Isolation of Fungi from Transplanted, Chemically Induced and Spontaneous Tumors

I. General Considerations*

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The microscopic study of a variety of tumors, by means of cytological technics not ordinarily applied to such tissue (19), has led to the observation of spores and other fungal structures in all types of neoplasms examined. These structures were noted initially in the debris of tumors degenerating in response to chemotherapeutic agents; later, by means of special stains, they were studied in the intact tissue and found to be intracellular or even intranuclear. Attempts to isolate the organisms from tissue were successful in so many types of neoplasm, including human tumors and spontaneous tumors of the mouse, that we have become convinced that they must play some significant role—even if only a secondary one; and studies were undertaken in the hope of elucidating these relationships. The present paper deals with our general observations; the study of specific tumors will be reported later.

MATERIALS AND METHODS

Conidia and other plant cells were demonstrated in tissue by means of smears and paraffin sections subjected to a variety of cytological technics. Among these were: acetic orcein (18), Benda-Kull (48), Robinow (37), Protargol (32), Papnicolaou (33), Chlorazol Black E. (13), and also such well known technics as Flemming’s tri-color following Flemming fixation, the Feulgen reaction, iron-hematoxylin (particularly in sections), and the Pianese technic for demonstration of fungus in tissue. Dry tissue imprints of blood-forming organs and of tumors were stained with Giemsa, May-Grünwald, and Robinow. Fixatives containing osmic acid were the most favorable for differentiating fungal elements, and Protargol was particularly good for the demonstration of delicate membranes and other characteristics of spores inside the host cells. The pre-hydrolysis employed in the Robinow technic produced extremely clear pictures of vegetative growths in situ (Figs. 1, 2) when used with smear preparations. Only dissociated fungal parts were revealed when the other technics were applied to smears. Sections were less useful than smears in these examinations except for the study of dissociated cells, since the patterns of the complex plant structures were difficult to reconstruct. This might explain why they have not been more commonly observed.

Tumors from animals previously immersed in an iodine-alcohol solution were dissected inside a Plexiglas transfer chamber. The tissues were streaked on Petri plates of Sabouraud’s agar (4 per cent glucose, 3 per cent peptone, and 2 per cent bacto-agar, adjusted to pH 5.5). Uninoculated control plates exposed to the air of the transfer chamber were incubated with every series of tests as an aid in detecting the presence of possible air-borne contaminants (usually Penicillium and Aspergillus.) Syncephalastrum and other forms which were recovered regularly from tumor tissue have never been found on the control plates. Sabouraud’s agar is not favorable for bacterial growth, and we but rarely found bacterial forms among tissue isolates and never in the controls. Littman’s ox-gall agar (38) has also been used with considerable success in primary isolations, although the fungi produce aerial mycelium more slowly on Littman’s than on Sabouraud’s. Adjustment of pH is not required with this medium. Early growth is colonial, which facilitates separation if more than one organism appears on the plate.

Initially, the cultures were incubated at approximately the body temperature of mice (37° C.). Later, it was observed that fungi were more readily isolated from tissue when the incubation temperature was slightly lower (room temperature to 33° C.), although the cultures, once isolated, could be transferred to slants, where they grew abundantly at 37° C.

* Supported in part by an institutional grant from the American Cancer Society, Inc.

Received for publication October 10, 1949.
Fig. 1.—Robinow smear of Sarcoma 37, showing fungus mycelium interspersed between tumor cells. × 900.

Fig. 2.—Robinow smear of a methylcholanthrene-induced fibrosarcoma (C3H strain mice) similar to Fig. 1. × 900.

Fig. 3.—Paraffin section, stained with hemalum and eosin, of a growth induced in the leg muscle of a C3H mouse by injection of a suspension of Alternaria spores. × 900.

