PERSISTENCE OF TYPE-SPECIFIC ANTIBODIES IN MAN FOLLOWING INFECTION WITH GROUP A STREPTOCOCCI

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A given individual once infected with a Group A streptococcus of any serological type rarely is reinfected with an organism of the same type. Presumably the low incidence of reinfection is due to type-specific immunity correlated with the persistence of type-specific antibodies. The duration of this immunity following a streptococcal infection has not been established. An approach for obtaining laboratory evidence for the duration of immunity is the study of the persistence of type-specific antibodies which are usually demonstrable in patients following streptococcal infections. It has been shown by others that type-specific antibodies in some individuals persist for as long as 3 years (1–6). There are few data available covering longer periods of time.

In this study immunological and clinical data are reported on individuals who had streptococcal infections as long ago as 32 years. Many of the individuals had experienced their streptococcal infections before sulfonamide or penicillin therapy was available, which is of importance because of the possible effect of therapy on antibody response (5, 7). The causative streptococci were isolated and typed at the time of the infection, and the cultures frozen and dried. Type-specific antibodies were demonstrated by means of bactericidal tests and, in a few instances, confirmed by mouse protection tests.

Materials and Methods

1. Bactericidal Test.—The presence of type-specific antibodies in human blood was established by one or more of the following methods.

(*a*) *Direct Tests.*—When fresh heparinized blood could be obtained at a time suitable for testing, the direct bactericidal test was performed by the technique previously described (8, 9). Results are frequently better with this method than with the indirect method described in the next paragraph since use of serum and blood from two different individuals is avoided. Errors due to slight incompatibility between serum and blood cells or to the undiscovered presence of streptococcal antibodies in the "normal" individual supplying the whole blood necessary to furnish phagocytic cells are thus avoided. The elimination of this source of error by using washed leucocytes has other obvious inherent errors, and was impracticable.

(*b*) *Indirect Tests.*—In the indirect test, serum or plasma is tested for the presence of antibodies which promote phagocytosis of streptococci by human phagocytes from another donor. Usually 0.2 cc. or 0.05 cc. of test plasma or serum was used, suitably controlled with plasma or serum taken at approximately the same date from normal individuals. Serial dilutions of culture in 0.05 cc. or 0.1 cc. volume and 0.3 cc. of fresh heparinized normal human blood were

employed as previously described (8, 9). Blood was considered normal for the purposes of the test if it did not contain demonstrable antibodies for the serological types of streptococci included in the experiment.

(*c*) *Replacement of Plasma in Normal Blood by Plasma To Be Tested.*—Occasionally the plasma of normal human blood was replaced by the test plasma, and the experiment then carried out in the same way as a direct test, using the mixture of normal blood cells and test plasma instead of whole untreated blood. It was found that this procedure had little, if any, advantage over the simpler method of merely adding a fixed amount of plasma to the whole normal blood. Due regard to compatibility of blood groups was observed by using Group O blood when blood of matching group was not available.

2. Passive Protection Tests in Mice.—In some instances the finding of type-specific antibodies by means of bactericidal tests was confirmed by passive mouse protection tests either with untreated serum or with gamma globulin concentrated by precipitation with sodium sulfate. Details are included with each experiment.

3. Collection of Blood.—Abbott's heparin sodium solution containing 1000 units/cc. (approximately 10 mg./cc.) was used: 0.1 cc. for 20 cc. of human blood. When blood was left over from direct tests, plasma was removed from the cells at the end of the day for further tests by the indirect method.

Blood for serum was collected in the usual way by venipuncture. Whenever practicable the blood was allowed to clot and retract in vaseline-lined tubes without rimming, in order to prevent hemolysis.

4. Streptococcal Cultures.—Many of the strains isolated from the patients at the time of their infections were preserved in our frozen and dried stock, and are still available. These cultures together with others of the same and heterologous types were used for testing. With the exception of cases 4, 21, and 23, all strains were typed in this laboratory. Most of them were identified at the time of the infection; others were typed later with sera not available earlier. The types of all available strains were checked at the beginning of these experiments

EXPERIMENTAL

Persistence of Type-Specific Streptococcal Antibodies in Man.—In Tables I *a*, I *b*, and I *c*, a record of the antibodies found in individuals selected for this study is presented.[1] Many of these persons were employees of this institution or were patients in the various departments of The Rockefeller Hospital. In half of the known infections, most of which occurred 15 to 32 years ago, before chemotherapeutic drugs were generally available, type-specific antibodies corresponding to the type known to have caused the infection were found. In the others with known infections, some of whom had been treated with sulfonamides or penicillin, type-specific antibodies were not demonstrable or were too weak to assess with certainty in current tests.

Tables I *a* and I *b* show the results after known infections. In addition to the findings on the presence or absence of antibody to strains of the homologous types associated with the preceding infection, data on the positive and negative reactions with strains of heterologous types are also included. Usually a number of strains of each type was tested and the experiments repeated several times.

[1] The cooperation of all these individuals who have so generously supplied me, often repeatedly, with samples of their blood is greatly appreciated.

In 12 individuals type-specific antibodies against 11 different homologous types following 14 separate infections persisted for 4 to 32 years (Types 2, 3, 6, 12, 13, 14, 15, 18, 22, 26, and 28) (Table I *a*). In 13 individuals, on the other hand, who also had had known infections 1 to 31 years previously (Table I *b*), no type-specific antibodies were demonstrable against 14 different types following 17 different infections (Types 1, 3, 8, 12, 15, 17, 18, 19, 23, 25, 36, 48, and strains H105 and 1RS85). In 3 of these individuals homologous type-specific antibodies, which had been present shortly after the infection (cases 14, 18, and 23), had disappeared at the time when these individuals were retested (footnote to Table I *b*). In 12 other individuals, although the strains causing the infections were not available, type-specific antibodies were observed. In some of these individuals no history of streptococcal infections was obtained, in others scarlet fever or severe pharyngitis was known to have occurred. Many in this group had antibodies against several types. Antibodies in various combinations were found against the following: Types 2, 3, 6, 8, 12, 13, 15, 19, 36, 43, and Strains B514, C36, and B393. One individual, case 30 (Table I *b*), had demonstrable antibodies against 7 specific types or strains, although none resulting from the only known preceding infection.

