STUDIES OF STREPTOCOCCAL CELL WALLS

VI. Effects of Adjuvants on the Production of Type-specific Antibodies to Cell Walls and Isolated M Protein

JAMES A. HAYASHI[1] AND GERALD WALSH[2]

Department of Biological Chemistry, University of Illinois College of Medicine, Chicago, Illinois

Received for publication May 10, 1961

ABSTRACT

Hayashi, James A. (University of Illinois College of Medicine, Chicago) and Gerald Walsh. Studies of streptococcal cell walls. VI. Effects of adjuvants on the production of type-specific antibodies to cell walls and isolated M protein. J. Bacteriol. **82**:736–742. 1961.—Mineral oil adjuvant was shown to assist in type-specific immunization of rabbits, when use was made of isolated cell walls or pH 2-extracted M protein from type 14 group A hemolytic streptococci. The antibodies produced were type specific, and passively protected mice that were challenged with virulent type 14 streptococci. A single injection each week for 4 weeks gave good levels of antibody. Simultaneous immunization with three different types of group A streptococci resulted in production of antibodies against all three types.

The importance of type-specific antibodies in immunity to group A β-hemolytic streptococcal infections has been demonstrated in studies with experimental animals, and is consistent with clinical experience (McCarty, 1954). Type specificity is a function of the serologically active M proteins of streptococci, and these proteins also play a significant role in the virulence of these organisms. The early work of Lancefield (1943) indicated that M proteins are located on the surface of the streptococcal cell. Studies of isolated cell walls confirmed this impression (Salton, 1953; Barkulis and Jones, 1957), and showed that the M proteins are a principal component of streptococcal cell-wall proteins. They may be readily extracted from isolated walls by heating in weakly acidic solutions, and can be purified by conventional techniques of protein fractionation (Lancefield and Perlman, 1952; Zittle, 1942; Barkulis and Jones, 1957). These purified preparations retain serological activity, but have been considered to be only weakly antigenic.

Barkulis, Walsh, and Ekstedt (1958) demonstrated that saline suspensions of isolated cell walls injected intravenously are capable of stimulating type-specific antibodies in rabbits.

This report presents a study of the effects of two adjuvants on the antigenicity of cell walls and of M protein administered by several routes. It is shown that, with mineral oil adjuvant, it is possible to use a simplified injection schedule and to immunize simultaneously against at least three different group A streptococcal types by injections of cell wall or M protein mixtures.

MATERIALS AND METHODS

Growth of organisms. The organisms used were: Group A, β-hemolytic streptococci, including a type 14 strain designated S23; a type 19 streptococcus designated J17D; and a type 12 organism designated SF42, all of which were grown in Todd-Hewitt broth with addded salts and glucose (Barkulis and Jones, 1957).

Preparation of cell walls and M protein. Cell walls were obtained by a slight modification of the method of Shockman, Kolb, and Toennies (1957). A 20-ml quantity of a 2% (wet weight) suspension of whole cells was placed in a large (5 by 1 and 3⁄16 in.) stainless steel capsule, and 20 ml of Ballotini no. 12 beads were added. The capsule was then attached to an International Shaker Head in a refrigerated centrifuge and shaken at a speed of 1,000 rev/min. Complete breakage of cells was obtained after 20 min of shaking. The cell walls were washed and treated as described previously (Barkulis and Jones, 1957).

M protein preparations were obtained from isolated cell walls by the method described by Barkulis and Jones (1957).

[1] Present address: Department of Biochemistry, Presbyterian-St. Luke's Hospital, Chicago, Ill.

[2] Present address: Graduate College, University of Hawaii, Honolulu, Hawaii.

Preparation of vaccines. Preparations for immunization were made following previously published methods:

1) Saline suspensions of cell walls (Barkulis et al., 1958) were prepared, with Merthiolate (1:10,000) added as a preservative. Solutions of M protein were made by dissolving weighed amounts of lyophilized protein in 0.85% saline.

2) Alum-precipitated vaccines were prepared by a method similar to that of Baer, Bringaze, and McNamee (1954). Cell walls were suspended in pH 7.4 phosphate buffer and sonically treated for 1 min in a Raytheon 9 kc sonic oscillator. To each 19 ml of suspension was added 0.25 ml of 10% $NaHCO_3$ solution and then 1 ml of 10% $KAl(SO_4)_2$ solution. The resulting precipitate was sedimented at 20,000 $\times$ g for 20 min, washed once with buffered saline, and then resuspended in the original amount of buffered saline. The suspension was transferred to a rubber-capped bottle, after addition of Merthiolate to a concentration of 1:10,000.