Fig. 4.—Robinow stained dry tissue imprint of Sarcoma 37 cell showing bodies that have penetrated into, or have been engulfed by, the cytoplasm. × 900.
The materials tested were transplantable mouse sarcomas 37 and 180, a transplantable carcinoma originating in our own Institute, transplantable leukemia, tumors induced in A and C3H strain mice by subcutaneous injections of methylcholanthrene, spontaneous mammary carcinomas from C3H mice, and spontaneous lymphatic leukemia from MacDowell’s C-58 stock. Normal tissues (heart, kidney, lungs, intestinal wall, stomach, liver, ovary and testis, mammary tissue, lymph nodes, spleen, blood, and thymus) of the host mice were also tested for the presence of fungus, both before and after the animals became tumor-bearing. Human tumor tissue (mammary carcinoma, lymph nodes of Hodgkin’s disease, fibro- and lymphosarcomas), taken in sterile Petri dishes directly from the operating room following biopsy or surgery, were also cultured. Normal tissues from human hosts were not tested. Care was taken to choose spontaneous lesions that had not been open to the surface, and, in the case of transplantable tumors, to use young (7 days or younger) non-necrotic implants.

Fungus cultures from the agar plates were studied in cotton blue preparations, and identifications were made by means of this technic as well as by direct observation in the phase and light microscopes. Samples of each tissue to be tested were also prepared for microscopic examination by more permanent methods.

### RESULTS

#### ISOLATIONS

Table 1 shows the types of microorganisms isolated from the different tumors and the results of culturing normal tissue controls. Occasionally, common laboratory contaminants such as *Aspergillus niger* and species of *Penicillium* were obtained from fibrosarcomas induced by carcinogens. It seems entirely possible that these forms were contaminants acquired through the ulceration so common in early stages of induction of chemically produced tumors.

<table>
<thead>
<tr>
<th>TUMOR TISSUE</th>
<th>Fungus isolated</th>
<th>No. of positives</th>
<th>TUMOR TISSUE Host</th>
<th>Fungus isolated</th>
<th>No. of positives</th>
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<tbody>
<tr>
<td>S-37 Trans.</td>
<td>Syncophalastrum</td>
<td>138</td>
<td>Intestine</td>
<td>Aspergillus</td>
<td>5</td>
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<td>S-180 Trans.</td>
<td>Syncophalastrum</td>
<td>14</td>
<td>Intestine</td>
<td>Aspergillus</td>
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<tr>
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<td>Syncophalastrum</td>
<td>43</td>
<td>Intestine</td>
<td>Alternaria</td>
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<td></td>
<td>Alternaria</td>
<td></td>
<td>Stomach</td>
<td>Syncopehalstrum</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Yeasts</td>
<td></td>
<td>Ovary</td>
<td>Alternaria</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Mammary tissue</td>
<td></td>
<td>Thymus</td>
<td>Syncopehalstrum</td>
<td>1</td>
</tr>
<tr>
<td>MC-l rhabdomyo- sarcoma Chem.-induced</td>
<td>Alternaria</td>
<td>3</td>
<td>Intestine</td>
<td>Penicillium</td>
<td>1</td>
</tr>
<tr>
<td>MC-lb fibrosar- coma Chem.-induced</td>
<td>Alternaria</td>
<td>18</td>
<td>Intestine</td>
<td>Penicillium</td>
<td>2</td>
</tr>
<tr>
<td>MC-lb fibrosar- coma Chem.-induced “A” strain</td>
<td>Alternaria</td>
<td>1</td>
<td>Intestine</td>
<td>Penicillium</td>
<td>2</td>
</tr>
<tr>
<td>Fibrosarcoma Chem.-induced</td>
<td>Imperfect (un-identified)</td>
<td>6</td>
<td>Stomach</td>
<td>Yeastlike</td>
<td>5</td>
</tr>
<tr>
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<td>Yeastlike</td>
<td>22</td>
<td>Blood</td>
<td>Yeastlike</td>
<td>4</td>
</tr>
<tr>
<td>Leukemia Trans.</td>
<td>Yeastlike</td>
<td>37</td>
<td>Spleen</td>
<td>Yeastlike</td>
<td>11</td>
</tr>
</tbody>
</table>

### TABLE 1

**ISOLATION OF FUNGI**

<table>
<thead>
<tr>
<th>TUMOR TISSUE</th>
<th>Fungus isolated</th>
<th>No. of positives</th>
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<td>37</td>
</tr>
</tbody>
</table>

Normal tissue controls.—None of the normal body tissues, except the intestine of Swiss mice used for the implantation of Sarcoma 37 and Sarcoma 180, produced any fungi on Sabouraud’s agar, either before or after the implantation of the tumors. The intestinal forms were usually species of *Aspergillus* and could be found in unimplanted as well as implanted mice, although never in the tumor. *Syncephalastrum*, always present in Sarcoma 37, was never recovered from intestinal or any other tissue of Carworth Farms mice. Apparently no infection of the normal tissue from the tumors occurred during the period of survival of the host after implantation.

