Effects of a Podophyllotoxin Derivative on Tissue Culture Systems in Which Human Cancer Invades Normal Tissue*

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In the search for clinically effective antitumor agents, numerous biologic systems have been considered as possible screening devices (4). Some investigators have preferred test systems with human tumors, with the thought that these may provide the greatest possibility of correlation with clinical cancer. Experiments have been conducted with chemical agents and human cancer in several ways, e.g., in the patient, in heterologous animal transplant, and in tissue culture. This report describes the development and application of methods in which cancer invades normal tissue in sponge-matrix tissue culture for the evaluation of chemotherapeutic agents.

Tissue culture approaches in experimental medicine have received increased attention recently as an outgrowth of the simplifications of methods, the ready commercial availability of a variety of media, the development of many lines of cells derived from human tissue, and, finally, the conclusive demonstration of the usefulness of such technics in the fields of virology and cell nutrition. Of great interest is the approach of Eagle and Foley (3). With almost completely defined nutritional conditions and with methods that permit quantitative studies on the nutrient as well as on the cell population, they have added potential chemotherapeutic agents to the medium and have observed their toxic effect upon the growth and nutrition of various cell lines.

In our work, several lines of cells derived from human cancer, when inoculated into cultures of normal human or chick embryonic tissues, have been seen to infiltrate and replace the normal tissues. These observations have given rise to the interesting possibility that significant indices of drug effect might be observed relating to the phenomena of invasiveness itself, in addition to the classical (and perhaps too limited) index of cytotoxicity alone. Such a possibility stems from the fact that combinations of tumor and normal tissue growing in sponge matrix provide a histologic picture comparable in many details to that seen in sections taken from the patient, in which cancer cells may be observed invading and replacing normal tissues. Among the indices that are theoretically observable as expressions of a chemical agent’s effect upon a host-tumor interaction are reduced rate of invasiveness, maturation or differentiation of the tumor, and responses of the host that limit the growth of the tumor, mechanically or otherwise.

Practical considerations govern the realistic use, for chemotherapeutic studies, of host-tumor interaction in sponge-matrix tissue culture. The expense involved in preparing serial histologic sections of many cultures in a drug screening project would make the use of such preparations almost prohibitive. A variation in method was required that could lend itself to the detection of promising chemical agents at a screening level. Once this technical problem was solved, a chemical agent selected on the basis of its activity in the screening could be studied by the sponge-matrix method in which serial sections are prepared. In this way differentiation and host responses limiting tumor invasion could be observed at a morphologic level. These two properties would, for the present, lend themselves to only the crudest quantitative meas-

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† Strain HeLa (Gey) from carcinoma of the cervix, Strain KB (Eagle) from oral carcinoma, and Strain J-96 (Osgood) from monocytic leukemia.
† Normal human fetal liver, adrenal, bone, lung, and brain, and adult heart and synovia.
urements. One factor, however, that might be measured is the rate of invasion, or at least the total amount of invasion, in a standard time interval. Experiments were conducted to develop a systematic approach to drug evaluation with these considerations in mind.

The drug acetylpoodylloletoxin-2-5-pyridinium chloride (NCI-3022), a water-soluble derivative of podophyllotoxin, was used in our studies. The source material podophyllin has exhibited an impressive capacity for inducing extensive necrosis in transplantable animal tumor test systems in vivo (2), as well as in tissue culture (11, 12). In addition to observing its effect on malignant and nonmalignant human cells in vitro, on single and on repeated applications, we used it as a tool in the development of sponge-matrix methods that would make the study of chemical agents on cancerous invasion practicable.

EXPERIMENTAL

MATERIALS

NCI-3022.—Ten mg. of the dry powder was dissolved in 100 ml. of glass-distilled water just before use and sterilized by filtration through a Bush-type fritted glass filter. The drug was then diluted with glass-distilled water to 10 times the desired final concentration. One part of this diluted NCI-3022 was combined with 9 parts of regular liquid nutrient so that the desired final concentration could be obtained. Controls consisted of 1 part of distilled water and 9 parts of medium.

HeLa.—Strain HeLa, derived from an epidermoid carcinoma of the uterine cervix in 1947 by Gee (5), grows readily in sponge matrix with the histologic appearance of a poorly differentiated epidermoid carcinoma. Stock cultures of HeLa were maintained in Gelfoam particle suspension (8). Gelfoam fragments were used as particulate explants and added to established host cultures in cellulose sponge. Gelfoam has a characteristic microscopic appearance that distinguishes it from cellulose sponge and serves to mark the site of tumor implantation.

