ANTIGENIC PROPERTIES OF THE TYPE-SPECIFIC SUBSTANCE DERIVED FROM GROUP A HEMOLYTIC STREPTOCOCCI

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Although the constituent of the hemolytic streptococcus cell, which determines specificity in each of the various types included within group A, has been considered a protein, attempts to demonstrate its antigenicity after separation from the cell have not been entirely successful. This paper gives a more detailed study of this subject. It seems probable that in some instances the chemical procedures used to isolate the type-specific substances tended to degrade the proteins, and thus render them less antigenic. The early experiments (1) with the type-specific protein M showed that although the sera produced by the injection of whole streptococci gave specific precipitin reactions with this partially purified extract, the injection of the protein solution itself failed to stimulate type-specific antibody production in rabbits or guinea pigs, as shown by the following negative tests: precipitin reaction, agglutination, passive protection in mice, passive and active anaphylaxis in guinea pigs.

By extracting ground streptococci with increasingly alkaline solutions, Heidelberger and Kendall (2) isolated a fraction which stimulated type-specific precipitin formation when injected into rabbits; but these sera were not tested for their ability to protect mice passively.

Mudd and his collaborators (3) have reported the isolation of an antigenic fraction from group A hemolytic streptococci which they have named labile antigen.[1] They postulated that the labile antigen is a complex molecule containing the type-specific protein M in addition to several other serologically active constituents. Besides absorbing type-specific agglutinins, opsonins, and protec-

[1] On the basis of recent unpublished findings of Mudd, Lackman, Pettit, and Morgan, Dr. Mudd has informed us that these authors now prefer to substitute the term "nucleoprotein agglutinogen" for "labile antigen."

tive antibodies from antibacterial serum, the labile antigen, when injected into rabbits, induced antibodies which gave partly type-specific precipitin reactions, but these antisera were not tested for protective action in animals.

In 1937, Stamp and Hendry (4) isolated a fraction from a group A type 3 hemolytic streptococcus (strain Richards), which produced active immunity when injected into mice. 47 per cent of the immunized animals were protected against 100 minimal lethal doses of the homologous organism. The specificity of this immune response, however, was not tested.

EXPERIMENTAL

As a starting point for obtaining the type-specific substance in antigenic form, it was thought promising to use bacteria ground in the cold, as suggested by Mudd and his collaborators. The first untreated saline extracts of such material, injected into rabbits, induced the formation of slight amounts of antibody, as demonstrated by precipitin and agglutinin tests and the passive protection of mice, but the antibody titers of these sera were very low.

Because of the large quantities of extract required to immunize rabbits, it was decided to immunize mice and to test their active immunity following the method of Stamp and Hendry, using the per cent of survival of actively immunized mice as the index of antigenicity of various preparations. In numerous experiments this technique proved to be a rough but convenient way of testing various fractions.

Methods

Selection of Strains.—Since the antigenicity of the extracts was to be tested by actively immunizing mice, it was essential to use only highly mouse-virulent strains which would be suitable for testing the degree of immunity in mice. It seemed probable, furthermore, that virulent cultures would yield larger amounts of antigenic material than avirulent ones. One strain each of three different types within group A was used for preparing antigenic extracts and one additional strain of each of these types was employed for testing the immunity.

Description of Strains.[2]—

Type 1:[3] 1. *Strain S118* was isolated in Texas in 1918 from the pleural fluid of a patient with bronchopneumonia following measles (6).

2. *Strain T1* is Griffith's type 1 strain, S. F. 130/2 (7).

Type 3: 1. *Strain D58* is the strain Richards, isolated by Colebrook from

[2] Strains T1 and T14 were kindly sent by Dr. F. Griffith, strain D58 by Dr. T. C. Stamp, and strain C203 by Dr. M. B. Kirkbride.

[3] Types are designated according to Griffith's classification (5).

puerperal septicemia (4). It was obtained in a virulent state following mouse passage.

2. *Strain C203*[4] was isolated by Dochez about 1921 from a patient with scarlet fever, and obtained in virulent form in 1927.

Type 14: 1. *Strain S23* was isolated in Texas in 1918 from the throat of a patient with lobar pneumonia (6).

2. *Strain T14* is strain Barker, a representative of type 14.

Virulence.—At the beginning of this experiment, three of the six strains, namely S23, C203, and D58, were virulent enough to kill mice in doses of 10^{-6} cc. to 10^{-8} cc. of 6 to 12 hour cultures. Strain T14 was moderately virulent and required only six to eight mouse passages to reach the same degree of virulence. The other two strains, S118 and T1, were so degraded that they produced typically glossy colonies and failed to yield demonstrable type-specific substance in extracts of ordinary concentration. They were so avirulent that 0.1 cc. to 0.5 cc. of a young culture was required to kill mice. By repeated mouse passage (25 passages for S118 and 60 passages for T1) it was possible to render these cultures virulent.

The chief method used in the preparation of antigenic extracts was as follows:—

Medium.—The bacteria used for preparing antigenic extracts were grown in the type of broth developed by Todd and Hewitt (8), modified chiefly by the substitution of beef heart for horse meat. The fact that the broth is sterilized by filtering through Chamberland B filters, rather than by heating, increases its value as a medium but also adds an element of danger in its use, since contaminants sometimes grow in uninoculated flasks which have been incubated for 3 or 4 days. By seeding the broth immediately after filtration with a very large inoculum, and then limiting the incubation to 4 hours, pure cultures were obtained in all except one instance.

