DEVELOPMENT IN TISSUE CULTURES OF THE INTRACELLULAR CHANGES CHARACTERISTIC OF VACCINAL AND HERPETIC INFECTIONS

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Plates 29 to 31

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In a preliminary note (1)* a method of obtaining in tissue cultures intracellular changes characteristic of vaccinal and herpetic infections was briefly described. The purpose of the present paper is to give a detailed report concerning this method and some of the results secured through its use.

In 1906, Aldershoff and Broers (3), in order to prove that Guarnieri bodies are not derived from leucocytes, inoculated rabbit corneas with vaccine virus, immediately removed them from the animals, and incubated them in sterile serum at 37°C. This method was unsuccessful, the corneas becoming contaminated with bacteria which in a liquid medium interfered with the work. Thereupon the workers altered their procedure and placed the inoculated corneas, suspended in physiological salt solution, in humid chambers. After incubation for different periods of time, the tissues were fixed, sectioned, stained, and examined for the presence of vaccine bodies. Guarnieri bodies were found in corneas treated in this manner.

Gins (4), in 1916, using the coverslip method for the cultivation of vaccine virus in corneal cells, obtained what he considered Guarnieri bodies. Later (5), however, he stated that the bodies observed by him in 1916 were probably not vaccine bodies.

The majority of workers, including Steinhardt, Israeli, and Lambert (6), Harde (7), and Haagen (8), who searched for Guarnieri bodies in their tissue

* Shortly after this note was sent in for publication, one of the authors (Rivers) received a letter from Dr. C. H. Andrews regarding the appearance of inclusions in cultures of testicular tissue infected with Virus III. His observations and our findings were made independently and approximately simultaneously. A preliminary note (2) of his work appeared in Brit. Jour. Exp. Path., 1929, 10, 188.
cultures infected with vaccine virus, failed to find them. It also appears that no investigator has observed nuclear inclusions in tissue cultures infected with herpetic virus. Andrewes (2), however, in a recent report, has described the appearance of characteristic nuclear changes in testicular tissue infected with Virus III and cultivated in vitro.

Intracellular changes, "inclusion bodies," either cytoplasmic or nuclear, are characteristically associated with certain virus infections as they occur within the living host. These alterations, although their exact nature has not as yet been determined, are frequently used for diagnostic purposes. In spite of the fact that inclusion bodies occur in infected cells within the host, no observer, until recently (1, 2) has found them in tissues infected and grown in vitro. Inasmuch as virus infections, virus immunity, and even the nature of viruses themselves are poorly understood, it seemed that, if it were possible to devise a means of securing visible evidences of virus activity in tissue cultures, definite information might be found concerning many obscure points in this field of work. After a number of unsuccessful attempts, the following method of regularly obtaining Guarnieri bodies and herpetic nuclear inclusions in tissue cultures was evolved.

**Materials**

_Viruses._—Vaccine virus and the virus of herpes simplex were employed because the rabbit is equally susceptible to both. Furthermore, the vaccine virus produces cytoplasmic changes while that of herpes induces nuclear alterations. Thus, one virus served as a control on the other.

Levaditi's neurovaccine propagated in the testicles of rabbits, and Noguchi's testicular virus, were the strains of vaccine virus used. The herpetic virus chosen for the work was the H. F. strain isolated by Flexner and Amoss. In all experiments save one, freshly prepared virus emulsions without glycerol were employed. For constant results, one must conduct the experiments with fresh potent virus to which no preservative has been added.

_Tissues._—For this work, rabbit corneal tissue was chosen because of the following facts: 1) rabbit corneal epithelium is equally susceptible to the viruses of vaccinia and herpes simplex; 2) Guarnieri bodies and herpetic inclusion in cells of this tissue have been extensively investigated; 3) in the past, corneal cells have frequently been used for the in vitro cultivation of vaccine virus; 4) many studies concerning vaccinal and herpetic immunity within the animal have been conducted in the cornea of rabbits.
Procedure

A rabbit, under light ether anaesthesia, was exsanguinated from the heart by means of a syringe into which 1 cc. of a 1/1,000 heparin solution for each 10 cc. of blood had been previously drawn. Then, to obtain a tissue extract, the spleen was excised, minced, added to a small amount of Ringer's solution, and centrifuged. Finally, both eyes were enucleated intact, after stout silk ligatures had been placed around the nerve and large vessels.

