SELECTION OF GROUP A STREPTOCOCCI RICH IN M-PROTEIN FROM POPULATIONS POOR IN M-PROTEIN

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In 1928 Todd [1] demonstrated that hemolytic streptococci freshly isolated from the bloodstream of patients with septicemia had the ability to multiply in normal human blood *in vitro*. Lancefield and associates [2] demonstrated that group A streptococci could be classified into serologic types on the basis of protein components, designated M-proteins, which determined the production of protective antibodies against each type. Kuttner and Lenert [3] observed that blood from patients convalescing from infection with group A, type 36 streptococci had greater bacteriostatic power against homologous than heterologous streptococci. Maxted [4] found that rabbit antiserums to group A streptococci enhanced the power of normal human blood to destroy streptococci of the type used for immunization. He used this method to identify serologic types of group A streptococci. The latter two observations indicated that neutralization of a type-specific factor, presumably M-protein, by antibody rendered the streptococci more susceptible to phagocytosis.

Lancefield [5] showed that resistance of group A streptococci to phagocytosis by human leukocytes *in vitro* is a sensitive indicator of M-protein content. Rothbard and Watson [6] found that progressive loss of M-protein occurred in many strains of group A streptococci that persisted for many weeks in the human nasopharynx. This loss correlated with increasing susceptibility of the streptococci to the bacteriostatic action of human blood. M-protein content of streptococcal cultures may also decrease or even disappear during growth in artificial media. In the laboratory, restoration of M-protein and resistance of streptococci to phagocytosis can often be accomplished by serial passage of the degraded micro-organisms in mice. This restoration is usually attributed to the survival in the mouse of streptococci resistant to destruction by phagocytes.

The purpose of this communication is to report results of experiments in which group A streptococci rich in M-protein and resistant to phago-

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cytic destruction were selected from populations of streptococci poor in M-protein and susceptible to phagocytic destruction during rotation with lightly heparinized human blood.

Material and Methods

Streptococci

Group A streptococci of serologic types 1, 3, 4, 12, 19, 27, 41, 49, and London were used. In addition a strain (A 648) of group A streptococci was used that could not be typed with the various antiserums in the laboratory of Dr. Rebecca C. Lancefield. Several of these strains had been subcultured on artificial media many times and had become difficult or impossible to type. Type 1 streptococci were originally isolated from the nasopharynx of a child with glomerulonephritis and were obtained through the courtesy of W. R. Maxted. Type 12 (B 225) streptococci were originally isolated from a patient during an epidemic at Bainbridge, Maryland. Types 1 (K 43), 19 (J 17 D), and London (I, II) streptococci were obtained through the courtesy of Dr. George E. Murphy. Types 3 (I, II), 4, and 27 (I, II) streptococci were obtained through the courtesy of Dr. Rebecca C. Lancefield. Type 49 (B 737) streptococci were originally isolated from the nasopharynx of a child with glomerulonephritis by Dr. Lewis Wannamaker. Type 41 streptococci and strain A 648 were isolated from the nasopharynges of children with pharyngitis.

Rotation of Streptococci with Human Blood

Lyophilized streptococci were grown overnight in 40 ml. of Todd-Hewitt broth. A sample of culture (0.2 ml.) was transferred to 5 ml. of Todd-Hewitt broth to which 0.2 ml. of defibrinated blood from a normal rabbit had been added. This culture was incubated for 4 hours and then diluted in 10-fold steps in Todd-Hewitt broth to a final dilution of 10^{-10}. Samples (0.1 ml.) of the 10^{-1} to 10^{-10} dilutions of culture were transferred to sterile Petri dishes, and 5 per cent rabbit blood agar was added. Hemolytic colonies were counted the following day. A loop of the 10^{-1} dilution of culture was streaked on the surface of 5 per cent rabbit blood agar to observe the form of colonies arising from bacteria of what will henceforth be referred to as the "parent" culture. Samples (0.1 ml.) of various dilutions of culture, ranging from 10^{-1} to 10^{-4}, were transferred to 2.4 ml. of freshly drawn, lightly heparinized blood from normal adult humans without antibodies to the types of streptococci used. The mixture of streptococci and human blood was placed in a 10 by 75 mm. Pyrex tube sealed with a siliconized rubber stopper and rotated end over end at 8 r.p.m. at 37° C. for 3 hours. Cultures of streptococci arising from microorganisms rotated for 2 periods of 3 hours each with fresh human blood will be referred to as "selected" cultures.

