IDENTIFICATION OF THE VIRUS OF LYMPHOCYTIC CHORIOMENINGITIS

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A relatively simple and rapid method of identifying the virus of lymphocytic choriomeningitis is of importance not only for special workers in the virus field but also for those called upon in a routine manner to isolate and identify etiological agents of human diseases. The availability of such a technique is especially desirable because this virus has been isolated from several species of animals used in experimental work, e.g., from white mice by Traub (1935, 1936), Findlay, Alcock and Stern (1936), Lépine and Sautter (1936), and Kasahara, Hamano and Yamada (1939), and from gray house mice by Armstrong and Sweet (1939) and Armstrong, Wallace and Ross (1940); from monkeys by Armstrong and Wooley (1935) and Coggeshall (1939); from guinea pigs by Kasahara and coworkers (1939); and from dogs by Dalldorf (1939) and Howitt (1939–40). Not infrequently the virus has been found in normal-appearing animals, and for that reason Andrewes (1939) has placed it in the group of "indigenous viruses." The capacity to produce an inapparent infection probably accounts for the relative ease with which the virus of lymphocytic choriomeningitis has been picked up as a contaminant during serial transmission of other viruses. For example, it has been encountered by Dalldorf (1939) during passage in dogs of the virus of canine distemper and by Casals-Ariet and Webster (1940) in serial tissue cultures of rabic virus. In the latter instance the workers believe that the contaminating agent was introduced by way of the monkey serum rather than the mouse embryo tissue used in the culture medium.

The classical technique for the identification of a virus, namely,

by means of cross-immunity and neutralization tests, is expensive and time-consuming. An abbreviated form of this method, which consists of inoculating the unknown agent into animals immune to known viruses, is available only to those workers who have on hand a constant supply of such immune animals. The complement fixation obtained with materials from animals with lymphocytic choriomeningitis (literature reviewed by Smadel and Wall (1940)) seemed to offer a satisfactory means for diagnosing this disease without recourse to more laborious techniques. The present report deals with the identification of the agent of lymphocytic choriomeningitis by the use of complement fixation; this is accomplished by the demonstration in infected animals of the specific soluble antigen associated with the disease and the subsequent development of anti-soluble-substance antibodies in animals that survive infection.

METHODS

Complement-fixation tests

Details of the technique of the test have been previously described (Smadel, Baird and Wall, 1939). Briefly, the procedure was as follows: 0.2 ml. of falling dilutions of the materials to be tested were mixed with equal amounts of hyperimmune serum (5 units) or of standardized antigenic solution (5 units), depending on whether antigen or antibody was being identified. Then 2 units of complement were added to each tube, and the mixtures were allowed to stand overnight at 3°C. The following morning 0.5 ml. of a 5 per cent suspension of washed sheep erythrocytes and 0.2 ml. of anti-sheep-cell amboceptor (2 units of hemolysin) were added to each tube. A final reading of the amount of hemolysis was made after the tubes had been in a water-bath at 37°C. for 30 minutes. Suitable anticomplementary and hemolytic controls, as well as one with a hyperimmune guinea pig serum of known titer, were included in each set of tests.

Antigen

Stock solutions of complement-fixing antigen were prepared according to the routine used in this laboratory (Smadel, Baird and Wall, 1939). A 10 per cent suspension prepared from 5 to

30 spleens taken from guinea pigs infected with the W.E. strain of virus was freed of large particles of tissue by ordinary centrifugation; after the supernatant fluid was spun in the ultracentrifuge (Bauer and Pickels, 1936) at 30,000 R.P.M. for 20 minutes, it was filtered through a Seitz pad. Then merthiolate[1] was added to a concentration of 1:10,000 in order to maintain bacteriological sterility during storage in the cold for periods of time up to 6 months. Antigenic solutions prepared in such a manner contained little or no virus and as a rule had a complement-fixing titer of 1:16 in the presence of hyperimmune guinea pig serum. At times when new or unidentified strains of viruses were being studied extracts were prepared from a single infected guinea pig spleen; when such small amounts of material were used Seitz filtration and in some instances ultracentrifugation were omitted. Ten per cent suspensions of infected spleens which had been cleared of gross particles by means of centrifugation at 3000 R.P.M. for 20 minutes were rarely anticomplementary and were employed successfully in the work reported in this communication. Such crude fresh preparations have a limited use, however, as has been pointed out elsewhere (Smadel, Wall and Baird, 1940).