In one patient multiple infections with different types were observed, case 10 (Table I *c*). The persistence of antibodies in this individual was not related to the severity of infection. Following 3 of the 5 infections, antibodies persisted for many years, whereas in the other two, none was demonstrable. In addition, in the absence of any known infections, antibodies to two other types were also present.

In the course of this study in some individuals no type-specific antibodies were demonstrated. These individuals were only tested against a limited number of strains, and type-specific antibodies might have been demonstrable if more types had been included.

These observations are not sufficiently extensive to draw conclusions as to the prevalence of streptococcal types. They are recorded to show that streptococcal antibodies are frequently present in normal adults and persist for many years.

Examination of the data and of the clinical records of the individuals studied gave no suggestion that the persistence of type-specific antibodies was correlated with the severity of the infection or with the serological type of the homologous streptococcus. This is in contrast to the findings of others that the *initiation* of type-specific antibody response is closely correlated with the failure to eradicate streptococci from the throats of patients (5, 7). The interval since the infection occurred was, however, found to be of some importance in the present study of persistence of type-specific antibodies. In the few instances in which data were available on this point, there was some evidence of a gradual decline in titer with time (cases 9, 10, 18, and 23). This finding is in agreement

TABLE I *a*

Type-Specific Antibodies in Human Sera Following Infections with Group A Streptococci of Known Types

(*a*) Presence as demonstrated in current bactericidal tests.

Case No.	Streptococcal infection: serolgical type	Interval since infection	Homologous type-specific antibodies	Heterologous type-specific antibodies	
			Present	Present	Absent
		yrs.			*Types tested*
1	Type 14	32	Type 14 (+++)		1, 3, 6, 18, 19, 26
2	" 26	22	" 26 (+++)	Type 3 (+++)	1, 6, 14, 18, 19
3	" 3	21	" 3 (++)		1, 6, 14, 19, 22, 26
4	" 13	22	" 13 (++)	Type 3 (±) " 48 (+)*	2, 6, 18, 22, 28, 49
5	" 12	32	" 12 (+)		1, 3, 26
6	" 15	21	" 15 (++)	Type 12 (++)	22, 49
7	" 22	21	" 22 (+++)	" 8 (++)	1, 4, 15, 6
	Strain D215‡	19	Not tested, strain lost		
8	Type 22	15	Type 22 (+++)		4
	" 41	6	Not tested, no serum		
9§	" 26	15	Type 26 (+++)	None tested	
10‖	" 2	22	" 2 (+)	Type 13 (+)	1, 4, 11, 12, 14, 17,
	" 6	17	" 6 (+++)	" 15 (++)	18, 19, 22, 23, 26,
	" 28	1	" 28 (+++)		27, 36, 38, 40, 41,
		4	" 28 (±)		43, 44, 48, 49
12	" 6¶	9	" 6 (+++)	Type 2 (+)	1950: 4, 5, 14, 22, 24 1951: 1, 5, 14, 24 1956: 4, 5, 13, 48, 49**
13	" 18¶	7	" 18 (++++)	Type 12 (++) " 19 (+++)	22

In all tables ± to ++++ indicates estimate of strength of reactions.

* Known cross-relationship between Types 13 and 48 (8).

‡ This strain, isolated at the time of the infection, could not be typed with the available sera. It has since been lost.

§ Serial bleedings were still available from case 9 (a patient in the rheumatic fever series) dating from 1949, 7 years after her infection in 1942, to the present. When tested in 1957 these showed a gradual decline in titer, but her blood still contained readily demonstrable antibodies.

Tests on her serum were made by Dr. Rothbard at the time of her infection with strain C118 (see Tables I, II, IV, and V of his paper (2)). These earlier sera had been used up so that none before 1949 was available for the current tests.

‖ See also Tables I *b* and I *c*.

¶ In this infection the patient was treated with penicillin.

** Dr. Floyd Denny kindly furnished records of tests on this patient which he made in 1950 and 1951. Type 6 was also positive in his tests, as well as in the current tests done in this laboratory in 1956.

TABLE I *b*

Type-Specific Antibodies in Human Sera Following Infections with Group A Streptococci of Known Types

(*b*) Absence as demonstrated in current bactericidal tests.

Case No.	Streptococcal infection: serological type	Interval since infection	Occurrence of homologous type-specific antibodies	Heterologous type-specific antibodies absent
				Types tested
10*	Type 8	22 yrs.	None present	See Table I *a*
	" 3‡	20 "	" "	
14	Type 12‡	1 "	Type 12 (++++)	6, 28
		3½ "	None present	
18	Strain 1RS85‡	9–22 wks.	Strain 1RS85 (++)§	1942: 6, 28§
		2 yrs.	None present§	1944: 6, 28§
		14 "	" "	1955: 28
19	Type 23	27 yrs.	None present	None tested
20	" 36	23 "	" "	28
21	" 1	31 "	" "	2
22	" 25	20 "	" "	8, 36
23	" 36	6 mos.	Type 36 (++)‖	None tested
		17 yrs.	None present	
24	" 17‡	12 yrs.	None present	None tested
25	" 48	1 "	" "	1956: 2, 13, 28, 44
26	" 3‡	14 "	" "	None tested
27	" 15	18 "	" "	3, 18, 19, 26
	" 1	16 "	" "	
	" 19	11 "	" "	
	" 18	10 "	" "	
30¶	Strain H105‡	1 yr.	" "	1, 3, 4, 12, 14, 15, 17, 18, 19, 22, 23, 27, 28, 38, 48, 49, strain B514

* See also Tables I *a* and I *c*.

