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THE ANTIGENIC STRUCTURE OF HEMOLYTIC STREPTOCOCCI OF LANCEFIELD GROUP A

XII. Antigenicity of the Type-specific M-protein[1]

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The chemical and physical properties of the type-specific M-protein have been extensively studied (9, 12–15). The studies presented in this paper are concerned with the antigenicity of this protein. Hirst and Lancefield (5) showed that the type-specific substance is the antigen primarily involved in the protection of animals against live streptococci. The isolation of a purified antigenic substance which would protect against subsequent streptococcal infection might be of value to preventive medicine, even if the protection were only type-specific. The antigenicity of the M-protein has been investigated with preparations from streptococcal cultures at different stages of growth, different temperatures of extraction and after adsorption of the protein on aluminium hydroxide. In these studies active immunization of mice, production of mouse-protective anti-M sera in the rabbit, and absorption of mouse-protective anti-streptococcal rabbit sera with the M-substance were used.

METHODS

Strains of hemolytic streptococci of Lancefield's group A used:

1. 1048, mucoid colony form (8)
2. 1685, mucoid colony form (8)
3. D 58, type 3, Colebrook's strain Richards (5)

The question as to which type strains 1048 and 1685 should be assigned is not settled. Strain 1048 was originally classified as type 6 in Dr. F. Griffith's laboratory (8). Dr. R. C. Lancefield and Dr. F. H. Fraser kindly studied strain 1048 and were unable to classify it according to any type of Griffith. Dr. Alice C. Evans kindly allows us to report the following experience: "For the third time I have carried out the agglutination tests with strain 1048. In the last test it agglutinated in serum of type 30, as well as in serum of type 23. This last test was done on a culture from which inoculations were made for a cross protection test. It was highly virulent, killing all 12 mice including those receiving the 10^{-8} dilutions. The serums of types 6, 15 and 19 protected the mice against strain 1048 fully as well as they protect their homologous strains (only one of 12 mice died in each of the three tests). On the other hand, serum of type 23 did not give full protection against this strain. Apparently strain 1048 is unstable in its agglutinin reactions, and in view of the fact that serum of type 6 gives complete protection against this strain, it is quite likely that it may have given complete agglutination in type 6 serum under some circumstances of its history."

In reporting the type of the 1685 strain, Dr. Griffith observed that in addition to colonies of type 1, colonies were obtained which reacted with both type 1 and type 3 antisera. He had not yet decided whether the reaction with type 3 antiserum was a group reaction. Dr.

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Lancefield put strain 1685 into type 3; agglutination with type 1 antisera did not occur. Likewise the isolated M-protein from 1685 reacted type-specifically with Dr. Lancefield's type 3 antiserum. Dr. Lancefield kindly supplied us with type 3 antiserum prepared with strain D 58. The mouse-protective tests performed with this serum, our 1685 serum and 1685 and D 58 cultures seemed to support the assertion that strain 1685 belongs in type 3 as far as mouse-protection is concerned. Recent typing kindly performed for us by Dr. F. H. Fraser, classed strain 1685 by slide agglutination in type 1. The reactions were slow and weak. Dr. Alice C. Evans (1) has studied strain 1685. She reports: "In cross protection tests carried out with strain 1685 against serums of six Griffith types which give protection in a broad range of types, the reaction of these serums with strain 1685 is quite different from their reaction with "SF 130," which is Griffith's type 1 strain, although my strain 1685 agglutinates to full titer in type 1 serum, prepared with "SF 130," and in no other serum of the Griffith types."

It may be recalled in this connection that Lancefield (6) discovered that strain C 203 contains both type 1 and type 3 T antigens, but M only of type 3.

Active immunization

a. *Immunization of mice and test of immunity.* White mice, weighing 20 to 23 grams, were injected intraperitoneally three times a week for four weeks with increasing doses of antigen. After a rest-period of one week, the test dose which consisted of an adequate dilution of an 18 hour blood broth culture in 0.5 ml of sterile broth was injected. For 1 MLD we chose the dilution which in preliminary mouse-titration killed all the mice in 24 hours. After the administration of the living organisms the mice were observed for 96 hours and those living at the end of this period were recorded as surviving.

