ANTIGENIC DIFFERENCES BETWEEN MATT HEMOLYTIC STREPTOCOCCI AND THEIR GLOSSY VARIANTS.

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In a previous communication (1) it was shown that hemolytic streptococci in the matt form contain the type-specific substance M and that when matt cultures are degraded to the glossy variant form the organisms lose their type-specific substance. The antigenic differences between matt and glossy cultures, which are described in the present paper, were demonstrated with four different strains of hemolytic streptococci.

Owing to individual differences between the four strains it is essential to describe each series of experiments separately.

A. Strain S43.

This strain was isolated from a case of bronchopneumonia and had been kept in stock cultures for 10 years before our work was commenced. In spite of this long interval, however, the culture was entirely composed of matt colonies. Virulence tests proved that 0.1 cc. killed mice regularly in 24 hours but mice receiving 0.01 cc. survived indefinitely.[1]

The culture was, therefore, in the matt attenuated state and efforts were made to obtain the matt virulent form and the glossy variant so

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[1] A standard technique was used in all the virulence tests recorded in this paper. Cultures were sown in tryptic digest broth and incubated for 16 hours. Tenfold dilutions of culture, in infusion broth, were injected intraperitoneally into a series of white mice, each mouse receiving 0.5 cc. of the appropriate dilution. In some cases counts were made by plating dilutions in blood agar and although this was not done as a routine in every test, a sufficient number of observations were made to show that the dose representing 0.000001 cc. of culture usually contained about 100 organisms.

that the three forms of the same strain could be compared and their antigenic relationships established.

The matt virulent form was secured by passing the original culture through a series of mice (0.1 cc. or 0.2 cc. of peritoneal washings being transferred directly from mouse to mouse), and, although there was an initial increase of virulence, after the first few passages, it required 51 passages before 0.000001 cc. of culture could be relied upon to kill mice regularly.

It was even more difficult to obtain the glossy variant from this highly stable matt strain, as prolonged subcultivation on agar slants did not alter the typical

TABLE I.

Precipitin Reactions of Extracts of S43 Matt Virulent, S43 Matt Attenuated and S43 Glossy with the Homologous Pure Anti-M Serum from Which Non-Type-Specific Antibodies Had Been Removed by Absorption with a Heterologous Strain.

Anti-M serum absorbed with heterologous strain*	Volume of extracts†	Precipitates with extracts from		
		S43 matt virulent	S43 matt attenuated	S43 glossy
0.2 cc.	0.3 cc.	+++	+++	−
0.2 cc.	0.1 cc.	++	++	−
0.2 cc.	0.025 cc.	+	+	−

* Serum R166, against Strain S43 matt attenuated.

† These volumes of crude HCl extract were made up to 0.3 cc. with saline.

The tests were incubated 2 hours at 37°C. and read after standing in the ice box overnight.

+++, ++, + represent degrees of precipitate.

− represents no precipitate.

matt appearance of the colonies. The original culture was, therefore, grown in undiluted, high titer anti-M serum, prepared by immunizing rabbits with the matt attenuated form, and after sixteen transfers in this medium the appearance of the colonies seemed to indicate that the culture had been reduced to the glossy state.[2] Table I shows the precipitin reactions of HCl extracts prepared from the three forms of culture with pure anti-M serum previously absorbed with a heterologous strain to remove antibodies to P and C.

Extracts of the matt virulent and of the matt attenuated cultures formed equally heavy precipitates with anti-M serum but a similar extract of the glossy

[2] Strain S43 in the matt state formed typical matt colonies, but colonies of the variant culture were characterized by a heaped up center surrounded by a wide flat margin and they never assumed the typical glossy appearance.

culture did not cause any precipitation.[3] This experiment showed that the variant contained little, if any, type-specific substance but subsequent examination of a concentrated extract proved that the culture was not entirely free from the type-specific fraction. An HCl extract was made from the deposit of 9 liters of culture, it was then concentrated by precipitation with alcohol and redissolved in 5 cc. of saline. This concentrated and purified extract caused some precipitation with the pure anti-M serum showing that the culture still retained traces of type-specific substance. Further attempts to reduce this strain to a completely degraded state were unsuccessful; colony selection was combined with more than 120 transfers in immune serum but even after this treatment traces of type-specific substance could still be detected.

Three kinds of antiserum were prepared by immunizing three groups of rabbits with the three forms of Strain S43. Four rabbits were included in each group and all received equal doses of the appropriate cultures on the same days—the whole series being finally bled on the same day. The immune sera were used for precipitin reactions and for mouse protection tests; in the latter tests, 0.5 cc. of serum was injected into the peritoneal cavity of a series of mice and the degree of passive immunity conferred was tested, on the following day, by inoculating the mice intraperitoneally with graduated doses of virulent culture.

