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Inhibition of β-haemolysis by opacity factor in group A streptococci

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SUMMARY

Group-A streptococci belonging to opacity-factor (OF)-positive M types were poorly haemolytic on horse-blood agar, but members of OF-negative M types, and M-negative variants of OF-positive strains gave good haemolysis. Horse-serum extracts of strains of OF-negative serotypes 6 and 12, and M-negative variant cultures of OF-positive serotypes 4 and 49, had higher titres of streptolysin S than did similar extracts of OF-positive, M-positive cultures of types 4 and 49. However, much larger amounts of streptolysin S could be extracted with ribonuclease (RNAase)-digested yeast ribonucleic acid (RNA) and M-positive OF-positive cultures treated in this way gave extracts at least as strong as did their M-negative variants or the OF-negative strains. Extraction of streptolysin S from OF-negative strains by serum could be inhibited by previous incubation of the serum with extracellular OF, suggesting that the production of diffusable OF by M-positive variants of OF-positive serotypes interferes with the extraction of streptolysin S by serum and leads to poor haemolysis on blood agar.

The haemolysis of all strains on blood agar was greatly improved by the incorporation of 0·1 % (w/v) RNAase-digested yeast RNA into the medium, but the improvement was most marked in OF-positive serotypes.

INTRODUCTION

Dillon (1968) reported that a large number of group-A streptococci isolated from the skin were poorly haemolytic on sheep-blood agar. Whether or not this was due to poorer production of streptolysin S by these strains than by respiratory strains was not established. We examined a collection of 153 strains of various serotypes on horse-blood-agar plates, incubated aerobically, and showed that the majority of 'skin' strains formed small zones of haemolysis with incomplete clearing and a fuzzy edge, and that most of the throat strains gave large, clear zones of β-haemolysis (Pinney, 1974). There were many exceptions in both groups, but the correlation between poor haemolysis and the production of OF was complete. Our previous work (Widdowson, Maxted & Grant, 1970; Maxted *et al.* 1973) indicated that OF production is restricted to all the members of certain M types and to some M-untypable strains. It has also been noted that OF-positive strains, many of them M untypable, predominate over OF-negative strains in isolates from skin lesions (Maxted *et al.* 1973; Pinney, 1974).

In the present study we confirm the correlation between poor haemolysis and the production of opacity factor by examining another collection of 75 OF-positive and 75 OF-negative strains isolated from throat and skin infections.

The substrate for the OF-mediated serum opacity reaction is considered to be serum α-lipoprotein (Krumweide, 1954; Rowen & Martin, 1963), which can also act as carrier and extracting agent for streptolysin S (see Ginsburg, 1970). The possibility that the poor haemolysis exhibited by OF-positive strains results from combination with, or destruction of, α-lipoprotein by OF, rather than from diminished lysin production by these strains has been investigated.

METHODS

Strains

Strains of group A streptococci were classified by T-typing (Griffith, 1934) and M-typing (Swift, Wilson & Lancefield, 1943). Details of the collection of 150 strains examined for haemolysis on blood agar are given in Table I. Approximately half the strains were isolated from the upper respiratory tract and the other half from streptococcal skin lesions. The strains were selected to cover a wide variety of serotypes including some more common M types, e.g. 2, 4, 6, 11, 12, 22, 49, 55, a selection of M-untypable strains of various T patterns, and a few more unusual strains such as the 'Potter 6' strain (see footnote of Table 1), an M-type 1 strain with the T-pattern 8/25 and an M-negative T11 strain. Paired M-positive and M-negative variants of OF-positive strains SF59 (M-type 2), R68/1115 (M-type 4), T11/54/4 (M-type 11), R69/2408 (M-type 11), R69/229 (T11, M-non-typable), R69/1805 (M-type 22), R69/3116 (M-type 22), R68/1544 (M-type 49) and Griffith's type B3264 (M-untypable) and OF-negative strains 1130 (M-type 12), S43/78 (M-type 6) were also examined for production of haemolysis on blood agar.

Cell-bound streptolysin S was extracted from M-positive and M-negative variants of OF-positive strains R68/1544 (M-type 49) and R68/1115 (M-type 4) and of OF-negative strains 1130 (M-type 12) and S43/78 (M-type 6) by a method based on that of Weld (1935). M-positive and M-negative variant strains were checked for the presence or absence of M antigen and opacity factor and their ability to survive in human blood as described by Widdowson *et al.* (1970).