Owing to environmental circumstances, a number of mice in each experiment were lost during the period of immunization. Only the animals which received the test dose of living organisms are here recorded.

Because most of the solutions of M substance used for vaccine were made up in M/20 phosphate buffer of pH 7.0, we inoculated our normal control mice with phosphate buffer throughout the immunization period of four weeks.

b. *Rabbit immunization with whole organisms and test of immunity.* Gray chinchilla rabbits, five pounds and over in weight, were injected with increasing amounts of heat-killed streptococci. The vaccine was prepared by centrifugation of 18 hour streptococcal cultures and resuspension of the sediment in sterile 0.85 per cent salt solution. The turbidity of the vaccine had to be adjusted according to the virulence of the strain for rabbits. The organisms were killed by heating to 60 C for 30 minutes. The rabbits were injected for five weeks with this vaccine; after this period seven injections of living vaccine prepared in the same manner were administered.

c. *Rabbit immunization with M-protein.*[2] We followed Lancefield's schedule of immunization (5). Increasing amounts of protein were given five times a week for five weeks until a total of 125 mg per rabbit had been injected. The animals were allowed to rest for one month after which the inoculations were continued during seven additional weeks.

Passive protection

White mice, weighing 20 to 23 gm, were injected intraabdominally with 0.5 ml of the serum under test. Twenty-four hours after the injection, the test dose, consisting of an adequate dilution of an 18 hour blood broth culture in 0.5 ml of sterile broth, was injected. The mice were observed for 96 hours and the animals living at the end of this period were recorded as surviving.

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[2] The term, M-substance, is retained for the material obtained from neutralized and dialyzed HCl-extracts by precipitation with acid, for historical reasons and also because to some degree it does represent a definite compound of protein and nucleic acid in salt-like union. The purified active proteic component of the M-substance is designated the M-protein.

Absorption technic for passive protection tests. In the experiments involving the use of absorbed sera we tried to remove the protective antibodies from anti-streptococcal rabbit sera, which were known to protect mice against streptococcal infection, by absorption with M-protein. We used for absorption dilutions of M in an amount corresponding to the optimal antigen-antibody proportion as found for another streptococcal antiserum on the basis of quantitative determinations of precipitin (13) and checked for this serum on the basis of the precipitative reaction.

Plate counts were performed on most of the test doses of living organisms. Usually three different dilutions were used and three plates were poured from each dilution, incubated overnight and the colonies counted and averaged. We found the average number of organisms to remain fairly constant for the same dilutions of the test doses.

Antigens

a. *Whole organism vaccine.* 18 hour streptococcal cultures were centrifuged, washed twice in sterile 0.85 per cent NaCl solution and the nitrogen content determined. The nitrogen content per ml multiplied by 7.4 gave the mg of whole organisms per ml, based on the nitrogen content of dried streptococci as found by Sevag, Smolens and Lackman (11).

b. *Crude M-substance.* The M-substance was extracted as described in previous papers (12, 13) from undried whole organisms from 4 hour or 18 hour cultures with N/20 HCl in 0.85 per cent NaCl at 37 C and 56 C.

c. *Purification of the M-protein.* The purification of the M-protein by precipitation with neutral $(NH_4)_2SO_4$ has been described (13). However a simpler and probably more specific method is the following: one gram of crude M substance is suspended in 25 ml of water and 58 ml of 95 per cent ethyl alcohol and 1 ml of N HCl added. The reaction will be acid to Congo red paper. The suspension is warmed to 56 C, thoroughly shaken, and all lumps broken up with a spatula. After 5 to 10 minutes the suspension is centrifuged at high speed. The M-protein is precipitated from the slightly opalescent supernate by the *careful* addition, (the M-protein readily goes into solution again when the isoelectric point is passed), of N and finally N/10 NaOH. Precipitation in the form of gelatinous floccules occurs at pH 5.2 to 5.5. The precipitate is dried with alcohol and ether. The yields of M-protein range from 10 to 20 per cent with a phosphorus content of 0.4 to 0.7 per cent (starting material contains about 4.5 per cent phosphorus). The phosphorus content, which represents nucleic acid (12), can be reduced further if desired by precipitation with neutral $(NH_4)_2SO_4$ (13). For serological studies it is significant that treatment with acid-alcohol removes extraneous protein material, since the M-protein is unusual in being soluble in acid-alcohol and at the same time retaining such serological properties as the capacity to precipitate with streptococcal antisera and, as will be shown in this paper, to protect mice against streptococcal infection. The gelatinous appearance of the precipitates from acid-alcohol was similar to that of the most purified preparations of the M-protein (13, 14). Lancefield had originally pointed out the solubility of the M-protein in acid-alcohol (7).

