THE TECHNIQUE AND PRACTICAL APPLICATIONS OF THE COMPLEMENT-FIXATION TEST FOR DIAGNOSIS OF INFECTION WITH ENCEPHALITIS VIRUSES[1]

J. CASALS

Rockefeller Institute for Medical Research, New York, N. Y.

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Some questions have arisen about the practicability of the complement-fixation test in the diagnosis of human encephalitides. These questions refer mainly to the following points:

Laboratory procedures. (1) Inactivation and preservation of antigens; use of irradiated and lyophilized material. (2) Production of animal hyperimmune sera for standardization of antigens. (3) Existence of cross reactions within certain groups of viruses, especially the equine encephalomyelitis group.

Practical applications. Use of the complement-fixation test with human sera, especially the detection of false positive and false negative reactions.

The methods for the preparation of antigens and hyperimmune sera as well as the technique of the test have been described in detail (Casals and Palacios, 1941; Casals, 1941, 1943). The only modification thus far introduced in the preparation of antigens is that centrifugation at 6,000 rpm in the Pickels (1942) centrifuge substitutes for the original method, centrifugation at 3,500 rpm in the Swedish angle centrifuge.

Use of Irradiated and Lyophilized Antigens. Inactivation and Preservation of Antigens

A Western equine encephalomyelitis (W.E.E.) antigen was irradiated by ultraviolet light and lyophilized in November, 1942. This antigen was kept at 4 C in 5-ml amounts in sealed ampules. In September, 1944, after about 2 years, several of these ampules were opened; the dried material was easily resuspended in distilled water and tested. Tests included determination of (a) the anticomplementary power, (b) the specificity, (c) the titer of hyperimmune sera in the presence of this antigen, and (d) the antigenicity. All these properties were compared with those of a freshly prepared W.E.E. antigen, with the result that the lyophilized antigen had the same properties as a freshly prepared one (table 1). W.E.E. antigen is so far the only one tested after such long storage. The results of our experience with other lyophilized antigens, Eastern equine encephalomyelitis (E.E.E.), St. Louis encephalitis, and West Nile virus used within 2 or 3 months of preparation, were equally favorable.

Animal Hyperimmune Sera

The general procedures used were, first, to inject formalin-inactivated virus intraperitoneally for several doses, then active virus by the same route.

[1] Work done under the Commission on Neurotropic Virus Diseases of the Board for the Investigation and Control of Influenza and other Epidemic Diseases in the Army, in the Preventive Medicine Service of the Office of the Surgeon General, United States Army.

Since guinea pigs are highly susceptible to Venezuelan equine encephalomyelitis (V.E.E.), E.E.E., and W.E.E. viruses, they are used in preference to other animals. Guinea pig sera are inactivated at 56 C for 20 minutes. Now and again cross reactions between E.E.E. and W.E.E. occur in 1:2 and 1:4 dilutions of serum, but such reactions arise in these dilutions (generally more often in a dilution of 1:2) with other antigens also, and are therefore likely to be nonspecific in nature. This will be discussed later.

St. Louis virus is pathogenic for mice and hamsters; consequently these two species are used for the production of hyperimmune serum. Our experience with St. Louis virus, hamster-immune serum is limited; we have, however, prepared hyperimmune sera which, following inactivation at 65 C for 20 minutes (the serum is heated at this temperature following dilution in order to prevent

TABLE 1

Comparison of lyophilized, irradiated antigen with freshly prepared antigen

ANTIGEN	ANTICOMPLEMENTARY POWER (AMOUNT OF GUINEA PIG SERUM 1:30 EQUAL TO 1 UNIT)	SPECIFICITY AND SERUM TITER												ANTIGENICITY				
		W.E.E.-immune guinea pig serum							E.E.E.-immune guinea pig serum							Titer of antigen Constant amount of serum				
		1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/4	1/8	1/16	1/32	1/64	1/128		1/1	1/2	1/4	1/8	1/16
	ml.																			
W.E.E. lyophilized, 1942.....	0.13	4	4	4	4	4	2	0	0	0	0	0	0			4	4	4	0	0
W.E.E. fresh, 1944.....	0.13	4	4	4	4	±	0	0	0	0	0	0	0			4	4	4	±	0
E.E.E. fresh, 1944.....	0.13	0	0	0	0	0	0		4	4	4	3	0	0		0	0	0	0	0
No antigen, saline.....	0.16	0							0							0				

4 = no hemolysis; ± = almost complete hemolysis; 0 = complete hemolysis.

coagulation), had titers of 1:64 to 1:128. Here also a partial nonspecific fixation of the complement occurs at times with the dilutions 1:2 and 1:4.

