serologically as a member of one of the specific types of Group B, the group composed chiefly of strains of bovine origin. It may be recalled that a C substance, specific for Group B, is elaborated by all members of this group and serves in the precipitin reaction to identify strains included in the group. More complete identification is afforded by classification into types by means of type-specific polysaccharides, the so called S substances. The strain under discussion, Streptococcus O 90, possessed in addition the distinguishing characteristic of elaborating a yellow-brown pigment. For several years it has been observed that certain strains, particularly those other than human in origin, form pigments varying in color from yellow-brown to lemon. The pigment is contained within the bacterial cells and is manifest in microorganisms grown in fluid medium and also in plain agar plates without dextrose or with dextrose in concentrations lower than 1 per cent. It is apparent only under conditions of partial or of complete anaerobiosis, as in deep broth cultures, in the deep colonies in poured plates, or in cultures grown in anaerobic jars. Under aerobic conditions the surface colonies on plain blood agar plates are colorless.

This relationship between color and oxidized state of the pigment has not, however, held true with every chromogenic strain. Among 29 strains of hemolytic streptococci classified in Groups B and D, nine were observed which produced pigment varying in amount and color. Pigment-forming strains occurred in all three serological types of Group B.[1] Although two of the chromogenic strains were of human origin, the remaining seven were derived from sources other than human. One of the strains of human origin was obtained in pure culture from the throat of a child, but in spite of this gave rise to no symptoms of disease. The other strain with a history of human derivation had been under laboratory cultivation for many years. All of its cultural, biochemical, and serological reactions were the same as those of the other Group B strains of non-human origin.

A considerable quantity of pigment was obtained from Strain O 90. Following acid extraction of the bacteria, a layer of precipitated pigment was observed on the upper surface of the bacterial sediment. On neutralization of the solution in which the previously extracted bacteria were suspended, the pigment dissolved and could be separated by throwing down the bacterial sediment in a centrifuge. The water-soluble pigment was deep red and markedly fluorescent. It was precipitated on the addition of hydrochloric acid, and was redissolved on neutralization; it was not soluble in 95 per cent ethyl alcohol, in ether, or in acetone in this extracted form, nor could it be extracted from intact bacteria which were suspended in these solvents. The dissolved pigment did not hemolyze rabbits' red blood cells.

The only record found in which the occurrence of chromogenic hemolytic streptococci was noted was that of Durand and Giraud (7). The eleven strains, out of 125 examined, in which they observed a yellow-brown pigment were all of human origin except one isolated from a rat. Some of these streptococci were the causative agents of disease and others were considered to be saprophytes. The chromogenic strains in the present study, on the other hand, were chiefly non-human in origin, as noted above, and were all in serological groups not usually associated with human disease.

Durand and Giraud found that the pigment was formed in the absence of oxygen, and that impoverishment of the medium with respect to starchy constituents

---

[1] The characteristics of these strains have been recorded in preceding papers (5, 6). Their distribution with respect to serological types was as follows: eight belonged to Group B: Strains O 90 and K 158 A were in Type I; Strains V 9, B 112, and B 132 were in Type II; Strains B 115 and K 198 were in Type III; and Strain B 126 was unclassified. Strain C 7 belonged to Group D.

resulted in diminution or disappearance of chromogenic power. Potato starch, glycogen, inulin, and dextrose favored the elaboration of pigment while certain other carbohydrates inhibited it. They found it difficult or impossible to restore the pigment-forming function after it had been suppressed. They state that they were able to demonstrate some serological relationship among the chromogenic strains by the reactions of agglutination and agglutinin absorption, but the details of these serological relationships are not reported. The pigment described by Durand and Giraud appears similar in its reactions to the pigment of Strain O 90 observed in the present studies.

The alterations in Strain O 90 occurred during the first or second passage of the culture through mice, but whether this procedure bore any causal relationship to the changes observed is undetermined. In subcultures in poured blood agar plates, made after the second mouse passage, a large majority of the colonies showed neither surrounding zones of hemolysis nor greenish discoloration of the blood. When preliminary serological tests demonstrated the improbability of the non-hemolytic variant being a contaminant, more extensive investigations were undertaken to establish the relationship of the two forms. It was observed, at once, that the non-hemolytic form did not elaborate pigment in either solid or liquid media when grown aerobically or anaerobically. There was, however no indication that hemolysin and pigment are the same substance, for in addition to numerous other differences, solutions of the pigment did not hemolyze red blood cells.

With the exception of hemolysin and pigment-producing properties, all cultural and biochemical characteristics of the variant were the same as those previously observed in the original organism (5, 6).

