STUDIES ON VIRULENCE OF GROUP A β-HEMOLYTIC STREPTOCOCCI[1]

JOHN M. LEEDOM AND S. S. BARKULIS

Department of Biological Chemistry, University of Illinois College of Medicine, Chicago, Illinois

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The fact that some, but not all, group A β-hemolytic streptococci are capable of producing lethal infections in man and animals has long been appreciated. The factor or factors primarily responsible for the ability to initiate infections is not known.

Several streptococcal exotoxins, including two hemolysins, a fibrinolysin, desoxyribonuclease and ribonucleases, a nucleotidase, a specific diphosphopyridine nucleotidase, erythrogenic toxin, hyaluronidase, proteinase, leukocidin, and leukotoxin have been demonstrated. Despite the fact that all of these enzymes or toxins are potentially injurious to a host animal, no one product is characteristically associated with virulent strains, and all may be elaborated by avirulent strains.

Todd (1928*a*) and Todd and Lancefield (1928) observed that avirulent nontypable group A β-hemolytic streptococci are of the smooth colonial morphology, whereas virulent strains have a serologically distinguishable M protein by which they can be typed and are largely of the mucoid or matt colonial morphology. However, not all M protein-containing streptococci are virulent (Lancefield, 1940). It has also been noted that virulence can be enhanced by mouse passage.

Wilson (1953, 1957) studied the dynamics of interaction between host phagocytes and many strains of group A β-hemolytic streptococci. He found that streptococci which have M protein tend to resist ingestion by human, mouse, and guinea pig leukocytes. Resistance to phagocytosis is not affected by the death of the organisms (Wiley and Wilson, 1956). Wilson also studied a property of certain strains of group A β-hemolytic streptococci which enables them to destroy a leukocyte after they have been ingested. This ability is not a function of any recognized extracellular elaboration of the streptococcus but is apparently brought about in an unknown manner by the chain after ingestion. This property is termed leukotoxicity. Wilson (1957) has not found a correlation between the leukotoxicity of a streptococcal strain and its virulence.

Most previous studies on the virulence of the group A β-hemolytic streptococcus have compared various virulent strains for their ability to produce M protein, hyaluronic acids, and various toxins or enzymes (Kass and Seastone, 1944; Quinn *et al.*, 1953; and Wasielewski, 1956). In the present study we have attempted to compare the properties of virulent variants with an avirulent parent culture. The variants were isolated after serial mouse passage of the avirulent culture.

MATERIALS AND METHODS

Strain of streptococcus. A streptococcal strain designated as Q496 isolated from a convalescent patient with pharyngitis was employed. We are indebted to Dr. Eugene Fox for this culture. The strain is of low virulence for mice and does not contain M protein. It has a glossy colonial morphology.

Mouse passage. Serial mouse passage of the avirulent Q496 culture was performed to facilitate the isolation of variants of streptococci of enhanced virulence. One ml of a Q496 stock culture was inoculated into 10 ml of brain heart infusion (Difco) broth and allowed to grow overnight. This culture (0.5 ml) was inoculated intraperitoneally into a mouse. After 24 hr, the mouse was killed, the spleen removed aseptically, and ground in a mortar and pestle with 3 ml brain heart infusion. This suspension (0.5 ml) of ground spleen was inoculated intraperitoneally into another mouse. One ml was pipetted into 10 ml brain heart infusion, incubated overnight, and the culture saved at 4 C. Plates were also made from the ground spleen to determine the purity of the culture. If the ground spleen suspension was contaminated with organisms other than hemolytic streptococci, the preceding 10-ml brain heart infusion culture was used to continue mouse passage. Stock cultures were grown in Todd-Hewitt broth (Difco) supplemented with 5 per cent sheep

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blood, and 1-ml amounts were sealed in thermal death point tubes which were kept frozen at −55 F. The organism isolated after 22 passages was designated as Q22× and after 43 passages as Q43×, and these were preserved as stock cultures.

