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Antibody to streptococcal opacity factor in human sera

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SUMMARY

Two tests are described for detecting antibody to the type-specific opacity factor (OF) of group A streptococci. This antibody was detected among patients convalescent from streptococcal sore throat in two communities in which outbreaks due to opacity factor-producing strains of group A streptococci occurred.

In an outbreak due to streptococci of M-type 22 there was a close correspondence between the distribution of anti-OF and of bactericidal M-antibody for the type. In a smaller outbreak due to M-type 58 streptococci, however, M-antibody was detected more often than antibody to OF.

INTRODUCTION

The streptococcal opacity factor (OF) is found in all members of 16 clearly differentiated M-types of group A streptococci and in a number of other strains in which the M-antigen has not yet been identified. It causes the appearance of opacity in horse serum (Ward & Rudd, 1938; Gooder, 1961) and antibody to it inhibits this opacity (Top & Wannamaker, 1968). The antigenic specificity of OF runs parallel with that of the M-antigen (Widdowson, Maxted & Grant, 1970), so that inhibition is observed only when OF is mixed with antisera against a member of the same M-type. The OF is closely associated with the M-antigen in the bacterial cell and the two may form part of the same complex protein (Widdowson, Maxted, Grant & Pinney, 1971).

Before the type specificity of the OF had been established, Krumweide (1954) reported the presence of antibody to it in human serum. However, no attempt to study the occurrence of the antibody in serum of patients with streptococcal disease has since been reported. The type specificity of the opacity factor and its undoubted close association with M-antigen suggested that the identification of antibody to OF might also be an indication of the presence of protective antibody to the M-antigen.

In the course of other work (Maxted *et al.* 1973) we obtained active preparations of the OF of most of the known opacity producing M-types and made rabbit antisera against some of these opacity factors. We were thus in a position to investigate possible tests for antibody against OF and then to test sera from patients who had recovered from streptococcal disease for antibody to the infecting type.

Two outbreaks of streptococcal infection of the upper respiratory tract, one due to M-type 22 and the other to M-type 58, provided the sera used in the investigation reported here. Both of these serotypes are OF positive.

MATERIALS AND METHODS

Preparation of opacity factor

Streptococci of each known OF-positive M type (types 2, 4, 9, 11, 13, 22, 25, 28, 48, 49, 58, 59, 60, 61, 62, 63 and provisional types 67/3875, 453, 3354, PS346, PS432) were grown overnight in Oxoid Todd Hewitt Broth to which 1% (w/v) Neopeptone had been added. The supernatant after centrifugation at 3500 *g* was tested for its activity against horse serum (see below); if this was satisfactory, thiomersal 1/5000 (w/v) was added and the supernatant kept at −20° C.

Two tests were used to establish the OF activity of each supernatant.

The tube test was done by incubating 0·2 ml. of supernatant with 1·0 ml. horse serum (Recalcified Plasma No. 2. Wellcome Research Laboratories, Beckenham, Kent) containing thiomersal 1/5000 (w/v) for 18 hr. at 37° C. Physiological saline 1·2 ml. was added to the tubes and the optical density (A_{475}) was read on a Unicam SP 600 spectrophotometer. A control tube containing 1·4 ml. saline and 1·0 ml. horse serum was included. An increase in the opacity of 0·1 or more over the control tube was taken as a positive reaction; strongly positive supernatants gave an increase of at least 0·2–0·3.

Serum-agar slides. Equal volumes of 2% Ion Agar (Oxoid) and horse serum were mixed, and 6 ml. of the mixture was poured onto 2 in. × 2 in. (5 cm. × 5 cm.) glass slides. The surface moisture was dried off by incubating the slides at 37° C. for 20 min. Supernatants were spotted on the slides with a 2-mm. diameter wire loop, and the slides incubated in a moist chamber overnight. Zones of opacity, varying in intensity with the OF activity of the supernatant, were seen in the area of application of the supernatant.

Sera

Preparation of anti-OF serum

These were prepared in rabbits. Three were sera made by injecting cell-wall fragments of streptococci, of M-types 2, 4 and 25 respectively (Widdowson *et al.* 1970). Other sera were unabsorbed M-typing sera, made in the conventional manner in the Streptococcus Reference Laboratory (Williams, 1958) and found to contain OF antibody that inhibited the serum opacity reaction of the homologous strain. To ensure that the antisera were specific, each serum was tested against the OF of several streptococci of the homologous type, a large number of OF positive strains with identical T-agglutination patterns but without identifiable M-antigen, and a selection of other strains that were quite unrelated.

