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PREPARATION OF SPECIFIC ANTISERA TO THE OPACITY FACTORS OF GROUP-A STREPTOCOCCI

CHERRY A. M. FRASER

Division of Hospital Infection, Central Public Health Laboratory, London NW9 5HT

SUMMARY. Type-specific antibody to the opacity factor (OF) of group-A streptococci can be demonstrated in human sera but the multiplicity of antibodies to different serotypes limits their usefulness in anti-OF typing. The antibody response in rabbits is inconsistent; only 61 of 138 (44%) of rabbit anti-M sera tested contained OF antibody. Of these only about half had titres of > 16 , and usable sera to only 11 of 23 OF-positive serotypes were obtained. On the other hand good anti-OF sera (titres > 16) to 27 of 28 serotypes resulted from the interperitoneal and subcutaneous inoculation of heat-killed whole-cell vaccines in guinea-pigs and the frequency of response in groups of animals given injection of the same vaccine was 100% for all but three serotypes. Antibody response was not obtained with M-type 13. A comparison of routes of inoculation for M-type 25 showed that the subcutaneous route alone could probably be used in the routine production of anti-OF typing sera. Use of the set of 27 sera in OF-inhibition tests confirmed the remarkable specificity of OF antigens and their parallelism with M-antigen specificity, with the exception of a reciprocal cross reaction between M-type 61 and provisional type PT3875.

INTRODUCTION

The production of opalescence in mammalian sera by a factor that interacts with α -lipoprotein (Krumwiede, 1954; Rowen and Martin, 1963) is a consistent property of the strains of some serotypes of group-A streptococci (Gooder, 1961; Top and Wannamaker, 1968*a*; Widdowson, Maxted and Grant, 1970). The antigenicity of opacity factor (OF) (Krumwiede, 1954; Top and Wannamaker, 1968*b*) and the

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parallel specificity of OF and M antigen (Widdowson *et al.*, 1970) have been used in the development of an anti-OF typing system based on the inhibition of opacity by specific antisera (Maxted *et al.*, 1973b).

Suitable antisera for routine use in the OF-inhibition test are difficult to prepare in rabbits because the immune response is inconsistent and, although human sera can be used, they often contain antibody to many serotypes (Maxted, Widdowson and Fraser, 1973a; Fraser, 1981) and interpretation can be complicated by the presence of antibody to new serotypes (Fraser and Maxted, 1979).

Guinea-pigs have been used in the preparation of antisera to the M-associated protein (MAP) antigen of group-A streptococci (Widdowson, 1980) and the sera from guinea-pigs inoculated with OF-positive serotypes also contained antibody to OF (Dr W. R. Maxted, personal communication). The aim of the present study was to assess the variability of the anti-OF response in rabbits by testing a large selection of anti-M sera for OF antibody and to investigate the possibility of using guinea-pigs for routine production of OF antisera. Comparisons of different vaccination procedures were made, to establish a method for the production of a complete set of typing sera.

MATERIALS AND METHODS

Streptococci. The strains used for the production of anti-OF sera in guinea-pigs are indicated in table I. The same strains were used for the routine production of M-typing sera in rabbits except that strains R68/1115, R79/2943 and R77/1604 were used in place of the strains cited for M types 4, 22 and PT3875 respectively. Four randomly selected strains each of M-type 61 and PT3875 from different sources were used to test cross reactions between guinea-pig anti-OF sera to these types.

Todd-Hewitt broth (Difco) was prepared according to the maker's instructions but it was supplemented with Neopeptone (Difco) 2% (w/v). Additional buffering was provided by the use of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 0.74 g/L and $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 0.13 g/L. The pH was adjusted to 7.4 and the medium was sterilised by autoclaving for 5 min at 115°C. Sterility was checked by incubation at 37°C for 18 h. The extra buffering capacity of the medium was necessary to prevent production of proteinase.

