INVESTIGATIONS OF MENINGOCOCCAL INFECTION. II. IMMUNOLOGICAL ASPECTS [1]

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The development of specific antibodies in human beings during recovery from meningococcal infection has been studied by a number of workers, who have employed a variety of methods, including agglutination (1 to 4), the opsonic reaction (5, 6), complement-fixation (7, 8), the bactericidal test (9, 10), and the mouse-protection test (11). Although immune bodies have been demonstrated with each of these tests during convalescence in some patients, the various reports show considerable irregularity in the results, especially with regard to the development of agglutinins and bactericidal antibodies. This irregularity may be due in part to differences between strains of meningococci used in the tests in different laboratories, as well as to differences in techniques. In some of the earlier investigations, the results were further modified by the use, in the majority of cases, of therapeutic antiserum. The nature of the immune response to meningococcal infection has therefore remained an unclarified problem.

It seemed possible that certain useful information might be secured by reinvestigating this problem in a group of patients, carriers, and normal contacts, by the concurrent use of a number of different methods for the estimation of specific antibody. The present study was undertaken in order to determine (*1*) to what extent and at what time, antibodies are produced following meningococcal infection; (*2*) by what serological tests such antibodies may most consistently be demonstrated; (*3*) what correlation, if any, exists between the development of antibodies and such factors as the severity of infection, the rapidity of recovery, or the biological characteristics of the infecting organism; and (*4*) what levels of antibody are to be found in the blood of carriers and of normal individuals in an epidemic area.

Thirty-four patients with Group I and 2 patients with Group II meningococcal meningitis were studied in detail. The clinical characteristics of some of these cases have been mentioned elsewhere (12). Serum was obtained at the time of admission to the hospital, which was usually within the first 2 or 3 days of the onset of the disease; another specimen was obtained during the second week and, in some cases, a third after several weeks. In the majority of cases, the temperature became normal within 2 or 3 days and convalescence was clinically apparent by the second week or earlier. Two deaths occurred. Sulfonamide drugs were used in the treatment of all of the patients; therapeutic antiserum was employed, in addition, in 3 cases of Group I infection. Serum was obtained from 4 Group I carriers, 6 Group II carriers, and 59 normal individuals with negative nasopharyngeal cultures who were in contact with cases during an epidemic of Group I meningococcal meningitis.

The following tests were employed in the study of these sera: agglutination, mouse-protection, quellung, plate precipitation (Petrie halo reaction (13)), complement-fixation, and the bactericidal test. Because of limited quantities of certain sera, it was impossible to perform each test with all specimens. In general, however, enough determinations were made in each instance to constitute a fairly representative group.

AGGLUTINATION

Method

Several preliminary tests with homologous strains of Group I meningococci were carried out in the usual fashion, *i.e.*, incubation for 2 hours at 37.5° C., followed by 18 hours in the icebox. By this method, agglutinins were detected in the convalescent serum of a number of patients. The titers were usually no higher than 1 : 16, how-

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TABLE I

Agglutination for Group I meningococcus by the serum of 10 patients during the acute and convalescent stage of the disease

Case	J		G		E		Ro.		Fl.		H		Eg.		Ec.		Wo.*		D	
Day of disease	3	15	1	17	1	16	5	19	1	7	1	7	2	8	2	8	10	24	3	24
Agglutinin titer †	0	32	4	32	0	64	32	64	0	128	0	128	32	128	0	32	32	8	0	128

† Titer = highest dilution of serum producing definite agglutination.
0 = No agglutination in 1:4 dilution.

* Early specimen of serum not available.

ever, and in order to obtain a more sensitive index of agglutination, a modification of the thread test (14) was employed. This consisted of the inoculation of a small number of organisms into serial dilutions of serum in a nutrient medium and the observation of agglutination with a hand lens during growth of the organisms. The medium in which the serum dilutions were made consisted of beef-heart infusion broth, containing 10 per cent rabbit serum, 2 per cent whole rabbit blood hemolyzed in an equal volume of distilled water, and 5 mgm. per cent of para-aminobenzoic acid. In tubes containing 0.25 ml. of this medium, serial twofold dilutions of the serum to be tested were made from 1 : 4 to 1 : 128. One-tenth ml. of a broth suspension, containing approximately 1,000 organisms, was added to each tube, after which they were incubated in a candle jar. Optimal agglutination was seen after from 10 to 12 hours' incubation. After 18 hours, agglutination was obscured by heavy growth of the organisms.

The sera of some patients were tested with strains of meningococci which had been cultured from their own cerebrospinal fluid, but it was found that the titers with these strains were the same as with a single stock strain of Group I meningococcus (strain No. 2). The latter strain was therefore used in tests with all of the sera from patients, contacts, and carriers. The sera of 2 patients with Group II meningococcal meningitis and of 6 Group II carriers were tested with homologous as well as with Group I organisms.

Results

Cases. Of 34 convalescent sera from Group I cases (obtained during or after the second week), agglutinins for Group I meningococci were present in all except 1. In 23 of these cases, the acute serum (obtained on admission to the hospital) was also tested. Agglutinins were present in some of these acute sera, but a definite rise in titer between the acute and convalescent sera was found in all except 2 patients. This increase in titer is shown in Table I, in which the detailed results with the sera of 10 patients are shown. The distribution of agglutinin titers in 23 acute sera and 34 convalescent sera is shown in Table II.