‡ In this infection the patient was treated with sulfonamides or penicillin.

§ Tested 1942 and 1944 by Dr. S. Rothbard.

‖ Tested 1942 by Dr. A. G. Kuttner (case 15 in reference 1). I am indebted to Dr. Rothbard and Dr. Kuttner for their records, and to Dr. Harrison F. Wood for supplying me with current serum from case 23.

Case 30 had antibodies without history of known infections for the following types: Type 2 (+), Type 6 (+++), Type 13 (++), Type 36 (+++), Type 43 (+++), strain C36 (+++), strain B593 (+++).

TABLE I *c*

Type-Specific Antibodies in Human Serum Following Infections with Group A Streptococci of Known Types

(*c*) Variable occurrence after multiple infections in one individual as demonstrated in current bactericidal tests.

Streptococcal infections Case 10		Interval since infection	Occurrence of type-specific antibodies	Heterologous type-specific antibodies absent
Clinical findings	Serological types			
		yrs.		*Types tested*
Severe pharyngitis	Type 2	22	Type 2 (+)	1, 4, 11, 12, 14, 17, 18, 19, 22, 23, 26, 27, 36, 38, 40, 41, 43, 44, 48, 49, and numerous unclassified strains
Superficial infection of finger	" 8	22	None present	
Severe pharyngitis and facial erysipelas*	" 3	20	" "	
Moderate pharyngitis	" 6	17	Type 6 (+++)	
" "	" 28	1	" 28 (+++)	
		2	" " (++)	
		4	" " (±)	
Preceding infection unknown		Unknown	Type 13 (+)	
" " "		"	" 15 (++)	

* In this infection the patient was treated with sulfanilamide.

with the findings of others (1, 2, 4, 5). On the other hand, several of the strongest reactions were obtained in individuals studied 15 to 32 years postinfection.

Two of the most severe infections resulted from cuts on the hand followed by heavy accidental inoculation of streptococci. Homologous type-specific antibodies were readily demonstrated 22 years later in one of these individuals (case 2). None was found, however, in current tests with the serum of the other individual (case 19), who had had a severe infection 27 years earlier. In another severe case (case 1), the patient was hospitalized for several weeks with a peritonsillar abscess. He still has good antibody after 32 years, while in case 10 with a severe Type 3 infection, the individual after 20 years has no demonstrable Type 3 antibody.

The persistence of antibody response to any given streptococcal infection was, therefore, entirely unpredictable: long persisting antibodies in some instances followed clinically mild infections, whereas severe infections might produce no response, or only one of low titer.

The serum of case 5 in current bactericidal tests showed weak, but definite

TABLE II

Examples of Tests for Type-Specific Antibodies in Human Sera Demonstrable Many Years after Known Infections with Group A Streptococci
Direct or Indirect Bactericidal Tests

Strains tested	Conditions of test*	Results of test						Interval since infection
		No. of colonies				Antibody found		
		Inoculum						
Type 14	No. of streptococci inoculated	28	8	3	1			
Strain S23/101 Homologous to case 1	No. of streptococci at end of test with human serum or plasma from:	*Bactericidal tests*						
	Case 1	7	0	9	2	+++	Type 14	32 yrs.
	" 2	∞	C	700	350	No	" "	—
	" 3	∞	∞	C	240	"	" "	—
	" 30	∞	C	1000	188	"	" "	—
		Inoculum						
Type 26	No. of streptococci inoculated	114	21	12	2			
Strain J17F/90 Homologous to case 2	No. of streptococci at end of test with human serum or plasma from:	*Bactericidal tests*						
	Case 1	∞	∞	225	200	No	Type 26	—
	" 2	500	40	3	0	+++	" "	22 yrs.
	" 3	∞	C	1000	400	No	" "	—
	" 30	∞	C	100	47	"	" "	—
		Inoculum						
Type 3	No. of streptococci inoculated	101	50	23	13			
Strain D121 Homologous to case 3	No. of streptococci at end of test with human serum or plasma from:	*Bactericidal tests*						
	Case 1	C	C	C	485	No	Type 3	—
	" 2	0	0	0	0	+++	" "	No known Type 3 infection
	" 3	24	0	0	0	++	" "	21 yrs.
	" 30	C	C	500	340	No	" "	—

The control, case 30, has antibodies to other types (infection unknown), Types 2, 6, 13, 36, 43, strains C36, B593.

* *For direct tests*, each tube contained:
0.1 cc. culture dilution.
0.3 cc. fresh heparinized blood.

TABLE II—(*Concluded*)

For indirect tests, each tube contained either:

(1) 0.2 cc. test plasma or serum.
0.05 cc. culture dilution.
0.3 cc. fresh heparinized normal blood.

or (2) 0.05 cc. test plasma or serum.
0.1 cc. culture dilution.
0.3 cc. fresh heparinized normal blood.

At the end of 3 hours' rotation at 37°C., 0.1 cc. of each mixture was plated in blood agar pour plates for counts of surviving organisms.

The culture dilutions were plated at the beginning of the test to determine the size of the inoculum.

In all tables:

∞ indicates innumerable colonies with blood completely hemolyzed.

C indicates confluent growth with some areas of unhemolyzed blood.

2000 to 500 colonies were estimated by comparison.

Colonies on plates with less than 400 were usually counted.

In the strain designations, the figure preceding the slanted line is the strain number, and the figure following the slanted line indicates the number of mouse passages.

For further details, see Methods.

Type 12 antibody. This case was of special interest because the infection was followed by an attack of glomerulonephritis in 1926. The strain (K235) obtained from Dr. W. T. Longcope in 1932, at that time could not be typed. When this culture was reexamined in 1958, no M antigen was demonstrable by precipitin reactions. The absence of M antigen was confirmed by the failure of the strain to grow in rotated tubes containing normal human blood (8, 9). In spite of 129 serial mouse passages made in an attempt to identify its type-specific component, no M antigen was demonstrable. The strain, therefore, could not be typed by the anti-M precipitin reaction. However, a 10 T antigen was identified by means of agglutination reactions. The 10 T antigen has never been observed except in Type 12 strains. Strain K235, therefore, was classified as probably belonging to Type 12. Dochez's widely used strain, N.Y.5, has a similar antigenic composition, 12 M and 10 T, as have several other known strains isolated during this period (10).

