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VIRUS MENINGO-ENCEPHALITIS IN SLOVENIA

3. Isolation of the Causative Agent

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SYNOPSIS

An organism was isolated from the blood of a patient clinically diagnosed as suffering from virus meningo-encephalitis; the organism causes illness and death in white mice. The antigen prepared from the brains of mice infected with this organism fixes complement with sera from typical cases of virus meningo-encephalitis. From its biological and serological characteristics, the isolated organism appears to belong to the group of neurotropic viruses and to be the causative agent of virus meningo-encephalitis in Slovenia.

A disease, which was clinically described as serous meningitis, has been observed for the past seven years or more in Slovenia, the north-western part of Yugoslavia.

The clinical and epidemiological features of this disease have been presented in the preceding papers (see pages 503 and 491). It is the purpose of this paper to report on the isolation of the etiological agent and to describe its characteristics.

Isolation of the Infectious Agent

From 1947 until 1953—during which time a clinical diagnosis of serous meningitis was frequently made in Slovenia—many attempts to isolate a bacterium were made. None of them was successful.

In the spring of 1953 we began virus isolation studies of the disease. In the course of these isolation studies 20 blood specimens, 6 spinal fluids, and 2 urine specimens from a total of 12 patients were inoculated into animals. The blood was inoculated as whole blood with heparin (in 8 cases), as a blood clot resuspended in saline (in 4 cases), and as serum (in 8 cases). An infectious agent belonging to the group of neurotropic viruses was isolated from the brain of one mouse which had been inoculated with blood drawn on the sixth day of disease from one of the patients (B. S.), suffering from what had been described as serous meningitis, and which we will henceforth refer to as virus meningo-encephalitis.

On 3 July 1953, the patient B. S. entered hospital with symptoms of virus meningo-encephalitis. On 6 July, six days after the onset of illness (while the patient was still in the first phase), whole blood was drawn. The blood was placed in a container kept at approximately -10^{o}C; 12 hours later, during which time the specimen was transported from Ljubljana to Zagreb, the specimen was thawed rapidly and the blood clot separated from the serum. The blood clot was ground up and resuspended in saline to its original volume. Each of five mice, 4-6 days old, was inoculated intracerebrally with 0.02 ml of this suspension and intraperitoneally with another 0.2 ml. Of these five mice two were later found dead, one on the ninth and the other on the nineteenth day after inoculation. Neither showed any signs of illness. These two mice could not be used for further passage. The third of the five mice disappeared on the seventeenth day after inoculation. On the nineteenth day after inoculation the fourth mouse showed agitation and tremor; the next day he had paralysis of the hind legs. The fifth mouse did not show any signs of illness during 30 days of observation. The paralysed mouse was autopsied on the day paralysis was observed. The brain was removed aseptically and kept at -40^{o}C. Cultures of the brain were made on blood agar and broth; both remained sterile. On the next day a 10% suspension in saline was made from one half of the brain and five more mice about 6 days old were inoculated in the same way as the first time. On the fifth day after inoculation two mice were found dead and three showed ataxia and tremor. These three mice were killed, their brains pooled, and a further passage was done as described above.

Behaviour of the Isolated Organism in White Mice

In all, 17 passages have so far been carried out in white mice. Baby mice as well as adult mice proved to be susceptible to the isolated organism. With baby mice the incubation period was shorter (3-4 days) than with adults (4-8 days). The mice proved to be as susceptible to the intracerebral-plus-intraperitoneal inoculation as to the intracerebral inoculation only. With the intraperitoneal inoculation alone, the incubation period proved

to be longer. The first signs of illness were usually hypersensitivity with ruffled fur, tremor, ataxia, convulsions, circling and running in circles, and then paralysis followed by death, which occurred 1-4 days after the first signs of illness. Occasionally mice died with paralysis after a period of hyposensitivity. Not all the symptoms described were found in every mouse inoculated; paralysis and death sometimes occurred without any previously noticeable signs. With increased passages we noticed a diminishing lethal effect of the virus in mice; fewer of them became sick and fewer died.