Haemolysis on blood agar

Strains were grown overnight at 37 °C in 5 ml of Oxoid Todd-Hewitt broth with 1 % (w/v) of added Neopeptone (Difco Ltd). A loopful of culture was streaked, so as to obtain some discrete colonies, on the surface of a quadrant of an agar plate containing 5 % v/v of horse blood (Oxoid Ltd) in Hartley-digest agar base. The plates were incubated aerobically at 37 °C for 18 h and the haemolysis designated 'good' if there were definite zones of clear haemolysis at least 1 mm wide around the colonies. Cultures were designated poorly haemolytic if the zones of haemolysis were difficult to measure, with incomplete clearing and a fuzzy edge.

Preparation of cultures for streptolysin S extraction

Strains were grown in 5 ml of brain–heart infusion broth (Difco) for 7 h at

37 °C and then inoculated into 100 ml of similar medium with the addition of 20 % (v/v) Burroughs Wellcome horse serum No. 5. After incubation at 37 °C for 16 h the cultures were adjusted to an opacity of 0·5–0·6 at 520 nm by the addition of brain–heart infusion broth. Cells from 100 ml of the standardized cultures were harvested by centrifugation. The supernatant was removed and the cells were suspended in 2 ml of extracting agent in sterile 5 ml screw-capped bottles containing glass beads, and rotated on a blood cell suspension mixer (Baird & Tatlock Ltd) at room temperature for 2 h. The cells were then removed by centrifugation and the supernatants stored at −10 °C.

Extracting agents

The following agents were used to extract streptolysin S.

(1) Burroughs Wellcome horse serum No. 5.

(2) Burroughs Wellcome horse serum No. 5 with the addition of 20 % (v/v) of filtered supernatant of a culture in Todd-Hewitt broth of the OF-positive strain PS346 (provisional M-type – see Maxted *et al.* 1973). Serum and supernatant were incubated at 37 °C for 16 h to give a positive serum-opacity reaction, then inactivated at 56 °C for 30 min.

(3) As 2, but with the growth supernatant of OF-negative strain R70/1648 (M-type 5) in the place of the growth supernatant of strain PS346.

(4) 0·01 M-$MgSO_4$ in physiological saline.

(5) 1 % (w/v) Ribonucleic acid (RNA); sodium salt from yeast; (British Drug Houses Ltd) 1 % (w/v) in physiological saline.

(6) Solution (5) digested with ribonuclease (RNAase; from pancreas, British Drug Houses Ltd) 0·001 mg/ml at 37 °C for 8 h, and inactivated at 56 °C for 30 min.

(7) 10 % (w/v) sodium deoxycholate in physiological saline.

(8) 2 % (v/v) Tween 80 in physiological saline.

Streptolysin-S assay

Streptolysin S was assayed by haemolysis of a standard suspension of rabbit erythrocytes. Fresh rabbit blood was defibrinated by gentle shaking with glass beads. The red blood corpuscles were washed in borate buffer, pH 8·0, and a 5 % (v/v) suspension of the packed cells in borate buffer was adjusted to give an E_{520} of 0·8–0·84 when diluted 1/4 with water. Amounts (0·5 ml) of this standard blood-cell suspension were added to 1·5 ml of dilutions of the streptolysin-S extracts and incubated at 37 °C for 2 h. The tubes were then centrifuged at 1000 rev./min for 3 min and the E_{520} of the supernatant read on the spectrophotometer. Since the percentage haemolysis is directly proportional to the optical density over a wide range (Liao, 1951), the 50 % haemolysis end-point was calculated from the optical density readings of tubes containing dilutions on either side of the end-point.

