INFECTION AND IMMUNITY, Jan. 1977, p. 104-108

Vol. 15, No. 1
Printed in U.S.A.

Homologous and Heterologous Protection of Mice with Group A Streptococcal M Protein Vaccines

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Received for publication 23 August 1976

Purified streptococcal M proteins precipitated with alum (APM) were used to immunize mice. A trivalent vaccine of serotypes 1, 3, and 12 protected mice against challenges by homologous live streptococci and also conferred protection against serotypes 6 and 14 but not against a strain of group B streptococci. Monovalent APM vaccines afforded homologous protection and restricted heterologous protection. The extent of heterologous protection was a function of serotype combinations and was also dose dependent. Rabbit antisera exhibiting strong opsonic activities were active in vitro and in passive mouse protection only for homologous serotypes. Mouse antisera did not passively transfer protection and were not bactericidal in vitro. It was concluded that homologous and heterologous active mouse protection was most likely a result of shared antigenic determinants of the various M proteins although protection of mice could not be measured as a function of circulating anti-M antibodies.

Immunization with group A streptococcal vaccines of purified M proteins induces type-specific antibodies in experimental animals and humans (3, 12). In vitro assays of these antibodies for opsonic (bactericidal) activity occasionally reveal some degree of heterologous, as well as homologous, anti-group A streptococcal activity. Evidence has accumulated that the extent of cross-reactions among serotypes is, in part, a function of antigenic determinants shared between selected M proteins (7, 19). The significance of this cross-protection is important in the development of M protein vaccines for human use (4, 14). Polyvalent vaccines administered to humans could protect against homologous infections and also afford a degree of protection against heterologous strains sharing antigenic M determinants. The purpose of the experiments presented here is to demonstrate simultaneous homologous and heterologous protection of mice immunized with a trivalent (types 1, 3, and 12) M protein vaccine. Homologous protection of mice with polyvalent vaccines has already been demonstrated (A. S. Armstrong, G. D. Hansen, H. H. Fricke, and J. C. Holper, Bacteriol. Proc., p. 84, 1971), and in vitro cross-reactions by antisera induced by similar vaccines are also documented (19). The present work presents evidence that some measure of heterologous protection of mice is afforded by vaccines that are identical to those already demonstrated as efficacious in human subjects.

MATERIALS AND METHODS

Streptococci and M protein vaccines. Purified M proteins were prepared from mouse virulent strains of group A streptococci. Washed cell walls of 18-h cultures were extracted twice at pH 2.0 and 95°C for 10 min and further purified as described previously (5). The M proteins were precipitated with alum and standardized for protein and aluminum hydroxide content (6).

Group A streptococci, M types 1, 3, 6, 12, and 14, were those described in a previous publication (19); group B *Streptococcus*, type Ia, was kindly supplied by T. Myoda, Dupont Institute, Wilmington, Del.

The trivalent alum-precipitated M protein vaccine of serotypes 1, 3, and 12 (APM-1, 3, 12) was suspended in sterile Ringer lactate buffer. One 0.25-ml dose contained 10 μg of each M protein absorbed onto a total of 250 μg of aluminum hydroxide. The monovalent vaccines (APM-1, etc.) contained either 10 or 30 μg of M protein on 250 μg of aluminum hydroxide, as indicated in the text. Merthiolate, at a concentration of 50 μg/ml, was present in all vaccine preparations. Control mice were injected with a placebo containing only aluminum hydroxide according to the immunization protocol.

Immunization and challenge. Male, Swiss, white outbred mice, 28 days old, weighing 18 to 20 g, and supplied by Carworth Farms (Wilmington, Del.), were injected subcutaneously in the dorsal cervical area with 0.25 ml of vaccine on days 1, 7, and 14; the mice were challenged on day 21 with 0.2 ml of streptococci by intraperitoneal injection. Streptococci for challenge were grown overnight in Todd-Hewitt broth (THB) (Difco Laboratories, Detroit, Mich.) from frozen inocula. One milliliter of the overnight culture was transferred to 10 ml of fresh broth,

grown for 4 h, and washed twice by centrifugation and decantation in cold THB. Serial 10-fold dilutions of suspensions standardized by spectrophotometric absorption (660 nm) were made in THB. Samples were spread on Difco heart infusion agar containing 5% defibrinated sheep blood to enumerate the number of colony-forming units (CFU) per milliliter of challenge culture.

