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ENS (1915) William J. Bowman, USNR
Univ. of Colorado Medical School

ENS (1915) Robert E. Daggett, USNR
Univ. of Illinois School of Medicine

ENS (1915) Thomas L. Davis III, USNR
Univ. of Colorado Medical School

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13. ABSTRACT

This report contains the final reports of the research projects done by the research clerks assigned to the Naval Medical Research Unit No. 4 during 1971. The titles of the projects are as follows:

1. The Effect of Heat and Kaolin Treatment of Serum Fractions Obtained by G-200 Gel Column Chromatography.

2. Isolation of Influenza Viral Subunits With Special Emphasis on Neuraminidase.

3. An Investigation on the Incidence of Acute Respiratory Disease Concomitant With Meningococcal Disease.

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PREFACE

This report includes papers presented by the research clerks assigned to the Naval Medical Research Unit No. 4 (NAMRU-4) in 1971, and gives the details of the investigations which they undertook. These young officers in the Naval Reserve were in medical school when they reported for 60 days of active duty. Due to limitations of time, preliminary plans and experimental designs for the studies were prepared by the preceptors for the clerks. However, insofar as possible, the clerks selected the divisions in which they worked and they chose specific projects from among numerous alternatives.

At NAMRU-4, the clerks experience the various important steps involved in research work, including: (1) review of the pertinent literature, (2) planning the work, (3) collection of specimens, (4) laboratory procedures, (5) collection of data, (6) analysis of data, and (7) preparation of the final report. Close supervision and direction were given the clerks during their work in order to insure their knowledge of the objectives and techniques needed to accomplish the study. Each paper was reviewed by the respective preceptors, but changes were kept to a minimum.

In order to broaden the clerks' experience, additional opportunities were offered. There were weekly seminars, field trips, and a formal lecture series, given by members of the Scientific Department. The lecturers included selected topics in biochemistry, biometrics, epidemiology, immunology, microbiology and virology which were intended to be of particular interest to the clerks.

It is hoped that the ideas and techniques learned were a useful supplement to the medical education of each officer, as well as a helpful introduction to service in the Naval Medical Corps.

Charles E. Miller
Captain, MC USN
Editor
1971 Research Clerkship Reports

FOREWORD

It is the intent of this publication to familiarize those responsible for medical education with the research clerks' 1915 program at Naval Medical Research Unit No. 4 (NAMRU-4). The environment in which the clerks produced the following reports is best portrayed by a description of the research unit.

The mission of NAMRU-4 is to conduct research on the epidemiology, etiology, and methods of prevention and control of acute communicable diseases of the respiratory tract.

NAMRU-4 offers a unique opportunity to the research clerks in offering field trials, laboratory study, and clinical evaluation, singularly or in combination, through the fine cooperation of other adjacent Naval activities including the Recruit Training Command, Service School Command, and the Naval Hospital.

The permanent staff at the research unit consists of approximately 80 individuals who are about equally divided between civilian and military ranks. The scientists are widely recognized as experts in their fields.

The consultant staff and affiliations represent such organizations as the World Health Organization, National Institutes of Health, Universities of Chicago, Illinois, Michigan, Northwestern, Tennessee, South Dakota and Wisconsin, and many of the Commissions of the Armed Forces Epidemiological Board, including those on influenza, acute respiratory diseases, streptococcus and immunization.

The unit offers its resources to the clerks in the process of conducting their investigations. Their projects are frequently not a means to an end in themselves, but are pilot studies for much larger programs. The clerks not only have the satisfaction of completing their own projects, but also may have the opportunity of realizing that they played a small part in a much larger scheme while being introduced to the philosphy and principles of the research methods.

Robert O. Peckinpaugh
Captain, MC USN
Commanding Officer

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THE EFFECT OF HEAT AND KAOLIN TREATMENT ON SERUM FRACTIONS OBTAINED BY G-200 GEL COLUMN CHROMATOGRAPHY*

Ensign William J. Bowman
University of Colorado Medical School

Preceptor: Robert P. Nalewaik
Mycoplasma Division

A widely recognized reaction of viruses is their ability to combine specifically with red blood cells (RBC) of certain species and agglutinate them. Antibody for these viruses can be determined by the hemagglutination-inhibition (HI) test. The inhibition of hemagglutination may not always be the result of specific antibody, but may be due to non-specific factors. Such a phenomenon, however, is not well documented in bacteria. Several bacterial species are listed in a review by Neter[7] which hemagglutinate RBC, and this list has since been expanded to include several other bacteria, pneumococci, for example[8]. Studies conducted on the L-phase of group B Neisseria meningitidis uncovered another hemagglutinin[4]. This alcohol soluble fraction directly agglutinated rabbit and rooster RBC but had no affect on guinea pig, sheep or human RBC. Using this hemagglutinin, HI tests on sera from 54 naval recruits studied for meningococcal carrier status demonstrated the presence of a serum inhibitor. Animal, chicken and other human sera tested, virtually all showed high inhibitory titers[5].

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*From Naval Medical Research Unit No. 4 (NAMRU-4), Great Lakes, Illinois. This investigation was done in connection with Research Project No. M4305.01-1014BFG6, Bureau of Medicine and Surgery, Navy Department, Washington, D. C. The opinions and assertions contained herein are those of the authors and are not to be construed as official or as reflecting the views of the Navy Department or the Naval Service at large. The use of commerically available products does not imply endorsement of, nor preference for these products. The experiments reported herein were conducted according to the principles enunciated in "Guide for Laboratory Facilities and Care" prepared by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council. The animal care facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

While testing recruit sera, an observation was made that the titer of the inhibitor dropped preceeding the acquisition of meningococcal infection. Correspondingly, a rise in complement-fixation titer was observed[5]. What this inhibitor is, and its role in the prevention of meningococcal infection is not known. In an attempt to shed some light on the inhibitor, a preliminary study was undertaken to uncover its nature. This report is the result of that study.

MATERIALS AND METHODS

Serum Source: Sera used in the course of this study were obtained from various individuals at the Naval Medical Research Unit No. 4. The blood was allowed to clot and then centrifuged at 2,000 rpm for 20 minutes. The clear sera was withdrawn and stored at -20°C until used.

Column Chromatography and Spectrophotometry: The method used was a modification of the procedure of Gaidamovich, et al[6]. Five grams of Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, New Jersey) was suspended in 200 ml distilled water and allowed to swell for 3 days with occassional gentle stirring. This swollen gel was slowly added to a Sephadex K15/90 column. The column was equilibrated with 1M NaCl in 0.1M Tris, pH 7.5. Three ml of serum, based on 1 ml of sample for every 30 cm of column height, was put on the column and eluted with 0.1M Tris, pH 7.5. The flow rate was approximately 6 ml/hr. Sodium azide (0.02%, w/v) was added to the eluant as a preservative. An ISCO Golden Retriever fraction collector and Volumeter (Instrumentation Specialties Company, Lincoln, Nebraska) were used to obtain 2 ml fractions. The fractions were diluted 1:10 and read for absorbance at 280 nm using a Beckman DU Spectromphotometer (Beckman Instruments, Inc., Fullerton, Calif.).

