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IMMUNOLOGIC INVESTIGATIONS OF
MENINGOCOCCAL DISEASE. I. GROUP-
SPECIFIC 'NEISSERIA MENINGITIDIS'
ANTIGENS PRESENT IN THE SERUM OF
PATIENTS WITH FULMINANT MENINGOCOCCEMIA

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13. ABSTRACT Data presented show that patients with fulminant meningococemia which resulted in death have a readily detectable level of soluble group-specific meningococcal antigen present in their serum. Patients with meningococcal meningitis and who recovered did not give a positive test for meningococcal antigen under the conditions of the test described. The test is rapid, sensitive and specific for meningococcal group-specific antigens. Three patients were found to have a level of 1.5 µg/ml to 4.5 µg/ml of meningococcal antigen present in their acute blood sample. These levels may signal the onset of fulminant meningococemia. An important relationship yet to be determined is the threshold level of circulating meningococcal antigen in the serum and the reversibility of the disease process. Preliminary observations made at this laboratory indicate such a level may exist. The rapidity and specificity of the counterimmunoelectrophoresis test for meningococcal antigen should permit its use in studying spinal fluids, as well as serum, for rapid identification of the infecting organism. To date, eight spinal fluids have been examined for <u>Neisseria meningitidis</u> antigen. The counterimmunoelectrophoresis test results all agreed with bacterial isolation and identification.			

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IMMUNOLOGIC INVESTIGATIONS OF MENINGOCOCCAL DISEASE

I. Group-Specific *Neisseria meningitidis* Antigens Present in the Serum of Patients with Fulminant Meningococcemia¹

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Patients with fatal fulminant meningococcemia had readily detectable levels of soluble group-specific meningococcal antigen in their sera. Patients with meningococcal meningitis who recovered did not give a positive test for meningococcal antigen with the test described (counterimmunoelectrophoresis). The test is rapid, sensitive and specific for meningococcal group-specific antigens. Three patients had levels of 1.5 $\mu\text{g}/\text{ml}$ to 4.5 $\mu\text{g}/\text{ml}$ of meningococcal antigen in their acute-phase blood samples. Such levels may signal the onset of fulminant meningococcemia. An important relationship yet to be determined is the threshold level of circulating meningococcal antigen in the serum and the reversibility of the disease process. Preliminary observations indicate such a level may exist. The rapidity and specificity of the counterimmunoelectrophoresis test for meningococcal antigen should permit its use in studying spinal fluids, as well as serum, for rapid identification of the infecting organism. Eight spinal fluids have been examined to date for *Neisseria meningitidis* antigen. The results all agreed with the isolation and identification of meningococci by bacteriologic methods.

The laboratory diagnosis of meningococcal disease is generally accomplished by isolating the organism from the spinal fluid, blood, petechiae and/or from joint fluids. *Neisseria meningitidis* is identified by the Gram stain, agglutination reactions with grouping antisera and biochemical tests. Cultural procedures are time consuming and technical problems often result in failure to

isolate or identify the infecting organism. A rapid, reliable procedure which could be used to identify group-specific meningococcal antigen directly in the body fluids would greatly aid the diagnosis of meningococcal infections. Several investigators have demonstrated meningococcal antigens in the spinal fluid of patients with meningitis by using a standard precipitin technique (1-3). Because of the generally poor agreement between the results so obtained and clinical diagnosis, this method has not been accepted as a routine laboratory procedure.

This paper describes detectable circulating soluble group-specific meningococcal antigen in the sera of patients with acute meningococcal disease. To date, circulating antigen has been demonstrated only in those patients who expired soon after hospitalization.

MATERIALS AND METHODS

Since June 1967, approximately 10 ml of blood have been taken within 24 hr of hospital admission from all patients with meningococcal disease. For our study the sera were stored at -20°C until they were tested, the time of testing ranging

¹ From Naval Medical Research Unit Number 4 (NAMRU-4), Great Lakes, Illinois. This investigation was done in connection with Research Project M4305.01-1013B, Bureau of Medicine and Surgery, Navy Department, Washington, D. C. The opinions and assertions contained herein are those of the author and are not to be construed as official or as reflecting the views of the Navy Department or the Naval Service at large. 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.

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from 1 week to 2.5 years from the time the sera were collected. Sera from 23 patients were available for study, five of whom had expired shortly after admission to the hospital. The sera of two of these five patients were collected post-mortem. None of these men had received antibiotics before blood was collected. Twenty-two of the 23 cases were confirmed by isolation of meningococci from the spinal fluid. None of the spinal fluids were available for the present study.

