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# The serum opacity reaction of *Streptococcus pyogenes:* frequency of production of streptococcal lipoproteinase by strains of different serological types and the relationship to M protein production*

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The serum opacity reaction of *Streptococcus pyogenes* (Ward & Rudd, 1938) has been found to be associated with a lipoproteinase which acts upon the $\alpha_1$ lipoprotein of the serum of various species to produce opalescence (Krumwiede, 1954; Rowen & Martin, 1963). Some observations on the general properties of the streptococcal factor and on the nature of the reaction in aged serum are recorded in an accompanying paper (Hill & Wannamaker, 1968). Although the nature of the reaction is not fully defined and other factors may possibly produce opalescence in serum, the terms serum opacity reaction (SOR) and lipoproteinase will be used interchangeably in this communication.

Data presented by Ward & Rudd (1938), Gooder (1961), and Köhler (1963) suggested to us that production of the serum opacity reaction was rather closely associated with serotype as determined by M and T antigens. Furthermore, both Gooder and Köhler concluded that strains which were difficult to type by the M-precipitin method generally produced a SOR but that M-typable strains rarely produced this reaction. If an inverse relationship between M-antigen and SOR could be substantiated, the serum opacity reaction might be useful as a preliminary test to characterize group A strains as M-positive or M-negative. Further investigation of the production of this enzyme in individual strains in relationship both to serotype and to production of an M-antigen and an investigation of the consistency of production of lipoproteinase by individual strains were therefore undertaken.

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## METHODS

### *Source of strains*

The strains examined were obtained from a wide variety of sources. The majority were obtained from an investigation of pyoderma at the Red Lake and Cass Lake Indian Reservations in Minnesota (Anthony, Perlman & Wannamaker, 1967), from a study of pharyngitis conducted at the St Paul-Ramsey Hospital (Top, Kaplan & Wannamaker, unpublished observations), and from the diagnostic bacteriology laboratory of the University of Minnesota Hospitals. Strains from our laboratory stock collection were examined, as were the prototype strains obtained from the Communicable Disease Center in Atlanta, Georgia. Other strains were obtained through the courtesy of Dr Rebecca Lancefield of the Rockefeller University, Mr W. R. Maxted of the Central Public Health Laboratory at Colindale, England, and Dr Hugh Dillon of the University of Alabama, Birmingham, Alabama.

All strains were examined concurrently for the serum opacity reaction, group specific carbohydrate, M-antigen, T-antigens, and, when indicated, the 28R-antigen.

### *Determination of streptococcal serotypes*

Grouping antisera were obtained from the Communicable Disease Center in Atlanta, Georgia, as were M-typing antisera of types 1–6, 8, 11–15, 17–19, 22–26, 28–33, 36–44, 46, and 47. M-typing antisera for types 27, 34, 48, 49, and 51 were generously supplied by Dr Rebecca Lancefield who also supplied 28R antiserum. Type 9 antiserum was obtained from the Central Public Health Laboratory, Colindale, England. T-antisera were most generously supplied through the courtesy of Dr M. T. Parker and Mr W. R. Maxted of the Central Public Health Laboratory. The presence of M and 28R antigens on group A strains was determined by the capillary precipitin technique of Swift, Wilson & Lancefield (1943). Group A strains were examined for T-agglutination pattern by the method described by Williams (1958).

### *Screening of streptococcal strains for the serum opacity reaction*

Production of the serum opacity reaction was determined as follows: strains were grown in 5 ml. of Todd–Hewitt broth* overnight at 37° C. and one drop of 1% merthiolate solution was added. After collection by centrifugation, the cells were thoroughly resuspended in 2 ml. of horse serum† plus one drop of 1% merthiolate solution and the suspension was incubated overnight at 37° C. After centrifugation, the presence of opalescence in the supernatant serum was determined visually by comparison with the opalescence produced by two control strains—one producing lipoproteinase, the other not producing lipoproteinase. Measurement of the opalescence produced was not attempted; the results were scored only as positive or negative.

* Obtained from Difco Laboratories, Detroit, Michigan.

† Obtained from Grand Island Biological Company, Grand Island, New York.

## RESULTS

### *Production of lipoproteinase by strains of different serological types*

Table 1 shows the production of the serum opacity reaction by streptococci of various groups. Only strains of group A streptococci produced a SOR, a finding previously noted by Ward & Rudd (1938) and by Köhler (1963). Streptococci of groups B, C, D, G and F did not produce a SOR.