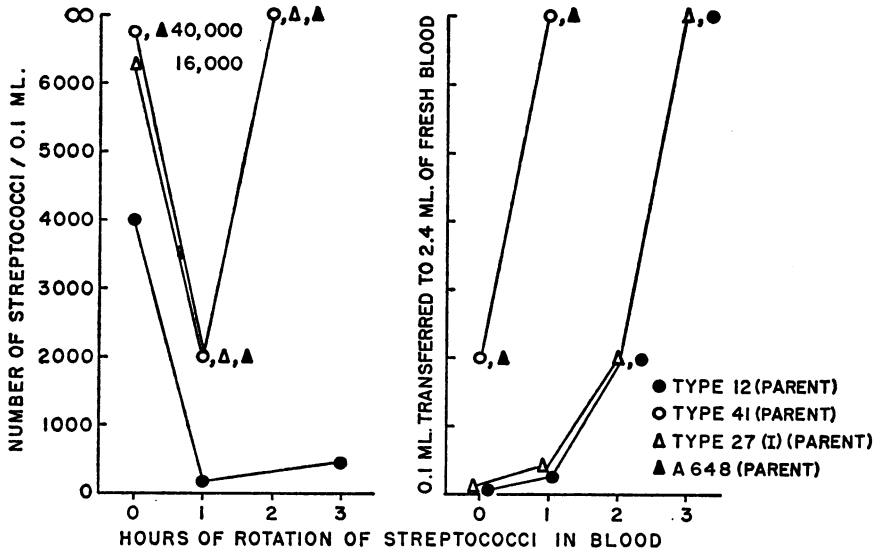
Sampling of Streptococci Rotated with Human Blood and Their Transfer to Fresh Blood

At hourly intervals rotation was stopped for approximately 1 to 2 minutes, during which a loopful of the mixture was streaked on the surface of a 5 per cent rabbit blood agar plate and 0.1 ml. of the mixture placed in a Petri dish with 5 per cent rabbit blood agar. Colonies were counted on the following day. Plates in which complete hemolysis had occurred were recorded as containing an infinite number of colonies. Colonies on the surface of blood agar plates were also examined after 18 hours' incubation at 37° C. and their surface was classified as glossy, matt or mucoid. After 1 period of 3 hours' rotation, 0.1 ml. of the mixture was transferred to 2.4 ml. of freshly drawn, lightly heparinized human blood, and rotation was carried out as described above for a second period of 3 hours. Occasionally tubes of blood for second or subsequent periods of rotation were inoculated with bacteria

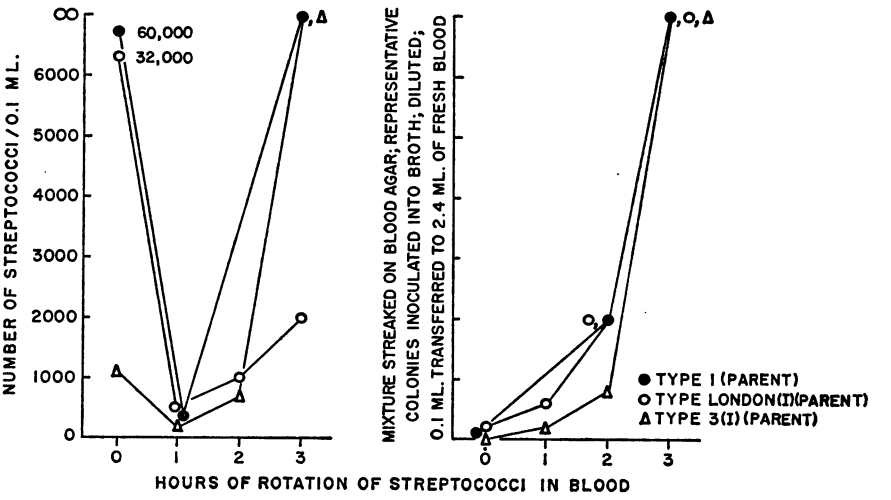
derived from micro-organisms streaked on blood agar after an earlier period of rotation with human blood.

Preparation of Extracts of M-Protein and Semiquantitative Determination of M-Protein Content

From blood agar plates streaked after 2 or more periods of rotation of streptococci with human blood, several colonies were inoculated into 40 ml. of Todd-Hewitt broth. Morphologically these colonies were representative of the majority of colonies on the plate. Colonies from the blood agar plate streaked with parent culture were treated similarly. After 18 hours' incubation the turbidity of all cultures was measured



TEXT-FIG. 1. Selection of streptococci resistant to destruction in normal human blood.