Strain D.—The genealogy of Strain D and the method of culture have been described (10). It is a connective tissue growth. (In the living culture, tumor

Methods

Thick sponge.—The method with which we have worked most extensively is one in which fragments of tissues are cemented to a rectangular slice of cellulose sponge by means of a plasma clot. The sponge slice measures approximately 12.0 × 6.0 × 1.0 to 0.5 mm. A culture of host tissue is established in this matrix in a roller tube, and masses of tumor cells are implanted in particulate form as fragments of Gelfoam matrix. These are cemented to a selected site on the host cellulose sponge with a plasma clot. The cultures are fed as indicated by changes in the pH of the medium. Microscopic examination of the living cultures is limited by the dense growth in the 0.6-mm. thick sponge. However, some information is obtained by inspecting the growth in the larger interstices as well as the growth in the plasma clot around the margins of the sponge. For detailed examination of the growth within the sponge, serial histologic sections are prepared and stained as desired.

Thin sponge.—A slice of cellulose sponge, 100 μ thick, is cemented to an 11 × 22 mm. coverslip with 1 percent Formvar and used in a flat tube (8). Host cultures of connective tissue are prepared by cementing several explants to the sponge surface with plasma clot. After good outgrowth has been established, particles of Gelfoam containing tumor are placed wherever desired—at the edge of the connective tissue outgrowth, as in this study, or on a bed of dense connective tissue near the original tissue explant. Living cultures on these thin sponge slices can be studied microscopically more easily than the thick sponge cultures. When fixed, stained, and prepared as total mounts, the over-all pattern of host and tumor growth, as well as the zone of their interaction, can be examined. When the fixed, stained preparation is being observed, it is often desirable to examine the surface that has been in direct contact with the medium during the life of the culture. This can be done easily by mounting the coverslip onto a larger coverslip. When the mounting medium hardens, the entire preparation can be reinforced by cementing the large coverslip over a piece of firm, punched cardboard or a large glass slide in which a hole has been drilled. Such preparations permit convenient examination of both surfaces of the culture.

Sponge ribbon.—A narrow strip of sponge 0.5–1.0 mm. thick, 35 mm. long, and 5–8 mm. wide was used in an effort to measure the progress of the tumorous invasion of the normal connective tissue. The sponge first received an extensive inoculation of connective tissue explants. When the outgrowth of connective tissue was luxuriant, tumor-bearing particles of Gelfoam were implanted at one end of the ribbon, and the progress of the tumor along the course of the ribbon was observed (see Chart 1). In some instances plasma clot alone was used to keep the sponge in place. In other cases the sponge was cemented to the wall of the tube with Formvar before the cultures were prepared.

Observations

The details of the experiments are arranged in tabular form (Table 1).

Series I: single application of the drug (thin sponge).—Twelve cultures of connective tissue from 9-day-old chick embryo heart were prepared in thin sponges which had been previously cemented to coverslips with Formvar. Gelfoam particles containing HeLa cells were added after 5 days and were placed at the edge of the established connective tissue growth. In the living culture, tumor

cells were seen to leave the Gelfoam inoculum in increasing numbers, growing radially, and infiltrating the connective tissue where the two kinds of cells met. On the 11th day after the addition of the HeLa cells, growth of the tumor cells into the connective tissue was extensive; the drug NCI-3022 was then applied in 1 ml. of fresh medium in the doses indicated in Table 1.

Six hours after the addition of the drug-medium mixture, many enlarged spherical HeLa cells containing scattered chromosomes could be identified. The cultures were fixed at this time in Zenker’s formalin and subsequently stained with hematoxylin and eosin, dehydrated, cleared, and mounted.

The drug had a dramatic cytologic effect at all concentrations studied (Figs. 1-3). Even at greatest dilution, 1 part in 10 million, disrupted metaphase figures almost completely replaced the usual bipolar and multipolar metaphase structure seen in dividing HeLa cells. The chromosomal material was irregularly clumped and was either splattered throughout the cytoplasm or appeared as an eccentric, coalescing, basophilic mass just beneath the cell membrane. The involved cells were swollen and irregularly vacuolated. In concentrations of 1 part in 1 million or higher, no normal dividing cells could be found. Cells showing this cytoplasmic pattern after exposure to podophyllin have been described by King and Sullivan (7). The presence of the scattered chromatin as diplococcus-like groups in many HeLa cells suggests that the drug affected mitosis after cleavage of the chromosomes. The percentage of cells involved in mitotic arrest was obviously much larger than the percentage of cells in normal division in the controls, although cell counts were not made. The connective tissue cells from the chick heart exhibited the same response to the drug that the tumor cells showed in all the concentrations studied.

**Series II: single application of the drug (thick sponge).**—Two cultures were prepared in thick sponge with the use of 9-day-old chick heart and HeLa as in Series I. One of them was used as a control. The other was given a dose of 1 μg/ml of the drug and was fixed after 6 hours of exposure. Microscopic sections showed the presence of the drug effect on dividing cells of both the host and the tumor through all levels of the thick sponge culture. This ruled out the remote possibility that the drug might be acting exclusively on the surfaces of the culture rather than on cells at all levels within the sponge (Figs. 4 and 5).

The abnormalities in cell division were confined specifically to treated cultures. In both the treated and the untreated cultures, however, a nonspecific degeneration was seen in areas with large populations of HeLa cells. In this degeneration nuclear pyknosis and shrunken, condensed, eosinophilic cytoplasm were present. Although there appeared to be somewhat more degeneration in the treated culture, this was uncertain.