The production of lipoproteinase by types of group A streptococci as determined by M-antigen is seen in Table 2. A distinct association between M-type and SOR was found; all strains examined of a given type invariably produced lipoproteinase or invariably failed to produce it. Types consistently producing lipoproteinase were types 2, 4, 8, 9, 11, 13, 22, 25, 27, 28, 35, 44, 48 and 49. Strains of types 1, 3, 5, 6, 12, 14, 15, 17, 18, 19, 23, 24, 26, 29, 30, 31, 32, 33, 34, 36, 37, 38, 39, 40, 41, 42, 43, 46, 47, 50, 51, and provisional types Schoenborn, Hanson and Kingbird (Top, Wannamaker, Maxted & Anthony, 1967) were never found to produce a SOR.

Table 1. *Serum opacity reaction (SOR) of streptococcal groups*

| Group | Serum opacity negative (SOR −) (no. of strains) | Serum opacity positive (SOR +) (no. of strains) |
|---|---|---|
| A | 733 | 602 |
| B | 14 | 0 |
| C | 20 | 0 |
| D | 3 | 0 |
| G | 36 | 0 |
| F | 5 | 0 |

Our results of lipoproteinase production by M-typable strains are in general agreement with the results reported by Gooder (1961). Among types which we invariably found to produce a SOR, Gooder encountered a few strains which were apparently SOR negative. Whereas we found that all types 2, 11, 27, and 35 strains examined produced lipoproteinase, Gooder found no SOR in 1 of 30 type 2 strains tested, 2 of 13 type 11 strains tested, 7 of 9 type 27 strains tested, and 2 of 3 type 35 strains tested. These differences may be due to differences in strain examined. In addition, both type 27 and 35 strains in our experience often produce weak serum opacity reactions, and it seems possible that the growth of certain weak SOR-producing strains such as these by Gooder may have been insufficient to demonstrate the reaction with these strains. Among strains of types which we have found never to produce lipoproteinase, Gooder encountered a few strains which were SOR positive. Aside from types 5 and 12 strains, the exceptions were limited to but one strain of each type tested. The unique production of lipoproteinase by types 5 and 12 strains will be examined more fully subsequently. With these infrequent exceptions, the earlier data of Gooder support the clear indication from our findings that there is a close relationship between lipoproteinase production and type among M-typable strains.



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The results of lipoproteinase production by group A strains not typable by the precipitin method are shown in Table 3. Among these non-M-typable strains, a close association between SOR and serotype as determined by T-agglutination was also apparent. Non-M-typable strains of T-patterns 2, 4, 5/27/44, 9, 11, 12 and 28, produced a SOR with but two exceptions. All non-M-typable strains of T-patterns 1, 6, 15/17/19/23/47 and 18 failed to produce the reaction. Variable

Table 2. *The serum opacity reaction (SOR) of strains typable by the precipitin method*

| M type | SOR− (no. of strains) | SOR+ (no. of strains) | M type | SOR− (no. of strains) | SOR+ (no. of strains) |
|---|---|---|---|---|---|
| 1 | 31 | 0 | 31 | 38 | 0 |
| 2 | 0 | 24 | 32 | 2 | 0 |
| 3 | 31 | 0 | 33 | 2 | 0 |
| 4 | 0 | 33 | 34 | 1 | 0 |
| 5 | 12 | 0 | 35* | 0 | 2 |
| 6 | 46 | 0 | 36 | 2 | 0 |
| 8 | 0 | 4 | 37 | 2 | 0 |
| 9 | 0 | 1 | 38 | 1 | 0 |
| 11 | 0 | 5 | 39 | 2 | 0 |
| 12 | 59 | 0 | 40 | 2 | 0 |
| 13 | 0 | 2 | 41 | 142 | 0 |
| 14 | 6 | 0 | 42 | 2 | 0 |
| 15 | 3 | 0 | 43 | 2 | 0 |
| 17 | 3 | 0 | 44 | 0 | 2 |
| 18 | 10 | 0 | 46 | 2 | 0 |
| 19 | 7 | 0 | 47 | 2 | 0 |
| 22 | 0 | 3 | 48 | 0 | 55 |
| 23 | 5 | 0 | 49† | 0 | 15 |
| 24 | 6 | 0 | 50‡ | 3 | 0 |
| 25 | 0 | 2 | 51 | 5 | 0 |
| 26 | 2 | 0 | Type Schoenborn§ | 65 | 0 |
| 27 | 0 | 5 | Type Hanson§ | 29 | 0 |
| 28 | 0 | 4 | Type Kingbird§ | 26 | 0 |
| 29 | 2 | 0 | | | |
| 30 | 2 | 0 | Total | 556 | 157 |

* Typing antiserum not available in our laboratory; strains originally typed by Dr Rebecca Lancefield. Type 35 is now believed to be identical with type 49 (Subcommittee on Streptococci and Pneumococci, in press).