During the early stages of growth in this broth, large capsule-like areas could be seen surrounding the organisms in moist India ink preparations. These capsules increased markedly in size and were maximal in about 4 hours, following which they rapidly became smaller. The bacteria at this early stage were very difficult to throw down in the centrifuge, but following heating at 56°, or after more prolonged incubation, the capsules disappeared and the bacteria were then easily packed on centrifugation, an observation also made by Seastone (9) and more recently by Loewenthal (10).

These capsules resembled those described by Seastone and later by Ward and Lyons (11), in young cultures of hemolytic streptococci grown in whole blood, in that they were present only in young cultures and were not correlated with

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[4] While Dr. Griffith has placed this strain in type 1 by means of slide agglutination, work carried out in this laboratory indicates that, on the basis of protection and precipitin tests, it falls in type 3. Further studies are being made here on the interrelationships of strain C203 and types 1 and 3.

mouse virulence, but they differed in that we could not stain them by ordinary methods. The difficult question of the significance of capsules for the hemolytic streptococcus has been reviewed by a number of authors (9, 10, 12), but in these experiments the presence of a capsule was taken to mean that the cultures were in a period of optimal physiological activity.

Collection of Bacteria.—The broth was filtered while still hot through Chamberland filters and was then cooled to 37°C. 64 liters were inoculated with 25 cc. per liter of an actively growing 4 hour culture. After 4 hours' growth, samples were removed from each flask and plated on blood agar to test the purity of the culture. The culture was then stored at 0°C. overnight, and kept chilled while running through a Sharples centrifuge on the following day. The caked bacterial sediment was suspended in about 200 cc. of cold saline by grinding it in a large, previously chilled mortar and pressing it through sterile gauze, stretched over a wire strainer, into another cold container in order to break up clumps and facilitate uniform heating during the process of heat-killing. The suspension was then transferred to a glass stoppered pyrex bottle, and the bacteria killed quickly by the following procedure: A thermometer was inserted into the bottle so that the temperature could be accurately adjusted. The temperature was raised within 1 minute to 56°C. by the addition, while shaking, of about 400 cc. of boiling saline. The stopper was then covered tightly with a rubber cap; and the bottle was completely immersed for 15 minutes in a 56°C. water bath. Sterility tests showed that all the bacteria in this heavy suspension were killed by the end of this time. The suspension was rapidly cooled by placing the bottle under running water and then centrifuged in 50 cc. wide mouthed tubes; the supernatant fluid was discarded. The tubes, containing not more than 3 gm. dry weight of bacterial sediment, were placed in a CO_2-ice-acetone mixture. While freezing, the bacteria were easily distributed over the sides of the tube with a spatula. The completely frozen organisms were dried by means of the Flosdorf-Mudd lyophile apparatus (13).

Extraction.—Preparatory to extraction, the bacteria were ground in a ball mill consisting of a 1 liter spherical heavy glass flask and 500 one-quarter inch stainless steel balls (14). At first the grinding was done at −73°C. as advocated by Mudd (15). Later, however, it was found that satisfactory results could be obtained by grinding 1 gm. of dried organisms in each flask for one-half hour at room temperature. This procedure rendered approximately 75 per cent of the cocci Gram-negative without causing much change in their morphology. The organisms from twelve flasks were collected in 500 cc. N/10 HCl and extracted at 37°C. for 24 hours. After centrifugation the supernatant extract was removed; 250 cc. N/10 HCl were added to the organisms which were then extracted for a second 24 hours. This procedure was repeated on a 3rd day. The yield of active material from the second and third extractions tended to be larger than that from the first; but since further extractions resulted in smaller yields, the bacterial residue was discarded.

Purification of the Extract.—On cautious neutralization of each of these acid

extracts with N/1 NaOH a precipitate began to form as pH 4.0 was approached and became maximal at about pH 4.5. In the case of the first acid extract it was necessary to bring the pH to 5.0 before flocculation occurred. After standing overnight in the ice box the precipitates were thrown down in a centrifuge and the supernatant fluids discarded. The combined precipitates from various extractions were taken up in M/15 phosphate buffer solution at pH 7.2 but most of the precipitate was insoluble and was discarded. N/1 HCl was slowly added to the supernatant fluid. As pH 5.5 was approached a precipitate began to form which was maximal at pH 4.5. This was allowed to stand overnight in the ice box and the precipitate separated the following morning. The supernatant fluid was discarded, and now nearly all the precipitate was dissolved in 50 cc. of M/15 phosphate buffer at pH 7.2. After removal of the small amount of insoluble material the solution was filtered through a Berkefeld N filter.

The filtrate was distributed in amounts suitable for one day's injections and then frozen and dried to prevent deterioration. In order to estimate the dosage, the total nitrogen and that precipitated by trichloroacetic acid were determined by means of the micro Kjeldahl method. The nitrogen precipitated by trichloroacetic acid was always between 50 and 60 per cent of the total nitrogen. Since it was felt that the antigenic activity probably resided in the protein fraction, the dosage in all experiments was calculated as 6.25 times the nitrogen content of the material precipitated by trichloroacetic acid; and the total yield calculated in the same way was about 0.6 per cent of the dry weight of the bacteria.

Serological Reactions Obtained with the Extract.—Different preparations of antigen reacted type specifically to about the same dilution, usually 1:200,000 in precipitin tests with homologous type-specific sera. The dilution was calculated in the same way as the dosage employed in immunization. No group-specific polysaccharide C could be detected in these extracts when tested with sera potent in anti-C precipitins nor could this carbohydrate be split off by heating the solution at 100°C. with N/20 HCl.

The method of extraction and partial purification outlined was adopted as the procedure of choice for the chief experiments of this investigation, but several less successful methods of preparation were tried in numerous other experiments.