New Zealand white rabbits, approximately 3 months old and weighing 2 to 2.5 kg, were immunized subcutaneously with 0.5 ml of APM vaccines containing, per dose, 100 μg of each M protein combined with 2.5 mg of aluminum hydroxide. Injections were on days 1, 14, and 30; immune sera were collected on day 45. Preimmune serum was collected from each rabbit on day 0.

Assay procedures. Serum was collected from ether-anesthetized mice via bleeding from the retroorbital venous plexus. Rabbit serum was obtained from the marginal ear vein. Passive hemagglutination of erythrocytes sensitized with M protein (2) and the Lancefield indirect bactericidal assay (11) were as previously described. Evaluation of bactericidal indexes was by the method of Fox et al. (8).

Each of five 10-fold serial dilutions of THB cultures was injected into a group of six mice, and the mean lethal dosages (LD_{50}) were determined by the Reed and Muench titration (15).

RESULTS

Six groups consisting of 30 mice/group were immunized with the trivalent APM vaccine containing M proteins, types 1, 3, and 12; an equal number of control mice were injected with the protein-free aluminum hydroxide carrier. The six strains of streptococci listed in Table 1 were used to challenge each group of mice 1 week after the third immunization. Each of five groups of six mice was injected with 10-fold dilutions of the live organisms previously titrated in groups of mice so that the third of six dilutions corresponded approximately to the LD_{50} dose. It may be seen in Table 1 that the trivalent vaccine simultaneously conferred both type-specific and heterologous (types 6 and 14) protection. LD_{50} ratios of immune over control challenges were considered significant at 10 or greater. A highly virulent culture of group B *Streptococcus* with an LD_{50} of 10 or fewer CFU was a control for non-group A streptococcal specificity of the trivalent vaccine. The latter conferred no protection on four groups of six mice receiving between 0.1 and 100 LD_{50} doses of the group B streptococci.

From the above observations it was of interest to determine to what extent each M protein contributed to the range of heterologous protection and whether the total protein in each dosage of vaccine was a factor. Mouse protection experiments with monovalent vaccines were therefore carried out. Groups of mice were immunized with monovalent M protein vaccines containing (per dose) the amount of protein (30 μg) in the trivalent vaccine, and additional groups of mice were immunized with monovalent vaccines containing the amount of M protein (10 μg) equivalent to the dosage of the individual M protein in the trivalent vaccine. Equal numbers of control mice inoculated with the protein-free aluminum hydroxide carrier were challenged along with the immunized mice. Procedures were as described for the trivalent vaccine; i.e., 30 immune mice and 30 control mice were used for each LD_{50} determination.

TABLE 1. *Active protection tests in mice immunized with a trivalent (APM-1, 3, 12) M protein vaccine*[a]

Challenging streptococcus	LD_{50} (CFU/dose)		LD_{50} ratio of immune to control
	Immune mice	Control mice	
Group A, M-1	$1.2 \times 10^{5.2}$	$1.2 \times 10^{4.4}$	19.9
Group A, M-3	$4.2 \times 10^{2.6}$	$4.2 \times 10^{1.6}$	10.0
Group A, M-12	$2.8 \times 10^{3.7}$	$2.8 \times 10^{2.7}$	10.0
Group A, M-6	$1.8 \times 10^{5.5}$	$1.8 \times 10^{4.0}$	31.6
Group A, M-14	$1.0 \times 10^{3.5}$	$1.0 \times 10^{2.2}$	19.9
Group B, type Ia	$<10^{1.0}$	$<10^{1.0}$	1.0

[a] One dose of trivalent APM-1, 3, 12 vaccine consisted of 10 μg of each M protein combined with a total of 250 μg of aluminum hydroxide in a volume of 0.25 ml of buffer. Three subcutaneous injections were given at weekly intervals. Control mice received only aluminum hydroxide in the above schedule.

