Arthropod-Borne Virus Plaques in Agar Overlaid Tube Cultures.*† (24902)

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The agar overlay technic as originally described by Dulbecco(1) and modified by Hsiung and Melnick(2), has been adapted for use in studying plaque formation of selected arthropod-borne (arbor) viruses in tube tissue cultures. It has become evident in working with this group that virus-induced cytopathogenic effects (CPE) in fluid cultures and plaque formation in parallel agar overlaid cultures did not always coincide. In a number of instances it was found that in similar tissue culture systems, virus multiplication proceeded in fluid cultures without evidence of CPE, but it could be detected by formation of plaques under an agar overlay. With use of tube overlay technic, it has thus been possible in many instances to detect multiplication of certain arbor viruses by formation of plaques when cytopathic reactions in fluid cultures were doubtful or not evident. This method may, therefore, be useful for recovering many members of this group of viruses which would have otherwise been missed if CPE were used as the only criterion for tissue culture susceptibility to virus multiplication. The method also lends itself quite satisfactorily to the performance of serum neutralization tests.

Methods. Tube cultures (16 x 100 mm test tubes) were prepared from freshly trypsinized Pekin duck and rhesus monkey kidney and chick embryo tissues in the usual manner(3, 4). Growth medium for all cultures consisted of 0.5% lactalbumin hydrolysate and 2% calf serum in Hanks' balanced salt solution(5). Earle's medium containing 2% calf serum was used to maintain cell monolayers once they had become confluent. Virus log or half log dilutions in Earle's medium were freshly prepared and inoculated in 0.2 ml amounts into pre-drained tube cultures. All tissue culture tubes were incubated 2 hours at 33-35°C to allow virus adsorption to the cell monolayer. After washing, each cell sheet was then covered with 2 ml of the agar overlay medium (2) which contained a final concentration of 2% agar and 2% calf serum. The tubes remained in slanted position during delivery of overlay. After the agar had solidified, the tubes were stoppered and incubated 2-8 days or until plaques became evident. Following initial incubation period of 12-14 hours, it was best to invert the tubes so that any condensed moisture could drain from the agar slants.

Results. Differentiation of arbor viruses within sero-groups A, B and C by plaque size or shape was extremely difficult or not possible. However, differences between these groups could be shown by plaque formation in specific tissue culture systems (Table I). Formation of plaques following virus seeding generally required 2-3 days for viruses of sero-group A in chick embryo, 5-6 days for group B in Pekin duck kidney, and 6-7 days for group C in rhesus monkey kidney tissue cultures. The period required for CPE, when it occurred, was usually the same as required for appearance of plaques.

The type of virus titration endpoint obtained by the plaque method in tube cultures of chick embryo is illustrated in Fig. 1. The plaques result in cells losing the neutral red stain and appear as white transparent colonies upon a pink background. As will be observed, number of plaques relates to dilution of the virus. The 10^{-6} dilution of Eastern equine encephalomyelitis (EEE) virus titration with normal mouse serum (upper figure) shows no isolated plaques as the plaques have become confluent and completely decolorized the cell layer. Whereas, distinct plaques appear as the endpoint of titration in the 10^{-10} dilution. Plaque-forming unit (PFU) deter-

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TABLE I. Comparative Titration of Representative Arthropod-Borne Viruses.

Sero-group	Virus strain	No. mouse pass.	Tissue culture system	Tube TC titer/0.1 ml		Mouse
				TCD_{50}	PFU*	LD_{50}/.02 ml
				Log		
A	EEE RF B-53677	3	Chick embryo	7.1	8.5	7.4
	Middleburg RF 254557	13		<2.0	7.4	6.0
B	West Nile Ar 248	11	Pekin duck kidney	6.2	7.0	6.4
	St. Louis RF B-50487	122		<2.0	5.8	5.1
C	Marituba† Be An 15	16	Rhesus monkey kidney	<2.0	6.6	6.0
	Oriboca† Be An 17	4		<2.0	7.0	6.5

* Plaque-forming units.

† Isolated by O. R. and C. E. Causey, reported by Theiler, M. and Casals, J., *Klin. Wochensch.*, 1959, v37, 59.

minations were found, in most instances, to yield higher titers than TCD_{50} (when CPE occurred) or infant mouse LD_{50} titers (Table I).

Fig. 1 also illustrates the application of this method in a serum neutralization test. Heat inactivated normal and EEE immune sera, each at a 1-20 dilution, were mixed with equal amount of increasing log dilutions of the virus. After one hour incubation at room temperature, the mixtures were inoculated into prepared tissue culture tubes. It will be noted that with the normal mouse serum-virus mixture, the tube containing 10^{-10} dilution of the virus shows plaques, while in immune mouse serum-virus mixtures, the highest dilution of virus to show plaques is at 10^{-6}. This indicates that approximately 4-5 logs of virus were neutralized by the immune serum. It was customary to run 3 tubes for each virus dilution, and average plaque endpoint was recorded as the product of average number of plaques formed and corresponding virus dilution.