Heat Treatment: The collected serum fractions were divided into three portions. One was to be unheated, another heated at 56°C, and the third heated at 90°C for thirty minutes. Hemagglutination-inhibition tests were conducted on these variously treated samples.

Kaolin Treatment: To the variously treated samples above, 0.1 ml of 25% (w/v) kaolin (acid washed, American standard, Fisher Scientific Company, Fair Lawn, New Jersey) was added. The tubes were allowed to stand at room temperature with occassional shaking for 20 minutes. The kaolin was removed by centrifugation at 1500 rpm for 30 minutes. The clear supernatants were removed by pasteur pipettes and tested for HI activity.

Hemagglutinin Titration: The alcohol soluble hemagglutinating antigen (HA) of the L-phase of group B N. meningitidis was previously extracted[5]. Its hemagglutinating potency was measured by direct titration using the microtiter procedure. Stock, lyophilized HA material was prepared by dissolving 10 mg/5 ml in sterile saline for injection (USP), with 0.9% benzyl alcohol as preservative (Abbott Laboratories, North Chicago, Illinois). As previously determined[5], rooster RBC were the erythrocytes of choice in this

test. To all the wells of a microtiter plate was added 0.025 ml of isotonic phosphate-buffered saline (pH 7.2). One 0.025 ml drop of the HA solution was added to the first well, and 0.025 ml was serially diluted omitting the last well which was for a saline control. The final concentration of HA antigen in the first well was 25 µg (100 µg/ml). This was followed by the addition of 0.05 ml of a 0.5% suspension of washed RBC. The plate was gently tapped to mix the RBC and incubated for 1 hour at room temperature. End-points were determined visually by using a boxed viewing mirror (Cooke Engineering Company, Alexandria, Virginia) with an overhead light. One unit of antigen was the greatest dilution at which a smooth blanket of RBC covered the bottom of the well with no evidence of "doughnut" formation or other doubtful reactions. Four HA units were used for the titration of the serum inhibitor.

Titration of Serum Inhibitor (HI) Test: The microtiter procedure was used for measuring the HI titer of the untreated and variously treated samples obtained from the column. All wells contained 0.025 ml of the phosphate-buffered saline diluent. A 0.025 ml volume of the sample was serially transferred from the first to the eleventh well. A 0.025 ml drop of HA antigen containing 4 HA units was then added to each well omitting well No. 12, the serum control. Finally, 0.05 ml of 0.5% chicken RBC was dropped into all wells. The controls consisted of serum fraction with RBC in the twelfth well for agglutination by the serum; diluent with RBC for autoagglutination; and a normal rabbit serum control titrated in the same manner as the test sera. Incubation was at room temperature for 1 hour. The end-point was the highest dilution of serum that resulted in a round "button".

RESULTS

Column Chromatography and Spectrophotometry: Samples collected from the column were diluted 1:10 and read at 280 nm in the Beckman DU. Examples of curves obtained are demonstrated in Figure 1 (top and bottom). The immunoglobulins IgM, IgA and IgG are present from left to right. The heavier globulin, IgM, is the first to be eluted, followed by the IgA and IgG. Differences in magnitude of adsorption curves may be the effect of packing the column, of the flow rate, or of individual serum.

Hemagglutination-Inhibition: All samples (fractions) collected were tested using the HI test. The antigen was the HA fraction of the L-phase of the group B meningococcal organism. In Figure 1 (top and bottom) are seen the reciprocal titers of each fraction of two separate sera as determined by the HI test. The inhibitor appears to be located throughout the immunoglobulin region.

Fractions subjected to 56°C were tested for HI activity. Figure 2 demonstrates that the HI activity is not lost. The activity remains essentially the same as that for untreated serum. One-tube variation is seen.

The pattern shown in Figure 3 (top) is the result of heating the serum

fractions at 90°C. This figure indicates that not only is the HI activity still found throughout the immunoglobulins, but that there is a 16-fold rise in the HI titers.

Treatment of the untreated and heat treated fractions with kaolin has resulted in changes in the HI activity. Addition of kaolin to the untreated fractions and to the 56°C heated fractions resulted in a loss of the HI activity throughout the immunoglobulin region. When kaolin is added to the 90°C heated fractions, the HI activity is lost in the IgM and IgA areas, but activity still remains in the IgG (Fig. 3, bottom).

DISCUSSION

A hemagglutinin has been extracted from the L-phase of group B N. meningitidis which directly agglutinates rabbit and rooster RBC but not erythrocytes from guinea pigs, sheep or humans. Such a hemagglutinin has not been demonstrated in parent bacterial organisms. When tested with sera from several sources, this HA elucidated the presence of a serum inhibitor. The loss of this inhibitor in sera of naval recruits appears to be related to the acquisition of meningococcal organisms. Several methods were available to shed light on the nature of this inhibitor. One such method was that of heat and kaolin treatment followed by the use of the HI test.

The HI activity was observed to be evenly distributed throughout the immunoglobulin pool. This does not imply that the inhibitor is an immunoglobulin or related to the immunoglobulins, but that it is a compound whose molecular weight range is similar to that of the globulins. The effect on this activity was nil when the fractions were heated at 56°C. This would indicate that the inhibitor was not labile at this temperature. Addition of kaolin, to the 56°C heated sera, however, resulted in the eradication of any HI activity. The inhibitor appeared to have been adsorbed out. There was an increase in HI activity when the fractions were heated at 90°C. Apparently, the inhibitor measured under these conditions is a compound. Whether this be a lipoprotein, mucoprotein or like complex is not known. The application of 90°C, however, appears to have affected the protein portion of the complex and unmasked more reactive sites for HA antigen attachment.

Kaolin had an unusual affect on the fractions which were heated to 90°C. The HI activity was removed from the IgM and IgA areas of the curve. The activity in the IgG peak, however, remained intact. The site of kaolin adsorption appears to have been removed. The inhibitor in this area may be different from that of the other two areas. This inhibitor would not be protein in nature as it would have been affected by the 90°C heat treatment.

For these initial studies, heat and kaolin were used. Future studies could incorporate other materials such as the receptor-destroying-enzyme or trypsin. It would also be of value to have enough serum from personnel who demonstrate a fall in inhibitory titer and observe the effect of heat and kaolin treatment. Chemical methods such as used by Brishammer and Philipson[1]

and Chanock and Sabin[2,3] to investigate the nature of viral inhibitors may be a feasible approach for investigating bacterial inhibitors. It may also be possible to utilize the ability of sodium amylosulfate to separate beta-lipoprotein to further elucidate this problem. Consideration could also be given to other chromatographic techniques. The use of electrophoretic techniques would not be practical as the amount of sample used and that recovered would not allow the use of the HI test.