TEXT-FIG. 2. Selection of streptococci resistant to destruction in normal human blood.

in a Coleman Jr. spectrophotometer. Cultures of approximately the same density were centrifuged. Acid extracts of the bacterial sediments were made according to the method of Lancefield,[7] brought to equal volume, and serially diluted in physiologic saline of pH 7.4 containing a phosphate buffer. Dilutions of these extracts were tested in capillary tubes for precipitation with standard homologous, absorbed, type-specific rabbit antiserums, graciously supplied by Dr. Rebecca C. Lancefield.

RESULTS

When large numbers of streptococci of parent cultures of types 1, 3, 12, 27 (I), 41, London (I) and strain A 648 were rotated with freshly drawn human blood, all but relatively few streptococci were destroyed. The survivors and their progeny were much more resistant to destruction in human blood than were the vast majority of micro-organisms of the parent cultures (Text-figs. 1 and 2). Colonies formed by streptococci rotated with blood for longer periods were mucoid, whereas those rotated for shorter periods formed matt colonies. Surviving micro-

TABLE I

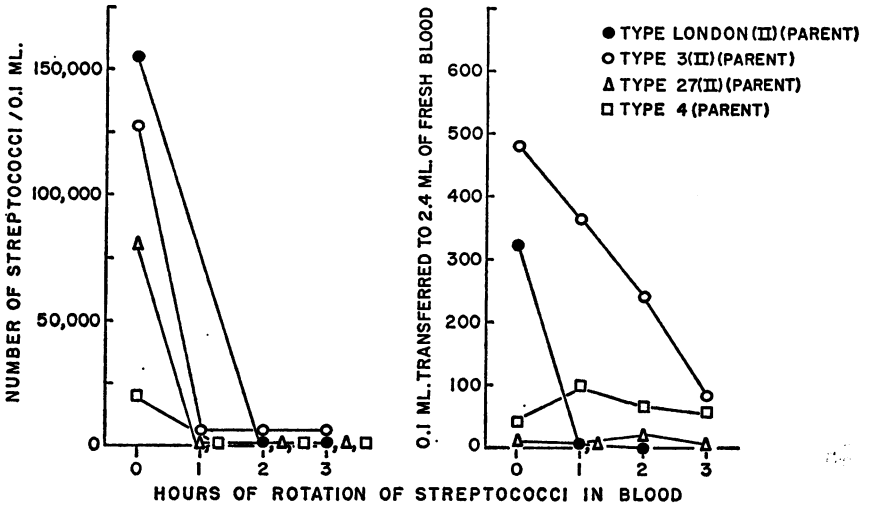
M-PROTEIN CONTENT AND COLONY FORM OF PARENT AND SELECTED STREPTOCOCCI

Streptococcus	No. of 3-hour periods of rotation with human blood *	Colony form	Greatest dilution of M-protein extract precipitating with type-specific antiserum
Type 1 (Parent)	0	Glossy	1:1
	1	Matt	
	2	Mucoid	1:64
Type 3 (I) (Parent)	0	Glossy	1:1
	1	Matt	
	2	Matt	1:16
Type 4 (Parent)	0	Glossy	1:1
	1		
	2	Glossy	No precipitate
Type 12 (B 225) (Parent)	0	Glossy	1:4
	1	Matt	
	2	Mucoid	1:128
Type 27 (I) (Parent)	0	Glossy	No precipitate
	1	Matt	
	2	Matt	1:4
Type 41 (Parent)	0	Glossy	1:1
	1	Glossy-matt	
	2	Glossy-matt	1:16
Type London (I) (Parent)	0	Glossy	No precipitate
	1	Matt	
	2	Mucoid	1:16

* Cultures of streptococci arising from micro-organisms rotated for 2 periods of 3 hours each with fresh human blood are referred to as "selected" cultures.

organisms and their progeny contained as much as 64 times more M-protein than streptococci of parent cultures (Table I). It was not possible to type streptococci of strain A 648 either before or after rotation with human blood. Because survivors of this strain and their progeny were more resistant to destruction in human blood than the vast majority of micro-organisms in the parent culture, it is probable that they contained significantly more M-protein which could not be measured because antiserum against this type was not available.