Lymphocytic choriomeningitis (L.C.M.) hyperimmune sera have been obtained in mice only. Our strain of L.C.M. virus, the IISF strain, is pathogenic for this species and not for others. Not at all times have we been able to obtain a complement-fixation reaction with this strain, owing to defective antigen or serum; it would therefore be advisable to investigate other strains of L.C.M. virus.[2]

Havens *et al.* (1943) found a definite crossing in the complement-fixation test between E.E.E. and W.E.E. viruses. On the other hand, we had not been able heretofore to find such definite crossing although we had not investigated the problem systematically. An experiment was undertaken in order to study this question, as well as to find out (a) whether any differences existed between two strains of W.E.E. virus, namely, the Rockefeller Institute (R.I.) strain and the

[2] Mouse sera contain a natural anticomplementary substance; heating at 60 C for 20 minutes will not only inactivate such sera but will also remove the anticomplementary factor.

McMillan strain, and (b) what relationship, if any, V.E.E. virus had to W.E.E. and E.E.E. viruses.[3]

Seventeen guinea pigs were placed in four groups, each having 4 or 5 animals. All the animals were vaccinated with V.E.E., W.E.E. Rockefeller Institute strain, W.E.E. McMillan strain, and E.E.E. viruses, respectively. The guinea pigs were bled on the following days after the first injection: 26th, 40th, 55th, and 95th. All sera from each bleeding were tested simultaneously against the antigens of W.E.E., E.E.E., V.E.E., and St. Louis encephalitis viruses.

The conclusions reached were that by complement-fixation test with guinea pig hyperimmune sera: (1) V.E.E. virus is not related to either E.E.E. or W.E.E. viruses. (2) Two strains of W.E.E. differ in their capacity to develop the complement-fixing antibody, i.e., the R.I. strain is superior to the McMillan. (3) The relationship between E.E.E. and W.E.E. is a doubtful one. Cross reactions which are repeatable rarely occur in 1:2 and 1:4 dilutions; more often they arise in the same dilutions as nonspecific partial reactions, since in that zone other antigens as well are reactive with the same sera (see the next section).

Use of the Complement-Fixation Test as Applied to Human Sera. False Positive and False Negative Results

For practical use, only avirulent lyophilized antigens should be considered. As already stated, such antigens can be prepared.

Two important questions concerning the complement-fixation test are (a) whether sera which are positive by neutralization test fail at times to give a positive complement-fixation, and (b) whether in the latter test false positive reactions occur.

In order to eliminate false positive, as well as anticomplementary, reactions, (a) sera should be collected and prepared for test in the accepted manner. If sera cannot be tested within a few days, they must be kept frozen in a dry-ice refrigerator at −76 C. (b) Sera must be inactivated at 60 C for 20 minutes. (c) When performing the test, a serum should not be tested aginst one single antigen, but against several antigens simultaneously (W.E.E., E.E.E., L.C.M., St. Louis, and others as indicated); and a serum control with no antigen, for anticomplementary power, should be included. Only when there is a clear-cut reaction with one antigen and none with the others can the result be called positive. Reactions with undiluted serum or even with serum in dilution 1:2 must be considered as doubtful, especially if incomplete, since they only acquire significance when found repeatedly.

If a nonspecific reaction takes place, it can often be eliminated by inactivating the serum at 65 C for 20 minutes instead of 60 C. This procedure reduces the titer of the specific antibody to some degree, but not enough to extinguish it entirely.