Thus, both forms attained a final hydrogen ion concentration in 1 per cent dextrose broth of pH 4.4, both hydrolyzed sodium hippurate and grew on bile agar plates, both fermented trehalose but not sorbitol, and both failed to reduce methylene blue in milk and were unaffected by streptococcus bacteriophage. Each form of this strain has, as far as observed, remained true to form for a period of almost 3 years under a variety of cultural conditions and during serial passages through mice.

Before the immunological tests were performed, the purity of the strains with respect to hemolytic properties was insured by cultivation in series with the selection of single colonies from blood agar plates. The two forms were then employed for preparing antisera.

Formalinized cultures, injected intravenously, were used as the immunizing agents. Rabbits R 32-61 and R 32-62 received the hemolytic form, and R 32-63 and R 32-64 the non-hemolytic form, in three weekly courses. The resulting serum was employed in agglutination, precipitin, and mouse protection tests.

Before use in the serological tests the variant and the original form of this strain were again tested for hemolytic and pigment-producing capacity, in both aerobic and anaerobic cultures. Tests for hemolysin in fluid cultures were also made in the usual manner by mixing broth cultures in varying dilutions with rabbits'

TABLE I

Cross-Agglutination Tests

Antisera and Cultures of the Hemolytic and the Non-Hemolytic Forms of Strain O 90

Antiserum		Cultures used in agglutinations	Final serum dilution						
Against	Serum No.	Form	1-20	1-40	1-80	1-160	1-320	1-640	1-1280
Hemolytic form........	R 32-61	Hemolytic	++++	++++	++++	++++	+	−	−
" "	R 32-61	Non-hemolytic	++++	++++	++++	++++	+	−	−
" "	R 32-62	Hemolytic	++++	++++	++++	±	−	−	−
" "	R 32-62	Non-hemolytic	++++	++++	++++	±	−	−	−
Non-hemolytic form....	R 32-63	Hemolytic	++++	++++	++++	+	−	−	−
" "	R 32-63	Non-hemolytic	++++	++++	++++	+	−	−	−
" "	R 32-64	Hemolytic	++	+++	+++	+++	±	−	−
" "	R 32-64	Non-hemolytic	++	+++±	+++±	+++±	±	−	−

In Tables I, II, and III ±, +, ++, +++, ++++ indicate degrees of reaction; — indicates a negative reaction.

In the agglutination reactions in Tables I and III, broth controls and controls with normal serum in the same series of dilutions as the immune serum were completely negative.

washed red blood cells. The original culture maintained its hemolytic and chromogenic functions; the variant lacked them.

Agglutination tests were performed as follows: The bacterial cells, after centrifugation from 18 hour broth cultures, were resuspended in broth and added in volumes of 0.5 cc. to equal volumes of serum diluted serially in broth. Readings were made after incubation for 2 hours at 56°C. .

The data presented in Table I demonstrate that an immune serum prepared with either form of this strain agglutinated both the original and the variant to the same titer. The non-hemolytic form agglu-

tinated somewhat more rapidly than did the hemolytic, but the final titers always corresponded and the agglutinated organisms in both instances took the form of compact discs. As a general rule, the agglutination reaction is not suitable for immunological identification of Group B hemolytic streptococci on account of the marked tendency towards non-specific cross-agglutination. However, this difficulty was not encountered in these experiments because organisms of Type I, to which these strains belong, agglutinate specifically. Consequently, the fact that reciprocal specific agglutination of both strains occurred was valid evidence in support of the view that they were immunologically identical.

Additional proof of this identity was furnished by precipitin reactions. It has been established that from hemolytic streptococci belonging to Group B two characteristic polysaccharides are extractable: (1) a group-specific C substance, and (2) a type-specific S substance. Since Strain O 90 belongs to Group B, Type I, it was possible to establish the serological identity of the non-hemolytic variant and the original hemolytic strain by testing for the presence of these two carbohydrates by means of the precipitin reaction.

Hydrochloric acid extracts (5) were made from the bacterial sediment of both varieties of organisms, using portions of the same cultures employed in the agglutinations. These extracts were tested for the group-specific C substance with an antiserum known to contain anti-C precipitin, but no Type I anti-S precipitin. The same extracts were tested for the Type I S substance by precipitin tests with the type-specific antisera used in the agglutination reactions.

The precipitin tests were performed as follows: To a series of tubes containing 0.4 cc., 0.1 cc., and 0.025 cc. of extract in a final volume of 0.4 cc., a constant volume of 0.2 cc. of serum was added. Final readings were made after 2 hours incubation at 37°C., and overnight in the ice box.

