VENEZUELAN EQUINE ENCEPHALOMYELITIS IN MAN

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The virus of Venezuelan equine encephalomyelitis was isolated and identified in 1938 by Kubes and Rios (1) and by Beck and Wyckoff (2). Although the disease produced in horses by this virus is similar to that caused by the Eastern and Western equine encephalomyelitis viruses, neutralization as well as cross-protection tests have shown that the Venezuelan virus is immunologically different from the other types. Venezuelan equine encephalomyelitis in man may have been suspected previously (3) but it has not been described. The observations reported herein constitute the first evidence that the virus of Venezuelan equine encephalomyelitis may be the causative agent of an illness in man; they also bring to light one of the few instances in which an encephalitis-producing virus has been isolated from the nasopharynx of a patient and from human blood.

Clinical Data

Case 1.—A., female, aged 24 years, a laboratory assistant who for 2 months had been working with Venezuelan equine encephalomyelitis virus, became ill on Nov. 6, 1942. That morning she felt chilly and during the day developed malaise, headache, and a "tight" feeling in her chest. In the afternoon she was seen by a physician who found that her temperature was 103°F. Her pulse rate was 112, her respirations 22 per minute. Examination revealed no other evidence of disease. White blood cells numbered 8,400 per c.mm., of which 88 per cent were granulocytes.

On Nov. 7, when she was admitted to the Hospital of The Rockefeller Institute,

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the patient insisted that she felt well except for mild headache. Her temperature was 100.2°F. The pulse and respiratory rates were 96 and 22 respectively. She was alert, cooperative, and in no apparent distress. The nasal mucous membranes were congested and the pharynx was slightly inflamed. Small, non-tender, cervical lymph nodes were palpable bilaterally. No disturbances referable to the central nervous system or other abnormal physical findings were detected at this time or later. Roentgenograms of the chest revealed no abnormalities on this or on subsequent occasions. Cultures of blood remained sterile. The erythrocyte sedimentation was 9 mm. at the end of 1 hour (Wintrobe method). The hematocrit was 40 per cent. The leucocytes numbered 3,850 per c.mm. Cultures from the nose and throat yielded no organisms of pathogenic significance. From a specimen of defibrinated whole blood and from nasopharyngeal washings obtained at the time of admission, a virus was isolated the nature of which was subsequently investigated.

The patient's illness was brief and uneventful. Her temperature rose to 102°F. on the day of admission but returned to normal on Nov. 8. Her headache subsided and no other symptoms or signs of disease appeared. She returned to her home on Nov. 9, where she experienced no difficulty other than mild headaches each morning for several days. She resumed work on Nov. 13. Although a physical examination on that day revealed no abnormalities, roentgenograms suggested clouding of the frontal and ethmoid sinuses. Subsequently, however, she was symptom-free and on repeated visits to the hospital seemed entirely well. Additional specimens of blood were obtained for serological studies.

Case 2.—C., male, aged 31 years, who also had been working with Venezuelan equine encephalomyelitis virus for 2 months in the same laboratory with A., likewise became ill on Nov. 6, 1942. His chief complaints were drowsiness, chilly sensations, and fever. He found his temperature was 102°F. and went to bed. On Nov. 7 his temperature rose to 103.5°F. and his pulse rate averaged about 100 per minute. Drowsiness continued, unaccompanied, however, by headache or any other complaint. On Nov. 8 his temperature was still 101°F. and he remained in bed, although by this time he felt well. On Nov. 10 he became ambulatory and on the following day returned to work. He did not consult a physician during his illness and it was not until much later that a specimen of convalescent serum was obtained. Although no blood had been drawn during the febrile period of the patient's illness, a specimen of serum obtained 8 months previously was fortunately available. During the interval between this bleeding and the onset of illness on Nov. 6, the patient had enjoyed uninterrupted good health.

Isolation of a Filterable Agent from the Blood

The supernatant serum of defibrinated blood drawn from patient A. (Case 1) on Nov. 7 was inoculated into six Swiss mice by the intracerebral route. 3 days later three of the mice were found dead, and the other three appeared to be sick. The sick mice were killed, their brains removed aseptically, and made into a 10 per cent suspension in 10 per cent rabbit serum saline. This material, hereinafter referred to as mouse passage No. 1, was stored in the CO_2 ice box at -76°C. and was used for a second mouse passage by the intracerebral route.