Antiserum

For the detection of antibodies in guinea pigs convalescent from infection with a particular strain of virus, serum was obtained by cardiac puncture while the animals were under ether anesthesia. Properly diluted hyperimmune serum from either guinea pigs or from mice was used in tests for the presence of soluble antigen in splenic extracts. It has recently been found that the injection of formolized soluble antigen into resting hyperimmune guinea pigs provides the simplest means so far available for obtaining complement-fixing serum of consistently high titer (Smadel and Wall, 1940).

RESULTS

It has previously been demonstrated by means of complement fixation that animals infected with any one of the 4 strains

[1] Product of Eli Lilly and Company.

of lymphocytic choriomeningitis virus maintained in this laboratory possess in their tissues a soluble antigen which is separable from the virus and is specific for the disease; moreover, no evidence of type specificity was encountered in the antigens associated with these strains (Smadel, Baird and Wall, 1939). During the last 1½ years, we have examined by means of complement fixation materials from animals infected with 9 additional strains of the virus. In each instance the soluble antigen was detected in the tissues of infected animals. Furthermore, antibodies for the soluble antigen were manifest in the serum of surviving guinea pigs. Identification of the virus was thus established in studies on animals of the first passage; results obtained by complement fixation were verified by cross-immunity tests. The classical method was used with several of the 9 strains, but with the remainder it seemed sufficient to show that guinea pigs or mice known to be immune to the W. E. strain of virus were not susceptible to infection with the agent in question.

The technique of rapid identification of the virus of lymphocytic choriomeningitis is illustrated by a summary of the work on one typical strain, S.M. Portions of a bacteriologically sterile mixture of cerebrospinal fluid and blood, taken 6 days after onset of illness from S.M. who was suspected of having lymphocytic choriomeningitis, were inoculated by intracerebral, intraperitoneal and subcutaneous routes into 2 guinea pigs (G,1-1 and G,1-2) and intracerebrally into 6 mice. Both guinea pigs developed fever on the 4th day after inoculation. On the 5th day one of the animals (G,1-1) was sacrificed by bleeding from the heart while under ether anesthesia, and 1 ml. of its heparinized blood was immediately inoculated subcutaneously into each of 2 normal guinea pigs (G,2-1 and G,2-2) and into a guinea pig (G,2–3) known to be immune to lymphocytic choriomeningitis. The brain of G,1-1 was removed aseptically and stored in the cold as a source of this strain of virus. A 1:4 dilution of an extract prepared from the spleen of this animal fixed complement in the presence of a known hyperimmune serum. The other guinea pig of the first passage, G,1-2, had fever for 8 days during which time it appeared ill and lost weight, but by the end of 2

weeks it had begun to improve. Serum taken from this animal 3 weeks after inoculation contained anti-soluble-substance antibodies in a titer of 1:16. Finally, this pig was subsequently found to be immune to a test dose[2] of the virus of lymphocytic choriomeningitis. The 2 guinea pigs (G,2-1 and G,2-2) inoculated with heparinized blood from the first passage animals died on the 16th and 17th days, respectively, after exhibiting typical signs of the disease in this host, i.e., fever, conjunctivitis, salivation, somnolence, loss of weight, labored breathing and prostration. The hyperimmune pig, G,2-3, which had been inoculated with a portion of the same infectious blood remained well. All of the 6 mice which received part of the mixture of cerebrospinal fluid and blood from S.M. died on the 6th day after inoculation, and a 1:16 dilution of an extract of their pooled spleens fixed complement in the presence of hyperimmune mouse serum.

In the manner described above, the specific soluble antigen of lymphocytic choriomeningitis was detected in guinea pigs and in mice on the 5th and 6th days, respectively, after inoculation with material from the acutely ill patient, S.M. The nature of the infectious agent was thus established before the patient was discharged from the hospital and approximately 2 weeks before complement-fixing antibodies for the soluble antigen of lymphocytic choriomeningitis were detectable either in the patient's serum (Smadel and Wall, 1940) or in the serum of a surviving guinea pig of the first passage. Identification of the agent by cross-immunity tests was comparatively rapid in this instance; nevertheless, data from these tests were not available for tentative classification until 2 weeks after receipt of material and were not complete until a month later.