The results are summarized in Table II. It was evident from the results of the direct precipitin tests that the group-specific polysaccharide, C, and the type-specific polysaccharide, S, were both present in the variant of Strain O 90 and in about the same amounts as in the original hemolytic form.

The data presented in the third column show that extracts of the original and the changed form of Strain O 90 reacted equally well with a serum of known anti-C content. The precipitates were disc-like but not heavy because this antiserum

was of only moderate titer. In order to eliminate the type-specific S reaction and to bring out the group-specific C reaction, the antiserum employed to detect the C substance was prepared with a strain of a serological type heterologous to Strain O 90. On the other hand the relationships due to the type-specific S substance in these two strains are shown when extracts are tested with serum of the homologous type, as indicated in the last four columns of Table II. Sera from Rabbits R 32-61 and R 32-62, immunized with the hemolytic form of Strain O 90, and sera from Rabbits R 32-63 and R 32-64, immunized with the non-hemolytic form of

TABLE II

Cross-Precipitin Tests

Antisera and Cultures of the Hemolytic and the Non-Hemolytic Forms of Strain O 90

Extracts		Anti-C serum (containing no Type I anti-S precipitins) against a Type III strain	Type I anti-S serum against			
			Hemolytic O 90		Non-hemolytic O 90	
Culture	Amount		Serum R 32-61	Serum R 32-62	Serum R 32-63	Serum R 32-64
	cc.					
Hemolytic O 90 (Type I)	0.4	++	++++	++++	+++±	+++
	0.1	++	++++	++++	+++	+++±
	0.025	±	+++	++±	+±	++
Non-hemolytic O 90 (Type I)	0.4	+	++++	++++	+++	+++
	0.1	++	++++	++++	+++	+++±
	0.025	++±	++	++	+±	+±

In the precipitin reactions in Tables II and III, the precipitates indicated by +++ and ++++ signs were in the form of unbroken discs. Controls of extract with normal serum and with saline alone were uniformly negative.

Strain O 90, gave equally good precipitates of the disc type with extracts of both the hemolytic and the non-hemolytic forms of the microorganism. Although these sera were not specifically absorbed, most of the reaction was undoubtedly due to the presence of the Type I specific antibody, for preliminary tests had shown them to be almost entirely devoid of anti-C precipitin.

Agglutinin and precipitin absorption experiments further substantiated the evidence for the serological identity of the two forms of Strain O 90.

Samples of serum from a rabbit immunized with the hemolytic form of Strain O 90, were mixed with equal parts of heat-killed bacteria packed by centrifugation

in a graduated centrifuge tube. One sample was absorbed with organisms of the hemolytic form and one with organisms of the non-hemolytic form of Strain O 90. After 45 minutes' incubation in a water bath at 37°C., the mixtures were centrifuged and the supernatant serum thus absorbed was used for agglutination and precipitin tests in the manner described above.

TABLE III

Absorption of Agglutinins and Precipitins by the Hemolytic and the Non-Hemolytic Form of Strain O 90

(a) Absorption of Agglutinins

Antiserum R 36-60	Culture used in agglutinations	Results of agglutinations Final serum dilutions			
	Form	1-10	1-20	1-40	1-80
Unabsorbed.......................	Hemolytic	++++	++++	—	—
"	Non-hemolytic	++++	++++	—	—
Absorbed with hemolytic O 90........	Hemolytic	—	—	—	—
" " " O 90........	Non-hemolytic	—	—	—	—
" " non-hemolytic O 90....	Hemolytic	—	—	—	—
" " " O 90....	Non-hemolytic	—	—	—	—

(b) Absorption of Precipitins

Antiserum R 36-60	Polysaccharides from hemolytic O 90 used in the precipitin test	
	C (group-specific)	S (type-specific)
Unabsorbed...	+++	++±
Absorbed with hemolytic O 90.........................	—	—
" " non-hemolytic O 90.....................	—	—

0.2 cc. of antiserum and 0.2 cc. of a 1–20,000 dilution of polysaccharide were used in these tests. Final readings were made after 2 hours at 37°C. and overnight in the ice box.

From Table III *a* it is evident that Serum R 36-60, which agglutinated to the same titer both varieties of Streptococcus O 90, lost all of its type-specific agglutinins after absorption with either variety of organism. A comparable removal of precipitins from the same absorbed serum was demonstrated (Table III *b*). Absorption with either form of the streptococcus removed both anti-C and anti-S precipitins.