Mice are also susceptible to intranasal inoculation. By this route, however, the incubation period was longer and the mice usually died without any signs of illness.

The pathology in white mice will be reported in a separate paper by A. Zimolo and J. Vesenjak-Zmijanac.

Unsuccessful Isolation Attempts

Whole blood with heparin, serum alone, and cerebrospinal fluid were also collected from patient B. S. These three additional specimens were obtained at the same time as the whole-blood specimen from which the organism was isolated as described above. All three of these additional specimens were preserved in exactly the same manner as the whole-blood specimen and inoculated into baby mice in the same way. All three of these additional attempts at isolation were unsuccessful, i.e., none of the inoculated mice became sick. All three specimens, and the whole-blood specimen from which an organism was isolated, were inoculated into guinea-pigs; none of the animals became sick.

In addition to these unsuccessful attempts at isolation, 17 additional blood specimens from 11 patients, 5 spinal-fluid specimens, and 2 urine specimens were inoculated into both mice and guinea-pigs. None of the inoculated animals became ill.

As mentioned in our epidemiological observations (page 491), ticks are strongly suspected of being the reservoir in nature of the causative agent of the disease. For this reason, isolation of the causative agent from the tick *Ixodes ricinus*—the only species found so far in the endemic area—was attempted. Ticks were collected three times; the first time in two lots of 75 ticks each and the second time in three lots of 60 ticks each. The ticks were washed in ether and saline and ground in a mortar with saline. About 1.5 ml of saline was used for 100 ticks. After the material had been triturated it was centrifuged. Five or six 20-day-old mice were inoculated intracerebrally and intraperitoneally with each tick emulsion as described under the blood-clot isolation attempt. The mice were observed for 30 days, but no signs of illness could be detected. On a third occasion 339 ticks were collected and divided into three lots : 100 females, 87 males, and

152 nymphs. They were treated and inoculated according to the method employed by the Encephalitis Laboratory of the Hooper Foundation and described in that Foundation's mimeographed document, *Procedures for encephalitis virus isolation from field-collected mosquitos or mites.* The mice did not show any signs of illness during a 30-day period of observation.

The Isolated Agent as a Complement-Fixing Antigen

To prepare the antigen from the isolated infectious agent for the complement-fixation test, Casals's method [2] was used with one modification—for technical reasons the dipping of the mouse-brain solution was done in a deep freeze at −20°C instead of a dry ice and alcohol mixture. The first antigen was made from the brains of the third mouse passage. The antigen proved to be stable for about three months when kept at 4°C. With the same method an antigen from normal mouse brains was prepared. The antigen thus prepared was titrated in the complement-fixation test against the convalescent sera of two patients (B. S. and U. A.) with virus meningo-encephalitis. The antigen had a titre of 1/2 with B. S.'s serum and 1/3 with U. A.'s serum; hence in the test the antigen had to be used undiluted. The antigen from normal mouse brains showed no fixation of complement. The antigens prepared from later mouse passages had higher titres (1/4 or 1/6).

Serological Reactions

Sera from acute and convalescent cases of virus meningo-encephalitis were tested against the Slovenian strain antigen in the complement-fixation test. Casals's method,[2] with some modifications, was used. The sera were inactivated at 64°C for half an hour. The high inactivation temperature was used in order to get results which were more specific and less anticomplementary.[1] We started the test with the undiluted serum; the serum controls were also carried out with undiluted serum. A total of 419 sera from 272 typical cases were tested. First, only the convalescent serum from each patient was tested in a screen test at 1/2 dilution. If a positive result was obtained, then all the sera from the same patients—if more than one was available—were included simultaneously in the same test in order to demonstrate a rising titre. Most of the tests were repeated two or three times in order to control the reproducibility of the results. The results in 16 positive cases are shown in table I. For the first four cases the confirmatory neutralization-test results with louping-ill virus are included.[a] The fifth case is the patient from whom the infectious agent was isolated.