Table 1. *Haemolysis on horse blood agar of strains of different serotypes of group A streptococci*

Opacity-factor-positive strains Haemolysis of all serotypes: poor†			Opacity-factor-negative strains Haemolysis of all serotypes: good†		
M type	T-type pattern	No. of strains examined	M type	T-type pattern	No. of strains examined
2	2	4	1	1	3
2	8/25/IMP 19	5	1	8/25	1*
4	4	4	3	3/13/B3264	2
9	9	4	5	5/27/44	4
11	11	3	6	6	4
22	12	4	12	12	5
22	22	3	14	14/49	2
25	8/25	3	18	−ve	1
28/28R	28	2	24	4	5
48	4	2	26	4	3
49	14/49	6	30	30	3
58	25/IMP 19	3	31	−ve	1
60	4	3	33	3/13/B3264	5
62	12	4	41	3/13/B3264	4
63	4	5	52	3/13/B3264	3
PS346‡	3/13/B3264	6	53	3/13/B3264	1
3354‡	25/IMP 19	1	54	15/17/23/47/19	1
3875‡	25/IMP 19	1	55	25/IMP 19	5
NT	4	6	56	3/13/B3264	2
NT	11	1	56	25/IMP 19	1
NT	14	1	57	25/IMP 19	1
NT	3/13/B3264	4	'Potter 6'	B3264	1*
			M −ve variant Blackmore	11	1§
			NT	4	2
			NT	9	1
			NT	25/IMP 19/3890	1
			NT	3/13/B3264	10
			NT	5/27/44/3/13/B3264	1
			NT	−ve	1
Total		75			75

NT = untypable with available M antisera

* Isolated in Trinidad from streptococcal pyoderma by Dr E. V. Potter. The 'Potter 6' strain was killed by M-type 6 antiserum in normal human blood but absorption of the serum with this strain did not remove true M-type 6 bactericidal activity. Extracts of 'Potter 6' failed to precipitate with M6 antiserum.

† Good = clear zone of complete haemolysis at least 0·1 cm wide around colonies, distinct edge to zone. Poor = indistinct zone of incomplete haemolysis with fuzzy edge. Zone size difficult to measure.

‡ Provisional M-types.

§ M-negative variant of type 11 which lacked extracellular opacity factor but cells of the culture were OF positive.

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Table 2. *Haemolysis produced by paired M-positive and M-negative strains* on horse-blood agar*

Opacity-factor (OF) category of serotype	M-antigen	OF		No. of strains examined‡	Haemolysis on blood agar§
		Cell-bound*	'Free'†		
Positive	Present	+	+	9	Poor
	Absent	+	−	9	Good
Negative	Present	−	−	2	Good
	Absent	−	−	2	Good

* Opacity factor detectable by incubation of whole cells or 'cell-wall membrane' fractions with serum(see Widdowson *et al.* 1970).

† Opacity factor detectable in broth-culture supernatants or in zone surrounding colonies in 50% horse-serum agar (see Widdowson *et al.* 1970).

‡ See text for list of strains.

§ See footnote on Table 1.

Table 3. *Titres* of cell-bound streptolysin S extracted from M-positive and M-negative variants of M-types* 49, 4, 6 and 12

Extracting agent	Titre* of streptolysin S in stated extract of strain no.							
	R68/1544		R68/1115		S43/78		1130	
	M49+	M49−	M4+	M4−	M6+	M6−	M12+	M12−
Normal horse serum	200	975	400	1 000	890	1 000	600	800
+OF-positive supernatant	<50	200	<50	80	115	320	<50	55
+OF-negative supernatant	N.D.	N.D.	N.D.	N.D.	850	750	580	690
RNAase-digested RNA solution 1% (w/v)	3 300	3 750	15 000	25 200	4 150	8 750	4 700	6 900

N.D., Not done

No haemolytic activity was detectable in extracts made with RNA 1% (w/v), 0·01 M-$MgSO_4$, sodium deoxycholate 10% (w/v) or Tween 80, 2% (v/v).

* Dilution causing 50% haemolysis

RESULTS

Haemolysis by OF-positive and OF-negative strains on horse blood agar

Table 1 shows the division of the 150 strains examined into those that caused 'poor' haemolysis, i.e. small zones of incomplete clearing with a fuzzy edge and those that gave large clear zones of β-haemolysis on horse-blood agar. All the OF-positive strains examined, regardless of source, gave small incomplete zones of haemolysis but all the OF-negative strains examined gave zones of 'good' haemolysis (see Plate 1 A).

