Effect of Vaccinia on Cells Grown in Tissue Culture. (20166)

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The cytopathogenic effect of viruses on cells in tissue cultures, as described by Enders, Robbins, and Weller(1) in connection with the poliomyelitis viruses represents a syndrome of pathologic changes in the cultured cells that in its end results can be broken down into the following symptoms: 1) degenerative changes in cell morphology due to the virus; 2) inhibition of cell migration due to the virus; and 3) changes in tissue metabolism as indicated by decreased acid production. These effects can all be observed by inspection of the cultures, as well as by observation of the changes in the gross morphology of the growing fragments under regular microscopy at magnifications ranging from 100 to 400. In the present study the cytopathogenic effect of the vaccinia virus on the cells cultured *in vitro* was followed principally in its manifestation of degenerative changes in the cell morphology beginning with cell injury through complete cell degeneration and destruction. The observations at this point were undertaken without the advantages offered by the use of phase microscopy with its opportunities for a dynamic study of the actual action of the virus on the cell or the use of electron microscopy and micrography.

The experiments presented in this paper were aimed at the establishment of the fact that the cytopathogenic changes caused by the vaccinia virus in the growing tissue *in vitro* were present, that they were definite and specific, and that they were not due to incidental or accidental influences. It is a well known fact to all workers in the field of tissue culture that for obvious reasons the necessary use of substrates and nutrients consisting primarily of so-called natural components present the investigator with an endless array of unknown variables. Extensive studies have been made of the physical, chemical, biochemical and physiological knowns and unknowns which may cause changes in cell morphology, theoretically and actually amounting to cytopathogenic effects. But in virology, where the growing tissue represents the substrate and the nutrient, *i.e.*, the host for the virus, one can only hope to strike an average in supplying the tissue and the virus with ingredients which permit sufficient multiplication for the first and, consequently, for both.

Methods and material. The roller tube experiments constituting the major portion of this study employed the usual technics and procedures, and a detailed description would only be repetitious(2). In several experiments the Maximow double cover slip method (3) was used, which permitted observation of the cultures under high power microscopy. In the roller tubes, the finely minced tissue fragments were planted in a single vertical row of explants at 10 to 15 mm intervals inside the tube on a thin coat of heparinized plasma. Clotting was induced by adding 2 to 3 drops of chick embryo extract. The liquid phase of the medium consisted of 1.5 ml of nutrient, composed of a balanced salt solution, ox serum ultrafiltrate, embryo extract, and horse serum. This liquid phase varied in the percentage composition of the single ingredients, not only for suitability to the different tissues used, but also to the virus. Since an extremely rapid rate of growth is not optional in virus studies, a more or less "lean" liquid phase consisting of 85 to 90% of balanced salt solution containing 1:4 ultrafiltrate of ox serum, 5 to 10% diluted embryo extract, and 5% of horse serum, inactivated at 56°C for 30 minutes was used as warranted. This composition closely follows that recommended by the Enders group(4). The balanced salt solution was prepared according to the formula of Hanks (5). Chick embryo extract and plasma were also prepared in our laboratory. Ox serum ultrafiltrate and horse serum were obtained commercially. The liquid phase also contained phenol red indicator in a final concentration in the medium of 2 mg %, with the final pH of the medium corrected by the addi-

tion of 1.4% isotonic sodium bicarbonate to 7.2. Penicillin was used in amounts resulting in final concentrations in the medium no higher than 100 to 300 units/ml—a concentration known to be well below inhibitory or toxic effect. Chloromycetin was also tried in several experiments in extremely small concentrations without apparent interference with the objectives of the study. The following tissues were used: (a) chick embryos at 10 to 11 days incubation, minced as a whole and fragments picked at random; (b) chick embryo hearts at 12 days incubation; (c) chick embryo skin and muscle from legs and backs of embryos incubated for 12 to 13 days; (d) human embryonic skin and muscle; and (e) human embryonic kidney tissue. With the use of material coming from human and animal sources in studies of virology, there is always the additional hazard of introducing unknown virus from the tissue source into the culture(6). To eliminate this hazard 40 series of experiments were run and the soundness of components and sources was determined before the validity for their use was established.

