Effects of an Antibiotic From *Aspergillus fumigatus* Fresenius on Tumor Cells *in Vitro*, and Its Possible Identity With Gliotoxin

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IN SPITE OF THE DIFFICULTIES NOW INherent in the supposition that a chemotherapeutic agent can be found which will act against cancer cells without harming their normal prototypes, much research has been directed toward this end in recent times. Detailed studies already completed in this laboratory have made it plain that the growth of Brown-Pearce rabbit carcinoma cells can be suppressed under a variety of experimental conditions by an antibody that reacts specifically with a distinctive constituent of the tumor cells but not with similar constituents of other rabbit cells, either normal or neoplastic (*5*, *6*). Subsequent work extending over a period of several years has shown that a mold, isolated originally from the air of an animal room and later identified as *Aspergillus fumigatus* Fresenius, provides culture filtrates that will regularly render the cells of various animal tumors incapable of further proliferation upon brief contact with them *in vitro*. Recently a crystalline substance identical with, or similar to, gliotoxin[2] has been isolated from the culture filtrates. Both this material and purified gliotoxin procured from another source have likewise proved highly active against tumor cells *in vitro*.

Effects of Culture Filtrates on the Cells of Various Animal Tumors

Following growth of the mold (designated A21 in our laboratory) on the surface of a modified Czapek-Dox medium in Blake bottles for 7–12 days at 28°–30°C., the culture liquids, which generally have a pH in the neighborhood of 3.5–4.5, are filtered through paper and stored in the refrigerator. To test for effects upon tumor cells, the culture filtrates are brought to a pH of 7.4 with NaOH, appropriately diluted with buffered Ringer's solution (pH 7.4) containing 250 mg. per cent of glucose, and incubated 2 hours at 37°C. with suspensions in the same medium of one or another of three types of tumor cells—a lymphosarcoma of C_3H mice (Gardner tumor), a sarcoma (RSI), and the Brown-Pearce carcinoma of rabbits.

Dilutions of the culture filtrates from 1:10 to 1:80 have usually prevented entirely the growth of the tumor cells upon implantation of the mixtures in suitable situations in susceptible hosts following the 2-hour sojourn *in vitro*; under these circumstances, dilutions of 1:160 and more have often affected the cells so that only small growths resulted, while control suspensions of the tumor cells in buffered glucose-Ringer's, incubated concurrently, have always given rise to large growths when implanted in corresponding situations in the same test animals. Later experiments have shown that incubation *in vitro* is unnecessary, the tumor cells being inhibited by a few minutes contact with potent culture filtrates at room temperature. The presence of broth or of serum has not diminished the tumor-inhibiting potency of culture filtrates.

Another strain of *A. fumigatus* Fresenius, procured from a different source and designated HA in our laboratory, although resembling A21 in appearance and being somewhat similar to it in cultural characteristics, has provided culture filtrates with comparatively little or no activity against tumor cells; the same has proved true of a variety of other molds.

Characteristics and Effects of an Antibiotic Substance Isolated From the Culture Filtrates

The A21 culture liquids have retained their activity apparently undiminished for several months when kept in the acid state in the refrigerator. When shaken with chloroform, the tumor-inhibiting substance has gone into the organic solvent, the remaining watery solution being devoid of inhibitory ability, while the chloroform extracts, evaporated to dryness, have yielded a gummy substance (about 0.1 mg. from each cubic centimeter of the original metabolite solution) which, redissolved in a small amount of alcohol and diluted appropriately with buffered glucose-Ringer's, has been quite as potent against tumor cells as was the original material.

As a first step toward learning whether the active material produced by *A. fumigatus* Fresenius (A21) might

[1] These investigations have been aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research. It is a pleasure to acknowledge the able assistance of Miss Rose Robbins in the work.

