Adaptation of Vaccinia Virus to Earle's L-Cells with Prolonged Noninfectious Period.* (25649)

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It is well known that vaccinia virus may be cultivated in tissue cultures of various types. Differences in abilities of several strains of the virus to proliferate in established cell cultures have been observed(1). L and LLC-M_1 cell cultures inoculated with Rivers and Minnesota vaccinia virus resulted in initial rounding and clumping of cells, but no morphological changes were noted in succeeding passages and tests for infectivity produced negative results. Previous to this study Scherer(2) made the observation that the Minnesota strain caused "agglutination" of L cells without viral multiplication. The phenomena of agglutination of L cells by vaccinia virus has been investigated further by Mayyasi(3). The present report is concerned with adaptation of a strain of vaccinia virus to L cells by a modification of the usual tissue culture method(4). The emergent strain has been designated VM1.

Materials and methods. Virus: The strain of vaccinia used in this study has been documented previously(1). The stock virus was cultivated in the chorioallantoic membrane of 10-11 day old chick embryos and stored at −45°C; 0.1 ml had an intradermal titer for rabbits of 10^{-5}. *Cell strain*: Earle's L cell strain used in this study was obtained from Dr. W. F. Scherer, Univ. of Minn. All cultures, whether normal or inoculated with virus, received the same nutrient fluid consisting of 40% Earle's salt solution, 20% chick embryo extract and 40% horse serum. *Adaptation experiments*: Two methods were attempted, both at 37°C. The first was the so-called conventional method of blind passage. In the primary trial 0.2 ml of stock virus was introduced into 7 day-old cultures. After incubation at 37°C for 3-4 days the cultures were frozen and ground, diluted 1:10 with their own culture fluid and centrifuged lightly to remove gross cellular debris. In this repetitive experiment 0.2 ml of each proceeding culture was inoculated into succeeding fresh cultures. In the 2nd method (modified procedure) cultures of the same age were inoculated as before but blind passage was not resorted to. Instead, the cultures after inoculation were maintained in the same manner as normal uninoculated ones. *Conventional method*: Within 24 hours following primary inoculation, the cultures showed the characteristic clumping into small masses described by Scherer(2). At 72 hours cells were rounded and granular with some sloughing, and each culture was harvested at this time. By the 3rd passage clumping could no longer be noted. Repetitive passage of material obtained from freezing and grinding the cells through succeeding fresh cultures did not result in demonstrable propagation of the virus as attested by 4 separate trials (10 passages each). Late passage(7-10) ground cells stored at −45°C were not infectious for rabbit skin either by intradermal inoculation or scarification, or for HeLa cell cultures. *Modified procedure*: The changes from 24-72 hours were consistent with those noted above. In 98 hours marked sloughing had ensued and upon gentle agitation most of the remaining cells were detached from the glass. The estimated 5% remaining were round, granular and dark appearing. The supernatant containing the cell debris was aspirated and fresh nutrient added to the parent culture. After several days the remaining cells began to show improvement and in 7 days appeared to be relatively normal with clear cytoplasm and numerous cell processes. In 2 weeks the proliferating cells had largely covered the glass surface. Cells were removed by scraping and transferred to a fresh Carrel flask and were subcultured every 7-10 days. The parent culture was scraped every 12 to 14 days, a portion saved and the remaining cells were dis-

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carded. Representative aliquots from the various procedures were stored at −45°C. Under low magnification (32×) the parent culture and the various subcultures arising therefrom did not appear unusual until the 8th week, when a few scattered gross plaques appeared in both the parent culture and the 6th subculture, but not in control cultures, and clumping of cells into granular masses was prominent. Within 48 hours after onset of plaque formation the remaining cells had largely sloughed from the glass. Cells and culture fluid from previous subcultures (except the first and the 5th, which was lost) or from those scraped from the parent vessel were not infectious for HeLa cells or rabbit skin by scarification or the intradermal technic until onset of cytopathogenic effects. Contamination of the cultures with another strain of vaccinia virus was considered, but can be ruled out for the following reasons. The parent cell culture and the subculture lines were tended by different individuals and identical supernatant was added to these lines and to several different sets of control cultures. It is emphasized that no cytopathogenic changes were observed in normal control cells during or following the study. Subsequent events are worthy of note. Fresh nutrient fluid was added to the few cells remaining adherent to the glass. Daily examination revealed a slow increase in cell population eventually covering the glass surface of the vessel in about 2 weeks. Most of the component cells were clear and did not appear unusual, but scattered cells were granular, rounded and relatively dark, and were interpreted as being infected. When cell population had largely covered the available surface widespread sloughing and necrosis occurred, leaving a few adherent cells which did not appear healthy. This cycle of growth and degeneration was repeated 2 additional times and then terminated. Material from this last procedure (VM1 strain) was supplied to Dr. R. F. Parker, Western Reserve Univ. who did not know of the cycle described above and made the entirely independent observation that infection with the U12 strain of tissue culture cells with VM1 and other strains of vaccinia virus leads to recurring "micro-epidemics"(5). The cycle of infected cell growth and degeneration appears to be similar if not identical to that described here and to studies described by Chambers with EEE virus in L cells(6). The agent obtained by the modified procedure now could be cultivated in usual manner, regularly produced intracytoplasmic inclusions in HeLa cells, L cells and rabbit cornea, and was neutralized specifically by immune sera prepared from 2 other strains(1). The adapted virus now readily infected L cultures and other host-cell systems to approximately the same degree as the highly infectious WR strain(1). Material from the 18th serial passage in L cells revealed an infectious titer of 10^{-4} and 10^{-5} per 0.1 ml for rabbit skin and L cultures respectively.

