

New Method of Preparing Immunizing Antigens for the Production of Anti-M Sera Against Certain Serotypes of Group A Streptococci¹

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Received for publication 14 September 1964

ABSTRACT

ASHWORTH, HELEN (Communicable Disease Center, Atlanta, Ga.), AND WILLIAM K. HARRELL. New method of preparing immunizing antigens for the production of anti-M sera against certain serotypes of group A streptococci. *J. Bacteriol.* **89**:141-145. 1965.—Antisera against types 4, 29, 41, and 46 streptococci have been prepared by use of a vaccine killed by adjusting the pH to 11.7 and heating for 1 hr at 56 C. Heating at the high pH destroyed the antigenic material responsible for the major reciprocal crosses present in cells of types 4, 29, and 46 without affecting the M protein. Antisera prepared with the "alkaline-treated" vaccine did not contain the major reciprocal crosses. Antibodies against the M protein were shown to be present by the bactericidal test, failure to react in the precipitin test with acid extracts of homologous trypsinized cells, and retention of the precipitin reaction after absorption with homologous trypsinized cells. The antisera did not react with the "B" antigen of the provisional type B 3264. Type 11 antisera prepared with alkaline-treated vaccine did not contain antibodies against the M protein. The usefulness of this type vaccine, particularly for the preparation of type 4 antiserum, is discussed, as well as its limitations.

The preparation of specific antisera against a number of serological types of group A streptococci is extremely difficult when using standard heat-killed vaccines. One of the problems frequently encountered with this method is the difficulty in removing cross-reactions from the antiserum. This is particularly true of antisera prepared against cells of types 4, 26, 29, and 46 which have reciprocal cross-reactions that are difficult and sometimes impossible to remove by absorption.

The vaccine most commonly used for making typing antisera is that of Lancefield (1955), prepared by suspending the cells in saline and heating at 56 C for 30 min. Conroy and Updyke (1954), using mechanically disrupted cells, obtained antisera against 16 serological types of streptococci which had few or no antibodies against the group carbohydrate. Rosendal (1956) used formalin-killed cells. Barkulis, Walsh, and Ekstedt (1958) and Hayashi and Walsh (1961) used cell-wall preparation suspended in saline. The latter authors also used, as antigens, cell-wall suspensions, which had been precipitated

¹ A preliminary report of this work was presented at the 64th Annual Meeting of the American Society for Microbiology, 6 May 1964, Washington, D.C.

with alum or suspended in mineral oil, as well as purified M protein. None of these investigators reported on the cells of types 4, 26, 29, and 46.

This investigation was intended to determine the feasibility of removing the antigenic material responsible for the major cross-reactions from the intact cells prior to the immunization of animals. Types 4, 11, 26, 29, 41, and 46 were selected for study.

Type 11 was selected, because it is one of the difficult types against which to prepare satisfactory antiserum. Specific antiserum against type 41 is usually not too difficult to prepare. Vaccines for the latter type were treated in the same manner as vaccines for the other five types in hopes of detecting any alterations of the M protein that might be reflected in the resulting antisera.

MATERIALS AND METHODS

Streptococcal strains. The following strains, obtained from Rebecca Lancefield, were used for the immunization of rabbits: type 4, strain T 4/56/0; type 11, strain T 11/54/3; type 29, strain D 23; type 41, strain C 101/10/1; and type 46, strain C 105.

Precipitin test. The capillary precipitin method of Swift, Wilson, and Lancefield (1943) was used. Crude hot acid extracts and alcohol-precipitated

M extracts (Lancefield, 1928) were used as antigens.

Preparation of "alkaline-treated vaccine." Cells from 1,000 ml of an overnight Todd Hewitt Broth culture were collected by centrifugation and resuspended in 35 ml of 0.85% saline. This suspension was adjusted to pH 11.7 with sodium hydroxide, and incubated for 1 hr at 56 C. After centrifugation, the cells were washed once with saline, resuspended in saline, and adjusted to pH 7.0 to 7.4 with hydrochloric acid. The cell suspension was diluted to a final volume of 120 ml and stored at 4 C.