[2] Gliotoxin is a sulfur-containing compound for which an unusual structure and the formula $C_{13}H_{14}O_4N_2S_2$ have been suggested (*1*, *2*, *4*); it was first isolated as a product of *Gliocladium fimbriatum* by Weindling and Emerson (*9*) and is prominent among the antibiotic substances produced by molds of the genus *Aspergillus* (*3*, *7*, *8*).

be similar to gliotoxin, tests were made of the effects of heat upon it, for it is known that gliotoxin is remarkably unstable when heated in alkaline solution (*7*, *9*). In two experiments it was found that culture filtrates having pH levels of 3.58 and 3.64, respectively, placed in a boiling water bath for 10 minutes and brought to pH 7.8 after cooling, were quite as potent against Gardner lymphosarcoma cells as were unheated specimens that had been adjusted to pH 7.8 and allowed to stand meanwhile at room temperature, all being inhibitory in every dilution up to and including 1:160; the same filtrates, however, brought to pH 7.8 and then heated at 100°C. for 10 minutes, smelled strongly of hydrogen sulfide and subsequently were found to have lost all demonstrable activity against tumor cells. It is noteworthy in this relation that a similar amount of heating in an alkaline medium inactivated the gliotoxin or gliotoxin-like compound produced by the strain of *A. fumigatus* employed by Menzel, Wintersteiner, and Hoogerheide (*7*).

A sample of purified gliotoxin prepared by Johnson, Bruce, and Dutcher in the Baker Laboratory of Chemistry, Cornell University, and made available by Prof. du Vigneaud, was next tested for activity against tumor cells. In all concentrations of 1.0 μg./ml. and greater, this completely inhibited the growth of Gardner lymphosarcoma cells when incubated with them for 2 hours at 37°C.; dilutions containing 0.1 μg./ml. and less were not notably effective.

As a further step in identifying the active substance, a small amount of crystalline material was isolated from an A21 culture filtrate by the method of Menzel, Wintersteiner, and Hoogerheide (*7*); this proved indistinguishable under the microscope from the reference sample of purified gliotoxin.[3] When dissolved in buffered glucose-Ringer's solution it proved to be about as active against Brown-Pearce carcinoma cells as was the known gliotoxin, both materials in concentrations of 5 μg. and more/ml. preventing completely or almost completely the subsequent growth of the carcinoma cells held in contact with them for 2 hours at 37°C. Neither was active against these cells, however, in concentrations of 1 μg. and less/ml. Both the A21 crystalline material and the purified gliotoxin smelled strongly of hydrogen sulfide when saturated solutions of them at pH 7.4 (containing less than 200 μg./ml.) were placed in a boiling water bath for 10 minutes, and both of the heated specimens proved devoid of activity when tested subsequently against Brown-Pearce carcinoma cells.

[3] The writer is indebted to Prof. du Vigneaud for advice and suggestions concerning the characterization of the crystalline material, and to Dr. Rachele for confirming the observation on the similarity of the isolated crystals and those of the purified gliotoxin. Dr. Melville generously determined the melting points on the crystalline A21 material and the sample of purified gliotoxin and also interpreted ultraviolet absorption curves on both. He reports that the micro melting points and a mixed micro melting point were not inconsistent with the possible identity of the sample of gliotoxin and that of the isolated compound, though the melting points were spread over a range of several degrees in all three cases. The ultraviolet absorption curves on both samples were quite similar to the published curves for gliotoxin.

Further Tests With the Antiobiotic and With Various Types of Cells

Culture filtrates having notable potency against tumor cells *in vitro* exhibited no activity against *Treponema pallidum*, *Plasmodium lophurae*, or *Trypanosoma lewisi* in a variety of similar tests, and they failed to inhibit growth in broth of a variety of microbes, including staphylococci, streptococci, pneumococci, meningococci, and *Escherichia coli*. They did not diminish the motility of human spermatozoa, as tested by John MacLeod, and had no influence on the ciliary movement of rabbit tracheal epithelium upon prolonged contact therewith *in vitro*, although the ciliary beating ceased abruptly upon contact with anisotonic solutions, fixatives, acids, and alkalis. Solutions containing large amounts of the tumor-inhibiting antibiotic did not bring about lysis of sheep's erythrocytes in 2 hours at 37°C., although they caused inflammation and necrosis in the skin and subcutaneous tissues of mice and rabbits—the effects being roughly proportional to their tumor-inhibiting potency—and they proved lethal to mice when injected in sufficient amounts.