Precipitin Tests.—Table II gives the results of precipitin reactions with the three kinds of sera and purified solutions of the nucleoprotein P, the carbohydrate C and the homologous type-specific substance M.

The non-type-specific fractions P and C precipitated the three kinds of sera to a similar extent though, as usual, the anti-glossy sera contained, in the aggregate, an excess of C antibody. The type-specific substance M, in contrast to the P and C fractions, formed heavy flocculent precipitates with the anti-matt sera but only traces of precipitate with the anti-glossy sera. The appearance of these traces of precipitate confirmed the observation, already noted above, that the S43 glossy form was not completely degraded by showing that it

[3] Crude HCl extracts of glossy cultures when fully degraded, are free from type-specific substance but they contain the non-type-specific fractions P and C and will consequently precipitate *unabsorbed* antibacterial serum prepared against any strain of hemolytic streptococcus. Precipitates due to the C fraction are easily recognized as they appear late and form compact discs quite unlike the flocculent precipitates of the type-specific fraction which begin to appear as soon as the extract comes in contact with the serum. Precipitates due to the nucleoprotein P are less flocculent than those of the type-specific fraction M and appear as a diffuse cloud; the use of absorbed serum is, however, necessary to distinguish between these two forms of precipitate.

TABLE II.

Precipitin Tests on Three Kinds of Sera, Prepared against the Three Forms of Strain S43 (Matt Virulent, Matt Attenuated and Glossy) with Purified Solutions of Nucleoprotein P, Carbohydrate C and Homologous Type-Specific Substance M.

	Volume of extracts and concentration of P*	Sera prepared with matt virulent culture				Sera prepared with matt attenuated culture			Sera prepared with glossy culture			
		A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11
Purified solution of homologous type-specific substance M from S43	0.4 cc.	++	++	+++	++	++	++++±	+±	±	±	±	+
	0.1 cc.	+±	++	++±	++±	++±	+++	++	±	+	±	+
	0.025 cc.	+±	+	++	+	++	+	+±	±	+	±	+
Purified solution of species-specific carbohydrate C	0.4 cc.	++	++	±	++	+	+±	+	+±	+++	+++	++±
	0.1 cc.	++	++	+	++	++	++±	+±	++	++±	+++	++
	0.025 cc.	+	+±	+	+±	+±	+±	±	+±	+±	+±	+
Purified solution of nucleoprotein P	1 in 1,000	+	+	+	+	+	+	±	+	+	+	+
	1 in 4,000	+	+	+	+	+	+	−	+	+	+	±
	1 in 16,000	−	±	±	±	±	+	−	−	−	±	±

* These volumes were made up to 0.4 cc. with saline, and 0.1 cc. of serum was added to each tube.

produced traces of type-specific antibody when used as an antigen for immunizing rabbits.

TABLE III *a*.

Passive Protection of Mice against S43 Matt Virulent by Three Kinds of Sera, Prepared against S43 Matt Virulent, S43 Matt Attenuated and S43 Glossy.

Test doses of virulent culture	Control normal mice	Sera prepared with matt virulent culture				Sera prepared with matt attenuated culture			Sera prepared with glossy culture			
		A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11
0.00001 cc.	†72	S	S	See	S	S	See	S	S	S	†24	S
0.0001 cc.	†24	S	S	Table	S	S	Table	†77	†90	S	†21	†27
0.001 cc.	†21	S	S	III *b*	S	†21	III *b*	S	S	†22	†21	S
0.01 cc.	—	S	S		†22	†21		S	†21	†21	†21	†21
0.1 cc.	—	†21	†29		†24	†21		S	†21	†21	†21	†21

In all tables the following symbols are used.

S indicates that the mice survived for 8 days and were then discarded.

† indicates death of mice.

Numerals indicate number of hours between time of injection and finding mice dead.

— indicates that the test was not done.

The mice received 0.5 cc. of serum intraperitoneally and 24 hours later test doses of virulent culture were injected into the peritoneal cavity.

TABLE III *b*.

Passive Protection of Mice against S60 Matt Virulent (Same Type as S43) by Three Kinds of Serum Prepared against S43 Matt Virulent, S43 Matt Attenuated and S43 Glossy.

Test doses of virulent culture	Control normal mice	Serum prepared with S43 matt virulent culture A3	Serum prepared with S43 matt attenuated culture A6	Serum prepared with S43 glossy culture A11
0.000001 cc.	†45	S	S	S
0.00001 cc.	S	†45	S	†69
0.0001 cc.	†45	S	S	†45
0.001 cc.	†21	S	S	†23
0.01 cc.	†21	S	S	†21
0.1 cc.	†21	†21	†21	S

Passive Protection of Mice.—Table III *a* shows the degree of passive immunity conferred on mice by these sera against a virulent culture

of the homologous strain and Table III *b* shows the protection against a virulent culture of S60, a strain identified by Dochez, Avery and Lancefield (2) as belonging to the same type as S43.[4]

TABLE IV *a*.