The results of these protection tests are summarized in Table 2. APM-1 vaccine at 30 μg/dose protected against challenges by types 1, 6, and 14 streptococci but not against types 3 or 12. However, the APM-1 vaccine at 10 μg/dose (the equivalent amount of M-1 protein in the trivalent vaccine) conferred only minimal protection (i.e., less than a 10-fold LD_{50} increment) against homologous or heterologous challenges. Protection tests with monovalent APM doses of 10 μg of protein were omitted when the 30-μg regimen was not significantly protective. This was the case just cited and with four of the five serotypes used to challenge mice immunized with the APM-3 monovalent (30 μg) vaccine. A 30-μg/dose vaccine of APM-12 showed significant protection against challenges by streptococcal serotypes 3, 6, 12, and 14; the 10-μg vaccine induced type-specific protection but little if any protection versus types 3, 6, or 14 streptococci.

Table 3 summarizes the results of the active mouse protection experiments. No single vaccine serotype conferred protection to all of the heterologous strains. Protection at these dosages was frequently nonreciprocal; i.e., M-1 vaccine protected against type 12, but M-12 vaccine did not protect against a type 1 chal-

TABLE 2. *Active protection tests in mice immunized with monovalent vaccines and effect of dosage on heterologous challenge*

Vaccine[a] (μg/dose)	Group A streptococcus challenge serotype	LD_{50} (CFU/dose)		LD_{50} ratio of immune to control
		Immune	Control	
APM-1, 30	1	$2.6 \times 10^{4.7}$	$2.6 \times 10^{3.4}$	19.9
	3	$1.5 \times 10^{1.0}$	$1.5 \times 10^{1.1}$	~1.0
	6	$4.8 \times 10^{5.0}$	$4.8 \times 10^{3.4}$	39.8
	12	$3.0 \times 10^{2.0}$	$3.0 \times 10^{2.2}$	~1.0
	14	$3.6 \times 10^{5.4}$	$3.6 \times 10^{3.6}$	63.1
APM-1, 10	1	$3.2 \times 10^{4.1}$	$3.2 \times 10^{3.5}$	4.0
	6	$4.8 \times 10^{3.9}$	$4.8 \times 10^{3.4}$	3.2
	14	$2.6 \times 10^{2.4}$	$2.6 \times 10^{1.6}$	6.3
AMP-3, 30	1	$2.6 \times 10^{3.4}$	$2.6 \times 10^{3.0}$	1.0
	3	$2.4 \times 10^{3.5}$	$2.4 \times 10^{1.0}$	316.0
	6	$2.1 \times 10^{1.8}$	$2.1 \times 10^{1.5}$	2.0
	12	$2.2 \times 10^{3.7}$	$2.2 \times 10^{3.0}$	5.0
	14	$4.6 \times 10^{2.3}$	$4.6 \times 10^{2.2}$	1.3
APM-12, 30	1	$1.6 \times 10^{5.0}$	$1.6 \times 10^{5.0}$	1.0
	3	$3.6 \times 10^{5.4}$	$3.6 \times 10^{3.6}$	63.1
	6	$1.8 \times 10^{5.5}$	$1.8 \times 10^{4.0}$	31.6
	12	$2.4 \times 10^{4.8}$	$2.4 \times 10^{2.8}$	100.0
	14	$1.7 \times 10^{2.5}$	$1.7 \times 10^{0.8}$	50.1
APM-12, 10	3	$2.4 \times 10^{2.6}$	$2.4 \times 10^{2.2}$	2.5
	6	$1.8 \times 10^{4.8}$	$1.8 \times 10^{4.0}$	6.3
	12	$1.0 \times 10^{4.0}$	$1.0 \times 10^{2.0}$	100.0
	14	$1.8 \times 10^{2.0}$	$1.8 \times 10^{1.6}$	2.5

[a] The schedule of injections was as noted in the footnote to Table 1.