Patient's serum

Sera were collected about 4 weeks after infection, and stored at −20° C immediately upon receipt in the laboratory.

Typing sera and typing methods

The methods used in the Streptococcus Reference Laboratory for the preparation of typing sera and for M & T typing were described by Williams (1958).

Specific inhibition of the serum opacity reaction

Tube method

Two-fold dilutions of anti-OF serum were made in physiological saline. To 0·02 ml. volumes of each dilution was added 0·1 ml. of the culture supernatant of an OF-positive streptococcus diluted to five times the concentration necessary to cause opacity. The tubes were shaken and incubated at 37° C. for 30 min.; 1 ml. of horse serum was added to each tube, the tubes again shaken and incubated overnight. A tube of horse serum without antiserum and another of horse serum without OF were always included as controls and the opacity was estimated visually or after dilution with an equal volume of saline. The A_{475} was read spectrophotometrically (Widdowson *et al.* 1971).

To identify the OF of a streptococcus, 0·02 ml. of each available anti-OF serum, in a dilution previously shown to inhibit the OF of the homologous strain, was added to a tube containing 0·2 ml. of the supernatant and 1 ml. of horse serum.

Solid agar method

Equal volumes of 2% Ion Agar and horse serum were mixed; to 6 ml. of the mixture, 1·2 ml. of diluted active OF supernatant was added and after thorough mixing poured on a 2 in. × 2 in. glass slide. A loopful (2 mm. diameter) of each antiserum, undiluted or diluted for titration, was placed on the dried agar surface. The slides were incubated overnight in a moist chamber. The reaction of the OF with the horse serum produced an opaque background against which clear areas of inhibition were seen.

The indirect bactericidal test

The bactericidal power of normal heparinized human blood for streptococci is enhanced by the addition of human serum containing M-antibody of the homologous streptococcus. The test was done by adding 0·02 ml. of a suitably diluted broth culture (50–500 viable units) to 0·02 ml. of patient's serum (undiluted, or after diluting 1/5 or 1/10 in an attempt to get a more quantitative estimate of the antibody present. A 0·3 ml. volume of heparinized blood from a donor previously shown to have no antibody to the test strain was then added to each tube. The tubes were sealed and rotated end-over-end at 37° C. for 3½ hr. and an estimate of the viable streptococci present made, by subculturing 0·02 ml. volumes of the mixture into molten blood agar, pouring blood agar plates and counting the colonies after 18 hr. incubation. The growth was recorded as:

\− = 0 to 9 colonies,
\+ = 10–49 colonies,
\+ + = 50–249 colonies,
\+ + + = 250–999 colonies,
\+ + + + = discrete but uncountable number of colonies.

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Table 1. *Presence of antibody to type 22 OF in human sera; 67 sera were tested by the tube and the slide method*

Serum no.	Depression of A_{475}	Slide reaction
377	0·22	+
378	0·25	+
387	0·28	+
389	0·32	+
390	0·33	+
395	0·32	+
396	0·21	+
398	0·28	+
403	0·35	+
421	0·33	+
434	0·25	+
436	0·26	+
54 other sera	0	−

+ = inhibition of serum opacity reaction.
− = no inhibition of serum opacity reaction.

RESULTS

Rabbit anti-OF sera to streptococcal OF

Active OF supernatants and corresponding antisera were available for M-types 2, 4, 9, 28, 48, 49, 58, 59, 62 and 63 and for four further provisional types (67/3875, 453, PS346, PS432). Each supernatant was tested for inhibition by each antiserum; in every case inhibition of opacity was observed with the antisera of the homologous M type and not with the heterologous antisera, with the single exception that the OF of type 9 was inhibited not only by the type 9 antiserum but to some extent also by the type 28 antiserum. The non-specific inhibition shown by this serum could be removed by absorbing the type 9 OF antiserum with type 28 cells. The type 9 OF-antiserum was then specific and inhibited type 9 OF only.