Active Immunization of Mice against S43 Matt Virulent by Vaccines Prepared with S43 Matt Virulent, S43 Matt Attenuated and S43 Glossy.

Test doses of virulent S43 culture	Control normal mice	Mice previously inoculated with S43 vaccines*			Controls Mice previously inoculated with heterologous vaccine prepared from S23 matt virulent and with broth	
		Vaccine prepared with matt virulent culture	Vaccine prepared with matt attenuated culture	Vaccine prepared with glossy culture	Vaccine prepared with heterologous matt virulent culture	Inoculated with broth
0.000001 cc.	†31	S	S	†24	†28	†71
0.00001 cc.	†28	S	†29	†23	†23	†27
0.0001 cc.	†29	S	S	†23	†23	†23
0.001 cc.	†23	†80	†23	†23	†23	†23
0.01 cc.	†23	†23	†23	†23	†23	†23
0.1 cc.	†23	S	†47	†23	—	†23

* Vaccines = undiluted 16 hour broth cultures heated at 56°C. for 1 hour.

TABLE IV *b*.

Showing that the Actively Immune Mice Surviving the Test Recorded in Table IVa Were Not Protected against a Matt Virulent Culture of a Heterologous Strain S23.

Test doses of virulent S23 culture	Control normal mice	Mice protected with S43 matt vaccines which had survived test doses of S43 matt virulent
0.000001 cc.	S	†23
0.00001 cc.	†23	†23
0.0001 cc.	†144	†23
0.001 cc.	†23	†23
0.01 cc.	†23	†23
0.1 cc.	†8	†23

The sera prepared against the matt virulent and matt attenuated cultures afforded an equal amount of protection to mice and the

[4] Most of the protection tests recorded in this paper were only done once and no attempt has been made to correct obvious discrepancies due to the natural resistance of individual mice by repeating the tests.

anti-glossy sera also showed some protective power. The small degree of protection conferred on mice by the anti-glossy sera corresponds to the small quantity of type-specific antibody contained in the sera (see Table II) and this coincidence becomes significant when we observe, from the study of strain S23 that serum, prepared with completely degraded glossy cultures which are devoid of type-specific substance, does not contain any type-specific antibody and does not protect mice.

Active Immunization of Mice.—Table IV records the results obtained when mice, actively immunized with the matt virulent, matt attenuated and glossy cultures, were tested for immunity.

Tryptic digest broth cultures of the three forms of Strain S43 were incubated for 16 hours and then killed by heating at 60°C. for an hour. These heat-killed cultures, undiluted and suspended in the broth in which they had grown, were inoculated intraperitoneally into three series of mice. During the 1st week the mice received 0.1 cc. doses on 4 successive days, during the 2nd week four 0.2 cc. doses and during the 3rd week four 0.4 cc. doses. 10 days after the final inoculation the active immunity of the mice was tested by intraperitoneal injection of graduated doses of the matt virulent culture. The controls for this experiment were (1) a series of normal untreated mice, (2) a series of mice inoculated with corresponding doses of tryptic digest broth, (3) a series of mice treated with the matt virulent vaccine of a heterologous strain, S23.

The mice vaccinated with the matt cultures in both the virulent and attenuated forms showed evidence of protection; but mice vaccinated with the glossy form were not protected against infection with the matt virulent cocci.

The specificity of active immunization is also demonstrated by this experiment. Vaccination with a heterologous matt virulent Strain S23 did not afford any protection against Strain S43 and Table IV *b* shows that there was no cross-immunity when protected mice surviving from the first experiment and immune to S43 were tested 10 days later for immunity to S23.

B. Strain S23.

This strain, isolated from a case of bronchopneumonia at the same time as Strain S43, had been used by Andrewes, Derick and Swift (3) as the test organism for protection experiments, and in the course

of their work its virulence had been increased by passage through a number of mice.

Virulence tests showed that 0.000001 cc. of culture killed mice regularly and occasionally 0.0000001 cc. and even 0.00000001 cc. proved a fatal dose.

The colonies had an undulating contour which made observations on the light-reflecting properties of their surfaces difficult and this peculiarity persisted when the strain was converted to the glossy state. Cultures of the virulent form usually contained many pseudo-glossy colonies and, although a large number of plate cultures were examined, it was only rarely that either matt or glossy colonies appeared in typical forms.

Owing to these peculiarities we relied entirely on precipitin tests, by which the quantity of type-specific substance in a culture can be assessed, to judge when this strain was completely degraded to the variant form. The matt virulent strain was cultivated in homologous high titer anti-M serum and after 55 transfers in 50 per cent serum, concentrated extracts of the culture did not precipitate the homologous anti-M serum; and a single mouse passage did not cause the reappearance of type-specific substance. This variant culture, which, measured by the absence of type-specific substance, was completely degraded, was nevertheless partially virulent, 0.01 cc. being the M.L.D. for mice.

