nounced, was still significant in concentrations of the antibiotic which were considerably lower than the therapeutic blood levels ordinarily employed in clinical practice.

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## Cytopathogenic Effect of Poliomyelitis Viruses *In vitro* on Human Embryonic Tissues.* (18202)

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We have previously described the cultivation of the Lansing and Brunhilde strains of poliomyelitis virus in suspended cell cultures of human non-nervous and nervous tissue (1,2). It was also noted that degenerative changes occurred in cells from cultures inoculated with the Lansing virus(1) that were more extensive than in tissues from uninoculated cultures. This phenomenon has been more extensively investigated. The most significant observations will be here summarized; a more detailed account will be published later.

*Materials and methods. Suspended cell cultures.* The procedures have been previously described(1,3). As routine 50 units of penicillin and 50 μg of streptomycin per cc have been included in the medium. *Plasma hanging drop cultures.* Fragments of tissue from suspended cell cultures were explanted in plasma hanging drop cultures. The medium in which each fragment was embedded consisted of 2 drops of heparinized fowl plasma, one drop of chick embryo extract and one drop of beef embryo extract. Usually 8 cultures were prepared in a petri dish(4). This method permits the study of large numbers of fragments with economy of time and effort. *Roller tube cultures.* The general method has been presented in detail(5). The medium used in these experiments consisted of 9 parts of the Hanks-Simms solution adopted as routine for the suspended cell cultures(1,3) and 1 part of beef embryo extract. Two cc of this mixture was employed in each culture. *pH determinations.* The pH of suspended cell cultures was determined colorimetrically by direct comparison of each culture with a set of standard buffer solutions. The latter were prepared in 25 cc Erlenmeyer flasks each containing 3 cc of solution and the same concentration of phenol red as the Hanks-Simms solution used as culture medium. *Sections of tissue fragments from suspended cell cultures.* At the end of an experiment, Zenker's solution without acetic acid was added to the fragments in each flask. After fixation the fragments from one set of cultures were combined, embedded and stained with hematoxylin and eosin. *Viruses.* The Lansing and Brunhilde strains of poliomyelitis virus have been used(1,2). *Antisera.* Specific monkey antiserum for the Brunhilde strain was obtained through the courtesy of Dr. Jonas Salk. Lansing immune serum was prepared in this laboratory by repeated intramuscular injection of a monkey with infected monkey cord. This serum diluted 1:1000 completely protected mice against 1-3 $LD_{50}$ of virus, but failed to protect in a dilution of 1:5000.

*Experimental. Changes in cell morphology*

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*in fragments from suspended cell cultures.* Sections of fragments from 12 serial cultures of the Lansing virus in human embryonic skin-muscle have been examined along with material from 11 successive cultures of another comparable series inoculated with the same virus. Sections from cultures to which no virus was added were, in every case, compared with the corresponding inoculated material. The results were as follows: Eleven cultures included in these 2 series were maintained for periods approximating 30 days (mean period of cultivation 29 days, range 21-32 days). In sections from these, few or no well preserved cells were seen. The changes observed consisted of loss of typical staining properties, nuclear pyknosis and fragmentation of cells. In contrast normal-appearing well-stained cells were present in one or more of the fragments that had not been exposed to the virus. The number of such cells varied but, in all but one instance, were recorded as fairly numerous to numerous. The cells that regularly presented the best state of preservation in uninfected cultures resembled young fibroblasts with large oval nuclei staining pale blue and containing many small chromatin granules. Moreover, the nuclei of muscle cells in many cases appeared to be in good condition. When the period of exposure to the virus was shorter, *i.e.* 16-20 days, differences between inoculated and uninoculated tissue were not always apparent. In certain experiments, however, even by the 16th day of cultivation sections of uninoculated fragments could be easily distinguished from those of infected tissue.

A comparable experiment was carried out with the Brunhilde strain. Sections of embryonic skin-muscle fragments from 10 successive cultures were studied. With the exception of material from the first and seventh culture, cellular degeneration was more marked in the infected fragments. Essentially similar results were recorded in 10 serial cultures of human embryonic intestine inoculated with the Lansing virus.

Examination of material from flask cultures of human embryonic brain inoculated with Lansing and Brunhilde viruses on the whole revealed more extensive cellular degeneration than was noted in control material. The differences, however, in some cases were not so distinct as with the other tissues.