The remaining 11 cases all showed an eightfold or greater rise in titre. There were 66 more positive cases, either with a twofold to fourfold rise

[a] We are indebted to Dr. D.G. ff. Edward of the Wellcome Research Laboratories, Beckenham, Kent, England, for carrying out the neutralization tests.

TABLE I. RESULTS OF SEROLOGICAL TESTS ON CASES OF VIRUS MENINGO-ENCEPHALITIS

Case	Day of disease on which blood was drawn	Complement-fixation titre *	Neutralization index against louping-ill virus
U. I.	4	0	0
	29	1/8	no test
	41	no test	60
	60	1/16	no test
	89	1/8	no test
J. F.	20	1/8	600
	44	1/32 (NE)	600
	63	1/32 (NE)	no test
C. A.	20	1/4	20
	37	1/8	no test
	81	1/16	200
	100	1/8	no test
B. M.	5	anticomplementary	0
	19	1/2	no test
	47	1/2	100
	68	1/2	no test
B. S.	6	0	
	17	1/16	
	34	1/16	
	33	1/3	
L. F.	unknown	0	
	24 days interval	1/8	
	19 days interval	1/4	
U. A.	unknown	0	
	24 days interval	1/8	
	39 days interval	0	
G. M.	16	0	
	35	1/8	
	184	1/4	

*** Antigen prepared from virus isolated from patient B. S.**
0 = negative with undiluted serum
NE = no end-point

TABLE I. RESULTS OF SEROLOGICAL TESTS ON CASES OF VIRUS MENINGO-ENCEPHALITIS (continued)

Case	Day of disease on which blood was drawn	Complement-fixation titre *	Neutralization index against louping-ill virus
A. M.	1	0	
	33	anticomplementary	
	69	0	
	133	1/8	
B. A.	5	0	
	26	1/4 (NE)	
	58	1/8	
P. J.	18	0	
	29	1/2	
	44	1/4	
	53	1/8	
O. S.	19	0	
	31	1/4	
	38	1/16	
	72	1/16	
R. M.	10	0	
	21	1/8 (NE)	
	38	1/8	
	49	1/4	
	80	1/4	
	166	1/2	
C. J.	10	0	
	25	1/16	
	32	1/8	
J. J.	unknown	0	
	16 days interval	1/8	
	7 days interval	1/16	
	38 days interval	0	
S. T.	unknown	0	
	11 days interval	1/8	
	4 days interval	1/8	
	33 days interval	1/32	

*** Antigen prepared from virus isolated from patient B. S.**
0 = negative with undiluted serum
NE = no end-point

in titre or with only one positive serum specimen; these cases are not included in the table. The other 190 cases were negative when tested in 1/2 serum dilution. Some sera which were negative or of low titre at the inactivation temperature of 64°C gave relatively high titres when inactivated at a lower temperature. Studies to determine the optimum inactivation temperature are continuing. However, it seems that we are probably dealing with two diseases. Extensive comparative studies of complement-fixation and neutralization tests as well as of some other serological problems of virus meningo-encephalitis are planned.

A few sera from known cases of typhoid, paratyphoid, poliomyelitis, and mumps were included in the test and all gave negative results in undiluted serum.

The convalescent sera from guinea-pigs inoculated with various types of specimens from patients gave negative results.

Almost all the sera were tested for Widal and Weil-Felix reactions, agglutination with leptospirae, cold agglutinins, complement-fixing antibodies against influenza, lymphocytic choriomeningitis, Q fever, and epidemic typhus.[b] They all gave negative results.

Cross-Immunity Complement-Fixation Tests

Preliminary cross-immunity complement-fixation tests were carried out with the Slovenian (B. S.) strain antigen of virus meningo-encephalitis, the Graz strain antigen out of virus meningo-encephalitis found in Austria,[c] and other encephalitis antigens against different hyperimmune rabbit and other animal sera.[d] Casals's method was used as described earlier.[2] The results given in table II, while fragmentary, definitely classify the organism with the encephalitis group of viruses.