The sera were also tested for inhibition of the OF of a number of strains of the homologous type, and of a variety of strains with similar T agglutination patterns but with an unidentifiable M-antigen. Each opacity factor was inhibited specifically by a single antiserum.

Antibody to opacity factor in human sera

Sera from an outbreak of M-type 22 infection

In the spring of 1970, there was a large outbreak of streptococcal sore throat in a boys' school. Bacteriological investigations were carried out late in the outbreak, and it appeared that two types of streptococcus were predominant; both had the T-typing pattern 12, and one had M-antigen 22 and the other the M-antigen 12. The former was OF positive and the latter OF negative. Cultures were received from 16 boys, of whom 11 yielded the type 22 streptococci and 5 the type 12 streptococci. Sera were collected from 67 boys about 4 weeks after the peak of the outbreak.

Table 2. *Titration of antibody to OF in patients' sera. Comparison of the tube and agar slide methods*

Serum no. 378			Serum no. 387			Serum no. 421		
Diluted	A_{475} depression	Slide reaction	Diluted	A_{475} depression	Slide reaction	Diluted	A_{475} depression	Slide reaction
1/5	0·21	+ + +	1/5	0·15	+	1/5	0·21	+ + +
1/10	0·22	+ +	1/10	0·10	tr	1/10	0·22	+ +
1/20	0·23	+	1/20	0	—	1/20	0·23	+ +
1/40	0·21	+	1/40	0	—	1/40	0·21	+
1/80	0·15	±	1/80	0	—	1/80	0·16	+
1/160	0·05	tr	1/160	0	—	1/160	0·1	±
1/320	0	—	1/320	...	—	1/320	...	tr
1/640	0	—	1/640	...	—	1/640	0	—

+ + +, + +, +, ±, tr = strength of inhibition of serum opacity reaction on an arbitrary scale.

— = no inhibition of serum opacity reaction.

... = not done.

The results of the initial screening of the sera for antibody to OF alone, done by the two methods, appear in Table 1. The optical density readings and agar slide results agreed well. It was found that horse serum + opacity factor + normal human serum, gave a higher reading (0·75) than OF and horse serum alone (0·6). It is apparent that a control mixture containing a human serum known to be devoid of OF antibody must be included in every batch of tests.

There was considerable variation in the strength of the OF inhibition, seen with the patients' serum (Plate 1). Three of these, each differing in the strength of the initial reaction, were diluted and the OF activity titrated on slides and in tubes. The two methods gave good agreement and the end points reflected the degree of activity seen in the initial test with the undiluted sera. In the tube test a difference of 0·1 in the A_{475} reading was taken as evidence of inhibition and this difference could be detected on the serum agar slides also (Table 2).

All the sera were treated similarly, and in Table 3 a quantitative estimate of the anti-OF titres are given together with the result of the bactericidal tests.

In the bactericidal test, 15 sera showed the presence of M-antibody for type 22 (22 % of the sera tested). Antibody to OF was detected in 12 of these 15 sera and in none of the remaining 52 sera.

The results of the bactericidal tests were derived from four separate experiments. Tests using either graded doses of patient's serum or graded streptococcal inocula did not always show the expected gradation of the killing effect. However, the sera could be classified semi-quantitatively into those that gave good, moderate, poor or no killing effect based on the growth seen under the conditions of the experiment as indicated in the footnote to Table 3.

The sera that consistently gave the best bactericidal effect when added to whole blood also had the highest antibody titres (1/640) against the OF of type 22. Similarly, 2 of the 3 sera that were negative in the anti-OF test were the weakest of the 15 sera that showed any bactericidal power at all.

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Table 3. *Patients' sera: comparison of OF antibody titre with bactericidal activity against M type 22 streptococci*

Serum no.	Bactericidal activity against M type 22*	OF antibody titre†
389	Good	> 640
378	Good	640
390	Good	640
421	Moderate	320
395	Moderate	80–160
434	Moderate	80–160
396	Moderate	20
380	Moderate	0
377	Poor	80–160
398	Poor	160
403	Poor	20
387	Poor	10
436	Poor	10
406	Poor	0
417	Poor	0
12 other sera fully tested	Nil	0
40 sera screened	Nil	0

* Good = 0·02 ml. of a 1/5 dilution of the serum reduced + + + + to < + + survival in the bactericidal test (see Methods).