Much of the outgrowth of connective tissue from the chick embryo heart consisted of organoid structures made up of endothelial-lined channels in an angiomatous pattern (Figs. 7 and 8). Such vascular structures were seen at all levels of the cultures, and connective tissue adjacent to these channels was infiltrated by occasional tumor cells. Nothing was found to suggest that the drug had a direct effect upon the endothelium.

**Series III and IV: single application of the drug**

There is evidence from some of our other tissue culture studies that the capillary outgrowth from some normal tissues is capable of morphologic changes in the presence of chemical substances. For instance, we have observed definite thickening, as well as other alterations, in the capillary walls of the outgrowth from normal, adult, human synovia upon the addition of 100 μg/ml of hydrocortisone (Solu-Cortef, Upjohn Company) to the medium.
and recovery (thick sponge).—The recovery from
the effect of a single 6-hour exposure to NCI-3022
was examined in two series of cultures with a dif-
ferent host connective tissue in each. In Series III
the host was connective tissue from chick embry-
onic heart; in Series IV it was Strain D. In each
series the concentration of the drug was 1 µg/ml.
Since the response observed in both interacting
systems was the same, they are described together.

Many HeLa cells
were found to be in all stages of mitosis, with con-
figurations that are normal for HeLa. In addition,
degenerating cells with scattered or coalescing
chromosomes were seen, some of which were the
same as those found immediately after a 6-hour
exposure to the drug (Fig. 9). Most of the degener-
crating cells could not be so definitely character-
ized and were similar to those ordinarily seen in

<table>
<thead>
<tr>
<th>Series and method</th>
<th>No. cultures</th>
<th>Host tissue</th>
<th>Age of host culture when HeLa added (days)</th>
<th>Age of interacting culture when drug added (days)</th>
<th>µg/ml of NCI-3022</th>
<th>Period of exposure to drug (days)</th>
<th>Recovery period (days)</th>
<th>Total age of interacting culture (days)</th>
</tr>
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<tr>
<td>I</td>
<td>1</td>
<td>9-day-old chick embryo heart</td>
<td>5</td>
<td>11</td>
<td>1000</td>
<td>6 hr.</td>
<td>none</td>
<td>11</td>
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<tr>
<td>Single application: thin sponge</td>
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<td>9-day-old chick embryo heart</td>
<td>14</td>
<td>8</td>
<td>1</td>
<td>6 hrs.</td>
<td>none</td>
<td>8</td>
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<tr>
<td>II</td>
<td>1</td>
<td>Strain D</td>
<td>2-3 mo.</td>
<td>14</td>
<td>1</td>
<td>6 hr.</td>
<td>none</td>
<td>6</td>
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<tr>
<td>Single application and recovery: thick sponge</td>
<td>2</td>
<td>2-3 mo.</td>
<td>21</td>
<td>none</td>
<td>21</td>
<td>27</td>
<td></td>
<td></td>
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<tr>
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<td>28 days</td>
<td>1</td>
<td>0.01</td>
<td>21 days</td>
<td>none</td>
<td>22</td>
</tr>
<tr>
<td>IV</td>
<td>2</td>
<td>Strain D</td>
<td>74 days</td>
<td>2</td>
<td>1</td>
<td>27 days</td>
<td>none</td>
<td>29</td>
</tr>
<tr>
<td>Repeated application: sponge ribbon</td>
<td>2</td>
<td>Strain D</td>
<td>51 days</td>
<td>2</td>
<td>0.1</td>
<td>27 days</td>
<td>none</td>
<td>28</td>
</tr>
</tbody>
</table>

The chemical agent was added to the cultures
as in the previous experiments, and after 6 hours
the medium containing the drug was replaced with
fresh medium. The nutrient was replenished sub-
sequently as indicated by the pH of the superna-
tant in the culture.

Five cultures were fixed 1 day after the drug was
administered—that is, 18 hours after the drug was
removed. On examination of histologic sections of
these cultures, it was found that the drug effect
had largely disappeared (Fig. 6). Many HeLa cells

Table 1

Data on Application of NCI-3022 to Sponge Cultures

<table>
<thead>
<tr>
<th>Series and method</th>
<th>No. cultures</th>
<th>Host tissue</th>
<th>Age of host culture when HeLa added (days)</th>
<th>Age of interacting culture when drug added (days)</th>
<th>µg/ml of NCI-3022</th>
<th>Period of exposure to drug (days)</th>
<th>Recovery period (days)</th>
<th>Total age of interacting culture (days)</th>
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<tr>
<td>I</td>
<td>1</td>
<td>Strain D</td>
<td>28 days</td>
<td>1</td>
<td>0.01</td>
<td>21 days</td>
<td>none</td>
<td>22</td>
</tr>
<tr>
<td>Repeated application: sponge ribbon</td>
<td>2</td>
<td>Strain D</td>
<td>74 days</td>
<td>2</td>
<td>1</td>
<td>27 days</td>
<td>none</td>
<td>29</td>
</tr>
<tr>
<td>VI</td>
<td>2</td>
<td>Strain D</td>
<td>51 days</td>
<td>2</td>
<td>0.1</td>
<td>27 days</td>
<td>none</td>
<td>28</td>
</tr>
</tbody>
</table>