Streptococci of parent cultures of types 3 (II), 4, 27 (II) and London (II) were destroyed during rotation with human blood. These cultures apparently contained no resistant streptococci (Text-fig. 3). The prog-



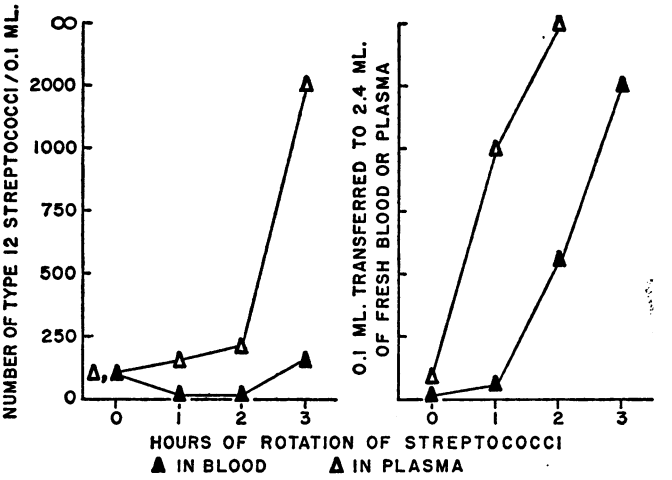
TEXT-FIG. 3. Destruction in normal human blood of streptococcal populations containing no resistant micro-organisms.

eny of a few type 4 streptococci that escaped destruction contained no more M-protein than the parent type 4 streptococci (Table I).

Parent cultures were rotated either with fresh blood or plasma from the same donor to ascertain whether the presence of phagocytes was necessary for the occurrence of the effects described above. In contrast with destruction of parent type 12 streptococci demonstrated at the end of 1 hour of rotation with human blood (Text-fig. 1), no demonstrable destruction of parent type 12 streptococci occurred during rotation with plasma (Text-fig. 4). Streptococci that had been rotated in plasma contained no more M-protein than streptococci of parent cultures and only 1/64 as much M-protein as streptococci that had survived rotation with freshly drawn human blood (Table II). Furthermore, colonies of strepto-

cocci of parent cultures and of micro-organisms that had been rotated with plasma were glossy. Colonies of streptococci that had been rotated with blood were slightly matt.

When types 1 (K 43), 19, and 49 streptococci, known before these experiments to form matt colonies and to resist phagocytic destruction



TEXT-FIG. 4. Growth of parent type 12 streptococci during rotation with normal human blood or normal human plasma.

TABLE II

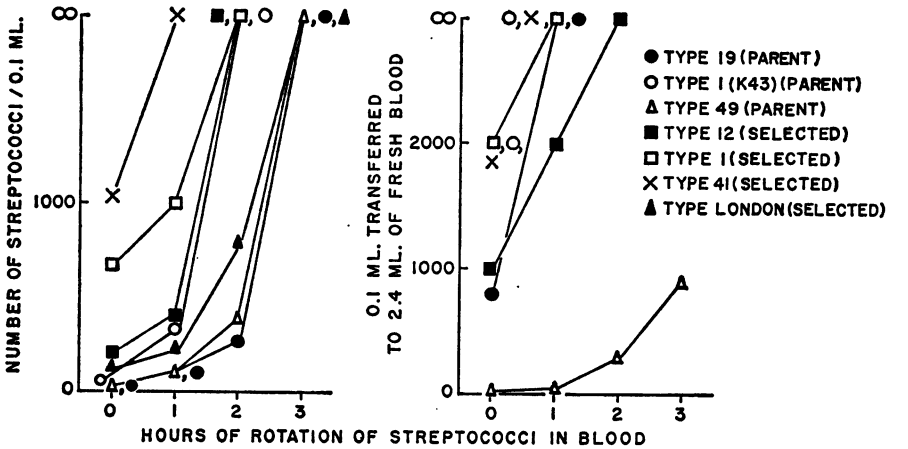
M-PROTEIN CONTENT AND COLONY FORM OF PARENT TYPE 12 STREPTOCOCCI AND PROGENY SURVIVING ROTATION WITH HUMAN BLOOD OR PLASMA

Streptococcus	Method of culture	Colony form	Greatest dilution of M-protein extract precipitating with type-specific antiserum
Type 12 (B 225) (Parent)	24 hr. growth in Todd-Hewitt broth	Glossy	1:1
	Two 3-hr. periods of rotation with human plasma	Glossy	1:1
	Two 3-hr. periods of rotation with human blood	Slightly matt	1:64

in human blood, were rotated with human blood, no demonstrable destruction of micro-organisms occurred (Text-fig. 5). These streptococci formed mucoid rather than matt colonies on the surface of blood agar but did not contain more M-protein than the streptococci of the parent cultures (Table III).