3) Mineral oil suspensions were prepared according to the method given by Freund (1947), except that the mycobacteria were omitted. An appropriate amount of cell walls was suspended in 30 ml of saline solution by sonic treatment for 1 min. The suspension was placed in a widemouth bottle containing 15 ml of Falba (Pfaltz and Bauer Company, New York), 30 ml of light mineral oil were added, and the entire mixture was stirred mechanically until a smooth emulsion was obtained. Merthiolate was added to a final concentration of 1:10,000, and the suspension was placed in a rubber-capped bottle and refrigerated. The bottle was warmed before use to facilitate flow of the viscous suspension. All immunizing preparations were tested for sterility prior to use.

Immunization procedures. Adult male rabbits were injected by a modification of the method of Lancefield (1947). The animals were injected on 3 consecutive days of a week and rested for 4 days. In the first week the dose was 0.5 ml per injection but was raised to 1 ml during the next 3 weeks. In several experiments (Tables 4, 5, 6), the animals were given 0.5 ml of mineral oil suspension once a week for 4 weeks.

Blood samples were obtained by intercardiac puncture, care being taken to maintain sterility so that the serum could be assayed for specific antibody in the bactericidal test.

Antibody determinations. Determinations of specific antibody were made using the bactericidal and mouse protection tests described by Barkulis et al. (1958).

RESULTS

Immunizations with cell walls. The results of immunizations with cell-wall preparations are shown in Table 1. Suspensions of cell walls in saline stimulate antibody formation only when given intravenously. Intramuscular injections of mineral oil suspensions of type 14 cell walls resulted in readily demonstrable type-specific antibody formation in rabbits. These suspensions induced a somewhat slower antibody formation than did saline suspensions of cell walls given intravenously, but antibody is demonstrable in the sera of the rabbits that received mineral oil suspensions 6 months after the last injection. The sera of animals immunized intravenously with saline suspensions showed no antibody 2 months after immunization.

Immunization with M protein. Type 14 M protein suspended in mineral oil was given to another group of rabbits. Previous experiments in this laboratory had shown that M protein injected intravenously was not effective in stimulating antibody formation in rabbits. The data in Table 2 show that small amounts of M protein suspended in mineral oil stimulate long-lasting, type-specific antibodies in rabbits. Six months after the last injection, type-specific antibody was still present in the sera of rabbits immunized with type 14 M protein in mineral oil.

Mouse protection studies. To demonstrate the protective character of the immune sera produced in these studies, mouse protection studies were made using the sera from all the rabbits numbered 89 through 124; this group included 20 rabbits. Sera were obtained from these animals 8 weeks after the last injection, and were used to protect, passively, mice challenged with an infectious dose of virulent type 14 streptococci. Table 3 shows results which are typical for the various groups used in this test. The sera from other rabbits in each immunization group gave results which were almost identical with those listed and are, therefore, omitted from the table. While the number of mice in each category shown in Table 3 was small, it was felt that enough mice were used to illustrate the correlation between mouse protection and the presence of type-specific antibody, as shown by the bactericidal test.

The serum from rabbit 68 was used as a posi-

TABLE 1. *Bactericidal activity of antisera against group A, type 14 cell-wall vaccines**

Vaccine and route of injection†	Rabbit no.	Type-specific antibodies after last injection (in weeks)‡						
		1	2	4	8	9	12	25
Saline suspensions:								
Intravenously	65	++						
	68	++						
	83	++						
	89		++	++	0		0	0
	91		++	0	0		0	0
Intramuscularly	61	++				0		
	82					0		
	84					0		
	92		0	0	0		0	0
	94		0	0	0		0	
	95		0	0	0		0	0
Alum suspensions:								
Intravenously	58	0				0		
	71	++				0		
	73	0						
Intramuscularly	59	0				0		
	60	0				0		
	74	0				0		
Mineral oil suspensions, intramuscularly	69	0				++		
	75	0				+		
	78	+				++		
	100		0	++	++		++	+
	101		++	++	++		++	+
	102		++	++	++			

* Combined results of two experiments.