It was difficult to obtain a matt attenuated culture as the peculiarities of the strain made selection of colonies impossible. After nine transfers in homologous immune serum, containing only a small amount of type-specific antibody, the virulence of the culture was reduced from 0.000001 cc. to 0.001 cc. although the quantity of type-specific substance, measured by precipitin reactions, remained unchanged. This culture probably represented the matt attenuated form of Strain S23 as it was only slightly more virulent than the glossy variant; but owing to its high virulence, compared with the matt attenuated cultures of other strains, it was not used in our experiments.

Precipitin Tests.—Two series of rabbits were immunized with the matt virulent and glossy forms of Strain S23; Table V shows the precipitin reactions of their sera. It will be seen that all the sera

TABLE V.

Precipitin Tests on Two Kinds of Sera, Prepared against S23 Matt Virulent and S23 Glossy, with Purified Solutions of Nucleoprotein P, Carbohydrate C and Homologous Type-Specific Substance M.

	Volume of extracts and concentration of P	Sera prepared with matt virulent culture					Sera prepared with glossy culture				
		B1	B3	B4	B5	B6	B7	B8	B9	B10	B12
Purified solution of homologous type-specific substance M from S23	0.4 cc.	++	++	++	±	+++	−	−	−	−	−
	0.1 cc.	++±	++±	++±	±	+++	−	−	−	−	−
	0.025 cc.	+±	++	+	+	+	−	−	−	−	−
Purified solution of species-specific carbohydrate C	0.4 cc.	−	+±	−	−	−	+	±	−	±	+
	0.1 cc.	−	++	−	−	−	++	++	++	+	+±
	0.025 cc.	−	++±	−	−	−	++±	++	++	+	++
Purified solution of nucleoprotein P	1 in 1,000	±	±	±	−	±	−	±	++	±	±
	1 in 4,000	−	−	−	−	±	−	−	+±	−	−
	1 in 16,000	−	−	−	−	−	−	−	±	−	−

prepared against the matt virulent culture were precipitated by an extract containing the homologous type-specific substance but the sera prepared against the glossy variant remained perfectly clear

TABLE VI.

Absorption of Anti-Matt and Anti-Glossy Sera with the Homologous Matt and Glossy Cultures. Strain S23.

	Volumes of extracts	Serum B6 prepared with matt virulent culture of Strain S23			Serum B9 prepared with glossy culture of Strain S23		
		Control unabsorbed	Absorbed with S23 matt virulent	Absorbed with S23 glossy	Control unabsorbed	Absorbed with S23 matt virulent	Absorbed with S23 glossy
Purified solution of homologous type-specific substance M from S23	0.4 cc.	++±	−	++±	−	−	−
	0.1 cc.	++±	−	++±	−	−	−
	0.025 cc.	+	−	+	−	−	−
Purified solution of species-specific carbohydrate C	0.4 cc.	−	−	−	−	−	−
	0.1 cc.	−	−	−	+±	−	−
	0.025 cc.	−	−	−	++	−	−

TABLE VII.

Passive Protection of Mice against S23 Matt Virulent by Two Kinds of Serum Prepared against S23 Matt Virulent and S23 Glossy.

Test doses of virulent culture	Control normal mice	Sera prepared with matt virulent culture					Sera prepared with glossy culture				
		B1	B3	B4	B5	B6	B7	B8	B9	B10	B12
0.0000001 cc.	†67	−	−	S	−	S	†30	†21	†31	†23	†29
0.000001 cc.	†23	S	S	S	S	S	†21	†24	†25	†20	†29
0.00001 cc.	†23	†23	†23	S	†23	S	†21	†21	†26	†20	†23
0.0001 cc.	†23	S	S	S	†26	S	†21	†21	†23	†20	†23
0.001 cc.	−	†28	S	†96	S	S	†21	†21	†23	†20	†23
0.01 cc.	−	†21	†21	†27	†21	†72	†21	†21	†23	†20	†23
0.1 cc.	−	†21	†21	†21	†21	S	†21	†21	−	†20	†23

when mixed with the same extract. Both kinds of immune sera were precipitated by the non-type-specific fractions P and C, the anti-glossy sera being as usual more uniformly rich in antibody to the carbohydrate fraction C.

The anti-matt serum (B6) and the anti-glossy serum (B9) were chosen from the two groups of sera for absorption experiments. Both sera were absorbed with homologous matt virulent cocci and also with homologous glossy cocci. Table VI shows the result of this experiment.

The type-specific antibody was completely removed from the anti-matt serum by absorption with the homologous matt culture but it was unaffected by absorption with the homologous glossy culture.