The exact stage of the disease at which agglutinins first appeared is unknown, since the second samples of sera were not obtained until at least the seventh day. Agglutinins were present in significant titer, however, in some patients at this time. In 1 patient (Table I, Ro.), the initial serum was obtained on the fifth day of the disease, and at this time, the agglutinin titer was 1 : 32. As is shown in Table II, 12 of 23 patients had

TABLE II

Distribution of agglutinin titers in Group I patients and non-carrier contacts during an epidemic of Group I meningococcal meningitis

Serum		Total number of persons	Serum dilution					
			0 to 1–4	1–8	1–16	1–32	1–64	1–128
Patients *	Acute	23	11	3	6	3		
	Convalescent	34	1	3	7	13	4	6
Non-carrier contacts	Total examined	59	33	7	12	4	3	
	Household A	6			3	2	1	
	Household B	5			3		2	
	Household C	9	9					

* Acute—Serum obtained during first 2–3 days of disease.
Convalescent—Serum obtained during or after the second week.

titers of 1 : 8 or higher in the first serum taken during the first 3 days of the disease. It is not known whether these early agglutinins appeared as a rapid response to infection or were present before the onset of infection.

In 1 patient (Table I, Wo.), the agglutinin titer diminished from 1 : 32 on the tenth day to 1 : 8 on the twenty-fourth day. This, however, was the only instance in which a diminution in agglutinins was observed. In 10 other patients, there was no change in titer between the second and third weeks. The latest serum to be tested was obtained on the twenty-eighth day, and in this patient, there was no diminution in agglutinin titer. The only negative serum after the first week of the disease was that of an infant, aged 2 years, who had been ill for 3 weeks and who died of obstructive hydrocephalus several days later. An earlier serum from this patient was not available.

The acute and convalescent sera of 2 patients with Group II meningococcal meningitis were tested with homologous strains. No agglutinins were detectable in the acute sera, while both had titers of 1:16 after 2 weeks. One of these patients also possessed agglutinins for Group I organisms, which increased from 1:16 in the acute serum to 1:64 in the convalescent serum. On the other hand, no agglutinins for Group II organisms were present in the convalescent sera of 6 Group I patients who were tested.

Contacts. The sera of 59 presumably normal persons who were living in the homes of 12 cases of Group I meningitis were tested for agglutinins against Group I meningococci. A single nasopharyngeal culture in each of the contacts failed to reveal meningococci. The distribution of titers in these sera is shown in Table II. It may be seen that 40 were 1:8 or lower. When the individuals with titers of 1:16 or higher were grouped according to their place of residence, it was found that 11 were concentrated in 2 households. Group A was composed of 6 longshoremen in a waterfront rooming house in which a Group I carrier also lived. Group B consisted of 6 seamen who had been living together in quarters ashore. In contrast, 9 women and children contacts occupying household C had negative agglutination tests. The comparative titers in these 3 groups are shown in Table II.

Carriers. The sera of 4 carriers of Group I meningococci were found to have relatively high agglutinin titers for Group I organisms (Table III). In contrast, 5 out of 6 Group II carriers had no detectable agglutinins for Group II organisms. The latter sera were also negative when tested with the Group I strain. One Group II carrier (A. D.) had agglutinins for Group I as well as for Group II organisms, in a titer of 1:16.

TABLE III

Agglutinins for homologous strains of meningococci in the sera of healthy carriers

Group of carrier strain	Carrier	Serum dilution					
		1–4	1–8	1–16	1–32	1–64	1–128
I	D.	4*	4	4	3	2	2
	F.	4	4	4	4	4	3
	C.	4	4	3	2	1	0
	S.	4	4	3	2	1	0
II	M. L.	1	1	0	0	0	0
	M. H.	0	0	0	0	0	0
	N. H.	0	0	0	0	0	0
	B. H.	1	0	0	0	0	0
	L. L.	0	0	0	0	0	0
	A. D.	4	3	2	0	0	0

* Figures refer to degree of agglutination:
4—complete agglutination.
1—slight agglutination.
0—no agglutination.

Comment. The agglutination test was found to be a useful method for demonstrating a rise in antibody during convalescence from meningococcal meningitis. It would appear, however, to be of little diagnostic value as far as active clinical infection is concerned because of the occurrence of appreciable levels of agglutinins in Group I carriers as well as in normal individuals. Furthermore, some of the patients possessed agglutinins early in the course of the disease and may have had them before the onset.

The findings in the group of normal contacts are of some epidemiological interest, in view of the concentration of persons with high agglutinin titers in the same households. Similar observations have been made by Finland and his co-workers (15, 16) in individuals exposed to cases of pneumonia and carriers of pneumococci. It is possible that these contacts may have been intermittent carriers, or may have had a clinically inapparent infection in the past. It is certain that they were repeatedly exposed to sources of meningococcal infection, in one instance a case and in the other a case and a carrier. The question arises as to whether such individuals as these are any less susceptible to meningitis than another group lacking agglutinins, as, for example, household C (Table II). In this regard, it may be noted that the patient who came from the longshoremen's rooming house (Table I, Eg.) had an agglutinin titer of 1:32 on the second day of his disease, which rose to 1:128 on the eighth day. The titer in the earlier serum is comparable with that in the other members of the household (Table II, A), and it is possible that this was the titer of the patient's serum before the onset of meningitis. If this is true, it would suggest that although repeated exposure may lead to the formation of agglutinins, this event does not confer immunity against meningitis. On the other hand, the presence of agglutinins early in the disease may merely mean that the day determined as the " first day of the disease " was not actually the first day of in-

fection. The nasopharynx may have been infected and bacteremia may have occurred some time before the clinical disease became severe enough to be recognized. Further studies along these lines seem to be indicated.