Mouse virulence. The mouse virulence of these three cultures of streptococci was determined. Ten ml of brain heart infusion broth were inoculated with each culture and allowed to incubate 21 hr. Optical density readings were made of samples of the three cultures at 660 mμ against a brain heart infusion blank. Serial dilutions of each culture were made and 0.5 ml of the appropriate dilutions (for Q496, 10^{-1} through 10^{-5} dilutions; for Q22× and Q43×, 10^{-4} through 10^{-8} dilutions) was inoculated intraperitoneally into five groups of six male, 15 to 20-g white Harlan mice. One-ml samples of the 10^{-8} dilution of each culture were used to make pour plates to determine the number of chains received by each mouse. A group of six mice inoculated intraperitoneally with 0.5 ml of sterile brain heart infusion served as a control. The animals were observed for deaths for 1 week following inoculation and the LD_{50} dosage determined for each culture.

Rate of multiplication or disappearance of streptococci in vivo. The fate of streptococci injected intraperitoneally into white mice was studied in the following manner. Eight-hour cultures of the Q496, Q22×, and Q43× strains were grown in Todd-Hewitt broth. The concentration of organisms in these cultures was determined by serial dilution and plating in the usual manner. Groups of four mice were injected intraperitoneally with 0.5 ml of the 10^{-2} dilution of the Q496, Q22×, and Q43× cultures. Mice of the same weight and age were killed at 1, 3, 5, and 7 hr, the spleens ground in Todd-Hewitt broth, and the number of organisms (chains) in each spleen determined by dilution and plating.

Resistance to phagocytosis and leukotoxicity. The ability of the Q496 and Q43× strains to resist phagocytosis was studied by the method of Wilson (1957). The preparations were also observed for evidences of leukotoxicity—that is, the ability of chains of each culture to cause the death of leukocytes following phagocytosis (Wilson, 1957).

Bacteriostatic test. Indirect bacteriostatic tests were carried out by the method of Rothbard (1945) with the slight modifications employed by Barkulis *et al.* (1958). Cocci from young cultures of Q496, Q22×, and Q43× were incubated with human blood (heparinized) to which was added serum from a normal rabbit, or antiserum from a rabbit immunized with a type 14 streptococcal culture. As will be shown, cultures Q22× and Q43× proved to be type 14.

M protein content. Cell walls of cocci grown in brain-heart infusion broth were isolated and extracted for M protein by the method previously described from this laboratory by Barkulis and Jones (1957). The M protein content of extracts obtained from a specified weight of cell walls from each culture was estimated semiquantitatively using ring tests against a reference type 14 rabbit antiserum. The extracts were diluted to determine the highest dilution which would give a positive ring test.

Comparative glycolytic rates of resting cells. Five hundred ml of Todd-Hewitt broth were inoculated with a 100-ml overnight broth culture of the strain to be studied. After 4.5 hr, cocci were harvested by sedimentation, washed 2 times with distilled water, and resuspended in 4 to 5 ml of distilled water. Samples of this suspension were dried at 100 C in previously weighed beakers to determine the weight of cocci per ml. An incubation mixture containing 0.3 ml of cell suspension, 10 μmoles K_2HPO_4, adjusted to pH 7.2, 3 μmoles $MgCl_2$, 4 μmoles adenosine triphosphate, and 1 mg of yeast extract in a final volume of 4.2 ml was placed in a 10-ml beaker and allowed to equilibrate with a water circulator maintained at 37 C. After 10 min, 0.2 ml of 0.1 M glucose was added to the beaker and the rate of lactic acid production determined microtitrimetrically by adding NaOH during glycolysis and maintaining the pH constant at 7.5 as determined with a Coleman pH meter. Anaerobic conditions were assured by bubbling helium through the incubation mixtures. Lactic acid Q values were calculated from the earliest 10-min portion of the run, during which acid production was progressing at a constant linear rate.

Streptolysin S and streptolysin O assay. The experimental conditions of Bernheimer (1949) for streptolysin S production and assay were employed with the minor modifications indicated by Younathan and Barkulis (1957).

Ten-ml brain heart infusion cultures, to which

20 mg of ascorbic acid had been added during growth to increase streptolysin O production (Slade and Knox, 1950), were incubated with freshly neutralized cysteine hydrochloride to activate any streptolysin O present, diluted, and assayed for hemolytic activity by the same method employed for streptolysin S. Dilutions through which oxygen had been bubbled were run in parallel and any hemolytic activity present in the aerobic samples was subtracted from the values obtained on the reduced samples to correct for any streptolysin S which might have been present.