Preparation of antisera. New Zealand white rabbits were immunized by use of the schedule recommended by Lancefield (1955).

Indirect bactericidal test. The procedure used by Lancefield (1957) was followed. Briefly, this was carried out as follows. Todd Hewitt Blood Broth was inoculated from an overnight Todd Hewitt Blood Broth culture and incubated for 2 to 2.5 hr at 37 C. A 10^{-5} dilution of the rapidly growing culture was prepared in Todd Hewitt Broth; from this, dilutions of 1:4, 1:16, 1:64, and 1:128 were prepared. The number of organisms present in the inoculum was determined by blood-agar pour plates with 0.1 ml of each dilution. To glass tubes (12 × 75 mm), 0.1 ml of the culture dilution, 0.05 ml of serum, and 0.3 ml of heparinized normal human blood were added. After stoppering, the tubes were rotated end over end at 6 rev/min for 3 hr at 37 C. A 0.1-ml amount from each tube was added to blood-agar and pour plates were prepared. After overnight incubation at 37 C, the number of colonies present was multiplied by 4.05 to obtain the total number of chains present in the rotated mixture.

RESULTS

Crude acid extracts of cells of types 4, 26, 29, and 46 had reciprocal crosses when tested by the precipitin reaction with adsorbed antisera. This observation was used as a tool to determine whether the antigenic material responsible for these cross-reactions could be removed from the intact cells without destroying the M protein. It was found that extracts of type 4 cells heated for 1 hr at 56 C, pH 11.7, did not contain crosses against antisera of types 26, 29, and 46, whereas extracts of type 4 that had been heated for 1 hr at 56 C, pH 7.0, gave weak to moderate crosses with these same antisera (Table 1). The precipitin reaction with type 4 antiserum was qualitatively the same with both extracts. Comparable results were obtained with cells of types 29 and 46. However, extracts prepared from alkaline-treated type 26 cells retained the crosses of types 4 and 46. For this reason, no attempt was made to prepare type 26 antiserum with this vaccine. Alkaline treatment of cells of types 11 and 41 prior to extraction did not qualitatively affect

their precipitin reaction with homologous antisera.

Test on antisera. Before absorption, antisera of types 4, 29, 41, and 46, prepared with the alkaline-treated vaccines, had the usual array of crosses with extracts of other serotypes, except for the major reciprocal crosses referred to above. The minor crosses could be removed by absorption, resulting in antisera specific for their homologous antigens.

In the indirect bactericidal test, the inhibition of growth in the presence of homologous antisera is indicative of an M anti-M system. To ensure that the antigenic response to the alkaline-treated vaccine was primarily to the M antigen, this test was run on each serum using homologous and heterologous cultures of types 4, 29, 41, and 46. Cells of types 29, 41, and 46 grew well in human blood, but were completely destroyed in the presence of their homologous antisera (Table 2). These same cultures were highly resistant to phagocytosis in the presence of heterologous antisera. Thus, these sera contain antibodies against their homologous M proteins.

Two different strains of type 4 cultures were used in the bactericidal test for the type 4 antisera (Table 3). Both cultures gave adequate growth in human blood. There was some multiplication in the presence of type 4 antiserum, although not nearly as much as in the presence of normal rabbit serum. This would indicate

TABLE 1. Cross-reactions of crude acid extracts and adsorbed antisera*

Crude acid extracts	Precipitin reactions† with antisera against types			
	4	26	29	46
Type 4				
Heat-killed.....	S	M	W	M
Alkaline-treated....	S	—	—	—
Type 26				
Heat-killed.....	W	S	—	?
Alkaline-treated....	W	S	—	W
Type 29				
Heat-killed.....	W	—	S	W
Alkaline-treated....	—	—	S	—
Type 46				
Heat-killed.....	W	—	—	S
Alkaline-treated....	—	—	—	S

* Extracts concentrated twofold compared with routine extracts.