The central portion of a large aggregate of HeLa
cells in sponge matrix. We have interpreted central
degeneration, seen usually in crowded aggregates
of cells, as the result of an inadequate exchange of
essential metabolites between the cells and the
medium, e.g., anoxia. That some of the morpho-
logically nonspecific degenerating cells were a
residue of the drug effect is suggested by their
presence at all levels in the covering of HeLa cells,
including the free surfaces of the growth, where
they are usually not seen (Fig. 10). Nothing suggestive of a drug effect was found at the next point of observation, 3 days after the single exposure, or in the later periods. After 2 weeks, both in the treated cultures as well as in the controls, the surfaces of the host cultures were completely surrounded by infiltrating HeLa cells (Figs. 11 and 12). This observation suggested that sponge slices prepared as long strips in the manner described under "Methods" (sponge ribbon) might permit us to measure the extent of invasion at varying time intervals and perhaps also to determine the rate of invasion.

Series V: repeated application of the drug (thin sponge).—As seen in Table 1, combinations of Strain D and HeLa were exposed to repeated application of NCI-3022 on alternate days, for a period of 3 weeks. Of the three concentrations used, only the largest, 1 μg/ml, produced an unequivocal effect. In these cultures there was no definite infiltrative growth of HeLa into the connective tissue. Only a few cells had survived in the Gelfoam explant, and these were enlarged and bizarre. No mitotic figures were seen beyond metaphase. The D cells were comparatively sparse, enlarged, and polyhedral rather than spindle-shaped. Nucleoli were prominent, and the nuclei hyperchromatic.

In the two lower concentrations, 0.01 and 0.1 μg/ml, and in the controls, there was extensive infiltration of the connective tissue by tumor cells. Both kinds of cells had the same appearance in all three groups.

Series VI: repeated application of the drug (sponge ribbon).—Cultures of Strain D cells were prepared in four ribbons of cellulose sponge by placing stringlike segments of D cells from established cultures in a row along the length of the ribbon and cementing these to the walls of culture tubes by means of a plasma clot. During the next few weeks, as connective tissue cells grew from the line of the inoculum laterally into the adjacent sponge, the ribbons curled, taking the shape of furrows. The cultures were transferred to a petri dish, and the "adhesions" produced by the connective tissue growth between the inoculum and the sponge ribbon were cut with a scalpel. Following this procedure the cultures were placed in fresh tubes and cemented flat with a new plasma clot (see Chart 1). When good growth of connective tissue had been established, the cultures were placed in fresh tubes, and particles of Gelfoam containing HeLa cells were planted at one end of the ribbon and again cemented in position with a fresh plasma clot. One pair of cultures served as controls, the other pair received 1.0 μg/ml of NCI-3022.

The medium of the cultures was replenished on alternate days. The volume of the nutrient mixture added at each feeding increased progressively during the course of the experiment from 1 ml/culture initially to 3 ml/culture by the end of the experiment. The cultures received a complete change of medium containing NCI-3022 every other day. On the intervening day cultures with a pH of 7.1 or less received a supplement of 2 or 3 drops of 1 per cent glucose and a like amount of 1.4 per cent NaHCO₃. It was very difficult to follow the progress of the tumor in the connective tissue because of the density of growth of the connective tissue within the sponge. However, at the lateral edges of the sponge ribbons and on the adjacent glass wall, differences between the treated and control cultures were obvious.

In the controls, spindle-shaped cells that appeared normal were seen migrating from the sponge into the thin plasma clot on the nearby glass surface. After the first 3 or 4 days, HeLa cells were observed growing out of the Gelfoam inoculum in ever increasing numbers. The growth of HeLa cells at first was contiguous with the Gelfoam particle, but in the last 10 days of the experiment, islands of HeLa cells appeared in several areas not in direct continuity with the mass of growth that extended from the explant. In the treated cultures, outgrowth of HeLa cells from the Gelfoam was meager. The outgrowth of Strain D cells from the edges of the ribbon was scanty and consisted of large, bizarre cells that were rounded rather than spindle-shaped.

Both in the controls and in the treated groups clot liquefaction was a problem. It was especially serious in the control. Sometimes it was so extensive that the sponge was no longer attached to the glass wall. In this circumstance the sponge was cemented to the wall of a new tube with fresh plasma, since it was almost impossible to make direct observations on a floating sponge.

The cultures were fixed after 27 days and embedded on edge, so that sagittal sections could be prepared as illustrated in Chart 1. Serial sections were made from the edge to the midline, i.e., the site of the connective tissue inoculum.