In recent years, Type 12 has been frequently associated with acute glomerulonephritis. These strains, however, in contrast to strain K235, are characterized by the antigenic composition 12 M, 12 T. Strains containing 12 M and 10 T antigens are extremely rare at the present time.

Examples of Type-Specific Bactericidal Reactions Demonstrable Many Years after Streptococcal Infections of Known Type.—As shown in Table II, the growth of Type 14 was specifically inhibited by the serum of case 1 obtained from an individual with a known infection due to this type which had occurred 32 years before. The sera of three other individuals failed to inhibit the growth of this strain.

TABLE III

Correlation between Results of Bactericidal and Protection Tests in Demonstrating Type-Specific Antibodies in Human Sera Following Infections with Group A Streptococci

Passive Protection Tests in Mice with Human Serum

Human serum 1 cc.	Group A streptococci tested	Results of protection tests* Dose of culture, *cc.*																					
		10^{-6}						10^{-6}												10^{-7}									
Antibody	Strain and type	Mouse No. 1	2	3	4	5	6	Mouse No. 1	2	3	4	5	6	7	8	9	10	11	12	Mouse No. 1	2	3	4	5	6	7	8	9	10
Type 3 (case 29)§	T3/60, Type 3	6	S	S	S	S	S‡	6	6	S	S	S	S	S	S	S	S	S		1	S	S	S	S					
	S43/137, " 6							1	1	1										1	1	1	1						
	T13/51, " 13	1	1	1	1			1	1	1	1	1	1	2	S					1	1	1	1	1	1	2	S		
Type 6 (case 10)‖	T3/60, Type 3	1	1	1				1	1	1	1	1	1	1	1	1	1			1	1	1	1	2					
	S43/137, " 6	1	1					1	S	S	S	S	S	S	S					1	S	S	S	S	S	S	S		
	T13/51, " 13	1	1	1	1			1	1	1	1	1	1	1	1	1	1	1	S	1	1	1	1	1	2	2	4	S	S
Type 13 (case 4)‖	T3/60, Type 3	1	S	S				1	1	1	1	6	6	S						1	1	1	2	5	S	S	S		
	S43/137, " 6							1	1	1	S																		
	T13/51, " 13	1	1	1	1			1	1	1	S	S	S	S	S					1	1	2	S	S	S	S	S	S	S

* Data collected from tests made in several different experiments.

‡ Results are recorded as follows: Numeral indicates day of death and S indicates survival of individual mice.

Mice were injected intraperitoneally with 1 cc. of human serum or of slightly concentrated gamma globulin, on the day before the culture dilutions in 0.5 cc. volume were injected by the same route.

After 10 days, surviving mice were discarded.

Suitable virulence controls were included in all tests, showing that these cultures in the absence of serum killed mice regularly in a dose of 10^{-7} cc.

Rabbit immune serum of homologous type protected mice against infection with the homologous type streptococcus but not with the heterologous types.

§ Case 29 had Type 3 antibody (by bactericidal tests), but no infection of known type. In 1927 he was hospitalized for 4 weeks with scarlet fever, but bacteriological studies were not made. In current studies bactericidal tests were positive with Type 3 strains and negative with 25 other types tested.

‖ Cases 10 and 4 had known infections (see Table I *a*) with streptococci of Types 6 and 13 respectively. Bactericidal tests showed slight Type 13 antibody in case 10 serum, and slight Type 3 antibody in case 4 serum.

The growth of Type 26 was specifically inhibited by the serum of case 2 obtained from an individual with a known infection 22 years before, due to this type. The sera of three other individuals failed to inhibit the growth of this strain.

The growth of Type 3 was specifically inhibited by the serum of case 3 obtained from an individual who had had a known infection 21 years before, due to this type. This strain was also inhibited by the serum of case 2 obtained from an individual who had had no known infection with this type. In contrast to the sera obtained from cases 1, 2, and 3, that of case 30 had no demonstrable antibodies for the three types studied, although this individual had antibodies for 7 other types or strains (Table I *b*).

Passive Protection Tests in Mice with Human Sera.—In a previous paper a close correlation between the occurrence of protective antibodies and antibodies promoting phagocytosis in bactericidal tests was demonstrated with rabbit immune sera (9). A similar correlation has now also been shown with human sera whenever these "bactericidal" antibodies are demonstrable in moderate titer, irrespective of whether the antibodies resulted from a known infection with the homologous type or appeared in the absence of such data on the preceding infection.

In Table III examples of these findings are given. In case 29 the serum contained Type 3 antibodies without bacteriological data on an infection of the individual with streptococci of this type. He had a history of scarlet fever in 1927, but no throat cultures were taken. His serum, as well as the gamma globulin fraction separated from it by standard procedures of sodium sulfate precipitation, protected mice against infection with Type 3 streptococci in doses of 10^{-5} to 10^{-7} cc. of culture. Further data not included in the table showed that half of the mice receiving 10^{-3} cc. and 10^{-4} cc. of culture also survived in these protection tests. The serum or gamma globulin fraction from cases 10 and 4 showed no significant protection against infection with Type 3 streptococci. Cases 10 and 4, however, possessed antibodies for Types 6 and 13 respectively with which they were known to have been infected 17 and 22 years before. In passive protection experiments, the sera of these two individuals each protected mice against their homologous strains, although not in high titer. The small amount of Type 13 antibody in case 10 serum, or the trace of Type 3 antibody in case 4 serum, detectable by bactericidal tests, was probably insufficient to give convincing evidence of passive protection in mice.