M-negative variants of OF-positive serotypes possess cell-bound opacity factor, but do not release it into culture supernatants or the area surrounding colonies on serum agar plates (Widdowson *et al*, 1970). M-negative variants of OF-positive serotypes were distinctly more haemolytic on blood agar than their

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M-positive counterparts (see Table 2) suggesting that the production of extracellular opacity factor in blood-agar plates might interfere with the extraction of streptolysin S from the cells by serum, or with the production of haemolysis by streptolysin S. Cultures of M-positive and M-negative variants of four serotypes, two OF-positive (M4 and M49) and two OF-negative (M6 and M12) were therefore treated with a variety of agents, suggested by other workers as means of extracting streptolysin S (see Ginsburg, 1970), and the titres of streptolysin S in the extracts compared (see Table 3).

Between 2·5 and 5 times more streptolysin S was extracted by horse serum from the M-negative variants of OF-positive serotypes 4 and 49 than from their M-positive counterparts, and the titres of streptolysin S in horse serum extracts from M-positive OF-positive cultures were lower than those obtained with M-positive or M-negative cultures of types 6 and 12. Undigested yeast RNA solution did not extract streptolysin S, but RNA predigested with RNAase (Okamoto, Shoin, Koshimura & Shimizir, 1967) extracted very large amounts of lysin from all cultures tested, and there was no difference in the titres given by M-positive and M-negative cultures of the OF-positive serotypes.

Horse serum that had been pre-incubated with opacity factor from a strongly OF-positive strain (in the form of crude growth supernatant) extracted very little or no detectable streptolysin S from the test strains; this suggested that the carrier for streptolysin S in serum was 'blocked' during incubation and so was no longer available to combine with streptolysin S. Pre-incubation of the same batch of horse serum with the growth supernatant from an OF-negative strain did not impair its ability to extract streptolysin S. No streptolysin S was extracted by 0·001 M-$MgSO_4$, 10% (w/v) sodium deoxycholate or 2% (v/v) Tween 80 by the methods employed.

Haemolysis on blood agar containing digested RNA

The incorporation of 0·1% (w/v) RNAase-digested yeast RNA into blood agar greatly enhanced haemolysis by all strains of group A streptococci, but the improvement was most marked among the OF-positive strains (see Plate 1A and B).

DISCUSSION

Differences in the extent of haemolysis shown by different serotypes of group-A streptococci on blood-agar plates has previously received little attention, although Dillon (1968) observed that many strains isolated from pyoderma lesions were poorly haemolytic. We have confirmed the observation of Pinney (1974) that the tendency to form small indistinct zones of haemolysis completely correlated with the production of diffusible opacity factor irrespective of the source of the strains. Although opacity-factor-positive strains are common among skin isolates, several well-known M-types of skin streptococci, e.g. 33, 41, 55 and 57 (see Wannamaker, 1970), as well as many untypable skin strains are OF-negative, and these consistently produce large well-defined zones of haemolysis on horse-blood agar. M-negative variants of OF-positive serotypes also produced large well-defined

zones of haemolysis, and this seemed to be correlated with their lack of diffusible opacity factor.

The extraction of M-positive and M-negative cultures of OF-positive and OF-negative strains by a variety of agents showed that the amount of streptolysin S extracted from the cells varied considerably with the extracting agent used. Serum extracts of OF-positive M-positive cultures apparently contained less streptolysin S than did extracts of their M-negative variants or of OF-negative strains, but all the strains tested yielded high titres of streptolysin S when extracted with RNAase-digested yeast RNA. This suggests that gross differences in haemolysis are not caused simply by lack of production of streptolysin S by OF-positive cultures, but possibly by an OF-mediated inhibition of serum extraction of streptolysin S. The impaired ability of serum that had been treated with opacity factor to extract streptolysin S from cells also suggests that the α-lipoprotein carrier for streptolysin S is 'blocked' or destroyed by reaction with opacity factor. RNAase-digested RNA contains guanylic-acid residues which have a high affinity for streptolysin S (see Okamoto *et al.* 1967) but do not appear to extract opacity factor (Pinney, unpublished).

Okamoto (1940) first observed that the incorporation of 1% (w/v) yeast RNA into blood agar plates greatly enhanced the production of β-haemolysis by certain streptococci. We have shown that the haemolysis of all strains tested was greatly enhanced by the incorporation of 0·1% (w/v) of RNAase-digested yeast RNA into horse-blood-agar plates. This enhancement was particularly striking with the poorly haemolytic opacity-factor-positive strains. Blood agar supplemented with digested yeast RNA may be useful for the primary isolation of β-haemolytic streptococci, especially those belonging to OF-positive serotypes.