Individual and pooled erythrocytes from 4 roosters were previously investigated for use in the HA titration and the HI test; individual and pooled RBC reacted satisfactorily, pooled cells produced a finer reaction[5]. When the HA titration was performed in the present study, it failed. It was determined, after careful examination of all factors involved, that the pooled RBC were not reacting properly. Upon testing the roosters individually, it was found that 2 of the 4 roosters used in the pool were not satisfactory sources of RBC, and their deficiency masked the ability of the erythrocytes from the other 2 roosters to react in the HA titration. Since the previous study, there was a turn-over of roosters in the animal house. This points out a need to test each animal individually prior to pooling their red blood cells.

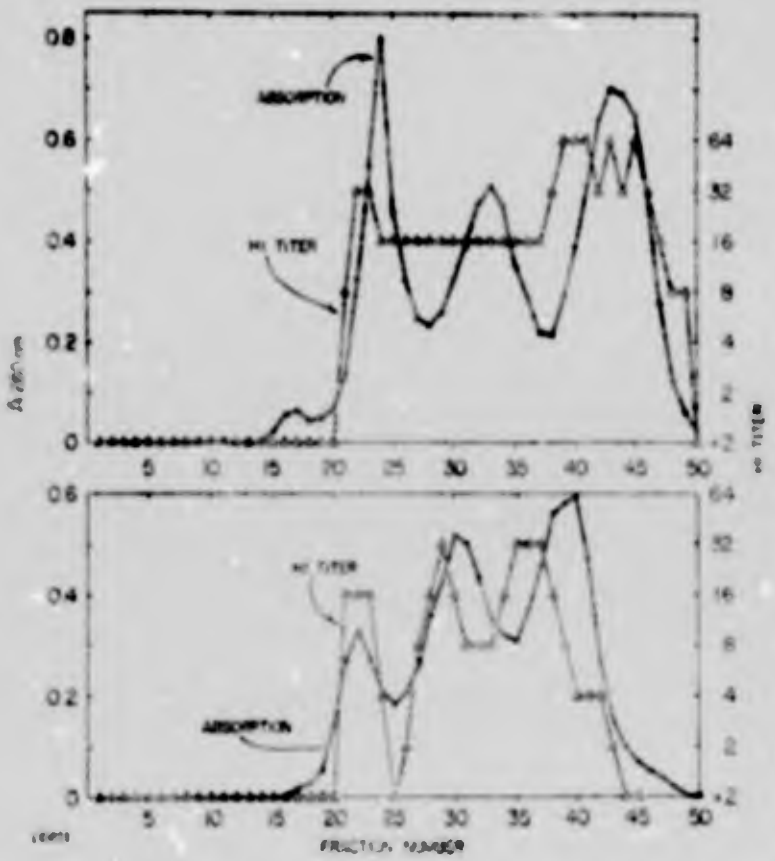

Fig. 1. Top and bottom: Absorbance curves obtained after passing human serum through Sephadex G-200 columns. Immunoglobulins are eluted according to their weight. IgM, the heavier immunoglobulin, is eluted first, followed by IgA and IgG (left to right). Also seen in this figure are reciprocal HI titers of each fraction obtained from the columns.

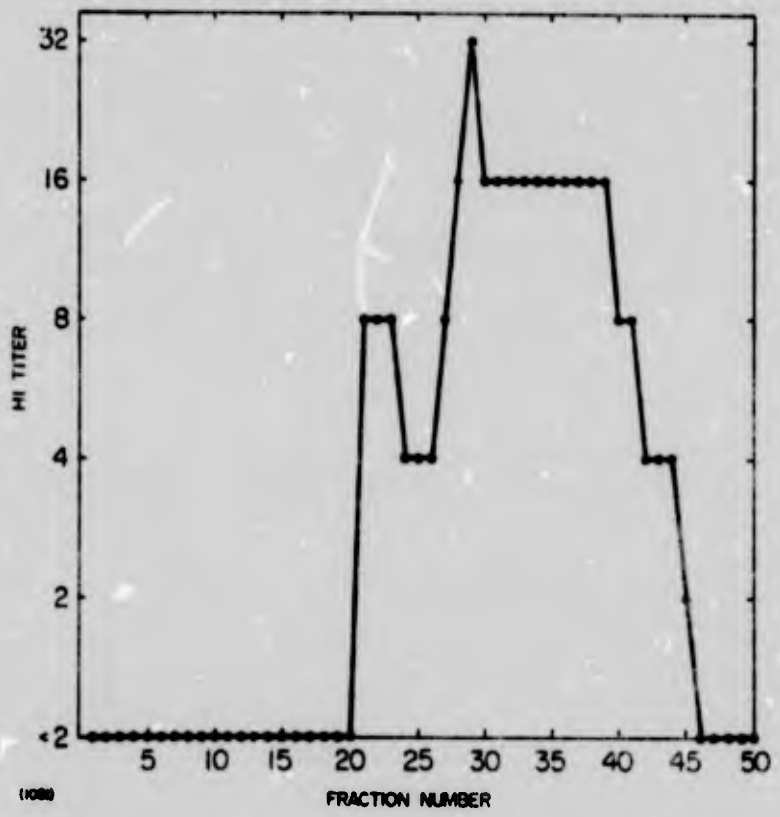

Fig. 2. Effect of 56°C heat treatment on the HI activity of fractions obtained from Sephadex G-200 columns.

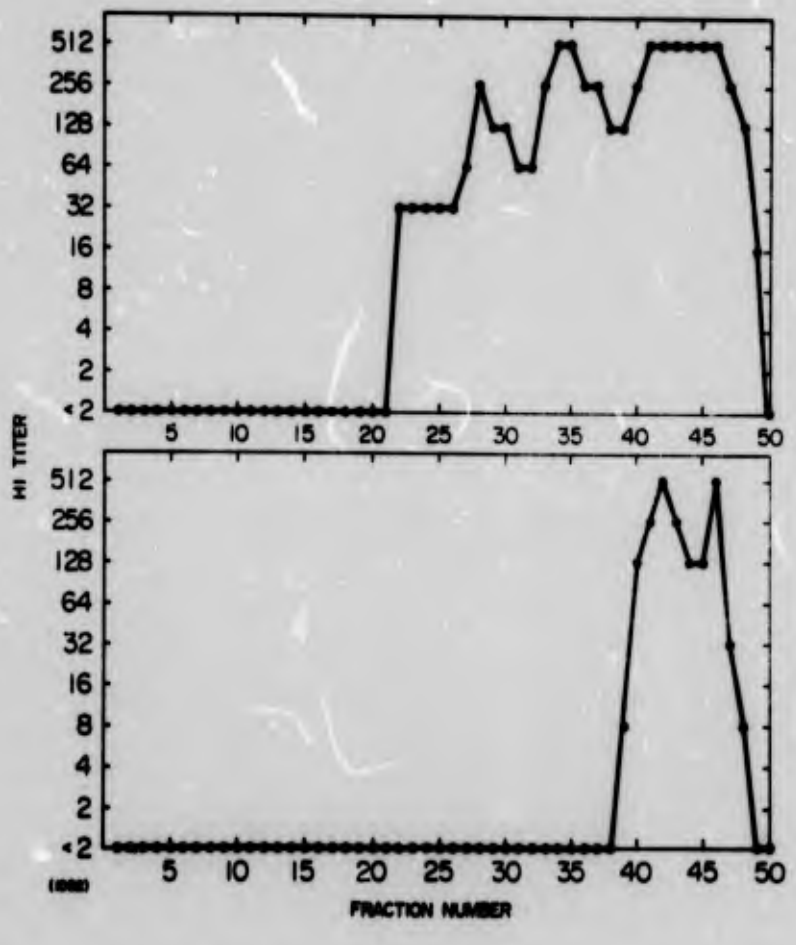

Fig. 3. Top: Effect of 90°C heat treatment on the HI activity of fractions obtained from Sephadex G-200 columns. Bottom: Effect of Kaolin on the HI activity of 90°C heat treated fractions obtained from Sephadex G-200 columns.