Methods of Measuring Content of M Antigen in Group A Streptococci.—

(*a*) *Ability to Resist the Bactericidal Effect of Normal Human Blood in Bactericidal Tests.*—In previous papers it was shown that the ability of Group A streptococci to produce M antigen determines whether or not they can resist phagocytosis and grow in normal human blood in bactericidal tests (8, 9).

These observations have now been extended by using this property as a rough quantitative measure of the M antigen produced by a strain.

Phagocytosis, in the bactericidal system used, only occurs when the leucocytes are in constant contact with the streptococci (11). A comparison is made in Table IV *a* between duplicate sets of tubes containing normal human blood

TABLE IV *a*

Methods of Measuring Content of M Antigen in Group A Streptococci

(*a*) Ability to resist bactericidal effect of normal human blood.

Direct Bactericidal Tests

Strains tested	Conditions of test*	Results of test (No. of colonies)			
		Inoculum			
Type 3 Strain D58X/11 M antigen ++++±	No. of streptococci inoculated	79	29	12	4
	No. of streptococci at end of test with normal human blood	*Bactericidal test*			
	(*a*) rotated tubes	∞	C	700	200
	(*b*) stationary tubes	∞	∞	700	146
		Inoculum			
Type 3 Strain C203/42 M antigen +++	No. of streptococci inoculated	162	41	6	2
	No. of streptococci at end of test with normal human blood	*Bactericidal test*			
	(*a*) rotated tubes	1000	500	7	0
	(*b*) stationary tubes	∞	C	400	201
		Inoculum			
Type 3 Strain D121 M antigen ++	No. of streptococci inoculated	113	44	3	1
	No. of streptococci at end of test with normal human blood	*Bactericidal test*			
	(*a*) rotated tubes	730	204	0	0
	(*b*) stationary tubes	∞	700	500	66
		Inoculum			
Type 3 Strain F208 No M antigen	No. of streptococci inoculated	∞	85	14	3
	No. of streptococci at end of test with normal human blood	*Bactericidal test*			
	(*a*) rotated tubes	6	0	0	0
	(*b*) stationary tubes	∞	1000	175	50

* Each tube in these tests contained
0.1 cc. culture dilution.
0.3 cc. fresh heparinized normal human blood.
For further details of tests, see Methods and Table II.

and serial dilutions of culture in which one set is rotated during incubation and the other set is kept stationary. The amount of growth in the pairs of tubes is a reflection of the amount of M antigen produced. With maximal amounts of M antigen, the streptococci are protected from phagocytosis even in rotated tubes, and growth in the two sets of tubes is identical and is initiated by very small inocula. With strains containing no demonstrable M antigen, the cultures in stationary tubes grow out normally from small inocula but in the rotated tubes even the cultures with large inocula are quickly sterilized.

Since intermediate growth occurs in the rotated tubes when the precipitin tests show intermediate amounts of M antigen, this technique can yield valuable information as to the M-producing properties of strains. The extremes of maximal M and complete absence of M have been illustrated in a preceding paper (9), but in the present experiments, useful strains were sought in the intermediate range. Table IV *a* shows the growth of different strains in rotated and stationary tubes compared with an estimate of M antigen from the precipitin reaction. With strain D58X/11 which gave the best precipitin reactions, the two sets of tubes were indistinguishable in growth. Strains C203/42 and D121 showed progressively less growth in the rotated sets, and the M^- strain (12), F208, only had 6 colonies left from the largest inoculum (rated ∞ in the table).

By means of this biological assay, therefore, as well as by M precipitin reactions, Strain F208 would be eliminated from any experiment requiring an M^+ strain (12). Strain D58X/11 would be used whenever maximal M antigen is required; for example, in bactericidal tests of sera with high antibody content, or for virulence or immunization experiments. Strains C203/42 and D121, which show an intermediate amount of M and growth in normal human blood, would be selected as test strains with human sera containing antibody in low concentration.

(*b*) *Determination of the Amount of Antibody Necessary to Inhibit Growth of Streptococci in Bactericidal Tests.*—The best M^+ strain, D58X/11 (Table IV *a*), and an intermediate strain, D121, were tested against serial dilutions of Type 3 rabbit antiserum. The results in Table IV *b* show that Strain D58X/11 requires at least 4 or 5 times as much antibody as Strain D121 for equivalent inhibition of growth in bactericidal tests: a 1:25 dilution of rabbit antiserum has essentially the same effect on strain D58X/11 as a 1:100 dilution has on strain D121. This indicates that Strain D58X/11 has more M antigen to combine with antiserum than is the case with Strain D121, a conclusion in agreement with the results of comparing the growth of these strains in rotated and stationary tubes.

In numerous tests not included in the table, it was observed that in the stationary sets the presence of antibody had no effect on growth even of M^+ cultures, the growth in comparable series of stationary tubes being indistinguishable whether they contained normal or immune serum. In rotated tubes, on the other hand, immune sera markedly enhanced the destruction of M^+ strains but had no more effect than normal serum on M^- strains.

TABLE IV *b*

Methods of Measuring Content of M Antigen in Group A Streptococci

(*b*) Amount of antibody necessary to inhibit growth in bactericidal tests.

Indirect Bactericidal Tests

Strains tested	Conditions of test*	Results of test		
		No. of Colonies		
		Inoculum		
Type 3 Strain D58X/11 M antigen +++±	No. of streptococci inoculated	68	23	2
	No. of streptococci at end of test with normal human blood and rabbit serum:	*Bactericidal test*		
	Type 3 anti-M serum 1:1	1	0	0
	" " " 1:5	48	0	0
	" " " 1:25	212	20	2
	" " " 1:50	500	104	120
	" " " 1:100	C	C	250
	" " " 1:200	∞	C	500
	Normal rabbit serum 1:1	∞	C	500
		Inoculum		
Type 3 Strain D121 M antigen ++	No. of streptococci inoculated	61	20	2
	No. of streptococci at end of test with normal human blood and rabbit serum:	*Bactericidal test*		
	Type 3 anti-M serum 1:1	0	0	0
	" " " 1:5	3	0	0
	" " " 1:25	1	6	0
	" " " 1:50	83	8	4
	" " " 1:100	129	18	0
	" " " 1:200	∞	500	31
	Normal rabbit serum 1:1	∞	C	500

* Each tube in these tests contained

0.05 cc. dilution of rabbit serum.