† S = strong reaction; M = moderate reaction; W = weak reaction; ? = questionable reaction.

that anti-M antibodies were present in this serum, but in a lower concentration than in the antisera of types 29, 41, and 46.

After the first series of immunizations (18 inoculations) with alkaline-treated vaccine, the type 11 antiserum gave only a moderate precipitin reaction with its homologous antigen. It contained a cross with extracts of type 9 cells that could not be removed by absorption, and in the bactericidal test, the cells were almost completely resistant to phagocytosis. Thus, it is apparent that alkaline treatment of this vaccine was of no value in preparing the type 11 antiserum.

The vaccines used for preparing the antisera of types 4, 29, 41, and 46 were treated in an unusual manner; thus, the following additional tests were carried out to ensure that the antigenic response was to the M protein rather than to some other antigenic material.

(i) Overnight cultures of each type were treated with 2% trypsin for 2 hr at 37 C. Crude acid extracts prepared from these cells did not give a precipitin reaction with homologous antisera. The same results were obtained with trypsinized crude acid extracts of untreated cells. Therefore, with the destruction of the M protein by trypsin, the antigens responsible for the precipitin reaction with these antisera were destroyed. This also indicated that the precipitin reactions were not due to the T antigen, because this was not destroyed by trypsin.

(ii) Cells of types 4, 29, and 46, together with types 24 and 26, contain a common T antigen (Stewart et al., 1944), and therefore are agglutinated by type 4 anti-T serum after the cells have been trypsinized. When cells of these three serotypes are alkaline-treated prior to trypsinization, they are not agglutinated by the type 4 anti-T serum. In these three types, the T antigen was destroyed by heating for 1 hr at 56 C, pH 11.7.

(iii) When the type 4 antiserum was absorbed with trypsinized type 4 cells, the antibodies responsible for the precipitin reaction were not removed or qualitatively diminished. Therefore, absorption of this antiserum with cells lacking the M protein, but containing the T antigen, had no obvious effect on the precipitin reaction of this antiserum.

(iv) Antisera against cells of types 4, 29, 41, and 46 did not give a precipitin reaction with an extract of the provisional type B 3264. According to Hambly (1958), hot acid extracts of these cells contain the "B" antigen which is commonly found in types against which it is difficult to prepare anti-M sera. Antisera prepared against the provisional type B 3264 culture gave a

TABLE 2. Bactericidal test on absorbed antisera of types 29, 41, and 46

Culture	Inoculum*	Plate count†	
		Normal rabbit serum	Homologous serum
0029-B ₁ (type 29)	100	12,000	0
	26	5,600	0
	3	1,200	0
	2	56	0
	1	0	0
0070-B ₂ (type 41)	350	16,000	0
	125	12,000	0
	35	4,000	0
	8	680	0
	2	560	0
0046-B ₃ (type 46)	136	16,000	0
	34	8,000	0
	7	7,000	0
	2	400	0
	1	500	0

* See text for dilutions used for inoculum.

† Except for inoculum, plate counts or estimate of plate count in 0.1 ml multiplied by 4.05.

TABLE 3. Bactericidal test on absorbed type 4 antiserum

Culture	Inoculum*	Plate count†	
		Normal rabbit serum	Type 4 serum
0106-M ₃	72	9,000	172
	15	6,000	60
	10	2,000	24
	2	1,200	8
	1	0	0
0126-M ₅	135	20,000	1,200
	37	12,000	192
	8	8,000	32
	4	500	4
	1	0	0

* See text for dilutions used for inoculum.

† Except for inoculum, plate count or estimate of plate count in 0.1 ml multiplied by 4.05.

strong precipitin reaction with extracts of these cells. Because these antisera do not react with an extract known to contain the "B" antigen, they do not contain antibodies against this antigen.