Moderate = 0·02 ml. of the serum reduced + + + + to < + + survival but 0·02 ml. of a 1/5 dilution had no effect.

Poor = 0·02 ml. of the serum reduced + + + + to + + or + + + survival.

Nil = 0·02 ml. of the serum had no effect on survival.

† = Reciprocal dilution in the tube test.

All the sera were also tested for bactericidal activity against M type 12 and this was found in 32 sera (47·7 %).

Sera from M-type 58 infections

In 1971, an outbreak of acute upper-respiratory tract infection occurred in a training centre for RAF cadets, in which it appeared that three M-types of streptococci were involved: type 5, type 18 and type 58. Type 5 and type 18 are OF negative but type 58 is OF positive, and attempts were made to find type 58 OF antibody in the serum of the patients by the same two methods as before.

Bactericidal tests were done on 142 sera and M-type specific antibody to M58 was found in four of them, but only one of these four had detectable antibody to type 58 OF (titre < 1/10). None of the 138 sera in which M58 antibody was not detected had antibody to type 58 OF. Bactericidal antibody to M-type 5 was found in 36 sera and to M-type 18 in 18 sera.

DISCUSSION

This investigation is the first to report the presence of antibody to OF in the serum of patients recovering from infection with a streptococcus of a known M-type.

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Two points appear to be of importance, the first concerns the antibody as a monitoring marker for the patients' response to the M-type specific antigen and the second the role the OF antibody itself might play in protection of the host. Consideration of these two possibilities is stimulated by the undoubted close relationship between OF and the type specific determinant of M protein. The two have so far resisted separation by physical or chemical means, and their antigenic specificity shows a strict parallelism.

Whole organisms, or fragmented cell-wall material, were used to produce antibody to OF in rabbits, and these contain both OF and M antigen. Many rabbit antisera prepared against OF-positive types – for example, some of the M-typing sera made in the Streptococcus Reference Laboratory – contain M antibody but show no activity when tested for inhibition of opacity produced by the vaccine strain. On the other hand, all sera with anti-OF activity showed some bactericidal effect, though some of them gave a poor tube-precipitation reaction with homologous antiserum.

In the patients' sera, 12 of the 15 in which type 22 M-antibody could be demonstrated by the bactericidal test also contained the corresponding anti-OF antibody. Two of the three sera in which anti-OF antibody could not be detected gave a weak bactericidal reaction. It may be that the poor response to either antigen is related to the severity of infection. Because of the very close relationship of the OF antigen with the type specific determinant, antibody to both might be expected and this was so in 80 % of the sera with type 22 M-antibody, but in only one of four sera with type 58 M-antibody. This may be a consequence of the rather feeble extracellular OF activity sometimes shown by this M-type, compared to the vigorous activity seen with type 22 strains.

The tests we used for detecting OF antibody seem to be precise and sensitive, and identified the antibody in sera showing a considerable variation in anti-OF titres. Although OF is limited in its distribution among streptococcal serotypes, such an additional sensitive marker for antibody to a part of the M protein may be of some advantage when investigating infections with OF-producing types. They are all types in which the M-antibody response is notoriously poor, a characteristic of many of the strains found in streptococcal pyoderma (Wannamaker, 1970).

Antibody to the type specific M-antigen persists for many years (Lancefield, 1959) and is of significance in protection against reinfection. It is of obvious interest to know whether OF antibody has a similar activity and whether it is also persistent.

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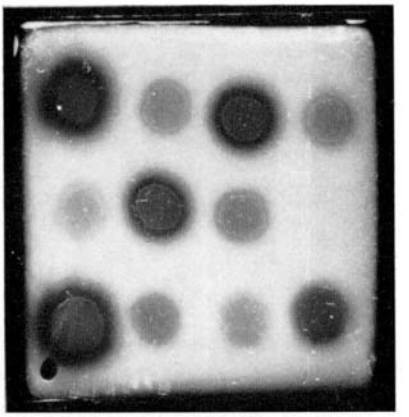
EXPLANATION OF PLATE

Inhibition of the opacity factor (OF) of type 22 by 11 of 12 patients' sera on agar containing type 22 OF and horse serum viewed against a black background. (× 1.)



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