d. *Aluminium hydroxide precipitation of the M extract.* These experiments were undertaken in order to decrease the rate of absorption and were prepared as described by Seibert (10).

EXPERIMENTAL

I. Vaccine composed of whole organisms

In one of our experiments we used 18 hour heat-killed organisms in order to obtain a standard of protection. All the vaccinated animals survived. This result led us to an experiment designed to test the solidity and specificity of this immunity. In table 1 the results of the immunization of mice with intact heat-killed streptococci from 18 hour cultures and their resistance to different amounts of the living blood broth culture are recorded.

The immunity obtained with this vaccine shows clearly that the protecting substance is present in an 18 hour culture and that a large number of animals are protected against 100 MLD.

The strain 1685 M-protein has shown a strong serological cross-reaction with rabbit sera prepared against 1048 whole organisms (13). The precipitation of 1048 M-protein with 1685 antisera has also been observed. Evidence has been presented (13) that the cross-reactivity is a property of the M-protein and is not due to contamination. The same cross-reaction as observed with rabbit sera vs. whole organisms was observed with rabbit sera against purified 1048 M-protein, as recorded in this paper. The question whether this cross-reaction was associated with cross-protection presented itself. The results of a group of mice immunized with heat-killed 18 hour organisms of

TABLE 1

Immunity conferred on mice by injections of whole heat-killed organisms against test doses of homologous and heterologous type organisms

INJECTED WITH	AMOUNT OF STREPTOCOCCI USED—MG PER MOUSE	NUMBER OF MICE USED	TEST DOSE OF				NUMBER OF MICE SURVIVING AT END OF			PER CENT SURVIVAL	REAL SURVIVAL (TEST GROUP LESS CONTROL GROUP), PER CENT
			MLD	1048M ml of 18 hr blood broth culture	MLD	1685M ml of 18 hr blood broth culture	24 hr	46 hr	96 hr		
1048 M heat-killed organisms.....	22.0	9	100	10^{-4}			9	9	7	78	78
Normal control animals.....		10	100	10^{-4}			0			0	
1048 M heat-killed organisms.....	22.0	9	10	10^{-5}			9	8	7	78	78
Normal control animals.....		10	10	10^{-5}			0			0	
1048 M heat-killed organisms.....	22.0	14			1	10^{-5}	13	10	2	14	7
Normal control animals.....		14			1	10^{-5}	14*	2	1	7	

Period of Immunization: 4 weeks.
* Sick.

strain 1048 and tested with 1 MLD of strain 1685 of heterologous type are given in table 1. Although immunization with the heterologous type seemed to delay somewhat the death of the animals, only 7 percent of this group survived—a small number compared with the mice which lived when homologous organisms were employed.

II. Comparison of M-substance extracted at 37 C and 56 C by active immunization of mice and test of immunity

Our experiments indicated that the protective substance was present in 18 hour cultures. This suggested that it was not the age of the culture in the Hirst-Lancefield (5) preparation of M-substance (extraction of 4 hour cultures at 37 C), which was responsible for the good protection. The question presented itself whether the temperature at which the extraction was performed had influenced

the antigenicity of the extract. An experiment was carried out to compare the protective value of a preparation of crude M-substance extracted from 18 hour cultures of strain 1048 at 56 C and of 18 hour cultures of the same strain extracted at 37 C. The results are recorded in table 2.

The M-substance extracted at 37 C protected 55 per cent of the animals as compared to none in the normal control group. Immunization experiments with M-protein extracted from 18 hour cultures at 56 C and M-substance extracted from 18 hour cultures at 37 C were run in parallel. According to the results recorded in table 2, it seems that the antigenicity of the M extract is about the same irrespective of which temperature is employed for extraction.