RESULTS

Serial mouse passage. Before serial mouse passage, the Q496 strain was of the glossy colonial morphology. Variants Q22× and Q43× isolated after serial mouse passage exhibit the matt colonial morphology. Table 1 shows the data obtained in assays of the virulence of these cultures in mice. It is apparent that a considerable enhancement of virulence occurred after 22 serial mouse passages. Some enhancement of virulence was also evident after an additional 21 passages, but this was not striking.

Fate of the Q cultures in vivo. The data in table 2 are the results of representative experiments undertaken to follow the fate *in vivo* of the Q cultures in the peritoneal cavities of white mice. The number of streptococci of each strain injected was approximately equal. Essentially equal numbers of viable streptococcal chains of the avirulent Q496 strain were recovered at 1 and 3 hr after injection. No viable organisms of the Q496 strain were recovered at 5 and 7 hr after injection. A large increase in the numbers of the virulent Q22× and Q43× organisms recoverable from the spleen was noted between 1 and 7 hr after injection.

TABLE 1

LD_{50} for mice of Q cultures

Culture	No. Mouse Passages	LD_{50}*
Q496	0	500,000
Q22×	22	100
Q43×	43	25

* Recorded as the number of streptococcal chains required to kill half of the animals during the observation period.

Resistance to phagocytosis and leukotoxicity. Preparations of the three cultures of streptococci studied under phase microscopy by the method of Wilson (1957) revealed that the Q496 organisms were readily ingested by human neutrophils. Individual phagocytes observed for as long as 30 min after ingestion of several chains remained actively motile and exhibited no evidence of leukotoxic injury. Other preparations of the Q496 culture and human leukocytes incubated at 37 C for 1 to 2 hr revealed actively motile neutrophils literally stuffed with cocci. The neutrophils did not exhibit any evidences of leukotoxic damage. At the end of 3 hr incubation, some of the neutrophils which had ingested cocci were motionless and apparently dead, although many other neutrophils which contained large numbers of cocci were still actively motile. Since extensive proliferation of the streptococci in the preparations occurred during the 3 hr incubation, the presence of a few motionless phagocytes does not constitute evidence of leukotoxicity. The extracellular elaborations and metabolites of proliferating streptococci are known to be capable of killing leukocytes (Wilson, 1957), but the cumulative effect of these substances is not to be confused with a specific leukotoxicity as defined by Wilson.

Observations on preparations of Q22× and Q43× for periods of up to 3 hr failed to disclose any ingestion of streptococcal chains by neutrophils. Phagocytes happening to contact chains of either the Q22× or Q43× cultures simply moved around them without any attempt at ingestion. Preparations incubated overnight showed extensive streptococcal proliferation with most of the leukocytes motionless, but extensive search failed to reveal any neutrophils which had ingested cocci. Thus, cocci of the Q22× and Q43× cultures show marked resistance to ingestion by phagocytes. Since cocci were not phagocytized, a statement as to the leukotoxicity of these cultures cannot be made.

Indirect bacteriostatic tests employing human leukocytes, type 14 rabbit antiserum, and cocci of the Q496, Q22×, and Q43× cultures gave the results shown in table 3. Neither the avirulent, easily ingested, Q496 culture, nor the virulent Q22× and Q43× cultures were affected in the absence of antibody. Addition of type 14 rabbit antiserum enabled the leukocytes to inhibit completely multiplication of the Q22× and

TABLE 2

Fate of Q cultures in vivo

Culture Injected	No. Chains Streptococci Injected	No. Chains Streptococci Recovered from Spleen at Various Times Postinjection			
		1 hr	*3 hr*	*5 hr*	*7 hr*
Q496	1.5×10^6	4.1×10^3	5.5×10^3	0	0
Q22×	5.0×10^6	1.4×10^4	1.9×10^5	6.3×10^5	5.3×10^6
Q43×	1.0×10^6	2.2×10^3	3.2×10^3	1.5×10^5	6.0×10^5

Experiments done using male white mice of the same weight obtained from the vendor, Harlan.

