A Simplified Method for Preparing Eastern and Western Equine Encephalomyelitis Complement Fixing Antigens. (32206)

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(Introduced by J. S. Finlayson)
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Suspensions of virus infected central nervous system (CNS) tissue are used as antigens in complement fixation (CF) tests. These suspensions may be anticomplementary (AC) unless treated to remove AC activity. Methods(1-4) developed for removal of AC activity result in decreased CF antigenic potency and sensitivity.

A procedure used for preparation of high titered CF antigens is described by Casals (5). Excellent hemagglutinating and CF antigens are prepared with this technique. However, the procedure is time-consuming, complex and necessitates the manipulation of highly infectious virus powder.

This paper describes a simplified method for preparing Western equine encephalomyelitis (WEE) and Eastern equine encephalomyelitis (EEE) CF antigens from borate stock buffer (BSB) suspensions of infective mouse brain. Ethylene oxide vapor is used to inactivate infective antigens. A modified CF test is presented using these antigens, and a comparison of antigenic potency is made with that of commercial products.

Materials and methods. Borate standard. Dissolve 6.202 g of H_3BO_3 (C.P.) and 7.456 g of KCl in 500 ml of distilled water. *Borate stock buffer* (BSB) pH 9.0—50 ml of borate standard, 27 ml of 0.2 M NaOH and 563 ml of 0.9% NaCl. The BSB is used for CF antigen preparation. *Veronal buffered saline* (VBS)—*Stock solution.* A. Dissolve 83.8 g NaCl; 2.52 g of $NaHCO_3$ and 3.0 g sodium barbital (Na 5,5-diethylbarbiturate) in approximately 1000 ml of distilled water. B. Dissolve 4.6 g barbital (5,5-diethylbarbituric acid), 1.0 g $MgCl_2 \cdot 6H_2O$ and 0.2 g $CaCl_2 \cdot 2H_2O$ in approximately 500 ml hot distilled water. Allow to cool. Mix A and B and dilute to 2 liters with distilled water. *VBS—Working solution.* Dilute 1 part stock solution with 4 parts distilled water and mix well. The mixed VBS working solution is used as diluent for all CF test procedures. *Antigens. Antigen preparation.* Brains of moribund suckling mice infected with EEE (Massachusetts strain 469th mouse brain passage) or WEE (California strain 58th mouse brain passage) were ground in a mortar with BSB to give a 20% suspension. After 24 hours incubation at 4°C the suspension was centrifuged at 2500 RPM for 5 minutes (International PR-2 centrifuge, No. 269 head). The supernatant fluid constituted the infective CF antigen. Aliquots of infective antigens in BSB ("BSB antigens"), not exceeding 70 ml, were dispensed in 125 ml Erlenmeyer flasks. The unstoppered flasks were placed in a Ben-Venue sterilizing chamber† (Model No. VIII) and exposed to ethylene oxide gas for 4 hours. After ethylene oxide treatment, infectivity as determined by titration in mice was negative. The inactivated BSB antigens were usable in CF tests immediately following the ethylene oxide exposure. *Commercial antigens.* Lederle EEE (Lot No. 2472-77) and Markham WEE (Catalogue No. M 3511) commercial antigens were used in this study. *Antisera preparation.* Rabbit antiserum to mouse brain was prepared by immunizing New Zealand rabbits with four 1.0 ml intraperitoneal inoculations at weekly intervals with a 20% suspension of normal mouse brain (NMB) in BSB. EEE and WEE hyperimmune mouse antisera were prepared using the method described by Casals(5) except ethylene oxide inactivated virus preparations were used for the first 2 immunizing inoculations.

Modified complement fixation tests. The CF test described by Bozicevich *et al*(6) was

* These studies were originally conducted in the Laboratory of Tropical Virology, Nat. Inst. of Allergy & Infect. Dis.

† Obtained from Scientific Products, Evanston, Ill.

TABLE I. Anticomplementary Reactivity of Antigens.*

Antigens tested	No. of 50% units of complement 1U	2U	3U & 4U
EEE (infectious)	++†	0‡	0
EEE (ethylene oxide)	"	"	"
EEE (commercial)	++++§	++	"
WEE (infectious)	++	0	"
WEE (ethylene oxide)	"	"	"
WEE (commercial)	++++	++	"

* All tests incubated 4°C for 18 hr.

† ++ = 50% hemolysis of indicator system. One 50% unit complement hemolyses 50% of the sheep red blood cells.

‡ 0 = Complete hemolysis of indicator system.

§ ++++ = No hemolysis of indicator system.

used with the following modifications: 1. The incubation period of the primary reactants was reduced from 18 hours at 4°C to 2 hours at 4°C preceding addition of the indicator system; 2. VBS was used as the diluent for all test procedures.

Results and discussion. Borate stock buffer virus suspensions of 20% mouse brain in the presence of 1, 2, 3, or 4 fifty percent units‡ of complement were not AC when incubated for 18 hours at 4°C (Table I).

Box titrations of infective and ethylene oxide treated BSB antigens showed optimal zone reactivity indicative of a potent CF antigen(3). Two-fold dilutions of commercial antigens mixed with 2-fold dilutions of serum gave results indicative of low antigenic potency (Table IIA) since maximum fixation of complement was observed either in antibody excess, or antigen excess.