Preparation of vaccines. Lyophilised cultures were resuscitated in blood-broth medium, incubated at 37°C for 18 h and then subcultured on to blood-agar plates. A single colony was picked from the blood agar into 5 ml of Todd-Hewitt broth, and grown for 4–6 h at 37°C. This culture was used to seed 250 ml of the same broth for an 18-h growth. The culture was then plated for purity on blood agar; the cells were harvested by centrifugation and washed three times in physiological saline, resuspended in 25 ml of 0.1M phosphate buffer, pH 7.8 and plated again for purity; 5 ml of the suspension was removed to make an acid extract (Lancefield, 1928). The vaccine was then heated in a water bath at 56°C for 30 min and plated once more as a test for sterility. A check of the potency and specificity of the vaccine was made by testing the M-antigen content of the Lancefield extract with an homologous anti-M serum and two control sera by the double-gel diffusion technique. The OF content was checked by spotting a loopful of the Lancefield extract on to a serum-agar slide (see below).

If the homologous M-antigen and OF reactions of the extract were very weak, or negative, a fresh extract was made from another 5-ml sample of the vaccine and the two extracts were tested in parallel for both reactions.

Phage-associated-lysin treatment of type-13 cells. A culture of M-type 13 was heated to 70°C and the cells were treated with the muralytic enzyme, group-C phage-associated lysin (Krause, 1958) so that group polysaccharide, M antigen and OF were released into solution (Maxted, 1964). The titre of OF in this preparation was 1024.

Test for opacity factor. Horse or pig serum (1 ml) adjusted to pH 6.0 was mixed with 1 ml of

2% (w/v) Oxoid Ion Agar in distilled water and the mixture was poured evenly over a 7.5 × 2.5-cm glass slide, allowed to solidify and dried for 15 min at 30°C. Supernates or extracts were spotted on to the slide with a 4-mm loop. Slides were left in a moist chamber overnight at 37°C. A positive reaction was indicated by the development of a zone of opacity.

Inoculation schedules. White Dunkin-Hartley guinea-pigs of 450–500 g weight were used. A primary sensitising dose was always given 5 days before commencement of the full course of immunisation, and in experiments with M-type 25, 4–6-week courses of weekly injections were given to groups of four or more animals as follows: (schedule i) 0.5 ml intraperitoneally (IP), (schedule ii) 0.25 ml subcutaneously into the loose skin at the back of the neck (SC), (schedule iii) 0.5 ml SC, (schedule iv) 0.5 ml IP and 0.25 ml SC.

For the other types, schedule iv, given once weekly for 4 weeks before a test bleeding, was used. Weekly injections continued until a satisfactory titre was obtained. If no response occurred within 8 weeks, the animal was rested for 2 weeks and a booster course of four weekly injections was given. Sera were stored at 4°C after the addition of thiomersal (1 in 5000 final concentration).

Rabbit anti-M sera tested for the presence of OF antibody. Existing rabbit anti-M sera prepared by conventional methods (Swift, Wilson and Lancefield, 1943) for the OF-positive types 2 to 78 and provisional M-types 180 and 3875 were tested for their OF-antibody content. These sera contained 1 in 5000 thiomersal as a preservative and had been stored at 4°C for up to 10 years. Anti-M sera were selected from stock to provide six sera representing each of 23 different serotypes; anti-M sera for type 13 were not available. When possible these were taken from six different rabbits but in some cases (types 2, 4, 9, 11, 22 and PT3875) they were sera from fewer, but never less than three, rabbits, and included more than one serum from the same rabbit taken at different stages in immunisation. For the latter, first and second bleedings were obtained after a primary 6–8-week course of inoculations and third and fourth bleedings were obtained after a series of booster inoculations of 3–4 weeks duration. Some sera taken from bleedings made after the third course of immunisation were often potent anti-M sera.

Estimation of OF antibody in immune-animal sera. Sera were tested by slide method (a) to determine the titre against the vaccine strain and then slide method (b) was used to screen the sera against the whole range of OF-positive group-A serotypes for the specificity of their inhibition reactions.