Microscopic preparations made by the Benda-Kull method showed occasional spores lodged in the tissue of the intestinal wall of nursling C3H.
mice, but no other tissues of these young mice showed spores, nor did they yield fungus of any kind when cultured. The intestine from 6-week-old mice was still the only source of fungus. After the first pregnancy, fungus could be cultured from mammary tissue, as well as from the intestine, even though the mouse was not yet tumor-bearing. The same fungus was once or twice derived from ovarian tissues in old age, after tumors had appeared; but at no time were we able to isolate fungus of any kind from the blood of C3H mice, and only once from the thymus. The remainder of the blood-forming organs and other body tissues yielded no fungus when streaked on Sabouraud's agar. Alternaria was found only in the tumor and in no mouse stock other than C3H.

Mouse tumors.—Two hundred and sixty-six Petri plate isolations of mouse tumors have been studied, with appropriate controls. All the spontaneous and chemically induced tumors and all but a few of the untreated transplanted tumors have yielded fungus. Mycelium is not always produced, but small glistening colonies usually can be detected microscopically in the tissues streaked on agar (Fig. 5), even in the absence of aerial filaments. The possibility that the organisms isolated on culture plates might be extrinsic contaminants is considerably decreased by such observations, since it is clear that the organisms are confined to the tumor tissue and do not appear on the surrounding medium.

In the case of mouse leukemias, all tissues (with the exception of the heart) tested from obviously moribund C-38 leukemic mice yielded a yeastlike growth that formed smooth, white, moist colonies (Fig. 6) on Sabouraud's agar and reluctantly produced aerial mycelium in cultures of lung, blood, and intestine. These grew so slowly that the agar became dry and cracked. When transferred to slants, they showed a tendency to revert to yeastlike forms. Microscopic examination of living unstained cultures showed that these are also very similar to the Fungi Imperfecti isolated from other mouse tumors. In most cases genera are still undetermined, but apparently they are all closely related. Similar yeastlike forms that produced a delicate nonsporulating mycelium have also been obtained from the lymph nodes, liver, spleen, and
kidney of AKm mice transplanted with leukemic spleen (courtesy of Dr. Burchenal of the Sloan-Kettering Institute).

**Human tumors.**—Our observations on human neoplasms are fragmentary and limited to random samples of specimens obtained from the biopsies of patients undergoing chemotherapy. This was further restricted by the necessity of using only tumors that were not obviously infected or superficially eroded. The most abundant materials available were mammary carcinoma, swollen nodes from positively diagnosed Hodgkin's patients, lymphosarcoma, and one fibrosarcoma. A suspected Hodgkin's node, tentatively diagnosed as a pyogenic granuloma, was also tested. The fungi isolated from human tumors were all of the group of Fungi Imperfecti (nonsporulating). Microscopic study of human mammary carcinomas and of several types of sarcoma suggests that they are allied to the Blastomycetes (Figs. 7–10).

From the granulomatous nodes and from several positively diagnosed Hodgkin's nodes, nonsporulating growths with abundant glistening white mycelium were obtained. The cultures turned a delicate pink after a few days' culture because of the production of numerous red droplets. Evidence of sporulation has been found in one or two of the specimens after numerous transfers, and these are now being grown on other media, in the hope of stimulating formation of identifiable spore structures.

All types of human neoplasms thus far tested have produced fungus growth when streaked on agar. Furthermore, spore forms are microscopically demonstrable in many types of human tumors that we have not yet tried to culture. Bacterially infected specimens, or specimens that had recently been subjected to some form of physical or chemical therapy, were usually negative. Preliminary indications are that, in biopsy material taken a few days after treatment with bacterial polysaccharide, fungal growth in culture is suppressed or much delayed. We are currently performing a series of experiments in mice, aimed at testing this further.