Although cross agglutination for Group II organisms was not noted in the convalescent serum of Group I cases, one of the patients with Group II meningitis developed agglutinins for both groups of organisms during convalescence. Also, a Group II carrier had agglutinins in a titer of 1:16 for Group I as well as for Group II organisms. It is possible that both of these individuals may previously have been exposed to Group I organisms.

MOUSE-PROTECTION TEST

Method

The serum to be tested, diluted 1:5, was injected intraperitoneally into mice in 0.5 ml. amounts, 30 minutes before the injection of organisms. The preparation of mucin suspensions was made in the same manner as that described for the virulence titrations in the preceding paper (17). White Swiss mice from a single breed, each weighing between 14 and 16 grams, were employed in all of the tests. A strain (No. 2) of Group I meningococcus was used which had been isolated from the cerebrospinal fluid of a patient; the virulence of this strain was such that 10 organisms produced 50 per cent mortality in mice. Each serum was tested for its protection against 100, 10,000, and 1,000,000 organisms, using 2 mice for each dose. Three groups of control mice were included with each test: one group received normal serum (1:5), the second, standard antimeningococcal serum (M19) of known protective action in 1:50 dilution,[3] and the third, no serum.

Tests were made with sera from 7 patients with Group I meningococcal meningitis and from 14 contacts and 2 Group I carriers in the Halifax epidemic. Since the sera of most of the patients contained sulfadiazine in varying amounts, para-aminobenzoic acid in a concentration of 5 mgm. per cent was added to all of the sera before injection.

Results

Patients. In tests with the sera of 7 patients, 2 showed no significant protection in either the acute or convalescent serum, 2 showed protection in both samples of serum against 100,000 50 per cent lethal doses, 2 showed protection against 100,000 and 1 against 1,000 50 per cent lethal doses in the convalescent serum.

[3] This standard antiserum was supplied through the courtesy of Dr. Sara E. Branham.

The 2 patients whose "acute" serum possessed protective action were known to have received sulfonamide therapy shortly before coming under our observation. It seemed possible that the apparent protection might be due to the presence of sulfadiazine in the serum. Because of this, separate experiments were undertaken to determine whether sulfadiazine could be inhibited *in vivo* by para-aminobenzoic acid, under the conditions of the mouse-protection test. The results of these experiments have been reported in detail elsewhere (18). In brief, it was found that good protection was provided by as little as 0.0025 mgm. of sulfadiazine per mouse, and that this effect was not inhibited by the presence of 10 mgm. per cent of para-aminobenzoic acid in the serum nor by a single subcutaneous injection of additional para-aminobenzoic acid at the time of the introduction of organisms. The protective action of sulfadiazine was eliminated only after repeated subcutaneous injections of para-aminobenzoic acid were made at 3-hour intervals. Presumably this was necessary to compensate for the rapid excretion of para-aminobenzoic acid (19).

The extremely small amounts of sulfadiazine required to induce protection of mice, and the difficulties involved in inhibiting this protection with para-aminobenzoic acid, render the mouse-protection test an unreliable method for assessing antibody in the sera of patients who have received sulfonamide therapy. False positive results occur when the concentration of sulfadiazine in serum is as low as 0.5 mgm. per cent. The test with patients' sera was therefore discontinued.

Carriers. One of the Group I carrier sera protected against 100,000 50 per cent lethal doses and the other against 10 50 per cent lethal doses.

Contacts. Seven contact sera possessed no protective action. Five showed slight or questionable protection. One protected against 100,000 and 1 against 10 50 per cent lethal doses.

These results with carrier and contact sera are shown in Table IV, in which the agglutinin titer of each serum for the Group I meningococcus is also indicated.

Comment. No conclusions can be drawn concerning the patients' sera in which protective action was demonstrated because of the possibility of interference by small amounts of sulfadiazine in the serum. The 2 negative results with convales-

TABLE IV

Mouse protection tests with the sera of Group I carriers and contacts during an epidemic of Group I meningococcal meningitis

Source of serum		Agglutination titer	Number of organisms injected		
			1,000,000	10,000	100
Carriers	F	1–128	2*	2	2
	S	1–32	0	1	2
Non-carrier contacts	McQ	1–32	2	2	2
	Cu	1–32	0	1	2
	T	1–64	0	0	1
	R	1–64	0	0	0
	Rd	1–32	0	0	0
	Pg	1–32	0	0	0
	K	0	0	0	0
	M	0	0	0	0
	McE	0	0	0	0
	D	0	0	0	0
	H	0	0	0	1
	Sw	0	0	0	1
	P	0	0	0	1
	McD	0	0	0	1
Controls	Normal serum	0	0	0	0
	No serum		0	0	0
	M–19 antiserum, dil. 1–50		2	2	2

* Figures refer to the number of mice surviving with each dilution of organisms. Two mice were inoculated in each group.

cent sera, on the other hand, are valid indications of the absence of mouse-protective antibody in the dilution employed. One of these sera possessed agglutinins in a titer of 1:16 and the other in a titer of 1:32. Both were negative in the quellung and complement-fixation tests, to be reported subsequently. Both were obtained on the eighteeenth day of the disease.