Eight additional infectious agents were shown to be strains of lymphocytic choriomeningitis virus by the demonstration of

[2] A test dose of virus is prepared as follows: A mixture of equal parts of normal guinea pig serum and of a 10^{-3} dilution of guinea pig brain infected with the W.E. strain of virus is held at 37°C. for 1 hour. Then 0.5 ml. of the mixture is inoculated subcutaneously into a guinea pig or 0.03 ml. is given intracerebrally into a mouse; such an inoculum contains from 100 to 1000 M.L.D. of virus. Animals receiving the infectious material are observed for 18 days and their temperatures are recorded daily during this period.

the presence of the soluble antigen specific for this agent in extracts of spleens prepared from febrile guinea pigs which had

TABLE 1

Demonstration of the presence of soluble antigen and anti-soluble-substance antibodies in guinea pigs infected with strains of lymphocytic choriomeningitis virus

STRAIN	ORIGINAL MATERIAL INJECTED INTO GUINEA PIGS	PRESENCE OF SOLUBLE ANTIGEN IN SPLEENS OF INFECTED ANIMALS	DAY OF SPLENIC HARVEST, 1ST PASSAGE	DEVELOPMENT OF C-F ANTIBODIES IN SERUM OF RECOVERED ANIMALS	CROSS-IMMUNITY WITH W.E. STRAIN OF VIRUS
S.M. (1)	Human spinal fluid and blood	+	5th	+	+
K.P. (2)	Human spinal fluid and blood	+	9th	+	+
M-2 (3)	Guinea pig brain	+	7th	+	+
A.S. (4)	Mouse brain	+	8th	+	+
McC. (5)	Human spinal fluid	+	4th	+	+
J.P. (6)	Guinea pig brain	+	6th	−*	+
J.L. (7)	Human spinal fluid	+	11th	+	+
S. (8)	Mouse brain	+	8th	+	+
II S.F. (9)	Mouse brain	+	12th	+	+

* Animals died before antibodies developed.

Strains isolated from human beings with lymphocytic choriomeningitis:

(1) S.M., patient of Dr. J. Schneider, Lincoln Hospital, New York.

(2) K.P., patient of Dr. J. S. Vanneman, Princeton Hospital, Princeton, New Jersey.

(3) M-2, isolated from patient and identified by Dr. H. L. Hodes, Sydenham Hospital, Baltimore, Maryland.

(4) A.S., isolated from patient by Dr. G. O. Broun, Firmin Desloge Hospital, St. Louis, Missouri.

Strains isolated from animals inoculated with human material:

(5) McC., patient in Rockefeller Hospital, New York.

(6) J.P., isolated by Dr. S. O. Levinson from a guinea pig inoculated with spinal fluid from a suspected case of lymphocytic choriomeningitis, Samuel Deutsch Convalescent Serum Center, Michael Reese Hospital, Chicago, Illinois.

(7) J.L., patient of Dr. Donald D. Parker, Presbyterian Hospital, New York.

Strains isolated from apparently normal animals:

(8) S., isolated from a "normal" mouse in the laboratories of the International Health Division of The Rockefeller Foundation.

(9) II S.F., isolated during serial tissue culture of rabic virus, probably from monkey serum (Casals-Ariet and Webster, 1940).

been inoculated with the original materials received in our laboratory. Moreover, the spleens of mice which received 3 of the

strains were found to contain the soluble antigen. In addition, anti-soluble-substance antibodies were demonstrable in animals that recovered from infection produced by 8 of the 9 strains of virus; animals inoculated with strain J.P. invariably died before antibodies developed. In each instance subsequent cross-immunity tests confirmed the identification based on data derived from *in vitro* reactions. Results obtained in studies of the 9 strains of virus are summarized in table 1.