When the mice of the second passage appeared to be sick on the 2nd day following

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inoculation, they were killed and their organs removed. Cultures of their brains, lungs, livers, spleens, and blood showed no bacterial growth. A 2 per cent suspension of brain tissue from these mice was prepared in 10 per cent rabbit serum saline and centrifuged at 2000 R.P.M. for 10 minutes. The supernate was then filtered through a Berkefeld N candle and the filtrate inoculated intracerebrally into six Swiss mice. 2 days later three of these mice were dead and the other three were sick. The sick animals were killed and their brains stored in the CO_2 ice box (passage No. 3—Berkefeld N-filtered).

Several mice were inoculated intracerebrally with the material designated as passage No. 1. These animals were killed when they appeared sick 2 days later. Their brains were removed and made into a 2 per cent emulsion in rabbit serum saline. This was centrifuged at 2000 R.P.M. for 10 minutes and then filtered through a Seitz pad. Some of this filtrate was inoculated intracerebrally into additional Swiss mice which became ill in 2 days and were killed. A 10 per cent emulsion was prepared with the brains of these mice in 10 per cent rabbit serum saline and stored in the CO_2 ice box as passage No. 3 (Seitz-filtered).

Essentially the same results have been consistently reproduced by intracerebral inoculation of Swiss mice, employing any of the materials designated as passage No. 1, passage No. 3 (Berkefeld N-filtered), and passage No. 3 (Seitz-filtered).

Isolation of a Filterable Agent from Nose and Throat Washings

Nose and throat washings in a menstruum consisting of equal parts of broth and physiological saline were collected from patient A. (Case 1). Part of this material was frozen and stored in the CO_2 ice box, a portion was cultured upon a blood agar plate, and 0.5 cc. was injected into a mouse by the intraperitoneal route. No colonies of pathogenic organisms were recognized in the bacterial flora which grew upon the blood agar plate. The mouse was found dead 2 days after injection; washings of the peritoneal cavity revealed no bacterial organisms in stained films and proved sterile on culture. The lungs of this animal were removed, made into a 2 per cent emulsion, and filtered through a Berkefeld N filter. The filtrate was inoculated intracerebrally into six Swiss mice which became ill and died within 3 days in a manner indistinguishable from that of the mouse inoculated with the strain of virus isolated from the blood of patient A. Subsequently both strains of virus were proved to be identical.

A filterable agent was thus isolated from the blood and from the nasopharynx of patient A., which was readily transmissible in and highly pathogenic for Swiss mice. The next problem was to establish its identity.

Identification of the Filterable Agent

As a preliminary step in the identification of the agent isolated from patient A., an investigation was undertaken to determine its degree of pathogenicity for available laboratory animals.

Two guinea pigs were inoculated intracerebrally with material from the previously described, Seitz-filtered, mouse passage No. 3. Each animal received 0.2 cc. of a 10^{-2}

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suspension; both were dead within 48 hours. A rabbit given 0.3 cc. of the same suspension intracerebrally likewise became sick and died within 48 hours. A second rabbit inoculated with the same preparation on the scarified cornea appeared sick during the morning of the 2nd day following inoculation and died that afternoon with no apparent local lesion in the cornea. In mice the agent was highly virulent and lethal, not only by the intracerebral route in dilutions of 10^{-9} and 10^{-10} but also on subcutaneous or intraperitoneal injection in dilutions of at least 10^{-4}.

The effect of this agent on experimental animals strongly suggested that it belonged in the equine encephalomyelitis group of viruses. Further identification of the agent isolated from patient A. was established by complement-fixation and neutralization tests. The complement-fixation tests were conducted according to a method previously described by Casals and Palacios (4).

In order to obtain an immune serum against the agent from patient A., a group of fifteen mice approximately 50 days of age were injected with formolized material from one of the early mouse brain passages. Each mouse received two injections of 0.5 cc. of a 10 per cent mouse brain suspension made inactive by the addition of 0.5 per cent formalin. 15 days later the mice were anesthetized with ether and bled from the heart. The blood specimens were pooled, and the serum, designated as *A.-immune mouse serum*, was stored in the CO_2 ice box. At the same time, complement-fixing antigens were prepared from both strains of virus isolated from patient A., employing the method described elsewhere (4).