The sera of 2 Group I carriers and 2 contacts (McQ. and Cu.) in which protective antibody was detected also possessed relatively high titers of Group I agglutinins. Four contact sera with equal or higher agglutinins had no protective effect, however, so that no reciprocal correlation between agglutinins and mouse-protective antibodies is to be seen. It is of interest, nevertheless, that no protection was exhibited by any of the sera lacking agglutinins.

QUELLUNG

Capsular swelling of the Group I meningococcus is best observed when very young cultures are used, and is enhanced by prolonged contact of the organisms with the antiserum. The standard method for demonstrating quellung, namely that of mixing a suspension of organisms with immune serum on a slide, has proved rather unsatisfactory on occasion because of the rapid loss of this property in older cultures. Some strains may exhibit good quellung in 6-hour cultures but none after 12 hours of growth. On the other hand, excellent capsular swelling occurs when the organisms are allowed to grow in specific antiserum for a few hours, regardless of the age of the culture at the time of inoculation.

Method

Each serum was inactivated by heating at 56° C. for 30 minutes. Para-aminobenzoic acid was added to the serum to make a concentration of 5 mgm. per cent. A single cerebrospinal fluid strain of Group I meningococcus was used in all of the tests. Of a 10^{-4} broth dilution of this organism, prepared from a 6-hour serum-dextrose agar growth, 0.05 ml. was added to 0.25 ml. of the serum. The mixture was then incubated in a candle jar, and a loopful examined on a slide, with methylene blue stain, at hourly intervals.

It was found that the optimal time for demonstrating quellung was between 2 and 4 hours of incubation. Quellung was less marked when excessive growth occurred and, similarly, was less evident when excessive inoculum was employed.

The sera of 23 patients with Group I and 2 patients with Group II meningococcal meningitis were tested in this manner. The same sera were tested for quellung by the standard slide method. Tests were also made with the sera of 4 Group I carriers.

Results

The convalescent sera of 6 Group I patients produced definite quellung of the Group I meningococcus. The acute sera of these patients showed no quellung. Acute and convalescent sera from 17 other Group I cases and 2 Group II cases, and sera from the 4 Group I carriers, all yielded negative results.

When the 6 positive sera were tested for quellung by the slide method, using young cultures of the same Group I meningococcus which was employed in the incubation tests, 1 produced questionable capsular swelling and the remaining 5 were negative.

Comment. The 6 convalescent sera which produced quellung of the Group I meningococcus also contained agglutinins. Two had titers of 1:128, 3 of 1:64, and 1 of 1:32. Three of these patients

had received specific antiserum, while 3 were treated with chemotherapy alone. The degree of quellung was about the same in both groups. One patient's serum showed good quellung 2 days after the injection of antiserum, but none 1 month later.

The results indicate that although sera from the majority of patients with Group I infection do not show capsular swelling during convalescence, the test may be of some value in estimating antibody levels in the course of treatment with therapeutic antiserum. The incubation technique of demonstrating quellung is easily performed and appears to be considerably more sensitive than the usual slide method; when positive, it indicates that a high antibody level has been established.

PLATE PRECIPITATION TEST (PETRIE HALO REACTION (13))

Method

Beef-infusion agar plates were prepared so as to contain the serum to be tested in a dilution of 1:20. These were inoculated at single points with heavy scrapings from a plate culture of Group I meningococcus and incubated in a candle jar for 5 days. Control plates containing Group I antimeningococcal rabbit serum showed definite halo formation after 24 hours and very pronounced halos after 5 days.

Results

Two acute sera and 4 convalescent sera from patients with Group I meningococcal meningitis were tested. No precipitation was observed, although 2 of the convalescent sera possessed agglutinin titers of 1:64 in the thread test and also produced quellung of the Group I meningococcus. Both were positive in the complement-fixation test.

Comment. Because of limited quantities of convalescent serum, it was not possible to carry out plate precipitation tests with higher concentrations of serum. In view of the negative results with sera which possessed relatively high antibody titers by other methods, the plate precipitation method was discontinued. It is possible that positive results might have been obtained if higher concentrations of serum could have been used.

COMPLEMENT-FIXATION TEST

The convalescent sera of 26 patients with Group I meningococcal infection were tested for complement-fixing antibodies with an antigen prepared from Group I organisms. The acute sera of 14 of these patients were also tested. Sera from 3 Group I carriers and from 30 normal contacts in the Halifax epidemic were examined. Two normal adults were injected with 25 ml. and 40 ml., respectively, of commercial Group I rabbit antiserum,[4] and complement fixation tests were made before and after these injections.

Method

The antigen was prepared according to a modification (20) of McNeil's method (21) for the preparation of gonococcus antigen. Six different strains of Group I meningococcus, isolated from spinal fluid and stored on carbon dioxide ice, were employed.

The procedure in the complement-fixation test was as follows: 0.25 ml. of varying dilutions of serum (inactivated by heating at 56° C. for 30 minutes) was mixed with 0.25 ml. of the antigen, diluted 1:40. Two units of complement contained in 0.5 ml. of normal saline were added, after which the mixtures were incubated for 30 minutes at 37.5° C. Five-tenths of a milliliter of 2 per cent sheep cells previously sensitized with two units of amboceptor was then added to each tube and again incubated for 30 minutes at 37.5° C. Appropriate anticomplementary and hemolytic controls were included in each titration. A positive rabbit antiserum of known titer (1–2048), and a normal human serum lacking in antibody, were run as controls with each test. The titer of the serum was regarded as the highest dilution producing definite fixation of complement. The results of the titrations have been expressed by the final dilution of serum employed.