The eyes, fixed in the grip of special forceps (Text-fig. 1) and suspended over a large Petri dish, were thoroughly washed with Ringer's solution to remove as many contaminating bacteria as was possible. With a sterile cataract knife the eyes to be used as controls were cross-hatched (Text-fig. 1) with closely spaced scarifications just deep enough to penetrate the epithelial layer. Test eyes were treated in a similar manner with the exception that prior to each scarifying stroke the knife was dipped in a virus emulsion. The cornea was then removed from each eye and divided into 4 to 8 pieces. The pieces from test eyes were further inoculated by immersion in an emulsion of virus for 1 to 3 hours at 37°C.

In some experiments antemortem inoculations were made. Following an instillation of a 2 per cent cocaine solution, the corneas were scarified and inoculated in the usual manner. 1 or 2 hours later the animals were sacrificed, the eyes were enucleated and washed with Ringer's solution, and the corneas were removed and divided into 4 to 8 pieces.

With the plasma, spleen extract, and bits of cornea, cultures were set up. Into a sterile 50 cc. pyrex centrifuge tube 2 or 3 cc. of plasma were introduced, to
which a few drops of tissue extract were added to induce clotting. On top of each clot was placed 1 piece of normal or infected cornea which was then covered with another thin clot of plasma (1 cc.) and spleen extract (Text-fig. 2). The tubes, sealed with sterile corks, were placed in an incubator at 37°C. for 24, 48, and 72 hours.

After incubation the clots, containing their bits of cornea, were removed from the tubes, fixed in Zenker's fluid, embedded in paraffin, sectioned, and stained according to Giemsa's method or with eosin and methylene blue. Phloxine at times was substituted for eosin. Then many sections from each block of tissue were examined for the presence of characteristic inclusions. As controls for the presence of active virus in the cultures, preparations similar to those studied histologically were emulsified and tested in the skin of rabbits.

RESULTS

Numerous observations have been made on the virus of vaccinia, and 2 sets of experiments have been performed with herpetic virus. Inasmuch as corneal tissue from adult rabbits was used, one might suppose that contaminating bacteria would have interfered with the work. Many of the tubes, however, showed no evidence of bacterial contaminations; others contained only a few colonies of organisms that did not disturb the activity of the viruses; and a few were
sufficiently infected with bacteria to be valueless. To circumvent the
invalidation of results by bacterial contaminants, experiments were
always set up in duplicate and occasionally in triplicate.

In this work Levaditi’s strain of neurovaccine produced satisfactory
lesions more consistently than did Noguchi’s testicular virus. Al-
though on one occasion positive results were obtained with a glyc-
erolated virus, it seemed advisable to use a freshly prepared virus
emulsion for each experiment. Corneas infected in vivo and in vitro
showed excellent lesions regularly. The latter, however, were less
frequently contaminated.

In each experiment, bits of cornea were fixed for examination after
24 and 48 hours of incubation. Characteristic lesions with inclusion
bodies were found in infected tissues after both periods of incubation.
If the virus were very potent, however, and if the inoculation were
highly successful, 24 hours post-inoculation was found to be a suitable
time for examination of the tissues, otherwise 48 hours proved to be
more favorable. Some preparations were allowed to remain at
37°C. for 3 days. In these the infected epithelium was extensively
disintegrated and unsuitable for histological examination.

Control tissues always revealed definite evidence of growth of
epithelial cells in the form of new epithelium filling in the defects
caused by scarification (Fig. 13). At times mitotic figures were
observed (Fig. 7). Frequently the tissues appeared to be almost as
normal as those treated in a similar manner with the exception of
being allowed to remain in the animal. If the inoculations were
made in vitro, the absence of leucocytes was striking. Significant
inclusions were never seen in the controls.

Corneas inoculated with vaccine virus showed either discrete
lesions separated by normal tissue or a diffuse infection involving all
the epithelium. At the sites of infection the epithelium was thickened
(Figs. 3, 6, 11, 14) because of an increase both in the number of cells
and in the size of individual cells. Numerous typical Guarnieri
bodies (Figs. 8, 9, 11, 12, 14) situated in clear spaces within the cyto-
plasm of epithelial cells were found. These intracellular changes
occurred both in the cells present at the time of inoculation and in the
new cells (Fig. 12) growing in to fill the defects produced in the corneal
epithelium by scarification. On several occasions cells undergoing
mitosis were observed to contain vaccine bodies (Fig. 9). In addition to the Guarnieri bodies, smaller, irregular, basophilic structures that were considered to be Paschen bodies at times studded the cytoplasm of many cells. The end result of the vaccinal infection as studied under the conditions of these experiments was a complete dissolution of the majority of the involved cells (Fig. 3). By testing the material on the skin of normal rabbits, active vaccine virus was demonstrated in the cultures exhibiting characteristic lesions.