Tumor cells of the three types employed in the present study, incubated for periods of 4–5 hours with excessive amounts of potent culture filtrates, have, upon scrutiny under the microscope, looked precisely like those incubated concurrently with buffered glucose-Ringer's solution, as repeated examinations have shown; this proved true also in the case of Brown-Pearce carcinoma cells that had undergone prolonged incubation with saturated solutions of the A21 crystalline material and of the purified gliotoxin before examination with the phase microscope by Hans Zollinger. The latter observation would seem to have added significance when contrasted with the fact, recently studied in this laboratory by Dr. Zollinger, that normal and neoplastic cells "killed" by fixatives, heat, molar NaCl, surface-active agents, etc. immediately manifest irreversible morphological changes that prove readily visible with the phase objective.

In an experiment to determine whether the activity of culture filtrates against tumor cells *in vitro* could be altered, perhaps differentially, by prior incubation with suspensions of various tissue cells, two culture filtrates were incubated 4 hours at 37°C. with suspensions of citrated mouse blood and with suspensions of washed mouse liver, spleen, kidney, and Gardner lymphosarcoma cells. The incubated mixtures were then centrifuged and the supernatant liquids tested in the usual way for activity against fresh Gardner lymphosarcoma cells. The various suspensions had not discernibly affected the more potent of the two filtrates, all of the incubated aliquots of this material inhibiting completely in the two dilutions tested (1:10 and 1:40) the subsequent growth

of the fresh lymphosarcoma cells with which they were held in contact during 2 hours at 37°C. The findings were otherwise, however, with the weaker of the two filtrates. The portion incubated with the suspension of Gardner lymphosarcoma cells was devoid of activity in the test with fresh tumor cells in dilutions of 1:10 and 1:40, and those portions incubated with citrated mouse blood and with washed mouse kidney remained inhibitory in the 1:10 but not in the 1:40 dilution; the aliquots incubated with suspensions of spleen and liver cells, by contrast, had retained much, and perhaps nearly all, of their tumor-inhibiting potency, being completely inhibitory in the 1:10 dilution and almost so at 1:40, although this potency was slightly diminished when compared with that of the control aliquot that had been incubated with Ringer's solution.

In manometric experiments performed by Dr. William H. Summerson it was found that anaerobic glycolysis proceeded equally in mixtures containing Gardner lymphosarcoma cells and (a) crystalline A21 material in saturated solution in buffered glucose-Ringer's, (b) gliotoxin, also in saturated solution in the same medium, (c) a potent culture filtrate in a dilution of 1:10, and (d) control buffered glucose-Ringer's solution. Further tests provided evidence that oxygen consumption and glycolysis under aerobic and anaerobic conditions of Gardner lymphosarcoma and Brown-Pearce carcinoma cells were not altered by the presence of amounts of an active culture filtrate more than sufficient, as subsidiary tests proved, to prevent subsequent growth of the cells.

Tests for Chemotherapeutic Activity

Tests for *in vivo* activity against tumor cells with whole filtrates and with chloroform extracts of them have not thus far yielded encouraging results. In one experiment, for example, a small "dose" of Gardner lymphosarcoma cells was injected intraperitoneally into five groups of C_3H mice, each containing four animals of uniform weight. Immediately afterward the mice of the control group were injected intraperitoneally with 1 ml. of buffered glucose-Ringer's solution, while each of the mice of the other four groups received 1 ml. of one or another of four potent culture filtrates that had been brought to pH 6.5–7.0, the amounts of the latter administered to each mouse being greatly in excess of what would have been required to inhibit the growth of the lymphosarcoma cells following *in vitro* contact. All of the mice developed intraperitoneal lymphosarcomata within 2 weeks, the growths appearing as promptly and growing as rapidly in the animals of the experimental groups as in the controls. In a number of other experiments, culture filtrates were administered repeatedly to C_3H mice in near-lethal doses, both before and after implantation of minimal effective numbers of lymphosarcoma cells; in no case has there been any evidence that the subsequent course of events was significantly altered by the injections. Chloroform extracts of the culture filtrates, evaporated to dryness and taken up in concentrated form in alcoholic and watery solutions, have also been administered repeatedly to mice in near-lethal amounts without influencing significantly the course of implanted lymphosarcoma cells. Toxicity tests have shown, however, that both the filtrates and the chloroform extracts contain toxic substances that produce effects distinct from those of the crystalline A21 material and of gliotoxin. Tests for *in vivo* activity against tumor cells will be undertaken with the crystalline A21 compound and with purified gliotoxin as soon as sufficient amounts of the materials can be procured.

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