Results

The results of the tests with the sera of patients, carriers, and contacts are summarized in Table V. It may be seen that none of the 14 sera from patients during the acute stage of the disease contained complement-fixing antibodies, while 16 of the 26 convalescent sera were positive. In the 14 patients whose acute as well as convalescent sera were tested, the actual development of antibodies was demonstrated in 8.

One of the 3 Group I carrier sera possessed antibody in a dilution of 1:32. The other 2 were negative. All of the 30 sera from normal contacts in the Halifax epidemic were negative in a final dilution of 1:4.

The majority of positive reactions occurred with convalescent sera obtained during the second week after the onset of the disease. In 2 pa-

[4] Furnished by Lederle Laboratories, Inc.

TABLE V

Titers of complement-fixing antibodies for Group I meningococcus in sera of patients, carriers, and contacts

Serum	Total number	Complement-fixation titer						
		<1–4	1–8	1–16	1–32	1–64	1–128	1–256
Patients acute	14	14						
Patients convalescent	26	10	2	5	6	2		1
Group I carriers	3	2			1			
Non-carrier contacts	30	30						

tients, a diminution in antibody titer was demonstrated later in convalescence. In 1 of these cases, the titer at 1 week was 1:16 and after 3 weeks, the test was negative. In the other case, the titer at 1 week was 1:256 and after 4 weeks, it had fallen to 1:64.

Three of the patients in this series were treated with therapeutic Group I antiserum. The sera of 2 of these patients were tested 3 days after the administration of antiserum, and the complement-fixation titer in each was 1:32. The third patient was tested 2 weeks after the administration of antiserum, and the test was negative at this time. In order to determine the increase in antibody titer which is produced by therapeutic antiserum, 2 normal adults were given antimeningococcal Group I rabbit serum intravenously. The first subject received 25 ml. of antiserum; after 20 minutes, the titer of his serum was 1:8. The second subject received a total of 40 ml. of antiserum in 3 divided doses at 2-hour intervals; 2 hours after the last dose, the titer was 1:64, and 18 hours later, it had fallen to 1:32. In the sera of both of these individuals, quellung of the Group I meningococcus was demonstrable by the incubation method following the injection of antiserum.

Comment. The results of the tests with passively immunized normal individuals suggest that the complement-fixation test as used here is not a highly sensitive index of specific antibody, since the titers are relatively low. There are, however, certain advantages in such a test. False or nonspecific positive results seem less likely to occur, and a positive result assumes considerable significance. This is supported by the fact that in the series of 73 sera tested, no positives were encountered except in convalescent patients and in 1 Group I carrier.

The titers in the convalescent sera may be correlated to some extent with the results of the agglutinin tests previously described. Of the 10 convalescent sera yielding negative results with the complement-fixation test, 9 were tested for agglutinins. Eight of the 9 sera had agglutinin titers of 1:32 or lower. On the other hand, of the 16 convalescent sera with positive complement-fixation tests, 12 had agglutinin titers of 1:32 or higher, and 5 of these had titers of 1:128. This correlation is shown in Table VI. It is not a

TABLE VI

Relation between complement-fixing antibodies and agglutinins (thread reaction) for Group I meningococcus in convalescent patients' sera

Complement fixation test	Number of sera	Titer of agglutinins				
		1–8	1–16	1–32	1–64	1–128
Negative	9	2	2	4		1
Positive	16	1	3	3	4	5

reciprocal relationship, however, since it may be seen that low agglutinin titers were present in some of the positive complement-fixation sera, while 1 of the negative sera had an agglutinin titer of 1:128. Furthermore, some of the contact and carrier sera, which showed no reaction in the complement-fixation test, had relatively high agglutinin titers.

BACTERICIDAL TEST

Method

The method was similar to that employed in previous bactericidal tests with the influenza bacillus (22). The organisms used in each test were grown for 6 hours on serum-dextrose agar slants and then suspended in beef-infusion broth from which serial dilutions from 10^{-1} to 10^{-6} were made in broth. The turbidity of the original suspension was adjusted by the use of a photoelectric turbidometer so that 0.1 ml. of the 10^{-6} dilution contained approximately 100 organisms.

In testing the bactericidal action of fresh serum, 0.25 ml. of serum was mixed with 0.05 ml. of each dilution of organisms, in small Pyrex tubes. In other tests, the sera were inactivated by heating at 56° C. for 30 minutes. Dilutions of these sera, in 0.05 ml. amounts, were mixed with 0.25 ml. of fresh normal human serum, diluted 1:2, or with fresh normal defibrinated blood, and 0.05 ml. of each dilution of organisms was added. The tubes were

sealed and rotated in an incubator for 20 hours. After incubation, the contents of each tube were thoroughly mixed and two loopfuls were inoculated on the surface of a blood agar plate. Para-aminobenzoic acid in a final concentration of 5 mgm. per cent was added to the materials used in all tests in order to inhibit the effect of any sulfonamide in the sera.

Results

Preliminary bactericidal tests with the convalescent sera of patients with Group I meningococcus infection yielded paradoxical results. It was found necessary to undertake a reinvestigation of the mechanism of bactericidal action in normal and immune sera before satisfactory tests could be performed with the sera of patients.