Immunodiffusion studies. An Ouchterlony double-diffusion precipitin test was performed using regular microscope slides covered with 3 ml of a 1% agarose (Fisher Scientific Co., Pittsburgh, Pa.) in 0.1 M barbital buffer, pH 8.6 (LKB 3276-VB Stockholm). A circular pattern of wells around a central well was made in the agar, the center-to-center distance of the wells being 7 mm. Each well was 3 mm in diameter. The slides could be used immediately or stored in a moist chamber for several days and remain satisfactory for use. The central well was filled with rabbit meningococcal group C antisera (Difco Laboratories, Detroit, Mich., lot 538071) and the wells around the central well were filled with the sera from patients with meningococcal disease.

Sera were applied to the wells in the agar at least three times over a period of 1 hr, since a single application did not prove satisfactory. The multiple applications were accomplished by adding more reagent to the wells as the reagents diffused through the agar. This occurred more rapidly if the slides were at least 24-hr old. The slides were then incubated at room temperature in a moist chamber.

Counterimmunoelectrophoresis. Slides were covered with agarose as outlined above. Kodak projection slides were also made, as described by Gocke and Howe (4). Parallel rows of wells, 3 mm in diameter, were cut 1.5 to 3 mm apart. Serum samples for testing for meningococcal antigen were diluted 1:1 with 0.15 M NaCl and 10 μ l of each serum were placed in one row of wells and 16 μ l of antisera in the opposite row of wells. The slides were placed in a simple electrophoresis apparatus (Kallested Laboratories, Minneapolis, Minn.) with the wells containing antiserum near the anode side of the chamber. Electrophoresis was carried out at room temperature for 15 min using a constant current of 3 mA/microscope slide or 12 mA/Kodak slide. Strips of Whatman No. 1 filter paper were used for electrode wicks.

Sensitivity of counterimmunoelectrophoresis.

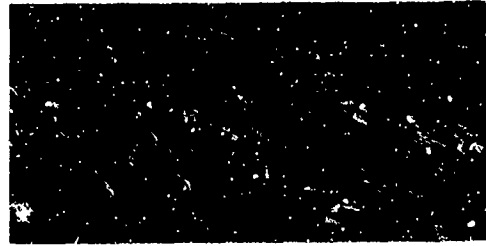


Figure 1. Demonstration of *Neisseria meningitidis* antigen in the acute sera of patients with meningococcal disease. The center wells contain rabbit meningococcal group C antisera. Each of the peripheral wells contains serum from a patient with meningococcal disease. The five positive precipitation bands occurred with the sera from patients with fulminant meningococemia who expired within 24 hr after hospitalization. Two sera were obtained post-mortem.

Twofold dilutions of group C meningococcal polysaccharide vaccine² were made and 10 μ l of each dilution were added to a row of wells cut on an aprotose-covered Kodak slide. Rabbit anti-meningococcal group C serum was added to the row of wells on the opposite side and electrophoresis carried out as described above. The highest dilution of the meningococcal antigen showing a precipitate which could be observed with the aid of a 3 X hand lens and a viewer (Kallested Laboratories), was considered to be the minimal concentration of this antigen that could be detected by this procedure.

RESULTS

Figure 1 shows that 5 of 23 sera tested had a level of meningococcal antigen that could be demonstrated by the Ouchterlony double-diffusion method. The five reacting sera were obtained from patients who expired rapidly (within 24 hr after admission). The 18 sera that failed to react were from patients who had recovered normally from their infection after receiving penicillin treatment. By close observation, precipitin lines were detectable within 6 hr, and were completely developed in 18 hr.

Figure 2 shows the pattern observed using counterimmunoelectrophoresis. This method is

² E. R. Squibb & Sons; meningococcal vaccine antigen prepared for Walter Reed Army Institute of Research, containing 100 μ g carbohydrate/ml, lot C, January 7, 1969.

more sensitive and rapid than the double-diffusion method, i.e., 15 min vs 4 to 6 hr (and longer depending upon the antigen concentration), and required only a single application of serum instead of at least three in the double-diffusion method. Five of the sera were from patients who had expired from 7 to 24 hr after admission with fulminant meningococemia. Two of these samples were obtained post-mortem. No difference could be seen in the precipitation of antigen from the serum collected before the patient expired and that collected post-mortem.

Figure 3 demonstrates the sensitivity of the counterimmunoelectrophoresis method. A level of 0.048 μg ml of polysaccharide could be detected by unaided visual observation. However, by using a 3 \times hand lens and a viewing box, this sensitivity could be increased at least fourfold.

Quantitative studies on sera from three patients who expired from fulminant meningococemia revealed levels of 1.5 to 4.5 μg ml of meningococcal antigen.