† Each animal received a total of 10.5 mg cell walls, according to the immunization procedure of Lancefield (1947).

‡ 0, no antibodies; +, low antibody titer; ++, high antibody titer.

TABLE 2. *Bactericidal activity of antisera against group A, type 14 M protein in mineral oil suspension**

Injection of vaccine, intramuscularly	Rabbit no.	Type-specific antibodies after last injection (in weeks)						
		1	2	4	8	9	12	25
3.8 mg	77	+						
	86	+				++		
	87	0				++		
4.2 mg	106		++	++	++		++	+
	107		+	++	++		++	+
	109		++	++				
0.52 mg	110		0	++	++		++	++
	123		+	++	++		++	++
	124		0	++	++			

* Combined results of two experiments.

TABLE 3. *Typical results from a mouse protection experiment*
(Sera obtained 8 weeks after immunization)

Antiserum	Mouse no.	Dose of culture medium, ml				
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Normal (rabbit 88)	1	D1*	D1	D1	D1	D1
	2	D1	D1	D1	S	D3
	3	S	S	D1	S	S
Cell walls, iv, (rabbit 68), bled 1 week after immunization)	1	D1	S	S	S	S
	2	D1	S	S	S	S
	3	S	S	S	S	S
Cell walls, iv, (rabbit 89)	1	D1	D1	D1	D2	D1
	2	D1	D1	S	D2	S
	3	D1	S	S	S	S
Cell walls, in mineral oil, im (rabbit 102)	1	D1	S	S	S	S
	2	D1	S	S	S	S
	3	D1	S	S	S	S
Cell walls, in saline, im (rabbit 92)	1	D1	D1	D1	D2	D2
	2	D1	D1	D1	D2	D3
	3	D1	D1	D1	D2	S
Type 14 M protein in mineral oil, im (rabbit 110), 4.2 mg	1	D1	D1	S	S	S
	2	D1	S	S	S	S
	3	D1	S	S	S	S
M protein in mineral oil, im (rabbit 124), 0.525 mg	1	D1	D1	S	S	S
	2	D1	S	S	S	S
	3	D1	S	S	S	S

* D, dead; the number following indicates the day of death; S, survived.

TABLE 4. *Results of a simplified injection schedule of one injection per week for 4 weeks*

Total antigen injected*	Rabbit no.	Type-specific antibodies 4 weeks after immunization
Cell walls, 4.0 mg	117	++†
	119	++
M protein, 1.6 mg	122	++
	147	0
M protein, 0.2 mg	148	+
	149	++

* A mineral oil suspension (1 ml) of type 19 J17D cell walls or M protein was injected intramuscularly each week for 4 weeks.

† 0, no antibodies; +, low antibody titer; ++, high antibody titer.

tive control, since such sera had been shown previously (Barkulis et al., 1958) to give good protection in mouse protection tests. Table 3 shows that intravenous injections of cell walls gave a rapid rise in protective antibody (rabbit 68), but the protective capacity was lost within 2 months (rabbit 89). Intramuscular injections of a similar saline suspension stimulated no antibodies. The two sera obtained from rabbits immunized with M protein in mineral oil gave protection comparable to antisera from rabbit 68, and further substantiated the results of the bactericidal tests in Table 2, which showed high levels of antibody for those two sera.

Use of a simplified injection schedule. The injection schedule of Lancefield (1947) was used in this study, to allow comparison of these results with earlier work (Barkulis et al., 1958). It seemed probable as this work progressed that a simpler immunization schedule might be followed when using mineral oil suspensions for injection. An experiment was designed to test whether a single injection each week for 4 weeks might give adequate levels of antibody in rabbits. The data summarized in Table 4 indicate that the simpler procedure, using cell walls or pH 2-extracted M protein, is effective in producing type-specific antibodies in rabbits. In addition, it is apparent that as little as 0.2 mg of M protein stimulates type-specific antibodies in rabbits.

TABLE 5. *Bactericidal activity of antisera from simultaneous injections of three types of group A cell walls in mineral oil*

Vaccine	Rabbit no.	Antibody response*		
		Type 12	Type 19	Type 14
Polyvalent†	170	++	+	+
	171	+	0	+
	172	+	+	+
	173	++	0	+
	174	++	+	+
	176	+	+	0
Type 12‡	181	++		
	182	0		
	183	+		
Type 19‡	184		++	
	185		+	
	187		+	
Type 14‡	177			++
	178			+
	180			++

* 0, no antibodies; +, low antibody titer; ++, high antibody titer.