The results of this investigation have been reported in detail elsewhere (17, 23), but the observations which are pertinent to the present study may be summarized as follows: (*1*) It was found that Group I meningococci could be divided roughly into two classes—those which were susceptible and those which were resistant to the bactericidal action of fresh normal human serum, as indicated by their failure or ability to survive (17). Some variation was also encountered in the bactericidal property of sera from different normal individuals; for example, occasional sera were found in which the usually susceptible strains were able to survive. (*2*) No bactericidal action could be demonstrated in the fresh serum of rabbits which had been intensively immunized against Group I meningococci, even for strains which ordinarily were killed by normal rabbit serum. When these immune sera were diluted in the presence of normal rabbit complement, however, strong bactericidal action against resistant strains was produced by dilutions as high as 1:60,000, while a prozone was produced by dilutions of 1:600 or lower. Furthermore, when immune serum was added to fresh normal serum, inhibition of the normal bactericidal action for susceptible strains was observed, suggesting that a prozone might be responsible. (*3*) When fresh normal human serum was used as complement, with specific rabbit antisera or human convalescent sera, no killing of the resistant strains took place. When defibrinated blood was used instead of serum as complement, killing of these strains was caused by high dilutions of immune sera, suggesting that another mechanism, presumably phagocytic, was operative. (*4*) When fresh serum which lacked bactericidal action was employed as complement with the susceptible strains, killing was produced by immune sera.

In the light of these findings, tests were made with the sera of patients with Group I meningococcus infection, with the following results.

Bactericidal effect of fresh serum. The undiluted sera of 3 patients were tested for bactericidal action against the homologous organism during the acute and convalescent stages of the disease. In each instance, the serum was strongly bactericidal during the acute stage and definitely less bactericidal during convalescence (Table VII).

TABLE VII

The bactericidal action of fresh serum for homologous organisms during the acute and convalescent stages of Group I meningococcus meningitis

Patient	Day of disease	Dilution of organisms					
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
R.	3	0	0	0	0	0	0
	14	+	+	+	+	+	+
E.	3	0	0	0	0	0	0
	11	+	+	0	0	0	0
L.	2	0	0	0	0	0	0
	14	+	+	+	+	+	+

0 = No growth. + = Growth.

Effect of patients' sera upon bactericidal action of fresh normal serum. These tests were carried out in the same way as the usual bactericidal test, except that the fresh normal serum used as complement was of itself bactericidal for the organisms. Of undiluted serum obtained during the acute and convalescent stages, 0.05 ml. was added to 0.25 ml. of normal serum, diluted 1:2. A susceptible strain (No. 2) was employed.

The sera of 7 patients produced no effect upon the bactericidal action of normal serum. The convalescent sera of 4 patients caused inhibition of bactericidal action, while the acute serum was without effect (Table VIII).

Bactericidal effect of convalescent serum in the presence of neutral human complement. When the susceptible strain (No. 2) was used with fresh serum from an individual lacking bactericidal action for this strain, the addition of convalescent serum produced striking results. Killing took

TABLE VIII

The inhibitory effect of convalescent serum on the bactericidal action of fresh normal serum

Patient	Day of disease	Dilution of organisms					
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
R.	3	+	0	0	0	0	0
	14	+	+	+	+	0	0
E.	3	+	+	0	0	0	0
	11	+	+	+	+	+	+
J.	2	0	0	0	0	0	0
	14	+	+	+	0	0	0
Hu.	2	+	0	0	0	0	0
	14	+	+	+	0	0	0
Normal serum alone		+	0	0	0	0	0

0 = No growth. + = Growth.

place with 1 : 6,000 dilutions of the immune serum, while a prozone occurred with 1 :6 dilutions of serum. In contrast, serum obtained during the acute stage had no effect (Table IX).

With a resistant strain (No. 21), human serum was found to be ineffective as complement. No bactericidal antibody was demonstrable either in convalescent serum or in rabbit antisera. When human defibrinated blood was used instead of serum as complement, however, the results were comparable with those obtained in the preceding test with the susceptible strain. Strong bactericidal action was caused by the convalescent sera of 4 patients, while the acute sera were negative.

TABLE IX

Bactericidal effect of acute and convalescent sera for a susceptible strain of Group I meningococcus (No. 2) with non-bactericidal human serum as complement

Day of disease	Dilution * of serum	Dilution of organisms					
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
3	1–6	+	+	+	+	+	+
	1–60	+	+	+	+	+	0
	1–600	+	+	+	+	+	0
	1–6000	+	+	+	+	+	0
10	1–6	+	+	+	+	+	0
	1–60	0	0	0	0	0	0
	1–600	0	0	0	0	0	0
	1–6000	+	+	+	0	0	0
Complement alone		+	+	+	+	+	+

0 = No growth. + = Growth.
* Figures indicate the final dilution of serum.

The results with the sera of 1 of these patients are shown in Table X.

