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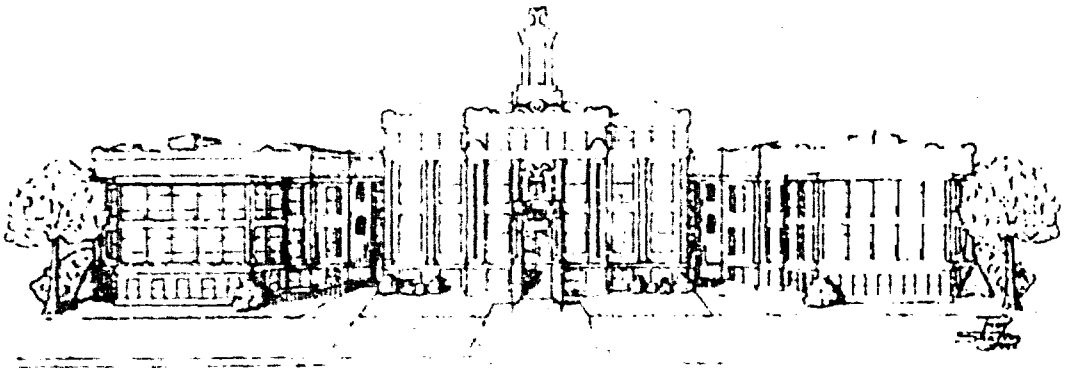

MENINGOCOCCAL PROGRESS REPORT--1968

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MENINGOCOCCAL

PROGRESS

REPORT

1968

FOREWORD

The papers included in this report were presented by NAMRU-4 staff members at a BuMed-ONR-sponsored Meningitis Workshop hosted by NAMRU-4, Great Lakes, Illinois, 10-12 June 1968. The reports are considered significant enough to publish for limited distribution in this form of publication to make the information available to other workers in this field. Some of these papers have been, or will be, submitted for publication in the open literature.

The incidence of new groups of the meningococcus in a military population has not previously been studied so extensively. The epidemiological study has been aided by newly developed serological tests which give a new depth to an understanding of the carrier and disease state. Further studies of the meningococcal L forms are reported, as well as lab characteristics of a CF antigen. All antigens have been studied in mouse protection tests with hopeful results.

A model for the screening of antimicrobial agents in the laboratory which requires a minimal number of volunteers is also described. It is hoped that this technic will eliminate large field trials until favorable results can be expected.

ROBERT O. PECKINPAUGH
CAPT MC USN
Commanding Officer

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THE EPIDEMIOLOGY OF MENINGOCOCCI IN NAVAL PERSONNEL AT GREAT LAKES

L. F. Devine, E. A. Edwards, F. G. Askin and W. E. Pierce

Naval personnel continuously arrive at Great Lakes from civilian life and from naval ships and stations around the globe. Their experience with the navy ranges from nil to a decade or more. Continuous sampling of large segments of these populations makes it possible to observe variations in the prevalence and the sulfadiazine sensitivities among the different serological groups of meningococci being brought in from various sections of the United States and from overseas.

Two major groups of personnel being studied for meningococcal infections at Great Lakes are recruits and Service School Command personnel (Table 1). Ten percent of all recruits are cultured upon arrival. This sample is obtained by random selection of the weekly input of recruits. Additionally, each month two companies of 80-100 men are cultured on arrival. One of these two companies is given sulfadiazine 1 g twice a day for 2 days. The other company receives no sulfadiazine and serves as a control. Five days later all meningococcal carriers in both companies are recultured. All men in the non-treated company are bled for serological studies at 0 day and again at 5 and 9 weeks after arrival. All recruits in both companies are recultured upon graduation at 9 weeks.

All personnel reporting to the Service School Command are cultured upon arrival and divided into categories on the basis of their immediate past duty station as indicated in Table 1. Personnel from San Diego and Great Lakes arrive at the Service School Command after 10 to 14 days of recruit leave. The naval reservists spend 2 weeks in Great Lakes recruit camp and return to civilian life for various periods of time up to a year before coming to the Service School Command.

Figure 1 shows the bacteriological procedures used in this study. Nasopharyngeal cultures are taken with a bent swab and plated on Mueller-Hinton agar containing 25 units/ml of polymyxin B and 10 μg/ml of ristocetin. The plates are incubated for 18 hours, and the meningococci are grouped by the slide agglutination method using either CDC or locally prepared rabbit antiserum. Sulfadiazine sensitivities of all meningococci isolated are determined by using the following procedure: one meningococcal colony is transferred to 2 ml of Mueller-Hinton broth in a 13x100 mm test tube and incubated for 18 hrs. A standard 2 mm loop is used to inoculate a series of Mueller-Hinton plates containing 4-fold increments of sulfadiazine. All cultures are confirmed to be Neisseria meningitidis by inoculating 0.1 ml of the 18-hour culture into 1/2% solutions of dextose, lactose, levulose and maltose in 2 ml volumes of Mueller-Hinton broth. The Mueller-Hinton broth contains 12.5 units of polymyxin B per ml and 5 μg/ml of ristocetin.

All meningococci are grouped by the slide agglutination method as indicated in Figure 2. The points of the arrow indicate the bacterial agglutination by the antiserum at the base of the arrow. The Z group is distinguished from 29E in that Z cells are agglutinated by antiserum to 17, Z, 29E, and 112, while 29E is agglutinated only by antisera to 112 and 29E.

Figure 3 shows the series of 9 sulfadiazine concentrations in milligram percent that is present in the Mueller-Hinton agar for determining the sulfadiazine resistance of the meningococci. These concentrations are in 4-fold increments ranging from 0.00098 to 64 mg%. When there is no growth on the 6th plate, the minimal inhibitory concentration (MIC) is 1 mg% or less and is considered sensitive, and when growth occurs on plate 6, the MIC is greater than 1 mg% and the organism is called resistant. The validity of this division into resistant and sensitive organisms is substantiated in the paper on chemotherapy (1).

Table 2 shows the prevalence of different groups of meningococci in all populations from June 1967 to March 1968. More than 83% of all isolates are identified. Ten percent agglutinate in 2 or more antisera. Seven percent are autoagglutinable in 0.15 molar sodium chloride. The Boshard group is the

most prevalent group and accounts for 59% of all strains isolated. The descending order of prevalence of other groups identified is B, C, 29E, Scar. Hospital, and X.

The percentages of meningococcal carriers in all populations from June 1967 through March 1968 are shown in Table 3. The ranges of the percentages of infections are shown on a monthly basis on the right. New arrivals, i.e. 0-day recruits, have the narrowest range, while graduating recruits at Great Lakes have the widest range. Great Lakes and San Diego recruit personnel have a wider range in carrier rates than all other categories of personnel arriving at the service school.

The predominant serological groups in the various categories of personnel from October 1967 through March 1968 are shown in Table 4. Group B meningococci predominates in 0-day recruits and sea duty personnel. The Boshard group is the predominant meningococcal group among all other populations. The highest percentage of group C is found among sea duty personnel. However, a rapid rise in group C occurs among Great Lakes graduates coming to the Service School Command increasing from 1.4% in December 1967 to 2.9% in January 1968 to 4.9% in February to 7.6% in March to 15.2% in April to 22.7% in the first two weeks of May.

The unusual serological groups of meningococcus are found most frequently in 0-day recruits and sea duty personnel as shown in Table 5. Despite the fact that more than 2,000 men from overseas have had nasopharyngeal cultures taken in the last year, a group A meningococcus has not been isolated. A sulfadiazine-sensitive group A strain was isolated from a case of urethritis from a long-term brig occupant. Two group D strains were found among 0-day recruits.

The seasonal trends of carrier rates are readily apparent among graduating recruits from both San Diego and Great Lakes and are contrasted with the absence of such a trend in 0-day recruits who show little, if any, increase in the winter months (Fig. 4). Likewise, carrier rates among sea duty, shore duty,

and naval reservists as shown by monthly ranges in the upper left show no marked seasonal variation.

The chronologic proximity to Recruit Training Centers is associated with increased prevalence of sulfadiazine-resistant meningococci among naval personnel. This is illustrated in Figure 5 by the low rate of 14.6% resistance meningococci observed from Great Lakes 0-day recruits. Three months later when these men arrive at the Service School Command, 88.8% of meningococci are sulfadiazine-resistant. Sixty-five percent of the meningococci isolated from the San Diego recruits arriving at the Service School Command are also resistant. Naval reservists coming to the Service School Command carrying meningococci have 42.7% of their strains resistant to sulfadiazine. Those coming from the Atlantic and Pacific carry meningococci of which 26.6% and 18.4%, respectively, are sulfadiazine-resistant.

The percentage of meningococci cultures resistant to sulfadiazine among the different sero-groups in various populations is lowest among 0-day recruits and sea duty personnel and highest among recent Great Lakes graduates (Table 6). However, more than 55% of all Boshard strains are resistant from carriers who have had recruit training compared to only 20% in men who have had no previous service in the navy. Sea duty personnel have a lower percentage of B and C meningococci resistant to sulfadiazine than 0-day recruits.

The effect of sulfadiazine prophylaxis on the carriage of N. meningitidis from July 1967 to April 1968 is shown in Table 7. The meningococcal carriers found at 0-day in the sulfadiazine-treated company and the non-treated control company are recultured 5 days after treatment. Sixty-five of 68 of the initial carriers of sensitive organisms in the treated companies have negative cultures 5 days after treatment, and 8 of 15 carrying resistant meningococci have negative cultures. Sixteen percent of the carriers of sensitive organisms in the non-treated companies are cured spontaneously, but spontaneous loss fails to occur in any of the 9 carriers of sulfadiazine-resistant organisms. Since more than 95% of the carriers of sulfadiazine-sensitive

organisms are cured with this treatment, it is apparent that the 1 mg% level, as used to distinguish sensitive from insensitive strains is valid. This is further discussed in the chemotherapy paper (1).

The effect of sulfadiazine prophylaxis on clearing the carrier and sulfadiazine resistance is illustrated in Table 8. The treated and non-treated companies of recruits are both cultured at 0-day and 9 weeks. The 0-day negative personnel acquire resistant organisms to the same degree whether or not prophylaxis is given. The non-treated group, which is composed of carriers at 0-day, is still positive 82% of the time at 9 weeks, while in the treated group, those positive at 0-day carry meningococci only 59% of the time upon graduation. Sensitive meningococci tend to persist in the non-treated group during training and be lost in the treated group. The net effect of prophylaxis is to lower the total carrier rate, thus producing an apparent increase in resistant organisms and, conversely, no treatment results in greater carrier rates with a smaller percentage of resistant organisms. The greater carrier rate among the non-treated group is obviously due to retention of sensitive organisms. There is a suggestion of minimal protection (5% difference) against acquisition of meningococci afforded by the 2 days of sulfadiazine prophylaxis at the beginning of training (Table 8).

The results of this exhaustive bacteriological sampling are useful to estimate the number of meningococcal infections occurring in nearly 70,000 recruits entering training during this study. Table 9 shows the estimated number of infections by group and sulfadiazine sensitivity recognized in this population together with the cases of meningococcal disease associated with them. About equal numbers of infections occur with groups B and C per case, while the Bo strain appears to be the least virulent. There are comparatively few infections with other groups.

A continuous serological survey of recruits at Great Lakes has been in effect since October 1964. Sera are collected from recruits on arrival, and again at mid-training and graduation. A new company has been sampled every 3 months from October 1964 to December 1965, and monthly thereafter. The percentages

of 4-fold CF serological conversions to N. meningitidis that occur in each of these companies are shown in Figure 6. Nasopharyngeal cultures have been collected upon arrival and upon graduation of these same men since June of 1967.

These data portray the high rates of infection that occur in this recruit population together with the pronounced seasonal alternation of summer depression followed by winter elevation in the incidence of these infections. This pattern parallels the seasonal incidence of clinical meningococcal disease in the United States since 1960 (2).

Another study was done to determine the relationship of bacterial isolations to CF serological conversions (3). One company of recruits was bled and cultured on arrival and at 10 additional times during the course of training. Figure 7 shows the results of selecting 52 men whose initial throat cultures were negative and for which all 11 specimens were collected.

A plus sign indicates the time of the first positive throat culture and the circle the simultaneous or subsequent 4-fold serological rise. Infection is not detected bacteriologically or serologically in 5 men. Positive throat cultures are found in two men who fail to respond serologically. The serological rise precedes the isolation of the organism in one instance. Three men who have a 2-tube serological rise prior to meningococcal acquisition subsequently develop 4-, 4-, and 6-tube rises, respectively. All of these men who acquired infections, except one, carry a meningococcus at the end of the study. The length of time required for a CF serological response to occur subsequent to the initial infection is apparently 8 to 10 days.

In summary, the analysis of continuous intensive bacteriological and serological sampling of different naval populations at Great Lakes reveals the following:

1. There is a nearly constant rate of infection among newly recruited civilians arriving at Great Lakes.

2. Eight-five percent of all meningococcal carriers

entering Great Lakes carry sulfadiazine-sensitive organisms.

3. The administration of 1 g of sulfadiazine twice a day for 2 days reduces the percent of sulfadiazine-sensitive organisms in carriers from 85 to 5%.

4. The Boshard group of meningococci dominates all other groups of meningococci in naval recruit camps.

5. The group B meningococci are found most frequently in civilians and sea duty personnel.

6. Clinical meningococcal disease occurs far less frequently following infection with the Boshard group than with either groups B or C.

7. Serological response follows meningococcal acquisition and is clearly associated with it.

8. Seasonal variation in meningococcal infections in recrui camps parallels the incidence of clinical meningococcal disease in civilian life. The prevalence of sulfadiazine-resistant meningococci is higher in all naval populations than among civilians.

FIGURE 1

MATERIAL FROM THE FIELD
IS PLACED ON
MUELLER-HINTON PLATES
CONTAINING
POLYMYXIN, 25 UNITS PER ml
RISTOCETIN, 10 μg PER ml

↓

STREAKED IN LABORATORY, INCUBATED 18 HOURS

↓

GROUPED

↓

ONE COLONY TRANSFERRED TO
MUELLER-HINTON BROTH
CONTAINING
POLYMYXIN, 12.5 UNITS PER ml
RISTOCETIN, 5 μg PER ml

↓

INCUBATE 18 HOURS

↓

USING 2 mm I.D. LOOP
8 MUELLER-HINTON SULFADIAZINE PLATES
CONTAINING SULFADIAZINE mg%

1 - 0.0009	5 - 0.25
2 - 0.0039	6 - 1.0
3 - 0.0156	7 - 4.0
4 0.0625	8 - 16.0

INOCULATE 0.1 ml IN
MUELLER-HINTON BROTHS
CONTAINING 0.5%

A - DEXTROSE	C - LEVULOSE
B - LACTOSE	D - MALTOSE

AND EACH CONTAINING
POLYMYXIN, 12.5 UNITS PER ml
RISTOCETIN, 5 μg PER ml

FIGURE 2

MENINGOCOCCAL SEROLOGIC RELATIONSHIPS BY SLIDE AGGLUTINATION

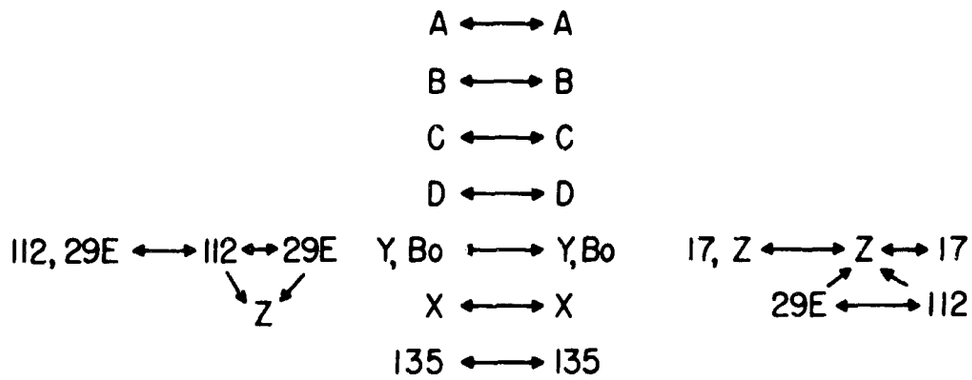

FIGURE 3

CODE FOR MEASURING SULFADIAZINE RESISTANCE

	Plate No.	Sulfadiazine Conc. (mg%)
SENSITIVE	1	0.00098
	2	0.0039
	3	0.0156
	4	0.0625
	5	0.25
	6	1.00
RESISTANT	7	4.00
	8	16.00
	9	64.00

WHEN NO GROWTH ON PLATE 6, THE MIC IS ≤6 (SENSITIVE).
WHEN GROWTH ON PLATE 6, THE MIC IS >6 (RESISTANT).