REFERENCES

1. Brishammar, S. and Philipson, L. Purification and Properties of a Non-specific Hemagglutination Inhibitor From Human Serum Against Picornaviruses. Biochem. Biophys. Acta. 1966, 127:140-150.

2. Chanock, R. M. and Sabin, A. B. The Hemagglutinin of St. Louis Encephalitis Virus. III. Properties of Normal Inhibitors and Specific Antibody, Use of Hemagglutination-Inhibition for Diagnosis of Infection. J. Immunol. 1953, 70:302-316.

3. Chanock, R. M. and Sabin, A. B. The Hemagglutinin of Western Equine Encephalitis Virus; Recovery, Properties and Use for Diagnosis. J. Immunol. 1954, 73:337-351.

4. Crawford, Y. E., Lytle, R. I. and Nalewaik, R. P. Hemagglutinin From the L-form of Neisseria Meningitidis, Group B. Bacteriol. Proc. 1970, M235.

5. Crawford, Y. E., Nalewaik, R. P., Lytle, R. I., and O'Connell, J. L. Hemagglutinin from the L-phase of Neisseria Meningitidis, Group B, with Observations on a Serum Inhibitor. Infect. Immun. 1971, 4:212-219.

6. Gaidamovich, S. Y., Mekler, L. B., and Kahaltayeva, G. G. Study of Certain Properties of Haemagglutination Inhibitors and Anti-hemagglutinins to Arboviruses. Acta Virol. 1967, 11:114-119.

7. Neter, E. Bacterial Hemagglutinations and Hemolysis. Bacteriol. Rev. 1956, 20:166-188.

8. Raunio, V. Haemagglutination Caused by Pneumococci. Ann. Med. Exp. Fenn. 1959, 37:243-250.

ISOLATION OF INFLUENZA VIRAL SUBUNITS WITH SPECIAL EMPHASIS ON NEURAMINIDASE*

Ensign Robert B. Daggett
University of Illinois School of Medicine

Preceptor: Ralph I. Lytle
Chief, Biochemistry Division

Since the catastrophic pandemic of 1918, there has been a great deal of interest in the subunits of influenza virus as to their antigenic structure and their ultimate use in vaccine preparations[1-5]. Gottschalk has proposed that the role of viral neuraminidase is to form an enzyme-substrate complex which is the initiation of the influenza virus infection. "The key reaction in the invasion of the natural host (man) by influenza virus is the formation of an enzyme-substrate complex. . . . . ., the substrate present (a) in the protective mucus layer of the respiratory tract and (b) in the outer membrane of the host cell. The complex undergoes activation yielding products (i.e., N-acetyl neuraminic acid [NANA]) and free virus particles. The enzyme-substrate complex is the mechanism underlying adsorption of the virus particle to the surface of the host cell"[6]. By the formation of an enzyme-substrate complex, the virus attaches to a red blood cell and passes throughout the body without causing agglutination.

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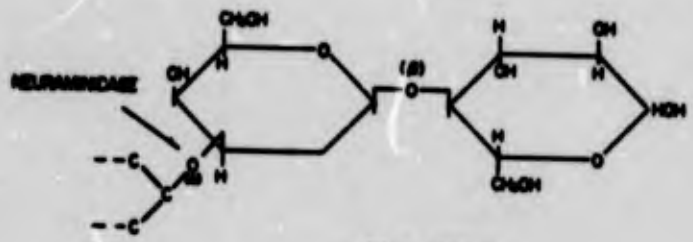
*From Naval Medical Research Unit No. 4 (NAMRU-4), Great Lakes, Illinois. This investigation was done in connection with Research Project No. MR04105.01-0011B2GE, Bureau of Medicine and Surgery, Navy Department, Washington, D. C. The opinions and assertions contained herein are those of the authors and are not to be construed as official or as reflecting the views of the Navy Department or the Naval Service at large. The use of commercially available products does not imply endorsement of, nor preference for these products. The experiments reported herein were conducted according to the principles enunciated in "Guide for Laboratory Facilities and Care" prepared by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council. The animal care facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

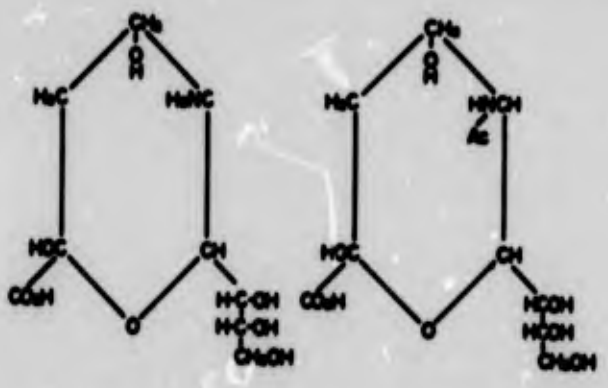
Neuraminidase (Fig. 1) is an enzyme which specifically splits the glucosidic bond between the keto group, liberating NANA as an end product, of the reaction. For example, the susceptible linkage in sialyl-lactose is the 2 ⟶ 3 type.



SIALYL-LACTOSE

Neuraminidase activity is measured by the presence of the end product, neuraminic acid (Fig. 2). Neuraminic acid and other members of the sialic family have been shown to occur in a variety of animal secretions, tissues and cells, and even in bacteria as conjugated protein[7].

Sialic acids are probably important constituents of oligosaccharides or polysaccharides. Neuraminic acid is a naturally occurring sialic acid, as are other substituted neuraminic acid derivatives (N-acetyl, N-glycolyl, N, O-diacetylneuraminic acid). These acids are essentially substitutions on the basic neuraminic acid chain, a 2-keto-3-deoxy sugar.



NEURAMINIC ACID N-ACETYL NEURAMINIC ACID (NANA)

MATERIALS AND METHODS

The Isolation of an Influenza Virus through Centrifugation. Centrifugation at 15,000 rpm for 45 minutes in a Model L Spinco Ultracentrifuge (Rotor Head #30) did not separate non-viral protein (egg protein) from the viral particles in PR-8 infected allantoic fluid, as may be seen from the results in Table 1. When centrifugation was increased to 30,000 rpm for 30 minutes, the influenza virus particles were sedimented (Table 2). In each regimen, 100 ml of infected allantoic fluid (IAF) were treated. The procedures were identical except for the rate of centrifugation.

