Propagation of Measles Virus in Non-Primate Tissue Culture. I. Propagation in Bovine Kidney Tissue Culture. (25372)

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Propagation of measles virus in human and primate tissue culture(1,2) as well as in chick embryo tissue culture(3) has been described. Attempts by Warren and Cutchins(4) to grow this virus in bovine kidney tissue culture (BKTC) were unsuccessful. This paper describes a method whereby measles virus can be propagated in BKTC, producing a cytopathogenic effect (CPE) typical of that for measles in other tissue cultures(5).

Material and methods. Virus. The Edmonston strain(6) of measles virus used was obtained from Enders.* This virus had been propagated 24 times in human kidney and 27 times in human amnion tissue culture (HATC) in Enders' laboratory. After 8 additional passages in HATC in our laboratory, a large seed pool of virus was prepared for the following experiments. *Tissue culture procedure.* BKTC was prepared by suspending 1 part of trypsinized kidney cortex cells from 8-9-months-old bovine fetuses in 200 parts of medium consisting of 0.5% lactalbumin hydrolysate, 5% horse or calf serum, and Earle's basic salt solution containing 200 units of penicillin and 200 μg of streptomycin/ml. This mixture was dispensed in 1 ml amounts in culture tubes and the medium changed at weekly intervals. Human heart cells (Girardi)† (HHTC) were used as indicator of virus in titration and identification tests; 200,000-500,000 cells/ml in Eagle's basal medium (EBM) plus 10% calf serum were dispensed into tubes. Regular medium changes were performed at 3-4 day intervals with EBM plus 2% calf serum. HATC were prepared according to the method of Zitcer *et al.* (7); 300,000-500,000 cells in 1 ml of EBM plus 10% calf serum were dispensed/culture tube, and handled as described for BKTC, using EBM plus 10% calf serum as maintenance medium. *Complement fixation (CF) test.* Tissue culture fluid from measles-infected HATC, inactivated at 56°C for 30 minutes, was used as antigen. Two units each of complement, antigen, hemolysin, plus 2% sheep cells, each in 0.2 ml amounts, were employed in the test. Sera inactivated twice at 56°C for 30 minutes were used in 2-fold dilutions. Complement fixation was carried out at 4°C overnight, after which the hemolytic system was added, with incubation at 37°C for 30 minutes. Endpoints were determined at 50% fixation of complement. Control antigens from non-infected HATC were included in each test. *Neutralization test.* A mixture of equal amounts of serum or serum dilutions and virus in final concentration of approximately 100 tissue culture infectious doses 50% ($TCID_{50}$) was incubated for 1 hour at room temperature, and 0.2 ml inoculated into each of several culture tubes. The final reading was recorded when approximately 100 $TCID_{50}$ were present in the simultaneous virus control titration. *Monkeys.* Cynomolgus monkeys of either sex were used, after an isolation period of 4 weeks following arrival from the Philippines. Their sera were tested for measles CF antibodies immediately and 4 weeks later. Only monkeys free of measles antibodies were used. After inoculation, the monkeys were observed daily for rash or other signs of illness, and rectal temperatures were taken. Serum specimens were drawn at weekly intervals for antibody studies. *Viremia and throat cultures.* Blood specimens for virus isolation were taken every 2nd day for 15 days, beginning with 3rd day post-inoculation. Whole, heparinized blood in amounts of 1.5-2 ml was inoculated into 2-5 tubes, using HATC and/or HHTC. Throat cultures were taken at the same intervals by swabbing the pharynx with sterile cotton which was then submerged and agitated vigorously in 2 ml of tissue culture fluid containing 2,000 units of penicillin, 2,000 μg of streptomycin,

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† Obtained from Microbiological Assoc.

TABLE I. Amount of Virus Present in Tissue Culture Fluid in the 1st Passage of Measles Virus in BKTC at Different Times, as Determined by Titration in HHTC.

Titer in logs/0.2 ml	Post-inoculation day	Titer in logs/0.2 ml	Post-inoculation day
2.3	14	2.5	51
1.8	23	1.8	59
2.3	37	2.5	81*
2.5	44	3.5	81†

* Tissue culture fluid only.
† After disrupting cells by freezing and thawing.

and 50-100 units of mycostatin ml. After 30 minutes at room temperature, the cotton was squeezed out and discarded. The fluid was inoculated in 0.2 ml amounts into each of 3-5 tubes. HATC was observed for approximately 27 days, and HHTC for 14 days. One blind passage was performed. Isolations were identified by serum-neutralization test.