Virus inoculum. The virus used in our experiments was prepared in two ways. One mode of preparation was to make a 20% suspension in buffered saline of the chorioallantoic membranes of chick embryos of 12-day incubation, inoculated with the 72nd egg passage of the CVI strain of vaccinia. The second preparation consisted of a 20% suspension in saline of the washed elementary bodies obtained from the same egg passage. The chorioallantoic membrane suspension was ultracentrifuged at a speed of 40,000 RPM and the sediment containing the elementary bodies was resuspended and recentrifuged at the same speed and, finally, made up to the original volume.

During the course of the study the identity and infectivity of the virus preparations used was confirmed in various ways: (a) inoculation of the chorioallantoic membrane of 12-day-old chick embryos with the suspensions as used in the tissue culture experiments, and close observation for the appearance of characteristic pock lesions; (b) inoculation of the material on the shaved skin of white rabbits. Titration of the virus content of the suspensions was carried out by hemagglutination tests. Specificity was confirmed by using antiserum from patients proven to have high anti-vaccinial titers in hemagglutination inhibition tests(7). In the Maximow double cover slip experiments the components of the substrates for the tissue culture, the tissues used, and the virus suspensions were the same as used for the roller tube experiments. Here, too, the tissue fragments were embedded in a thin plasma clot, and the feeding solution consisted of 3 drops of liquid composed of ultrafiltrate of serum, serum and diluted embryo-extract. Of all the possible variations available in inoculating tissue cultures with virus, the simplest method was used, *i.e.*, the addition of virus suspensions to the liquid nutrient phase. In the case of the roller tubes containing a liquid nutrient of 1.5 ml volume, the virus suspension consisted of 0.1 to 0.2 ml. In the Maximow slides, one drop of the suspension was added.

Results. (See Table I).

TABLE I. Days after Inoculation of Cultures of the 73rd Egg Passage of CVI Vaccinia Virus in the Form of (a) Washed Elementary Bodies and (b) Chorioallantoic Membrane Suspensions. 15 experiments.

Vaccinia inoculated cultures			
Age of cultures at inoculation	Cytopath. effect first noticeable after inoculation, days	Complete degeneration cytopath. effect at its max.	Uninoculated control cultures—day of discard
10	6	12	50
8	6	10	40
6	3	9	19*
6	3	9	19
10	3	14	28
10	5	14	28
4	4	9	24
6	3	6	26
6	3	6	26
6	3	8	26
5	3	6	19
6	4	7	16
6	4	7	16
5	5	8	33
6	3	8	21
Avg 6.6	3.9	8.9	

* Except for Exp. 1 and 2 in the control experiments there was no all over degeneration at conclusion of experiments. Many fragments in the control series still showed healthy growth.

Characteristic degenerative changes comparable to cytopathogenic changes as produced by poliomyelitis and other viruses (1,8,9), first became evident between the third and sixth day after introduction of the virus. It should be noted here that for purposes of standardization in all experiments, the tissue fragments were allowed to grow out to a strong, healthy tangle of fibroblast-like cells, or characteristic sheets of epithelial cells or wide strands of muscle cells, dependent on the material used. No histologic identification of cell material was attempted. Another point kept in mind was that in all experiments only original explants were followed, studies on subcultures will be made at a later date.

The earliest noticeable degenerative change, occurring between the third and sixth day, was the appearance of fatty refractile globules and liquid droplets causing swelling of the cells, amounting—somewhat later—to distortion into a variety of bizarre shapes. It is established that the vaccinia elementary bodies contained in the inclusions observed in the stained preparation of vaccinia infected cells, as well as by electron microscopy, are connected with lipoid material(10). It is possible that the tendency of cells to deposit fat droplets in certain conditions of tissue culture enhances the chance of the virus to invade the cell. As the cells swell and inflate, the continuity of the outgrowing aureoles around the explants is disrupted and the contact between the cells broken. The fatty refractile material in the cells is surrounded by dense granules, and the fusiform cytoplasm of fibroblast-like cells becomes less and less evident. The next stage seems to be a release of the large droplets, complete granulation, balling and rounding of the cells, and finally complete fragmentation and disintegration until only cell debris is left in the field. This process seems to work from the periphery of the radial outgrowth toward the center and the original implant and takes place within 8 to 10 days.