Active Immunization of Mice

The mice used in these experiments were the Rockefeller strain. When possible, mice weighing 24 to 26 gm. were used for active immunization, since they seemed to give better results than smaller ones. The dried antigenic extracts were dissolved in physiological sodium chloride solution just before injection, which was made intraperitoneally on 3 successive days with a rest period of 4 days between courses. Usually three or four courses were given. The immunized and untreated control mice were inoculated with the test culture 1 week

after the last injection of the antigen. Experiments were terminated after 2 weeks' observation.

The mice in the first experiment (Table I) were immunized for 4 weeks with an

TABLE I

Active Protection Test in Mice

Titer of Homologous Immunity

Mouse No.	Mice immunized with extract of type 1 strain, S118						Untreated controls	
	Inoculated with the following amounts of S118							
	10^{-2} cc.	10^{-3} cc.	10^{-4} cc.	10^{-5} cc.	10^{-6} cc.	10^{-7} cc.	10^{-7} cc.	10^{-8} cc.
1	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day
2	D 1 "	D 1 "	D 2 days	D 1 "	D 3 days	D 3 days	D 1 "	D 1 "
3	D 1 "	D 1 "	D 2 "	D 1 "	D 3 "	D 5 "	D 1 "	D 1 "
4	D 1 "	D 1 "	D 4 "	D 2 days	D 5 "	S	D 1 "	D 1 "
5	D 1 "	D 1 "	D 6 "	D 2 "	D 6 "	S	D 1 "	D 1 "
6	D 1 "	D 1 "	D 7 "	D 2 "	S	S	D 1 "	D 1 "
7	D 1 "	D 1 "	D 10 "	D 3 "	S	S	D 1 "	D 1 "
8	D 1 "	D 1 "	S	D 3 "	S	S	D 1 "	D 1 "
9	D 1 "	D 1 "	S	D 3 "	S	S	D 1 "	D 1 "
10	D 1 "	D 1 "	S	D 3 "	S	S	D 1 "	D 1 "
11	D 1 "	D 1 "	S	D 3 "	S	S	D 1 "	D 1 "
12	D 1 "	D 3 days	S	S	S	S	D 1 "	D 1 "
13	D 1 "	D 4 "	S	S	S	S	D 2 days	D 1 "
14	D 1 "	S	S	S	S	S	D 2 "	D 2 days
15	D 1 "	S	S	S	S	S	D 3 "	D 3 "
16	D 1 "	S	S	S	S	S	D 3 "	D 3 "
17	D 1 "	S	S	S	S	S	S	D 3 "
18	D 1 "	S	S	S	S	S	S	D 3 "
19	D 1 "	S	S	S	S	S	S	D 5 "
20	D 1 "	S	S	S	S	S	S	S

The mice were immunized for 4 weeks: 3 injections of 0.01 mg. each were given the 1st week, 3 of 0.02 mg. each the 2nd, 3 of 0.04 mg. each the 3rd and 4th.

The protection tests were performed as follows: A fresh 16 hour broth culture was serially diluted with broth so that the amount inoculated was contained in 0.5 cc. The inoculations were intraperitoneal. The untreated controls corresponded in age and weight with the immunized animals. The number of streptococci injected was estimated in colony counts from poured blood agar plates containing 10^{-6} cc., 10^{-7} cc., and 10^{-8} cc., respectively, of the culture used for inoculating the mice. The number of colonies in 10^{-8} cc. varied from two to six in different experiments.

In all experiments S indicates animals which survived at least 2 weeks, and D indicates death on the day stated.

extract of strain S118 in the manner indicated in Table I. A total dosage of 0.33 mg. was given to each animal. 1 week after the last injection, they were divided into six groups of 20 mice each, and each group was injected with a different dose of an overnight culture of strain S118. None of the mice receiving 1,000,000 M.L.D. (10^{-2} cc.) survived, but in all the groups receiving smaller doses enough animals survived to show definite protection against the homologous organism.

The second experiment (Table II) was designed to test whether the active immunity induced by these antigens was type-specific. Three sets of 84 mice were immunized for four courses with extracts of streptococci of three different types (types 1, 3, and 14). The mice received the same amounts of antigenic extract as those in the first experiment. 1 week following the last dose of antigen each group was subdivided into six subgroups of 14 mice each. Six strains were used as test inocula, two each of types 1, 3, and 14. Each subgroup received 10^{-6} cc. of culture diluted in broth. This small dose, containing 10 to 100 M. L. D., which killed all the control animals regularly, was selected in order to detect even slight evidence of cross protection.

In each case, good protection was demonstrated against 10 to 100 lethal doses of streptococci of the homologous type, as there were 93 per cent survivors in type 1, 93 to 100 per cent in type 3, and 43 to 50 per cent in type 14. On the other hand, comparably immunized mice, when inoculated with heterologous strains, showed only slight or no immunity. Some protection against the type 1 cultures was afforded to mice immunized with heterologous extracts, but little if any cross immunity was found where types 3 and 14 were used as test inocula. In every case type-specific immunity was clearly greater than the immunity against heterologous types. It is probable that if a larger dose of culture had been used, the immunity induced by immunization with extracts would have appeared strictly type-specific.

Type-Specific Immunization of Rabbits with Extract

After completing the experiments on active immunity in mice, an attempt was made to immunize rabbits with the same antigenic extract. The immunity was tested by protection of mice by the sera of these animals. The rabbits were treated as shown in Table III. During immunization the response to the antigen was determined by precipitin tests with the homologous M extract.