*The inhibition of cell migration.* Tissue fragments in which certain other viruses have been propagated fail to exhibit migration or peripheral growth of cells characteristic of normal tissues in plasma cultures(4,6,7). In view of these observations and our histologic findings the migratory capacity of cells removed from flask cultures of virus was determined. Plasma hanging drop cultures were prepared with fragments removed from flask cultures at intervals following inoculation of virus. Control cultures were made with uninoculated fragments. Skin-muscle tissue from embryos of not over four months gestation has alone yielded, with regularity, a zone of migrating or growing cells sufficiently dense to provide a reliable indicator system. Other tissues examined were embryonic brain and intestine, as well as foreskin and kidney from young children. The results of a number of experiments were essentially the same. Fragments of skin-muscle from flasks in which virus multiplication was demonstrated by animal inoculation either failed to show cell migration or, if this occurred, it was scanty and within 5 days the emigrating cells exhibited pronounced degenerative changes. In contrast nearly all fragments from control uninoculated flasks developed within 48-72 hours well defined zones of normal-appearing cells which continued to increase and presented a normal appearance for at least 7 days. The effect of the virus on cell migration was not clearly demonstrable until the flask cultures had been maintained for 8 to 21 days.

In Table I are summarized the results of an experiment in which the minimal quantity of virus was determined that exerted a definite inhibitory action on cell migration. Each of a group of 3 flask cultures of human embryonic skin-muscle tissue were inoculated with 0.1 cc of a dilution of the Lansing or Brunhilde strain. After these cultures had been

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7. Plotz, H., and Ephrussi, B., *Compt. rend. Soc. Biol.*, 1933, v112, 525.

TABLE I. Inhibition of Cell Migration in Plasma Explants of Tissues from Flask Cultures Infected with Poliomyelitis Virus.

| Virus strain | Dilution of virus inoculated into flask cultures | | | | | |
|---|---|---|---|---|---|---|
| | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | No virus |
| Lansing* | 0/6‡ | 0/6 | 1/6 | 2/6 | 6/6 | 10/10 |
| Brunhilde† | 0/6 | 0/6 | 0/6 | 5/6 | 6/6 | 10/10 |

* Inoculum: infected mouse brain.

† Inoculum: pooled supernatant fluids from 9th flask subculture in skin-muscle.

‡ No. explanted fragments showing normal cell migration after 5 days incubation / Total No. fragments explanted from flask cultures

TABLE II. Cytopathogenic Effect of Brunhilde Virus in Roller Tube Cultures of Human Embryonic Skin-Muscle.

| Dilutions of Brunhilde virus inoculated. | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $10^{-2}$* | | $10^{-3}$ | | $10^{-4}$ | | $10^{-5}$ | | No virus | | | |
| + | + | + | + | + | + | + | 0 | 0 | 0 | | |

* 2 cultures were inoculated with each dilution of virus.

\+ Culture showing widespread destruction of cells the 5th day after inoculation.

0 = Culture showing normal appearing active cell growth the 5th day.

maintained in the usual manner for 22 days, tissue fragments were removed and plasma hanging drop cultures prepared. From the table it is evident that both viruses, when diluted $10^{-4}$ and $10^{-3}$ respectively affected normal cell migration. In the case of the Lansing strain, the pooled fluids removed from the flasks inoculated with $10^{-4}$ dilution produced paralysis and death in mice whereas those from the cultures inoculated with $10^{-5}$ dilution caused no disease. With the Brunhilde virus, similar confirmatory experiments in animals have not yet been performed.

The effect of the virus on cell migration revealed by the explant method suggested that the roller tube technic which provides large numbers of actively growing cells might furnish a more convenient and rapid procedure for the demonstration of cell injury. Roller tube cultures of human embryonic skin-muscle were prepared and after 4 days incubation at 37°C were each inoculated with 0.1 cc of a dilution of Brunhilde tissue culture virus. Two roller tube cultures were inoculated with each dilution of virus. Uninoculated cultures were included for comparison. Incubation was continued and the cultures examined daily. The findings on the 5th day after inoculation and which were constant thereafter are summarized in Table II. Lansing tissue culture virus appears to behave in a similar manner in roller tube cultures. These observations indicate that poliomyelitis virus adapted to growth in human tissues rapidly destroys the cells growing out from human skin-muscle.