FIGURE 4

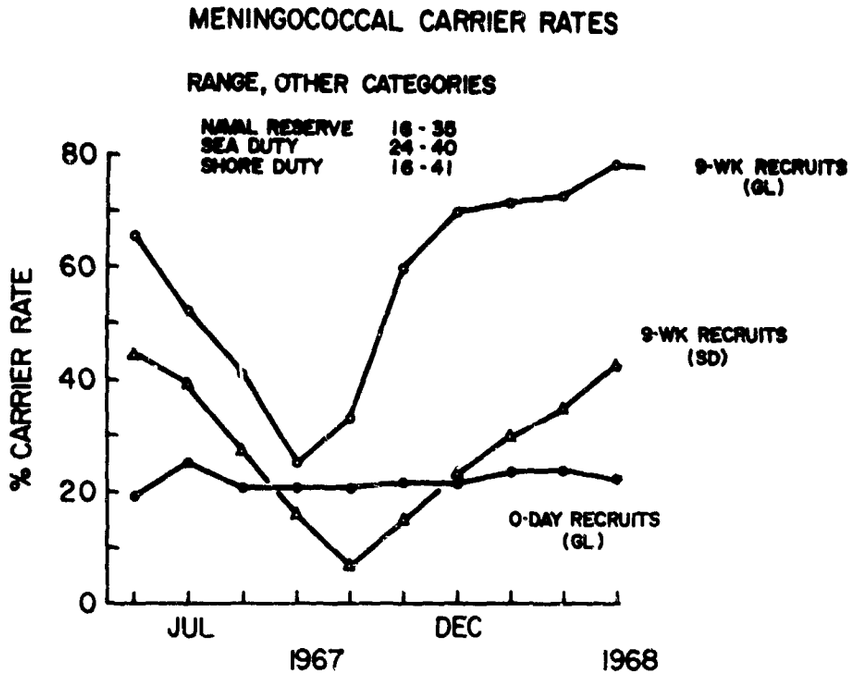

FIGURE 5

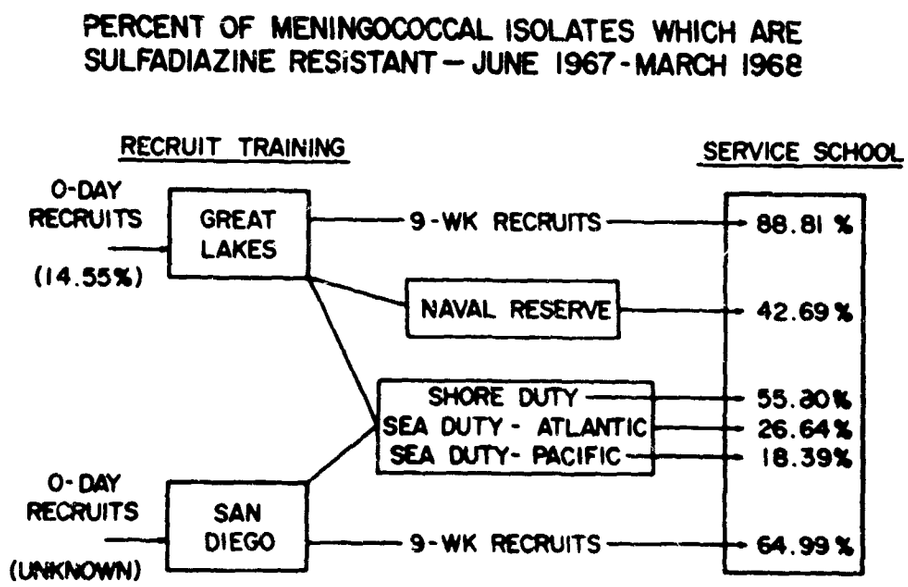

FIGURE 6

PERCENT SEROCONVERSION TO *N. MENINGITIDIS* - 1964-1968
AND PERCENT CARRIERS AT 9 WEEKS - 1967 - 1968

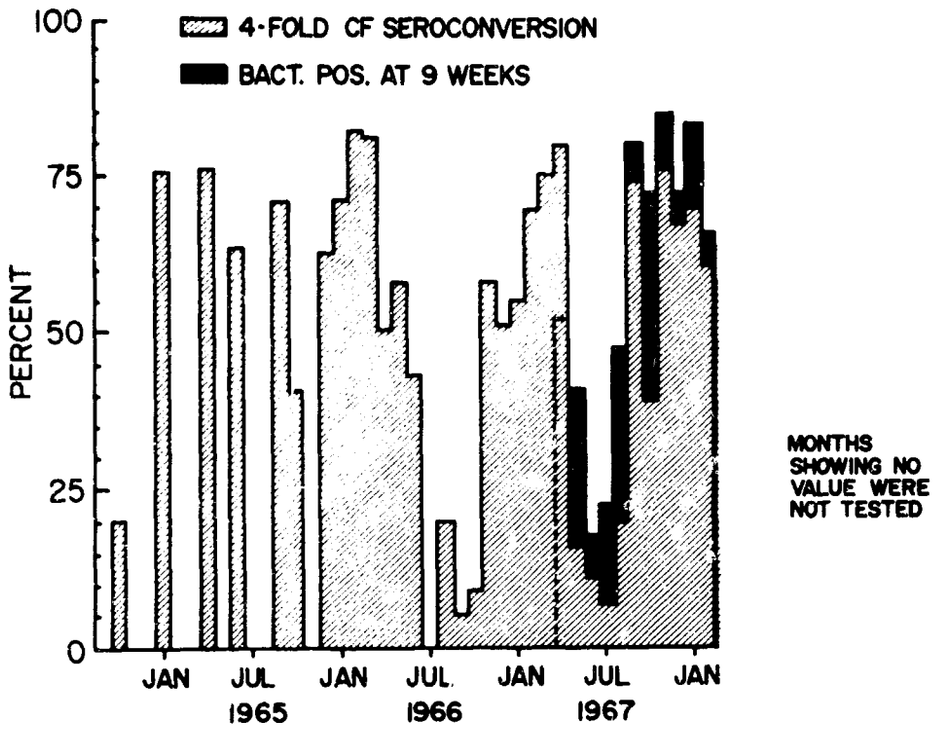

FIGURE 7

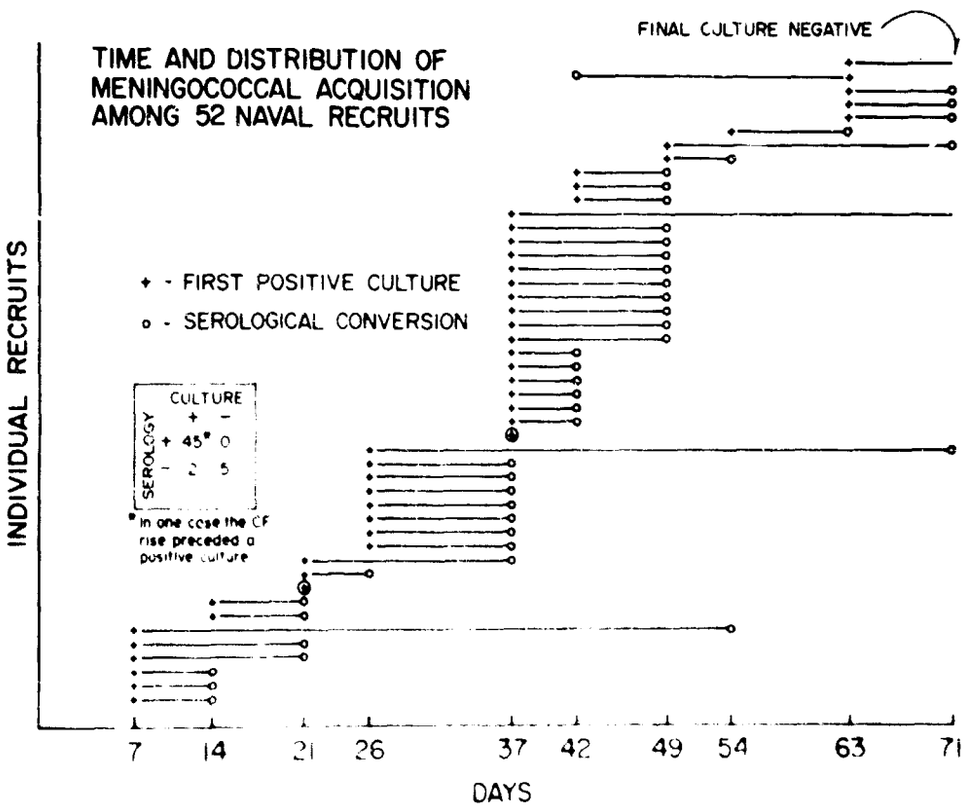

TABLE 1

CATEGORIES OF NAVAL PERSONNEL CULTURED FOR MENINGOCOCCI

RECRUITS

0-DAY RECRUITS: GREAT LAKES, DAY OF ARRIVAL

9-WK RECRUITS (NO Rx): GREAT LAKES GRADUATES, NO SULFADIAZINE PROPHYLAXIS

9-WK RECRUITS (Rx): GREAT LAKES GRADUATES, SULFADIAZINE PROPHYLAXIS

SERVICE SCHOOL COMMAND - FROM (LAST DUTY STATION)

GREAT LAKES: RECRUIT TRAINING, GREAT LAKES

SAN DIEGO: RECRUIT TRAINING, SAN DIEGO

SEA DUTY: SHIP OR STATION OVERSEAS (ATLANTIC OR PACIFIC, AS INDICATED)

SHORE DUTY: DUTY IN U.S.

NAVAL RESERVE (INACTIVE): GREAT LAKES RECRUIT TRAINING, FOLLOWED BY CIVILIAN LIFE UP TO ONE YR.

TABLE 2

SEROGROUPS ISOLATED IN ALL POPULATIONS JUNE 1967 - MARCH 1968

BOSHARD	4167
B	943
AGGLUT. IN ≥ 2 ANTISERA	712
C	497
AUTOAGGLUTINATING	476
NOT IDENTIFIED	107
29E	83
Z	43
SCAR. HOSP.	38
X	24
135	15
17	12
109	9

TABLE 3

PERCENT MENINGOCOCCAL CARRIERS IN NAVAL POPULATIONS JUNE 1967 - MARCH 1968

	POPULATION	PERCENT	RANGE BY MONTHS
RECRUITS	0-DAY RECRUITS	21.80	19.23 to 25.24
RECRUITS	9-WK RECRUITS (NO Rx)	56.75	21.73 to 82.66
RECRUITS	9-WK RECRUITS (Rx)	46.02	4.00 to 72.05
SCHOOL	GREAT LAKES	54.12	24.93 to 79.49
SCHOOL	SAN DIEGO	29.05	6.80 to 44.17
SERVICE	SEA DUTY (ALL AREAS)	33.04	26.97 to 40.19
SERVICE	SHORE DUTY	33.96	25.00 to 41.11
SERVICE	NAVAL RESERVE (INACTIVE)	28.66	22.89 to 35.48

TABLE 4

PREDOMINANT SEROLOGICAL GROUPS OF MENINGOCOCCI IN ALL POPULATIONS - FALL-WINTER 1967-1968

	POPULATION	% POSITIVE B	% POSITIVE C	% POSITIVE Bo
RECRUITS	0-DAY RECRUITS	6.26	1.64	4.59
RECRUITS	9-WK RECRUITS (NO Rx)	5.17	1.44	40.09
RECRUITS	9-WK RECRUITS (Rx)	5.75	2.80	33.04
SCHOOL	GREAT LAKES	1.94	3.02	41.53
SCHOOL	SAN DIEGO	3.61	3.61	16.66
SERVICE	SEA DUTY - ATLANTIC	12.36	4.45	7.70
SERVICE	SEA DUTY - PACIFIC	13.13	3.38	5.88
SERVICE	SHORE DUTY	7.40	2.59	14.57
SERVICE	NAVAL RESERVE (INACTIVE)	4.93	1.82	13.40

TABLE 5

UNUSUAL GROUPS OF MENINGOCOCCI
JUNE 1967 - MARCH 1968

	POPULATION	NO. OF ISOLATIONS 29E	Z	135	X	SCAR. HOSP.
RECRUITS	0-DAY RECRUITS	42	21	4	6	15
RECRUITS	9-WK RECRUITS (NO Rx)	5	1	1	0	0
RECRUITS	9-WK RECRUITS (Rx)	0	1	0	0	0
SERVICE SCHOOL	GREAT LAKES	7	3	6	3	13
SERVICE SCHOOL	SAN DIEGO	5	1	0	3	0
SERVICE SCHOOL	SEA DUTY - ATLANTIC	7	7	2	4	1
SERVICE SCHOOL	SEA DUTY - PACIFIC	11	4	1	7	3
SERVICE SCHOOL	SHORE DUTY	2	3	0	0	5
SERVICE SCHOOL	NAVAL RESERVE (INACTIVE)	4	2	1	1	1
	TOTAL	83	43	15	24	38

TABLE 6

PERCENT OF MENINGOCOCCAL ISOLATES RESISTANT TO SULFADIAZINE
JUNE 1967 - MARCH 1968

	POPULATION	% RESISTANT B	C	Bo	ALL SERO-GROUPS
RECRUITS	0-DAY RECRUITS	12.65	25.00	20.18	14.55
RECRUITS	9-WK RECRUITS (NO Rx)	50.00	50.00	87.27	79.25
RECRUITS	9-WK RECRUITS (Rx)	92.31	84.21	97.14	95.41
SERVICE SCHOOL	GREAT LAKES	52.46	72.49	92.47	[illegible]
SERVICE SCHOOL	SAN DIEGO	64.00	63.16	68.44	64.99
SERVICE SCHOOL	SEA DUTY - ATLANTIC	10.62	17.07	70.77	26.64
SERVICE SCHOOL	SEA DUTY - PACIFIC	10.89	7.41	55.81	18.39
SERVICE SCHOOL	SHORE DUTY	32.26	33.33	77.78	55.80
SERVICE SCHOOL	NAVAL RESERVE (INACTIVE)	15.91	23.53	60.91	42.69

TABLE 7

EFFECT OF SULFA PROPHYLAXIS ON CARRIAGE OF *N. MENINGITIDIS*

JULY 1967 - APRIL 1968

2nd CULTURE	SULFA Rx		NO SULFA Rx	
	SENSITIVE	RESISTANT	SENSITIVE	RESISTANT
POSITIVE	3	7	47	9
NEGATIVE	65	8	9	0
TOTAL.	68	15	56	9

CURED		SPONTANEOUS LOSS	
SENSITIVE	RESISTANT	SENSITIVE	RESISTANT
65 / 68	8 / 15	9 / 56	0 / 9
95.59%	53.33%	16.07%	0.0%

TABLE 8

EFFECT OF SULFADIAZINE PROPHYLAXIS ON MENINGOCOCCAL CARRIER RATE AND SULFA RESISTANCE FALL-WINTER 1967-1968

CATEGORY	NO Rx		Rx	
	% OF POP. POSITIVE	% OF + ISOLATES RESISTANT	% OF POP. POSITIVE	% OF + ISOLATES RESISTANT
0-DAY RECRUITS	24	7	23	18
9-WK RECRUITS +	76	91	67	96
− to +	74[1]	96[2]	69[1]	97[2]
+ to +	82[3]	72[4]	59[3]	91[4]

CONCLUSIONS

NO Rx	Rx
[1] NEGATIVES TEND TO BECOME POSITIVE.	[1] NEGATIVES NOT AS LIKELY TO BECOME POSITIVE.
[2] NEGATIVES TEND TO ACQUIRE RESISTANT ORGANISMS.	[2] SAME
[3] POS. TEND TO REMAIN POS.	[3] POS. TEND TO BECOME NEG.
[4] SENSITIVE ORG. TEND TO REMAIN.	[4] SENS. ORG. TEND TO BE LOST.
>CARRIER RATE WITH < % OF RESISTANT ORG.	<CARRIER RATE WITH > % OF RESISTANT ORG

TABLE 9

ESTIMATED NUMBER OF MENINGOCOCCAL INFECTIONS* IN NAVAL RECRUITS BY GROUP AND SULFADIAZINE SENSITIVITY. APRIL 1967 - MAY 1968

	SULFADIAZINE					
	SENSITIVE		RESISTANT		TOTAL	
TYPE	CASES	ESTIMATES	CASES	ESTIMATES	CASES	ESTIMATES
B		626	1	706	1	1,332
C		573	2	2,277	2	2,844
Bo	1	2,061	1	27,511	2	29,572
29E		66		11		77
Z		28		0		28
135		0		56		56
NO TYPE		342		0		342
AA		1,204		574		1,778
R4S		229		2,713		2,942
X		28		0		28
Scar Hosp		50		89		139
17		28		14		42
109		7		0		7
TOTAL	1	5,242	4	33,945	5	39,187

*BASED ON 10.5% SAMPLE OF 69,713 RECRUIT GRADUATES

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HEMAGGLUTINATION AND COMPLEMENT FIXATION ANTIGENS FROM NEISSERIA MENINGITIDIS. I. SOME LABORATORY CHARACTERISTICS

E. A. Edwards, W. S. Driscoll, P. M. Muehl,
R. I. Lytle and L. F. Devine

The serodiagnosis of meningococcal meningitis has been attempted by a number of workers during the past 60 years. The methods used were the precipitin test (1), bactericidal and agglutination tests (2), and the opsonin test (3). Additional epidemiological information on meningococcal meningitis has been obtained by fermentation and agglutination reactions of isolates from the patient and his contacts. The complement fixation (CF) and indirect hemagglutination test (IHA) have seldom been used for the diagnosis of meningococcal meningitis. Cruickshank (4) was one of the first to use what he called a protein antigen as a CF antigen. Ross and Stevenson (5) made a study using the CF test to diagnose aseptic meningitis. Their antigen was a modification of Price's GC CF antigen. Sanborn and Vedros (6) have recently used both the CF and IHA tests for determining antibody levels in clinical meningitis and studying carriers of Neisseria meningitidis.

This report will describe two antigens that we have been working with the past year. One of these antigens is a CF antigen (7) and the other an antigen which attaches to an unmodified erythrocyte, causing the erythrocyte to agglutinate in the presence of homologous antisera (8). Our experience with these antigens over the past year and performing over 6,000 tests indicate that they possess the sensitivity and specificity required of serologic tests in determining seroresponse to N. meningitidis infections.

Several of the laboratory characteristics, chemical composition, and toxicity studies will be shown as well as their behavior

in detecting antibodies in serum from clinical meningitis cases, and finally in carriers of N. meningitidis groups encountered in recruit training at Great Lakes.

Figure 1 shows a flow diagram demonstrating simple laboratory procedures used in antigen preparation. An 18-hour Mueller-Hinton broth culture is inactivated with a final concentration of 1% beta-propriolactone overnight at 6°, the sediment collected by centrifugation, the supernatant is saved for the preparation of a potent, non-antiC' CF antigen using the steps as shown on the right and a good hemagglutinating antigen obtained from the sediment using the steps on the left hand of the Figure (7). The HA has not caused spontaneous agglutination or lysis of the erythrocytes.

The remarkable specificity of the HA antigens is shown in Tables 1 and 2. The data in Table 1 demonstrates the specificity of N. meningitidis HA antigens group A, B, C, Bo, and 29E tested against homologous and heterologous hyperimmune rabbit antisera. This specificity has been repeated many times by different lots of N. meningitidis HA antigens. The data in Table 2 is a test for the specificity of the inhibitory capabilities of the HA antigens. Briefly, the test was performed on 3 separate microtiter plates. Two-fold dilutions of hyperimmune rabbit antisera to N. meningitidis HA antigens A, B, C, Bo and 29E were made in 0.01 M PBS with 1:200 normal rabbit serum added (the diluent for all N. meningitidis HA testing) (7). One drop of HA N. meningitidis group A antigen was added to each well of the plate marked group A; one drop of N. meningitidis group B antigen to the plate marked group B, and one drop of N. meningitidis group C HA antigen to the plate marked group C. The plates were mixed and allowed to stand for 1 hour at room temperature. Optimally sensitized sheep erythrocytes (7) were added to their homologous diluted hyperimmune antisera in each of the plates. The plates were ready to read in about 45 minutes (or when the controls had properly dropped). As the data indicate in Table 2, N. meningitidis group A antigen inhibited only antisera to group A and did not reduce the titer of the antisera to N. meningitidis groups B, C, Bo and 29E. Likewise, N. meningitidis antigens group B and group C inhibited only their homologous antisera

and did not reduce the titer to any of the heterogous group antisera. This is interpreted to indicate extreme specificity of these group-specific N. meningitidis HA antigens.

Figure 2 shows the optimal time required for the antigen to adsorb to the cells and, as can be seen, by 10 minutes, all antigens have satisfactorily sensitized the cells with the exception of group B antigen which takes at least 30 minutes for sensitization. This is not the only discrepancy that group B has shown. Efforts are being intensified to improve this (group B) HA antigen.

Because of the number of meningococcal groups now in the N. meningitidis array of Neisseria, it is obvious that it would be impossible for the laboratory to support large epidemiological studies. Following the lead of Landy (9), who in 1954 clearly demonstrated that an erythrocyte can be sensitized with more than one lipopolysaccharide (although he used lipopolysaccharides from different gram-negative bacilli), it was found, as shown in Table 3, that the erythrocyte could be sensitized with multiple antigens either sequentially or as shown in Table 4 simultaneously, with no loss of sensitivity or specificity. Optimal sensitizing dilutions of each antigen were added to sensitize the erythrocyte (8).

Turning to the chemical analysis of the HA antigen, the crude antigen recovered after alkaline hydrolysis alcohol precipitation was further purified by phenol-water extraction and acetone precipitation (Fig. 3). The results of this analysis are shown in Table 5. The carbohydrate (CHO) free fraction has never shown HA activity and the "pure" CHO fraction has always contained HA activity.

The CF antigen in contrast to the HA antigen has failed to show any group-specificity, thus permitting the use of a CF antigen made from any one of the meningococcal groups to detect Neisseria antibody. Table 6 demonstrates the genus specificity, but no group specificity is indicated.