† Vaccine: 0.75 mg/ml of each type in mineral oil. One im injection of 1 ml each week for 4 weeks; bled 4 weeks after last injection.

‡ Vaccine: 0.75 mg/ml of mineral oil; injection schedule same as above.

TABLE 6. *Bactericidal activity of rabbit antisera after injection of three types of group A, M protein in mineral oil*

Vaccine	Rabbit no.	Antibody response*		
		Type 12	Type 19	Type 14
Polyvalent†	188	0	+	+
	189	+	+	++
	190	++	+	+
Type 12 M protein‡	198	0		
	199	+		
	200	+		
Type 19 M protein‡	201		+	
	202		++	
	203		+	
Type 14 M protein‡	195			0
	196			0
	197			+

* 0, no antibodies; +, low antibody titer; ++, high antibody titer.

† Vaccine: 0.125 mg/ml of each M protein. A single 1.0-ml injection per week for 4 weeks and bleeding 2 weeks after the last injection.

‡ Vaccine: 0.125 mg M protein/ml. Injection schedule as above.

Simultaneous immunization with three streptococcal M types. It was of interest to determine whether the simultaneous injection of several different types of group A streptococcal cell walls could induce type-specific antibodies to all the injected types. A limited experiment to test this point was made using mineral oil suspensions of types 12, 14, and 19 cell walls. The results in Table 5 show that, in general, these strains of type 14 and type 19 cell walls, when injected alone, are better antigens than the cell walls of the type 12 strain. When given simultaneously, however, the response was best to type 12, and least to type 19. Because of the limited numbers of animals used, however, no conclusions may be drawn other than that rabbits do respond to all the injected cell-wall types by producing type-specific antibodies.

A similar investigation using acid-extracted M protein was made (Table 6). Two of the three rabbits tested showed a good response to all three antigens, and the third animal showed a response to two of the three antigens.

DISCUSSION

The use of a mineral oil suspension for enhancing the antigenic properties of M protein has many parallels (Freund, 1947), and has been reported recently by Kantor and Cole (1960) in their immunizations using M protein obtained by the phage-associated lysin of Krause (1958). The importance of the adjuvant used is apparent when comparing the immunizations using mineral oil suspensions and the alum-precipitated vaccines. The latter adjuvant preparation with type 14 cell walls does not stimulate significant antibody production in rabbits when injected intravenously or intramuscularly. In a somewhat related instance, Schmidt (1960) used aluminum phosphate as an adjuvant when injecting humans with acid-heat-extracted M protein. The fact that only a small number of patients showed significant antibody production may be ascribed, in part, to the adjuvant used, which is somewhat similar to the alum-vaccine described in this work.

A second example of the importance of the adjuvant is seen in Table 2. Past experiments in our laboratory indicate that intramuscular injections of type 14 M protein in saline solution do not produce type-specific immunity in rabbits. Similarly, Lancefield and Perlman (1952) have

shown that type 1 M protein obtained by acid-heat extraction gives uncertain immunization even in amounts as high as 45 mg for a single rabbit. With mineral oil suspensions of type 14 M protein, as little as 0.2 to 0.5 mg of protein will immunize an adult rabbit.

The data in Table 1 indicate that the route of injection must have some importance in the antigenicity of streptococcal cell walls. Intravenous injections of cell walls in saline produce type-specific antibodies in rabbits (Barkulis et al., 1958), but intramuscular injections of the same vaccine fail to produce any detectable amounts of antibody. Intramuscular injections of M protein in mineral oil not only stimulate good levels of type-specific antibody, but support such levels for as long as 6 to 10 months after immunization, as contrasted with 1 to 2 months for antibodies produced by intravenous injection of saline solutions.