The 9 strains of virus investigated in this work varied considerably in their pathogenicity for guinea pigs. Three of them, J.P., McC. and S., were so highly virulent that the survival of an animal, even when inoculated with only a few infective doses, occurred rarely or not at all. On the other hand, strain II S.F. induced such a moderate disease in guinea pigs that they displayed only a slight fever for a few days. Inasmuch as the amount of antigen is small (titer not above 1:2) in spleens of guinea pigs infected with a mild strain such as W.W.S. (Smadel, Baird and Wall, 1939) or II S.F., the interpretation of results of the complement-fixation test may be difficult. In such instances, identification of the virus rests on the detection of anti-soluble-substance antibodies in the serum of convalescent animals, or on some other type of procedure. On the other hand, the titer of soluble antigen in extracts prepared from spleens infected with a highly virulent strain, e.g., W.E. (Smadel *et. al.*, 1939) or J.P., is sufficiently high (1:16 to 1:32) for the result to be of diagnostic value.

DISCUSSION

The identification of the virus of lymphocytic choriomeningitis by means of complement fixation is an adequate, simple and inexpensive procedure. Solutions of specific soluble antigen and complement-fixing antiserum for use in the test may be kept for several months at ordinary ice box temperatures without appreciable loss of activity. Moreover, both materials when dried *in vacuo* from the frozen state and stored in the ice box remain active for longer periods of time.

The data presented in this report deal chiefly with the identi-

fication of the virus of lymphocytic choriomeningitis rather than with the significance that may be attached to the recovery of the virus from animals inoculated with supposedly infectious material. It should be emphasized that isolation of the virus from one of a number of animals inoculated with material from human beings does not constitute proof that this virus is the etiological agent of the human disease. The frequency with which apparently normal animals from certain stocks develop clinical manifestations of infection with the virus of lymphocytic choriomeningitis after intracerebral injection of sterile material (Traub, 1935, 1936, and Findlay and coworkers, 1936) should make one attach little significance to the illness of a single inoculated animal. The simultaneous illness of a number of the inoculated animals and the development of immunity in most of those surviving infection contribute to the possibility that the virus in question was isolated from the patient and not encountered fortuitously in the experimental animals. The opinion held by workers in this laboratory, which has been expressed elsewhere (Scott and Rivers, 1936), is that proof that a given human illness is caused by the virus of lymphocytic choriomeningitis rests ultimately on the demonstration of the development of antibodies in the serum of the patient. Thus, examination should show that a specimen of serum taken at the onset of the disease does not contain complement-fixing or neutralizing antibodies and that these antibodies appear in the patient's blood during convalescence. A strict adherence to these criteria may occasionally result in a falsely negative diagnosis, since MacCallum and Findlay (1939) and Howard (1939, 1940) have reported that the appearance of neutralizing antibodies in human beings may be delayed beyond the usual time of appearance—6 to 8 weeks—or fail entirely to develop. It should be pointed out that 2 strains of virus identified in our study, i.e., McC. and J.L., were recovered from 1 out of 6 and 1 out of 8 animals, respectively, inoculated with material from patients who developed neither complement-fixing nor neutralizing antibodies during convalescence; furthermore, none of the remaining animals became immune to the virus. Consequently these strains of virus were not regarded as

the cause of disease in these two individuals. Their origin is uncertain but two possible sources are obvious. In the first place, guinea pigs from our Institute-bred stock which is known to be free from lymphocytic choriomeningitis are occasionally not available; consequently, animals from commercial sources received material from one of these two patients. In the second place, the manipulation and trauma incident to the taking of daily rectal temperature in a large group of guinea pigs, some of which are suffering from experimentally induced lymphocytic choriomeningitis, offer a means of cross-infection. The foregoing experience emphasizes the importance of employing animals from stocks known to be free from infection with the virus of lymphocytic choriomeningitis and of following strict precautions of isolation in the housing and care of experimental animals.

SUMMARY

Nine strains of the virus of lymphocytic choriomeningitis were identified by means of complement fixation. The soluble antigen associated with the disease and responsible for fixation was detected in the spleens of infected guinea pigs and mice. In addition, the development of anti-soluble-substance antibodies was demonstrated in guinea pigs surviving infection with the agents. The significance which may be attached to the isolation of the virus from animals inoculated with human material is discussed.

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