*Effect of virus on tissue metabolism as indicated by decreased acid production.* The degenerative changes in the infected cells suggested that cellular metabolism might be impaired. Furthermore, in the case of tissues infected with certain other viruses a diminution in acid production and in their capacity to reduce methylene blue have been described (8,9).

Experiments have shown that acid production in flask cultures of human embryonic brain, intestine and skin-muscle inoculated with Lansing and Brunhilde strains, after a variable interval, declines more rapidly than in control cultures without virus. The time after addition of the virus at which significant differences become apparent has been found to vary according to the amount of virus inoculated and the lot of tissue employed. Moreover, the pH of individual cultures inoculated with the same material may occasionally vary within a fairly wide range. A frequent cause of this variation is failure to insert the stopper tightly. As an example, pH readings recorded in one experiment are given in Table III. Various dilutions of a suspension of mouse brain infected with Lansing virus were inoculated into a series of flask cultures of human embryonic brain. Each figure represents the average of readings of the pH of the medium in three flasks at the end of periods of 3 to 5 days before the addition of fresh medium. By the 15th day the cultures inoculated with the three lowest dilutions exhibited a pH differential of 0.2 to

8. Huang, C. H., PROC. SOC. EXP. BIOL. AND MED., 1943, v54, 1943.

9. Plotz, H., *Compt. rend. Soc. Biol.*, 1935, v118, 1400.

TABLE III. Determinations of the pH of the Fluid Phase of Flask Cultures of Human Embryonic Brain Inoculated with Lansing Virus.

| Dil. of virus† inoculated | Days after inoculation of culture | | | | | | |
|---|---|---|---|---|---|---|---|
| | 3 | 7 | 11 | 15 | 20 | 24 | 28 |
| $10^{-1}$ | 6.8* | 6.8 | 6.9 | 7.1 | 7.2 | 7.3 | 7.3 |
| $10^{-2}$ | 6.8 | 6.8 | 6.9 | 7.1 | 7.2 | 7.3 | 7.3 |
| $10^{-3}$ | 6.8 | 6.8 | 6.8 | 7.0 | 7.1 | 7.3 | 7.3 |
| $10^{-4}$ | 6.8 | 6.8 | 6.8 | 6.9 | 6.9 | 7.2 | 7.3 |
| $10^{-5}$ | 6.8 | 6.8 | 6.8 | 6.8 | 6.8 | 6.8 | 6.9 |
| No virus | 6.8 | 6.8 | 6.8 | 6.8 | 6.8 | 6.8 | 6.9 |

* Avg of individual pH readings of the medium in three flasks. Supernatant fluids were removed and fresh medium added on the days indicated. Readings were made just before this was done. The pH of the fresh medium varies from about 7.7-7.5.

† The virus consisted of a suspension of infected mouse brain. Three cultures were each inoculated with 0.1 cc of each dilution.

TABLE IV. Specific Inhibition by Immune Serum of the Cytopathogenic Effect of Poliomyelitis Virus as Indicated by Cell Migration.*

| Virus inoculum | Type of serum | | |
|---|---|---|---|
| | Lansing immune monkey | Brunhilde immune monkey | Normal monkey |
| Lansing† | 12/12‖ | 0/12 | 0/12 |
| Brunhilde‡ | 0/12 | 12/12 | 0/12 |

* The technic employed was as follows: 0.1 cc of serum inactivated at 56°C for 30 min. was added to each flask culture of human embryonic skin-muscle. One-half hour later 0.1 cc of virus inoculum was added to each flask. The cultures were maintained in the usual manner. Explant preparations were made on the 26th day after inoculation of the flasks.

† Inoculum: undiluted pooled supernatant fluid of 13th subculture of Lansing virus in skin-muscle flask cultures.

‡ Inoculum: undiluted pooled supernatant fluid of 9th subculture of Brunhilde virus in skin-muscle flask cultures.

‖ No. explanted fragments showing normal migration after 5 days incubation / Total No. fragments explanted from flask cultures

0.3 as compared with the controls. We have regarded as significant a difference of 0.2. By the 20th day those inoculated with dilution $10^{-4}$ likewise showed a significant difference. No differential was observed in the cultures receiving $10^{-5}$ dilution. Intracerebral injection of mice with the pooled fluids removed on the 15th day showed that virus was present in the cultures inoculated with virus dilutions $10^{-3}$ and $10^{-4}$ but not with $10^{-5}$. It should be pointed out that the endpoint of viral activity obtained by the explant method as recorded in Table I agrees with that based on pH differential. In general this criterion for the presence of virus in cultures has proved useful and reliable, although in a few instances a significant difference has not been recorded during the period of observation.