In the control cultures the entire length of the 3.5-cm. sponge ribbon containing connective tissue was intermittently covered by tumor cells from the
Gelfoam inoculum (Fig. 13). The depth of the invasion was variable, and in a few places tumor infiltrated to about one-third of the thickness of the sponge. The spatial relationship of the two cell strains varied in different areas (Figs. 14–19). In some areas HeLa cells were on the surface of normal-appearing D cells. Elsewhere, condensed extracellular fibers of the D cells, which stained intensely with the periodic acid-Schiff reaction, appeared to provide a limiting membrane to the further infiltration of HeLa. In other areas HeLa cells were seen infiltrating the connective tissue cells singly, as short columns, or as compact masses of cells. Groups of HeLa cells were also seen beneath a covering layer of D cells. The location of these variations was not in any definite way related to the distance from the HeLa inoculum in the Gelfoam particle, except that the thickest zones of the HeLa cells were usually near the Gelfoam explant. Occasional large Strain D cells were seen, with coarse cytoplasmic processes and large nucleoli (Fig. 18).

The picture presented by the treated cultures was quite different (Fig. 20). The HeLa cell population in the Gelfoam was very small, and there was no evidence of outgrowth (Fig. 21). Those cells that had survived in the Gelfoam were large, with bizarre multiple or multilobate nuclei. Occasional cells with metaphase arrest and a scattering of chromosomes in the cytoplasm were seen. Actually, their identification as HeLa cells is presumptive and was based on their location in the Gelfoam. The host connective tissue cells had tolerated exposure to the chemical agent better than the HeLa cells, although there were somewhat fewer connective tissue cells than in the controls. Most of the connective tissue had a strikingly altered appearance, both in the extracellular fibrillary pattern and in the morphology of the individual cells (Figs. 22–26). The treated cells were very large, plump, and stellate, rather than spindle-shaped, and many fibers extended from them in all directions into the fibrillary intercellular space. Some cells had nuclei that were multilobular; others were multiple and varied considerably in size within the same cell. Dividing figures were found easily, and these were all abnormal metaphase forms. Clear vacuoles were seen in the cytoplasm in some cells, and in rare cells in the nucleus. Such vacuoles did not stain with the periodic acid-Schiff reaction. Most of the connective tissue in the treated cultures was altered as illustrated, although in some areas the connective tissue cells and extracellular fibers had a normal appearance similar to the controls. Strain D was 20 months old at the time these cultures were fixed.

**Series VII: repeated application of the drug** (sponge ribbon with Formvar).

In view of the difficulties caused by clot liquefaction, requiring repeated use of a fresh clot to keep the sponge ribbon against the wall of the tube, the sponge ribbons used in this series were first cemented to the walls of the glass tubes with 1 per cent Formvar dissolved in ethylene dichloride. Since in Series VI repeated applications of 1 μg/ml of the drug had produced a profound effect on the HeLa inoculum as well as on the D cells, in this series 0.1 μg/ml, as well as the larger dose, was studied. The cultures were otherwise prepared in the same manner as in the previous experiment.

The sponges remained in position more satisfactorily in this series, although in the latter part of the experiment segments of the ribbon had become detached from the wall, and a plasma clot was sometimes required to keep the sponge in place. The tubes with the lower concentration and the control tubes were similar to each other in the character of the growth on the glass adjacent to the sponge. Some observers believed that with 0.1 μg/ml the HeLa cells looked more bizarre in the group receiving the drug than in the control.

In the group receiving the larger dose, 1 μg/ml, there was, as in Experiment VI, almost no outgrowth of HeLa cells from the Gelfoam explant. Large, bizarre D cells emerged from the edges of the sponge ribbons.

Sagittal serial sections were prepared as in Experiment VI. The control cultures in Series VII were similar to the controls of the previous series. HeLa cells were found throughout the length of the sponge ribbon. There was, however, a comparative lack of continuity of the covering of HeLa cells on the surface of the ribbon. There were large gaps on the surface of the sponge where only D cells were seen; but there were many zones scattered along the entire length of the sponge surface in which HeLa was infiltrating the D cells in patterns similar to the previous series. Disturbing alterations were noted in the “normal” Strain D cells that made for an additional complicating factor in interpretation of the controls. In each of the controls there were seen, in the connective tissue, some large, distorted spindle-shaped cells which sometimes had large, coarse cytoplasmic processes. The nuclei of these cells were large, multiple, or multilobate and often contained enormous nucleoli as in Figure 18. Such cells, seen in Series VI, were more numerous in Series VII. Strain D was 22 months old when these cultures were fixed.7

7 The most reasonable interpretation of these puzzling large cells emerged gradually over a period of time (10). As living contemporary cultures of stock Strain D cells and of Strain D cells from other experiments were observed, the behavior of the cultures was seen to be changing. In some instances, as
In the cultures receiving 1 µg/ml, the findings were identical with those in Series VI. In the lower dosage, 0.1 µg/ml, the connective tissue resembled the control; the tumor cells, however, were affected by this dosage. There was a small population of HeLa cells that was limited to the Gelfoam and to a narrow zone of nearby connective tissue. Many ghost forms of HeLa cells were seen in the Gelfoam. Dividing forms were found; all were abnormal, and none was beyond metaphase. The connective tissue resembled that of the controls except for abnormal dividing cells interrupted in metaphase.