Results. Each of 6 BKTC tubes was inoculated with 0.5 ml of virus. Three tubes were harvested at 14 days after freezing and thawing, even though no CPE could be noted, and 0.5 ml of fluid and cell mixture transferred into each of several new BKTC. Additional transfers were usually made at 14-16 day intervals, regardless of a CPE, and are referred to as blind passage line (b-line). In the remaining 3 cultures, CPE was observed 70 days after inoculation. Fluid from these cultures was tested for virus in HHTC at different intervals. Production and release of virus were continuous, since amount of virus in the fluid was relatively constant during 81-day period, despite 11 medium changes after inoculation (Table I). On 81st day, although 10%-20% of the cells appeared unaffected, a passage was performed in the manner described for the b-line. This initiated a series of passages in BKTC, referred to as normal, or "n" line, in which transfers were made, when at least 50% of the cells exhibited a CPE. The time required for appearance of CPE was considerably shortened after a few passages (Table II).

TABLE II. Appearance of Definite CPE at Different Passage Levels in BKTC in Relation to the Amount of Virus Inoculated.

Passage level (n-line)	Day post-inoc. CPE appeared	Amt of virus in 0.5 ml inoc. as titered in HHTC, log
1	70	3.9
2	24	3.9
3	8	4.7
4	15	4.2

Cytopathic effect. Cytological changes observed in infected cultures resembled those described by Milovanovic *et al.*(5) for measles CPE in HATC, and were characterized by formation of syncytium-like, or multinucleated giant cells. Eosinophilic nuclear inclusions were seen, as well as strongly eosinophilic amorphous material in the cytoplasm. A comparison of normal and infected BKTC cells can be seen in Fig. 1. In the b-line, 8

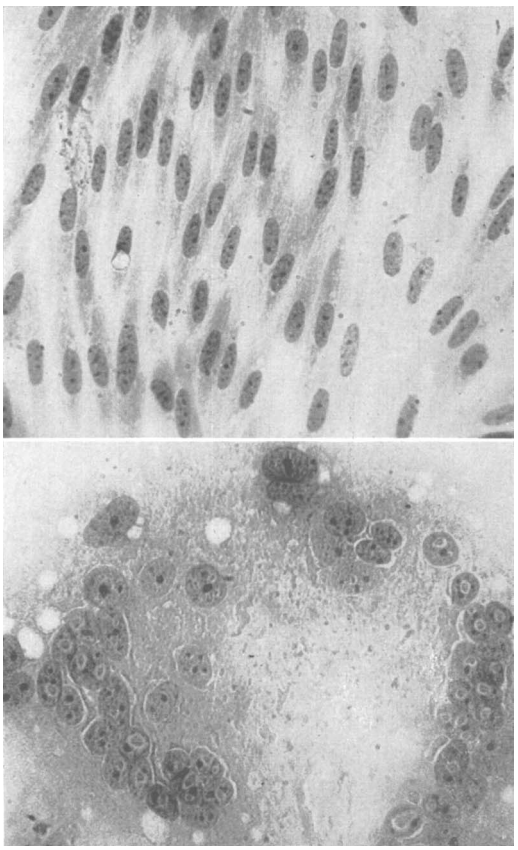

FIG. 1. Comparison of normal bovine kidney tissue culture (above) with a similar culture (below) showing CPE following inoculation with measles virus. Stained with hematoxylin eosin, magnification 192×.

passages were required before a fairly consistent CPE occurred (Table III). The 16th b-line passage in BKTC yielded $10^{4.3}$ $TCID_{50}$/0.2 ml of virus when titrated in HHTC, which indicated that the virus multi-

TABLE III. Appearance of a Definite CPE at Different Passage Levels of the Blind-Passage Line in BKTC.

Passage level (b-line)	Day post-inoc. CPE appeared*	Amt of virus in 0.5 ml inoc. as titered in HHTC, log
1-4	0	+†
5	14	3.4
6	0	3.4
7	0	+
8	0	+
9	14	3.9
10	11	4.2
11	10	3.2
12	0	2.7
13	13	3.7
14	13	3.9
15	0	3.9
16	7	4.2
17	7	4.7
18	5	5.7

* Tubes passed after approx. 14-16 days.
† Virus present as tested in HHTC, but not titrated.

plied in the BKTC, since this endpoint of titration represents a dilution of $10^{-15.5}$ of original inoculum which titrated only $10^{-3.5}$/0.2 ml.

TABLE IV. Results of Neutralization Test with Human and Monkey Measles Sera and 100 $TCID_{50}$ after 4th Normal Bovine Measles Virus in HHTC.