Table 7 demonstrates the reaction of sera collected from

clinical meningitis patients admitted to the Naval Hospital. Sera collected at approximately weekly intervals and tested against CF antigens made from 6 N. meningitidis groups or types show equivocal serology. That sera from clinical meningitis were not unique in their CF activity, sera collected from recruits who became infected during recruit training al o show equivocal serology to CF antigens made from 5 different groups or types as shown in Table 8. These are representative specimens selected from a company of 75 men.

Returning to the 4 clinical meningitis cases referred to, the antibody response from sera collected as measured by the IHA, is shown in Table 9. Meningococcus group Bo was isolated from the spinal fluid and throat culture of patient 226. Meningococcus group C was isolated from the spinal fluid and throat cultures of patients 521 and 782. No spinal fluid isolate was made on patient 749, but one colony, typed as a group Bo, was identified from the throat culture. Antibody responses, both CF and HA, are rapid and detectable by day 7 post-hospitalization. Patient 749 showed a marked CF response by day 14, but HA antibody was markedly delayed until a 44-day blood sample. The reason for the rather unusual serology of this patient is not presently known.

Absorption studies show that the removal of HA antibody does not reduce CF antibody levels. We must assume, therefore, that these antibodies represent two distinct antibody molecules, each with its specificity and not one antibody having dual specificity. If this assumption is true, it would be expected that the serology observed on patient 749 might occur with some degree of frequency in a random population. This has been observed in about 3% of our carrier studies.

To determine endotoxic and cytotoxic properties of a CF antigen, a group C CF antigen containing 50 mg in 15 ml was charged onto a Sephadex 200 column equilibrated with .01 M phosphate buffered saline (PBS) pH 7.2 and 3 ml fractions were collected through 40 fractions. Rabbit skin assay for endotoxic activity was performed on each fraction as described by Larson (10). The generalized Shartzman phenomena was performed by the method of Thomas and Good (11) and tissue cell cytotoxicity was made using

HEp-2 and WI-38 tissue culture cells in the microplate. There was no evidence of tissue culture toxicity in either cell line. The results from the other toxicity test are shown in Figure 4. Skin tests were from maximum reactivity of 22 mm with only slight edema, but distinct erythema; no necrosis was evident. The CF and HA activity were eluted off the column immediately following the void volumn indicating a molecular weight of over 200,000. The generalized Schwartzman was positive in fractions 12 through 18, following the same pattern as the skin assay tests for endotoxin. That the toxicity was not due to the protein or nucleic acid is shown in Figure 5. As the data in this Figure show, the major portion of the nucleic acids and total proteins are eluted after the HA and CF activity and gave negative skin tests and Schwartzman reaction.

Finally, concrete relationship of serology to bacteriology acquisition has recently been completed. A company of men were selected in February of this year (1968) for determining N. meningitidis acquisition by both bacteriology and serology. Weekly samples were taken. Fifty-two men from whom 11 samples were taken, and who entered recruit training without N. meningitidis on their initial culture, were analyzed. The data in Figure 6 show the correlation between bacterial isolation and CF serology (4-fold or greater). That this correlation is highly significant is obvious with the antibody seroconversion occurring about 9 days after the first bacterial isolation. Figure 7 shows the correlation between N. meningitidis group Bo isolation and group Bo hemagglutinating antibody response; the correlation is highly significant. Figure 8 shows the correlation between N. meningitidis group C and group C antibody response; again, the correlation is highly significant.

SUMMARY

Two antigens are described that detect antibody in persons with clinical meningitis and/or who became carriers of N. meningitidis. They seem to possess the sensitivity and specificity required of serologic antigens, and their ease in preparations using simple laboratory procedures should make them adaptable to nearly any laboratory situation.

FIGURE 1

PREPARATION OF COMPLEMENT FIXING AND HEMAGGLUTINATING ANTIGENS FROM *N. MENINGITIDIS*

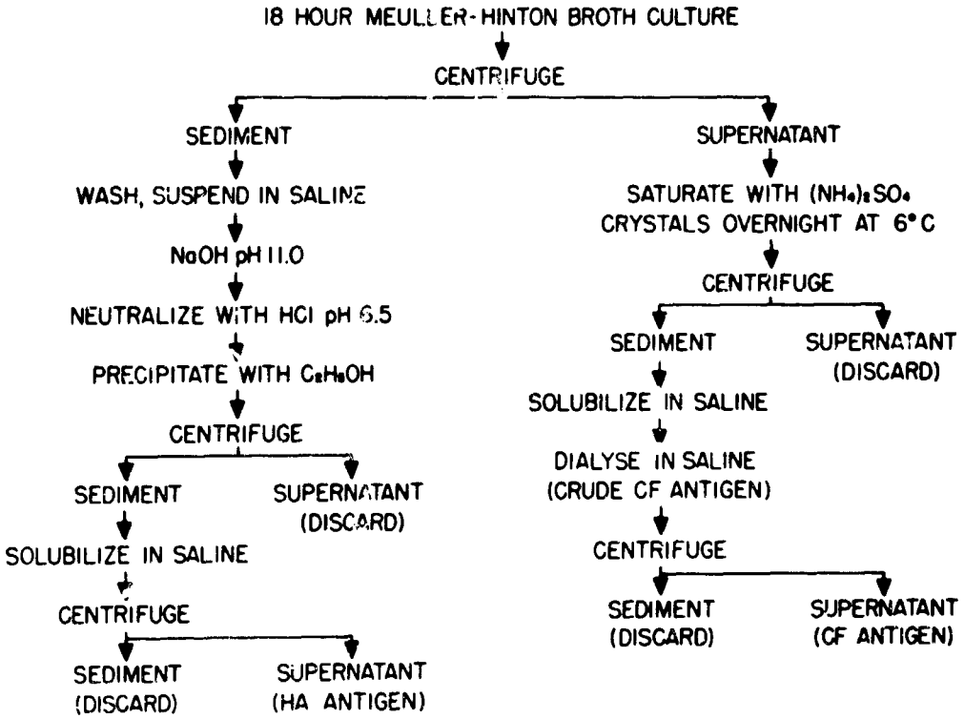

FIGURE 2

RATE OF SHEEP CELL SENSITIZATION BY VARIOUS *N. MENINGITIDIS* HEMAGGLUTINATING ANTIGENS

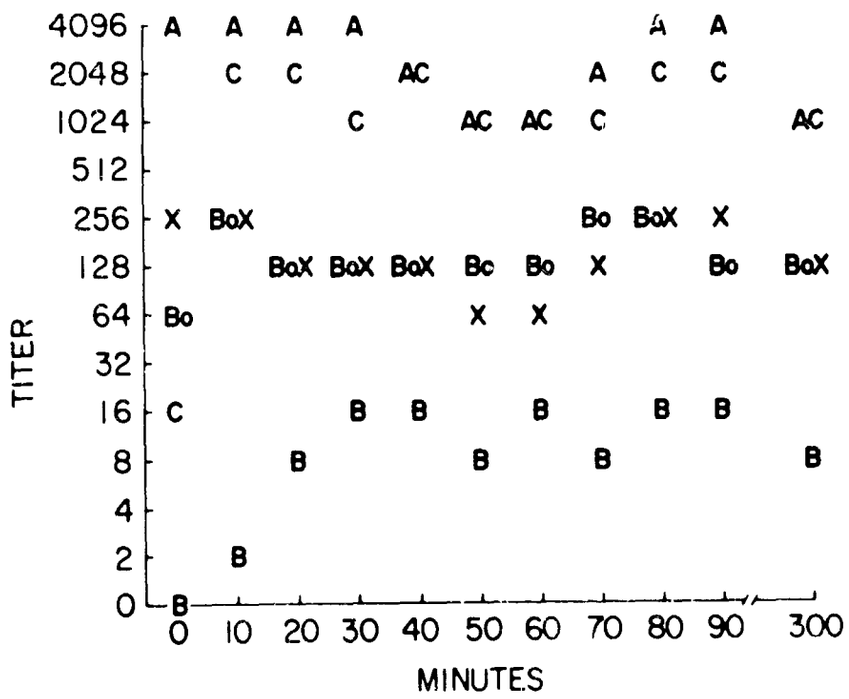

FIGURE 3

CHEMICAL CHARACTERIZATION OF HEMAGGLUTINATION ANTIGENS FROM *N. MENINGITIDIS*. A METHOD OF PURIFICATION

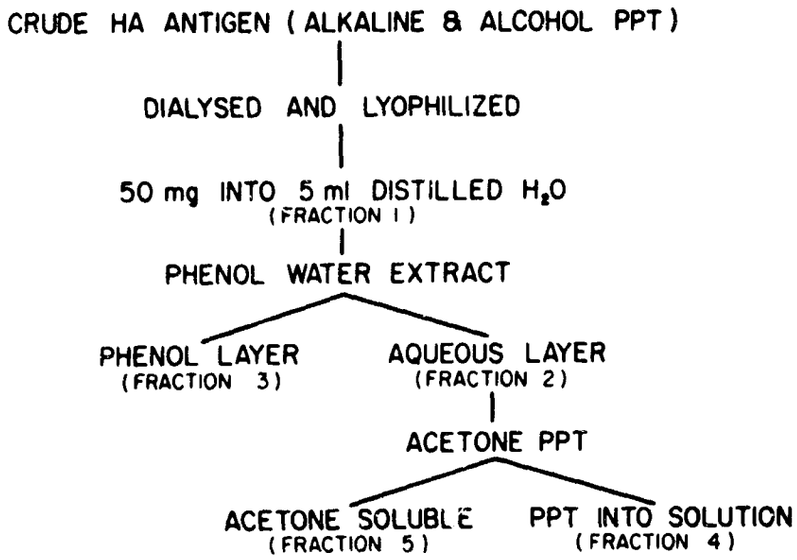

FIGURE 4

IMMUNOLOGICAL CHARACTERISTICS OF COMPLEMENT FIXATION *N. MENINGITIDIS* ANTIGEN

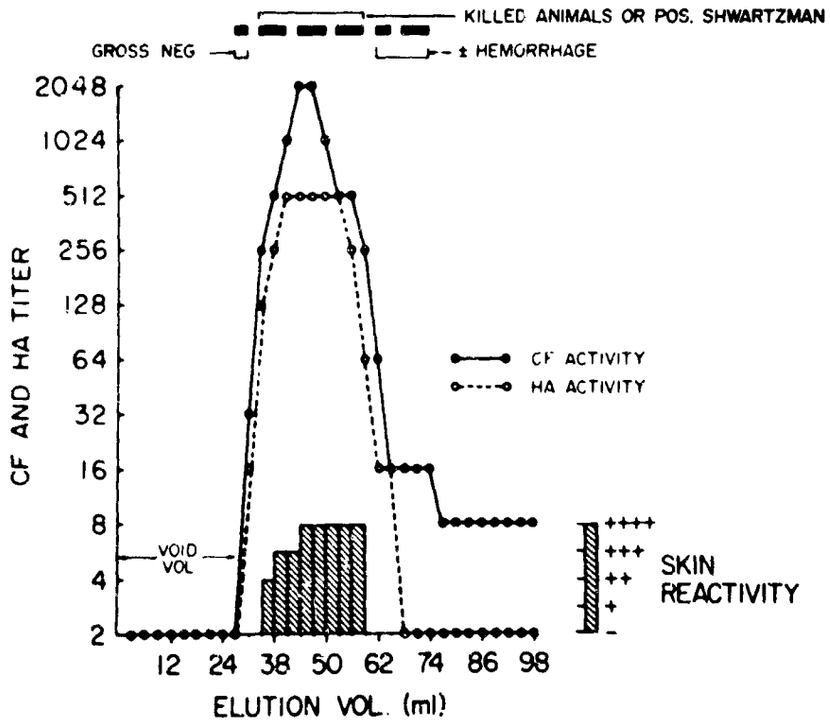

FIGURE 5

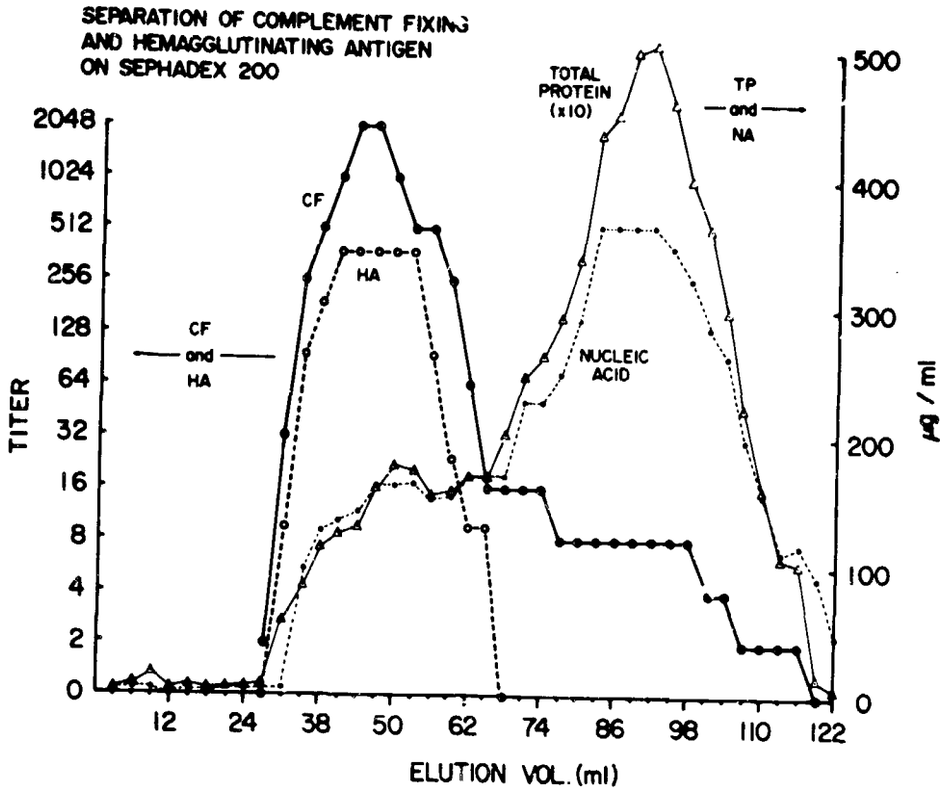

FIGURE 6

COMPARISON OF CUMULATIVE PERCENTAGE OF MENINGOCOCCAL ACQUISITIONS AND CF SEROLOGICAL CONVERSIONS DURING TRAINING

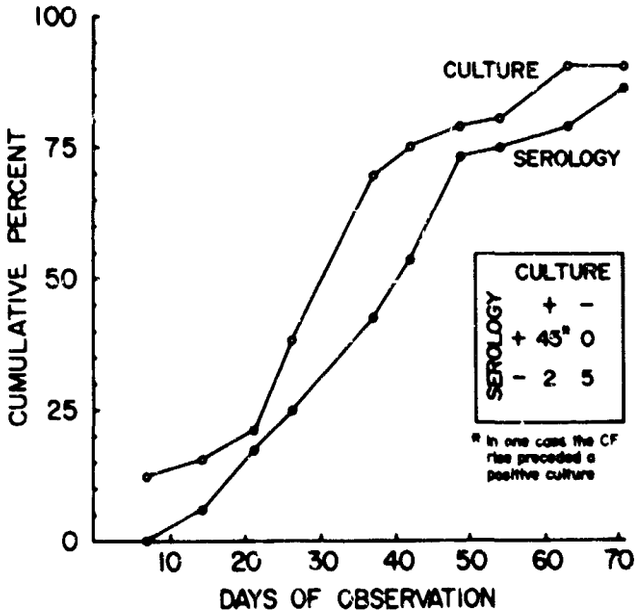

FIGURE 7

COMPARISON OF CUMULATIVE PERCENTAGE OF MENINGOCOCCAL ACQUISITIONS OF Gr. Bo AND HA SEROCONVERSIONS TO Gr. Bo DURING RECRUIT TRAINING

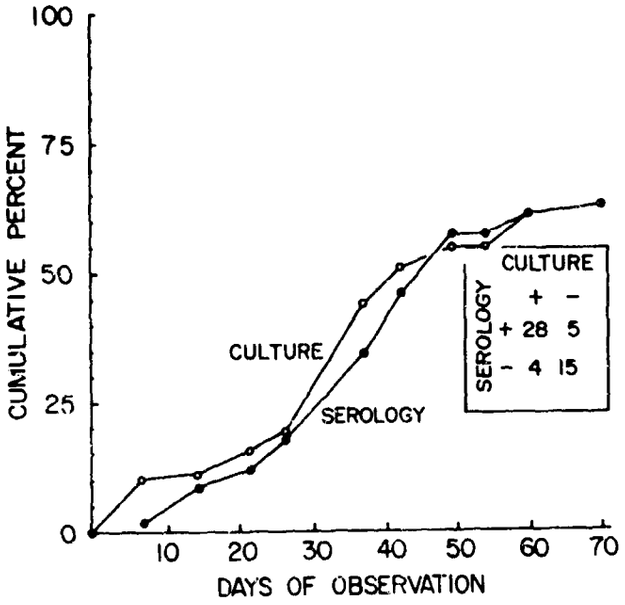

FIGURE 8

COMPARISON OF CUMULATIVE PERCENTAGE OF MENINGOCOCCAL ACQUISITIONS OF Gr. C AND HA SEROCONVERSIONS TO Gr. C DURING RECRUIT TRAINING

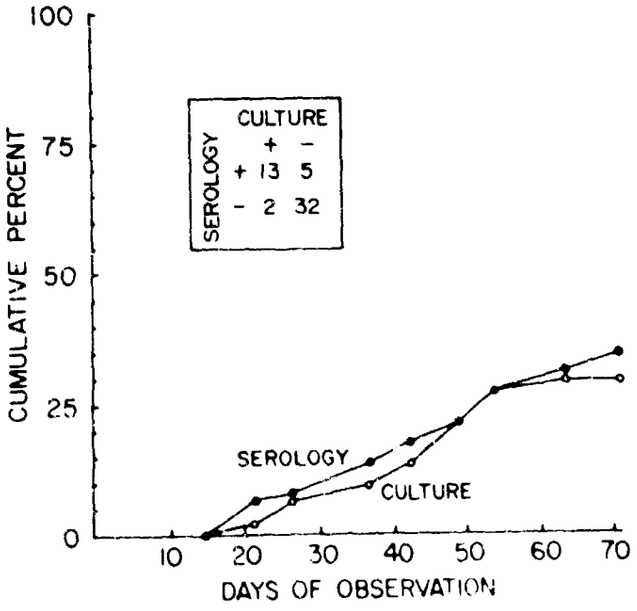

TABLE 1

SPECIFICITY OF THE INDIRECT HEMAGGLUTINATION ANTIBODY TEST USING VARIOUS ANTISERA TYPES AGAINST HOMOLOGOUS AND HETEROLOGOUS SENSITIZED ERYTHROCYTES

	ERYTHROCYTES SENSITIZED WITH ANTIGEN FROM *N. MENINGITIDIS* TYPE:				
ANTISERUM	A	B	C	Bo	29E
ANTI-A	1:256	1:8	<1:2	<1:2	<1:2
ANTI-B	1:2	1:32	<1:2	<1:2	<1:2
ANTI-C	<1:2	<1:2	<1:256	<1:2	<1:2
ANTI-Bo	<1:2	<1:2	<1:2	1:512	<1:2
ANTI-F	<1:2	<1:2	<1:2	<1:2	1:512
ANTI-*N. gonorrhoeae*	<1:2	<1:2	<1:2	<1:2	<1:2
ANTI-*N. perflava*	<1:2	<1:2	<1:2	<1:2	<1:2
ANTI-*N. flavescens*	<1:2	<1:2	<1:2	<1:2	<1:2

ANTISERA A, B AND C LOTS DIFCO NO. 499231, 496356 AND 491435, RESPECTIVELY. ANTISERA-F, *N perflava* AND *N flavescens* KINDLY SUPPLIED BY LT. NEYLAN VEDROS. ANTI-*N. gonorrhoeae* KINDLY SUPPLIED BY WM PEACOCK, JR, CDC, AND ANTI-Bo SUPPLIED BY LCDR LEONAND DEVINE, NAMRU-4.