Types 1, 41, London (I), and 12 streptococci that had survived 2 periods of rotation with human blood contained more M-protein and were more resistant to phagocytosis than streptococci of the parent cultures. When these selected micro-organisms were rotated with human blood for additional periods, no demonstrable destruction of streptococci occurred (Text-fig. 5).

The amount of M-protein in type 12 streptococci surviving these additional periods of rotation with human blood was less than in type 12 streptococci that had survived the first 2 periods of rotation. The amount of M-protein in selected types 1, 41, and London (I) streptococci that survived periods of rotation additional to the first 2 periods was the same as in streptococci of these types that survived the first 2 periods of rotation (Table III). Types 1, 12, 41, and London (I) streptococci



TEXT-FIG. 5. Growth of selected streptococci and parent streptococci resistant to destruction in normal human blood.

that survived periods of rotation additional to the first 2 periods formed matt and mucoid colonies on the surface of blood agar (Table III).

DISCUSSION

Streptococci rich in M-protein emerged from populations of group A streptococci poor in M-protein during rotation with freshly drawn, lightly heparinized blood from normal adult humans without antibodies to the types of streptococci used. The large majority of streptococci were destroyed. The progeny of those few streptococci that survived were rich in M-protein. When streptococci rich in M-protein were rotated in human blood, the large majority of micro-organisms survived and their progeny had no more M-protein and, on one occasion, less.

These observations indicate that the quantity of M-protein present in the progeny of streptococci surviving rotation with human blood is determined by the amount of M-protein present in those streptococci which have at least enough M-protein to survive phagocytic destruction. They further indicate that rotation of streptococci with blood does not induce M-protein production.

Many investigators have demonstrated that streptococci with hyaluronic acid capsules were somewhat more resistant to phagocytosis than nonencapsulated streptococci containing a similar amount of

TABLE III

M-PROTEIN CONTENT AND COLONY FORM OF PARENT AND SELECTED STREPTOCOCCI RESISTANT TO PHAGOCYTIC DESTRUCTION BEFORE AND AFTER ROTATION WITH HUMAN BLOOD

Streptococcus	No. of 3-hour periods of rotation with human blood	Colony form	Greatest dilution of M-protein extract precipitating with type-specific antiserum
Type 1	0	Glossy	1:4
(Selected)	1	Matt	
	2	Matt-mucoid	1:4
Type 1 (K 43)	0	Matt	1:4
(Parent)	1	Matt	
	2	Matt-mucoid	1:4
Type 12 (B 225)	0	Glossy	1:64
(Selected)	1	Matt	
	2	Mucoid	1:4
Type 19 (J 17 D)	0	Matt	1:64
(Parent)	1	Matt	
	2	Matt-mucoid	1:64
Type 41	0	Matt	1:16
(Selected)	1	Matt-mucoid	
	2	Mucoid	1:16
Type 49 (B 737)	0	Matt	1:2
(Parent)	1	Matt-mucoid	
	2	Mucoid	1:2
Type London (I)	0	Mucoid	1:16
(Selected)	1	Mucoid	1:16

M-protein.[8-12] Wilson [13] has demonstrated that the surface configuration of colonies of group A streptococci depends largely upon whether or not the cocci form capsules. In his experiments matt colonies contained fewer encapsulated cocci than did mucoid colonies, and nonencapsulated streptococci formed glossy colonies. In experiments reported here streptococci rich in M-protein that were streaked on blood agar formed matt and mucoid colonies. Matt colonies were formed by micro-organisms

rotated with blood for fewer hours than those that formed mucoid colonies. This observation suggests that streptococci capable of forming capsules were selected from populations composed largely of micro-organisms less capable of forming capsules in much the same way that micro-organisms rich in M-protein were selected from populations composed largely of micro-organisms poor in M-protein.

It is noteworthy that streptococci poor in M-protein were rapidly eliminated from the population during rotation with human blood. As a result, streptococci rich in M-protein and resistant to phagocytic destruction began to predominate in the population within 1 to 2 hours. If a similar process of natural selection occurs when a human being is infected with group A streptococci, the future of the infection, including the development of type-specific immunity, production in the host of other streptococcal substances, formation of antibodies to these products, and perhaps the development of nonsuppurative sequelae may all to some extent be determined during the first few hours of infection.

Summary

Streptococci rich in M-protein and resistant to phagocytic destruction emerged from populations of group A streptococci poor in M-protein and susceptible to phagocytic destruction during rotation with freshly drawn, lightly heparinized human blood. These selected streptococci tended to grow in matt and mucoid colonies on the surface of blood agar, whereas colonies of the parent micro-organisms were glossy.

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