These tests showed that the sera of the two rabbits immunized with S118 extract (rabbits R47-08 and R47-09) contained no precipitins against the homologous extract at the end of the third course, but both had precipitins and protective antibodies at the end of the fifth course. The rabbit given seven courses showed no increase in antibody titer over that reached at the end of the fifth series. The sera of the two rabbits (R47-22 and R47-23) similarly immunized with an

TABLE II

Active Protection Test in Mice

Type Specificity of Immunity

Immunized mice							
Immunized with extract of strain	Mouse No.	Inoculated with 10^{-6} cc. of					
		Type 1 strains		Type 3 strains		Type 14 strains	
		S118	T1	D58	C203	S23	T14
		(Homologous)					
S118 (type 1)	1	D 7 days	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day
	2	S	S	D 1 "	D 1 "	D 1 "	D 1 "
	3	S	S	D 1 "	D 1 "	D 1 "	D 1 "
	4	S	S	D 1 "	D 1 "	D 1 "	D 1 "
	5	S	S	D 1 "	D 1 "	D 1 "	D 2 days
	6	S	S	D 1 "	D 1 "	D 2 days	D 2 "
	7	S	S	D 1 "	D 1 "	D 2 "	D 2 "
	8	S	S	D 2 days	D 1 "	D 3 "	D 2 "
	9	S	S	D 2 "	D 1 "	D 3 "	D 3 "
	10	S	S	D 2 "	D 2 days	D 3 "	D 3 "
	11	S	S	D 2 "	D 2 "	D 5 "	D 3 "
	12	S	S	D 2 "	D 2 "	D 5 "	D 3 "
	13	S	S	D 3 "	D 2 "	D 9 "	D 3 "
	14	S	S	D 3 "	D 2 "	S	D 5 "
				(Homologous)			
D58 (type 3)	1	D 1 day	D 1 day	S	D 3 days	D 1 day	D 1 day
	2	D 1 "	D 1 "	S	S	D 1 "	D 2 days
	3	D 1 "	D 1 "	S	S	D 1 "	D 2 "
	4	D 2 days	D 2 days	S	S	D 1 "	D 2 "
	5	D 2 "	D 2 "	S	S	D 2 days	D 2 "
	6	D 2 "	D 2 "	S	S	D 2 "	D 2 "
	7	D 2 "	D 2 "	S	S	D 2 "	D 2 "
	8	D 2 "	D 2 "	S	S	D 2 "	D 2 "
	9	D 3 "	S	S	S	D 3 "	D 2 "
	10	D 3 "	S	S	S	D 3 "	D 2 "
	11	S	S	S	S	D 3 "	D 3 "
	12	S	S	S	S	D 7 "	D 3 "
	13	S	S	S	S	D 7 "	D 3 "
	14	S	S	S	S	S	D 8 "
						(Homologous)	
S23 (type 14)	1	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day
	2	D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 2 days
	3	D 1 "	D 1 "	D 1 "	D 1 "	D 2 days	D 2 "
	4	D 1 "	D 1 "	D 1 "	D 1 "	D 5 "	D 2 "
	5	D 1 "	D 1 "	D 1 "	D 1 "	D 5 "	D 3 "
	6	D 2 days	D 2 days	D 1 "	D 1 "	D 6 "	D 3 "
	7	D 2 "	D 2 "	D 1 "	D 1 "	D 7 "	D 3 "
	8	D 2 "	D 2 "	D 1 "	D 1 "	S	D 9 "
	9	D 2 "	D 2 "	D 1 "	D 1 "	S	S
	10	D 2 "	D 2 "	D 1 "	D 2 days	S	S
	11	D 2 "	D 3 "	D 1 "	D 2 "	S	S
	12	S	D 3 "	D 1 "	D 2 "	S	S
	13	S	S	D 2 days	D 2 "	S	S
	14	S	S	D 2 "	D 2 "	S	S

TABLE II—*Concluded*

Untreated virulence control mice							
Mouse No.	Inoculated with						
	Dose	Type 1 strains		Type 3 strains		Type 14 strains	
		S118	T1	D58	C203	S23	T14
	cc.						
1	10^{-6}	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day
2		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 1 "
3		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 1 "
4		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 1 "
5		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 1 "
6		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 1 "
7		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 2 days
8		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 2 "
9		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 2 "
10		D 1 "	D 1 "	D 1 "	D 2 days	D 2 days	D 2 "
11		D 1 "	D 1 "	D 1 "	D 2 "	D 2 "	D 2 "
12		D 1 "	D 1 "	D 1 "	D 2 "	D 2 "	D 2 "
13		D 1 "	D 1 "	D 1 "	D 2 "	D 2 "	D 2 "
14		D 5 days	D 1 "	D 2 days	D 15 "	D 2 "	D 7 "
1	10^{-7}	D 1 day	D 1 "	D 1 day	D 1 day	D 1 day	D 2 "
2		D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	D 2 "
3		D 1 "	D 1 "	D 1 "	D 1 "	D 2 days	D 2 "
4		D 1 "	D 1 "	D 1 "	D 2 days	D 2 "	D 2 "
5		S	D 1 "	D 3 days	D 9 "	S	D 2 "
1	10^{-8}	D 1 day	D 2 days	D 1 day	D 2 "	D 2 days	D 1 day
2		S	D 2 "	D 1 "	D 2 "	S	D 2 days
3		S	D 2 "	D 2 days	D 2 "	S	D 2 "
4		S	S	D 2 "	D 2 "	S	D 2 "
5		S	S	S	S	S	S

extract of strain D58, contained demonstrable type-specific precipitins and protective antibodies after 3 weeks' immunization; and the titer was increased following 2 weeks' further immunization.