TABLE 2

Comparison of the protective value of M-extracts from 18 hour cultures of strain 1048 at 56 C and 37 C

INJECTED WITH	AMOUNT OF ANTIGEN USED—MG PER MOUSE	NUMBER OF MICE USED	NUMBER OF MICE SURVIVING AT END OF 96 HOURS	PER CENT SURVIVAL
1048 M extracted from 18 hr cultures at 56 C.....	5.4	15	10	67
1048 M extracted from 18 hr cultures at 37 C.....	5.5	17	8	47
1048 M extracted from 18 hr cultures at 37 C.....	5.8	20	11	55
Phosphate buffer solution pH 7.0.....		20	0	0
1048 M heat-killed organisms.....	28	16	13	81
Normal control animals.....		10	0	0

Period of Immunization: 4 weeks.

Test dose: 10^{-6} ml of an 18 hour blood broth culture of homologous 1048 organisms (1 MLD).

Preparation of M-protein used: M-protein extracted from 18 hour cultures at 56 C. was purified by alcohol precipitation; it contained 0.7 P.

M extracts from 18 hour cultures at 37 C were crude preparations.

III. Experiment with M-substance extracted from 18 hour cultures at 56 C adsorbed on aluminium hydroxide

We attempted to enhance the antigenicity of the M-protein by adsorbing it on aluminium hydroxide; since we felt that by delaying the absorption of the antigenic material the formation of antibody would be rendered more effective (2–4, 10). Seibert (10), for example, was able to increase the activity of her tuberculin fractions considerably by this method.

Three groups of mice were inoculated with different amounts of the crude M-substance extracted from 18 hour organisms at 56 C and adsorbed on aluminium hydroxide. Our standard of protection consisted in the survival of all the animals in a group of mice immunized with whole heat-killed 18 hour organisms and inoculated with the test dose of living cocci. The results are in agreement with those of all our experiments since they indicate that whole heat-killed organisms give considerably better protection than the purified type-specific protein No superiority of the antigen precipitated by aluminium hydroxide

over the antigen in solution was revealed. It must be noted, however, that a single experiment with adsorbed extracts was performed. The discouraging result led us to abandon this method.

IV. Immunization of rabbits with M-protein

Two rabbits, #90 and #91, were injected five times a week with a total of 127 mg of M-protein obtained from 18 hour cultures of strain 1048 extracted at 56 C. Following a rest-period of one week after the last injection the rabbits were bled. Neither of the sera contained precipitins against the M-protein. After one month's rest these two rabbits and another rabbit (#88) were injected with 1048 M-protein extracted at 37 C from 18 hour cultures of strain 1048. The injections were given daily five times a week over a period of seven weeks. An intervening rest-period of three weeks was allowed during the

TABLE 3

Protection of mice with anti-M rabbit sera

SERUM	TEST STRAIN	TEST DOSE	TOTAL NUMBER OF MICE TESTED	TOTAL NUMBER OF SURVIVORS	PER CENT SURVIVAL
		ml			
Rabbit serum #88, anti-1048 M.	1048	10^{-6}	12	9	75
	1685	10^{-6}	6	0	0
Rabbit serum #91, anti-1048 M.	1048	10^{-6}	12	6	50
Rabbit serum #90, anti-1048 M.	1048	10^{-6}	6	0	0
Normal rabbit serum	1048	10^{-6}	12	1	8.3
	1685	10^{-6}	6	1	16

The test dose of heterologous type (1685) organisms consisted approximately of 400 organisms per mouse.

The test dose of homologous type (1048) organisms consisted approximately of 600 organisms per mouse.

course of immunization. Each rabbit received a total of 123 mg of M-protein during this second course of injections. Sera obtained from the test-bleedings of the rabbits gave no precipitative reaction with 1048 M-protein in the case of rabbits #90 and #91; the serum of rabbit #88 gave precipitation with 1048 M-protein and 1685 M-protein.

