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Cytopathogenicity and Plaque Formation with Lymphocytic Choriomeningitis Virus.* (25616)

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During experiments(1) aimed at a better understanding of pathogenesis of lymphocytic choriomeningitis (LCM) virus infection in mice and ability of infant mice to develop immunological tolerance to it, an attempt was made to obtain cytopathic effects (CPE) and a plaque assay with this virus. Previous work indicated that LCM virus is not readily cytopathogenic in tissue culture, except in KB cells(2). An earlier attempt to obtain a plaque assay with these cells was not successful. In our hands several other cell lines including HeLa, Detroit 6, human intestine, human liver, human kidney, FL, primary human amnion and L cells have shown no effects in spite of virus multiplication. However chick embryo tissue cultures appeared to have possibilities since infected cultures, kept 3 weeks or longer, showed decreased acid production with less luxuriant growth than controls. Experiments were therefore set up in which LCM mouse brain virus (the WE strain obtained originally from Rockefeller Inst.)† was passed in series through chick embryo tissue cultures.

Methods. Cells were prepared from 9-day chick embryos using Dulbecco's method(3). One ml of tissue suspension at a concentration of 300,000 cells/ml in growth medium was used to seed 16 × 125 mm screw-capped test tubes. The nutrient medium consisted of 20% calf serum, 5% chick embryo extract, 75% Medium 199 with penicillin and streptomycin. After 2 days' incubation, cultures were washed with Hanks tris pH 7.2 (M/50 tri (hydroxymethyl) amino methane in Hanks saline) and infected with LCM virus. A cytopathic effect was produced in the first chick embryo tube passage. In 6 days LCM infected cultures appeared slightly more granular than controls and the sheets were beginning to break. In 9 days the sheets in infected cultures were completely broken up, only single cells remaining, whereas cell sheets in control tube were intact. At this time pH of control culture fluid was 6.8 and pH of LCM culture fluid was 7.5 or higher. Forty serial passages of this virus have been made using at first 3- or 4-day and then 7-day fluid from LCM infected cultures as inoculum; the final passes produced a moderate CPE in 5 days and a moderate color change in 7 days. Control culture fluid has been passaged similarly, and has shown no CPE.

Results. Mice inoculated intracerebrally with LCM culture fluids at various passage levels, diluted 10^{-3} or 10^{-4}, produced typical LCM convulsions and deaths. Undiluted control fluids never caused illness or death of mice. Titrations indicated that maximum virus titer in tubes was reached by 7th day. CPE was obtained in terminal dilution (10^{-4}) tubes after 12 days; no new CPE appeared later. In a neutralization test in mice, virus

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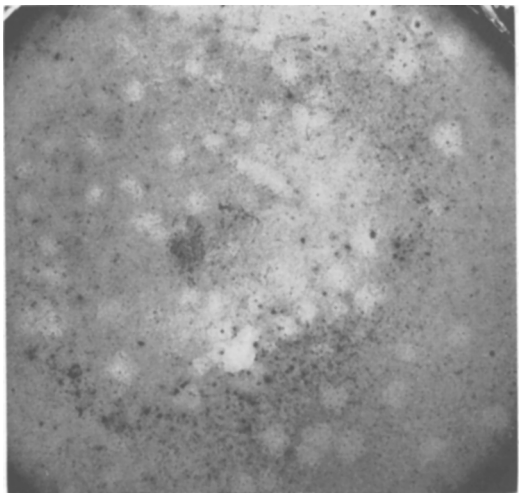

FIG. 1. Photograph of 10 cm Petri plate showing LCM plaques 13 days after inoculation of chick cell monolayer.

in 7-day fluid was neutralized at 10^{-2} but not 10^{-1} by anti-LCM guinea pig serum, but not by normal guinea pig serum. The 7-day fluid gave a positive result by complement-fixation test without anticomplementary effects with specific antiserum.

An attempt was made to produce plaques on chick embryo monolayers with the chick cell adapted strain. Monolayers were made by seeding 10 ml of cells at concentrations of 1.2-1.5 $\times$ 10^6/ml in 10 cm Petri plates. Monolayers were inoculated with 0.5 ml of inoculum and incubated for ¾ hour adsorption period. Overlay (9 ml/plate) was composed of 15% tryptose phosphate broth and 1% agar in M 199. On 2nd, 6th, and 10th days, additional overlays (3 ml) were added, the last one containing 1/8000 neutral red. Round plaques approximately 5 mm in diameter appeared on 12th day (Fig. 1). On 4 occasions 3 plaques and 3 adjacent "non-plaque" areas were removed, each emulsified in 1 ml of 10% normal guinea pig serum in saline and inoculated intracerebrally (0.03 ml) into 5 mice. All mice inoculated with undiluted plaque suspensions died with typical LCM convulsions, whereas mice receiving control area extracts remained healthy.