The capacity of these sera to protect mice was tested with six different strains of hemolytic streptococci, representing three types (Table IV). The S118 serum was a pool of bleedings from rabbit R47-08 after the fifth and seventh courses of injections of antigen. The D58 serum was taken from rabbit R47-23 after the fifth course. Although the cultures used in the passive protection experiments were

somewhat less virulent than usual, as shown by the survival of some control mice, nevertheless, there was distinct protection against 1,000 to 100,000 M.L.D. of the homologous type streptococci with some irregular deaths. The few survivals among animals tested with heterologous strains were very irregular, and the results in general show strict type specificity in these passive protection tests.

TABLE III

Protocol of Immunization of Rabbits with Extracts

Course of injection	Rabbits R47-08 and R47-09* with S118 extract	Rabbits R47-22 and R47-23 with D58 extract
1st	2.5 mg. daily for 5 days	2.5 mg. daily for 5 days
2nd	4.3 " " " " "	2.5 " " " " "
3rd	5.0 " " " " "	5.0 " " " " "
	Test bleeding	Test bleeding
4th	10.0 mg. daily for 5 days	5.0 mg. daily for 5 days
5th	10.0 " " " " "	10.0 " " " " "
	50 cc. bleeding	Final bleeding
6th	10.0 mg. daily for 5 days	
7th	20.0 " " " " "	
	Final bleeding	

Immunizing material was dissolved in saline, 1 to 2 cc. and given intravenously. There were 2 days of rest between each course except where a bleeding was taken, in which case the interval was a week.

* The final bleeding from rabbit R47-09 was taken after the sixth course of injections.

Precipitin Reactions with Anti-Extract Sera

In Table V are shown the precipitin reactions with samples of the same sera used in the passive protection tests recorded in Table IV. The M substances used as reagents were extracted with N/20 HCl in a boiling water bath as previously described (16). The immediate reactions were strikingly type-specific, but on standing overnight in the ice box the somewhat confusing cross reactions appeared. The latter are recorded in the table. The type 1 serum was not very potent but reacted most strongly with extracts of the homologous type strains, S118 and T1. It also gave weak reactions with all three of the type 3 extracts used but none with the type 14 extracts. The much more potent type 3 serum gave good immediate precipitates

with extracts of all the homologous type strains (T3, D58, C203), and weaker reactions with the type 1 extracts. With type 14 extracts, the type reactivity of which had been previously established by testing with antibacterial sera, this serum (R47-23) gave only traces of precipitin reaction. Neither serum contained group-specific antibody, as indicated by their failure to precipitate with a solution of group-specific C polysaccharide which, in the dilutions used, regularly precipitated sera known to contain the group-specific anti-C precipitin.

It is highly probable that the cross reactions in the precipitin tests are due to the presence of non-type-specific antibodies in the rabbit serum and of non-type-specific precipitinogens in the M extracts used.

Absorption of Antibacterial Serum with Extracts

In order to test the evidence identifying this antigen with the substance in intact streptococci which stimulates the production of protective antibodies in rabbits, the antigen was used to absorb protective antibody from a serum made by immunizing a rabbit with whole streptococci. Since Mudd and his collaborators (3) state that the type-specific protective antibody cannot be absorbed by the M fraction, extracted with N/20 HCl in a boiling water bath, another sample of the serum was absorbed with the M substance so prepared.

A rabbit was immunized chiefly with heat-killed but also with living culture of the type 1 strain, T1. Its serum gave a strong precipitin reaction with the homologous M antigen, and regularly protected mice against 1,000,000 M.L.D. of type 1 strains. One portion of serum was absorbed with a known antigenic extract of strain S118 (lot 61, made in the same manner as that used as antigen for the active and passive immunization experiments). Another portion was absorbed with an M extract of strain S118 made with N/20 HCl in a boiling water bath. Neither solution contained demonstrable group-specific C polysaccharide. The optimal proportions point for the precipitation of the serum by each antigen was determined by titration (17), and the solutions of antigens were added to the respective sera in double the optimal proportions. After incubating the mixtures at 37° for 2 hours and keeping them in the ice box overnight, the precipitates were removed and discarded and more of the respective antigen was added to the partially absorbed sera. Practically complete absorption was indicated by the lack of further precipitation with the lot 61 antigen and by the mere trace of precipitate with the M extract prepared by the older method. Serial dilutions of each absorbed serum and of a control lot of the same serum unabsorbed were

TABLE IV

Passive Protection Test in Mice—Type Specificity of Immunity

Dose	Mouse No.	Type 1 strains		Type 3 strains		Type 14 strains	
		S118	T1	D58	C203	S23	T14
		Serum from rabbit R47-08 immunized with S118 (type 1) extract					
		Mice inoculated with test culture					
cc.		(Homologous)					
10^{-3}	1	D 1 day	D 1 day				
	2	D 1 "	D 1 "				
	3	S	S				
	4	S	S				
10^{-4}	1	D 3 days	S				
	2	S	S				
	3	S	S				
	4	S	S				
10^{-5}	1	D 3 days	S	D 1 day	D 1 day	D 1 day	D 1 day
	2	D 4 "	S	D 1 "	D 1 "	D 2 days	D 1 "
	3	S	S	D 1 "	D 1 "	D 4 "	D 8 days
	4	S	S	D 2 days	D 1 "	D 4 "	S
10^{-6}	1	D 2 days	S	D 1 day	D 1 "	D 3 "	D 2 days
	2	S	S	D 1 "	D 1 "	D 3 "	D 4 "
	3	S	S	D 2 days	D 1 "	D 9 "	D 5 "
	4	S	S	D 2 "	D 2 days	S	S
10^{-7}	1	S	S	D 2 "	D 1 day	D 2 days	D 3 days
	2	S	S	D 2 "	D 1 "	D 3 "	D 3 "
	3	S	S	D 3 "	D 2 days	D 4 "	D 6 "
	4	S	S	D 3 "	D 2 "	S	S
		Virulence controls: no serum					
10^{-5}	1	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day	D 1 day
	2	D 1 "	D 1 "	D 1 "	D 1 "	D 1 "	S
	3	D 1 "	D 1 "	D 2 days	D 1 "	D 4 days	S
	4	D 1 "	S	D 2 "	D 1 "	S	S
10^{-6}	1	D 1 "	D 1 day	D 1 day	D 1 "	D 1 day	D 1 day
	2	D 2 days	D 1 "	D 2 days	D 1 "	D 1 "	D 1 "
	3	D 2 "	D 1 "	D 2 "	D 1 "	D 2 days	D 2 days
	4	D 2 "	S	D 2 "	D 1 "	S	S
10^{-7}	1	D 1 day	D 3 days	D 1 day	D 1 "	D 1 day	D 1 day
	2	D 2 days	S	D 2 days	D 1 "	D 1 "	D 2 days
	3	D 2 "	S	D 2 "	D 1 "	S	S
	4	S	S	D 2 "	D 10 days	S	S
10^{-8}	1	D 3 days	D 1 day	D 5 "	S	S	S
	2	S	S	S	S	S	S
	3	S	S	S	S	S	S
	4	S	S	S	S	S	S