† Typed as type 49 strains by Dr Rebecca Lancefield.

‡ Typing antiserum not available; strains examined originally typed as type 50 in other laboratories.

§ Provisional types 52, 53, 54 respectively (Top *et al.* 1967).

production of lipoproteinase was, however, evident among strains in the T-agglutination patterns 3/13/B3264, 8/25/imp.19, and 14. Since these latter T-patterns include two or more established M-types within each pattern, it seems reasonable to consider that the variation in lipoproteinase production may be due to the inclusion within them of currently undefined but distinct strains, only some of which produce lipoproteinase.

The majority of established types of group A streptococci were thus found not to

produce lipoproteinase. The types which failed to produce a SOR are types for which the production of M-antisera has been achieved without undue difficulty. However, the M-types found to produce a SOR—types 2, 4, 8, 9, 11, 13, 22, 25, 27, 28, 35, 44, 48 and 49—are types for which the production of M-antisera has generally been difficult (Williams & Maxted, 1953). The majority of strains which could not be typed by the M-precipitin method were also found to produce lipoproteinase.

Table 3. *The serum opacity reactions (SOR) of non-M-typable strains*

| T-agglutination pattern | SOR− (no. of strains) | SOR+ (no. of strains) |
|---|---|---|
| 1 | 7 | 0 |
| 2 | 0 | 5 |
| 3/13/B 3264 | 16 | 43 |
| 4 | 0 | 16 |
| 5/27/44 | 0 | 85 |
| 6 | 3 | 0 |
| 8/25/imp. 19 | 112 | 52 |
| 9 | 0 | 5 |
| 11 | 0 | 117 |
| 12 | 0 | 21 |
| 14 | 2 | 16 |
| 15/17/19/23/47 | 4 | 0 |
| 18 | 1 | 0 |
| 22 | 0 | 3 |
| 28 | 2 | 80 |
| NT | 30 | 2 |
| Total | 177 | 445 |

Table 4. *Serum opacity reaction of matt and glossy variants of strains*

| | Matt variant | | | Glossy variant | | |
|---|---|---|---|---|---|---|
| Strain | M | T | SOR | M | T | SOR |
| S 43 | 6 | 6 | − | NT* | 6 | − |
| T 12/126/3 | 12 | 12 | − | NT | 12 | + |
| Colindale 1130 | 12 | 12 | − | NT | 12 | + |
| 6184 | 15 | 15/17/19/23/47 | − | NT | 15/17/19/23/47 | − |
| 6186 | 14 | 14 | − | NT | 14 | − |
| 6188 | 44 | 5/27/44 | + | NT | 5/27/44 | + |

* NT = Not typable.

*Lipoproteinase production in relationship to M-antigen production*

Gooder (1961) reported that glossy strains of types 5 and 12 produced a SOR, whereas matt colonies of those strains failed to produce the reaction. Köhler (1963) reported a type 26 strain originally SOR negative which on subculture failed to produce M-antigen but produced lipoproteinase. These observations of an inverse relationship between M-antigen and lipoproteinase production suggest that many strains which fail to produce this enzyme might become lipoproteinase producers when they revert to the glossy state. In order to investigate this possibility, M-

positive and M-negative variants of individual strains were examined for M-antigen, T-antigens, and SOR. The results are shown in Table 4. Variants of a type 6, a type 14 and a type 15 strain were found not to produce a SOR in either the matt or glossy phase. The type 44 strain gave a SOR in both the matt and glossy phase. The two type 12 strains examined produced a weak SOR in the glossy phase but did not give this reaction in the matt phase, and so did exhibit the inverse relationship described by Gooder. We were unable to produce a glossy variant of a type 5 strain to confirm Gooder's observations of SOR variation in type 5 strains. Since all type 5 strains that we examined were found to be SOR negative, while all non-M-typable strains of T-pattern 5/27/44 were SOR positive, an inverse relationship between SOR and M-antigen production seems possible. With the exception of these two types, we found no difference in the SOR between M-positive and M-negative variants of individual strains.