In the first experiment to show definite and striking pathogenic cell degeneration, the virus was introduced in the form of washed elementary bodies 8 days after the start, with complete degeneration 6 days later. In this experiment besides morphological cytopathogenicity, another aspect of the syndrome was evident, *i.e.*, decrease of acid production due to metabolic changes. At the conclusion, the inoculated roller tubes showed a pH average of 7.2, while uninoculated cultures indicated a pH of 6.6. However, it should be pointed out that in roller tubes the pH differences in infected and normal cultures do not follow the course observed in suspended cell cultures(1), since there are additional factors present which may play a part. Healthy, strongly metabolizing tissue fragments in roller tubes often cause rapid liquefaction of the surrounding plasma clot substrate, which cannot be remedied with patching. If this happens the fragment seems to be cut along a tiny sharp line of incision, often crescent-shaped. Consequently, fragment particles and portions of fresh outgrowth drop into the medium and are inadvertently carried off at the next nutrient change, reducing the total amount of tissue present in the tube. In contrast to this, it was frequently observed that infected and degenerated fragments with their outgrowing coronae seemed to be fixed in position in their original rough outlines. Thus, the healthy controls with less tissue did not produce the rapid pH decline, while the infected tubes still continued to show pH changes, even though diminished.

In order to compare the action of washed elementary bodies and chorioallantoic membrane suspension, a number of experiments were run in parallel series—one part of the cultures being inoculated with washed elementary bodies, and the parallel run with the membrane suspension. In all experiments, *i.e.*, roller tubes and Maximow slides, control preparations equal in number to inoculated preparations were always set up in identical procedures, except for the addition of the virus.

Discussion. The vaccinia virus, both in the form of washed suspensions and in the membrane suspension, seemed to affect the tissue cultures and effect cytopathogenic changes. At the risk of pushing a point too far it may be stated that the washed elementary bodies seemed to go to work somewhat more rapidly. The time table of changes effected followed a

close sequence of events and complete degeneration was attained about 10 days after inoculation. At the peak of the changes effected, the contrast between healthy and infected cultures was very striking. As is well known, eventually healthy cultures will also undergo degeneration and necrosis and the gross appearance of these changes may closely resemble the changes called cytopathogenic when referring to infected cultures. However, this normally happens several weeks after injury and destruction of cells in virus infected cultures takes place.

Specificity of virus action was observed in several experiments, where specific antiserum from patients known to have a high antibody titer to vaccinia, was allowed to act on the virus and apparently had a decelerating and, in some cases, completely inhibitory action. This aspect of the study, and the question of survival and quantitation of the virus in supernatant fluids, is being studied at present.

Conclusions. The cytopathogenic effect of vaccinia virus in roller tubes and Maximow double cover slip tissue cultures was established in its principal aspect of degenerative and pathological changes in cell morphology. Specificity of this effect was corroborated as far as possible at the present with coordinated serological tests and animal experiments. Further studies and analysis of visual changes by phase microscopy and photomicrography are planned.

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Eosinophile in Human Skin Homografting.* (20167)

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The revival of interest in skin homografting is evidenced by the increase in the number of papers which have appeared in the medical literature within the past several years. Many of these reports explain the sloughing of most skin homografts on the basis of an *acquired immunity reaction.* In reviewing the literature(1-4) it becomes apparent that skin homograftings have been permanently successful only between identical twins(5), or between members of the same family(6,7). That the compatibility of blood types between donor and recipient remains an important factor necessary for permanent survival of homografts is indicated by other recent reports (4,8,9). Extensive skin homograft research in animals has been described(10-15). Brown and McDowell(16) found "many eosinophiles" in biopsies taken of the sloughing homograft in split-thickness homografting. Split-thickness homografting in a child re-

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