The herpetic virus produced striking changes in the infected corneas. In certain areas (Figs. 4, 15) the increase in the thickness of the epithelium produced hummocks. The intracellular bridges disappeared, the cells were swollen, many amitotic giant cells appeared, and numerous typical acidophilic nuclear inclusions were present (Fig. 15). Structures resembling Guarnieri bodies were never seen.

DISCUSSION

The in vitro production of pathological pictures similar to those caused in vivo by infectious agents has rarely been accomplished. Maximow (9, 10) and Lang (11), however, have reported that certain tissues cultivated and infected in vitro with tubercle bacilli evince pathological changes resembling those observed in infected hosts. In spite of the fact that the viruses of vaccinia and herpes simplex can be cultivated in tissue cultures, no one has been able previously to show definitely that characteristic cytologic lesions of these diseases occur in such cultures. Therefore, the method described above is the first one evolved by which typical vaccinal and herpetic lesions may be regularly produced in tissues inoculated and cultivated in vitro. Rabbit corneas handled in a special manner were used for this purpose. In view of Andrew's (2) work, it seems that other viruses and other tissues may be employed for similar studies. The paper that immediately follows indicates that such methods offer a new means of studying and analyzing virus infections and virus immunity.

SUMMARY

Characteristic vaccinal and herpetic lesions, including Guarnieri bodies and acidophilic nuclear inclusions respectively, regularly occur in rabbit corneas infected and cultivated in vitro according to the method here described.
BIBLIOGRAPHY


EXPLANATION OF PLATES

PLATE 29

Fig. 1. Section of a cornea immediately after in vitro scarification and inoculation. Note the breaks in the epithelial cells induced by scarification. Giemsa. × 155.

Fig. 2. Section of a bit of normal cornea after cultivation for 48 hours in normal plasma and spleen extract. Eosin and methylene blue. × 155.

Fig. 3. Section of a 48-hour vaccinal lesion in a cornea infected and cultivated in vitro. Note thickening of the layer of epithelial cells which are beginning to disintegrate. Compare with Figs. 1 and 2. Eosin and methylene blue. × 155.

Fig. 4. Section of a 48-hour herpetic lesion in a cornea infected and cultivated in vitro. Giemsa. × 155.

Fig. 5. Corneal epithelial cells 2 hours after inoculation with vaccine virus. Compare with Fig. 6. Eosin and methylene blue. × 380.

Fig. 6. Corneal epithelial cells 48 hours after in vitro inoculation with vaccine virus and cultivation in normal plasma. Compare with Fig. 5. Eosin and methylene blue. × 380.
FIG. 7. Mitotic figure in a 48-hour corneal culture. Giemsa. \( \times 1,000 \).

FIG. 8. Section of a 48-hour corneal culture infected with vaccine virus. Note Guarnieri bodies and mitotic figure. Giemsa. \( \times 1,000 \).

FIG. 9. Section of a 48-hour corneal culture infected with vaccine virus. Note Guarnieri bodies, cell with mitotic figure and Guarnieri body, and beginning dissolution of the epithelial cells. Giemsa. \( \times 1,000 \).

PLATE 30

FIG. 10. Section of a cornea infected (2 hours) with vaccine virus \textit{in vivo}. Note the defect caused by scarification. A bit of the cornea was fixed as a control immediately after removal from the rabbit. Compare with Figs. 11 and 12. Giemsa. \( \times 380 \).

FIG. 11. Section of a piece of cornea similar to that shown in Fig. 10, with the exception that it was cultivated \textit{in vitro} for 48 hours. Note the difference in thickness exhibited by the layers of epithelium in the two figures. Insert (\( \times 1,000 \)) shows a Guarnieri body in one epithelial cell engulfed by another. Giemsa. \( \times 380 \).

FIG. 12. Section of a piece of cornea similar to that shown in Fig. 10, with the exception that it was cultivated \textit{in vitro} for 48 hours. Note that the defect in the layer of epithelial cells has been filled in with new cells, many of which contain Guarnieri bodies. Insert (\( \times 1,000 \)) shows a Guarnieri body in one cell engulfed by another. Giemsa. \( \times 700 \).

PLATE 31

FIG. 13. Section of a bit of normal cornea cultivated for 48 hours in normal plasma and spleen extract. Note growth of cells filling in defects caused by scarification. Eosin and methylene blue. \( \times 225 \) and \( \times 1200 \).

FIG. 14. Section of a 48-hour culture of a vaccine virus infected cornea. Note the thickening of the layer of epithelial cells, many of which contain vaccine bodies. Giemsa. \( \times 225 \) and \( \times 1200 \).

FIG. 15. Section of a 48-hour culture of a herpes virus infected cornea. Note hummock formation, amitotic giant cells, and acidophilic nuclear inclusions. Giemsa. \( \times 225 \) and \( \times 1200 \).