Five milliliters was set aside for later study, and the viral activity, as measured by hemagglutination inhibition, of the remaining volume was determined by microtiter (Fig. 3) using 0.5% suspension of chicken red blood cells. Neuraminidase levels were determined by the method of Warren[8] except that 6% HCl in butanol was substituted for cyclohexanone. The IAF was then centrifuged at 5,000 rpm for 10 minutes to remove urate precipitates. After removal of urate precipitates, the fluid was centrifuged at 30,000 rpm for 30 minutes. The top 3/5 of the supernatant was removed with a syringe, labelled "original supernatant", and stored at 2 C. The sediment (virus concentrate) was resuspended to an equal volume in phospate buffer (pH 6.7 in 0.1 M concentration), thoroughly mixed and centrifuged at 30,000 rpm for 30 minutes. Again, the top 3/5 of the supernatant was removed, labelled "1st wash", and refrigerated at 2 C. The procedure was repeated a third time, the top 3/5 of the supernatant being labelled "2nd wash". The sediment was resuspended to its original volume in phosphate buffer. The viral activity and neuraminidase levels were measured in the original supernatant, the 1st and 2nd wash, and in the final virus suspension.

The Isolation of an Influenza Virus by Methanol Extraction[9]. The reserved 5 ml sample of the IAF was immersed in a dry ice and methanol bath and, with constant agitation, 2.5 ml of cold (-64 C) methanol was added, drop by drop, over a 10 minute period. The mixture was removed from the cold bath, allowed to stand 3 1/2 hours at -2 C, then centrifuged for 10 minutes at 3,500 rpm. The supernatant containing the methanol soluble fraction was decanted and allowed to evaporate at -2 C. Five milliliters of phosphate buffer was added to the sediment, shaken, allowed to stand at room temperature for 10 minutes, then centrifuged for 10 minutes at 1,500 rpm. The supernatant was removed, labeled "#1" and refrigerated at -2 C. The sediment was again treated in the same manner, the supernatant removed and labelled "#2", and refrigerated. The sediment was discarded. Viral activity and neuraminidase levels were measured in the methanol soluble fraction and in the phosphate buffer washings.

Additional Work on the Centrifugation Supernatants. More work was done on the supernatant, 1st wash, and 2nd wash obtained from the original procedure. A 30 ml sample was taken from each of these and centrifuged at 30,000 rpm for 1 hour. This procedure was repeated a second time. Each of the original supernatants, 1st and 2nd washes, and the final suspensions were measured for neuraminidase levels and viral activity.

RESULTS

Infected allantoic fluid was centrifuged at 15,000 rpm for 45 minutes in an attempt to separate non-viral protein from the influenza virus particles. The results shown in Table 1 indicate that, although the original HA titer was low (32), neither the HA activity nor neuraminidase were satisfactorily separated under these conditions. Another attempt was made in which the rate of centrifugation was increased to 30,000 rpm for 30 minutes. The resuts are shown in Table 2. Of interest were the neuraminidase levels observed in the supernatant with low or not detectable levels of HA activity. Also, as shown, the highest concentration of viral particles now appeared in the sediment. As indicated from these observations, greater separation of the enzyme from the virus might be forthcoming by the methanol extraction method of Lytle et al[9]. As outlined in Table 3, no HA activity was apparent in the methanol soluble fraction, while following the first elution of the sediment with phosphate buffer an HA titer of >4096 with only 13.1 µg/ml of NANA were released from a standard substrate. Of further interest was the possible cleaner separation of the HA in the second buffer extraction. Again, this is evidence following the second elution of the methanol insoluble fraction. Ratio of µmoles of NANA to tubes of HA indicate a much higher ratio before purification -- 3.1 µmoles/tube as compared with 1.09 µmoles/tube 1st elution and only 0.9 µmoles/tube following the 2nd elution.

DISCUSSION

Methods concerning the isolation of influenza neuraminidase indicate that the neuraminidase might disassociate from the viral particles. This could possibly explain the finding of moderate levels of NANA after culturing samples in a fetuin environment at 37 C. It is important to note the low level of NANA before the culturing technique. This technique shows that neuraminidase and not a foreign source of NANA is present (see Table 2). It is also important to note that units of viral activity are reduced as the amount of NANA being released is decreased. Further interest as to effects of methanol precipitation on neuraminidase activity awaits further study.

SUMMARY

Neuraminidase can be separated from influenza virus particles by centrifugation as described or may be extracted with the infected particles by the methanol procedure. Generally speaking, less viral hemagglutinating neuraminidase is lost by the methanol extraction procedure than by centrifugation. The enzyme neuraminidase can be separated with little or no hemagglutinating activity. As seen from the test, it is neuraminidase, not neuraminic acid, that is being separated. Neuraminic acid is the end product of the action of neuraminidase.

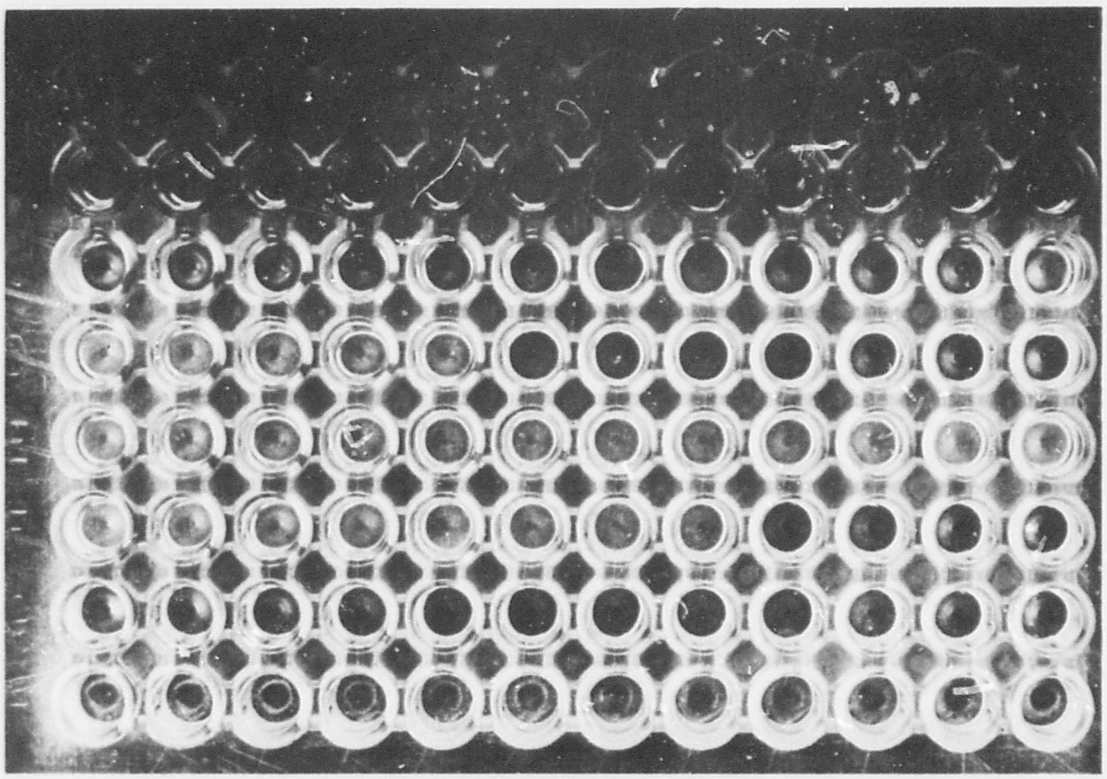


FIGURE 3. AGGLUTINATION TEST

C - Supernatant (Centrifugation 30,000 rpm 30")
D - Final Volume (Centrifugation 30,000 rpm 30")
E - 1st Phosphate Buffer (CH_3OH EXT)
F - 2nd Phosphate Buffer (CH_3OH EXT)
G - CH_3OH Layer (CH_3OH EXT)
H - Nothing