*Specific inhibition of cytopathogenic effect of virus by immune serum.* To obtain additional evidence for the specificity of the degenerative changes noted in infected tissues, the effect of the addition of specific immune serum was determined. Two procedures were employed. In the first, virus and serum were added to flask cultures of skin-muscle and, after incubation, tissue fragments were explanted to plasma drop cultures. In the second, virus and serum were added to freshly prepared roller tube cultures of skin-muscle.

In Table IV the results of an experiment carried out by the first method are presented. It is evident that (a) normal monkey serum fails to protect the cells against the cytopathogenic action of the virus; (b) the antiserum prevents the cytopathogenic effect as indicated by the capacity of the cells to migrate in a normal manner; (c) the effect of the antiserum is type specific.

In this series to which the Lansing strain was added virus was demonstrated by mouse inoculation in the supernatant fluids of cultures containing normal and Brunhilde immune serum but not in those from cultures containing homologous immune serum. Permanent inhibition of viral multiplication by homologous antiserum, however, has not always been observed in this type of experiment.

An experiment in roller tube cultures with Brunhilde virus and antisera against this virus and the Lansing strain has demonstrated a similar capacity of the homologous serum to prevent cell degeneration. Clear cut results were obtained by this method within 5 days after inoculation of the cultures.

Since this inhibitory effect of antiserum appears to be type specific it can be concluded that the degenerative changes observed were produced by the action of the virus on the cells.

*Summary and discussion.* The experiments which have been described demonstrate the capacity of the Lansing and Brunhilde strains of poliomyelitis virus to cause cell injury and death. This cytopathogenic property is revealed (1) by degenerative changes produced in infected tissue fragments in flask cultures and apparent on histologic examination; (2) by failure of such tissue fragments to exhibit normal cell migration when explanted to plasma cultures; (3) by degeneration of newly emigrated cells in roller-tube cultures; (4) by decreased acid production by infected cells. The conclusion that certain of these manifestations of injury are induced as a result of infection by the virus is further supported by the fact that type specific immune serum prevents their development. These phenomena are of interest from two general points of view. First, they leave no doubt that poliomyelitis virus *in vitro* can multiply in cells other than those of the nervous system and cause profound injury of such cells. Secondly, they provide criteria by which the presence of the virus can be recognized *in vitro* and hence may afford a basis of technics for isolating virus from patients or animals, for the quantitative assay of virus, for serologic typing and possibly for the screening of chemotherapeutic and antibiotic substances. Further study will be required of the reliability and practicability of the application of these phenomena to such ends.†

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## Biological Studies on *Entamoeba histolytica*. IV. Direct Action of the Antibiotic, Prodigiosin.* (18203)

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Antibiotics have been applied against *Entamoeba histolytica* as part of the search for more effective chemotherapeutic agents. Although various accepted antibiotics (penicillin, streptomycin, aureomycin, chloromycetin, etc.) have been tested *in vitro*, no clear-cut evidence has been presented of their direct action upon the amoebae. A recent report from Anderson's laboratory aptly summarizes the situation: "All antibiotics tested to date have shown some degree of bacteriostatic activity, and death of the ameba in cotton-stoppered tubes is shown to be due to inhibition of growth of the associated bacteria rather than to direct action of the antibiotic on *E. histolytica*"(1). The present experiments were designed to meet these objections, and the data presented below are submitted as the first proof of direct action by an antibiotic on *E. histolytica*.

Our original interest in prodigiosin sprang both from the long-known antagonism exhibited by chromogenic strains of *Serratia marcescens* against various microorganisms, and the feasibility of using the corresponding antibiotic pigment, prodigiosin, whose extraction and structure had been elucidated by Wrede and co-workers(2). Antibacterial activity has been demonstrated against gram-positive types, including *Bacillus anthracis* (3), *Corynebacterium diphtheriae* and *Neisseria gonorrhoeae*(4), *Pasteurella pestis*(5),

* Part of a project supported by a research grant from the National Institutes of Health, Public Health Service.

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