Although the data presented here seem to indicate no essential difference in the antibody-invoking capacity of cell walls and a corresponding amount of acid-heat-extracted M protein, there is evidence that the state of degradation of M protein is important in its antigenic capacity. Kantor and Cole (1960) immunized rabbits with M protein which was obtained by lysing cell walls with the phage-associated lysin of Krause (1958). Their results indicate that intradermal injections of saline solutions of lysin-obtained M protein do stimulate a low level of type-specific antibody. Mineral oil adjuvant enhances the antigenicity of such M protein preparations, and the antibody is detectable even 12 weeks after immunization. In the work reported here, M protein was prepared by the acid-heat extraction method of Lancefield and Perlman (1952). These preparations in saline solution gave no antibodies after injection into rabbits, but upon incorporation into mineral oil suspension caused the stimulation of type-specific antibodies which persisted at least 6 months and possibly as long as 10 months. The essential difference in this work and that of Kantor and Cole (1960) appears to lie in the M protein preparations used, and may explain many of the differences noted in the results.

When cell walls, or M protein from types 12, 14, and 19 group A streptococci, were incorporated into a single vaccine and injected into rabbits, the resultant antisera were active against all of the types injected. Although these results were obtained using a limited number of animals, it appears probable that at least three and possibly many more streptococcal M proteins could be incorporated into a single vaccine to give immunity against a number of different types of group A streptococci. The possible use of such a polyvalent vaccine in immunity against group A streptococcal infection is obvious, and further work in this direction would be of great interest.

ACKNOWLEDGMENTS

We are grateful for the technical assistance of Pearl Smith.

This investigation was conducted under the sponsorship of the Commission on Streptococcal Diseases, Armed Forces Epidemiological Board, and was supported by the Office of the Surgeon General, Department of the Army, Washington, D. C.

LITERATURE CITED

Baer, H., J. K. Bringaze, and M. McNamee. 1954. The immunochemistry of blood group O. I. The production of precipitating and agglutinating antibodies in chickens to various human and hog blood group substances. J. Immunol. **73**:67–80.

Barkulis, S. S., and M. F. Jones. 1957. Studies of streptococcal cell walls. I. Isolation, chemical composition and preparation of M protein. J. Bacteriol. **74**:207–216.

Barkulis, S. S., G. Walsh, and R. D. Ekstedt. 1958. Studies of streptococcal cell walls. II. Type-specific antigenicity in rabbits. J. Bacteriol. **76**:109–115.

Freund, J. 1947. Some aspects of active immunization. Ann. Rev. Microbiol. **1**:291–308.

Kantor, F. S., and R. M. Cole. 1960. Preparation and antigenicity of M protein released from group A, type 1 streptococcal cell walls by phage-associated lysin. J. Exptl. Med. **112**:77–96.

Krause, R. M. 1958. Studies on the bacteriophages of hemolytic streptococci. II. Antigens released from the streptococcal cell wall by a phage-associated lysin. J. Exptl. Med. **108**:803–821.

Lancefield, R. C. 1943. Studies on the antigenic composition of group A hemolytic streptococci. I. Effects of proteolytic enzymes on streptococcal cells. J. Exptl. Med. **78**:465–476.

Lancefield, R. C. 1947. Technical instructions and procedures involved in the production of

streptococcus grouping and typing sera. Brochure of the Hospital of the Rockefeller Institute for Medical Research, New York.

LANCEFIELD, R. C., AND G. E. PERLMAN. 1952. Preparation and properties of type-specific M antigen isolated from group A, type 1 hemolytic streptococcus. J. Exptl. Med. **96**:71–82.

MCCARTY, M. 1954. The antibody response to streptococcal infections, pp. 130–142. *In* McCarty, M., [ed.], Streptococcal infections. Columbia University Press, New York.

SALTON, M. R. J. 1953. Studies of the bacterial cell wall. IV. Composition of the cell walls of some gram-positive and gram-negative bacteria. Biochim. et Biophys. Acta **10**:512–521.

SCHMIDT, W. C. 1960. Type-specific antibody formation in man following injection of streptococcal M protein. J. Infectious Diseases **160**:250–255.

SHOCKMAN, G. D., J. J. KOLB, AND G. TOENNIES. 1957. A high-speed shaker for the disruption of cells at low temperature. Biochim. et Biophys. Acta **24**:203–205.

ZITTLE, C. A. 1942. The antigenic structure of hemolytic streptococci of Lancefield group A. VII. Separation of the protein and nucleic acid of the type-specific M substance and some chemical and serological properties of the purified type-specific protein. J. Immunol. **43**:31–46.