Table 5. *SOR of types whose T-antigens are as specific as their M antigens*

| | M-typable | | Non-M-typable | |
|---|---|---|---|---|
| T pattern | SOR− (no. of strains) | SOR+ (no. of strains) | SOR− (no. of strains) | SOR+ (no. of strains) |
| 1 | 31 | 0 | 7 | 0 |
| 2 | 0 | 24 | 0 | 5 |
| 6 | 46 | 0 | 3 | 0 |
| 12 | 59 | 0 | 0 | 21 |
| 22 | 0 | 3 | 0 | 3 |

Further evidence bearing on the suggested inverse relationship between M-antigen and SOR production was sought from examination of M-typable and non-M-typable strains with specific T-antigens. In some strains—types 1, 2, 6, 12 and 22, the T-antigen appears to be as strain specific as the M-antigen in that only a single M-antigen has been identified among strains with the particular T-antigen. Within these T-patterns, strains not typable by homologous M-antiserum can be considered with some confidence to be M-negative variants of that type. The results of serum opacity determinations of such strains is shown in Table 5. Both typable and non-typable strains with T-antigens 1 or 6 failed to give a SOR, while both typable and nontypable strains with T-antigens 2 or 22 did produce a SOR. Variation in lipoproteinase production was evident only among strains with T-antigen 12. Our data would then suggest that an inverse relationship between SOR and an M-antigen is uncommon and has been documented only in types 5 and 12 strains; the production of an SOR by other types appears to be unrelated to their production of an M-antigen.

The majority of SOR positive strains examined in our laboratory (445 of 602 or 74%) were not typable by the precipitin method. Lack of M-typability is of course not necessarily equivalent to lack of M-antigen or lack of virulence. The inability to type a strain may be due to other factors, such as: (1) loss of M-antigen production on serial transfer in standard media, (2) destruction of M-antigen by streptococcal proteinase, (3) poor antigenicity of certain M-antigens, and (4) production

of an M-antigen of an as yet undefined type. Many of the non-M-typable strains which produced a SOR were indeed clinically virulent strains. A total of 20 strains of group A streptococci isolated in pure culture from the blood of patients with septicaemia were examined in our laboratory shortly after their isolation. Sixteen of these strains gave a SOR and despite their virulence only two of them were M-typable, a type 2 and a type 48 strain. The SOR positive strains not typable by the precipitin method included 7 strains of T-pattern 28, 5 strains of T-pattern 3/13/B 3264, and one strain each of T-patterns 8/25/imp. 19 and 22. Virulence of other currently non-M-typable strains producing a SOR is suggested in that representative SOR positive, non-M-typable strains of 5 different T-patterns (4, 11, 28, 3/13/B 3264, and 5/27/44) grew well in rotated human blood and hence presumably contain an M-antigen (Lancefield, 1957; Maxted, 1956). Our data would then suggest that although lipoproteinase-producing strains are difficult to type by the precipitin method, many of these strains are clinically virulent and possibly produce M-antigens which presently are difficult to characterize.

Table 6. *Serum opacity reaction of strains of identical serotype isolated from individual patients at 3-week intervals*

| | Initial isolate SOR negative | | Initial isolate SOR positive | |
|---|---|---|---|---|
| Interval | Subsequent isolate SOR− (no. of strains) | Subsequent isolate SOR+ (no. of strains) | Subsequent isolate SOR− (no. of strains) | Subsequent isolate SOR+ (no. of strains) |
| 3 weeks | 67 | 0 | 0 | 24 |
| 6 weeks | 27 | 0 | 0 | 21 |
| 9 weeks | 18 | 0 | 0 | 3 |
| 12 weeks | 10 | 0 | — | — |
| 15 weeks | 5 | 0 | — | — |
| 18 weeks | 6 | 0 | — | — |
| 21 weeks | 1 | 0 | — | — |

*Consistency of lipoproteinase production by individual strains*

With the exception of the two type 12 strains previously discussed, we have not encountered a SOR positive strain which on subsequent examination failed to produce this reaction. Likewise we have not encountered a strain giving a negative SOR which later was found to give a positive reaction. Individual strains maintained a consistent SOR under laboratory conditions.