To determine the identity of the two strains of virus isolated from patient A., complement-fixation tests were carried out in which both strains were tested with the homologous mouse serum described above, as well as with a number of sera obtained from animals which had been hyperimmunized against other neurotropic viruses. These included the viruses of Venezuelan equine encephalomyelitis, lymphocytic choriomeningitis, Russian spring-summer encephalitis, louping ill, Japanese B encephalitis, St. Louis encephalitis, West Nile encephalitis, and Eastern and Western equine encephalomyelitis. Similarly the homologous mouse serum described above was tested against antigens prepared from the two strains of A. virus and from the heterologous viruses specified. The results of these tests are given in Table I.

It is evident that antigens prepared from the laboratory strain of Venezuelan equine encephalomyelitis virus[1] and the two strains of virus isolated from patient A. cross-reacted with their respective sera and failed completely to react with immune sera prepared against other neurotropic viruses. Similarly, the immune sera prepared against the standard Venezuelan strain and

[1] The strain of Venezuelan equine encephalomyelitis virus used in this study was kindly sent to Dr. John R. Paul by Dr. Vladimir Kubes, Director, Institute of Veterinary Research, Caracas, Venezuela, and made available to us through the agency of the Commission on Neurotropic Virus Diseases of the United States Army.

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the strain of A. virus cross-reacted only with their respective antigens and failed to react with antigens prepared from other neurotropic viruses. These

TABLE I

Results of Complement-Fixation Tests with Hyperimmune Animal Sera and Neurotropic Virus Antigens

Hyperimmune serum against	Titers of sera against indicated antigens										
Virus	A. (from blood)	A. (from nose and throat)	V.E.E.	L.C. M.	Russian	Louping ill	Jap. B	St. Louis	W. Nile	W.E. E.	E.E.E.
Venezuelan E.E.	1/32	1/32	1/64	0			0	0		0	0
Patient A. (from blood)	1/4	1/8	1/16	0	0		0	0		0	0
L.C.M.	0	0		1/128							
Russian s-s encephalitis	0	0			1/64						
Louping ill	0	0				1/64					
Japanese B encephalitis	0	0					1/32				
St. Louis encephalitis	0	0						1/32			
West Nile encephalitis	0	0							1/128		
W.E.E.	0	0	0					0		1/128	0
E.E.E.	0	0	0					0		1/2	1/128

The hyperimmune sera were prepared by the immunization of mice in the case of all the viruses listed with the exception of Western and Eastern equine encephalomyelitis viruses for which specific sera were prepared in guinea pigs.

The virus antigens indicated include A. from blood and A. from nose and throat, strains of virus isolated respectively from the blood and nose and throat washings of patient A.; V.E.E. (Venezuelan equine encephalomyelitis); L.C.M. (lymphocytic choriomeningitis); Russian (Russian spring-summer encephalitis); louping ill; Jap. B. (Japanese B encephalitis); St. Louis (St. Louis encephalitis); W. Nile (West Nile encephalitis); W.E.E. (Western equine encephalomyelitis), and E.E.E. (Eastern equine encephalomyelitis).

The serum titers are given as a fraction representing the highest dilution of serum showing a 2+ or better fixation of complement. 0 indicates no fixation. Blank spaces represent combinations of sera and antigens that were not tested. The first tube in all tests contained serum undiluted or in a dilution of 1 to 2.

observations indicate that the two strains of virus derived from patient A. were indistinguishable by means of complement-fixation tests from the virus of Venezuelan equine encephalomyelitis.

Further evidence suggesting that both strains of the agent isolated from patient A. were, indeed, Venezuelan equine encephalomyelitis virus was provided by the results of complement-fixation tests with human sera.

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Blood specimens were obtained from patient A. after intervals of 1, 14, 40, and 68 days from the onset of illness. A sample of serum obtained 2 months prior to illness was also available.

As noted previously, patient C. escaped clinical attention until long after he had recovered. Fortunately a specimen of serum obtained 8 months before his illness had been preserved in the CO_2 ice box and hence was available for testing. Another specimen of serum from patient C. was taken 62 days after the onset of illness.

Three sera for control purposes were obtained from persons who gave no history of illness but who at one time or another had worked in the laboratory with the virus of Venezuelan equine encephalomyelitis.