(v) Lancefield (1958) reported that when extracts containing the 3 R and 28 R antigens are heated at pH 2 in a boiling-water bath for ten min, and then neutralized, they no longer precipitate with their homologous anti-R antisera.

Extracts of cells of types 4, 29, 41, and 46 subjected to this treatment gave strong precipitin reactions with their homologous antisera prepared with alkaline-treated vaccines.

DISCUSSION

It is apparent that alkaline-treated vaccines are not of value for preparing antisera against all of the serological types of group A streptococcus. The negative results reported above for type 11 are ample demonstration of this. In addition, preliminary experiments with a type 6 culture indicate that most of the M protein is extracted from the cells by this treatment. However, the method has proven to be of definite value in preparing specific antisera against types 4, 29, 41, and 46. Antisera prepared against types 4, 29, and 46 with a routine heat-killed vaccine usually contain reciprocal crosses that are difficult or impossible to remove by absorption. The removal from the cells of the antigenic material responsible for these crosses, prior to the immunization of animals, results in antisera containing only minor cross-reactions. The latter are easily removed by absorption.

The use of the alkaline-treated vaccine for preparing type 4 antiserum appears to be particularly useful. Hambly (1958) discussed the problems relating to the preparation of this antiserum in detail. She reported that heat-killed vaccines from four freshly isolated strains and one laboratory strain of type 4 group A streptococcus failed to stimulate the production of M antibodies in rabbits, and she suggested the following possibilities for this: (i) the M antigen is masked by some other antigen such as the T or B antigen, (ii) the M antigen is readily lost from type 4 strains in laboratory media, or (iii) the type 4 strains do not contain an M protein comparable to that of other types. The work reported here eliminates the last possibility, inasmuch as an M anti-M system has been demonstrated in the type 4 serum prepared with an alkaline-treated vaccine. Additional evidence for this might have been obtained if mouse-protective studies and the long-chain phenomena could have been carried out. Unfortunately, to our knowledge, a type 4 culture virulent for mice does not exist, and all of the type 4 cultures we have tested grow in long chains in the presence of normal rabbit serum. Thus, neither of these tests could be used for testing the type 4 antiserum.

Perhaps one of the most useful criteria for evaluating the type 4 antiserum is its performance in the diagnostic laboratory. During the past year, some seventy diagnostic cultures have

given a positive precipitin reaction with this type 4 serum in the Streptococcus and Staphylococcus Unit, Laboratory Branch, Communicable Disease Center.

This still leaves unsettled the question of what antigenic material is removed or destroyed by the alkaline treatment. It was shown that the T antigen in types 4, 29, and 46 is destroyed by this procedure which would suggest that this antigen is responsible for these major crosses. On the other hand, hot acid extracts of cells of types 4, 26, 29, and 46 have reciprocal crosses with absorbed antisera. Also, following trypsinization of type 4 cells, the supernatant fluid contains antigenic material that reacts with antisera of types 4, 26, 29, and 46. The T antigens of most types are destroyed by extraction with hot acid, whereas trypsin supposedly does not affect this antigen. Thus, under conditions where the T antigen should theoretically not be involved, the reciprocal crosses between types 4, 26, 29, and 46 are still present. If the T antigen is responsible for these crosses, or, in the case of type 4, masks the M antigen, it must be different from the T antigens of the other types. Maxted (1953) has already pointed out that the type 2 T antigen is not typical of other T antigens in its sensitivity to heat, and, under certain conditions, is destroyed by trypsin. A comparable situation may exist regarding the T antigen common to cells of types 4, 26, 29, and 46.

ACKNOWLEDGMENTS

The authors wish to thank Rebecca Lancefield, Rockefeller Institute, New York, N.Y., and Armine Wilson, Alfred I. du Pont Institute, Wilmington, Del., for their helpful suggestions and testings of the type 4 antiserum. Also we thank Max D. Moody, Laboratory Branch, Communicable Disease Center, for the type 4 anti-T serum, and W. R. Maxted, Central Public Health Laboratory, London, England, for the anti-B serum.

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