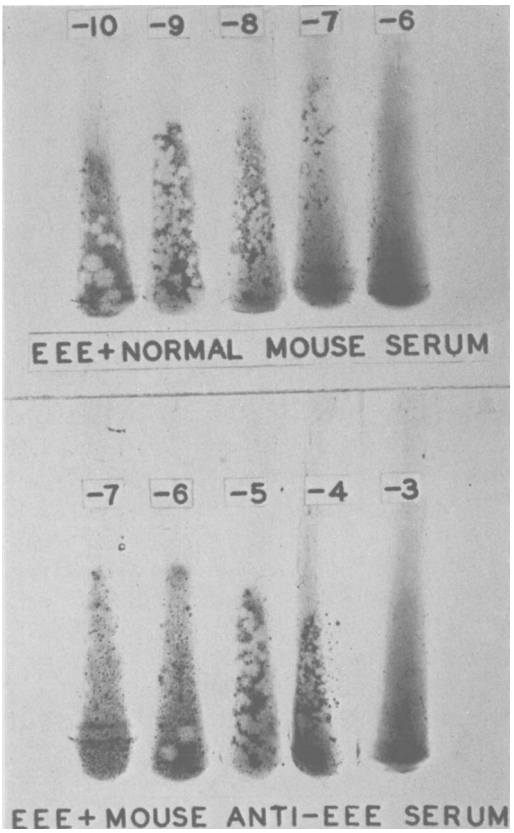

FIG. 1. Serum neutralization test of eastern equine encephalomyelitis virus in chick embryo tube cultures. Upper: Increasing virus $\log_{10}$ dilutions mixed with heat inactivated normal mouse serum, 1-20 dilution. Lower: Increasing virus $\log_{10}$ dilutions mixed with heat inactivated anti-EEE mouse serum, 1-20 dilution.

Summary and conclusions. A tissue culture tube overlay method for detecting virus growth and for performing titrations and serum neutralization tests with members of the arthropod-borne viruses has been described. This method was more consistent and more sensitive than cytopathogenic effects for detecting multiplication of certain

arbor viruses in tissue culture. Plaque-forming unit endpoint titrations were generally higher than the cytopathogenic endpoint (TCD_{50}) even when CPE was evident, or the endpoint (LD_{50}) in infant mice inoculated intracerebrally. It is suggested that the tube overlay technic may be an aid in large scale screening of clinical or field specimens and in conducting serum neutralizing antibody surveys for many arthropod-borne viruses.

1. Dulbecco, R., *Proc. Nat. Acad. Sci.,* 1952, v38, 747.

2. Hsiung, G. D., Melnick, J. L., *Virology,* 1955, v1, 533.

3. Rappaport, C., *Bull. W.H.O.,* 1956, v14, 147.

4. Dulbecco, R., *J. Exp. Med.,* 1954, v99, 183.

5. Melnick, J. L., *Diagnostic Procedures for Virus and Rickettsial Diseases,* 2nd Ed., 1956, APHA, N. Y. City, 140.

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Investigation of Rejection of Canine Renal Homotransplants by Fluorescent Antibody Technic.* (24903)

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It is widely held that rejection of transplants of homologous tissue results from an acquired immunity expressed by host to donor tissue. However, identification and classification of this type of immune process has remained enigmatic. Attempts to relate circulating antibody with homograft rejection have been conflicting(1,2,3). No changes in serum complement(3) or properidin(4) have been found. It appeared worthwhile to study renal homotransplants in various phases of rejection for presence of recipient's globulin by fluorescent antibody technic of Coons and Kaplan(5). Utilization of so-called indirect modification of the latter also allowed for possible detection of circulating antibodies in recipient's serum.

Materials and methods. Renal homotransplantation was performed on adult mongrel dogs by uniform surgical procedure used in renal homotransplant investigations. The right kidney and a segment of ureter of donor was severed with a cuff of aorta and vena cava attached to renal artery and vein respectively. After removal of recipient's right kidney, anastomosis of the vascular cuff with recipient's aorta and vena cava just above trifurcation was performed using standard vascular anastomotic technics. The ureter was implanted into recipient's urinary bladder. Left kidney of recipient was removed either simultaneously or 24 hours later through separate flank incision. Renal homotransplants were obtained by sacrificing 10 dogs prior to exhibition of rejection as indicated by normal BUN, 8 in an early phase of rejection (moderately elevated BUN) and 8 with moderate to marked elevation of BUN, often of several days duration. Portions of transplanted kidneys and their renal arteries, as well as from untreated controls were quickly frozen on dry ice. Sections were cut at 5 μ in a cryostat and stained with antisera containing either anti-dog globulin (anti-DG), anti-dog albumen (anti-DSA), or anti-dog fibrinogen (anti-DF) which had been conjugated with fluorescein isocyanate. Antisera were obtained by immunizing rabbits with fractionated canine plasma proteins prepared by continuous flow electrophoresis. Technical controls consisted of sections similarly treated except that they were first flooded with their respective non-fluorescent antisera. In some instances sections were stained with non-fluorescent anti-DG prior to application of anti-DF. Alternate frozen sections as well as those prepared after routine formaline fixation were stained with hematoxylin and eosin. In the indirect

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