Samples read from left to right as dilutions: $(1/2)^n$ n = 1 to 12
[1/2, 1/4, 1/8, 1/16, 1/32 . . . . . etc]

TABLE 1. EFFECTS OF ULTRACENTRIFUGATION, 15,000 RPM FOR 45 MINUTES ON INFLUENZA PR-8 AND NEURAMINIDASE ACTIVITY

	HA Agglutination	µmoles of NANA	
		Free	Free & Bound
Normal allantoic fluid (NAF)	<2	1.1	1.5
Infected allantoic fluid (IAF)	32	-	17.84
Supernatant	8	0.46	9.84
1st wash	8	0.63	4.63
2nd wash	<2	0.05	3.96
Final volume	2	0.00	4.59

TABLE 2. EFFECTS OF ULTRACENTRIFUGATION, 30,000 RPM FOR 30 MINUTES ON INFLUENZA PR-8 AND NEURAMINIDASE ACTIVITY

	HA Agglutination	µmoles of NANA*	
		Free	Free & Bound
Normal allantoic fluid	<2	1.1	1.5
Infected allantoic fluid	256	1.85	25.40
Supernatant	2	1.32	12.10
Supernatant	4	1.42	14.48
1st wash	<2	0.31	3.54
Final volume	2	0.00	4.36
1st wash	<2	0.00	4.61
Supernatant	<2	0.18	6.38
1st wash	<2	0.61	3.67
Final volume	<2	1.57	3.81
2nd wash	2	0.00	3.36
Supernatant	<2	0.00	4.98
1st wash	<2	0.00	3.16
Final volume	<2	0.00	5.03
Final volume (sediment)	64	0.88	5.13

*In a fetuin environment at 37°C for 30 minutes µmoles of N-acetylneuraminic acid (NANA)

$$= (E3/(E2E3 - E1E4) \times O.D._{549} - E4/(E2E3 - E1E4) \times O.D._{532}) \times (TV/TxV)$$

$$= ((0.021 \times O.D._{549}) - (0.00758 \times O.D._{532})) \times (TV/TxV)$$

$$= ((0.021 \times O.D._{549}) - (0.00758 \times O.D._{532})) \times 1360.92$$

E1 = 26 Extinction Coefficient x 10^{-3}, NANA 532 mu TV = Total volume
E2 = 57 Extinction Coefficient x 10^{-3}, NANA 549 mu T = Time (min)
E3 = 133 Extinction Coefficient x 10^{-3}, Deoxyribose 532 mu V = Volume
E4 = 48 Extinction Coefficient x 10^{-3}, Deoxyribose 549 mu (virus)

TABLE 3. EFFECTS OF METHANOL PRECIPITATION ON THE HEMAGGLUTININ (HA) AND NEURAMINIDASE, SUBUNITS OF INFLUENZA VIRUS PR-8

	HA Agglutination	μmoles of NANA	
		Free	Free & Bound
Normal allantoic fluid	<2	1.1	1.5
Infected allantoic fluid	256	1.85	25.40
Methanol soluble extract	<2	0.54	3.85
Fraction eluted from methanol insoluble fraction			
PO_4 buffer (1) 1st elution	<4096	0.00	13.12
PO_4 buffer (2) 2nd elution	256	0.00	2.35

REFERENCES

1. Laver, W. G. and Kilbourne, E. D. Identification in a Recombinant Influenza Virus of Structural Proteins Derived from Both Parents. Virol. 1966, 30:493-501.

2. Webster, R. G. and Pereira, H. G. A Common Surface Antigen in Influenza Viruses from Human and Avian Sources. J. Gen. Virol. 1968, 3:201-208.

3. Schild, G. C. and Pereira, H. G. Characterization of the Ribonucleoprotein and Neuraminidase of Influenza A Viruses by Immunodiffusion. J. Gen. Virol. 1969, 4:355-363.

4. Kilbourne, E. D., Laver, W. G., Schulman, J. L. and Webster, R. G. Antiviral Activity of Antiserum Specific for an Influenza Virus Neuraminidase. J. of Virol. 1968, 2:281-288.

5. Laver, W. G. and Webster, R. G. The Structure of Influenza Viruses IV. Chemical Studies of the Host Antigen. Virol. 1966, 30:104-115.

6. Gottschalk, A. The Chemistry and Biology of Sialic Acids and Related Substances. Cambridge University Press. 1960.

7. Burnet, F. M. Influenza Virus Isolated from an Australian Epidemic. Med. J. Aust. 1935, ii, 651-653.

8. Warren, L. The Thiobarbituric Acid Assay of Sialic Acids. J. Biol. Chem. 1959, 234:1971-1975.

9. Lytle, R. I. and Woolridge, R. L. Purification and Stabilization of Influenza Virus Antigens for use in Hemagglutination-Inhibition or Complement Fixation Antibody Titrations. J. Lab. & Clin. Med. 1953, 41:290-295.

AN INVESTIGATION ON THE INCIDENCE OF ACUTE RESPIRATORY DISEASE CONCOMMITANT WITH MENINGOCOCCAL DISEASE*

Ensign Thomas L. Davis III
University of Colorado Medical School

Preceptor: Earl A. Edwards
Chief, Immunology Division

Meningococcal infection, in a human population is manifested in one or more of several clinical entities. These include (1) carrier state, (2) meningococcemia, (3) meningococcal meningitis, and (4) inflammatory reactions of body fluids and/or membranes. In the carrier state, which is the most common infection of Neisseria meningitidis, the organism is recovered from cultures of the nasopharynx. The carrier has no apparent clinical evidence of disease. However, in disease, in addition to positive nasopharyngeal cultures, the organism can be recovered from the blood (Meningococcemia) and/or the cerebrospinal fluid (Meningococcal Meningitis[1]). In our experience at NAMRU-4, the organisms have also been recovered from pericardial fluid (1 case) and joint fluid (3 cases).