Bactericidal effect of sera from Group I carriers. The sera of 2 Group I contact-carriers were tested with the resistant strain, using defibrinated blood as complement. Two samples of serum were obtained from each individual, one at the time of the first nasopharyngeal culture and another after 3 weeks, at which time cultures were negative in both. Both carriers had received a short course of sulfadiazine treatment during this 3-week interval. In one of the carriers, both samples of sera were negative. In the other, the first serum was negative but the second showed bactericidal antibody in a dilution of 1 : 60. It is of some interest that this individual underwent

TABLE X

Bactericidal effect of acute and convalescent sera for a resistant strain of Group I meningococcus (No. 21) with normal human defibrinated blood as complement

Day of disease	Dilution of serum *	Dilution of organisms					
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
3	1–12	+	+	+	+	+	+
	1–60	+	+	+	+	+	0
	1–600	+	+	+	+	+	0
	1–6000	+	+	+	+	+	0
10	1–12	+	+	+	+	+	0
	1–60	0	0	0	0	0	0
	1–600	0	0	0	0	0	0
	1–6000	+	+	+	0	0	0
Normal defibrinated blood alone		+	+	+	+	+	+

0 = No growth. + = Growth.
* Figures indicate the final dilution of serum.

an upper respiratory infection with rhinitis and pharyngitis during the 3-week period. The agglutinin titer in the serum of the first carrier was 1 : 32 and in the second, 1 : 128. No change in agglutinins was noted in the 2 samples of sera in either individual.

Effect of sulfadiazine on the bactericidal test. In the preceding experiments, para-aminobenzoic acid was used in a concentration of 5 mgm. per cent. In the absence of this drug, small amounts of sulfadiazine exerted a misleading effect in bactericidal tests with the serum of patients. For example, a serum containing 8.0 mgm. per cent of sulfadiazine produced killing in a dilution of

1:120, but was entirely non-bactericidal when para-aminobenzoic acid was added.

Comment. The diminution in the bactericidal property of fresh undiluted serum during convalescence from Group I meningococcal infection may be analogous to the absence of bactericidal action in the undiluted serum of heavily immunized rabbits. In both instances this appears to be a manifestation of the prozone, since adequate dilution of the convalescent serum or of the immune rabbit serum results in bactericidal action when neutral complement is added. Similarly, inhibition of the bactericidal action of normal serum, which was observed with the convalescent sera of 4 patients, has also been shown to occur when immune rabbit serum is added to fresh normal rabbit or human serum. The mechanism of this action is not completely clear, but seems also to be related to the prozone phenomenon.

The strong bactericidal action in the fresh sera of 3 patients during the acute stage of the illness is difficult to explain. The same degree of bactericidal action is, however, encountered in many normal individuals and may represent the normal state in these 3 patients. If this is true, one might assume that this property does not constitute an adequate barrier against infection with the meningococcus, at least as far as the subarachnoid space is concerned. There are two alternative explanations which should also be considered: (*1*) The fresh serum of acutely ill, febrile patients has been shown by Tillett (24) to be bacteriolytic for hemolytic streptococci. It is possible that an analogous property may have caused the bacteriolysis of meningococci in the early serum of these 3 patients. Evidence is lacking to substantiate this at the present time. (*2*) It is conceivable that bactericidal antibody may have begun to develop very early in the course of the disease, perhaps even before the actual invasion of the meninges, and by the second or third day of florid symptoms, there may have been enough antibody to produce bacteriolysis in the fresh, undiluted serum. This is suggested, without direct supporting evidence, because it has been observed that after light immunization (*e.g.*, a single intravenous dose of living organisms), rabbits may develop bactericidal property in fresh, undiluted serum for resistant strains of meningococci. As has been mentioned, further immunization results in the disappearance of this property.

For practical purposes, the bactericidal test employing human defibrinated blood as complement with a resistant strain of meningococcus was found to be the most convenient method for estimating bactericidal antibody. The tests in which fresh normal serum was used as complement are of interest chiefly because of the differences which were brought out between meningococci of the same group. It was shown that if a strain was usually, but not always, susceptible to bacteriolysis in normal serum, it was also susceptible to bacteriolysis by immune serum when normal serum was used as complement. On the other hand, if a strain was generally resistant in fresh normal serum, it was also resistant when antiserum and normal serum were combined, but was destroyed when defibrinated blood was used as complement. Except for those differences in susceptibility, the strains were indistinguishable in their general characteristics, including morphology, agglutinability, quellung, and virulence for mice (17).

The presence of bactericidal antibody in the convalescent serum was in each instance associated with specific agglutinins and complement-fixing antibodies. The number of tests performed was not sufficient to determine a quantitative relation between the results. The bactericidal test appears, as might be expected, to be a more highly sensitive method than the agglutination or complement-fixation tests. The time when bactericidal antibody begins to develop has not been shown in this study. It has been shown, however, that antibody of high titer is present between the seventh and fourteenth days of the disease.

The appearance of bactericidal antibody in the serum of a Group I carrier, 3 weeks after a previous negative test, is of some interest. Unfortunately, the duration of the carrier state in this individual is not known. It is possible that it may have begun shortly before the first test, and the positive result in the second test may have represented a delayed response to infection. If, however, the carrier state had been of longer duration (which the agglutinin titer of 1:128 in the first serum might suggest), it is also possible that the appearance of bactericidal antibody may have been the result of a transient reinvasion during

an upper respiratory infection, unaccompanied by the usual symptoms of meningococcal infection.

DISCUSSION

The appearance of specific antibody during convalescence from meningococcal meningitis has been demonstrated by all except two of the methods employed. Negative results were obtained in the plate precipitation and mouse-protection tests. In the first, this may have been due to insufficient concentrations of serum. In the mouse-protection test, some of the positive results may actually have been due to the presence of antibody, but the potential interference by small amounts of sulfadiazine in the sera renders the test inconclusive.