TABLE II

Results of Complement-Fixation Tests with Human Sera and Neurotropic Virus Antigens

Serum		Titers of sera against indicated antigens						
Subject	Day of bleeding	Venezuelan E.E.	A. virus (from blood)	Japanese B	Russian encephalitis	Louping ill	L.C.M.	St. Louis encephalitis
Patient A.	Pre-infection: 2 mos.	0	0	0			0	
" "	Post-infection: 1 day	0	0	0			0	0
" "	" " 14 days	0	0	0			0	
" "	" " 40 "	1/8	1/2	0			0	
" "	" " 68 "	1/2	1/2			0	0	0
" C.	Pre-infection 8 mos.	0	0	0			0	0
" "	Post-infection 62 days	1/4	1/2	0	0		0	0
No. 3*		0	0	0		1/8	0	0
" 4*		0	0				0	0
" 5*		0	0				0	0

The serum titers are given as a fraction representing the highest dilution of serum showing a 2+ or better fixation of complement. 0 indicates no fixation; blank spaces represent combinations of sera and antigens that were not tested. The first tube in all tests contained undiluted serum.

* Control human serum.

The results of complement-fixation tests with these human sera are presented in Table II.

It is apparent that the sera collected from patient A. 2 months before as well as 1 and 14 days after the onset of illness failed to react with any of the virus antigens tested, including the homologous strain isolated from the blood of patient A. and the standard laboratory strain of Venezuelan equine encephalomyelitis virus. On the other hand, sera obtained from patient A. 40 and 68 days after the onset of illness did react positively but only with the antigens of the homologous A. and the standard Venezuelan strains of equine encephalomyelitis virus. The 40th day serum reacted in dilutions of 1/8 and 1/2 respectively against the standard and homologous virus antigens, whereas the 68th day serum reacted in a dilution of 1/2 against both.

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The serum obtained from patient C. 8 months prior to illness gave no reaction with any of the antigens tested, whereas serum obtained 62 days after the onset of illness reacted specifically with the standard Venezuelan and A. strain antigens in dilutions of 1/4 and 1/2 respectively. The control human sera Nos. 3, 4, and 5 were found to have no complement-fixing antibodies against any of the virus antigens tested with the exception of serum No. 3, which reacted in a dilution of 1/8 with louping ill virus. This result was expected, however, as serum No. 3 was derived from a person who had had a laboratory infection with louping ill virus in 1933 (5) and his blood serum was known to contain specific complement-fixing antibodies (4). The results of these complement-fixation tests with human sera further identify the agent isolated from patient A. as a strain of Venezuelan equine encephalomyelitis virus. Of still greater significance, the development of specific antibodies in the sera of patients A. and C. following an acute febrile illness strongly suggests that the virus isolated from patient A. was the causative agent of the disease in both instances.

Additional evidence was provided by the results of neutralization tests which showed that specific antibodies not only appeared in the sera of these patients following their febrile illnesses but also attained exceedingly high titer. Sera obtained from patient A. on the 1st and 40th day and from patient C. on the 62nd day after the onset of illness, as well as a control specimen (No. 5), were tested in mice for the presence of neutralizing antibodies against the standard strain of Venezuelan equine encephalomyelitis virus.

Technique of Neutralization Tests

48 hours after intracerebral inoculation with active Venezuelan virus, two mice were killed; their brains were removed, ground in a mortar, and emulsified in 10 per cent rabbit serum saline to a concentration of 10^{-1}. This emulsion was centrifuged at 2000 R.P.M. for 10 minutes. The supernate was drawn off and in the same diluent made into serial tenfold dilutions beginning with 1 to 50. These virus dilutions were then mixed with undiluted sera in equal volumes of 0.2 cc. each, making a series of final virus dilutions ranging from 10^{-3} to 10^{-10}. The serum-virus mixtures were incubated for 2 hours at 37°C. in a water bath and finally were inoculated intracerebrally into 3 weeks old Swiss mice, each mouse receiving an inoculum of 0.03 cc. Surviving mice were discarded after 21 days of observation.

The results of the neutralization tests are shown in Table III. The titer (50 per cent mortality end-point) of Venezuelan equine encephalomyelitis virus combined with normal, human control serum No. 5 was 10^{-10}. The serum from patient A. obtained 1 day after the onset of illness did not contain any neutralizing antibody against the virus since all mice inoculated with this virus-serum combination died, including those receiving the 10^{-9} virus dilution. On the other hand, the serum obtained from patient A. on the 40th day

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from the onset of illness contained sufficient antibody to reduce the titer of virus to $10^{-5.67}$, indicating a neutralization of more than 10,000 lethal doses. In a similar fashion, the serum obtained from patient C. on the 62nd day following the onset of his illness had sufficient antibody to reduce the titer of Venezuelan virus to $10^{-4.84}$—a neutralization of over 100,000 lethal doses. The high neutralizing antibody titers of the convalescent sera from the two patients are unequivocal, and are especially significant in the case of A. from whom strains of the virus were isolated and whose serum during the acute phase of illness contained no antibody whatever.