The anti-glossy serum contained no type-specific antibody but its non-type-specific antibody was completely removed by absorption with both forms of the homologous strain.

TABLE VIII.

Active Immunization of Mice against S23 Matt Virulent by Vaccines Prepared with S23 Matt Virulent and S23 Glossy.

Test dose of virulent S23 culture	Control normal mice	Mice previously inoculated with S23 vaccines		Controls Mice previously inoculated with heterologous matt virulent Vaccine S43 and with broth	
		Vaccine prepared with matt virulent culture	Vaccine prepared with glossy culture	Vaccine prepared with heterologous matt virulent culture	Inoculated with broth
0.000001 cc.	S	S	†24	†41	†41
0.00001 cc.	†63	†41	†21	†41	†26
0.0001 cc.	†23	†41	†22	†22	†21
0.001 cc.	†21	†110	†21	†21	†41
0.01 cc.	†21	†17	†17	†17	†17
0.1 cc.	†17	†13	†17	†22	†17

Passive Protection of Mice.—The sera prepared against the two forms of Strain S23 were used for mouse protection experiments with the homologous matt virulent culture as the test organism.

All the sera prepared against the matt virulent form afforded some degree of protection to mice but the anti-glossy sera did not confer any protection.

Active Immunization of Mice.—Two series of mice were inoculated with vaccines prepared from the matt and glossy forms of Strain S23; the dosage and technique were the same as those used in a similar experiment, previously recorded, with Strain S43. Controls con-

sisted of a series of normal mice, a series of mice inoculated with a heterologous matt virulent vaccine and a series of mice inoculated with broth (Table VIII).

In this experiment vaccines prepared from both forms of Strain S23 failed to confer immunity on the mice.

C. Strain C203.

This strain, from the collection of the Laboratories of the New York State Department of Health, was originally isolated from a

TABLE IX.

Precipitin Tests on Two Kinds of Sera, Prepared against C203 Matt Virulent and C203 Glossy with Purified Solutions of Nucleoprotein P, Carbohydrate C and Homologous Type-Specific Substance M.

	Volume of extracts and concentration of P	Sera prepared with matt virulent culture			Sera prepared with glossy culture			
		C1	C2	C3	C4	C5	C6	C7
Purified solution of homologous type-specific substance M from C203	0.4 cc.	+++±	++	++	±	±	−	±
	0.1 cc.	++	+±	+	−	−	±	−
	0.025 cc.	+	+	±	−	−	−	−
Purified solution of species-specific carbohydrate C	0.4 cc.	−	−	−	−	+	−	+±
	0.1 cc.	−	−	−	++	+++±	−	+++
	0.025 cc.	±	−	−	++±	++	−	++±
Purified solution of nucleoprotein P	1 in 1,000	+±	±	+	++	++±	+	++±
	1 in 4,000	+	±	±	+±	+	±	+±
	1 in 16,000	−	−	−	±	±	−	±

case of scarlet fever and had been kept in stock culture for a considerable time before our experiments commenced. It was found to be entirely composed of typical matt colonies; and virulence tests showed that the M.L.D. for mice was 0.0000001 cc. or 0.00000001 cc.

The glossy variant form of this strain was obtained by selection of colonies combined with cultivation in 50 per cent homologous anti-M serum of high titer. After 27 transfers the virulence for mice had fallen from 0.0000001 cc. to 0.1 cc. and the culture formed typical glossy colonies; but traces of type-specific substance could still be

detected in unconcentrated HCl extracts. Continued efforts to secure a completely degraded culture did not cause any further decrease in the quantity of type-specific substance which the organisms contained.

Attempts to reduce this strain to the matt attenuated form were unsuccessful as any reduction in virulence was always accompanied by a partial loss of type-specific substance and by the appearance of atypical matt colonies which tended to assume the characteristics of glossy colonies.

Precipitin Tests.—Rabbits were immunized with the matt virulent and glossy forms of this strain; Table IX gives the precipitin reactions of the immune sera.

In this instance a departure was made from the technique used for immunizing rabbits with the other three strains; the organisms were washed to remove the toxin as the use of unwashed broth cultures of highly toxic strains involved the loss of a relatively large proportion of rabbits during immunization.

The results of precipitin tests were similar to those previously recorded in reference to other strains; the sera prepared with the matt virulent organisms gave good precipitates with the homologous type-specific substance M but only traces of precipitate with the non-type-specific fractions C and P; on the other hand sera prepared with glossy organisms gave only traces of precipitate with the type-specific substance M and comparatively good precipitates with the non-type-specific substances C and P.

The traces of type-specific antibody in the anti-glossy sera were a natural sequel of failure to secure this strain in the completely degraded form.