0.1 cc. culture dilution.

0.3 cc. fresh heparinized normal human blood.

For further details of tests, see Methods and Table II.

The results of these tests show that Strain D58X/11 requires 4 to 5 times as much antibody as Strain D121 for equivalent inhibition of growth in bactericidal tests: *e.g.* the 1:25 serum dilution has about the same effect on Strain D58X/11 as the 1:100 dilution has on Strain D121.

Optimal Proportions of M Antigen and Antibody Required for Demonstraling Antibody in Human Sera.—From these considerations it is obvious that for studying the content of type-specific antibodies in human sera, which vary from high concentrations to barely discernible levels, the selection of a suitable strain for the assay is of first importance. Examples of the influence of this factor are shown in Table V. Four strains with varying ability to produce Type 3 M antigen were tested in serial dilution against the blood of 3 individuals known to have circulating Type 3 antibody and one control blood with no antibody. A rough estimate of the amount of M antigen produced by these strains as indicated by M precipitin tests is recorded by + signs for each strain in the first column. Strain B930/24 with ++++ M antigen shows a strong (+++) bactericidal reaction when tested with case 29 blood. It is negative with the other three bloods. Strain D58X/11 with slightly less M antigen (+++±) gives strong reactions with case 29 but is also good with case 32. Strain C203/42, still lower in the scale according to the precipitin reaction, has a suggestion of reaction with case 3, as well as good reactions with cases 32 and 29. Finally Strain D121 with the lowest capacity to produce M antigen is the best indicator strain in all 3 positive cases. As shown in the preliminary tests in Tables IV *a* and IV *b*, this strain has sufficient growth in rotated tubes as compared with stationary so that it can be used in these bactericidal tests for specific antibody.

It is apparent, therefore, that human sera which contain an excess of type-specific antibody can be successfully tested with strains producing maximal amounts of M antigen. In selecting strains with an intermediate capacity to produce M antigen in order to provide a more sensitive test organism for the low levels of antibody present in many human sera, the comparison of rotated and stationary sets of tubes, illustrated in Table IV *a*, is especially useful. By this means the optimal proportion of M antigen to antibody can be assured for these sera of low titer. Occasionally it may be necessary for best results to examine the strain further by titrating it against serial dilutions of homologous type rabbit antiserum to ensure its possession of enough M antigen for the test.

Selection of a Type 28 Strain Suitable for Following the Rise and Fall of Antibody in a Human Serum.—These tests were done with 2 of the Type 28 strains employed to study the rise and fall of antibody in a patient after a streptococcal infection. Table VI *a* illustrates both of the biological tests described above to determine the relative amounts of M antigen produced by these 2 strains. Although there is a difference between Strains T28/150A and B574 in their ability to grow in normal human blood in bactericidal tests, as indicated by the comparison of rotated and stationary series, this difference is not great. When, however, the 2 strains are compared for ability to combine with antibody, it is obvious that Strain B574, with less M antigen, would be a better assay strain than Strain T28/150A with its higher content of M antigen, which would require more antibody to cause inhibition of growth.

TABLE V

Example of Optimal Proportions of M Antigen and Antibody Required for Demonstrating Minimal Antibody Levels in Human Sera
Direct Bactericidal Tests with Human Blood

Strains tested	Conditions of test*	Results of test				
		No. of colonies				Antibody found
		Inoculum				
Type 3 Strain B930/24 M antigen ++++	No. of streptococci inoculated	97	34	8	1	
	No. of streptococci at end of test with whole human blood from:	*Bactericidal test*				
	Case 29	16	8	1	0	+++ Type 3
	" 32	∞	700	500	300	No " "
	" 3	∞	∞	C	200	" " "
	" 10	∞	∞	500	138	" " "
		Inoculum				
Type 3 Strain D58X/11 M antigen +++±	No. of streptococci inoculated	157	41	12	2	
	No. of streptococci at end of test with whole human blood from:	*Bactericidal test*				
	Case 29	14	0	0	0	+++± Type 3
	" 32	500	190	9	7	++ " "
	" 3	∞	∞	C	261	No " "
	" 10	∞	∞	C	700	" " "
		Inoculum				
Type 3 Strain C203/42 M antigen ++±	No. of streptococci inoculated	131	26	16	1	
	No. of streptococci at end of test with whole human blood from:	*Bactericidal test*				
	Case 29	7	0	0	0	+++± Type 3
	" 32	390	41	20	0	++± " "
	" 3	∞	∞	47	43	+ " "
	" 10	∞	∞	700	0	No " "
		Inoculum				
Type 3 Strain D121 M antigen ++	No. of streptococci inoculated	250	50	7	3	
	No. of streptococci at end of test with whole human blood from:	*Bactericidal test*				
	Case 29	3	0	0	0	+++± Type 3
	" 32	98	0	0	0	++± " "
	" 3	∞	1000	0	0	+ " "‡
	" 10	∞	500	32	43	No " "

* For further details of tests, see Methods and Table II.

‡ See Table II for better reaction between Strain D121 and case 3.

Rise and Fall of Type-Specific Antibody in Case 10 Following Untreated Pharyngitis with Strain B574.—The results of tests with these 2 strains, shown in Table VI *b*, substantiate the findings with regard to their properties. Case 10 (see Table I *c*) acquired a laboratory infection with Strain T28 before the M anti-

TABLE VI *a*

Detection of Minimal Concentrations of Type-Specific Antibody in Human Serum Following Infection with Group A Streptococci of Known Type

(*a*) Selection of strains suitable for testing.