TABLE IV—*Concluded*

Serum from rabbit R47-23 immunized with D58 (type 3) extract							
Mice inoculated with test culture							
Dose	Mouse No.	Type 1 strains		Type 3 strains		Type 14 strains	
		S118	T1	D58	C203	S23	T14
cc.							
				(Homologous)			
10^{-1}	1			D 1 day	D 1 day		
	2			D 1 "	D 1 "		
	3				D 1 "		
	4				S		
10^{-2}	1			D 1 "	D 1 day		
	2			D 1 "	D 1 "		
	3			D 1 "	S		
	4			S	S		
10^{-3}	1			S	D 2 days		
	2			S	D 3 "		
	3			S	S		
	4			S	S		
10^{-4}	1			D 1 day	D 1 day		
	2			D 7 days	S		
	3			S	S		
	4			S	S		
10^{-5}	1	D 1 day	D 1 day	S	D 10 days	D 1 day	D 1 day
	2	D 2 days	D 1 "	S	S	D 1 "	D 1 "
	3	D 2 "	D 1 "	S	S	D 2 days	D 1 "
	4	D 2 "	D 1 "	S	S	D 2 "	D 1 "
10^{-6}	1	D 1 day	D 12 days	S	S	D 1 day	D 1 "
	2	D 2 days	S	S	S	D 1 "	D 1 "
	3	S	S	S	S	D 1 "	D 1 "
	4	S	S	S	S	S	D 2 days
10^{-7}	1	D 3 days	S	S	S	D 1 day	D 1 day
	2	D 3 "	S	S	S	D 1 "	D 2 days
	3	D 3 "	S	S	S	D 2 days	D 3 "
	4	S	S	S	S	S	D 5 "

18 to 20 gm. mice were injected intraperitoneally with 0.5 cc. of the serum indicated, the day before inoculation. Fresh 12 hour blood broth cultures were diluted serially with broth so that 0.5 cc. contained the desired dose. The amounts recorded were injected intraperitoneally into four mice in each set. Similar sets of mice which had received no serum were included as virulence controls.

given to mice. On the following day all the mice were inoculated with varying amounts of strain S118.

TABLE V

Precipitin Reactions

Antisera Prepared with Extracts

Serum		M extracts for precipitin tests							Group-specific C fraction
		Type 1 strains		Type 3 strains			Type 14 strains		
		S118	T1	D58	C203	T3*	S23	T14	
	cc.								
Rabbit R47-08 anti-S118 extract (type 1)									
0.2 cc.	0.4	−	++	±	±	+	−	−	−
" "	0.2	−	++	+	+±	+	−	−	−
" "	0.1	+	+	+	±	−	−	−	−
" "	0.05	+±	+±	−	±	−	−	−	−
" "	0.025	+±	++	−	−	−	−	−	−
" "	0.013	+±	++	−	−	−	−	−	−
Rabbit R47-23 anti-D58 extract (type 3)									
0.2 cc.	0.4	++	++	+++	++	++	+	±	−
" "	0.2	+±	+±	+++	+++	+++	+	+	−
" "	0.1	+±	+±	++±	++±	+++	±	+	−
" "	0.05	+	+	+	+±	++	±	−	−
" "	0.025	±	±	±	+	+±	±	−	−
" "	0.013	±	±	±	+	±	−	−	−

The M extracts used here were prepared by heating at 100°C. with N/20 HCl and after alcohol precipitation were made up in a final concentration of about one-fifth of the original volume of extracts. Serial dilutions were made with saline in 0.4 cc. volume. To each tube 0.2 cc. of serum was added, they were then incubated at 37° for 2 hours and kept in the refrigerator overnight before reading. Readings were made on a scale of ++++ to ±. The C fraction used here was made from the type 6 strain S43 by acid extraction and the protein removed with $HgCl_2$. In the concentrations used here it gave strong precipitin reactions with sera potent in anti-C.

Strain S43 was isolated in Texas in 1918 from the throat of a patient with measles.

* Strain T3 is Griffith's strain "Lewis opaque," kindly sent by Dr. Griffith as a representative of type 3. It was given 40 passages through mice to increase its virulence and its content of type-specific substance.