**Series VIII: repeated application of the drug to HeLa and to Strain D alone (thick sponge).**—This experiment is not included in the table. Three subcultures were prepared from one stock culture of Strain D, and three were prepared of HeLa. One culture of each type was used as a control. The remaining ones were given NCI-3022 over a 4-week period in a pattern of repeated application similar to that in Series VI and VII. The dose for one culture of each of the cells was 0.1 µg/ml, and for the other, 1.0 µg/ml.

In serial histologic sections it was observed that each of the cell strains had responded in the absence of the other in a manner similar to the responses seen in Experiment VII. This indicated that the action of NCI-3022 that was observed in the combinations of cells was a direct one on the individual cell lines.

**DISCUSSION**

The podophyllin preparations have been of recurrent interest in biology and medicine for several hundred years. A comprehensive review on the biologic effects and chemical composition of podophyllin was published in 1954 by Kelly and Hartwell (6). The mechanisms of the action of these subcultures were prepared, instead of a luxuriant outgrowth of spindle-shaped cells with the accompanying pH change in the medium as previously observed, the metabolic activity declined and outgrowth often consisted mostly of very large, sluggish, plump or elongated stellate cells. In sections of such stock cultures only small numbers of cells were seen, and these were very large and bizarre.

We observed a precipitous change in one Strain D stock culture at this time, 23 months after original explantation. On section part of the culture we found areas of dense cellularity with large bizarre cells, as well as many smaller cells that also appeared malignant. In subsequent subculture the small cells have grown most vigorously and are designated as Strain D-189. At about the same time a second culture of Strain D exhibited unexplained rapid growth of small cells. Giant cell forms were not seen at the birth of this line (Strain D-189), and the possibility was entertained at first that the rapidly proliferating cells were in reality a HeLa contamination. Recent experiments with the direct action of hydrocortisone on a spectrum of cell lines have indicated that D-184 is not HeLa but is closely related to D-189 (unpublished data).

Substances on tumors is incompletely understood. Waravdekar et al. (15) have studied the enzyme changes induced in normal and malignant tissue with NCI-3022. Their observations suggest that this tumor-damaging agent acts on the cytochrome system. Ormsbee and Cormann (11, 19) found in their tissue culture studies that crude podophyllin has a much more damaging effect on some mouse tumors than on normal mouse epithelium and connective tissue. Belkin (2), working with transplantable tumors in mice, observed extensive necrosis of the tumors after the administration of podophyllin. The concept that the primary action of the podophyllin derivatives, in vivo, is directly upon cancer cells has been questioned by Algire (1). He has measured blood pressure by indirect means and has described hypotension coincident to damage of the vascular bed of tumors in animals bearing transplantable cancers. He suggests that the major effect of these drugs on tumors in vivo is the result of ischemic necrosis rather than one of direct cytotoxicity. On the other hand, Pradhan et al. (18), measuring carotid arterial blood pressure directly, found no correlation between tumor-necrotizing activity and the effect on arterial blood pressure of several podophyllin and colchicine derivatives.

In Experiment II, one culture of chick embryo heart and HeLa, given a single 6-hour exposure to 1.0 µg/ml of NCI-3022, was studied in serial sections. No sign of endothelial damage was recognized in the capillary outgrowth from the heart, yet an extensive drug effect on the tumor was found. This culture offered no evidence to augment Algire's hypothesis that the primary locus of action of the drug is the vascular system.

The pattern of our observations follows those of Ormsbee and Cormann. We found that the HeLa cells were much more markedly affected than were connective tissue cells when the two kinds of cells alone or in combination were exposed to a concentration of 1.0 µg/ml of NCI-3022 for 4 weeks. Most of the HeLa cells were dead. In addition, an alteration in the histologic pattern and the cytologic characteristics of the host connective tissue was seen which we cannot find previously described. The nature of this alteration remains to be determined, particularly whether it extends to the mucopolysaccharides of the ground substance and to capillaries, and whether it is reversible.

In concentrations of 0.1 µg/ml, the drug effect was completely absent in some cultures. Where detected, the effect was limited to a reduction in the growth of HeLa cells and abnormalities of mitosis in both the HeLa and Strain D cells.

Screening for minimally effective concentrations
of an antitumor agent in combined matrix of plasma clot and cellulose sponge has been made possible by the cementing of a thin sponge to a cover-slip as described. The thin sponge gives an over-all view of the pattern of interaction of the two tissues being studied, while the thick sponge method provides ample material in serial sections for the detailed study of the interaction with the use of all the special stains desired, e.g., reticulum, collagen, and mucopolysaccharides.