Serum 2-fold dilution	Acute or pre-inoc.	Convalescent, or post-inoc.
Human	1:13	1:604
Monkey	0	1:256

Identification. Virus propagated in BKTC, when tested at different passage levels in HHTC or HATC, still demonstrated the typical characteristics of measles virus. The 4th BKTC n-line virus was identified by neutralization test in HHTC, using acute and convalescent sera from a measles patient, and also pre- and post-sera from a monkey inoculated with Edmonston strain of measles virus passed in HATC only. The virus was neutralized to any significant degree only by convalescent and post-inoculation sera (Table IV). The 15th b-line virus was also identified by a neutralization test in BKTC, HATC and HHTC, using monkey pre- and post-inoculation sera. Post-inoculation sera neutralized the virus in all 3 tissue culture systems, while the negative sera had no inhibitory effect.

Tests in monkeys. Two monkeys were inoculated with 2nd passage n-line virus, and 3 with 20th passage b-line virus. Each monkey received 1 ml of undiluted, infected tissue culture fluid intramuscularly. No evidence of disease occurred, but the results (Table V) showed that virus could be isolated from throat and blood on several occasions.

Discussion. The above experiments show conclusively that measles virus can be propagated in BKTC, with production of a typical CPE which can be prevented by known measles immune sera. This study indicates, however, that to obtain a CPE for some viruses in certain tissue cultures either a prolonged period of observation, or more than 1 or 2 blind subcultures are necessary. Furthermore, it emphasizes the value of a good virus detection system, such as HHTC, for this particular strain of virus.

Inoculation of monkeys with BKTC-propagated virus provided further evidence that the CPE agent was measles, by development of measles complement-fixing and neutralizing antibodies, even though no illness or rash was observed. The fact that the virus could be recovered from the throat and blood of inoculated monkeys might be interpreted as a sign of virulence, but the final answer to this question can only by found by inoculation of

TABLE V. Intramuscular Inoculation of Bovine Kidney-Propagated Measles Virus into Cynomolgus Monkeys.

Passage No. and amt of virus inoc.*		Virus recovery, days post-inoc.				Antibody			
		Human amnion T.C.		Human heart T.C.		C.F.		Neutralization	
		Blood	Throat	Blood	Throat	Pre	Post	Pre	Post
2nd	5.0	5	N.T.	N.T.	N.T.	0	1:256	0	1:320
"	"	3, 7, 9	"	"	"	0	>1:1024	0	1:320
20th	5.2	3, 5, 7	0	3, 5, 7, 9	7, 9	0	1:512	0	1:32
"	"	3, 5, 7	7	3, 5, 7	5, 9	0	1:2042	0	1:320
"	"	5, 7	5, 7	5, 7	5, 7	0	1:1024	0	1:64

* In logs. N.T. = not tested.

susceptible humans. Regardless of the possible virulence of the present BKTC virus, propagation of measles virus in this tissue culture system may prove valuable as a means of further modification. It would also permit economical production of large quantities of measles virus for use in either modified or killed vaccines. Bovine embryonic tissue would be superior to monkey tissue since it is more readily available and, to our knowledge, contains no latent viruses; it further would be superior to chick embryo tissues to which many people are already sensitive.

Summary. 1. The Edmonston strain of measles virus was propagated in BKTC for 20 passages, and produced a CPE typical of measles, characterized by multinucleated giant cells and eosinophilic intranuclear inclusion. 2. Identification of this cytopathogenic agent was confirmed by neutralization test with known measles immune serum. 3. BKTC-propagated virus was inoculated intramuscularly into susceptible cynomolgus monkeys without causing clinical illness, but the virus could be recovered from throat and blood. All inoculated monkeys developed complement-fixing and neutralizing measles antibody. The significance of these results is discussed.

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Failure to Transfer Delayed Tuberculin Sensitivity from Human to Animal Species.* (25373)

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Transfer of delayed cutaneous sensitivity to tuberculin and to other materials with leucocytes obtained from specifically sensitive donors has been reported by Chase in the guinea pig and by Lawrence in man(1). In animals, the transfer of sensitivity is successful only when intact leucocytes are employed. However, in man, it has been shown that the factor in sensitive leucocytes concerned with transfer of delayed sensitivity retains its activity following leucocyte disruption and enzymatic treatment(2). This finding has been confirmed recently by Freedman, Fisher and Cooke(3). A valuable approach to the study of transfer factor would be provided by transfer of delayed sensitivity from man to animals —particularly if leucocyte extracts could be used. Lawrence, as well as Rammelkamp, and Thomas and Stetson(4) have repeatedly attempted to transfer tuberculin sensitivity to guinea pigs and rabbits with intact as well as disrupted human leucocytes obtained from sensitive donors. Their results have been consistently negative. The present study is based on the assumption that species barrier in transfer of sensitivity might be crossed more readily within a closer evolutionary relationship, as exists between man and the monkey. This report describes a series of attempts to

* The opinions or assertions contained herein are the private ones of the authors, and are not to be construed as official, or reflecting the views of the Navy Department or of the Naval Service at large.

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