The results of the absorption experiment recorded in Table VI, indicate that the lot 61 extract removed the protective antibody from the antibacterial serum so that even in doses of 0.5 cc. the absorbed serum no longer protected against as little as 100 M.L.D. (10^{-5} cc.) of a strain of homologous type. The absorption with the M substance, extracted at 100° with N/20 HCl, was only slightly less complete: 0.5 cc. of this absorbed serum protected only against 100 and 1,000 M.L.D. and not against larger doses of culture. This amount of protection was insignificant when compared with the original titer of the serum which was high enough for 0.06 cc. to protect a mouse against 100,000 M.L.D.

Active Immunization of Mice with M Extracts

The hypothesis that the M substance is essentially similar to the antigenic substance in extracts active in inducing immunity was also tested by the following experiment.

Mice were immunized actively with M extracts prepared by heating living streptococci with N/20 HCl for 15 minutes in a boiling water bath. The antigen was further purified by reprecipitating twice from saline solution with three to four volumes of 95 per cent ethyl alcohol. A neutral solution of the antigen was filtered through a Berkefeld N filter; and the amount to be injected was calculated on the basis of the trichloroacetic acid precipitable fraction. The antigen was distributed in tubes, each containing enough for one day's immunization, and dried from the frozen state on the Flosdorf-Mudd lyophile apparatus.

In Table VII are recorded the results of testing mice immunized with the extract. One set of animals received 0.33 mg. of material, the same dosage as those recorded in Tables I and II. Only 20 per cent of these survived an inoculation of 100 M.L.D. of the homologous strain S118. In another set which was immunized with 6.6 mg., 60 per cent survived a similar inoculum. A preliminary test with a similar M extract, but unfiltered, also showed 60 per cent survival when large immunizing doses were given for 4 weeks.

Preliminary Chemical Studies of the Antigenic Extract

Total nitrogen and phosphorus analyses on three typical extracts are given in Table VIII. Since the high phosphorus content and the precipitability at pH 4.5 suggested that nucleic acid might be present,

spectroscopic examination of several preparations was made.[5] All showed an absorption spectrum characteristic of nucleic acid, that is, a wide band with maximal absorption at about 2,600 Å. Quantitative spectroscopic estimates, using yeast nucleic acid as a standard, in-

TABLE VI

Absorption Experiment

Passive Protection Test in Mice

Type 1 antibacterial serum		Culture: Type 1, strain S118			
		10^{-2} cc.	10^{-3} cc.	10^{-4} cc.	10^{-5} cc.
	cc.				
Unabsorbed	0.5	S	S	S	S
	0.25	S	S	S	S
	0.12	S	S	S	D 2 days
	0.06	S	D 3 days	D 2 days	D 12 "
	0.03	D 3 days	S	D 2 "	S
Absorbed with type 1, S118 extract: Lot 61 antigen used for active and passive immunization tests	0.5	D 1 day	D 3 days	D 1 day	D 1 day
	0.25	D 1 "	D 2 "	D 2 days	D 2 days
	0.12	D 1 "	D 1 day	D 1 day	D 2 "
	0.06	D 1 "	D 1 "	D 2 days	D 2 "
	0.03	D 1 "	D 1 "	D 2 "	D 1 day
Absorbed with type 1, S118 M extract, made with N/20 HCl at 100°C.	0.5	D 1 "	D 1 "	S	S
	0.25	D 1 "	D 2 days	D 2 days	D 2 days
	0.12	D 1 "	D 1 day	D 2 "	D 5 "
	0.06	D 1 "	D 1 "	D 1 day	D 3 "
	0.03	D 1 "	D 1 "	D 2 days	D 3 "

Virulence controls: Inoculated with strain S118

20 mice inoculated with 10^{-6} cc.	6 mice inoculated with 10^{-7} cc.	6 mice inoculated with 10^{-8} cc.
10 mice D 1 day 8 " D 2 days 2 " S	3 mice D 2 days 1 mouse D 5 " 2 mice S	6 mice S

dicated that usually 25 to 30 per cent of the material in the extracts was nucleic acid. The biuret test and the Sakaguchi test for arginine were positive in high dilutions of the extracts. Since the relationship

[5] We are indebted to Dr. George Lavin for the spectroscopic determinations on these preparations.

TABLE VII

Active Protection Test in Mice

Immunized with M Extract of Strain S118*

Immunized mice: Immunity tested by inoculating with 10^{-6} cc. strain S118		
Mouse No.	Each mouse immunized with total dosage 0.33 mg. of extract	Each mouse immunized with total dosage 6.6 mg. of extract
1	D 1 day	D 1 day
2	D 1 "	D 1 "
3	D 1 "	D 2 days
4	D 2 days	D 4 "
5	D 4 "	S
6	D 4 "	S
7	D 4 "	S
8	D 4 "	S
9	S	S
10	S	S

Virulence controls: Inoculated with strain S118		
20 mice inoculated with 10^{-6} cc.	5 mice inoculated with 10^{-7} cc.	5 mice inoculated with 10^{-8} cc.
16 mice D 1 day	2 mice D 1 day	4 mice D 2 days
2 " D 2 days	3 " D 2 days	1 mouse S
1 mouse D 5 "		
1 " D 12 "		

Immunization was carried out in the same manner as with the mice reported in Table I, each set receiving four series of injections.

* Extract prepared by heating at 100°C. with N/20 HCl.

TABLE VIII

Chemical Analysis of Typical Fractions Used for Immunization

Lot No.	Extract from		Organisms heated at 56°C.	N	P	N precipitated by trichloroacetic acid	Nucleic acid
			min.	*per cent*	*per cent*	*per cent*	*per cent*
61	Strain S118	Type 1	15	16.73	3.97	52.06	33.3
62	" D58	" 3	15	15.60	3.69	50.61	6.9
65	" S118	" 1	5	16.25	3.40	58.72	30.2

of these findings to the constitution of the active agent in the extract is not certain, further work is being carried on in an attempt to clarify this point.