Efforts to measure the amount of invasion with sponge ribbons cut in sagittal section have led to an increased appreciation of the variety of morphologic invasive patterns that can be found in a single culture. Sections prepared in this way provided a better opportunity to observe the possible role of metabolic gradients on the morphology of the host and tumor, since the cells on or near the surface have their required metabolites in greater abundance than do those deeper in the culture. The pattern of spread was not always that of a continuous membrane of tumor cells infiltrating the implantation of dispersed cells, especially after the first 2 weeks of growth in combination. This quality of growth from disseminated cells made the effort to measure the amount of contiguous infiltration extremely difficult. The extent to which one may be able to study in pure form one or the other of the phenomena, direct extension or metastasis-like spread, can probably be controlled in part by the selection of the appropriate tumor cell. We have evidence from preliminary experiments that the cell strains with which we have worked can be classified in terms of their relative ability to break off from contiguous masses and to form new centers of growth. Chang's conjunctiva and Earle's skin grow mainly by direct extension; Osgood's J-96 and Gey's HeLa show a moderate ability to disperse; Eagle's KB and our D-189 disperse so readily that estimation of the amount of growth by direct extension is completely blurred in 3 or 4 days. Besides the selection of the most suitable cell strain, variations in the conditions of culture (stationary tubes or roller tubes) or in the composition of the medium might be expected to influence the extent of spread by dissemination. In the last instance, variations in the composition of the medium that would selectively reduce this dispersion and distant implantation of tumor cells might in itself be of some interest in cancer chemotherapy.

SUMMARY

Sponge-matrix tissue culture systems in which cells derived from a carcinoma (Strain HeLa) invade normal tissue (either chick embryonic heart or Strain D) were designed for the study of chemotherapeutic agents. Acetylpodophyllotoxin-a-pyridinium chloride (NCI-3022) served as the test agent. In one system, suitable for screening, thin (100 µ) slices of cellulose sponge cemented to coverslips with Formvar were employed to support the interacting tissues. Total mounts were prepared from these coverslips, and the effect of varying concentrations of the drug was studied. The details of drug effect at selected concentrations were seen in serial histologic sections of thick (0.5–1.0 mm.) sponge cultures. A ribbon-like sponge was used to obtain some measure of the spread of cancer. After the growth of normal cells was seen throughout the sponge ribbon, tumor cells were inoculated at one end. Contiguous growth, infiltration, and dispersion with distant implantation contributed to the spread of cancer seen along the ribbon.

The effect of the drug, NCI-3022, was observed after a single application and after repeated applications during a 4-week period. A 6-hour exposure to 1.0 µg/ml produced complete metaphase arrest in both the normal and the tumor cells. Removal of the drug at this time resulted in an almost complete disappearance of the effect in 18 hours. A dose of 1.0 µg/ml was given to combinations of HeLa and Strain D on alternate days for 4 weeks. The effect on the HeLa cells was much more severe than that on the normal tissue. A few bizarre HeLa cells survived at the site of inoculation only. The connective tissue cells appeared viable, although they were enlarged, stellate, and contained large cytoplasmic or nuclear vacuoles.

REFERENCES

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Fig. 1.—Series I. Thin sponge coverslip, total mount preparation. Control culture. At the extreme right the area of the explant of chick heart is seen as a black zone. A diffuse growth of connective tissue extends across the field from the explant of embryonic tissue. Entering the scene from the left are invading HeLa cells which appear as dense-staining, punctate clusters of cells. The cellulose sponge is not prominent, but is seen as continuous irregular lines across the center of the field. Hematoxylin & eosin. X21.

Fig. 2.—Series I. Thin sponge coverslip, total mount preparation. Control culture. A zone of interaction between HeLa and outgrowth from chick embryonic heart. The HeLa cells are spherical, large, and hyperchromatic. They are seen in the right upper corner as a large aggregate of cells, and in the remainder of the field in smaller aggregates, or singly. Three normal mitoses, probably all in HeLa cells, are in focus. The connective tissue outgrowth from the chick embryo heart covers the entire field. The chick cells are spindle-shaped and stain faintly; in this field most of them are not in focus. Hematoxylin & eosin. X288.

Fig. 3.—Series I. A zone of interaction, comparable to that seen in Figure 2, from a culture that has been exposed to NCI-3022 for 6 hours. Many abnormal mitoses in HeLa cells are seen. Hematoxylin & eosin. X288.

Fig. 4.—Series II. Microscopic section of a thick sponge culture in which HeLa and cells of chick embryo heart were combined. In this control culture, HeLa cells are undergoing normal division. Regaud's iron hematoxylin. X780.

Fig. 5.—Series II. Microscopic section of a thick sponge culture treated with 1.0 μg/ml of NCI-3022 for 6 hours. Metaphase arrest is seen in many HeLa cells with a splattering of chromosomal substance in the cytoplasm. Regaud's iron hematoxylin. X780.