TABLE 3. *Summary of homologous and heterologous protection conferred by monovalent and trivalent APM vaccines*

APM vaccine serotype	Protection[a] against streptococcal serotype challenge				
	1	3	12	6	14
1 (30 μg)	+	−	+	−	+
3 (30 μg)	−	+	−	−	−
12 (30 μg)	−	+	+	+	+
Trivalent (10 μg each)	+	+	+	+	+

[a] +, LD_{50} ratio of immune to control >10-fold.

lenge. One could infer from the data in Table 3 that a divalent vaccine of M proteins 1 and 12, each at protein concentrations between 15 and 20 μg, would have accomplished the same results with the streptococcal serotypes selected for these experiments.

The role of circulating antibody in protection was investigated via passive mouse protection tests. Six rabbits were immunized with three doses of the trivalent APM vaccine at concentrations 10-fold greater than doses used for mouse immunization. All six rabbits produced precipitating antibodies to the three soluble M proteins, which were observed by Ouchterlony double diffusion. Anti-M titers measured by passive hemagglutination were also recorded (Table 4). In vitro bactericidal activities of the six immune sera were assayed, and it may be seen that homologous, but no heterologous, activity was found in some of the sera.

Passive protection of mice was measured with one of the antisera (rabbit no. 5) as well as with pooled mouse sera. The latter were obtained from 10 mice immunized with three doses of the trivalent APM vaccine at dosages shown to afford adequate active protection. Table 5 compares these two lots of antisera. Mice were injected intraperitoneally with 0.2 ml of serum at 6 h prior to challenge. Five groups of six mice each were used to determine the LD_{50} doses of streptococci. The rabbit serum had a passive hemagglutination titer of 1:6,400 and strong anti-12 in vitro bactericidal activity and induced protection at an LD_{50} ratio of immune to control of 25. On the other hand, the pooled mouse sera had no in vitro bactericidal activity and did not protect the mice against a type 12 streptococcal challenge. It was therefore concluded that humoral antibody in mice, as measured under these circumstances, was not a

TABLE 4. *Homologous and heterologous antibody activities of rabbit sera after trivalent (APM-1, 3, 12) M protein immunization*

Rabbit no.	Passive hemagglutination titer (reciprocal × 10^{-2}) of erythrocytes sensitized with M types:					Bactericidal index vs. streptococcal M serotypes				
	1	3	12	6	14	1	3	12	6	14
1	1	4	4	0	1	1	0	1	0	0
2	4	4	32	1	4	1	2	1	0	0
3	16	64	128	8	32	4	2	4	0	0
4	16	16	32	2	8	4	2	4	0	0
5	8	32	64	8	8	3	3	4	0	0
6	64	64	64	32	32	4	1	4	0	0

TABLE 5. *Passive mouse protection comparing rabbit to mouse antisera versus the trivalent APM vaccine*

Immune serum	LD_{50} vs. type 12 streptococci of passive immunization with:		LD_{50} ratio of immune to preimmune
	Immune sera	Preimmune sera	
Rabbit (no. 5) anti-APM-1, 3, 12	$3.6 \times 10^{4.7}$	$3.6 \times 10^{3.3}$	25.1
Pooled mouse anti-APM-1, 3, 12	$3.4 \times 10^{4.0}$	$3.4 \times 10^{3.9}$[a]	1.1

[a] Preimmune mouse sera were pooled from 10 normal mice sacrificed at the time of bleeding.

significant factor in the mechanism of protection afforded by the vaccine.

DISCUSSION

In 1939 Hirst and Lancefield (10) immunized mice with crude acid extracts of group A streptococci. These M antigen extracts induced type-specific protection and some degree of heterologous protection also. The authors attributed the nonspecific protection to "other antigens in the immunizing extracts having a broader specificity than the type-specific substance or that there are chemical and antigenic relationships among the type-specific substances themselves."