The most consistently positive results were obtained by the agglutination test, in which 35 of 36 convalescent sera were positive. Agglutinins were also encountered, however, during the early stage of the disease in some patients, as well as in the sera of Group I carriers and contacts. The significance of a single observation during the course of the disease is therefore doubtful, and the chief value of the test lies in the demonstration of an actual increase in titer between the acute and convalescent stages. The complement-fixation test yielded fewer positive results in the convalescent sera, but, with the exception of 1 Group I carrier, it was only positive within this group. The quellung test, by the incubation method, was positive in only 6 of 25 convalescent sera, and probably indicates a high level of antibody. The bactericidal test, although beset with technical difficulties, seemed to be the most sensitive method for demonstrating antibody and provided the most striking differences between acute and convalescent sera.

No consistent correlation could be seen between the degree of antibody response and the severity or duration of illness in this series. Patients with relatively mild clinical courses and prompt recovery could not be distinguished from more severely ill cases on the basis of these tests. It should be mentioned, however, that the general course in the majority of cases in this series was approximately the same, and the number of patients at either extreme was relatively small. The response to sulfonamide treatment was rapid in most cases, and only 3 cases of resistant infection were observed. One of these patients, who eventually recovered, developed a high level of antibody, as measured by agglutination, complement-fixation, and bactericidal tests. The second, an infant aged 2 years, had no detectable antibody by any of the tests after 3 weeks and died shortly thereafter. A third patient, who received therapeutic antiserum, died after 3 days; the serum on the day of death possessed agglutinins in a titer of 1:64 and complement-fixing antibody in a titer of 1:32. The development of specific antibody was in no way related to the virulence of the infecting organisms for mice, nor to their ability to survive in fresh normal human serum.

The results of tests with carrier sera seem to cast some light on the problem of the carrier state. In the preceding paper (17), it was shown that the Group I carrier strains were of the same order of virulence for mice as the Group I strains from the cerebrospinal fluid of cases. In the present study, 4 Group I carriers were found to possess antibody levels which were as high as or higher than those in convalescent patients. Agglutination tests were positive in all, mouse-protection tests in 2, and the bactericidal test and complement-fixation test each in 1. These observations indicate that the Group I carrier state is, as Rake (11) has suggested, an actual infection which may remain limited to the nasopharynx. On the other hand, the absence of detectable antibody in Group II carriers, whose organisms were avirulent, indicates that such organisms may be passive saprophytes without potential invasiveness. This probably does not apply to Group II carrier strains during outbreaks of Group II cases, since Silverthorne and his co-workers (10) have shown that these organisms may be virulent.

The results of the agglutination tests with contact sera have implied that there is some relation between the repeated exposure to meningococci and the development of specific antibody. It has not been shown, however, that this process prevents the development of meningitis.

SUMMARY

1. Several methods have been employed for the demonstration of specific antibody in the sera of 36 patients with meningococcal meningitis during the acute and convalescent stages of the disease,

and in the sera of 4 Group I carriers, 6 Group II carriers, and 59 Group I contacts.

2. The *agglutination test* was positive in 33 of 34 convalescent sera from Group I cases and in 2 convalescent sera from Group II cases. Agglutinins were also present in 12 sera in the acute stage, but a definite rise in titer between the acute and convalescent sera was found in all except 2 patients. Agglutinins were shown to appear as early as the seventh day and to persist as long as the twenty-eighth day. The sera of 4 carriers of Group I organisms had agglutinin titers of between 1:32 and 1:128. The sera of 5 of 6 carriers of Group II organisms had no agglutinins for homologous or Group I strains. The serum of 1 Group II carrier had agglutinins for both strains in a titer of 1:16. Of 59 normal Group I contacts, 40 had titers of 1:8 or lower; 11 of the 19 contacts with higher titers were found to be concentrated in two households.

3. The *mouse-protection* test was found to be impractical for use with sera from patients who had received sulfonamide therapy, because of the protective action of small amounts of drug and the difficulty in inhibiting this action with para-aminobenzoic acid. Of 2 Group II carrier sera, 1 protected against 100,000 and the other against 10 50 per cent lethal doses. Of 14 Group I contact sera, 1 protected against 100,000 and 1 against 10 50 per cent lethal doses.

4. *Quellung* was produced by 6 convalescent sera from Group I cases when the organisms were incubated in the serum for from 2 to 4 hours.

5. The *plate precipitation* test was negative with 4 convalescent sera which possessed Group I antibody by other methods.

6. The *complement-fixation* test was positive in 16 of 26 convalescent sera from Group I cases. Fourteen sera were negative during the acute stage of the disease. One of 3 Group I carrier sera was positive. Thirty sera from Group I contacts were negative.

7. *Bactericidal* tests with the fresh, undiluted sera of 3 Group I patients against their own strains showed a diminution in bactericidal property during convalescence. The bactericidal action of fresh normal serum for a susceptible strain of Group I meningococcus was inhibited by the addition of convalescent sera from 4 patients, while the acute sera of these patients was without effect. This effect was apparently due to a prozone, as will be discussed in a later paper on the mechanism of bactericidal action against the meningococcus (23). With neutral fresh human serum or defibrinated blood as complement, strong bactericidal action was demonstrable with the convalescent sera of 4 patients in dilutions ranging from 1:600 to 1:6,000. In simultaneous tests, no bactericidal action was exerted by serum in the acute stage of the disease. One Group I carrier developed bactericidal action in a serum dilution of 1:60, during a 3-week period of observation.

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