During the year, April 1970 - March 1971, the carrier rate of Group C Neisseria meningitidis ranged from 1.5 to 42% in recruits at Great Lakes Naval Training Center. These figures represent 4,500 to 1[illegible],500 recruits processed. During this period 25 recruits, 0.5% were shown to have Group C meningococcemia or meningitis[2] (Fig. 1).

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*From Naval Medical Research Unit No. 4 (NAMRU-4), Great Lakes, Illinois. This investigation was done in connection with Research Project No. MF12.524. 009-4013BE6I, Bureau of Medicine and Surgery, Navy Department, Washington, D. C. The opinions and assertions contained herein are those of the authors and are not to be construed as official or as reflecting the views of the Navy Department or the Naval Service at large. The use of commerically available products does not imply endorsement of, nor preference for these products. The experiments reported herein were conducted according to the principles enunciated in "Guide for Laboratory Facilities and Care" prepared by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council. The animal care facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Comparison of these two figures suggests several possibilities that could account for a break down in the host-parasite relationship where only a few recruits acquire clinical disease: (1) a genetic defect in complement production[3], (2) a deficiency in the concentration of immunoglobulins, (3) immunodepression caused by drugs, (4) immunodepression related to concurrent viral infections[4-8].

Previous studies have shown that a number of viral diseases cause an immunodepressive state. Rubella has been shown to cause tuberculin positive children to become negative and then become positive immediately prior to the appearance of the rash[9]. It also causes depression of lymphocyte transformation and may be responsible for activation of TB.

This investigation was undertaken to demonstrate a possible relation between clinical disease caused by Neisseria meningitidis, meningococcemia, and/or meningitis, and common respiratory diseases. There are no previous reports of such a study.

MATERIALS AND METHODS

Sera samples were taken from patients on admission to the hospital (acute) and three weeks after admission. The samples were stored at -20°C. The three-week time period between samples was selected to allow significant antibody response to either bacterial or viral agents.

Several serological tests were used in conjunction with the clinical findings to verify meningococcal disease. These included the complement fixation test and the passive hemagglutination test[10] for antibody rises (four-fold or greater) and the counterimmunoelectophoresis technique[11] for presence of antigen in the acute phase.

Acute and convalescent sera from 40 patients showing clinical evidence of disease and one or more positive serological tests for Neisseria meningitidis were tested against 13 agents that might be etiologically related to acute respiratory disease in a recruit population (Table I).

Neutralization, complement-fixation, and hemagglutination tests were carried out using the micro-technique[10,12].

RESULTS

Of the agents tested, Mycoplasma pneumoniae, adenovirus and influenza type B showed the greatest number of four-fold rises between the acute and convalescent sera. The seroconversion rates to these agents were 22.5, 17.5 and 17.5% respectively (Table II). However, there was an overall seroconversion rate of 65% against those agents used in this study.

DISCUSSION

The reason for the low incidence of meningococcal disease in a population that is known to become infected with Neisseria meningitidis (20 - 80%) is unknown. It may be due to the low virulence of the meningococcal organism or certain individuals may be deficient in host factors essential to resistance. The immunodepressive effect of microbial diseases cannot be excluded as the possible mechanism for initiating a disease by an agent that appears to be low in virulence. Since from 60 - 80% of the recruits experience microbial infections while in training and if this infection predisposes the recruit to the susceptibility of meningococcal disease when the organism is acquired, the question arises of why there is not a much higher incidence of meningococcal disease. Previous studies, using experimental animals, indicate a critical timing and duration of administration of antigen to cause immunodepression[13]. Such a critical timing of events in experiencing microbial infection and acquiring meningococcal infection might be necessary to "cause" this relatively avirulent organism to become virulent in those individuals who have meningococcal disease. This concept is illustrated in Fig. 2.

The fact that 65% of the recruits gave serological evidence of simultaneous infection with Neisseria meningitidis and other microbial agents suggests that such infections may contribute to the susceptibility of these recruits to meningococcal disease. A prospective study investigating several parameters of immunocompetence during the acute stage of the disease would be necessary to prove this concept.

SUMMARY

A study was made to determine if concomitant respiratory infections in patients admitted with meningococcal disease may have been a possible contributing factor towards meningococcal disease. Of the 40 men studied, Mycoplasma pneumoniae, 22.5%, adenovirus, 17.5%, and influenza B, 17.5%, were most frequently associated with a four-fold increase in antibody titer. Sixty-five percent of the individuals with meningococcal disease showed a four-fold increase in antibody titer to one or more microbial agents. Such concomitant infections may play a role in increasing susceptibility to meningococcal disease.

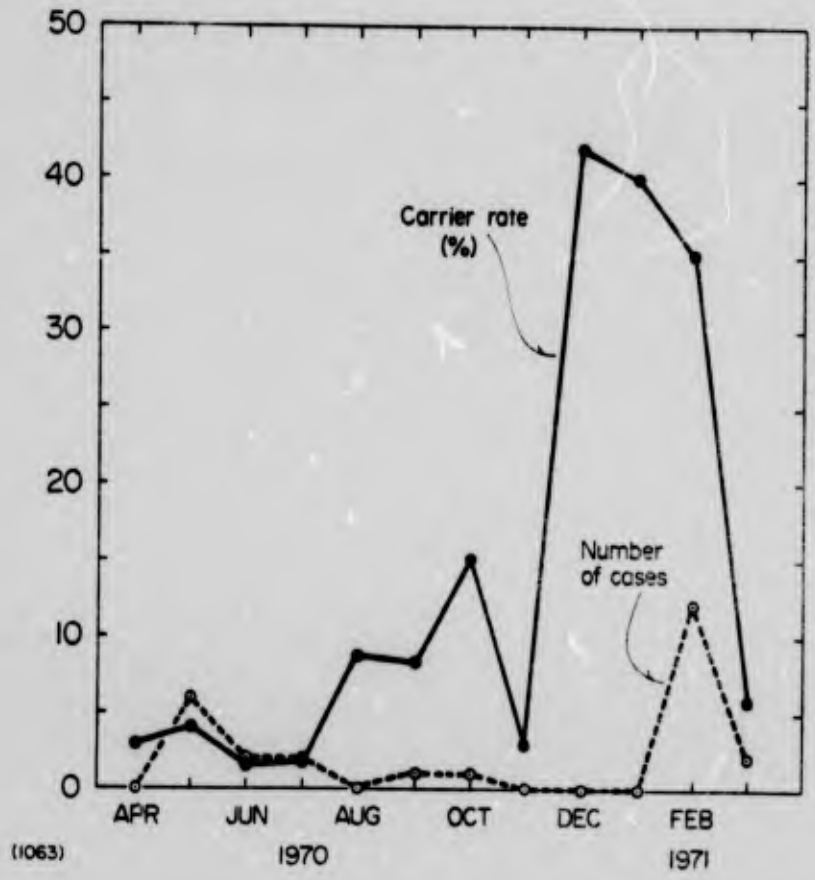

FIGURE 1. N. MENINGITIDIS GROUP C CARRIER RATE AND THE NUMBER OF RECRUITS WITH GROUP C MENINGOCOCCAL DISEASE, April 1970-March 1971