TABLE II. CROSS-IMMUNITY COMPLEMENT-FIXATION TESTS WITH VARIOUS STRAINS OF MENINGO-ENCEPHALITIS

Serum	Antigen					
	Slovenian	Graz	St. Louis	Jap. B	EEE	WEE
Slovenian	++	+	+	+	+	+
Graz	++	+	++	+	+	+
St. Louis	++	±	±	+	—	±
RSSE	+	—	+	—	+	+

— = negative at 1/2 ± = positive at 1/2 to 1/4 + = positive at 1/8 to 1/64
++ = positive at 1/128 or greater

b Widal, Weil-Felix, and leptospira-agglutination tests were carried out at the Microbiological Institute, Faculty of Medicine, University of Ljubljana.

c The strain was received by the courtesy of Dr. J. D. Verlinde, Nederlands Instituut voor Praeventieve Geneeskunde, Leyden, who isolated it from a case of similar virus meningo-encephalitis in Austria in 1953.

d Sera were gratefully received from Dr. J. D. Verlinde, Nederlands Instituut voor Praeventieve Geneeskunde, Leyden ; Dr. H. von Magnus, Statens Seruminstitut, Copenhagen ; and Dr. H. Cox, Viral and Rickettsial Research Division, Lederle Laboratories, New York.

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RÉSUMÉ

Dès 1947, maints essais ont été faits en vue d'isoler l'agent causal de la méningite survenue assez fréquemment en Slovénie durant ces dernières années et diagnostiquée actuellement comme méningo-encéphalite. C'est en 1953 que les auteurs sont parvenus à isoler le virus du cerveau d'une souris inoculée avec le sang d'un patient, prélevé au 6e jour de la phase prodromique de la maladie. Dix-sept passages sur souris blanche ont été effectués. Les souris adultes et les souriceaux sont sensibles aussi bien à l'inoculation intracérébrale qu'à l'inoculation intrapéritonéale. La période d'incubation a été de 4-8 jours chez les souris et de 3-4 jours chez les souriceaux. L'effet létal du virus pour la souris diminue à mesure qu'augmente le nombre des passages. Aucun autre isolement de l'agent causal n'a pu être réalisé. L'inoculation à la souris de broyats de tiques *(Ixodes ricinus)* supposées être porteuses de virus n'a donné aucun résultat positif.

L'antigène préparé à partir du cerveau de souris au 3e passage, utilisé dans la réaction de fixation du complément avec le sérum de malades sur lesquels l'agent causal avait été isolé, présentait un titre de 1:2 à 1:3. Des sérums prélevés sur des malades au stade aigu ou en convalescence ont été soumis au test de fixation du complément avec cet antigène. On a soumis à ce test 419 sérums provenant de 272 cas typiques. Une augmentation du titre au cours de la maladie a été constatée chez les patients sur lesquels plusieurs échantillons de sang avaient été prélevés. A la dilution 1:2, 190 sérums étaient négatifs. Certains sérums négatifs ou de faible titre qui avaient été inactivés à 64°C, ont donné de meilleurs résultats lorsqu'ils ont été inactivés à une température plus basse. La température optimum d'inactivation n'a pas encore été établie. Mais il semble, quoi qu'il en soit, que l'on ait affaire à deux maladies distinctes.

Les sérums des cas de méningo-encéphalite ont donné des résultats négatifs aux épreuves de Widal et de Weil-Felix, aux tests d'agglutination des leptospires, de fixation du complément avec l'agent causal de la grippe, de la chorio-méningite lymphocytaire, de la fièvre Q et du typhus épidémique.

Les tests d'immunisation croisée, par fixation du complément, avec diverses souches de virus méningo-encéphalitiques, permettent d'affirmer que la maladie en question appartient au groupe des méningo-encéphalites.

REFERENCES

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