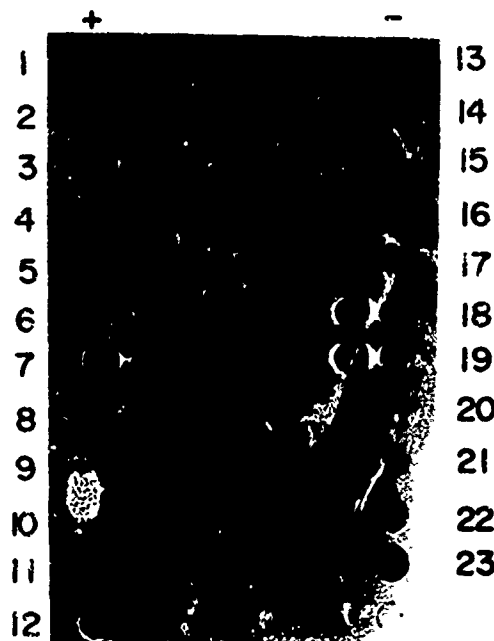


Figure 2. *Neisseria meningitidis* antigen in acute-phase sera demonstrated by counterimmunoelectrophoresis. Sera 2, 3 and 7 were from patients who expired within 24 hr after hospitalization. Sera 18 and 19 were obtained post-mortem. The remaining sera were from patients who recovered with routine therapy.

The specificity of the counterimmunoelectrophoresis method is shown in Figure 4. The *Neisseria meningitidis* A and B group antigens used were those described by Edwards and Driscoll

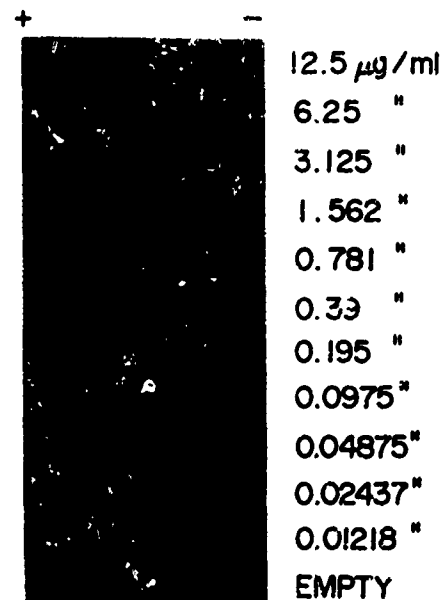


Figure 3. Sensitivity of counterimmunoelectrophoresis in detecting *Neisseria meningitidis* group-specific polysaccharide antigen. Each well contained 10 μl of reagent. Anode side contained *N. meningitidis* group C antiserum. Cathode side contained dilution of meningococcal polysaccharide antigen.



Figure 4. Specificity of counterimmunoelectrophoresis in identifying *Neisseria meningitidis* antigens. Left-hand wells in rows 1, 2, 3 and 4 contain rabbit anti-meningococcal grouping sera A, B, C and Y, respectively. Right-hand wells in columns I, II and III contain *N. meningitidis* groups A, B and C antigen, respectively.

(5). The *N. meningitidis* group C antigen was from an acute-phase blood sample taken from a patient who expired 9 hr later. As is seen, the test provides a rapid and specific method of detecting and identifying meningococcal antigen.

DISCUSSION

Vincent and Bellot (1) were among the first to demonstrate the precipitation of soluble meningococcal antigen from the spinal fluid of individuals with meningococcal meningitis. They performed a precipitin test with the supernatant of centrifuged spinal fluid and polyvalent horse meningococcal antiserum. This test required 6 to 12 hr before a final diagnosis could be made. Using a similar technique, Alexander (2) was able to relate the rapidity of the precipitin reaction to the prognosis in cases of meningitis. The more rapid the appearance of the precipitin (within 10 min), the greater the mortality rate. Rake (3) found a good correlation between the intensity of the precipitin reaction and the number of organisms present in the spinal fluid sediment.

Of the two techniques used here, counterimmunoelectrophoresis provided a more rapid and sensitive test for detecting meningococcal group-specific antigens. The detection of soluble antigen in the serum of patients in the acute phase of meningococcal disease is probably related to the concentration of organisms in the blood. The findings in this study, however, strongly suggest that if the serum from a patient with meningococ-

cal disease shows a precipitate under the conditions of testing described, the prognosis is poor. Three patients were found to have levels of 1.5 $\mu\text{g}/\text{ml}$ to 4.5 $\mu\text{g}/\text{ml}$ of meningococcal antigen in their acute-phase blood samples. These levels may signal the onset of fulminant meningococemia. An important relationship, yet to be determined, is the threshold level of circulating meningococcal antigen in the serum and the reversibility of the disease process. Preliminary observations indicate that such a level may exist (E. A. Edwards and P. Muehi, to be published).

The rapidity and specificity of the counterimmunoelectrophoresis test for meningococcal antigen should permit its use in studying spinal fluids, as well as serum, for rapid identification of the infecting organism. To date, eight spinal fluids have been so examined for *N. meningitidis* antigen. The results all agreed with the isolation and identification of meningococci by bacteriologic methods. However, the results using counterimmunoelectrophoresis were available within 30 min after the samples were received.

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