This serum from rabbit #88 behaved like that prepared by the injection of whole organisms of strain 1048, in showing the prozone with the homologous and the characteristic cross reaction (13) with the heterologous 1685 M-protein. aera from rabbits #88 and #91 were tested for complement fixing antibodies Sgainst homologous 1048 M-protein. The former possessed complement fixing antibodies, while the latter did not.

V. Protection of mice by anti-M rabbit sera

The anti-M rabbit sera were tested for their content of mouse protective antibodies. The results given in table 3 represent a summary of two experiments

performed at different times. Of a group of animals injected with serum from rabbit #88 75 per cent were protected against subsequent infection with 1 MLD of strain 1048 (homologous). This protection compares favorably with that conferred by whole organism-rabbit serum (table 4). Because the serum contained precipitative antibodies against homologous and heterologous (1685) M-protein, we tested it for protective antibody against the heterologous type. This, however, was not demonstrated.

Of the two sera of rabbits #90 and #91, both of which did not contain precipitative antibodies against the homologous M-protein, #91 protected 50 per cent of a group of animals tested, while #90 conferred no protection at all.

TABLE 4

Absorption of protective antibodies from homologous antiserum with 1048 M-protein

TEST SYSTEM	TEST DOSE	TOTAL NUMBER OF MICE TESTED	TOTAL NUMBER OF SURVIVORS	PER CENT SURVIVAL
	ml			
Virulence control	10^{-6}	24	2	8
	10^{-5}	20	0	0
	10^{-4}	4	0	0
1048 streptococcal anti-serum	10^{-6}	29	22	76
	10^{-5}	20	18	80
	10^{-4}	4	0	0
1048 anti-serum absorbed with 1048 M-protein	10^{-6}	20	3	15
	10^{-5}	20	4	20
	10^{-4}	4	0	0

For absorption of the protective antibodies a 1:5,000 dilution of the 1048 M-protein in M/20 phosphate buffer, pH 7.0 was added to an equal amount of anti-streptococcal rabbit serum, giving a final antigen-dilution of 1:10,000.

The test dose is given in dilutions of an 18 hour blood broth culture of homologous type (1048) organisms.

According to plate counts the average number of streptococci contained in 10^{-6} ml of 1048 was 600.

It can be observed from these experiments that it is difficult to prepare serologically reactive antisera against the M-protein in the rabbit. If the attempt, however, is successful, the serum is about equivalent in protection to anti-streptococcal rabbit serum.

VI. Absorption of protective antibodies from homologous antiserum with the M-protein

The serum used for protection was a pool of 1048 whole organism-rabbit serum which reacted with homologous M-protein up to a final antigen-dilution of 1:100,000; the optimal precipitation seemed to occur at a final antigen-dilution of 1:10,000. This is approximately the same as required for another serum on the basis of quantitative determination of precipitin (13). For the standard of

protection, in these experiments, equal amounts of the anti-streptococcal rabbit serum and 0.85 per cent NaCl were incubated at 37 C for 20 minutes and left in the refrigerator for 16 hours. Of this mixture 1 ml was injected 24 hours before the culture. For the negative control untreated stock mice were used in some experiments. In others, equal amounts of normal rabbit serum and 0.85 per cent NaCl solution were incubated as indicated above and 1 ml injected 24 hours before the culture. The results represent a summary of these experiments performed at different times. The preparation of the M-protein used for absorption was extracted from 18 hour organisms of strain 1048 at 56 C and purified by precipitation with neutral $(NH_4)_2SO_4$. From table 4 it is evident that the 1048 M-protein in the dilution employed removed effectively the protective antibodies from the antiserum. An antiserum which in amounts of 0.5 ml protected a mouse against 10 MLD of streptococci no longer protected against 1 MLD. The fact that fewer deaths occurred among the mice which received 10^{-5} ml than took place among those receiving 10^{-6} ml may have been due to the difference in weight of the animals. The weight of the mice was specified to be 21 to 22 g but they were not always within this limit.

Similar experiments with the 1685 M-protein were not as successful. The unabsorbed antiserum was not nearly as potent as the 1048 antiserum and the preparation of M-protein from strain 1685 was not highly purified (13). It showed, however, typical serological reaction i.e. cross reaction with 1048 antiserum and typical behavior with homologous 1685 antiserum. The preparation was type-specific for type 3.