Method (a). When only a small amount of OF antiserum was available, 1 ml of horse or pig serum, 0.2 ml of a selected culture supernate or extract and 1 ml of 2% (w/v) Ion Agar were mixed and poured on to a slide as described below. After drying, OF antisera were spotted on to the slide with a loop and the slide was incubated at 37°C overnight. Inhibition was shown by a clear area on a slightly opaque background. It was essential to use these slides immediately after preparation.

Method (b). Either horse or pig serum (1 ml) was added to 0.2 ml of OF antiserum, which was sometimes diluted depending on its titre, and this was mixed with 1 ml of 2% (w/v) Ion Agar and the mixture poured evenly over a 5 × 5-cm glass slide. The slide was dried and a 4-mm loopful of broth-culture supernate or Lancefield extract of the strain under test was placed on the slide, which was then incubated at 37°C in a moist chamber. Up to 25 strains were tested on one slide. Strongly active supernates or extracts were diluted fourfold. Control slides without OF antiserum were included. When an opaque area was seen on the control slide and there was no opacity on the antiserum slide this was taken as indicating inhibition.

Bactericidal tests were done by the method described by Maxted *et al.* (1973a). Strains, previously tested for their ability to survive in the donor blood, were suitably diluted (50–500 viable units/0.02 ml) and 0.02 ml was added to the same volume of antiserum; 0.3 ml of fresh human blood containing heparin 5 units/ml was added. In all tests with guinea-pig serum, asparagine 0.01% was added because group-A streptococci do not multiply in serum of this species unless this is done. Inhibition of the growth of group-A streptococci by guinea-pig serum was attributed by Dr D. C. J. Bassett (unpublished work) to the action of asparaginase in the serum, which apparently removed an essential nutrient. Asparagine, in certain conditions, has been shown to be necessary for the growth of group-A streptococci (Mickelson, 1964). Alternatively the inhibitory effect was overcome by chemical fractionation of the guinea-pig serum (Cohn *et al.*, 1950). The α -globulin fraction was used in the bactericidal tests.

RESULTS

The immune response to opacity factor in rabbits

The aim of these experiments was to assess how many usable anti-OF sera could be derived from our existing stock of unabsorbed anti-M sera, without resorting to the immunisation of more rabbits. A total of 138 undiluted sera (see *Materials and methods*) were screened for presence of OF antibody. Of these, 61 (44%) contained OF antibody to the homologous M type but 77 (56%) had none. The 61 positive sera were diluted in a series of doubling dilutions from 1 in 2 to 1 in 256. Of the 61 tested, 55 (90%) gave a titre greater than 2. However, closer examination of the range of titres (table II) demonstrates that only eight serotypes gave rise to sera with titres of greater than 32; three gave some moderately good titres (16–32) and 12 types gave sera that had titres that were in all instances less than 16.

A titre of at least 16 is necessary, if the anti-OF serum is to be incorporated in an

TABLE I
*Antibody response produced in guinea-pigs by inoculation with opacity-factor-positive strains**

Vaccine strains		Number of animals		OF inhibition by sera	
M-type	Strain no.	immunised	with OF antibody	Mean† titre of homologous reaction	Heterologous inhibition reaction
2	T2/44/Rb4	6	2‡	16	None
4	R68/1115	6	6	64	None
9	T9/101/4	6	6	32	None
11	T11/54/8	6	6	64	None
13	SS31	6	0	0	Not tested
22	R79/2943	8	7	64	None
25	Matthews PZH	6	6	128	None
28	Small	6	6	64	None
48	B403/48/1	6	6	16	None
49	B737/34/3	6	6	16	None
58	R67/3884	6	6	256	None
59	R68/2991	6	6	256	None
60	R69/2709	6	6	128	None
61	R69/2714	6	6	128	PT3875
62	R66/3489	8	8	128	None
63	SF2	6	6	128	None
66	R72/3333	8	8	1024	None
73	R67/239	6	6	32	None
75	R68/3354	6	6	256	None
76	R72/943	6	6	128	None
77	R72/2773	6	6	128	None
78	R72/3085	10	4§	64	None
PT180	R72/180	8	8	1024	None
PT3875	R77/1604	6	6	128	M-type 61

* Immunisation schedule: 0.5 ml of vaccine intraperitoneally and 0.25 ml subcutaneously weekly for 4–6 weeks (see text).