Direct and Indirect Bactericidal Tests

Strains tested	Conditions of test*	Results of tests (No. of colonies)			
		Inoculum			
Type 28 Strain T28/150A M antigen ++++	No. of streptococci inoculated	200	88	19	6
	No. of streptococci at end of test with normal human blood and the following:	*Bactericidal test*			
	(1) *Broth*				
	(*a*) Rotated tubes	∞	2000	262	112
	(*b*) Stationary tubes	∞	2000	500	123
	(2) Type 28 anti-M serum‡				
	diluted 1:1	302	26	41	0
	1:5	2000	300	152	0
	1:25	C	700	161	118
	(3) Normal rabbit serum‡				
	diluted 1:1	C	2000	500	138
		Inoculum			
Type 28 Strain B574 M antigen ++	No. of streptococci inoculated	200	55	12	3
	No. of streptococci at end of test with normal human blood and the following:	*Bactericidal test*			
	(1) *Broth*				
	(*a*) Rotated tubes	∞	C	500	74
	(*b*) Stationary tubes	∞	∞	C	200
	(2) Type 28 anti-M serum‡				
	diluted 1:1	1	0	0	0
	1:5	8	8	0	0
	1:25	500	85	0	0
	(3) Normal rabbit serum‡				
	diluted 1:1	C	2000	750	230

* For further details of tests, see Methods and Tables II, IV *a*, and IV *b*.

‡ Rotated tubes.

TABLE VI *b*

Detection of Minimal Concentrations of Type-Specific Antibody in Human Serum Following Infection with Group A Streptococci of Known Type

(*b*) Influence of optimal proportions of M antigen and antibody In demonstrating rise and fall of circulating antibody.

Bactericidal Tests with the Blood of Case 10 Following Untreated Pharyngitis due to Type 28, Strain B574

Date of serum or blood tested	Blood or serum from case No.	Results with varying culture dilutions of several Type 28 strains								"Bactericidal" antibody found and estimate of concentration
		Strains with ++ M antigen				Strains with ++++ M antigen				
		Strain No.	No. of colonies			Strain No.	No. of colonies			
1–18–55	10*	B574	∞	C	438					None (preinfection)
Control	29	"	∞	C	124	[Date of infection 1–31–55]				
5–3–55	10	B574	1000	254	67	T28/63A	C	1000	140	First suggestion—3–2–55—Less evidence of antibody with ++++ M strain than with ++ M strain
Control	30	"	∞	C	190	"	∞	C	292	
12–13–55	10	B574	118	2	0	0	T28/131A	C	500	0	0	First convincing evidence, 10–27–55. Maximum antibody found 10–27–55 to 4–17–56‡
Control	30	"	∞	C	1000	1	"	∞	C	196	9	
7–18–56	10	B574	260	11	0	0	T28/131A	700	173	57	33	Slight decline in titer
Control	30	"	C	500	208	91	"	C	700	80	123	
6–18–57	10	B574	750	350	122	0	T28/131A	∞	2000	500	155	++ M strain still gave variable slightly positive reactions from 1–18–57 to 10–2–58
Control	29	"	C	2000	750	360	"	∞	2000	500	216	
1–20–59	10	B574	C	438	69		T28/131A	C	400	267		No inhibition
Control	30	"	C	500	70		"	C	111	187		
1–20–59	10	T28	C	159	44							Trace of inhibition with poorest M strain
Control	30	"	2000	424	226							

Tests with bleedings at dates intermediate between those recorded were consistent with the slope of the curve for those recorded in the table.

* Strain B574 from the throat of case 10 after accidental infection with Strain T28.

For further accounts of the Type 28 strains used here, see also (8).

‡ On 10–27–55 several different Type 28 strains were all strongly inhibited by case 10 blood (see "La," same individual, tests recorded in Table III, reference 8). Most of these strains gave the same results as Strain B574 in later tests also.

In retrospect, early weak reactions prior to 10–27–55 (beginning about 3–2–55) can be interpreted as being positive, as well as some of the weaker reactions during the period of antibody decline. These may be compared with some of the cases tested currently in which earlier bleedings were not available.

For details of tests, see Methods and Table II.

gen of this strain was enhanced by serial mouse passage to yield Strain T28/150A with a higher content of M antigen. Strain B574 was isolated from the throat culture of case 10. Chemotherapy was not employed. Bactericidal tests with these two strains were carried out at frequent intervals. Although a suggestion of positive reaction was obtained with Strain B574 a month after the occurrence of the infection, it was 8 months before strong bactericidal reactions were observed even with this ++ M strain (B574). Nor did the ++++ M strain, T28/150A, begin to show traces of inhibition with this individual's blood until a month later than the first reactions with Strain B574. Maximal reactions with both strains were obtained during the period 9 to 15 months after the infection, followed by a more rapid decrease in the phagocytosis-promoting properties of the serum with respect to Strain T28/150A than for the weaker M^+ strain, B574.

These differences in effect of serum taken at different intervals following infection reflect the rise and fall in antibody level in the patient's blood. The results are comparable to the differences shown in Table VI *a* which reflect differences in relative concentrations of antigen and antibody demonstrated by serial dilution of rabbit immune serum.

It was also found that the non-passage strain, T28, was comparable to Strain B574 in its reactions both with rabbit antiserum and with case 10 serum. At the present time it would be questionable whether the weak reaction with Strain T28 could be considered evidence of antibody in this individual without the knowledge that this is the declining part of an antibody curve which has previously been unequivocally positive.

It seems possible that weak reactions in some of the other cases with earlier known infections, in which the higher part of the curve is missing may, in fact, be representative of the same kind of situation. In some of these, a survey of other strains of the homologous type by the use of the tests described has led to the finding of assay strains suitable for establishing the presence of low concentrations of antibody in these individuals tested many years after infection.