DISCUSSION

Our experiments with 18 hour heat-killed streptococci indicate that the presence of the protective substance is not restricted to organisms in the early stages of growth.

The intact streptococcal cell has proven to be of much higher antigenicity than the isolated M-protein. The adsorption of the M-protein on aluminium hydroxide was an attempt to simulate a simplified streptococcal cell. The results were, however, discouraging.

The change of the temperature of extraction from 56 C to 37 C did not seem to have any influence on the antigenicity of the protein.

The preparation of anti-M-protein rabbit sera and the subsequent mouse-protection confirms that this protein is antigenic and concerned with the stimulation of protective antibodies (5, 7). The latter is confirmed by the absorption of mouse-protecting rabbit sera with M-protein which rendered them ineffective for protection.

SUMMARY

The studies described lead to the conclusion that the M-protein is definitely antigenic even though subjected to drastic methods of isolation and purification. The isolated M-protein is not nearly as antigenic, however, as intact streptococci which are believed to contain a fiftieth or less of their weight as M-protein.

REFERENCES

(1) EVANS, ALICE C.: Personal communication.
(2) GLENNY, A. T., AND WADDINGTON, H. 1928 The immunity index method of testing antigenic values. J. Path. and Bact., **31,** 403–421.
(3) GLENNY, A. T., POPE, C. G., WADDINGTON, H., AND WALLACE, U. 1926 Immunological notes. XXIII. The antigenic value of toxoid precipitated by potassium alum. J. Path. and Bact., **29,** 38–39.
(4) HEIDELBERGER, M., KENDALL, F. E., AND SOO HOO, C. M. 1933 Quantitative studies on the precipitin reaction. Antibody production in rabbits injected with an azoprotein. J. Exp. Med., **58,** 137–152.
(5) HIRST, G. K., AND LANCEFIELD, R. C. 1939 Antigenic properties of the type-specific substance derived from Group A hemolytic streptococci. J. Exp. Med., **69,** 425–445.
(6) LANCEFIELD, R. C. 1940 The significance of M and T antigens in the cross reactions between certain types of Group A hemolytic streptococci. J. Exp. Med., **71,** 539–550.
(7) LANCEFIELD, R. C. 1941 Specific relationship of cell composition to biological activity of hemolytic streptococci. The Harvey Lectures, Series XXXVI, 251–290.
(8) MUDD, S., AND LACKMAN, D. B. 1940 The antigenic structure of hemolytic streptococci of Lancefield Group A. VI. The immunological behavior of the nucleoprotein extracted at neutrality. J. Immunol., **39,** 495–510.
(9) PAPPENHEIMER, A. M. JR., WILLIAMS, J. W., AND ZITTLE, C. A. 1942 The antigenic structure of hemolytic streptococci of Lancefield Group A. IX. Some physical properties of the M-protein. J. Immunol., **43,** 61–63.
(10) SEIBERT, F. B. 1935 The chemical composition of the active principle of tuberculin. XIX. Differences in the antigenic properties of various tuberculin fractions; adsorption to aluminium hydroxide and charcoal. J. Immunol., **28,** 425–432.
(11) SEVAG, M. G., SMOLENS, J., AND LACKMAN, D. B. 1940 The nucleic acid content and distribution in Streptococcus pyogenes. J. Biol. Chem., **134,** 523–529.
(12) ZITTLE, C. A. 1939 The antigenic structure of hemolytic streptococci of Lancefield Group A. IV. Nucleoprotein components: some chemical and serological properties, and changes in both caused by certain enzymes. J. Immunol., **37,** 1–16.
(13) 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.
(14) ZITTLE, C. A., AND SEIBERT, F. B. 1942 The antigenic structure of hemolytic streptococci of Lancefield Group A. VIII. Electrophoretic studies of the type-specific M-protein and other isolated fractions. J. Immunol., **43,** 47–59.
(15) ZITTLE, C. A., AND MUDD, S. 1942 Purification and properties of the protein of the "M substance" of Group A hemolytic streptococcus. Ann. New York Academy of Sciences, XLIII, Art. 2, 47–62.