TABLE 3

Bacteriostatic test

Strain of Streptococcus	Inhibition of Growth	
	Antiserum absent*	Antiserum present†
Q496	None	Slight
Q22×	None	Complete
Q43×	None	Complete
Type 30 control	None	None

* Tubes contained sterile serum drawn from a normal unimmunized rabbit.

† Antiserum produced by immunization of rabbit with a culture of type 14 group A hemolytic streptococcus designated S-23.

TABLE 4

Type 14 serologic reactivity of extracts of cell walls to Q cultures

Culture	Mg Dry Weight of Cell Walls Extracted into 1 Ml	Highest Dilution Showing Serologic Reactivity*
Q496	5.35	0
Q22×	2.86	1:40
Q43×	4.20	1:80

* Ring tests against type 14 rabbit antiserum.

Q43× cultures. Growth of the Q496 culture was only slightly inhibited by the addition of type 14 M antiserum.

M protein content. Table 4 depicts the results of ring tests for M protein on portions of extracts of Q496, Q22×, and Q43×. No detectable M protein was found in the Q496 culture. The Q22× and Q43× cultures contain approximately the same amount of M protein. Increase in virulence was correlated with the acquisition of serologically detectable type 14 M protein.

TABLE 5

Glycolytic rates

Strain of Streptococcus	Q Values of Lactic Acid*
Q496	322
Q22×	331
Q43×	220

* Microliters lactic acid produced from glucose per hour per mg dry weight of organism.

TABLE 6

Streptolysin production: comparisons between cultures adjusted to equal optical densities

	Organism	Units
Streptolysin S	Q496	1500
	Q22×	500
	Q43×	200
		Units/ml Culture Supernatant
Streptolysin O	Q496	2.3
	Q22×	1.0

Comparative glycolytic rate of resting cells. Table 5 gives the average values for Q lactic acid determinations in resting young cultures of the three strains. No significant difference was found in the glycolytic abilities of the Q496 and Q22× cultures. In fact, values for different runs on these two organisms overlapped. The average Q value of lactic acid for the Q43× culture is definitely lower. These results indicate no change in glycolytic rate with the increase in virulence between the Q496 and Q22× cultures, and a definite, but moderate, decrease in glycolytic ability of the Q43× culture.

Streptolysin S and O assay. Table 6 shows the

streptolysin S production of equal numbers of cells of the three cultures in a resting suspension. It also depicts the amount of streptolysin O in supernatant fluid of the Q496 and Q22× cultures adjusted to equal optical density.

There is a marked decrease in streptolysin S production by the virulent cultures as compared to the avirulent culture. The same picture is found with respect to streptolysin O production, although the ratio of the difference encountered between the virulent and avirulent cultures is less marked.

DISCUSSION

Before discussing several aspects of the preceding data, it is pertinent to summarize some experiments conducted by Fox with the Q496 culture which he kindly called to our attention, and which were presented as part of his doctoral dissertation (1955). After 17 mouse passages of the Q496 culture, he was able to isolate a virulent type 14 culture. Acid-heat extraction of the typable variant by the method of Zittle (1942) was used to isolate type 14 M protein which was further purified using the anion exchange resin Amberlite XE-64. Under defined conditions, the M protein emerged as a single symmetrical peak. When similar extracts were obtained from the Q496 organism and subjected to chromatography, a protein fraction was obtained which eluted in similar fashion to the type 14 M protein from the virulent variant, had the same qualitative amino acid composition, but did not react serologically with type 14 antiserum. He was, however, able to show that when type 14 antiserum was adsorbed with Q496 whole cells, there was an appreciable loss of type 14 antibody. This was specific in that the type-specific antibody of a heterologous antiserum was not diminished on adsorption with Q496 cells. Finally, Fox was unable to detect any metabolic differences in glycolytic rates and breakdown of arginine to ornithine by resting cell suspensions of Q496 and the type 14 culture derived from it.