† Titres in guinea-pigs inoculated with the same serotype did not vary by more than one doubling dilution from the mean.

‡ 100% response after immunisation with strain 1000s.

§ Two more animals responded after prolonged immunisation.

|| Potent sera were later prepared in three guinea-pigs each against strain 2768 (type 68), strain R74/2015 (type 79), strain R74/1658 (PT1658) and R76/2841 (PT2841). These sera gave no heterologous inhibition reactions.

TABLE II
Highest titre of OF antibody in rabbit anti-M sera for 23 serotypes

Serotypes yielding one or more sera with an anti-OF titre of		
< 16	16-32	> 32
2	22	4
9	60	49
11	PT180	59
25	...	61
28	...	63
48	...	66
58	...	77
62	...	PT3875
73	...	...
75	...	...
76	...	...
78	...	...

agar-gel slide for the OF typing of up to 25 strains on one slide (method (b)—see *Materials and methods*). Therefore only 11 OF-positive serotypes gave rise to rabbit sera with sufficiently high anti-OF titres to be used in a routine typing method.

Wide variation in the response of different rabbits to the same serotype was also a problem, and in some cases titres ranged from <1 to 128 in different animals inoculated with the same serotype.

The immune response to opacity factor in guinea-pigs

An M-type 25 vaccine was prepared and administered to groups of guinea-pigs as described in *Materials and methods*. Intraperitoneal injection alone did not promote an OF-antibody titre of greater than 4 whereas subcutaneous injection of 0.25 ml produced titres of 32 which could be boosted to 128 after a further 2 weeks. No increased response was noted if a 0.5-ml dose was used. Simultaneous injection by both routes stimulated a twofold increase over that from the subcutaneous route alone in the initial titres in the first 4 weeks, but after a further 4 weeks without injections the serum titres were identical with those of animals inoculated subcutaneously (see the figure). The greatest antigenic stimulus therefore appeared to be by the subcutaneous route with a slight synergistic effect in the early stages when the combined routes of inoculation were used. The combined routes of inoculation were used in the routine preparation of antisera to the other serotypes.

Preparation of guinea-pig antisera against all available OF-positive serotypes

When an optimal schedule of immunisation was used, nearly all the guinea-pigs responded by producing OF antibody at titres considerably greater than those obtained in rabbits. Potent sera were produced against the OF of 27 of the 28 OF serotypes (table I). The one exception was an M-type 13 vaccine which failed to produce any OF-antibody response by the method described, or by subcutaneous injection of phage-lysate-treated type-13 cells that had an OF titre of 1024. Prolonged immunisation for five courses failed to promote a response although the enzyme in the

FIG.—Time course of type-25 OF-antibody response after weekly immunisation of guinea-pigs by different routes. ○ = 0.5 ml intraperitoneally and 0.25 ml subcutaneously; ● = 0.25 ml subcutaneously; × = 0.5 ml intraperitoneally.

preparation was known to be active. There was a good immune response to OF for all the other serotypes tested but in two instances, M-types 22 and 78, not all the animals in the group responded to the vaccine (table I). One of the guinea-pigs receiving type-22 vaccine was ill intermittently and lost weight. This animal had a serum titre of only 4 after four weeks' inoculation. After a rest of 4 weeks, booster injections produced a type-22 titre of 8 which did not rise further. Of the 10 animals given type-78 vaccine, four responded well and two others showed an improvement in anti-OF titre from 16 to 64 after a third series of inoculations for 4 weeks. The remaining four animals had a final serum titre of 8.