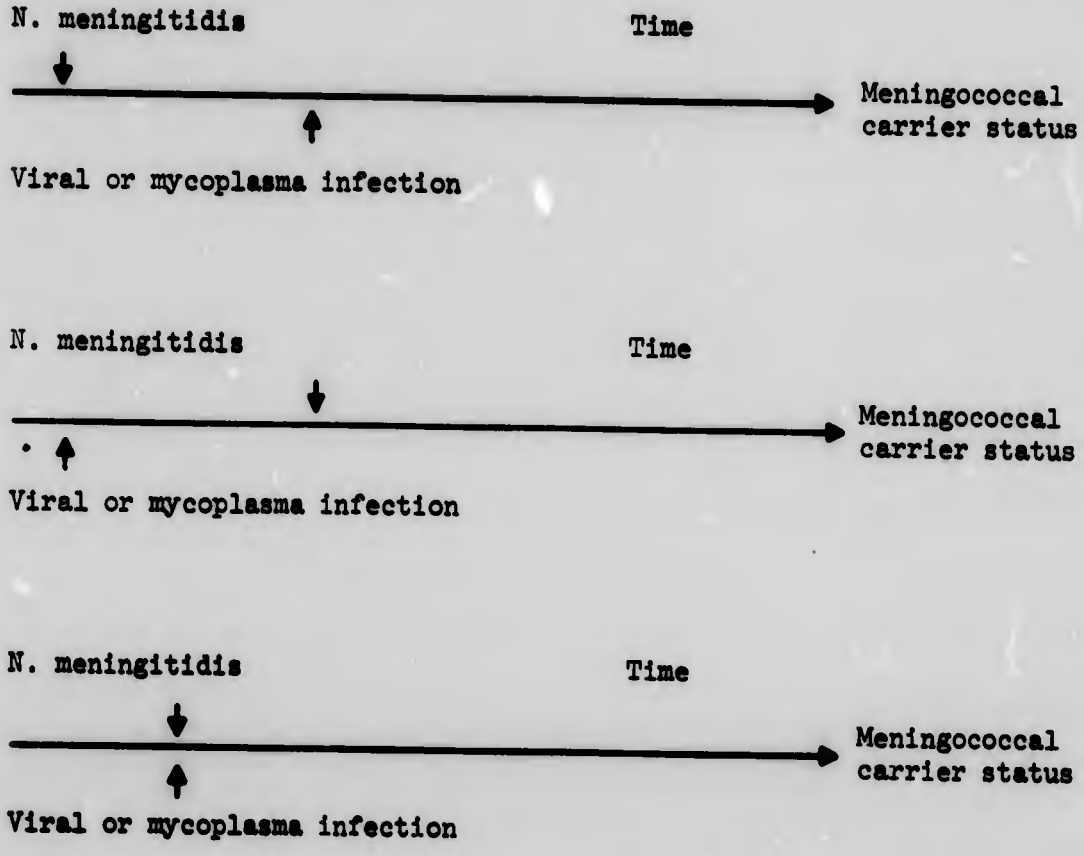

FIGURE 2. RELATIONSHIP OF INFECTIONS BY N. MENINGITIDIS AND VIRAL OR MYCOPLASMA AGENTS TO CARRIER STATE OR DISEASE. SCHEME SUGGESTS A CRITICAL TIME-RELATIONSHIP BETWEEN THE ACQUISITION OF N. MENINGITIDIS AND OTHER UPPER RESPIRATORY INFECTIOUS AGENTS AND EVENTUAL DISPOSITION OF THE MENINGOCOCCAL INFECTION

TABLE I. SEROLOGICAL TESTS USED TO DETECT ANTIBODY RESPONSE TO VARIOUS VIRAL AND MYCOPLASMA AGENTS

Antigen	Neut	CF	HI
Adenovirus		X	
Adenovirus Type 4	X		
Adenovirus Type 7	X		
Rhinovirus Type 2060	X		
Rhinovirus Type 1122	X		
Rhinovirus Type 779	X		
Mycoplasma pneumoniae		X	
Rubella			X
Respiratory Syncytial Virus (RSV)		X	
Influenza A		X	
Influenza B		X	
Parainfluenza I			X
Parainfluenza III			X

TABLE II. ANTIBODY RESPONSE IN CONVALESCENT SERA COMPARED TO ACUTE SERA

Antigen	Antibody rise 4-fold or greater
Mycoplasma pneumoniae	22.5%
Adenovirus (CF)	17.5
Adenovirus Type 4	17.5
Influenza B	17.5
Rhinovirus Type 779	15.0
Influenza A	12.5
Parainfluenza I	10.0
Adenovirus Type 7	5.0
Rubella	2.5
Rhinovirus Type 2211	2.5
Rhinovirus Type 2060	-
Parainfluenza III	-

REFERENCES

1. Vedros, N. A. and Hottle, G. A. Neisseria and Neisserial Infections. Infectious Agents and Host Reactions. W. B. Saunders Co., Philadelphia, Penn. 1970, pp 271-285.

2. Devine, L. F. Department of Bacteriology, NAMRU-4, Great Lakes, Ill. Personal Communications. 1971.

3. Cellular Immunity in Infectious Diseases. Lancet, 2 Aug, 1969, pp 253-255.

4. Thomas, J. W., Clements, D. and Norman, S. C. Lymphocyte Transformation by Phylohemagglutinin. IV. In Acute Respiratory Infections. Canadian Med. Assn. J. 1968, 99:467-468.

5. Turk, J. L. and Waters, M. F. R. Cell Mediated Immunity in Patients with Leprosy. Lancet. 2 Aug. 1969, pp 243-246.

6. Salaman, M. H. Immunodepression by Viruses. The Immune Response and Its Suppression. E. Sorkin, Davos (ed), S. Karger, New York, vol 15, 1968.

7. Meadzon, E. L. and Vas, S. I. Studies on In Vitro Antibody Virus. II The effect of Newcastle Disease Virus on Antibody Synthesis. Canad. J. Microbiol. 1964, 10:535-541.

8. Smithwick, F. M. and Berkovitch, S. In Vitro Suppression of Lymphocyte Response to Tuberculin by Live Measles Virus. Proc. Soc. Exp. Biol. 1966, 123:276-278.

9. Pirquet, C. von. Des Verhalten der kutanen Tuberculin reaktion während der masern. Dtsch. med. Wschr. 1908, 34:1297-1300.

10. Edwards, E. A. Serological Methods Manual for the Microtiter Technique. NAMRU-4, Research Project MF12.524.009-4019, 1969, pp 1-50.

11. Edwards, E. A. Group Specific Neisseria meningitidis Antigens Present in the Serum of Patients with Fulminant Meningococcemia. J. Immunol. 1971, 106:314-317.

12. Sever, J. L. Application of Microtechnique to Viral Seroligical Investigations. J. Immunol. 1963, 88:320-329.

13. Birenbaum, M. C. Immunosuppressive Agents and the Cellular Kinetics of the Immune Response. Immunity Cancer and Chemotherapy, New York, 1967, pp 217-241.