In our experiments, serial mouse passage of the glossy Q496 culture resulted in the isolation of variants of matt colonial morphology. Todd (1928*a*) obtained glossy forms after many transfers on chemically defined media of matt cultures. His attempts to reconvert these glossy forms to the original matt configuration by mouse passage were not successful. Lancefield and Todd (1928) studied this problem further and reported that only one of the strains tested, S43, reverted from glossy to matt. When they tested a concentrated extract of the S43 glossy form, they found a trace of serologically reactive M protein. Concentrated extracts from glossy cultures which failed to revert did not have any detectable M protein. They concluded that matt cultures might be degraded either to stable, glossy variants which had lost all the M protein and would not revert, or to a metastable, glossy form which retained traces of M protein and could be reconverted to matt morphology on serial mouse passage. By these criteria our glossy Q496 strain might have been expected to be stable in view of the failure to detect M protein. However, since the potency of type-specific antisera and judgments as to trace amounts of M protein in extracts might readily vary in different laboratories, the possibility that the Q496 culture is an unstable, glossy variant in the sense of Lancefield and Todd cannot be excluded.

Our experience with the Q496 culture also differed from that of Lancefield and Todd in that reversion of their S43 unstable, glossy variant to the matt form was not accompanied by an increase in mouse virulence. By contrast the matt variants in our study killed half of the mice injected in a dosage which was 3 to 4 logs lower than that required of the Q496 culture. Since increase in the virulence of matt forms following repeated mouse passage is well documented (Lancefield, 1940), this might have been observed by Lancefield and Todd had they continued beyond 8 mouse passages with the S43 strain.

Resistance to ingestion by host phagocytes is traditionally accorded an important role in determining the virulence of bacteria, particularly of the gram-positive cocci. Two substances, the M antigen of the cell wall and the hyaluronic acid capsule, seem to be the principal determinants in the ability of group A hemolytic streptococci to resist ingestion by phagocytes, with the former playing by far the more important role (Rothbard, 1948). The glossy Q496 organisms were readily phagocytized by human leukocytes when observed under the phase microscope; the matt variants were not. These findings parallel those of other investigators and are consistent with the thesis that resistance of a

culture to phagocytosis is associated with the presence of the M antigen on the cell walls.

The results of the bacteriostatic tests, however, seem to be at variance with the observations of phagocytosis. Organisms of all three cultures survived and multiplied to the same degree following 3 hr incubations with whole blood. Apparently, bacteriostasis of the glossy organisms did not occur despite the readiness with which they were phagocytized. Additions of type 14 antiserum effected a slight, but definite, inhibition of the growth of the glossy from and a marked inhibition of the matt forms. These results differ from those of Rothbard (1948) who found considerable bacteriostasis of glossy organisms in the absence of antiserum. The Q496 culture is not leukotoxic and does not seem to injure the neutrophil after phagocytosis. Thus death of the leukocyte following phagocytosis of the cocci does not account for the failure in inhibition. It seems that streptococci may be ingested by leukocytes, remain viable, and even proliferate intracellularly for considerable periods during which they will grow out, if plated. If this interpretation is correct, it also follows that something more than increased susceptibility to phagocytosis is involved in the bacteriostasis of M-containing streptococci in the presence of type-specific antibody and human leukocytes. The reaction of type-specific antibody with the M antigen on the streptococcal cell wall must not only promote phagocytosis of the organism, but also renders the ingested cocci more susceptible to the bacteriocidal mechanisms of the phagocyte. The reason for the slight inhibition of the growth of the Q496 culture in the bacteriostatic test in the presence of type 14 antiserum is not known. It might be due to the ability of these organisms to bind some type 14 antibody as suggested by the findings of Fox.