TABLE 2

SPECIFICITY OF MENINGOCOCCUS HA ANTIGENS IN INHIBITING THE IHA TEST FOR MENINGOCOCCAL ANTIBODY

	INHIBITORY EFFECT OF HA ANTIGEN																	
	GROUP A						GROUP B						GROUP C					
ANTI-	A	B	C	Bo	29E	CONTROL	A	B	C	Bo	29E	CONTROL	A	B	C	Bo	29E	CONTROL
A	1:8					1:512	1:512					1:512	1:1024					1:512
B		1:64				1:32		1:4				1:32		1:32				1:32
C			1:1024			1:1024			1:1024			1:1024			1:16			1:1024
Bo				1:256		1:512				1:256		1:512				1:512		1:512
29E					1:512	1:512					1:256	1:512					1:512	1:512

TABLE 3

SEQUENTIAL SENSITIZATION OF SHEEP ERYTHROCYTES WITH DIFFERENT *N. MENINGITIDIS* ANTIGENS

	SHEEP ERYTHROCYTES SENSITIZED WITH *N. MENINGITIDIS* ANTIGENS				
ANTISERA	A	A+B	A+B+C	A+B+C+Bo	A+B+C+Bo+29E
ANTI-A	2048	2048	2048	2048	2048
ANTI-B	-	64	64	64	64
ANTI-C	-	-	1024	2048	1024
ANTI-Bo	-	-	-	256	256
ANTI-29E	-	-	-	-	256

(- = <2)

TABLE 4

SIMULTANEOUS SENSITIZATION OF SHEEP ERYTHROCYTES WITH FIVE *N. MENINGITIDIS* ANTIGENS

	SHEEP ERYTHROCYTES SENSITIZED WITH *N. MENINGITIDIS* ANTIGENS					
ANTISERA	A	B	C	Bo	29E	A, B, C, Bo, 29E
ANTI-A	1024	-	-	-	-	1024
ANTI-B	-	64	-	-	-	64
ANTI-C	-	-	512	-	-	1024
ANTI-Bo	-	-	-	256	-	256
ANTI-29E	-	-	-	-	512	256

(- = <2)

TABLE 5

CHEMICAL CHARACTERIZATION OF CRUDE AND PURIFIED HA ANTIGEN

ANTIGEN 10mg/ml	CRUDE ANTIGEN TOTAL PROTEIN* mg/ml	NUCLEIC ACID** mg/ml	CHo†	CF	HA	'CLEAN' ANTIGEN TOTAL PROTEIN	NUCLEIC ACID	CHo	CF	HA
A	5.15	1.5	87	256	512	—	—	.73	64	256
B	4.75	2.27	59	256	128	—	—	.51	32	32
C	3.95	86	63	256	256	.25	—	.59	64	256
Bo	4.75	46	48	128	1024	—	—	.44	64	512
29E	5.15	72	45	256	512	10	—	.40	128	2048

* TOTAL PROTEIN — LOWRY METHOD
** NA — DIPHENYLAMINE METHOD (D595 - D650)
† CHo — INDOLE METHOD - ALL CARBOHYDRATES EXCEPT AMINO SUGARS

TABLE 6

COMPLEMENT FIXATION TITERS* OF VARIOUS HYPERIMMUNE RABBIT *N. meningitidis* ANTISERA USING ANTIGEN PREPARED FROM DIFFERENT *N. meningitidis* GROUPS AND STRAINS

IMMUNE SERUM	CF Antigen Prepared from *N. meningitidis* Groups & Strains (4 antigen units/unit volume) A	B	C	Bo	29E	135	Z
N. meningitidis group A	128	128	128	64	128	64	128
N. meningitidis group B	128	256	128	256	256	256	256
N. meningitidis group C	64	[illegible]	4	128	64	256	256
N. meningitidis strain Bo	64	128	64	128	128	256	256
N. meningitidis strain 29E	128	512	128	128	128	512	256
N. meningitidis strain Z	64	128	64	64	64	256	256
N. meningitidis strain 135	64	64	64	64	32	128	256
N. gonorrhoeae	32	64	32	64	64	32	32
N. catarrhalis	ND**	16	16	16	32	32	ND
N. flava	32	16	64	64	64	64	ND
N. flavescein	32	64	64	32	64	64	ND
N. sicca	32	16	32	32	16	32	ND
N. perflava	16	16	64	64	128	64	ND
N. subflava	ND	32	64	32	32	64	ND
D. pneumoniae	4	<4	4	4	8	<4	4
H. influenzae	<4	4	4	<4	8	4	<4
S. typhi (O=1:2530; H=1:80)	4	4	8	4	4	4	8
S. typhi (O=1:640; H=1:5120)	4	4	4	4	4	4	4

* The reciprocal of initial dilution of serum showing a 4+ fixation.

** Not Done.

TABLE 7

COMPARISON OF COMPLEMENT FIXATION SERUM ANTIBODY TITERS* IN NAVY RECRUITS WITH CLINICAL MENINGITIS USING 6 COMPLEMENT FIXATION ANTIGENS

		CF Antigen from *N. meningitidis* Groups & Strains					
PATIENT	ADMISSION DAY	A	B	C	29E	135	Bo
226	0	<	4	4	4	4	4
	10	128	256	128	256	256	128
	19	256	128	128	128	128	128
	31	64	64	128	64	64	64
521	0	<	<4	<4	<4	4	<4
	7	8	16	16	8	8	8
	14	32	64	64	64	64	64
	21	32	64	64	64	32	64
782	0	<	<4	<4	<4	4	<4
	7	32	64	128	64	64	64
	14	32	64	128	64	32	64
	21	32	64	128	32	64	64
749	0	<	<4	<4	<4	<4	<4
	7	4	4	4	4	4	4
	14	64	128	128	128	64	64
	21	32	64	64	64	32	64

*Reciprocal of the highest dilution of serum giving a 4+ fixation.

TABLE 8

COMPARISON OF COMPLEMENT FIXATION SERUM ANTIBODY TITERS* OF NAVY RECRUIT CARRIERS OF *N. meningitidis* STRAIN Bo USING 5 COMPLEMENT FIXING ANTIGENS

			CF Antigen for *N meningitidis* Groups & Strains				
STUDY RECRUIT NO	WEEK IN TRAINING	SAMPLE NO	29E	C	B(Scarborough)	B(16B6)	Bo
1053	0	1	1:4	<1:4	<1:4	<1:4	<1:4
	5	2	1:4	<1:4	<1:4	<1:4	<1:4
	9	3	1:64	1:32	1:32	1:64	1:64
1090	0	1	<1:4	<1:4	<1:4	<1:4	<1:4
	5	2	1:64	1:32	1:64	1:64	1:32
	9	3	1:32	1:16	1:32	1:32	1:16
1132	0	1	<1:4	<1:4	<1:4	<1:4	<1:4
	5	2	<1:4	<1:4	<1:4	<1:4	<1:4
	9	3	1:16	1:16	1:32	1:32	1:16

*Serum titers expressed as the reciprocal of the highest dilution giving a 4+ fixation in the presence of 4 units of antigen.

TABLE 9

INDIRECT HEMAGGLUTINATION AND COMPLEMENT FIXATION TITERS IN NAVY RECRUITS WITH CLINICAL MENINGITIS

PATIENT	SAMPLE BLOOD	INDIRECT HEMAGGLUTINATION *N. meningitidis* GROUP A	B (BC)	C	Bo	i35(Pool)	COMPLEMENT FIXATION
226	0 Day	NT	<	NT	<	<	< 4
	10	NT	<	NT	256	2	128
	19	NT	<	NT	256	2	256
	31	NT	<	NT	128	4	64
	106	NT	4	NT	16	2	16
521	0	4	<	<	<	2	< 4
	7	8	<	512	64	2	8
	14	8	<	512	32	32	64
	21	8	<	256	16	16	64
782	0	2	<	64	<	2	4
	7	8	<	2048	<	2	128
	14	8	<	1024	<	2	128
	21	4	<	512	<	4	128
	29	4	<	128	2	4	64
	70	4	<	64	2	4	32
749	0	<	<	4	<	<	< 4
	7	<	<	8	<	<	4
	14	<	<	8	<	<	128
	21	<	<	8	<	<	64
	44	<	<	4	64	NT	128

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MENINGOCOCCAL L FORMS: CULTURAL STUDIES AND SEROLOGICAL REACTIONS

D. W. Beno, D. D. Bradley and Y. E. Crawford

Among bacteria known to produce L forms are members of the genus Neisseria. Dienes was first to attempt the isolation of L forms from these organisms, but he found it impossible to transfer and propagate the tiny colonies he watched develop in his plates. More recently, at the University of Chicago, Bohnhoff and Paige, and at Walter Reed, Roberts and Wittler, isolated and propagated L forms from many strains of Neisseria meningitidis. In response to the need for a broadened view of the meningococci, a study of Neisserial L forms was undertaken to supplement bacteriological and immunological information being collected from naval recruits at Great Lakes, Ill. This report will concern isolation data and serological reactions with emphasis on complement fixation and newly observed growth inhibition reactions. The immunizing capabilities of an L form antigen, prepared in our laboratory, are reported in a subsequent paper (1).

METHODS

All meningococci used for parent strains were isolated, identified, or propagated by usual techniques. The method of penicillin inducement used by Bohnhoff and Paige was adapted for transforming bacteria to the L form (2).

RESULTS

Isolation from bacteria:

To date, 164 strains of N. meningitidis have been cultured for

L colonies and 159 or 97% were found capable of growing in this form. Table 1 shows the serological groups and sources of meningococci that were converted to the L form. The group in which a strain belongs appeared to have no bearing on the transforming ability. The source of the strain, however, seemed of significance since the diplococci from actual infections produced L forms more readily than cocci from carriers. All strains from infections shown here developed into L forms within 48 hours, whereas carrier strains required from 3 to 5 days before L's are noted.

Figure 1 represents a low power field of L colonies derived from a group B strain. The fried egg shape is typical of bacterial L colonies and is typical of mycoplasma colonies as well. With the organism in this form, carbohydrate fermentation is delayed 3 to 5 days, although it duplicates the pattern of the parent bacteria. The oxidase reaction is irregular, and agglutination by antibacterial rabbit sera is rarely observed. Through the courtesy of Dr. William Donnolan of Childrens' Memorial Hospital in Chicago, a sectioned colony was studied under the electron microscope. Figure 2 shows one section magnified on the original slide 17,300X. It was thought, and quite rigidly, that L form units lack cell walls and are bound instead by a single, triple-layered membrane approximately 75 A thick. With Proteus, recent photographs published by Dienes, show that single unit membranes occur in one type L colony, labeled "A", while a double membrane occurs in a second type of L colony, labeled "B" (3). This section of an N. meningitidis L colony shows, instead, organisms having both single and double unit membranes in the same colony.

Isolation from nasopharynx:

Whether or not bacteria exist in the L form in clinical material, and can be isolated as such, has remained a challenging question for many years. Attempts to grow them from the nasopharynx were unsuccessful on the high serum-sucrose medium used for meningococcal L forms. However, agar-penetrating, fried egg shaped colonies were detected among the normal meningococcal colonies in primary cultures. They grew out whether one used Thayer-Martin, Mueller-Hinton, or Trypticase Soy Agar without antibiotics. A

growth of these colonies is shown in Figure 3.

They become apparent in 12-16 hours, but with continued incubation, lose the fried egg appearance due to overgrowth by bacteria. Ten well-isolated fried eggs were picked and transferred to determine the bacteria they would yield. From these 10 colonies, 2 categories of bacteria were recovered --- the genus Neisseria and the genus Streptococcus. Five of the 10 were consistent with being Neisseria and were tested with agglutinating antisera. Three agglutinated as Boshard and 2 as N. flava. Of the 5 remaining streptococci, presumptive identifications were made on mitis-salivarius agar. Two grew as S. mitis, 2 as S. sanguis and 1 as S. salivarius. It was thought interesting and significant to the L form hypothesis that 2 genera of bacteria were derived from fried egg colonies of identical appearance.

Filtration experiments:

To determine if filter passing micro-organisms resembling L forms were present in the nasopharynx, several experiments were done. Membrane filters of 0.45μ porosity were placed on Mueller-Hinton agar and the edges sealed with agar. The center of the filter was touched with a nasopharyngeal swab and the plate incubated. A finely granular agar-penetrating growth often appeared beneath the filter with no evidence of normal bacterial cells. This growth was identical in all respects to growth obtained from known L forms which also pass the filter. It was similar also to the early growth of the fried egg colonies previously mentioned. In order to obtain information on the incidence of the filter passing forms, a series of 3 cultures were taken on a meningococcus study company as it passed through training, using membrane filters on the surface of the agar. The results are shown in Figure 4.

The incidence of the N. meningitidis bacterial form is shown to have increased steadily as recruits passed through training. The incidence of filter passing forms was always greater, but did not vary markedly as these recruits advanced through training. Transferring 153 of the filtered forms onto Thayer-Martin medium resulted in the appearance of meningococci 29 times (white bars).

These data indicate that a very high percentage of recruits harbor agents that pass 0.45μ filters, and it is interesting to speculate that these are sub-bacterial forms somewhat akin to the L forms of bacteria as we know them. It is noteworthy to the aims of this study that some of these filter-passing microbes reproduced as meningococci on appropriate selective medium. It was a matter of interest that 2 meningococci appearing from the filtered material typed as Z strains, but not until 11 passages on Mueller-Hinton medium and 2 additional passages in sheep blood. Delays in acquiring groupability have been commonly noted with meningococci when reverted from known L forms.

Complement fixation:

The antigenic properties of meningococcal L forms are of interest for the possibility of serological diagnosis and immunization. However, for reasons not clear, growth in fluid medium is rarely observed. One L-strain, derived from a group B meningococcus, grew well in broth, but not until more than 50 passages on agar medium were made. Our studies indicate that this organism contains complement fixing antigen that reacts strongly to animal and human sera. The antigen is extractable in hot saline, is free of anticomplementary properties, and may be stored on the shelf for long periods of time with phenol preservative.

Examples of reactions observed with rabbit diagnostic antisera and sera from recruits are shown in Table 2.

The groups A, B, and C rabbit antisera were commercially prepared and reacted rather weakly to both L and bacterial extracts. However, the strain Boshard rabbit antisera gave fixation out to a 1:128 dilution. This serum was prepared by LCDR L. F. Devine, at Great Lakes, using a special immunization procedure. It may be observed that the human sera reacted equally to both L and bacterial antigens. A surveillance company was tested by the Kolmer method for CF antibody titers. Of 76 recruits on whom tests were completed, 54 or 71% showed 4-fold or greater rises in titer to the L form antigen. Of these 54 rises, 14 were followed by falls. In other words, the CF antibody in 25% of these persons was of short duration. Of the 76 men, 9 entered the Navy from civilian life with a pre-existing titer of 1:8 or greater. Of these 9 persons, only 1 reverted to the negative status. Table 3 presents examples of the rising titers observed in these recruits as

they passed through training.

A chemical analysis of L form CF antigen and bacterial form CF antigen was carried out by Mr. R. Lytle to determine if differences exist and to characterize the reactive material. These studies indicated that the L form and bacterial extracts, as we prepare them, are mixtures of polysaccharide, lipo-polysaccharide, protein, nucleoprotein and nucleic acids. It was not possible to obtain complement fixation with any of these isolated components; however, it was observed that the CF reactivity of the L form extract could be abolished with trypsin, whereas the bacterial extract was not affected by this enzyme. It thus seems possible that the L-reactive material may be a protein complex differing structurally from the CF antigen of the bacterial form.

Growth inhibition:

It is well known that mycoplasma species stimulate growth inhibiting antibodies in animals and in man, and that this antibody is linked with immunity against mycoplasma infections. These antibodies may be detected by placing serum-containing paper disks on the surface of seeded agar plates (4). The antibody inhibits growth and a clear zone develops around the disc. An example of this growth inhibiting activity against Mycoplasma pneumoniae is shown in Figure 5 by the clear zone adjacent to the disc (dark area).

The question posed was whether growth inhibiting antibodies to meningococcal L forms can be demonstrated with the same test. From Baylor University, Dannis and Marston have reported on growth inhibiting antibodies to the L forms of Staphylococcus aureus (5). From Russia, Kagan has described growth inhibition with paper discs against the L form of Streptococcus pyogenes (6).

Kagan reports that the reaction is species-specific, that is, her antisera did not inhibit L forms of Staphylococcus or of a Proteus.

Attempts to demonstrate growth inhibition against the L form of a group B N. meningitidis strain were consistently negative with 6 rabbit antisera. However, substituting a serum pooled from mice immunized with the homologous L form, and who had

survived a challenging dose of N. meningitidis bacteria, led to better results. The zone produced by this serum pool is shown in Figure 6.

It was next of interest to react sera of recruits against the L form by the growth inhibiting paper disc test. It came as a surprise to note that good reactions were easily obtainable with certain human sera. A positive test with a recruit serum is shown in Figure 7.

We are presently engaged in testing sera from surveillance companies to determine if associations exist with bacterial isolations, complement fixing, and other Neisserial antibodies. One company has been completed and it shows that 72% of 86 men possessed sera that were active by growth inhibition against a group B N. meningitidis L form.

In this group of 86 men, only 2 arrived from civilian life with this antibody; however, by the 5th week, 55 men had acquired positive status. Further studies are underway on this phenomenon which is new to the biology of the meningococcus.

SUMMARY

The vast majority of N. meningitidis strains tested were capable of transforming to the L form when induced with penicillin. Under the electron microscope, one B strain L colony showed both single and double outer membranes. To our knowledge, this is an exception to L forms of other bacteria. A group B meningococcus L form has yielded complement fixing antigen that reacts to animal and human sera and detects antibody responses in up to 72% of recruits as they pass through training. The sera of immunized mice and the serum of up to 72% of recruits contains growth inhibiting antibodies to the L form of a group B meningococcus.