Discussion. It is significant that the variation in tissue culture method described resulted in adaptation of a strain of vaccinia virus. The explanation is not now apparent although it appears logical that allowing an uninterrupted association of cell and virus permits a more favorable environment for adaptation. During the prolonged non-infectious phase the cells were in contact with complete growth medium so that nutritional depletion of cells was not a factor in latent infection as demonstrated by Morgan with psittacosis virus(7). It is questionable that the present work is an example of latency. It is plausible that a few undetected cells were infected, liberating virus and the end result was adaptation, the mechanism of which is poorly understood. Shope(8) has summarized a number of latent viral infections in tissue culture but the virus was present in pathogenic form capable of detection by infectivity tests either in animal or tissue cultures. It was cited further that in certain persisting infections of HeLa cells with poliovirus the virus did not always exhibit the infectious property. Under certain conditions the latently-infected cells were capable of producing fully active virus.

Finally, the cycles of growth and degeneration described here pose a fundamental problem as to whether an apparently infected cell which presumably has elaborated virus is able to divide. The data do not answer this ques-

tion but it is curious that the few remaining cells prior to initiation of the cycle of regeneration have the cytological appearance of infection.

Summary. It has been shown with a modification of the usual tissue culture technic that the Minnesota strain of vaccinia virus has been adapted to Earle's L strain fibroblasts. Following establishment of the infection, several cycles of cell growth and degeneration occurred. The adapted strain was identified by conventional cytological and serological criteria. The method described might lend itself to adaptation of other viruses.

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Potentiation of the Local Shwartzman Phenomenon by Trypsin.* (25650)

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It has been reported that extracts of bacteria, tumors and normal mammalian tissues produced the Shwartzman Phenomenon in rabbits and hemorrhage into sarcoma 180(1, 2,3) and sarcoma 37(4,5) in mice. It was subsequently found that a number of other preparations of animal, plant and bacterial origin could effect many host responses including the Shwartzman Phenomenon and tumor damaging ability(6,7,8). In these preparations trypsin was employed in the extraction process(7), therefore the final products could contain residual trypsin which was not removed by dialysis. On the other hand we were able to obtain potent extracts, without use of trypsin, from pancreas and testes(2), organs which normally contain proteolytic enzymes(9). Accordingly, it did not seem unreasonable to assume that trypsin *per se* might play a role in production of lesions by bacteria or tissue extracts.

Material and methods. Stock Albino female rabbits were maintained on Rockland rabbit ration pellets which did not contain antibiotics. The abdominal skin was shaved with an electric clipper. Various concentrations of *E. coli* endotoxin (Lipopolysaccharide extract prepared by Difco Laboratories) were injected at different sites in the right flank. The same concentrations in combination with 0.070 mg crystalline trypsin (Takamine Laboratories, Clifton, N. J.) were injected into corresponding sites of the left flank. Other rabbits were treated similarly with *P. vulgaris* polysaccharide(1) instead of *E. coli* endotoxin. A provocative intravenous injection of 0.5 mg *E. coli* endotoxin was administered 24 hours later and the skin reaction was read as in the classical Shwartzman Phenomenon. All solutions were made with pyrogen free saline; the final volume injected in each site was 1 cc. The trypsin solutions were cultured and were not used if found to be contaminated by bacteria.

Results. In a characteristic experiment, in 16 out of 20 rabbits, *E. coli* endotoxin or *P. vulgaris* polysaccharide in combination with trypsin evoked a stronger skin reaction than was produced without use of trypsin. In 10 animals at least one concentration of the bacterial extract was positive in combination with trypsin but did not produce any reaction at the site without added trypsin (Fig. 1). Various concentrations of bacterial extracts in the remaining 6 animals produced skin reaction

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