A surprising result was obtained when monolayers were infected with 20% LCM infected mouse brain suspension (original virus unadapted to tissue culture) and treated as above. Plaques appeared on 12th day and were apparently the same as those produced with tissue culture adapted virus. A comparison was made /ml of virus preparation of sensitivities of the following methods of assay: plaque assay expressed as plaque-forming units (PFU), tissue culture assay expressed as 50% infective dose ($TCID_{50}$) as determined by cytopathogenicity, and 50% mouse lethal dose assay (LD_{50}) by intracerebral inoculation. Five different preparations were assayed by both PFU and LD_{50}, and 7 by both $TCID_{50}$ and LD_{50} (Tables I, II). Average ratio LD_{50}/ml: $TCID_{50}$/ml: PFU/ml was 1:0.73:0.23, indicating a very close agreement between the 3 methods, with descending sensitivity between mouse, tissue culture, and plaque methods.

A disadvantage of this assay has been the difficulty experienced in regularly keeping the monolayers alive for a minimum of 12 days. Duration of cell viability varied between a maximum of 20 and a minimum of 10 days.

Summary and conclusions. At first it was thought that 40 passages of the virus in chick tissue had increased its cytopathogenicity to the point where plaque production could oc-

TABLE I. Relationship Between Plaque Titer (PFU/ml) and Mouse Titer (I.C. Inoculation, LD_{50}/ml) of LCM Virus Preparations.

Avg No. of PFU/ml	Mouse titer expressed in LD_{50}/ml
1.6×10^4	3.3×10^4
3.7×10^3	$1.65 \times$ "
1.6×10^4	1.04×10^5
2.9×10^3	*Idem*
2.6×10^4	"

TABLE II. Relationship Between Mouse Titer (LD_{50}) and Tissue Culture Titer ($TCID_{50}$) of LCM Virus Preparations.

No. of days harvested	Mouse titer expressed as LD_{50}/ml	Titer expressed as $TCID_{50}$/ml
1	3.3×10^4	7.2×10^3
2	5.2×10^3	$1. \times 10^4$
5	1.65×10^4	$1. \times 10^5$
6	*Idem*	*Idem*
7	5.2×10^5	7.2×10^4
8	$1.65 \times$ "	*Idem*
9	$5.2 \times$ "	$1. \times 10^5$

cur. However ability of non-adapted strain to produce plaques refutes this conclusion. In retrospect it appears that no actual change in virus had occurred during serial passage, but the observation that CPE could be regularly produced after as long as 12 days led to use of above procedure and successful production of plaques appearing with this virus at relatively late time of 12 days after inoculation. The sudden appearance of plaques seems unusual; apparently the final change of CPE, resulting in loss of neutral red staining, must occur very suddenly with this system, resulting in overnight appearance of plaques.

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Agar Diffusion Method for Detection and Bioassay of Antiviral Antibiotics. (25617)

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The discovery of antibacterial and antifungal antibiotics clearly indicates that microbial cultures contain a wealth of diverse biochemicals useful in infectious disease. Unfortunately, there has been little progress in the exploitation of such microbial products for treatment of human and animal viral diseases. A major reason for this failure may lie in the time-consuming, cumbersome and insensitive technics that until recently were a necessary feature of virus research. Such technics did not permit large-scale screening of microbial cultures important to the discovery of clinically useful antibiotics. Furthermore, even if a naturally occurring antiviral agent were discovered, there were no satisfactory bioassay systems available to cope with the hundreds of assays essential to successful fermentation and isolation of the active principle. The following is a description of a filter paper disc-agar plate technic, similar to that used in bacteriology as applied to screening and assay of antiviral agents. The suggestion that such application was possible was made by De Somer and Prinzie(1) in their description of a similar method used for measurement of poliovirus antibody. More recently, Porterfield (2) applied this approach to interferon bioassay.

Materials and methods. Preparation of virus-infected agar plates. Trypsinized chick embryo cells were prepared as described by Dulbecco(3), suspended in Melnick's lactalbumin hydrolysate-calf serum medium(4) with Earle's bicarbonate balanced salt solution, to produce 3.5×10^6 cells per ml. The cell suspension was adjusted to pH 7 with CO_2, then poured into acid dichromate cleaned, sterile Pyrex baking dishes ($10 \times 6\frac{1}{2}$ inches), 85 ml per dish. The dishes were sealed air tight with Saran Wrap (Dow Chemical Corp.) and the medium readjusted with CO_2 to a pH 7.5 if required. This second gassing is often necessary because of the rapid loss of CO_2 from the relatively high bicarbonate containing medium during incubation period. About 5 to 10 chick embryos, depending on their age, were required to produce enough cells for a single dish. Eleven-day-old embryos seemed to produce the best cell yield. After 48 hr incubation at 36°C the chick embryo cells grew into a confluent sheet; the medium was discarded and the cell culture infected with a 5 ml suspension of one of the following viruses: West Nile virus (Egypt 101 strain from Dr. Joseph Melnick, Baylor Univ.), vaccinia (WR, mouse neurotropic), herpes simplex (HF378) and Newcastle disease (California No. 11914) viruses obtained from American Type Culture Collection. The virus was incubated with the cells for 1 hr, then 65 ml of overlay medium con-