A review of current investigations indicate that the latter speculation of Hirst and Lancefield is more likely (3). Since those early studies, no protective protein antigens other than the M proteins have been demonstrated in group A streptococci. Multiple M protein serotypes present in single strains have been found, but occurrences of multiple serotypes are in all likelihood rare events. For example, Wiley and Wilson (18) found that a few strains of type 14 contained a second M protein, at that time serologically unclassified, which they gave the new designation type 51. Harrell et al. (9) also described a type 46 strain that contained a type 51 M antigen. Recent work from a number of laboratories gives more convincing evidence that M proteins do, in fact, share antigenic determinants between serotypes. Wiley and Bruno (17) prepared rabbit antisera to selected strains of group A streptococci, including types 33, 41, 43, and 52. They demonstrated that, in the indirect bactericidal assay, these antisera, in addition to possessing strong type-specific opsonic activity, also exhibited reciprocal and "one-way" cross-reactivity. Similar data were also reported by Bergner-Rabinowitz et al. (1). Fox and Wittner (7) described shared antigenic determinants between M types 3 and 12 as evidenced by spurred precipitin lines with purified M proteins reacted in immunodiffusion with either of the type-specific rabbit antisera. In vitro, the sera were only homologously bactericidal; however, more recently Wittner (19) observed a wider range of cross-reactions among a number of serotypes by demonstrating the ability of many heterologous M proteins or whole streptococcal cells to absorb bactericidal antibodies from rabbit anti-M sera.

Within the relatively short-term immunization period prior to challenge in the present experiments, actively induced circulating antibody was negligible although the mice were protected against homologous and heterologous streptococci. Type-specific, as well as extratypic, protection appears to be a function of the antigenic determinants distributed among the M proteins in the trivalent vaccine. Nonspecific immunity is a less likely explanation in view of the limited ranges of protection afforded by each of the serotypes in the monovalent vaccines. Moreover, there was no evidence of induced nonspecific resistance when immunized mice were challenged with group B streptococci. Michael and Massell (13) studied streptococcal immunity in mice and observed that in the short term (48 h), nonspecific resistance

could be induced with cell walls, but not with M protein. These observations corroborated the earlier work of Rotta et al. (16), who enhanced nonspecific active resistance in mice with streptococcal cell wall mucopeptide. The present experiments appear to be the first demonstration of immunity in mice to selected heterologous streptococci, induced by purified M protein vaccines.

During the past 10 years this laboratory has been engaged in experimental clinical studies on the production of type-specific immunity in humans (4, 8, 14). Purified M proteins combined with aluminum hydroxide were administered subcutaneously, and soluble M protein solutions were applied topically to the nasopharynx. Both routes of administration were effective in producing immunity to subsequent infection although protection was not necessarily correlated with the induction of circulating antibody. Although subjects who developed opsonic antibody resulting from the vaccine were in most cases protected, many subjects without significant titers of bactericidal antibody were also protected. Although no alternative mechanisms of protection in mice or humans have been shown in either the present or previous studies, one must consider the possibilities of the mouse model for further studies. In devising a polyvalent streptococcal M protein vaccine for human use, selected serotypes of upper respiratory strains must be considered with respect to the endemic distribution of these streptococci in the population in question. The current studies indicate that a judicious selection of serotypes for inclusion in a polyvalent vaccine could possibly provide protection extended beyond the range of the specific serotypes comprising the vaccine. Continued studies similar to those presented here could delineate combinations of M protein serotypes capable of extending the protection of polyvalent vaccines beyond the range of the serotypes from which the vaccines are prepared.

ACKNOWLEDGMENTS

We thank Peggy Kelly and Sophia Fuerst for their excellent technical assistance.

This work was supported by Public Health Service grant AI-04342 from the National Institute of Allergy and Infectious Diseases.

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