Experiments on the fate of organisms of the three cultures following intraperitoneal injection into mice have shown that up to 3 hr following injection, there is little detectable difference in the ability of the matt and glossy forms to gain entry to the circulation and reach the spleen. In the succeeding 2 hr, however, the glossy organisms are rapidly destroyed while the matt organisms rapidly proliferate. The observations on phagocytosis and the bacteriostatic test showing resistance to phagocytosis and failure of bacteriostasis of the virulent Q22× and Q43× variants in the absence of type-specific antiserum are quite compatible with the rapid proliferation *in vivo* of these cultures. As mentioned above, streptococci of the avirulent Q496 culture are easily phagocytized, but are not inhibited in the absence of type-specific antibody by a number of leukocytes sufficient to completely inhibit the two virulent cultures when type-specific antibody is present. These results seem to indicate that the Q496 strain is capable of proliferation after ingestion by phagocytes. Why then do streptococci of the Q496 culture disappear 3 hr after injection into mice? Two explanations seem logical. (a) Some factor *in vivo* other than phagocytosis may have been operative. (b) Wilson (1953) showed that streptococci could be phagocytized, egested, and reingested. Perhaps a streptococcal chain must be ingested, egested, and reingested several times to be inactivated in the absence of type-specific antibody. The number of leukocytes available *in vitro* might have been insufficient to permit several ingestions, whereas larger numbers of phagocytes were available *in vivo*.

The difference in cell wall structure which is implied by the presence of M protein in the matt forms and its absence in the glossy will be further documented in a subsequent publication on the quantitative amino acid composition of cell walls from Q496 and Q43× organisms (Tepper, Hayashi, and Barkulis, *in preparation*). Since phagocytosis must begin by surface contact between the leukocytes and the streptococci, it is tempting to ascribe the insusceptibility of the matt forms to a changed cell wall structure from the glossy.

Todd (1928*b*) observed that some matt strains of streptococci lost their hemolytic properties on serial mouse passage. He found that culture filtrates of a glossy culture had much greater hemolytic activity than did filtrates from the parent matt culture. Similarly, assay of the supernatants of our glossy culture showed slightly more streptolysin O activity than did supernatants from the Q22× culture. The glossy form also produced much more streptolysin S in the resting state than did the Q22× culture, and the latter produced more than the Q43× culture. One might expect the more virulent forms to produce larger amounts of these highly toxic hemolysins, but it has long been known that there is no correlation between differences in virulence

and the toxicity of the extracellular elaborations of streptococcal cultures. The decreased hemolysin production and decreased glycolytic ability (of the Q43× culture) may reflect a reduction of metabolic potentialities *in vitro* which is often seen in fastidious, highly virulent microorganisms which have become well adapted to a parasitic existence.

In this paper the Q cultures have been variously referred to as forms and variants, presumably of the same strain. Whether the conversion of a glossy form to the matt form or the increase in virulence of the latter on serial mouse passage takes place by a gradual adaptation of the whole culture, or represents a selection of mutants with increasing ability to survive in the animal host, is not known. Although this is an important question from the genetic viewpoint, it is incidental to these studies.

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SUMMARY

A strain of streptococcus of low virulence for mice has been subjected to serial mouse passage and virulent variant cultures recovered. There was reversion of colonial morphology from the smooth to the matt form on serial mouse passage. The virulence of the recovered organisms was markedly increased over the original culture.

Studies on the fate *in vivo* of the three Q cultures showed quick disappearance of viable cocci of the Q496 culture when injected intraperitoneally into mice and rapid multiplication of cocci of the virulent cultures. The Q496 strain was readily phagocytized by human neutrophils, but cocci of the Q22× and Q43× strains were not ingested. The Q496 strain was found to be nonleukotoxic. In the absence of ingestion of the other two cultures by phagocytes, no statement as to leukotoxicity can be made. Study of the three cultures with human neutrophils in the bacteriostatic test showed only slight inhibition of the Q496 culture by type 14 antibody and virtually complete inhibition of the Q22× and Q43× cultures. None of the three cultures were inhibited in the bacteriostatic test in the absence of type 14 antibody.

M protein was not found in extracts of Q496 cell walls. M protein was present in extracts of the cell walls of the Q22× and Q43× variants.

The glycolytic rate of the Q43× strain was significantly lower than that of the other two strains. Streptolysin S production decreased with increase in virulence. The Q22× culture produced less streptolysin O than the avirulent Q496 strain.

These results were discussed with the tentative conclusions that the outstanding feature accompanying increased virulence on mouse passage was a marked change of gross colonial morphology associated with a considerably changed cell wall structure. Accompanying these changes, and perhaps caused by them, was the great resistance of the virulent forms to phagocytosis which helped allow them to proliferate faster than the avirulent glossy forms upon injection into mice. Evidence was presented indicating that something more than increased susceptibility to phagocytosis is involved in bacteriostasis in the presence of type-specific antibody and living leukocytes. It also appeared that the virulent forms had decreased metabolic capacities as evidenced by lower glycolytic rates and extracellular hemolysin production.