FIGURE 1

FIGURE 2

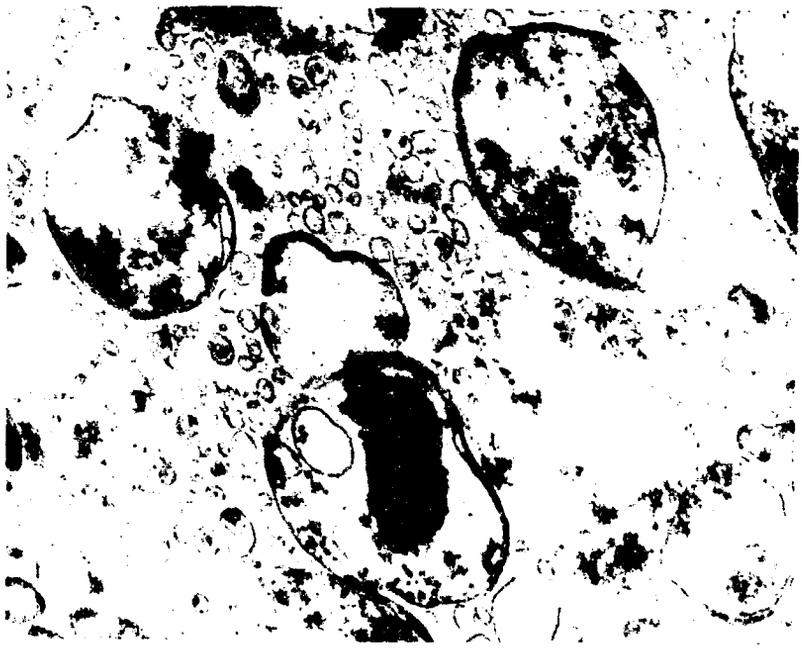

FIGURE 3

FIGURE 4

PERCENT OF CULTURES POSITIVE FOR *N. MENINGITIDIS*, FILTERED FORMS AND "REVERTED" MENINGOCOCCI
NAVAL RECRUITS, GREAT LAKES, ILLINOIS, APRIL-MAY, 1968

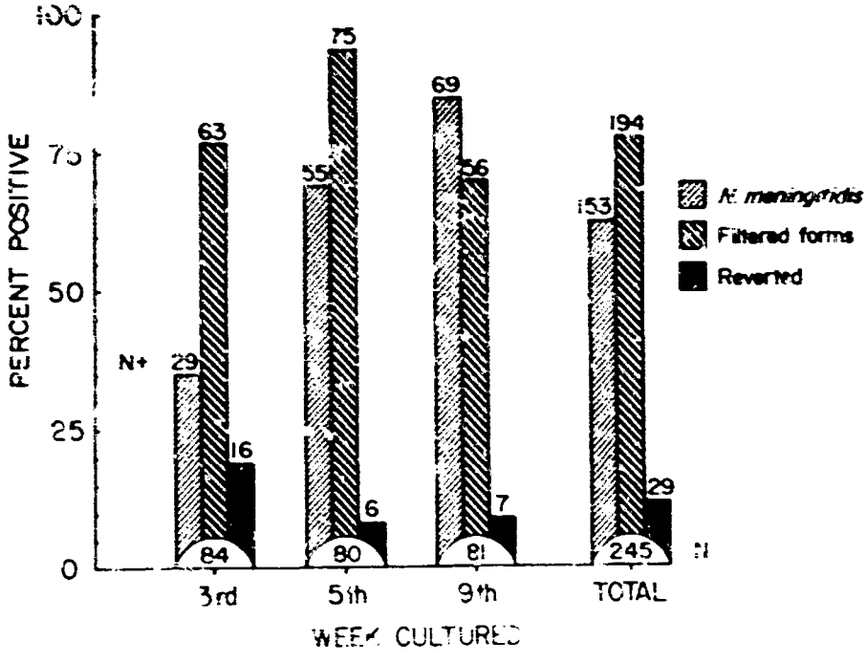

FIGURE 5

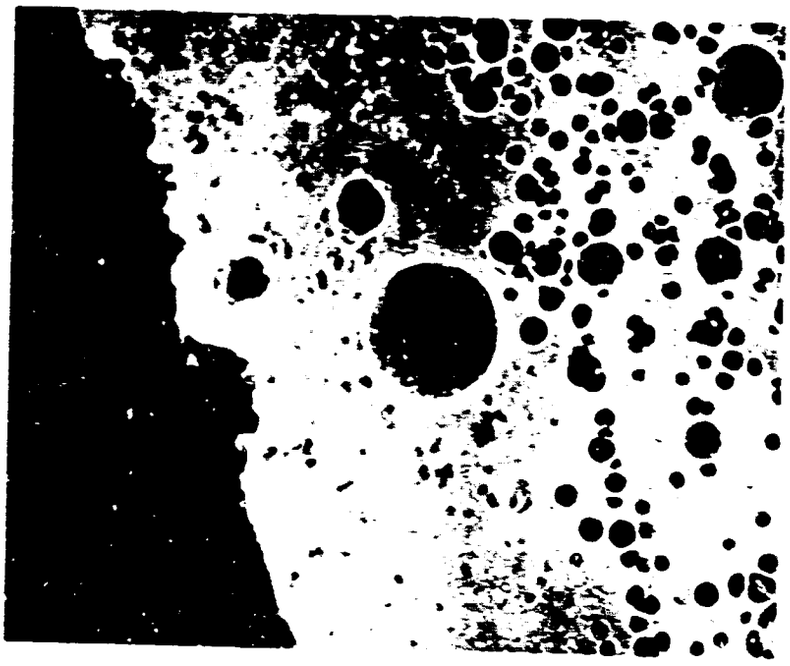

FIGURE 6

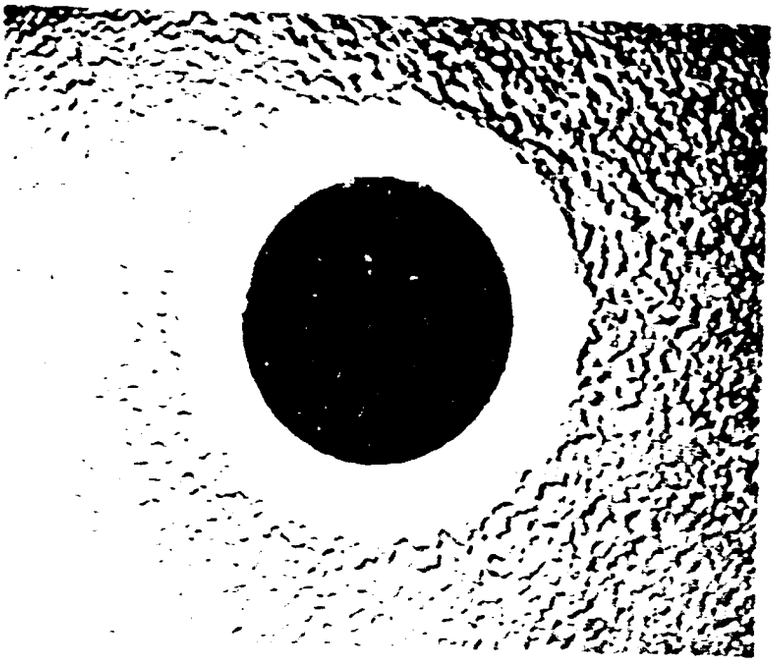

FIGURE 7

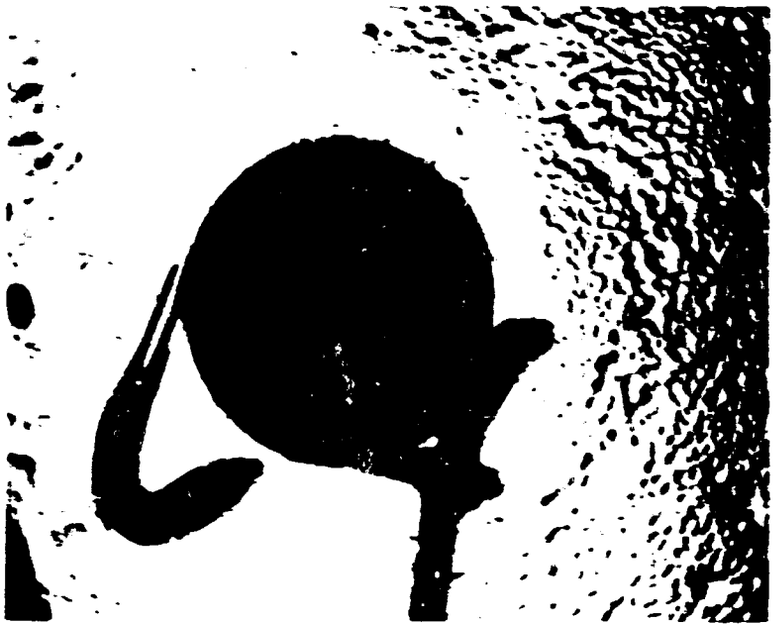

TABLE 1

MENINGOCOCCI PRODUCING L FORMS UPON EXPOSURE TO PENICILLIN

GREAT LAKES, ILLINOIS - 1966-1967

SEROLOGICAL GROUP OF *N. MENINGITIDIS*	NO. POSITIVE / NO. TESTED CARRIER	DISEASE	TOTAL
A	0/0	10/10	10/10
B	77/81	7/7	83/87
C	56/57	2/2	58/59
Bo	5/5	2/2	7/7
TOTAL	138/143	21/21	159/164

TABLE 2

CF REACTIONS OF 2-FOLD FALLING DILUTIONS OF RABBIT AND HUMAN SERA TESTED AGAINST L AND BACTERIAL FORM ANTIGENS OF *N. MENINGITIDIS* GROUP B GREAT LAKES, ILLINOIS - APRIL 1967

SERUM	CF WITH INDICATED ANTIGEN L FORM	BACTERIAL FORM
GROUP A RABBIT	430000*	330000
GROUP B RABBIT	442000	432000
GROUP C RABBIT	432000	432000
STRAIN BOSCHARD	444430	333210
POSITIVE HUMAN	444000	444000
NEGATIVE HUMAN	000000	000000

*EACH DIGIT REPRESENTS DEGREE OF FIXATION IN THAT SERIAL DILUTION OF SERUM TESTED, STARTING WITH 1:8. 0=NO FIXATION, 1=25%, 2=50%, 3=75%, 4=100% FIXATION

TABLE 3

GROUP B MENINGOCOCCAL L-FORM *vs* SERA OF NAVAL RECRUITS IN CF TEST: EXAMPLES OF RISING TITERS GREAT LAKES, ILLINOIS-1967

RECRUIT	WEEK SERUM OBTAINED 1	5	9
	FIXATION IN 4-TUBE TEST*		
418	0000*	4430	0000
421	2000	4420	3200
423	1000	4431	4441
424	1000	4420	4442
427	2000	4444	-
429	1000	3210	3200
432	1100	4444	4441
433	1100	1000	4441
443	4410	4440	4440

*0 = NO FIXATION 1=25% 2=50%
3=75% 4=100% FIRST TUBE = 1:8 DILUTION

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MOUSE PROTECTION STUDIES

A. R. Banknieder, L. F. Devine, T. C. Brewster,
and C. A. Faust

At NAMRU-4, we have devoted our studies in animals to both passive and active protection in mice. The passive studies were based on a study that showed antimeningococcal serum from human carriers and cases protected mice from challenge with Neisseria meningitidis (1). The work of Greenberg and Young provided the basis for attempting active protection studies (2). They were able to actively immunize mice with meningococcal antigen and protect these same mice against a challenge.

Passive mouse protection tests were undertaken to determine if measured antibody titers of carriers could be related to protection. We hoped to be able to determine to what degree each carrier is protected by relating antibody titer in man to protection in mice.

Active protection studies were undertaken to determine which of the three antigens available offered the most protection against homologous challenge. These antigens were also to be evaluated on the basis of their ability to protect the mice against heterologous challenges.

MATERIALS AND METHODS

In the passive protection studies, we utilized the method of Branham for determining the protective value of antimeningococcal serum (3). Six mice per point were used, both in the controls and the passively immunized mice. Four-fold dilutions beginning at 1:16 and ending at 1:1024 were made with the serum to be tested. The passively immunized mice received a 1-ml dose of the serum 1 hour prior to challenge. The challenge consisted of 1 ml of organisms in mucin in 10-fold dilutions ranging from 10^{-4} to 10^{-9} dilution. Aliquots of these dilutions were placed on plates and after 18 hours, the colonies were counted to

determine the number of organisms in each challenge dose. Deaths were recorded every 24 hours for 96 hours. Mucin controls and serum controls were also included. The mice used in these studies were of the ICR strain weighing 16-18g when challenged.

Antimeningococcal sera used in the passive protection studies were from hyperimmunized rabbits and human carriers of group C meningococci. The antigens used for the hyperimmunization of rabbits were:

1. L form antigen: A broth culture washed 3 times, suspended in saline, and stored at 4°C until no viable organisms could be recovered.

2. French Pressure Cell antigen: Consisted of cells washed in saline, suspended in saline, and put into a French Pressure Cell at 500 p.s.i. The effluent, containing the disrupted cells was then used as the antigen.

3. HA antigen: Prepared by Mr. E. Edwards of Immunology (5).

The following antigens, produced at NAMRU-4, were used in the active protection studies:

1. Formalized whole cells in 0.5% formaldehyde concentrated to 1.0 x 10^9 organisms per ml, supplied by LCDR L. F. Devine of Bacteriology.

2. Hemagglutinating antigen containing 50 mg of polysaccharide, supplied by Mr. E. Edwards of Immunology.

3. L form group B whole cells, 1.3 mg/ml in buffered saline prepared by Messrs. Crawford and Beno of Mycoplasma Research.

ICR mice, 6-8g, about 2 weeks old, were immunized with 0.1 ml of the undiluted antigen. One week later, the mice were given a second immunization with 0.1 ml of the same antigen. These mice were challenged one week after the second immunization, when they weighed 16-18g.

Calculations of LD_{50} for the controls, 50% serum neutralization endpoint for the passively immunized mice, and neutralization index for the actively immunized mice were determined using the formulae of Reed and Muench (4). The 50% neutralization endpoint is the dilution of serum which protects 50% of the mice against the challenge. The neutralization index, reported as protection in the Tables, is the number of LD_{50} challenges survived by the immunized mice.

RESULTS

The results of passive protection studies indicate that the hemagglutination (HA) antibody is the protective factor in serum. Sera with higher HA titers could be diluted more than sera with low titers and still protect 50% of the mice against a comparable challenge.

The results of mouse protection tests using rabbit antimeningococcal serum appear in Table 1. In the upper group of data, the sera were tested in mice that were subjected to a challenge of 2-3 LD_{50}'s or 0.3 to 0.5 log LD_{50}'s. The 50% endpoint for the L form B and French Pressure Cell antisera were the same (127.9 or 2.1 log). The French Pressure Cell antiserum had a higher HA titer and protected mice against a greater challenge. The HA antiserum had a HA titer of 512. This serum was tested in mice subjected to an intermediate challenge between the challenges used in testing the other antisera and showed a 50% endpoint of 363.1 or 2.5 log. In other words, the HA antiserum could be 3 times as dilute as the L form B and French Pressure Cell antisera and still protect 50% of the mice against a comparable challenge.

The same degree of protection was afforded mice given the HA antiserum even when subjected to a greater challenge. In the lower group of data in Table 1 where the challenges are comparable, the HA antiserum 50% endpoint was 3 times higher than that of the French Pressure Cell antiserum.

It is obvious from comparing the CF titers of the sera and their respective 50% endpoints (Table 1) that the CF titer is not related to passive protection. If the CF titer of the antimeningococcal serum were related to protection, then the L form B

antiserum would have provided the most protection.

Table 2 contains the results of studies using very high and very low titered human antimeningococcal serum. The serum from the same human carrier was used with its full HA antibody titer and after the HA antibody had been absorbed out with HA antigen. It should be noted that as the log challenge decreased by 1.0 log, the 50% endpoint increased by 0.5 log. This was true for only the high titered serum. By comparing the mouse protection afforded by high titered serum and low challenge with the absorbed serum, its 50% endpoint was also lower.

Human antimeningococcal serum of various HA titers, but similar CF titers, were studied and the results appear in Table 3. The LD_{50} challenge is essentially equal differing by only 0.2 log, but the 50% endpoint differs by as much as 1.0 log. It is notable that as the HA titer increased, the 50% endpoint also increased.

Table 4 contains data extracted from the previous study only grouping it by equal LD_{50} challenge. The only variables are the HA titer and 50% endpoint. As the HA titer increased, the 50% endpoint increased. The 50% endpoint for the high serum is 10 times higher than the same value for the low serum.

Table 5 contains the data of the study subjecting high HA titered and medium HA titered antimeningococcal serum to a challenge of 1.3 log LD_{50}'s. Again, it can be observed that as the HA titer increased, the 50% endpoint also increased. The medium serum had a reciprocal of dilution of 182.4 which is approximately one-half that of the high serum.

The results of the active protection studies have indicated that each of the described antigens can be used to immunize mice and protect them from meningococcal challenge. Table 6 contains the results of the study to determine the best route of immunization. One group of mice received two subcutaneous immunizations, while the other group received a subcutaneous followed by an intraperitoneal injection. The protection values of 158.5 and 100.0 were essentially the same indicating that both methods of immunization were equally effective.

One study was completed using heterologous challenges. The antigens used to immunize the mice were formalized A, B, C, and Bo. The vaccinated mice were challenged with a group C organism, strain PTS-5. The results of this study in Table 7 show that the two C antigens and the Bo antigen afforded equal protection against a C group challenge. The mice vaccinated with the A and B antigens survived 39.8 and 11.2 $LD_{50's}$, respectively, and were considered only slightly protected against a group C challenge.

A study was undertaken to compare the hemagglutinating (HA) antigen with the formalized antigen and the results appear in Table 8. The HA antigen was slightly more protective than the formalized antigen. The mice that received the HA antigen survived a challenge of 35.5 $LD_{50's}$, while the mice vaccinated with the formalized antigen survived 5.25 $LD_{50's}$.

The last study completed was a comparison of the three group B antigens. The HA antigen, Table 9, afforded the mice protection against a challenge of 436.5 $LD_{50's}$ and was equivalent to the protection (398.1 $LD_{50's}$) of the L form B antigen. The formalized antigen protected the mice against 158.5 LD_{50} challenge doses.

DISCUSSION

These limited studies have shown that the hemagglutinating antibody is the protective factor in both rabbit and human antimeningococcal serum. The higher titered serum could be diluted more than the lower titered serum and still protect 50% of the challenged mice. This was true in carrier sera of various HA titers and in the same sample when the HA antibody content was high and when it was low following absorption with HA antigen.

This work was devoted mainly to the study of group C antimeningococcal serum. The other groups of N. meningitidis antiserum should be evaluated in a similar manner.

Further studies are needed to determine if meningococcal groups or strains exist which can be used to protect against challenge by heterologous groups. In addition, it would be

desirable to make a determination of whether heterologous group protection can be demonstrated only in actively immunized mice or also in mice passively immunized in the mouse protection test.