Since mouse brain antigens could cause non-specific fixation of complement it was desirable to study their reactivity using antisera prepared against NMB. Non-specific CF by NMB is shown in Table IIA. By altering the test procedure, *viz.*, omitting the incubation of the primary reactants (Table IIB), the mouse brain CF antibody titer in rabbit sera was reduced or eliminated while EEE and WEE CF antibody of the mouse sera was still demonstrable.

‡ By definition, one 50% unit of complement incubated in the presence of antigen, followed by addition of the indicator system, hemolysed 50% of the sheep red blood cells.

Greater sensitivity and potency of BSB antigens compared with that of a commercial preparation were shown when serial bleedings from an EEE infected horse were tested in box titrations (Table III). The commercial antigen did not fix complement when tested without incubation, with 2 hours' incubation, or with overnight incubation of the primary reactants. Both the infective and ethylene oxide treated EEE BSB antigens showed identical CF antibody titer during the same incubation periods. These CF results indicate that reactivity during incubation of the primary reactants is dependent upon the potency and sensitivity of the antigen as well as the avidity of the antiserum.

There was no evidence of cross reactivity between EEE and WEE antigen-antiserum systems (Table IIA and B), and non-specific complement fixation with Wasserman and malaria positive sera was not demonstrable.

After a 4-hour exposure to ethylene oxide gas, the BSB-antigen contained no detectable virus by MLD 50 intraperitoneal titrations using 12-14 g NIH general purpose mice. Prior to ethylene oxide exposure the BSB-antigens gave MLD titers of 1×10^9 per g of mouse brain. The protective capacity of ethylene oxide treated EEE virus was determined by challenging mice, previously immunized with ethylene oxide treated virus, with 0.2 ml of 1×10^{-1} dilution of live virus. Since none of these mice became moribund this would suggest a possible use of ethylene gas in the preparation of inactivated EEE vaccine. The antigenic potency of ethylene oxide treated EEE and WEE CF antigens were the same (Table IIA) as infective mouse brain suspensions.

Results of CF tests utilizing BSB antigens without incubation of the primary reagents suggest differences in the rates of fixation of complement by antibody produced to heterologous tissue and antibody produced by virus inoculation. Tissue CF reactivity was no longer detectable when the incubation period was eliminated, while antibody to the specific virus was demonstrable. Under these conditions the reactivity of an antiserum with its corresponding antigenic factor

TABLE II. Complement Fixation with Various Antigens and Antisera.

Antisera tested	EEE (inf.) and (eth. ox.)	EEE (com.)	WEE (inf.) and (eth. ox.)	WEE (com.)	NMB‡
	(A) 2-hr incubation of the primary reactants				
EEE mouse	32/128*	8/32	0/0	0/0	0/0
WEE "	0/0 †	0/0	32/8	16/32	"
Mouse brain rabbit	64/32	16/32	16/64	128/16	64/32
	(B) No incubation of the primary reactants				
EEE mouse	32/16	0/0	0/0	0/0	0/0
WEE "	0/0	"	8/8	"	"
Mouse brain rabbit	"	"	0/0	"	"

* The highest dilution of antigen (1:32) and antiserum (1:128) giving a 2 plus or greater fixation of complement. Values shown are those at the maximum of optimal reaction zone.
† No fixation of complement using a 1:4 dilution of antigen and a 1:4 dilution of antiserum.
‡ Normal mouse brain BSB antigen.

would appear to be dependent only on antibody avidity.

Summary. 1. Western equine encephalomyelitis and Eastern equine encephalomyelitis CF antigens were prepared by grinding the brains of infected suckling mice in a borate stock buffer (BSB), allowing the 20% suspension to stand for 24 hours at 4°C, centrifuging, and harvesting the supernate. The "BSB Antigens" prepared in this manner were shown to be of greater potency and sensitivity than commercial products. Inactivation of viral antigens with ethylene oxide gas affected neither the potency nor the sensitivity of the antigen when compared to untreated BSB virus suspension. Ethylene oxide treated BSB mouse brain EEE and WEE CF antigen-antiserum systems did not cross react. 2. Reduction of the incubation time for primary reagents in CF tests from 18 hours at 4°C to 2 hours at 4°C gave little if any change in antiserum or BSB antigen titer. Elimination of the incubation period altogether vitiated the activities of commercial antigens or normal mouse brain, but did not appreciably affect that of the BSB antigen.

TABLE III. Complement Fixation with Sera from a Horse Infected with EEE Virus.*

Horse sera, days after infection with EEE	EEE (inf.) and (eth. ox.)	EEE (com.)
0	0/0*	0/0*
7	"	"
14	40/40†	"
20	20/20	"
30	40/10	"
60	0/0	"

* Identical titers were obtained with no incubation, 2-hr incubation, or overnight incubation of the primary reactants.
† Highest dilution of antigen (numerator) and antiserum (denominator) giving a 2 plus or greater fixation of complement. Values shown are those at the maximum of optimal reaction zone.

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