REFERENCES

BARKULIS, S. S. AND JONES, M. 1957 Studies on streptococcal cell walls. I. Isolation, chemical composition, and preparation of M protein. J. Bacteriol., **74**, 207–216.

BARKULIS, S. S., WALSH, J., AND ECKSTEDT, R. 1958 Studies on streptococcal cell walls. II. Type specific antigenicity in rabbits. J. Bacteriol., **76**, 109–115.

BERNHEIMER, A. W. 1949 Formation of bacterial toxin (streptolysin S) by resting cells. J. Exptl. Med., **90**, 373–392.

FOX, E. N. 1955 Studies on the M antigen of group A hemolytic streptococci. Doctoral Thesis, Department of Microbiology, Western Reserve University.

KASS, E. H., AND SEASTONE, C. V. 1944 The role of the mucoid polysaccharide (hyaluronic acid) in the virulence of group A streptococci. J. Exptl. Med., **79**, 319–330.

LANCEFIELD, R. C. 1940 Specific relationships of cell composition to biological activity of hemolytic streptococci. Harvey Lectures, Ser. **36**, 251–290.

LANCEFIELD, R. C. AND TODD, E. W. 1928 Antigenic differences between matt hemolytic

streptococci and their glossy variants. J. Exptl. Med., **48,** 769–790.

QUINN, R. W., SEASTONE, C. V., AND WEBER, W. R. 1953 The relationship of the antigenic characteristics of streptococci and specific antibody responses following streptococcal infections. J. Infectious Diseases, **93,** 57–64.

ROTHBARD, S. 1945 Bacteriostatic effect of human sera on group A streptococci. I. Type specific antibodies in sera of patients convalescing from group A streptococcal pharyngitis. J. Exptl. Med., **82,** 93–106.

ROTHBARD, S. 1948 Protective effect of hyaluronidase and type-specific anti-M serum on experimental group A streptococcus infections in mice. J. Exptl. Med., **88,** 325–342.

SALTON, M. R. J. 1952 The nature of the cell walls of some gram-positive and gram-negative bacteria. Biochim. et Biophys. Acta, **9,** 334–335.

SLADE, H. D. AND KNOX, G. A. 1950 Nutrition and the role of reducing agents in the formation of streptolysin of a group A hemolytic streptococcus. J. Bacteriol., **60,** 301–310.

TODD, E. W. 1928*a* Further observations on the virulence of haemolytic streptococci with special reference to the morphology of the colonies. Brit. J. Exptl. Pathol., **9,** 1–6.

TODD, E. W. 1928*b* The conversion of hemolytic streptococci to non-hemolytic forms. J. Exptl. Med., **48,** 493–511.

TODD E. W. AND LANCEFIELD, R. C. 1928 Variants of hemolytic streptococci, their relation to type-specific substance, virulence, and toxin. J. Exptl. Med., **48,** 751–768.

WASIELEWSKI, E. 1956 Nucleasaktivität und Virulenz von A-Streptokokken. Arch. Hyg. u. Bakteriol., **140,** 581–596.

WILEY, G. AND WILSON, A. T. 1956 The ability of group A streptococci killed by heat or mercury arc irradiation to resist ingestion by phagocytes. J. Exptl. Med., **103,** 15–36.

WILSON, A. T. 1953 The egestion of phagocytized particles by leukocytes. J. Exptl. Med., **98,** 305–310.

WILSON, A. T. 1957 The leukotoxic action of streptococci. J. Exptl. Med., **105,** 463–484.

YOUNATHAN, E. S. AND BARKULIS, S. S. 1957 Effect of some antimetabolites on the production of streptolysin S. J. Bacteriol., **74,** 151–158.

ZITTLE, C. A. 1942 Antigenic structure of hemolytic streptococci of Lancefield group A. VII. Separation of the protein and nucleic acid of the type specific M substance and some chemical and serological properties of the purified type specific protein. J. Immunol., **43,** 31–46.