Passive Protection of Mice.—Table X*a* shows the results of protection tests with the different kinds of sera. All the anti-matt sera showed evidence of protective power and the anti-glossy sera with one exception also protected mice to some extent. This protection by sera prepared against the glossy form of Strain C203 is in harmony with the results of precipitin tests and with the original observation that the culture was not fully degraded but still contained type-specific substance. Table X*b* shows the specificity of protection by the anti-glossy sera. On reference to Table X*a* it will be seen that

TABLE X *a*.

Passive Protection of Mice against C203 Matt Virulent by Two Kinds of Sera Prepared against C203 Matt Virulent and C203 Glossy.

Test doses of virulent culture Strain C203	Control normal mice	Sera prepared with matt virulent culture			Sera prepared with glossy culture				Control mice inoculated with normal rabbit sera	
		C1	C2	C3	C4	C5	C6	C7	N1	N2
0.0000001 cc.	†24	S	S	S	†26	†25	S	S	S	†22
0.000001 cc.	†21	†24	S	†120	†22	S	S	S	†24	†28
0.00001 cc.	†21	S	S	S	†21	†25	S	†21	†21	†26
0.0001 cc.	†21	S	†168	S	†21	†25	†69	†21	†21	†21
0.001 cc.	†21	†24	†21	S	†21	S	†93	†21	†21	†21
0.01 cc.	—	†21	†21	†24	†21	†21	†21	†21	†21	†21

TABLE X *b*.

Specificity of Passive Protection of Mice by Anti-Glossy (C203) Serum.

Test doses of virulent culture Strain S23	Control normal mice	Serum prepared with glossy culture of Strain C203 C6
0.0000001 cc.	†53	†51
0.000001 cc.	†51	†22
0.00001 cc.	†22	†26
0.0001 cc.	†22	†56
0.001 cc.	†22	†22
0.01 cc.	—	†22

TABLE XI.

Active Immunization of Mice against C203 Matt Virulent by Vaccines Prepared with C203 Matt Virulent and C203 Glossy.

Test dose of virulent C203 culture	Control normal mice	Mice previously inoculated with C203 vaccines		Mice previously inoculated with heterologous matt virulent Vaccine S43 and with broth	
		Vaccine prepared from matt virulent culture	Vaccine prepared from glossy culture	Vaccine prepared from heterologous matt virulent culture	Inoculated with broth
0.0000001 cc.	†25	S	—	†40	†40
0.000001 cc.	†21	S	†24	†20	†25
0.00001 cc.	†22	S	†64	†16	†18
0.0001 cc.	†16	S	†20	†21	†16
0.001 cc.	†16	S	†16	†16	†16
0.01 cc.	†16	S	†17	†16	†16
0.1 cc.	†16	†20	†16	†16	†16

Serum C6 was probably the best anti-glossy serum for protection against the homologous matt virulent form of Strain C203; a series of mice was, therefore, injected with this serum and their immunity to a heterologous matt virulent Strain S23 was subsequently tested. Table X*b* shows that Serum C6 afforded no protection against the heterologous Strain S23.

Active Immunization of Mice.—Mice were vaccinated with the different forms of Strain C203 in the manner previously described; and their immunity was tested by inoculating graduated doses of a matt virulent culture of the same strain.

Table XI shows that vaccination with matt virulent organisms produced immunity against subsequent infection by the same strain; but vaccination with homologous glossy organisms, or with heterologous matt virulent organisms did not cause any immunity.

D. Strain London.

This strain, isolated by blood culture from a case of puerperal septicemia, had been kept in stock for about a year before our experiments began; and plate cultures showed that it was composed of a mixture of matt and glossy colonies. A pure matt culture, capable of killing mice in a dose of 0.01 cc., was obtained by selecting a suitable colony and the matt virulent form (M.L.D. 0.000001 cc.) was then prepared by passing this culture through 47 mice.

The matt attenuated culture, which was entirely composed of typical matt colonies indistinguishable from those of the virulent form and failed to kill mice when 0.5 cc. was injected intraperitoneally, was obtained by combining the selection of typical matt colonies with cultivation at 41°C.

The glossy variant was obtained by daily subcultivation of the original strain on agar slants without any selection of colonies or cultivation in immune serum. After 148 transfers on agar the culture was entirely composed of typical glossy colonies, and it was avirulent for mice (M.L.D. 1.0 cc.); but precipitin tests with concentrated HCl extracts of the organisms showed that this culture still retained traces of the type-specific substance M.

Precipitin Tests.—Table XII gives the precipitin reactions of the sera of three sets of rabbits immunized with the three forms of this

TABLE XII.

Precipitin Tests on Three Kinds of Sera, Prepared against the Three Forms of Strain London (Matt Virulent, Matt Attenuated and Glossy) with Purified Solutions of Nucleoprotein P, Carbohydrate C and Homologous Type-Specific Substance M.