Fig. 6.—Series III. Microscopic section from a culture of chick heart and HeLa fixed 18 hours after a single 6-hour exposure to the drug. Recovery from the drug effect is indicated by the presence of many normal dividing figures, in both the HeLa cells and the embryonic cells. One normal division in a HeLa cell is seen below the center of the field. Regaud's iron hematoxylin. X480.
Fig. 7.—Series II. Control culture. Microscopic section of an area of angiomatous outgrowth from the chick embryo heart. Hematoxylin & eosin. ×288.

Fig. 8.—Treated culture. An area of angiomatous outgrowth from the chick embryo heart. Endothelial cells lining vascular spaces do not appear to be altered by a 6-hour exposure. Hematoxylin & eosin. ×288.

Fig. 9.—Series IV. Microscopic section of a culture of HeLa and Strain D in combination, fixed 18 hours after a single 6-hour exposure to NCI-304. A residue of the drug effect is seen in the lower half of the field in a large cell with coarsely clumped, scattered chromosomes. The presence of many normal dividing HeLa cells is evidence of recovery from the drug effect. One cell in telophase is seen in the center of the field. Hematoxylin & eosin. ×480.

Fig. 10.—Series IV. The surface of a sponge culture 18 hours after the removal of the drug. Degenerating and dividing HeLa cells are seen throughout the densely populated surface of the tissue culture. Hematoxylin & eosin. ×480.

Fig. 11.—Series IV. Low magnification of a sponge culture 2 weeks after removal of the drug. The surface of the host culture is completely covered by infiltrating HeLa cells. Hematoxylin & eosin. ×30.

Fig. 12.—Series IV. Microscopic section of a sponge culture 2 weeks after the exposure to the drug. Degenerating HeLa cells are not found on the surface in contact with the medium but are confined to the depths of the crust of infiltrating cells. Hematoxylin & eosin. ×480.
Fig. 13.—Series VI. Histologic section prepared in a sagittal plane through a ribbon-shaped cellulose sponge culture (see Chart 1). The surface bathed by the medium is on the right. At the upper end, at the site of the Gelfoam particle inoculum (arrow), is a thick zone of dense growth of HeLa. HeLa cells have spread and infiltrated along the entire surface of the connective tissue in the ribbon, which before fixation was 3.5 cm. long. Hematoxylin & eosin. Approx. ×20.

Fig. 14.—Series VI. Dense growth of HeLa cells at the level of the Gelfoam inoculum. Hematoxylin & eosin. ×180.

Figs. 15–19.—Series VI and VII. Varied pattern of interaction between HeLa and Strain D is illustrated. Hematoxylin and eosin. ×180. In Figure 15 HeLa cells appear as compact tumor tissue. In Figure 16 tumor cells are seen in rows between spindle-shaped connective tissue cells, both parallel and perpendicular to the surface of the sponge. Degenerating cells are numerous among the deeper infiltrating HeLa cells. In Figure 17 the population of infiltrating cells is small, and degeneration is scanty. In Figure 18 a nest of HeLa cells is seen well below the connective tissue surface. In addition a bizarre Strain D cell is seen with coarse spindle-shaped processes, a large nucleus and very prominent nucleolus. In Figure 19 the connective tissue is not invaded, but a thick crust of HeLa cells rests upon a condensed, thick hyaline membrane, which stains intensely with the periodic acid-Schiff reaction.
Fig. 20.—Series VI. Histologic section through a ribbon of sponge exposed to 1.0 μg/ml of NCI-3022 on alternate days for 47 days, and prepared and oriented in the same way as Figure 13. There has been no proliferation of HeLa cells at the site of the Gelfoam particle (arrow), and no invasion or spread by the tumor. An effect of the drug upon the connective tissue itself is seen even at very low magnification when compared with Figure 13. Hematoxylin & eosin. ×20.

Fig. 21.—Series VI. One of the more cellular areas of the Gelfoam inoculum is seen. The cells in the Gelfoam, presumably HeLa because of their location, are large and bizarre. Hematoxylin & eosin. ×180.

Fig. 22.—Series VI. One of many areas in the cellulose ribbon where the connective tissue cells are large and almost spherical. Hematoxylin & eosin. ×180.

Fig. 23.—Series VI. An area in the cellulose ribbon, stained with the periodic acid-Schiff reaction. The Strain D cells are large. The extracellular fibers at the surface stain prominently, while in the remaining portions of the sponge the fibers stain very faintly. Periodic acid-Schiff reaction. ×180.

Fig. 24.—Series VI. In an area in the depths of the sponge ribbon, argentophilic fibers produced by the Strain D cells are seen in a lacy pattern. Reticulum stain. ×468.

Fig. 25.—Series VI. A section from a control ribbon stained with the PAS reaction, for a comparison with Figure 23. ×180.

Fig. 26.—Series VI. A section from a control ribbon for comparison with Figure 24. Reticulum stain. ×468.
Effects of a Podophyllotoxin Derivative on Tissue Culture Systems in Which Human Cancer Invades Normal Tissue

Joseph Leighton, Ira Kline, Morris Belkin, et al.

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