As for negative complement fixation with human sera that are significantly

[3] The R.I. strain corresponds with Howitt's original California virus; the McMillan was isolated from the CNS of a person who succumbed to W.E.E. virus infection during the 1941 Winnipeg epidemic.

positive by neutralization test, it is our experience as well as that of others that this is to be expected under certain conditions. Thus following vaccination with either W.E.E. or St. Louis viruses, human sera show few or no antibodies of the complement-fixing type, while neutralizing antibodies are present (Casals, 1944). In sera from patients with lymphocytic choriomeningitis, complement-fixing antibodies are present early, at a time when neutralizing antibodies are not found; on the other hand, complement-fixing antibodies disappear within a few weeks whereas neutralizing antibodies persist for several years (Smadel *et al.*, 1939). In sera from patients infected with W.E.E., virus-neutralizing antibodies are found earlier than complement-fixing antibodies (Howitt, 1943), and it is our experience that they remain for a longer time. With St. Louis encephalitis virus, Howitt (1943) found that complement-fixing antibodies appeared earlier than neutralizing antibodies, but the determination of the latter was a more reliable method for sera obtained at a long period after the onset.

In the past 2½ years we have received over 500 sera labeled with clinical diagnoses of encephalitis. Only an occasional one reached our laboratory in a condition suitable for a complement-fixation test. With the exception of about 10, all these sera that could be tested have given negative results. It should be recalled that the number of proved human cases of encephalitis or central nervous system infections caused by St. Louis, W.E.E., E.E.E., and L.C.M. viruses has been low in this country.

CONCLUSIONS AND RECOMMENDATIONS

Avirulent lyophilized antigens that retain their properties, specificity, antigenicity, and lack of anticomplementary power, for at least two years can be prepared with W.E.E. virus.

Animal hyperimmune sera can be obtained in guinea pigs for W.E.E., E.E.E., and V.E.E viruses, and in mice for St. Louis and L.C.M. viruses. These sera, when properly prepared and tested, have specific titers from 1:32 to 1:256 or higher. When used undiluted or in dilution 1:2, they may give a nonspecific reaction; therefore, it is advisable to use them in dilution 1:4 or higher.

Human sera for complement-fixation tests must be properly collected and prepared for testing. The patient should be bled after a period of several hours of fasting; the blood should be drawn under sterile conditions, not allowed to hemolyze, and should be centrifuged a half to one hour later. Sera should be tested as soon as possible, being kept at 4 C until the test is performed. Whenever sera cannot be tested within a few days, they should be frozen and kept in a dry-ice refrigerator. Sera should be inactivated at 60 C for 20 minutes.

Sera should be tested simultaneously against several antigens, and, in addition, for their anticomplementary effect. At present, the antigens of W.E.E., E.E.E., L.C.M., and St. Louis viruses should be used as a routine practice. If the serum is anticomplementary or if a nonspecific reaction occurs, the test should be repeated with serum inactivated at 65 C for 20 minutes. If this procedure does not eliminate the nonspecific reaction, the serum must be classified as unfit for the test.

It is advisable to start the dilutions of human serum at 1:2. Even at this dilution, specific partial fixation of the complement is of little significance unless it is obtained on repetition.

Specific fixation of the complement is not found in certain instances when the neutralizing antibody is present. This occurs in sera following vaccination with W.E.E. and St. Louis viruses, and in sera obtained too long after convalescence from certain types of encephalitis (W.E.E., St. Louis, and L.C.M.).

Our experience with a large number of sera sent us by physicians from casual cases having a clinical diagnosis of encephalitis shows that a positive complement fixation is found only in rare instances. On the other hand, positive reactions have been found in a large percentage of cases in definite epidemics of Eastern and Western equine encephalitis.

A comparison of the complement-fixing and the neutralizing antibodies in human sera from acute cases, tested against several viruses for each serum, should be first undertaken in order to establish the indication of the complement-fixation test as a routine procedure in any particular epidemic.

Since the number of technical variables involved is a large one, the test should be performed by trained, experienced persons, preferably those who have access to standardized antigens and positive sera.

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