**Pathogenicity of Organisms Obtained from Tumors**

Spore suspensions of both Syncephalastrum and Alternaria, when freshly isolated from tumor tissue and injected either intraperitoneally or intramuscularly into our albino or CSH mice, were pathogenic. Animals thus injected died within 4 days, without exception. All normal tissues from these mice, cultured post mortem on Sabouraud's agar, yielded abundant colonies of fungus. There was a marked shrinkage of the spleen in each case. Virulence was reduced when the organisms were subcultured for several generations. Suspensions of spores from subcultures injected into mice produced firm lumps of tissue (granulomas?) but no deaths. Figure 3 shows partially disintegrated sporangia and spore cells (at tips of arrows) growing in the lesions thus produced.

**Microscopic Observations**

The tissues of tumors from which fungi were isolated were prepared for microscopic study by means of one or more of the technics mentioned above. In the control tumors, sectioned in paraffin and stained with hemalum and eosin, there were always found, scattered throughout the tissue, small round or oval cells that resembled small lymphocytes. These were never observed to divide mitotically, but in acetic orcein smears of Sarcoma 37 it was noted that some of them appeared to be subdividing within a membrane; and they were also recognized in 7-day implants stained with hemalum and counterstained with fast green. Moreover, the structure that had been mistaken for a nucleus surrounded by a very minute bit of cytoplasm was found to be an entire cell with a double-contoured or thickened wall. In addition, there were present smaller round bodies with deeply staining rims, each containing centrally located, endosome-like concentrations. These either became invasive or were phagocytized and were found in the cytosome (Fig. 4), or occasionally in the nucleus, of the tumor cells. In Benda-Kull preparations of smears of Sarcoma 37, delicate hyphal filaments were shown to run from one cell to another, sometimes penetrating into the cell and attaching to the nucleus, which then became distorted and at times was withdrawn completely from the cell body. Such enucleated cells are found frequently in areas of the tumor usually described as degenerating by "spontaneous necrosis."

**DISCUSSION**

No references to the demonstration of mycelium in tissue cultures, or even to any structures which were recognized as fungal, have come to our attention, although Grand (23) grew Hodgkin's lymph nodes in tissue culture, and by means of vital staining demonstrated the presence of specific cytoplasmic inclusions which were not found in the cells of corresponding normal tissues grown in vitro. Cell-free supernatant fluid from the Hodgkin's node cultures, added to cultures of normal lymph nodes, caused the appearance, in the cytoplasm of normal cells, of inclusions similar to those found in the original cultures. In personal talks with those who study tissue cultures of
Fig. 7.—Paraffin section, stained in hemalum and eosin, of a human uterine carcinoma. Parasitized nucleus (organism unidentified) at tip of arrow. × 900.

Fig. 8.—Papanicolaou smear of a primary carcinoma of the human cervix uteri. Note parasitized cell at tip of arrow. × 900.

Fig. 9.—Paraffin section of a spontaneous mammary tumor from an "A" strain mouse showing budding spore, greatly magnified, as demonstrated by the McManus stain for carbohydrates, in which background tumor cells remain completely unstained. × 1,800.

Fig. 10.—Paraffin section of a human fibrosarcoma. Yeastlike fungus spores, enclosed in capsules, at tips of arrows. × 900.
DILLER AND FISHER—Fungi in Tumors. I

neoplastic cells I have learned that it is standard practice to treat cultures of tumor tissue with antibiotics and to grow them at high temperatures in order to arrest the growth of fungal “contaminants.” The media employed are, furthermore, quite different from those needed to further isolation of fungus from tissues. Tissue cultures are ordinarily grown at high pH, whereas isolation of most fungi is accomplished on an acid medium. Voegtlin (54) has shown that tumor tissue is definitely more acid than the surrounding normal tissues. In hepatomas the pH dropped to 6.4 in rats (6.2 in mice), when starved host animals were supplied with excess glucose, while the pH of the liver (7.4) remained unchanged. Essentially the same findings were made earlier by Cori and Cori for blood passing through tumors.

The presence of bodies in cancer cells which did not appear to be normal cytoplasmic constituents was noted by many investigators in the nineteenth century (1, 2, 4, 11, 20, 34, 36, 43, 49, 50, 51, 52) and, more recently, by Burns and Schenken (7). Some of these authors considered that the inclusions were of fungal origin, while many others who observed similar inclusions (references not given) thought they were protozoa.