Discussion

Type-specific antibodies in some individuals persist for as long as 32 years. The persistence, in contrast to the initiation (5, 7), of these antibodies could not be correlated directly with the severity of the streptococcal infection or with the serological type of the causative streptococcus. Neither could this persistence be explained on the basis that it was characteristic of the particular individual to produce large amounts of antibody, because in some instances the same individual had had several infections of apparently equal severity with varying type-specific antibody response.

Following Group A streptococcal infections, the appearance of circulating type-specific antibodies is delayed compared to the immune response to other

streptococcal products, such as streptolysin-O (5, 7, 14). An example of the slow appearance of type-specific antibody is given in Table VI *b* of this report. In the case cited the concentration of type-specific antibody did not reach a high level until 8 months after the occurrence of the streptococcal infection, although the antistreptolysin-O peak was reached in 1 month and was declining within 2 months. The significance of this delay in relation to immunity and to long-term persistence of type-specific antibodies is unknown.

Evidence was obtained in a few instances by mouse protection tests that the type-specific antibodies for Group A streptococci in human sera are protective antibodies and, therefore, probably a reliable indication of immunity. In the past the use of human sera for protection tests in mice against Group A streptococci has often given equivocal results because the content of type-specific antibodies in the sera was not known. In this study by using sera in which the presence of type-specific antibodies had been previously determined, it was possible to demonstrate some protection, although the antibody titer was usually not high. Human sera with demonstrable antibody for Types 3, 6, and 13 protected mice against infection with streptococci of the homologous type to an extent roughly proportional to the relative concentration of antibody demonstrated by bactericidal tests.

Although little definite proof is available, it seems unlikely that an individual with type-specific antibodies would be susceptible to infection with Group A streptococci of that type. Pertinent findings are those of the group who worked at the Streptococcal Disease Laboratory of the Armed Forces Epidemiological Board at Warren Air Force Base, Wyoming (13, 14). These investigators showed by means of bacteriostatic tests that the presence of type-specific antibodies did not seem to influence the transitory acquisition of streptococci of the same or of heterologous types. In these instances the transitory acquisition of streptococci was not associated with antibody response. The persistence of organisms and the incidence of clinical and subclinical infections were, nevertheless, strikingly influenced by the presence of type-specific antibody but were unaffected by the presence of heterologous types of antibody. In their study the duration of these antibodies was not established, but it was thought probable that type-specific antibodies last for some years.

The stimulus which causes antibodies to persist for many years is not known. Repeated subclinical infections would account for persistence of antibodies, but the available evidence, such as the above, indicates that reinfections with with the same serological type of Group A streptococcus rarely occur. It is difficult to ascribe the long-continued occurrence of type-specific antibodies against Group A streptococci to the persistence of the organism in some unknown location in the body, perhaps in unrecognizable form. In an individual such as case 30, it would be necessary to assume that he harbored at least 7 different types of Group A streptococci. It is possible that streptococcal prod-

ucts may remain in the tissues for long periods after the infecting streptococci have been eliminated. The extreme susceptibility of the type-specific M antigen to proteolytic digestion, however, would make it readily susceptible to destruction in the body, and there is no evidence to indicate that any split products of the M antigen retain antigenic specificity. The simplest hypothesis is that the original infection modifies the antibody-producing cells permanently so that they continue to produce antibody (15). If this assumption is correct, then even individuals who have lost antibodies to a particular type would probably still be immune because contact with the original antigen would probably elicit an immediate response.

The suppression of type-specific antibody formation for Group A streptococci by penicillin or other chemotherapeutic drugs has been reported in a few studies (5, 7). It is possible that the current wide-spread use of penicillin will lead to the frequent occurrence of reinfections with the same serological type of Group A streptococci due to the absence of these antibodies, in contrast to the former rarity of such occurrences. In the present series most of the patients were untreated since their infections occurred before chemotherapeutic drugs were available.

The selection of suitable strains of streptococci for testing human sera is particularly important in bactericidal tests. Since their suitability depends on the amount of M antigen produced by the streptococci (Tables V and VI *b*), the technique developed for selection of strains also provides a method for estimating the amount of M protein contained in these organisms. Two biological and immunological methods are suggested. Both are only roughly quantitative, but they are effective measures for the selection of strains capable of furnishing the optimal proportions of M antigen for tests with sera of low antibody content. Comparison of ability of different strains of streptococci to resist phagocytosis and grow in normal human blood in rotated and stationary tubes is one of these methods (Table IV *a*). Further information may be obtained by a comparison of the amount of homologous type-specific antibody required to inhibit the growth of different strains in bactericidal tests (Tables IV *b* and VI *a*). Since quantitative methods for estimating M antigen contained in streptococci have previously been lacking, these biological assays have proved of use in the present bactericidal experiments, and they are also of value in certain other procedures connected with this work. For example, Group A streptococci which grow poorly in normal human blood in rotated tubes as compared to stationary tubes because they lack M antigen, are also avirulent for mice (9). One of the usual procedures for reestablishing their M production is to pass them serially through mice. The acquisition of M antigen and often of mouse virulence can be followed simply by testing these passage strains at intervals for growth in normal human blood in rotated and stationary tubes. Strains which grow equally well in the two sets of tubes have invariably pro-

duced maximal amounts of M antigen and are usually suitable antigens for preparing anti-M sera by immunization of rabbits or for use in virulence and protection experiments. Thus methods devised for one type of immunological assay have been useful for further elucidation of the properties of Group A streptococci.

SUMMARY

Whole blood or sera were collected from individuals who had had infections with Group A streptococci of known serological type as long ago as 10 to 32 years. Most of these patients had not been treated with chemotherapeutic drugs. By means of bactericidal tests with all these sera, and mouse protection tests with some, type-specific antibodies could be demonstrated in at least half of them after a lapse of many years, the longest interval being 32 years.

Two biological methods for estimating the amount of M antigen produced by Group A streptococci are described. By selecting strains for use by these methods, optimal proportions of M antigen and antibody could be employed in the tests and small amounts of antibody were, therefore, demonstrable.

The assay methods for M antigen are also of value for other experimental purposes.

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