Antibody titres to the homologous vaccine strain in all strains tested by slide method (a) ranged from 16 to 1024 for different serotypes after immunisation for a total of 6 weeks. Antibody titres of different animals immunised with the same serotype did not vary by more than one doubling dilution from the mean titres shown in table I. Test bleedings were taken after 4 weeks, after an interval in immunisation of 4 weeks and again at the 10th week. Final bleedings were in most cases taken either at this point or when the serum showed a further rise in the specific anti-OF titre.

Specificity of anti-OF reactions

All 27 anti-OF sera were tested against the whole range of supernates from OF-positive group-A serotypes by slide method (b). The results are shown in table I. All except two serotypes reacted specifically when tested at a dilution of 1 in 5. Type 61 and PT3875 showed reciprocal cross reaction at equal titre. A selection of strains of both serotypes was tested against both sera for OF inhibition and all were inhibited

equally to identical titre. Reciprocal absorptions were done with heat-killed and washed whole cells of streptococci for each type. The OF-neutralising activity was completely removed from both sera. In a similar manner, absorption with OF-containing supernates was performed by allowing the OF and serum (final concentration of OF, 1 in 5) to react at 37°C for 15 min and then spotting loopfuls onto the surface of dried slides containing horse serum, agar and supernate of either type 61 or PT3875. Antibody to both types was again removed. Control serum of an unrelated type was included in the absorption experiments but depletion of antibody was not observed. Bactericidal tests were performed with type 61 and type PT3875 sera to determine whether the M antibody also cross reacted and promoted reciprocal phagocytosis. Bactericidal tests of these guinea-pig anti-OF sera against the two vaccine strains showed no evidence of non-specific killing of either strain but in the presence of homologous antiserum each strain was specifically killed. Rabbit anti-M sera had also previously never shown any cross reactivity or related M-precipitin reactions and the two types have different T-typing patterns; type 61 possesses antigen T11 and PT3875 gives the T-pattern reaction 8/25/IMP 19.

Bactericidal tests on type-25 guinea-pig sera

A serum from a representative of each of the three sets of guinea-pigs inoculated by different routes with M-type 25 was used in a bactericidal test to determine whether any difference in M-antibody levels could be detected. The results of these tests, with the three type 25 sera, IP + SC, SC and IP, are shown in table III. A strain of M-type 1 was included as a control for non-specific activity by the test sera. Specific killing of the type-1 strain by polymorphs in the presence of type-1 anti-M serum showed that the conditions of the test were satisfactory. All cells of M-type 25 were killed in the presence of homologous hyperimmune rabbit serum as expected, and also by two of the guinea-pig sera (IP + SC and SC), and there was no apparent multiplication of the inoculum with the third serum (IP), indicating the presence of traces of M antibody. These results correlated with the respective anti-OF titres of 128 after IP + SC inoculation, 128 after SC, and 4 after IP alone.

TABLE III

Effect of anti-OF sera from guinea-pigs, inoculated with M-type 25 by different routes, on the bactericidal power of normal human blood

M-type	Inoculum (cfu)	Growth* of indicated strain in the presence of							
		rabbit serum		control guinea-pig serum		immune guinea-pig serum with 0.01% asparagine			no serum
		M1	M25	N	YGP	M25 IP+SC	M25 SC	M25 IP	
25	192	++++	—	++++	++++	—	—	++	++++
1	200	—	++++	++++	++++	++++	++++	++++	++++

N = normal guinea-pig serum with 0.01% asparagine; YGP = serum from 22-day-old guinea-pig, asparagine not added; IP = intraperitoneal inoculation; SC = subcutaneous inoculation.

* Colony-forming units counted. Survivors recorded as: — = 0–9 cfu, + = 10–49 cfu, ++ = 50–249 cfu, +++ = 250–999 cfu, ++++ = discrete but uncountable number of colonies.