TABLE 1. -- Rabbit Antimeningococcal Serum from Three Antigens Tested in Mouse Protection Tests

Antiserum	Rabbit Serum Titers		Challenge		50% endpoint reciprocal of dilution	
	HA	CF	LD_{50}	(log)	Arithmetic	Logarithmic
L form B	0	1024	1.995	(0.3)	127.9	(2.1)
French Pressure Cell	16	512	3.162	(0.5)	127.9	(2.1)
HA	512	256	2.512	(0.4)	363.1	(2.5)
French Pressure Cell	16	512	31.62	(1.5)	36.31	(1.5)
HA	512	256	25.12	(1.4)	96.38	(1.9)

TABLE 2. -- Antimeningococcal Serum from a Human Carrier with High HA Titer Tested in Mouse Protection Test before and after Absorption

Antiserum	Human Serum Group "C" Titers HA	CF	Challenge LD_{50}	(log)	50% Endpoint Reciprocal of Dilution Arithmetic	Logarithmic
High C	1024	64	2512.0	(3.4)	27.80	(1.4)
High C	1024	64	251.2	(2.4)	75.16	(1.8)
[High C	1024	64	25.12	(1.4)	202.3	(2.3)]
[Absorbed C	4	32	3.98	(0.6)	42.07	(1.6)]

TABLE 3. -- Comparison of Three Levels of HA Antibody Titered Antimeningococcal Serum Tested with Equivalent Challenges in the Mouse Protection Test

Human Serum Group "C"

Serum	Titers		Challenge		50% Endpoint Reciprocal of Dilution	
	HA	CF	LD_{50}	(log)	Arithmetic	Logarithmic
Low C	16-32	16	13.80	(1.14)	49.20	(1.69)
Medium C	64-128	16	19.95	(1.3)	182.4	(2.26)
High C	256	16	13.80	(1.14)	579.4	(2.76)
High C	256	16	19.95	(1.30)	393.6	(2.59)

TABLE 4. -- Low and High Titered HA Antimeningococcal Serum Tested with a 13.80 LD_{50} Challenge in the Mouse Protection Test

Human Serum Group "C"

Serum	Titers		Challenge		50% Endpoint Reciprocal of dilution	
	HA	CF	LD_{50}	(log)	Arithmetic	Logarithmic
Low C	16-32	16	13.80	(1.14)	49.20	(1.69)
High C	256	16	13.80	(1.14)	579.4	(2.76)

TABLE 5. -- Medium and High Titered HA Antimeningococcal Serum Tested with A Challenge of 19.95 $LD_{50's}$ in the Mouse Protection Test

Human Serum Group "C"

Serum	Titers		Challenge		50% Endpoint Reciprocal of Dilution	
	HA	CF	LD_{50}	(Log)	Arithmetic	Logarithmic
Medium C	64-128	16	19.95	(13)	182.4	(2.26)
High C	256	16	19.95	(13)	393.6	(2.59)

TABLE 6. -- Active Protection Study Comparing the Relative Effectiveness of Different Routes of Immunization

Active Protection Study

Antigen: Group C

Challenge: PTS5 Group C Count: 7.4 at 10^8

Organism	Number of Mice Dead/Challenged		
		Immunizing Antigen	
	Controls	Formalized *	Formalized**
Dilution			
10^4	6/6		
10^5	6/6	2/4	3/4
10^6	5/6	0/4	0/4
10^7	5/6	0/4	0/4
10^8	0/6		
10^9	0/6		
LD_{50}	$10^{-7.2}$	$10^{-5.0}$	$10^{-5.2}$
Protection			
(LD_{50})		158.5	100.0

*Immunizations subcut. subcut.

**Immunizations subcut. IP

TABLE 7. -- Active Protection Study: Heterologous Challenge with Group C for A, B and Bo Vaccinated Mice, and Homologous Challenge for Group C Vaccinates

Antigen: Formalized groups A, B, C Bo

Challenge: PTS5 Group C Count: 7.4 at 10^8

Organism dilution	Controls	Number of Mice Dead Immunizing antigen C	C	Bo	B	A
10^{-4}	6/6					
10^{-5}	6/6	2/4	3/4	2/4	2/4	3/4
10^{-6}	5/6	0/4	0/4	0/4	2/4	2/4
10^{-7}	5/6	0/4	0/4	1/4	1/4	2/4
10^{-8}	0/6					
10^{-9}	0/6					
LD_{50}	$10^{-7.3}$	$10^{-5.0}$	$10^{-5.3}$	$10^{-5.2}$	$10^{-5.7}$	$10^{-6.24}$
Protection		158.5	100.0	125.9	39.8	11.2

TABLE 8. -- Active Protection Study: Comparison of the Effectiveness of Two Different Group A Antigens

Active Protection Study

Antigen: Group A (MKO1-A)

Challenge: MKO1-A Count: 4.75 at 10^{-9}

Organism dilution	Controls	HA Antigen	Formalized Antigen
	Number of mice dead/challenged		
10^{-4}	5/6	0/4	5/5
10^{-5}	4/6	0/4	2/5
10^{-6}	3/6	0/4	0/4
10^{-7}	0/6	0/4	
10^{-8}			
10^{-9}			
LD_{50}	$10^{-5.55}$	$10^{-4.0}$	$10^{-4.83}$
Protection		35.5	5.25

TABLE 9. -- Comparison of the Effectiveness of Three Different Antigens of Group B to Homologous Challenge

Antigen: Group B

Challenge: Williams B Count: 2.2 at 10^{-9}

Organism dilution	Number of Mice Dead/Challenged Immunizing Antigen			
	Controls	HA	L Form	Formalized
10^{-4}	6/6	4/4	2/4	2/4
10^{-5}	5/6	1/4	2/4	4/4
10^{-6}	5/6	0/4	1/4	1/4
10^{-7}	5/6			
10^{-8}	1/6			
10^{-9}	0/6			
LD_{50}	$10^{-7.3}$	$10^{-4.66}$	$10^{-4.7}$	$10^{-5.1}$
Protection		436.5	398.1	158.5

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A MODEL FOR SCREENING ANTIMICROBIAL AGENTS FOR POTENTIAL USE IN ELIMINATING MENINGOCOCCI FROM THE NASOPHARYNX OF HEALTHY CARRIERS

L. F. Devine, R. C. Knowles, W. E. Pierce, R. O. Peckinpaugh, C. R. Hagerman and R. I. Lytle

Sulfadiazine has been effective in the control of meningococcal infections in the past. Since the recognition of sulfadiazine-resistant strains of meningococci in 1963 (1), a substitute prophylaxis has been sought. Several drugs which showed promise in vitro have been tested in the field (erythromycin, Vibramycin, sulfadiazine + Daraprim). However, only sulfadiazine has been effective in reducing the carrier rate. Field trials are expensive and laborious, therefore, a more reliable method of predicting the in vivo effectiveness of a drug was needed to facilitate the selection of the best prospective drugs for field testing. A model has been developed utilizing data from in vivo studies with antibacterial agents. Concentrations of these agents in the serum and in the saliva of carriers has been determined by either chemical or biological methods. The data suggest that to effectively eliminate Neisseria meningitidis from the nasopharynx, the drug must be secreted in the saliva at a concentration approaching the minimal inhibitory concentration (MIC) as determined by in vitro plate dilution methods.

The antibacterials were administered to the subjects orally. The paired serum and saliva samples were collected at various times during prophylaxis. Blood was collected by venipuncture. The subjects were asked to chew paraffin gum in order to induce salivation and facilitate the collection of saliva samples. Both samples were placed in insulated containers with dry ice to prevent deterioration of heat labile antibiotics while returning to the laboratory. The sera were separated by centrifugation, and the saliva was centrifuged to remove suspended material. Both substances were then frozen at -60°C until assayed.

The biological assay of the sulfadiazine concentrations in serum and saliva were determined by the tube dilution method using Mueller-Hinton broth. The sera were sterile and required no pretreatment. The saliva samples were centrifuged to remove precipitates and 25 units/ml of polymyxin B and 10 μg/ml of

ristocetin were incorporated into the Mueller-Hinton medium to prevent the growth of other organisms. A strain of N. meningitidis, 29E, isolated at NAMRU-4, was used as the indicator organism. A standard 2 mm loop of a 10^1 dilution of a 6-hour culture grown in an incubator shaker was used as an inoculum. The tubes were incubated for 16 hours. The endpoint was designated as the tube containing the highest dilution of the sample having no turbidity. The minimal inhibitory concentrations of sulfadiazine for the meningococci isolated from the subjects were determined as in the previous epidemiology paper (2). The chemical determinations were done by the Biochemistry Division of NAMRU-4. A modification of the method of Bratton and Marshall was used in that trichloracetic acid precipitation and filtration of the serum and saliva preceded the assay.

Concentrations of erythromycin in serum were determined biologically by the tube dilution method using Mueller-Hinton broth. A 10^1 dilution of a 6-hour Mueller-Hinton culture of strain No. 5 of N. meningitidis group B grown on an incubator shaker was used as an indicator organism. Saliva samples were centrifuged to eliminate precipitates, lypholized and treated with ethylene oxide overnight and again for two hours in the morning in a Ben Venue Model No. 8 sterilizer*. The saliva samples were reconstituted to original volume in distilled water before being assayed. A 10^1 dilution of a 6-hour culture of Sarcina lutea ATCC No. 9341, grown in Mueller-Hinton broth in an incubator shaker, was used as the indicator organism.

The biological assay of the samples of serum and saliva, containing Vibramycin, was carried out with Bacillus cerus ATCC 11778 as the indicator organism, and the saliva was prepared for assay as described for the erythromycin samples. The 0.1 ml inocula used in the serum and saliva assays were also prepared from 6-hour cultures of the indicator organisms grown in Mueller-Hinton broth in an incubator shaker.

Weinstein and Samet (3) reported that antibacterial activity of sera containing sulfadiazine was not correlated with sulfadiazine concentrations as determined by the method of Bratton and Marshall. This phenomenon was not observed in our laboratory for either the sulfadiazine assay of the serum or of the

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*Ben Venue Lab., Inc.,
Bedford, Ohio

saliva. Weinstein and Samet used an indicator organism which had a sensitivity of 4 μg/ml. A good correlation between the two methods has been observed in our laboratory when an indicator organism is used which allows the endpoint to occur at 0.02 μg/ml. This represents a 200-fold dilution beyond where the endpoint occurred in their experiments. In our laboratory any normal or specific antibodies, to the indicator organism, are diluted beyond the point where they might interfere with the reading of the endpoint.

The following series of figures illustrate that chemical and biological assay methods show good correlation and may be used for determining sulfadiazine levels in serum or saliva. They also show that there is a good correlation between the concentrations of sulfadiazine found in serum to the concentrations of sulfadiazine found in saliva irrespective of the method of assay.

The comparison of the chemical and biological assay of sulfadiazine in serum is shown in Figure 1. Excellent correlation is observed between the two methods. Figure 2 shows a statistically significant correlation between the chemical assay of saliva and the biological assay of saliva. Figure 3 again shows excellent correlation between chemical assay of serum and chemical assay of saliva. Consequently, one can predict the saliva levels from the serum levels. Figure 4 shows a statistically significant correlation between chemical assay of serum versus biological assay of saliva. The correlation coefficients involving chemical assay have a higher r value than those derived from biological determinations, because biological assays of necessity are derived from serial 2-fold dilutions, whereas, chemical assays involve a continuous scale.

The ratios of the mean concentrations of sulfadiazine in sera to the mean concentrations of sulfadiazine in salivas are 8.91 and 5.78 to 1, respectively, for chemical and biological methods.

Now that it has been shown that chemical or biological methods of assay may be used for determining sulfadiazine concentrations in serum and saliva, we will progress to the actual distribution of MIC's of sulfadiazine for specific serological categories of meningococci in naval personnel. The MIC's of more than 4,000 meningococcal cultures have been determined by the plate dilution method using log 4 increments of sulfadiazine spanning the entire range of sulfadiazine sensitivities of the meningococci as shown in

Figure 5. Regardless of the serological category studied, a bimodal distribution of sulfadiazine sensitivities are observed. The category "all other" includes all the other unusual serological groups. The modes of these two distinct peaks are at 0.06 and 16 mg%. Group C is the only exception among all the serological categories tested in that a slightly higher percentage of MIC's of sulfadiazine were found at a concentration of 0.25 mg% than at 0.06 mg%. Only about 2% of the MIC's of sulfadiazine for meningococci are observed at the intermodal point of 1 mg%.

Figure 6 shows there is a characteristic percent distribution of sulfadiazine sensitivity of meningococci for each of several categories of naval personnel. More than 45% of all cultures in any population studied have MIC's of sulfadiazine of either 0.06 or 16 mg%. The highest percentages of cultures with a MIC of 1 mg% occur where the sulfadiazine-sensitive organisms predominate in the population, and the lowest percentages occur where sulfadiazine-resistant organisms are predominant.

Figure 7 shows that meningococcal cultures can be categorized as sulfadiazine-sensitive or resistant by inoculating one plate containing a concentration of 1 mg% sulfadiazine. The test can be done with 96% confidence for sensitive organisms and 99% confidence with resistant organisms. This confidence is derived from computations based on estimates derived from the "area method of fitting a normal curve"(4).

Figure 8 shows the relationship of the levels of saliva and serum 6 to 8 hours after last dose to the MIC's of sulfadiazine for the meningococci inhabiting the nasopharynx of the carriers. The concentrations of sulfadiazine in the saliva of the carriers are shown at the upper left and range from 0.2 to 1.4 mg%. The serum concentrations of sulfadiazine are shown on the upper right and range from 2 to 11 mg%. The serum concentrations of sulfadiazine are shown on the upper right and range from 2 to 11 mg%. The MIC's of sulfadiazine for the meningococci actually infecting the carriers are shown on the bottom of the figure and range from 0.015 mg% to 16 mg%.

The range of sulfadiazine concentrations in the saliva shown in the upper left is superimposed on the scale of MIC's of sulfadiazine for the isolates on the bottom of the figure. The serum range of sulfadiazine concentrations is also superimposed on the same scale. The cross-hatched bars show the number of people

carrying meningococci which are inhibited by the different levels of sulfadiazine in the plate dilution test. The solid bars depict what the comparable number of carriers of meningococci and the sulfadiazine resistance of their meningococci 5 days after sulfadiazine treatment. Only 1 of 30 carriers of a sulfadiazine-sensitive meningococcal strain remained infected after treatment, while 3 of 4 carriers of sulfadiazine-resistant meningococci continued to be infected with sulfadiazine-resistant meningococci after treatment.

These data show that when the concentration of sulfadiazine in saliva, 6 to 8 hours after the last dose, is equivalent to that required to inhibit the growth of meningococci in the plate dilution test as conducted in our laboratory, then more than 95% of carriers of sulfadiazine-sensitive meningococci are cured.

The data in Figure 9 further substantiates these observations. A larger population was divided into two groups and the MIC's of sulfadiazine for the meningococci of individual carriers were determined. The numbers of people carrying meningococci are plotted on the ordinate, and the MIC's of the sulfadiazine for the meningococci, which they actually carried, were plotted on the abscissa. One group depicted on the top of the figure was treated with sulfadiazine and the group shown on the bottom of the figure received no treatment. Five days after treating one group with sulfadiazine, both groups were recultured and the MIC's of the final cultures were determined. The remaining carriers and the sulfadiazine sensitivities of their organisms were plotted as before and are indicated by the solid portions of the bars. Three of 59 subjects in the treated group still carried sulfadiazine-sensitive organisms, and 32 of 45 in the control group carried sulfadiazine-sensitive meningococci at similar times after originally detecting the meningococcal carriers. There were some spontaneous cures in the control group. The combined effect of treatment plus the spontaneous loss of meningococcal infection can be seen in the top part of the figure.

An extension of the sulfadiazine model, in Figure 10, shows why erythromycin is not effective in eliminating meningococci from carriers. Fifty-nine pairs of serum and saliva samples were collected at various times from individuals receiving erythromycin prophylaxis. These samples were assayed for erythromycin concentrations. The figure of erythromycin levels in serum versus erythromycin levels in saliva show good correlation. The ratio of the mean concentration of erythromycin in sera to the ratio of the

mean concentration in salivas is 75.8 to 1.

The range of erythromycin in μg/ml found in the saliva in these 59 samples was from 0.0037 to 0.12 μg/ml (Fig. 11). The range of erythromycin concentrations in the sera was 0.8 to 12.8 μg/ml. The range of the MIC's of erythromycin for the meningococci isolated from the carriers was 0.25 to 1 μg/ml. The range of saliva concentrations in μg/ml of erythromycin on the upper left and serum concentrations of erythromycin on the upper right are superimposed on the lower part of the figure on the MIC's of erythromycin for meningococci found in the carriers.

Only 8 of the 59 of the serum concentrations of erythromycin fell below the MIC's of erythromycin for the most highly erythromycin resistant meningococci. Eight serum concentrations were 0.8 μg/ml and none of the MIC's of erythromycin for the meningococci were above 1 μg/ml. The 59 saliva concentrations of erythromycin were all less than the concentrations of erythromycin required to inhibit the meningococci in the plate dilution method.

Ninety-one strains of different serological groups of meningococci were tested for the minimal inhibitory concentrations of Vibramycin required to inhibit their growth in the plate dilution test (Table 1). The Vibramycin sensitivites range from 0.125 to 1.0 μg/ml for all of the serogroups tested. Only 1 of 36 strains of serological groups B, C, and Bo had a MIC of Vibramycin of less than 0.25 μg/ml. Seventy-seven of 91 strains tested had minimal inhibiting concentrations of Vibramycin at 0.25 or 0.5 μg/ml. Ten of 11 strains with the lowest MIC value of 0.125 μg/ml were serological categories 29E, X, and Z.

A field trial of the effectiveness of Vibramycin was conducted to determine if it might be effective in eliminating meningococci from the nasopharynx of healthy carriers. Nineteen serum and saliva samples were collected at either 6 or 24 hours after the previous medication after having at least 3 days of medication. Paired serum and saliva samples were assayed as previously described.

The concentration of Vibramycin in μg/ml in the serum samples are plotted against the concentrations of Vibramycin

in μg/ml of the corresponding saliva samples (Fig. 12). There is a good correlation. The ratio of the mean concentrations of Vibramycin in sera to the mean level in the salivas is 16 to 1.

Figure 13 extends the sulfadiazine model to treatment of meningococcal carriers with Vibramycin. The distribution of the concentrations of Vibramycin in the salivas in the 19 samples is shown in the upper left of the figure and ranges from 0.0144 μg/ml to 0.115 μg/ml. The concentrations of Vibramycin in the 19 sera are shown in the upper right and range from 0.46 μg/ml to 1.84 μg/ml. The lower part of the slide shows the distribution of the MIC's of Vibramycin for the meningococci acutally infecting the carriers and the distribution ranges from 0.124 to 1.25 μg/ml. The saliva concentrations of Vibramycin on the upper left are superimposed on the lower part of the figure and are all lower than the MIC's of Vibramycin for the most sensitive meningococcal culture. The serum concentrations of Vibramycin on the upper right are superimposed on the MIC's of Vibramycin for the meningococci isolated from the carriers. Most of the serum concentrations are higher than is required to inhibit the meningococci in the plate dilution test. None of the concentrations of Vibramycin in saliva are equivalent to the concentrations required to inhibit meningococci in the plate dilution test.

An extension of the sulfadiazine model to potentiation of sulfadiazine by Daraprim was similarly studied. There were no cures among 41 men carrying sulfadiazine-resistant meningococci. An analysis of Daraprim concentrations in saliva showed that the concentrations of the drug were inadequate to potentiate the sulfadiazine activity.

These data appear to confirm the legitimacy of the proposition that when MIC's of a drug for meningococci are determined in the plate dilution test conducted in our laboratory and when the attainable levels of drug found in saliva are approximately equivalent, there exists a reasonably good prospect of eliminating meningococci from the nasopharynx of healthy carriers in a field test.

The results obtained are summarized in Table 2. The mean serum and saliva concentrations of individual drugs, the ratios of the concentrations found in serum and saliva, the probable concentration required to effectively cure meningococcal carriers and on the right the ratio of the concentration of the drug required

in saliva to effect a cure to that concentration of drug actually found.

The value of the probable concentration of 10 μg/ml, i.e., 1 mg% of sulfadiazine required for cure may not be realistic in this table since in reality there would be less than 4% of all sulfadiazine-sensitive meningococcal strains in nature inhibited by sulfadiazine concentration in the range of 0.25 mg% up to and including 1 mg%.

Probably only a few percent of the 4% of sulfadiazine-sensitive meningococci are actually resistant to more than 0.6 mg or 0.7 mg% which is the actual sulfadiazine concentration found in the saliva. Additionally, a large percent of this few percent might also be cured.