A number of authors, believing the intracellular bodies to be fungi, were successful in culturing the organisms outside the body (5, 6, 8, 14, 15, 29, 34, 35, 38, 39, 40, 41, 42, 44, 45, and 46 as well as many others listed in the Index Medicus from 1895 to 1900). An excellent review of the literature on the relation of yeasts to malignant tumors is that of Defendorf (17). Within the past few years, several papers on the association of Blastomyces with lymphosarcoma and Hodgkin’s nodes have appeared (10, 12, and 16). Contemporary authors who have isolated organisms other than Blastomyces are Leyton (27), Mori (31), and Gerlach (21). The latter author has obtained, from hundreds of human and animal tumors, microscopic fungi which he calls "Mycetogenes blastogenes," following the terminology of Mori. Both Mori and Gerlach believe that these fungi have a filterable phase during which they behave like viruses, a hypothesis similar to that of Glover (22). Wuerthele-Caspé (56) is currently isolating from all types of human and animal tumors organisms which appear to be similar to those described by Gerlach. However, since they are acid-fast in some phase of their life cycle, she believes they are Mycobacteria.

These are but a few examples of the many that might be chosen from the literature, showing how commonly inclusions—which some investigators regard as structures intrinsic to the malignant cell, others as metabolic products, and still others as microorganisms—have been observed in cancer tissue. Whatever their significance, their presence must be reckoned with, not only in terms of the cytological picture, but also of the chemical relationships or differences existing between the tumor tissue, in which these organisms are carrying out their life processes, and the normal tissues of the tumor host. Preliminary investigations made by us on the mode of action of chemotherapeutic agents have shown that the fungi respond to some of them, adding their debris to that of degenerating host cells and complicating the evaluation of experimental results.

What influence do these organisms have on tumor growth? Microscopic observations indicate that the destructive action of these parasites may account for much of the “spontaneous necrosis” observed in transplanted tumors. Are they merely contaminants that find a favorable environment (e.g., pH) in tumor tissue? If so, what are the properties of tumor tissue that render it particularly susceptible to invasion by this specific type of organism? Wharton (55) reported that, after injection of various antigens, tumor-bearing mice have a lower antibody titer than nontumor-bearing mice. Has this any bearing on the problem? Antigenic studies have been begun in collaboration with him to investigate some of these possible relationships.

At the present stage of our study of these phenomena we are not prepared to attempt any explanations of the presence or role of fungi in tumor tissue. The evidence of past investigations and of our own observations, however, point to their prevalence in many, if not all, types of tumor; and an extensive survey of the literature has revealed no conclusive evidence that microorganisms may not be causative agents. The large number of earlier papers were referred to by Haddow (25) or "a depressing catalogue of allegedly specific pathogens." “Nevertheless,” he continues, “the logical possibility exists and must therefore very willingly be admitted, that the biology of cancer may in some way be bound up with processes of infection.” The recent reports of Mann and Dunn (30) in England, that they have been able to transmit several mouse tumors from completely desiccated tissue following exposure to low temperatures (—79° C.), focus attention still further on the possibility of what these investigators call “a continuing cause.”

SUMMARY

The microscopic study of smear preparations of tumor tissue, prepared by a variety of cytological
rations made by routine histological technics, for developing conidia may be easily mistaken, in preparations made by routine histological technics, for lymphocytes and other blood cells.

Mycelial forms of the fungi were readily isolated from transplanted, induced, and spontaneous tumors of the mouse and from human tumors. Fungi Imperfecti related to the Blastomyctaceae group have been isolated from all types of tumor, including human neoplasms, and from mouse leukemias. In mice, a second form of fungus, apparently specific for the host strain, is frequently encountered.

ACKNOWLEDGMENTS
The authors wish to acknowledge particularly the help of Dr. W. G. Hutchinson of the Department of Microbiology, University of Pennsylvania, in identification of organisms; of Dr. Hope Hibbard of the Department of Biology at Oberlin College, for microscopic studies of living cultures and unstained tumor tissues; and of Dr. William F. Diller, in interpreting microscopic phenomena and parasitological relationships. We also thank Dr. George A. Clark of the Scranton Clinical Laboratory, Scranton, Pa., for access to a bibliography of early papers on parasites of tumors which he had prepared for use in radio addresses.

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