DISCUSSION

The production of antisera to the OF of group-A streptococci has hitherto been attempted only on a small scale. All previous work has been confined to a few serotypes because the object has been to examine antigenic structure and immunological relationships, rather than to produce a complete set of typing sera. Twelve immunologically distinct strain-specific opacity factors were reported by Top and Wannamaker (1968*b*) who prepared rabbit antisera to cell-wall-membrane fractions, but the relation of OF to the corresponding M antigens was not established. Widdowson *et al.* (1970) confirmed the serological specificity of the OF and showed that it corresponded exactly to that of the M type that produced it, but less than half of the vaccine strains tested in rabbits produced usable anti-OF sera. Preparations of OF purified by isoelectric focusing and then combined with adjuvant were used by Martinez *et al.* (1978) to immunise rabbits for the preparation of OF antiserum. Extensive immunisation resulted in the production of only weakly reactive sera. This was attributed to the poor immunogenicity of the OF. Varying degrees of success in the production of antisera to OF in rabbits have been ascribed to the preparation of the inoculum and to the differences in antigenic stimulus provided by some strains.

In the present study, rabbit anti-M sera showed great variability in content of OF antibody and only a third of all serotypes gave rise to antibody titres greater than 32. OF antibody was not detected in more than half of all the M-antisera prepared for OF-positive serotypes. However, investigation of the time-course of antibody formation indicated that when anti-OF was formed it was often detected before antibodies to the M protein (Maxted, Widdowson and Fraser, 1974). Some sera contained M antibody but no OF antibody at the same stage of immunisation and others contained antibody to M and to OF antigens simultaneously.

The diversity of the immune response to OF in rabbits appears to be related in the main to serotype but also in part to the variation in individual animals because consistently reproducible results were not always obtained. The results of examining existing rabbit M-antisera maintained in this laboratory showed that only 44% of these sera could be used in an OF-inhibition typing system and a set of only 11 typing sera was obtained.

The anti-OF response in guinea-pigs was very consistent and provided a satisfactory method of preparing a complete set of sera. The once-weekly injection of a whole-cell vaccine produced good antisera against 27 of 28 strains of different serotypes in almost 100% of the animals tested. Antibody titres usually reached a maximum after only six injections. Failure to prepare a satisfactory serum was encountered with only one strain, type 13, which is also one of the particularly difficult types with which to raise M antisera. It appears to be poorly antigenic for OF although it is able to survive and multiply in normal human blood, which suggests that it possesses an M antigen with phagocytosis-inhibiting properties. A search for alternative strains of this serotype might reveal a more antigenic vaccine strain.

It was cheaper to immunise six guinea-pigs for each serotype than to use two rabbits and the same amount of cage space was occupied. Routes of inoculation and evaluation of doses could therefore easily be compared. Our set of typing sera was prepared by giving inoculations simultaneously by the subcutaneous and intraperitoneal routes, because more detailed investigations into an M-type 25 strain showed

that although good titres of anti OF could be produced after six 0.25-ml injections by the subcutaneous route alone, a slight synergistic effect was noted in the early stages when the combined route of inoculation was used.

Bactericidal M-antibody was produced by the guinea-pigs inoculated with M-type 25 but at a lower level than OF antibody. The latter always appeared first.

Although the results with guinea-pig sera in general confirmed the remarkable specificity of the OF antigens and their parallelism with M-antigenic specificity, the use of these potent sera made it possible to establish one clear exception to the rule. The reciprocal cross reaction encountered in two types, M-type 61 and PT3875, does not correspond with other known antigenic relationships of T or M antigens but was reproducible in subsequent batches of sera prepared in different animals with fresh batches of vaccine. These strains may share a major antigenic determinant for OF but differ in an individual minor antigen.

The close relationship between M antigen and OF has been extensively investigated by several workers but the above results clearly demonstrate the need for more studies on antigenic determinants.

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