	Volume of extracts and concentration of P	Sera prepared with matt virulent culture					Sera prepared with matt attenuated culture						Sera prepared with glossy culture					
		D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16	D17
Purified solution of homologous type-specific substance M	0.4 cc.	++	+±	++	++	++	+±	+	+±	±	+	+	±	−	±	±	−	±
	0.1 cc.	+±	+	+±	+±	++	+	±	+±	±	+	+	−	−	−	−	−	−
	0.025 cc.	+	±	−	±	±	−	−	−	−	−	±	−	−	−	−	−	−
Purified solution of species-specific carbohydrate C	0.4 cc.	±	−	−	−	−	−	++	−	−	−	−	−	+±	−	++	++	−
	0.1 cc.	++	−	−	−	+	−	++	−	−	+±	−	±	++±	++	+++	+++	++±
	0.025 cc.	++±	−	−	+±	++	−	++	−	−	++	++	++	++±	++±	+++	++±	++±
Purified solution of nucleoprotein P	1 in 1,000	+±	+	+±	+	+	+	+	±	±	+	+	+	+	+	+	+	+
	1 in 4,000	+±	+	±	+	++	+	+	±	−	−	+	+	±	±	±	±	±
	1 in 16,000	+	±	−	±	+±	−	±	−	−	−	−	−	−	±	−	−	−

TABLE XIII.

Passive Protection of Mice against Matt Virulent Form of Strain London by Three Kinds of Sera Prepared against the Three Forms of Strain London (Matt Virulent, Matt Attenuated and Glossy).

Test dose of virulent culture	Control normal mice	Sera prepared with matt virulent culture					Sera prepared with matt attenuated culture						Sera prepared with glossy culture						Control mice inoculated with normal rabbit serum
		D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16	D17	
0.0000001 cc.	S	S	S	S	S	S	S	S	S	S	S	S	†69	†69	†45	†45	S	S	†30
0.000001 cc.	S	S	†45	†90	S	S	S	S	S	S	S	S	†90	†45	†30	†21	†29	S	†76
0.00001 cc.	†21	S	S	†52	S	S	S	†90	†90	S	S	S	†55	†51	†31	S	†31	†55	†45
0.0001 cc.	†21	S	S	†31	†45	S	S	†45	S	S	S	†90	†99	†45	†21	†31	†29	†30	†53
0.001 cc.	†21	S	†120	S	S	†140	S	†26	S	S	†160	S	†27	†21	†29	†21	†21	†21	†21
0.01 cc.	†21	S	†140	S	S	†26	†26	†26	S	S	†21	†21	†21	†21	†21	†21	†21	†21	†21

strain; and it will be seen that, as in previous similar experiments, the anti-matt sera were relatively rich in antibody to the type-specific substance M; while the anti-glossy sera had a preponderance of antibody to the non-type-specific fraction C.

Passive Protection of Mice.—Table XIII gives the results of protection tests with the sera prepared against the different forms of Strain London: both kinds of anti-matt sera protected mice against infection with the homologous matt virulent organisms but the anti-glossy sera, although they contained traces of type-specific antibody had little protective power.

TABLE XIV.

Active Immunization of Mice against the Matt Virulent Form of Strain London by Vaccines Prepared from the Three Forms of Strain London (Matt Virulent, Matt Attenuated and Glossy).

Test dose of matt virulent culture of Strain London	Control normal mice	Mice previously inoculated with vaccines prepared from Strain London in the following forms			Mice previously inoculated with heterologous vaccine prepared from S23 matt virulent and with broth	
		Matt virulent	Matt attenuated	Glossy	Heterologous matt virulent	Broth
0.0000001 cc.	†41	—	†89	—	†41	†41
0.000001 cc.	†27	†89	†41	†41	†25	†41
0.00001 cc.	†22	†70	†22	†24	†41	†27
0.0001 cc.	†22	S	†22	†22	†24	†17
0.001 cc.	†24	†65	S	†22	†22	†17
0.01 cc.	†17	†22	†17	†22	†17	†17
0.1 cc.	†17	†17	†17	—	†17	—

Active Immunization of Mice.—Table XIV shows the results of vaccinating mice with the three forms of Strain London and with matt virulent organisms of a heterologous strain. In this instance a final dose of 0.2 cc. of heat-killed culture was substituted for 0.4 cc. as used in other experiments because the mice began to lose weight during immunization. In this experiment, although there was little evidence of immunization by any of the vaccines, the mice vaccinated with the matt virulent organisms survived longer than any of the controls.

DISCUSSION.

Matt and glossy cultures of four strains of hemolytic streptococci, belonging to different serological types, were used to immunize rabbits. Only one of the four glossy cultures, Strain S23, was completely degraded to the point at which no trace of type-specific substance could be detected in highly concentrated HCl extracts of the organisms. The glossy cultures of the other three strains were not completely degraded as extracts from these organisms contained traces of the type-specific substance M.