TABLE III

Results of Neutralization Tests with Venezuelan Equine Encephalomyelitis Virus and Human Sera

Serum		Fate of mice following intracerebral injection of serum-virus mixture*								Titer of virus‡	Neutralization index§
		Dilution of virus									
Subject	Day of bleeding	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}		
Patient A.	Post-infection: 1 day			4/4	4/4	4/4	4/4	4/4		$10^{-9.50}$ (or more)	<3
" "	" " 40 days	4/4	4/4	4/4	1/4	0/4	0/4			$10^{-5.67}$	21,380
" C.	" " 62 "	4/4	4/4	1/4	1/4	0/4	0/4			$10^{-4.84}$	144,600
No. 5 (control)				4/4	4/4	4/4	4/4	4/4	2/4	$10^{-10.0}$	1

* Fate of mice is indicated by a fraction, the denominator of which signifies the number of mice injected with each serum-virus combination and the numerator the number of mice which died.

‡ Titer of virus is given as the highest virus dilution giving a 50 per cent mortality.

§ Neutralization index is the ratio between the titer of virus obtained with each of the sera tested and the titer of virus found with the normal human control serum.

These data conclude the evidence to show that the agents isolated from the blood and from nasopharyngeal washings of a patient (A.) during an acute febrile illness were strains of Venezuelan equine encephalomyelitis virus and that this virus was very probably the causative agent not only of her disease but presumably also of the similar illness contracted simultaneously by her fellow laboratory worker (C.).

DISCUSSION

Although the occurrence of equine encephalomyelitis in man was suspected as early as 1931 (6), it was not until 1938 that the diagnosis was established independently by actual isolation of Eastern equine encephalomyelitis virus from the brains of fatal human cases by Fothergill, Dingle, Farber, and Connerley (7) and by Webster and Wright (8). Somewhat later, Howitt (9) reported

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that she had isolated the virus of Western equine encephalomyelitis from the brain of a fatal human case. In these instances, as in many subsequent reports, equine encephalomyelitis viruses isolated from human sources were usually derived from tissues of the central nervous system following death of the patient. In 1939, however, Fothergill, Holden, and Wyckoff (10) reported a fatal case of proved Western equine encephalomyelitis, whose spinal fluid obtained on the day before death produced paralysis and death when injected intracerebrally into young mice and guinea pigs. In the same year, Howitt (11) succeeded in isolating Western equine encephalomyelitis virus from the blood of a patient during life.

No evidence is available, however, concerning the occurrence of Venezuelan equine encephalomyelitis in man. Prior to this report, the virus had not been isolated from a human source. Furthermore, no data have been published to indicate whether disease due to this agent occurs in human beings or whether circulating antibodies are demonstrable in persons living in areas where the disease is prevalent in horses.

Certain implications of the observations herein presented seem worthy of additional comment. In the first place, the illness produced by Venezuelan equine encephalomyelitis virus in the two patients reported was brief and mild, not at all so severe as the disease occurring naturally in horses nor that produced experimentally in other animals. Moreover, it was strikingly less severe than in most of the reported clinical cases of human infection with other types of equine encephalomyelitis virus.

Finally, the concept that a virus, ordinarily considered to be primarily neurotropic and encephalopathic, can produce a mild form of generalized infection which nevertheless promotes an immune response in the human host is not new (12), but in the present instance (of patient A.) is strikingly exemplified. The fact that the virus was readily isolated not only from the patient's blood but also from the nasopharynx would seem to bear in a significant way upon the epidemiology of neurotropic virus diseases in general, and the equine encephalomyelitis group in particular.

SUMMARY AND CONCLUSION

A filterable agent was isolated from the blood and from washings of the upper respiratory passages of a young laboratory worker during a mild, acute, febrile illness. This agent was identified as a strain of Venezuelan equine encephalomyelitis virus. Circulating specific complement-fixing and neutralizing antibodies not present in sera withdrawn during the acute phase of illness were demonstrated in sera obtained during convalescence. A fellow laboratory worker who became similarly ill simultaneously also developed during convalescence specific circulating antibodies not present prior to illness.

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