Consequently, from a practical standpoint, one should rarely encounter meningococci having a MIC of sulfadiazine of 10 μg/ml that will not be eliminated by sulfadiazine levels of 0.6 to 0.7 mg%. Consequently, the dividing line of 1 mg% does indeed separate meningococci into two distinct populations ---- the sulfadiazine-sensitive and the sulfadiazine-resistant organisms.

The only way to incontrovertibly settle this issue would be to have several hundred carriers of meningococci whose organisms are inhibited by MIC's of sulfadiazine in concentrations ranging from more than 0.25 mg% up to and including 1 mg%. This would require sampling 1,000 people to get 110 cases in this range and probably only one of these cases would be in the critical range 0.6 to 1 mg%.

SUMMARY

1. Regardless of the origin of the population or the serologic group studied, a bimodal distribution with identical modes of sulfadiazine sensitivity is observed.

2. The modes of these two distinct peaks are at MIC's of sulfadiazine for meningococci of 0.06 and 16.0 mg% and include more than 60% of all meningococci studied.

3. Only 2% of the MIC's of sulfadiazine for all meningococci studied occur at 1 mg%.

4. Using one plate containing 1 mg% of sulfadiazine for large numbers of meningococcal isolates, sensitive and resistant organisms may be separated with 96 to 99% confidence.

5. A sulfadiazine model for estimating the efficiency of a drug in the elimination of meningococci from the nasopharynx of healthy carriers has been presented. This model has been extended and applied to erythromycin, Vibramycin, and Daraprim plus sulfadiazine and may be useful in screening other drugs. The ratio of serum to saliva concentrations of a given drug are characteristic of that drug. However, with each drug tested, increasing levels of concentrations of that drug in serum are associated with increasing concentrations of that drug in saliva. Therefore, the effectiveness of a drug in the elimination of meningococci from the nasopharynx is a function of the concentration of the drug attainable in saliva and the sensitivity of the meningococci to the drug. These drugs eliminate meningococci only when the concentration of these drugs attained in saliva are equivalent to the MIC's of these drugs in the plate dilution test as performed in our laboratory.

TABLE 1

MIC OF VIBRAMYCIN FOR VARIOUS STRAINS OF MENINGOCOCCI PLATE DILUTION METHOD

SEROGROUP	MICs OF VIBRAMYCIN IN μg/ml 0.125	0.25	0.5	1.0	TOTAL NO. SEROGROUPS
B		5	3		8
C		3	7		10
Bo	1	7	17	3	28
135		2	3		5
29E	1	5			6
X	3	6			9
Z	6	16	3		25
TOTALS	11	44	33	3	91

TABLE 2

SERUM AND SALIVA CONCENTRATIONS OF ANTIBACTERIALS AND MIC OF DRUG IN AGAR PLATE

ANTIBACTERIAL	μg/ml in SERUM	SALIVA	RATIO	PROBABLE MIC REQUIRED FOR CURE	ACTUAL	RATIO
SULFADIAZINE CHEM. ASSAY	52.68	5.91	8.91	10.0	5.91	1.69
SULFADIAZINE BIOL. ASSAY	39.2	6.78	5.78	10.0	6.78	1.47
ERYTHROMYCIN ESTOLATE	2.9186	0.0385	75.8	0.8	0.0385	20.8
VIBRAMYCIN	1.0413	0.0651	16.0	0.5	0.0651	7.7
PYRIMETHAMINE	2.5	0.38	6.6	3.0	0.38	7.9

FIGURE 1

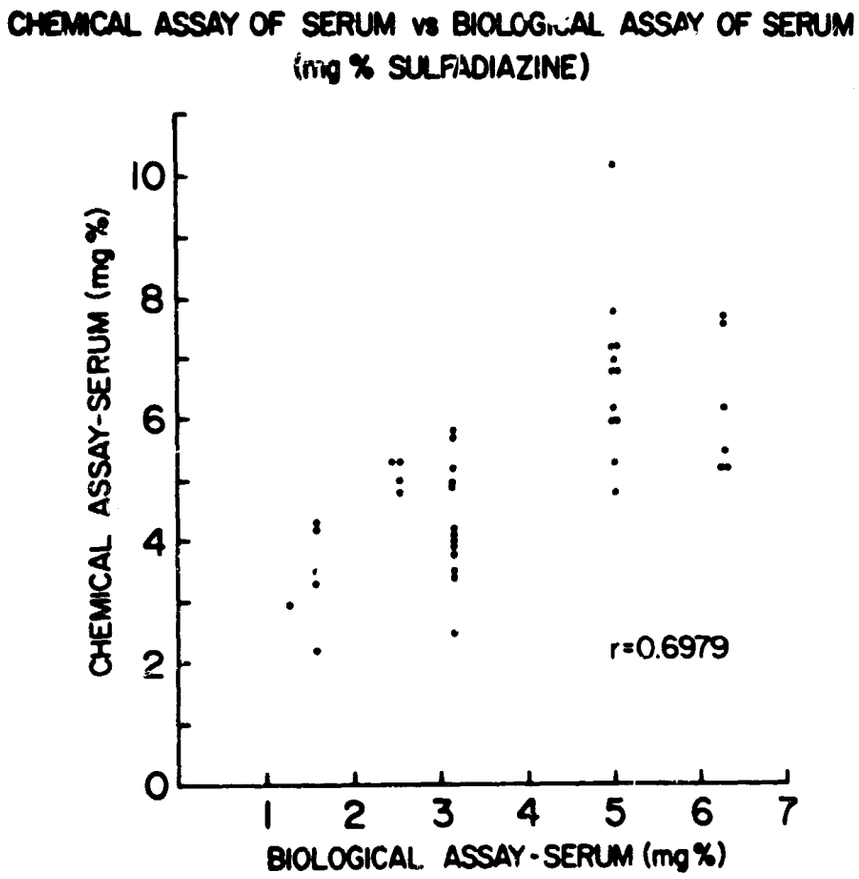

CHEMICAL ASSAY OF SERUM vs BIOLOGICAL ASSAY OF SERUM
(mg % SULFADIAZINE)
CHEMICAL ASSAY-SERUM (mg %)
BIOLOGICAL ASSAY-SERUM (mg %)
r=0.6979
0
2
4
6
8
10
1
2
3
4
5
6
7

FIGURE 2

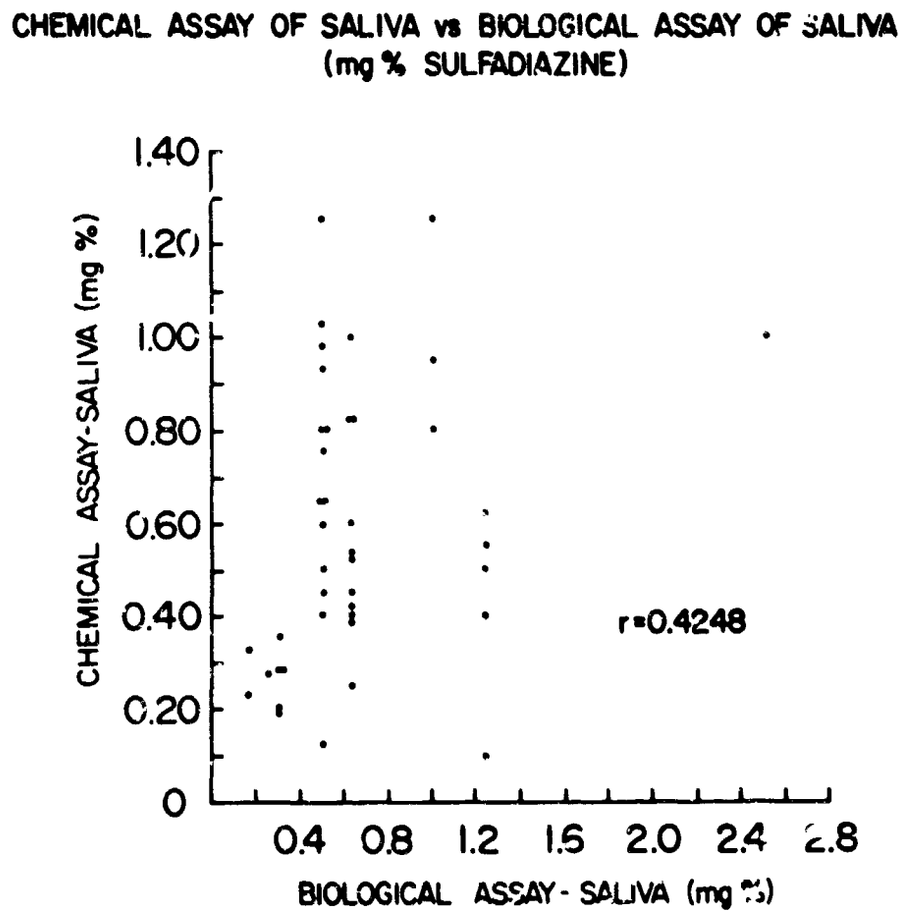

CHEMICAL ASSAY OF SALIVA vs BIOLOGICAL ASSAY OF SALIVA
(mg % SULFADIAZINE)
CHEMICAL ASSAY-SALIVA (mg %)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0
r=0.4248
0.4
0.8
1.2
1.6
2.0
2.4
2.8
BIOLOGICAL ASSAY - SALIVA (mg %)

FIGURE 3

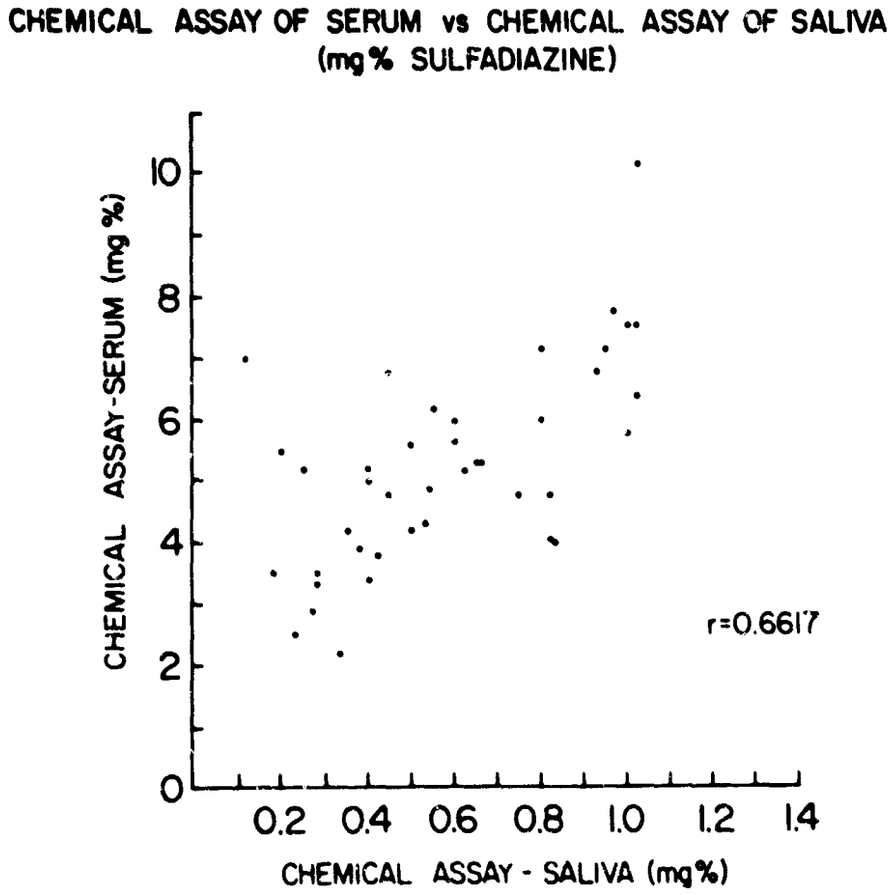

CHEMICAL ASSAY OF SERUM vs CHEMICAL ASSAY OF SALIVA
(mg% SULFADIAZINE)
CHEMICAL ASSAY-SERUM (mg%)
10
8
6
4
2
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
CHEMICAL ASSAY - SALIVA (mg%)
r=0.6617

FIGURE 4

CHEMICAL ASSAY OF SERUM vs BIOLOGICAL ASSAY OF SALIVA
(mg % SULFADIAZINE)

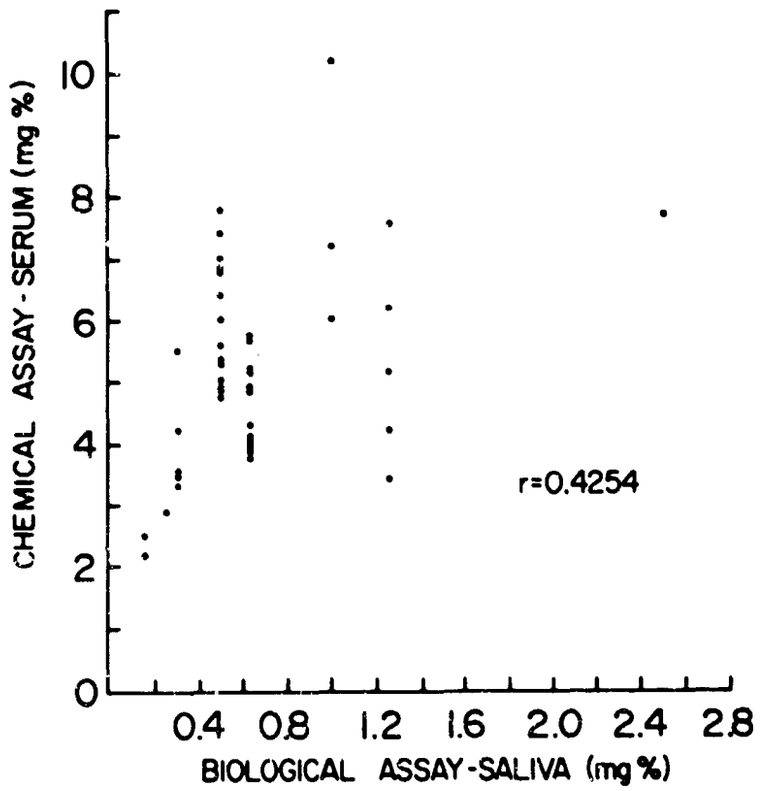


FIGURE 5

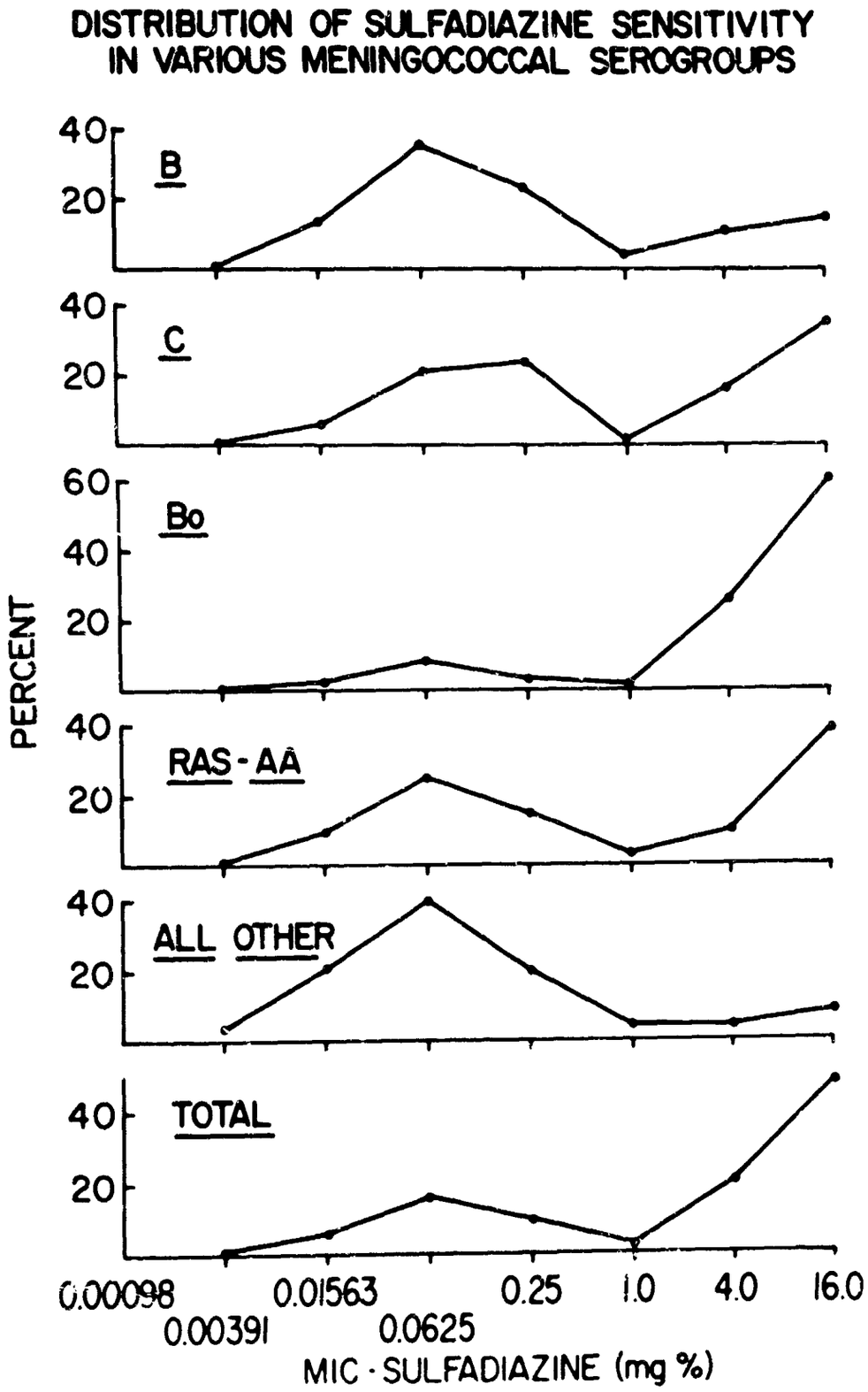

DISTRIBUTION OF SULFADIAZINE SENSITIVITY
IN VARIOUS MENINGOCOCCAL SEROGROUPS
PERCENT
B
C
Bo
RAS-AA
ALL OTHER
TOTAL
40
20
60
0.00098
0.00391
0.01563
0.0625
0.25
1.0
4.0
16.0
MIC - SULFADIAZINE (mg %)