Comparative virulence and cross-protection tests were also made with the variant and the original form of this strain as follows:

16 hour broth cultures were diluted serially with broth so that 0.5 cc. contained the amount of original culture required. Dilutions ranging from 10^{-1} cc. to 10^{-8} cc. of original culture were inoculated intraperitoneally into white mice of about 18 gm. weight. Other sets of mice were inoculated simultaneously with similar dilutions of the culture together with 0.5 cc. either of normal serum or of the sera

TABLE IV

Cross-Protection Tests in Mice

Antisera and Cultures of the Hemolytic and the Non-Hemolytic Forms of Strain O 90

Culture	Virulence controls		Antiserum against			
	No serum	Normal serum	Hemolytic O 90		Non-hemolytic O 90	
			R 32-61	R 32-62	R 32-63	R 32-64
cc.						
*Hemolytic O 90						
10^{-8}	S	D 42 hrs.	S	S	S	S
10^{-7}	S	D 42 "	S	S	S	S
10^{-6}	D 19 hrs.	D 19 "	S	S	S	S
10^{-5}	D 67 "	D 42 "	S	S	S	S
10^{-4}	D 19 "	D 19 "	S	S	S	S
10^{-3}	D 19 "	D 19 "	S	S	S	S
10^{-2}	—	D 19 "	S	S	S	S
10^{-1}	—	D 19 "	D 19 hrs.	D 19 hrs.	D 19 hrs.	D 19 hrs.
†Non-hemolytic O 90						
10^{-8}	S	D 120 hrs.	S	S	S	S
10^{-7}	D 47 hrs.	S	S	S	S	S
10^{-6}	D 45 "	D 96 "	S	S	S	S
10^{-5}	D 69 "	D 96 "	S	S	S	S
10^{-4}	D 45 "	D 72 "	S	S	S	S
10^{-3}	D 27 "	D 216 "	S	S	S	S
10^{-2}	—	D 72 "	S	S	S	S
10^{-1}	—	D 72 "	S	D 10 days	S	S

S indicates survival for 12 days.

D indicates death within the number of hours recorded.

— indicates test omitted.

* Estimated by plate counts as 461 million colonies per cc.

† Estimated by plate counts as 401 million colonies per cc.

of rabbits immunized with the hemolytic or the non-hemolytic form of Strain O 90. The serum was injected simultaneously with the culture, for it was known (6) that mice infected with Group B streptococci are effectively protected by this method. The mice were observed for 12 days and animals surviving for this period were considered effectively protected and were recorded as survivors.

The number of streptococci in the three highest dilutions was estimated by mixing the respective dilutions with blood agar in plates and counting the colonies after 48 hours' incubation.

The data recorded in Column 2 of Table IV show the relative virulence of the two forms of Streptococcus O 90. While the minimal lethal doses of the infecting organism were practically the same, the hemolytic form uniformly killed mice from 12 to 24 hours earlier than the non-hemolytic form in the same dilution. In addition to the virulence test just described, other controls (Table IV, third column) were included to show that normal rabbit serum did not protect mice against infection with these cultures. Samples of the same immune sera which were used in the agglutination and precipitin tests, however, protected mice against infection with the original culture and its variant.

SUMMARY

1. A variant arising in a culture of hemolytic streptococcus was shown to have lost the properties of producing pigment and hemolyzing blood. Despite the loss of these two functions, it had in common with the strain from which it was derived certain other distinguishing biochemical characteristics, as follows: Both attained the same hydrogen ion concentration in dextrose broth; both hydrolyzed sodium hippurate, grew on bile agar, and fermented trehalose but not sorbitol; both failed to reduce methylene blue in milk cultures, and were insusceptible to the action of streptococcus bacteriophage. In addition, the virulence of the variant remained the same as that of the original culture.

2. The antigenic and serological specificity of the variant was identical with the group and type specificity of the original strain (Group B, Type I). These specificities were established by the use of immune sera prepared by immunization of rabbits with each form. The immunological reactions employed were reciprocal agglutination, precipitation, agglutinin and precipitin absorption, and passive mouse protection.

BIBLIOGRAPHY

1. Valentine, E., and Krumwiede, C., *J. Exp. Med.*, 1922, **36**, 157.
2. Todd, E. W., *J. Exp. Med.*, 1928, **48**, 493.
3. Lancefield, R. C., and Todd, E. W., *J. Exp. Med.*, 1928, **48**, 751.
4. Fry, R. M., *J. Path. and Bact.*, 1933, **37**, 337.
5. Lancefield, R. C., *J. Exp. Med.*, 1933, **57**, 571.
6. Lancefield, R. C., *J. Exp. Med.*, 1934, **59**, 441.
7. Durand, P., and Giraud, P., *Compt. rend. Acad.*, 1923, **177**, 1333.