Precipitin tests with the antisera of the four strains gave the following results: (1) All the anti-matt sera, whether prepared with virulent or attenuated organisms, contained antibody to the type-specific substance M. (2) The anti-glossy sera, prepared with cultures which were not fully degraded, were relatively deficient in type-specific antibody and gave either negative or weakly positive precipitin reactions with the type-specific substance M. (3) The five sera prepared with the completely degraded glossy form of Strain S23 did not contain any type-specific antibody. (4) The type-specific antibody was completely removed by absorption with homologous matt organisms but was unaffected by absorption with homologous glossy organisms.

Experiments on passive immunization of mice showed that all the anti-matt sera had some protective power against infection with homologous matt virulent organisms. Some of the anti-glossy sera, prepared against strains which were not fully degraded, also protected mice but none of the anti-glossy sera prepared against the completely degraded glossy form of Strain S23 afforded any protection against infection with the homologous virulent organisms.

No exact parallel was established between the anti-M titer and the protective power of immune sera but high titer anti-M sera usually gave good protection.

It might be supposed that protection against infection with matt organisms by anti-matt sera and the absence of protection by anti-glossy sera merely represented specificity which could be demonstrated between different forms of the same strain and that anti-glossy sera would protect against infection with glossy organisms

better than anti-matt sera. Three of the strains used in this investigation were unsuitable for experiments to test this possibility because the glossy organisms were completely avirulent for mice. The completely degraded glossy variant of Strain S23 was, however, relatively virulent for mice (M.L.D. 0.01 cc.) and comparison was, therefore, made between the protective power of anti-matt and of anti-glossy sera against infection with the homologous glossy organisms. No evidence was obtained that either the anti-matt sera or the anti-glossy sera had any protective power against infection with glossy organisms.

There is considerable evidence that both passive protection and active immunity were antibacterial and not antitoxic; this evidence may be summarized as follows:

1. All the strains produced toxin to some extent but the protective action of the antisera was type-specific; sera prepared with toxigenic strains did not protect against infection with heterologous strains of equal toxigenicity. The immunity due to vaccination was also type-specific.

2. The toxigenicity of the matt form and of the glossy form of Strain S23 was determined quantitatively by intracutaneous tests in human subjects, and it was found that the matt and the glossy forms were approximately equal in their power to produce skin-reactive toxin. In spite of this equality, anti-glossy sera possessed no protective power against infection with homologous matt virulent organisms.

3. The anti-matt sera against Strain C203 were prepared with washed bacteria, and the production of antitoxin was, therefore, limited, but in spite of this limitation the sera protected against the highly toxigenic homologous strain but not against the weakly toxigenic heterologous Strain S23.

4. Active immunization of mice with whole broth cultures of the matt and of the glossy forms of the scarlet fever Strain C203 showed that, although the two forms were approximately equal in toxigenicity, the matt vaccine produced a high degree of immunity while the glossy vaccine produced no immunity against infection with homologous virulent organisms.

These observations, which show that the matt varieties of hemolytic

streptococci are type-specific while the glossy variant forms are not type-specific, are in agreement with the work of Andrewes. He also isolated two varieties of hemolytic streptococci corresponding to our matt and glossy forms, which, in a preliminary communication (4), he designates "rough" and "smooth" with the reservation "that they must not be supposed to correspond with the rough and smooth forms of *B. coli* and Salmonella." Using a special technique, he was able to establish by agglutination and absorption experiments that the "rough" forms exhibit considerable specificity; that the "smooth" forms of different strains are all alike serologically, and that the "rough" and "smooth" forms of a given strain are serologically distinct.

A comparison of his results with those recorded in this paper shows that his type-specific agglutination with hemolytic streptococci of the matt variety is in agreement with our observation that large quantities of the type-specific substance M are found in matt organisms; also his observation that anti-glossy serum agglutinates all strains of hemolytic streptococci, when in the glossy form, with complete impartiality is in agreement with our observation that anti-glossy sera contain more antibody to the non-type-specific fractions than anti-matt sera.

SUMMARY.

The matt and the glossy forms of four strains of hemolytic streptococci were used to immunize rabbits.

Precipitin tests showed that rabbit sera prepared against matt organisms, whether virulent or avirulent for mice, contained type-specific antibody while sera prepared against completely degraded glossy organisms contained no type-specific antibody.

Type-specific antibody was removed from the sera by absorption with homologous matt organisms but was unaffected by absorption with homologous glossy organisms.

Passive protection experiments on mice showed that anti-matt sera were protective and anti-glossy sera non-protective against infection with homologous virulent organisms.

Vaccination of mice with matt organisms rendered them immune to subsequent infection with homologous virulent cultures; but vaccination with glossy organisms established no active immunity.

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