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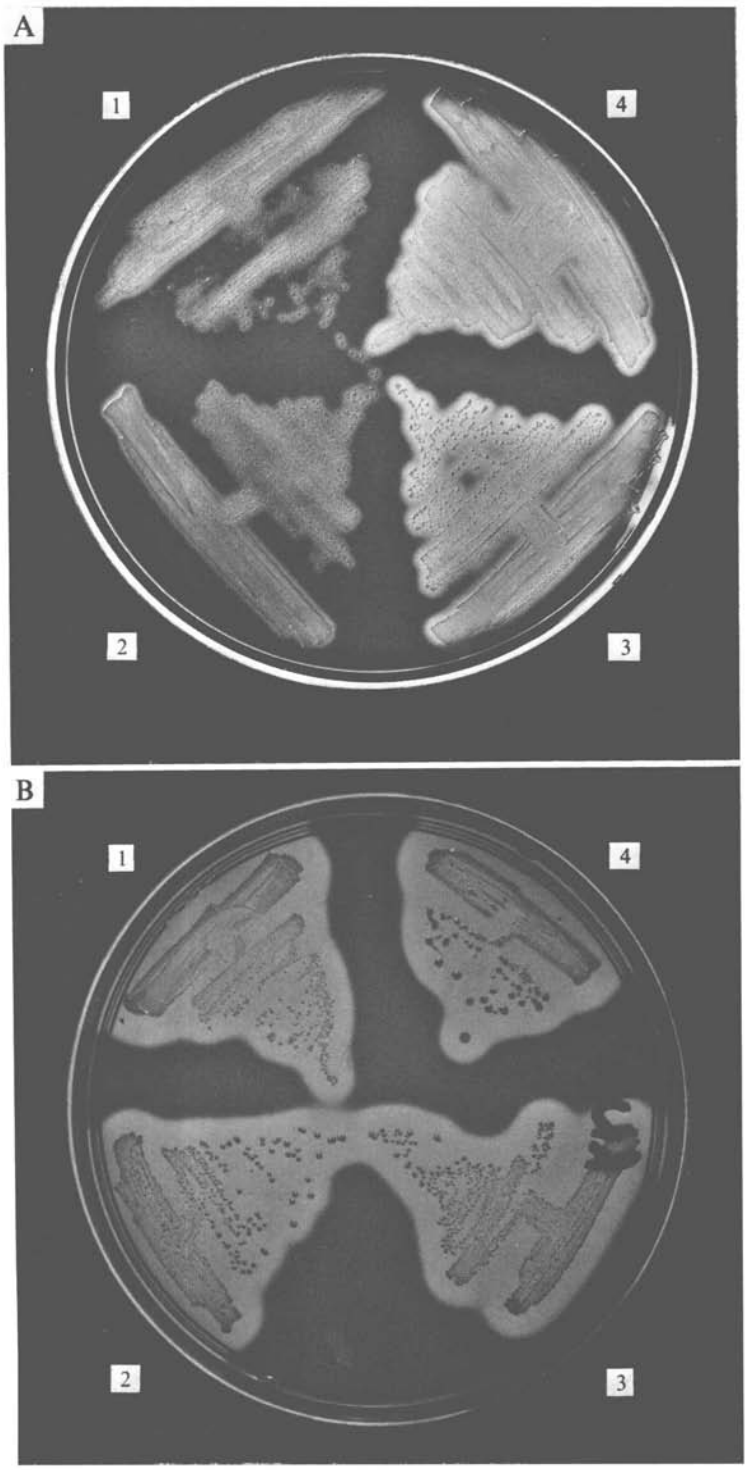
EXPLANATION OF PLATE

PLATE 1

(A) Four strains of group A streptococci on 5% horse blood agar. 1, R69/3116 (M-type 22, OF+); 2, R69/2408 (M-type 11, OF+), 3, 1130 (M-type 12, OF−), 4, S43/78 (M-type 6, OF−). (B) Four strains of group A streptococci on 5% horse blood agar with 1% RNAase-digested yeast RNA. Position of strains as in (A).



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