A similar consistency of SOR production was evident in strains isolated directly from patients. Two or more isolates of the same serotype as determined by precipitin test or T-agglutination which were obtained from an individual patient at one examination were tested for SOR. In all 98 patients from whom one isolate was SOR negative, the second isolate of the same serotype was also SOR negative. From all 58 patients from whom an SOR positive strain was isolated, the second isolate of the same serotype was likewise SOR positive.

In order to determine whether lipoproteinase production by a strain might vary

after prolonged colonization of a patient, strains of identical serotype isolated from patients with pyoderma or pharyngitis at intervals of three weeks were examined for SOR; the results are seen in Table 6. When the original strain isolated was SOR negative, all subsequent isolates of the same serotype gave a negative SOR, even if recovered as long as four months after the initial isolation. When the original strain produced a positive SOR, all subsequent isolates of the same serotype also gave a positive SOR. Our experience thus indicates that the production of lipoproteinase by individual strains is consistent both in strains maintained in the laboratory and in strains chronically carried by patients.

## DISCUSSION

Data obtained from the examination of a large number of stock and clinical strains from a variety of sources confirm the relationship between M-typability and lipoproteinase production previously reported by Gooder (1961) and by Köhler (1963). Types of group A streptococci for which the production of M-typing antisera has been accomplished without undue difficulty have been found not to produce a SOR. On the other hand, types for which the production of M-typing antisera has been difficult, as well as the majority of non-M-typable strains, have been found to produce lipoproteinase. The reasons for the relationship between poor M-typability and SOR production in group A streptococci remains unclear. Gooder found that extracts containing lipoproteinase did not destroy the antigenicity of M-protein on streptococcal cells or in Lancefield extracts. Further investigations of the mechanisms underlying the association of these two streptococcal proteins seem indicated.

A consistent association between serotype and SOR was found in the group A strains examined. Among strains typable by the more specific M-precipitin method, this association was absolute in that all strains examined of a given type either uniformly produced a SOR or uniformly failed to produce this reaction. When strain classification could only be accomplished by the T-agglutination method, a consistent association between SOR and serotype was also apparent. Only in the more complex T-agglutination patterns such as 3/13/B 3264, 14, and 8/25/imp.19 was variation in SOR production seen among members of a serotype. Such variation may be due to the inclusion within these few serotypes of distinct, but currently unclassifiable strains, only some of which produce this enzyme.

The association between lipoproteinase production and serotype of group A streptococci is indeed so close as to suggest a relationship between them. One possibility would be that lipoproteinase and one of the proteins determining serotype—the M or T antigen—may be identical or closely related proteins. Subsequent studies have indicated that, like the M and T antigens, the lipoproteinases from different types of group A streptococci are antigenically distinct, but no direct relationship between the lipoproteinase antigens and other well recognized cellular antigens, such as the M, T, and R antigens, has been demonstrated (Top & Wannamaker, unpublished observations). The full significance of the close association between serotype and lipoproteinase production must await further investigation of these relationships.

The serum opacity reaction does not appear to be generally useful as a screening test for the presence or absence of M-antigen in individual strains of group A streptococci; 157 of the 713 M-typable strains (22 %) tested produced a SOR and 177 of the 622 non-M-typable strains (28 %) failed to produce the reaction. In view of the close association of the SOR and serotype, however, this simple test might serve as a preliminary screening test to determine which M-typing antisera to use in typing individual strains in laboratories not equipped to classify strains by the T-agglutination method. Moreover, because of the often inverse relationship between M protein and lipoproteinase production, we have found it helpful, along with the ability to survive in rotated normal human blood, as a means of screening strains which are non-typable with current M antisera in order to select those which merit further investigation as possible new M types (Top *et al.* 1967).

## SUMMARY

The serum opacity reaction (SOR) is produced by some streptococci of group A, but not by streptococci of groups B, C, D, F and G. The production of this reaction was found to be closely related to serotype as determined by M and T antigens. The SOR of an individual strain was found to be consistently stable over a period of time both in strains maintained in the laboratory and in strains isolated sequentially from individual patients following streptococcal infections. Strains for which the demonstration of an M-antigen by the precipitin method was difficult or impossible in general produced a SOR, while strains more easily typable generally failed to produce this reaction. Laboratory selected variants of type 12 strains showed an inverse relationship between M protein and lipoproteinase production, whereas M positive and M negative variants of other serotypes showed no variability with respect to lipoproteinase production.

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