DISCUSSION

A substance has been obtained from extracts of group A hemolytic streptococci which induces active immunity in mice. On injection into rabbits it leads to the production of relatively type-specific antibody with which mice may be passively protected against infection with strains of the homologous type. Although the immunity in general was predominantly type-specific in nature, some non-type-specific reactions were also observed. It is important to realize that the methods employed for extracting the streptococci would probably not yield any single substance in a form approaching purity. This conception is strengthened by the previous work which suggests that the hemolytic streptococcus contains many proteins which are precipitable at a pH near 4.5 (2) and this idea is, moreover, confirmed by the cross reactions seen in precipitin tests with some of the antisera obtained from rabbits immunized with this material. Although the first experiment (Table I) indicated active protection against the homologous strain, the second experiment (Table II) showed that this was not strictly type-specific. This cross immunity may be explained by assuming either that it was induced by 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.

In the passive protection experiments, on the other hand, type-specific immunity was striking, and the indication of cross protection among types so slight that its existence is questionable. In the precipitin tests with the immune sera prepared in rabbits and used for passive protection tests in mice (Table V), both type specificity and cross reaction were observed. The cross reactions here are open to the same interpretation as in the experiment on active immunity. Although precipitin absorptions were not performed in the present experiments, previous work based on absorption experiments indicates that cross reactions observed in the precipitin test are probably due to an admixture of antigens in the extract used to immunize animals, and, correspondingly in the M extract used as reagents in the test tube.

In developing a method of extracting the antigen, different procedures were tried, and the extracts were compared in their ability to

produce immunity in mice. While the method used in the present experiments was the best of those tried, there are certain steps in the procedure which were used empirically. In the light of experience gained since this method was adopted, it may be that heat-killing and grinding the bacteria prior to extraction are not essential steps in obtaining the best antigens. We feel that it is important, however, to use young, actively growing cultures, prepared by the method described. The comparative experience in immunizing rabbits, which shows that large doses of antigenic extract did not induce as potent antisera as smaller doses of whole streptococci, makes us feel that the antigenicity of the active substance had been impaired by extraction. Possibly some refinement of these extraction methods or a different procedure may furnish an antigen with unimpaired or little diminished activity.

The nitrogen and phosphorus analyses of the extracts, the positive protein tests, and the spectroscopic analyses, as well as the behavior of the extracts in precipitating at pH 4.5, all suggest the presence of a nucleoprotein. It is impossible to say at present whether a nucleoprotein is the agent active in inducing the immunity observed, since the extracts were undoubtedly impure preparations. Conceivably a very small admixture of some other substance may have been responsible for the antigenic activity.

The antigen we have extracted cannot, as yet, be compared with the labile antigen described by Mudd and his coworkers,[1] since they have studied that fraction mainly in its ability to absorb precipitins, agglutinins, opsonins, and of protective antibodies from antibacterial serum, while we have confined ourselves principally to the production of active immunity in mice and protective antibodies in rabbits. Sevag, Lackman, and Smolens (18) have recently stated that the labile antigen is a nucleoprotein. While the active agent in the extracts described by us may be a nucleoprotein, it does not necessarily follow that it is the same as the labile antigen, since it is known that there are many nucleoproteins in the streptococcus (2), some of which are undoubtedly non-type-specific in nature.

The extract studied by Stamp and Hendry, however, is similar to the one described here, since both are made with N/10 HCl at 37°C. Although the subsequent method of purification is different, both ex-

tracts produce active immunity in mice against the homologous strain of streptococcus.

The active protection experiment in mice with the type-specific M substance extracted with N/20 HCl in a boiling water bath, shows that antigenic material is present, even though it is necessary to give larger amounts of it to obtain a degree of protection comparable to that elicited by the extract described in this paper. Both kinds of extracts react similarly in precipitin tests with specific immune sera, and both are also similar in the way they absorb the protective substance from immune sera. This last finding is at variance with the conclusions of Mudd and his collaborators, who were unable to absorb the protective substance from antibacterial sera with M substance prepared as described above.

The evidence presented, together with the general characteristics of the substance and manner of isolation, leads us to believe that the active principle in the extracts used for active and passive immunization in the present experiments is essentially similar to the active principle in the M extracts as formerly prepared, and probably also to the antigenic substances obtained by Stamp and Hendry, although different preparations exhibit varying degrees of purity and degradation from the native state. This substance, immunologically distinct for each serological type of group A hemolytic streptococcus, is probably the type-specific constituent in at least partially antigenic form.

SUMMARY

1. A substance extracted from group A hemolytic streptococcus is described, which induces active immunity in mice, and in rabbits gives rise to precipitins and to protective antibodies passively transferable to mice.

2. The active immunity in mice is principally type-specific, but some degree of non-type-specific immunity is also developed. The passively transferable protective antibodies are type-specific with only a slight suggestion of non-type specificity. In the precipitin test, the rabbit immune sera give both type-specific and non-type-specific reactions which have not been fully analyzed serologically.

3. Substances contained in the extract absorb the protective antibodies from the serum of rabbits immunized with whole hemolytic streptococci.

4. The most satisfactory method of extraction so far developed is fully described. Chemical tests on the material are consistent with the presence of protein and nucleic acid.

5. The type-specific M substance, prepared as previously described, was compared in some of its antigenic properties with the above mentioned substance. It was found capable of inducing active immunity in mice and of absorbing protective antibody from antibacterial immune serum in a manner qualitatively similar to that obtained with the preparations made by the newer methods.

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