FIGURE 6

DISTRIBUTION OF SULFADIAZINE SENSITIVITY OF MENINGOCOCCI FROM VARIOUS POPULATION CATEGORIES

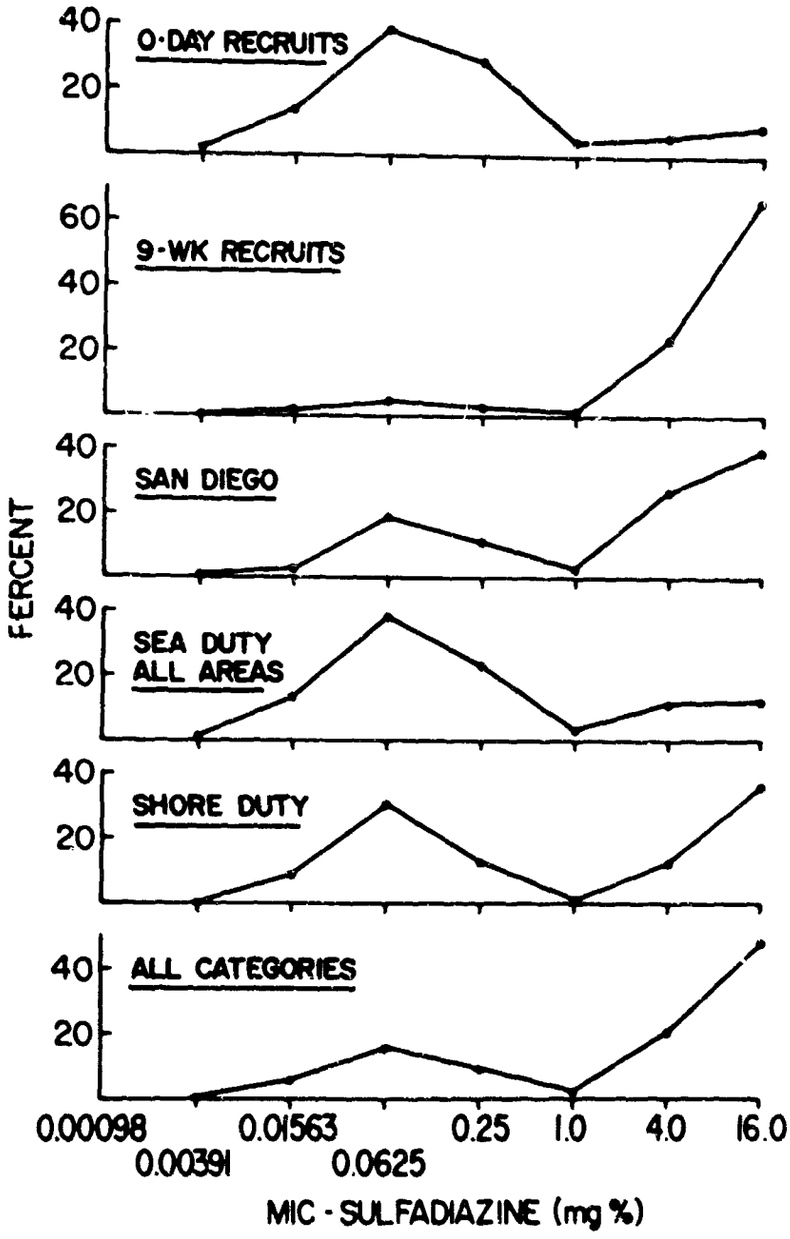


FIGURE 7

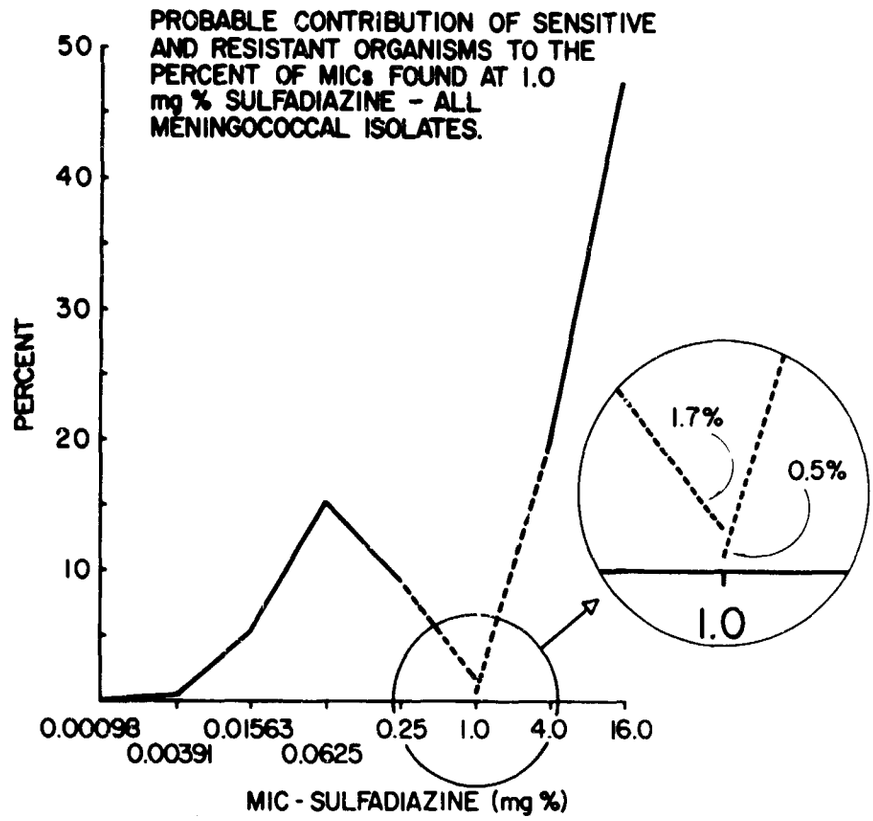


FIGURE 8

RELATIONSHIP OF SULFADIAZINE LEVELS IN SALIVA AND SERUM TO THE MICs OF SULFADIAZINE FOR THE MENINGOCOCCI OF CARRIERS

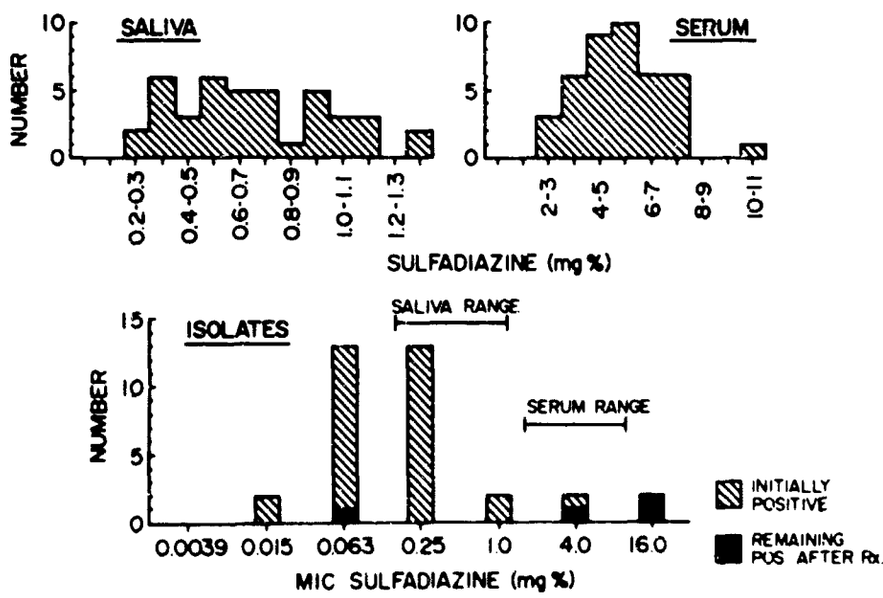


FIGURE 9

EFFECT OF SULFADIAZINE* ON CARRIAGE OF *N. MENINGITIDIS*
OCT 1967-APR 1968

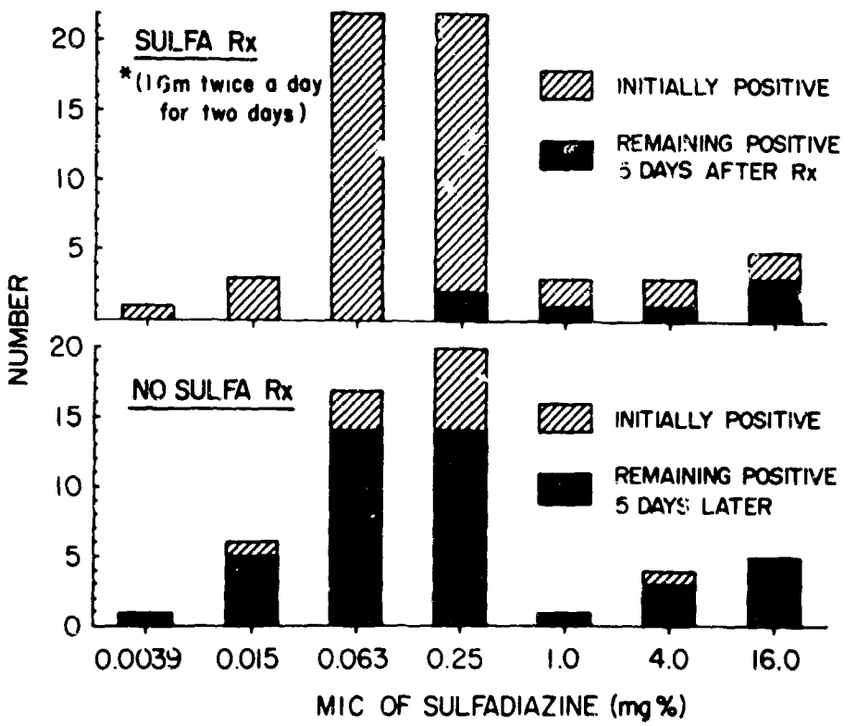


FIGURE 10

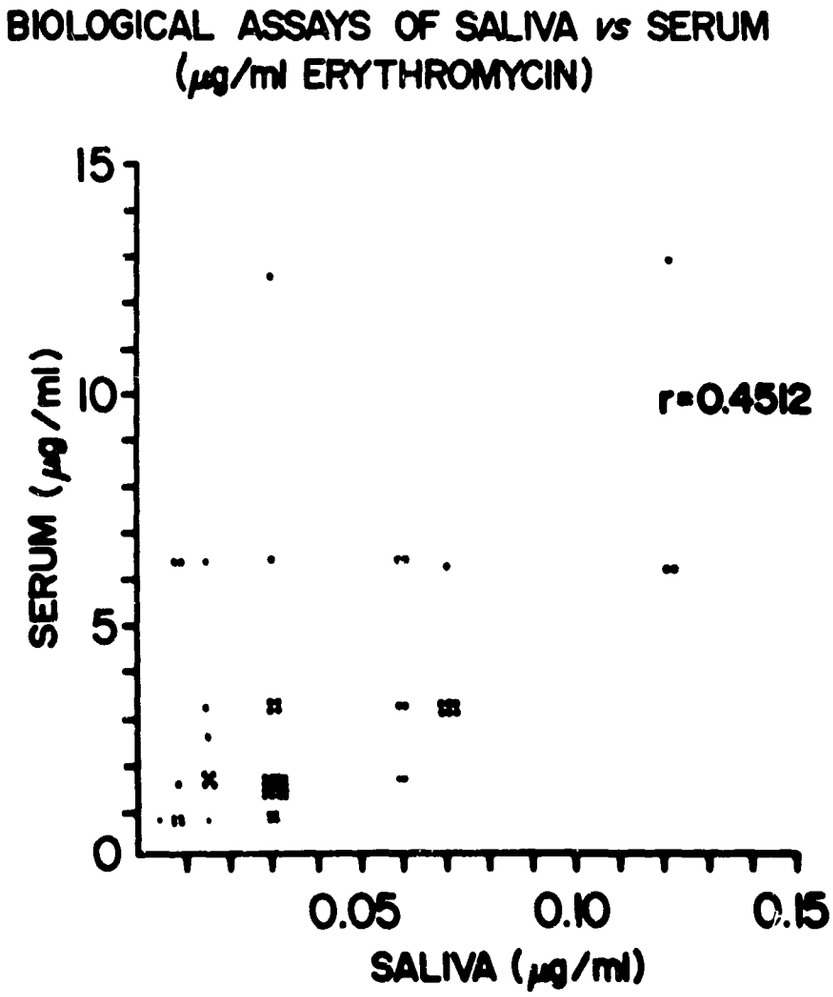

BIOLOGICAL ASSAYS OF SALIVA vs SERUM
(μg/ml ERYTHROMYCIN)
15
10
5
0
SERUM (μg/ml)
r=0.4512
0.05
0.10
0.15
SALIVA (μg/ml)

FIGURE 11

RELATIONSHIP OF ERYTHROMYCIN LEVELS IN SALIVA AND SERUM TO THE MIC OF ERYTHROMYCIN FOR THE MENINGOCOCCI OF CARRIERS

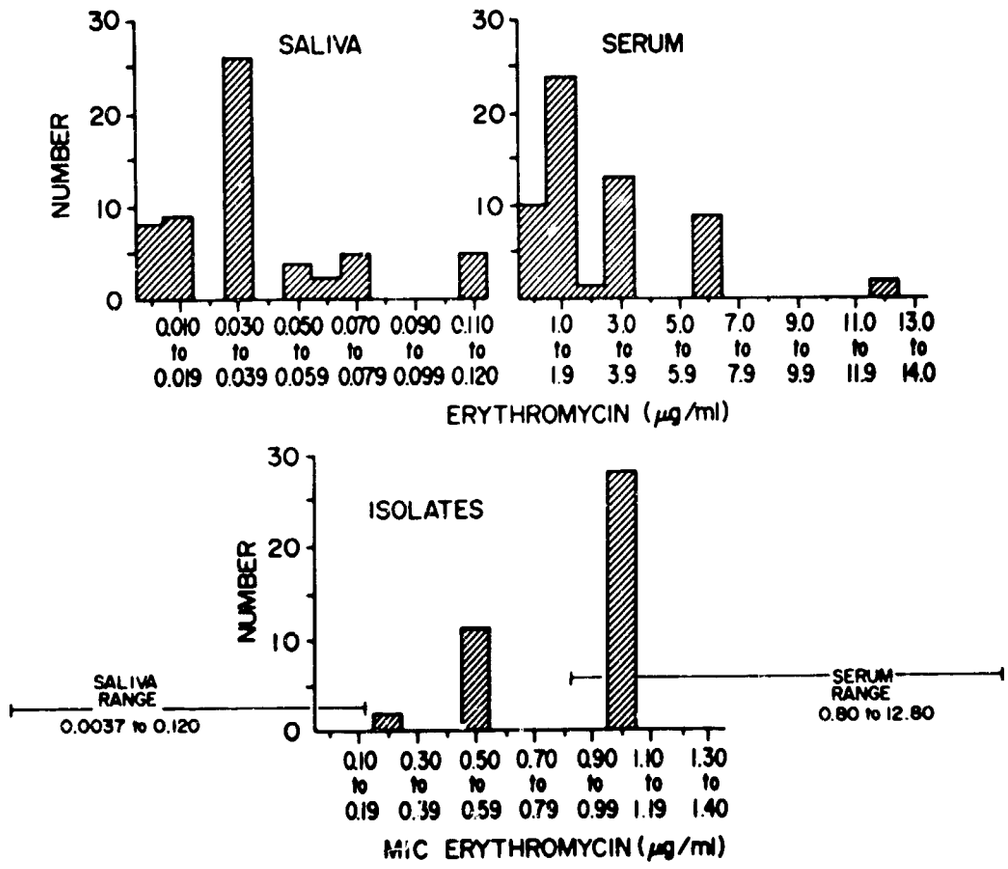


FIGURE 12

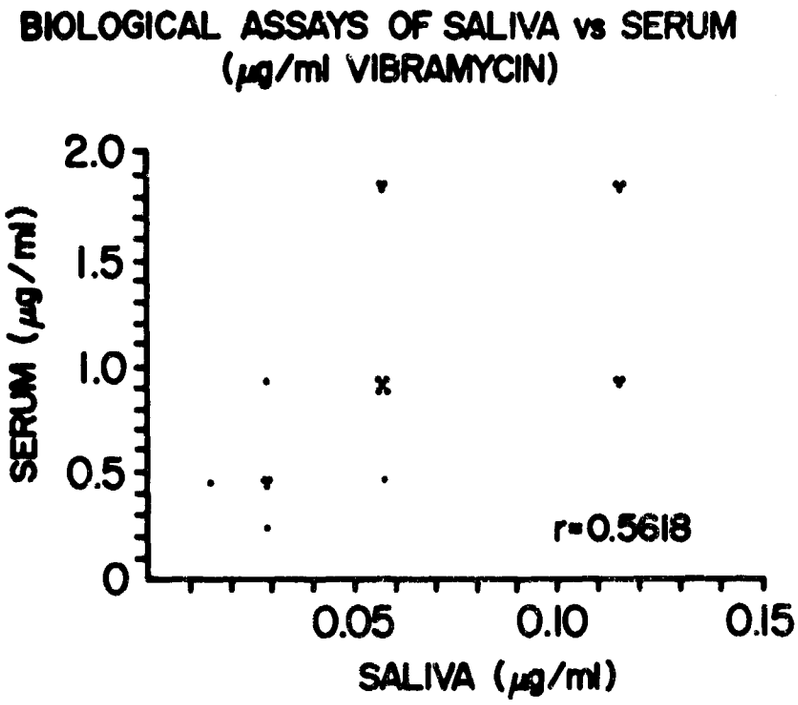
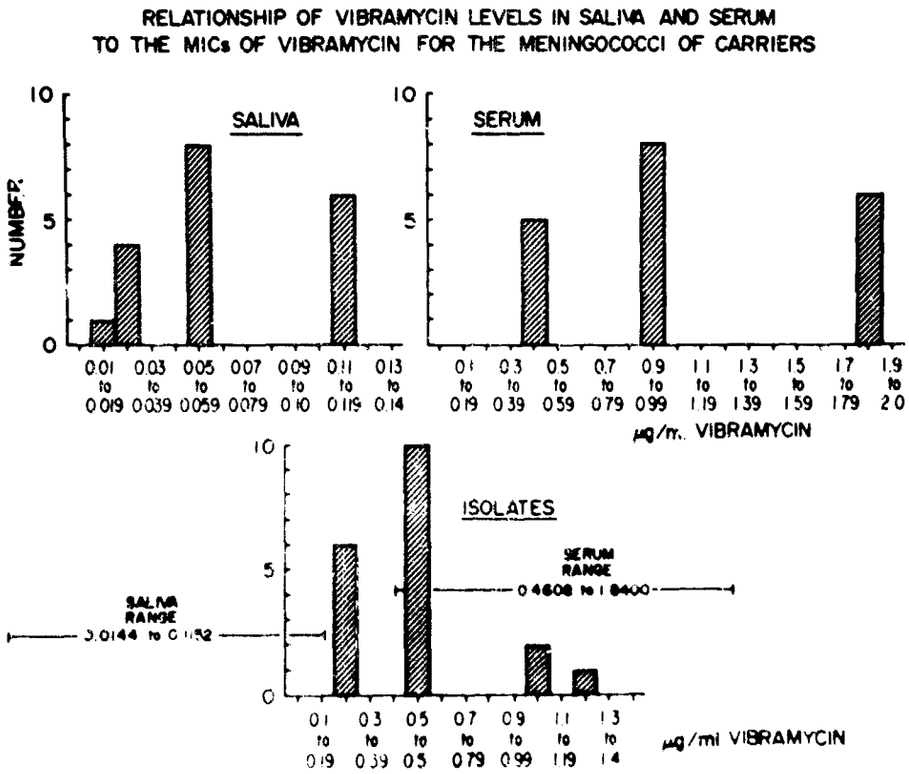


FIGURE 13



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The above papers were presented at a BuMed-ONR-sponsored Meningitis Workshop hosted by NAMRU 4, Great Lakes, Ill., 10-12 June 1968. The incidence of new groups of the meningococcus in a military population has not previously been studied so extensively. The epidemiological study has been aided by newly developed serological tests which give a new depth to an understanding of the carrier and disease state. Further studies of the meningococcal L forms are reported, as well as lab characteristics of a CF antigen. All antigens have been studied in mouse protection tests with hopeful results. A model for the screening of antimicrobial agents in the laboratory which requires a minimal number of volunteers is also described. It is hoped that this technic will eliminate large field trials until favorable results can be expected.

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4. Indirect hemagglutination serological tests						
5. Sulfadiazine sensitivities						
6. Mouse protection studies						
7. Neisserial L forms						
8. Neisseria meningitidis						

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