Studies on a Tumor-retarding Agent Produced by

*Aspergillus fumigatus*

H. CHRISTINE REILLY AND C. CHESTER STOCK

(Division of Experimental Chemotherapy, The Sloan-Kettering Institute for Cancer Research, New York, N.Y.)

For nearly 4 years a search has been conducted in our laboratories among materials of natural origin for agents having the ability to retard the growth of tumors. Early in the course of this investigation crude culture filtrates of the fungus *Aspergillus fumigatus* were found to retard the growth of the Crocker mouse sarcoma 180 and of the mammary adenocarcinoma E 0771 in mice (4). Meanwhile, Kidd (1) reported that this fungus produced a substance capable of destroying the viability of animal tumor cells in vitro. Chemical evidence indicated that this agent was identical with or similar to gliotoxin. Preliminary investigations of the in vivo activity of our preparations had indicated that gliotoxin was not the active principle. Thus, studies on the production, extraction, purification, and chemical nature of this new agent were undertaken.

**METHODS**

All testing was carried out in vivo with Sarcoma 180 by the use of an implantation technic (3). Small cubes of 7-day-old tumor, measuring approximately 2 mm. in all dimensions, were implanted, by means of a trocar, subcutaneously into the axillary region of RF or CFW Swiss mice weighing between 18 and 22 gm. Twenty-four hours later administration of the materials under test was begun. Solutions of the active agent were injected intraperitoneally in divided doses over a period of 7 days. Control mice bearing tumors received equivalent amounts of physiological saline. On the eighth day after implantation of the tumors fragments, the tumors on all mice were measured in two diameters by means of calipers. The following arbitrary system of grading was adopted:

- **Marked inhibition** (+ +).—The average diameters of the tumors in the treated animals were one-quarter or less than that in the control animals.
- **Good inhibition** (+).—Average tumor diameters in treated animals were one-fourth to one-half of those of control tumors.
- **Slight inhibition** (+).—Average tumor diameters in treated animals were one-half to three-fourths of those of control tumors.
- **No effect** (−).—Average tumor diameters in treated animals were three-fourths or greater than those of control tumors.

Each test was run on five animals, five animals serving as controls. Each preparation was tested at least 3 times with consistent results.

**Production and extraction of the tumor-retarding agent.**—Several strains of *A. fumigatus*, in addition to No. 1 (ATCC 9197) and No. 89\(^1\) (4), were found to produce an agent or agents having the ability to inhibit tumor growth, but for detailed study one strain, namely, 943,\(^2\) was chosen.

Three hundred and fifty ml. portions of a medium, consisting of 2 per cent glucose and 1 per cent Bacto casitone in distilled water, were dispensed in 2-l. Erlenmeyer flasks and sterilized at 120° C. for 10 minutes. The flasks were inoculated with an aqueous suspension of fungus spores and allowed to incubate at 25 ± 1° C. in a stationary state. In several preliminary experiments the maximum production of active material appeared on the seventh to the ninth day of incubation, accompanied by a drop in pH from an initial pH of about 6.6 to pH 3.7–4.2. Further incubation resulted in a rapid loss of activity with a rise in pH. Thus, in the preparation of large lots, cultures were harvested after 7 or 8 days' incubation. The liquid portion was filtered through paper to remove gross cell material. In order to extract the tumor-retarding agent, the crude culture filtrate was saturated with ammonium sulfate at 6° C., as indicated in Chart 1. The resulting insoluble material was collected on a Büchner filter, suspended in 1/40 the original volume of distilled water and

\(^1\) The authors are indebted to Dr. Kenneth B. Raper for the identification of this culture which in previous publication has been erroneously classified as *Pencillium*.

\(^2\) Dr. Waksman's No. 35. The authors are grateful to Dr. S. A. Waksman for supplying this organism.
dialyzed against running tap water for 2 days and then against frequent changes of distilled water, brought to pH 4 with HCl, until the dialysate no longer gave a positive test for sulfate ions. The nondialyzable fraction was filtered through paper. Further purification was accomplished by the addition of an equal volume of cold acetone to a 1 per cent aqueous solution of the \((\text{NH}_4)_2\text{SO}_4\) precipitated material. The mixture was allowed to stand overnight at 6°C. The resulting insoluble portion was removed by centrifugation, redisolved in a minimum volume of distilled water, and dialyzed to remove residual acetone. The nondialyzable fraction was filtered through paper and lyophilized. This procedure has been used satisfactorily to obtain material from lots of from 1 l. to 22 l. of culture filtrate.

The mycelial pads of the fungus were also found to contain an appreciable amount of a tumor-retarding agent. The pads were ground in a Waring Blender with 0.1 N HCl (10 ml. per pad). After standing overnight at 6°C, the mixture was filtered and the residue was extracted overnight a second time with the same volume of fresh acid and filtered. The filtrates were combined, adjusted to pH 4 with NaOH, and saturated with \((\text{NH}_4)_2\text{SO}_4\). The insoluble portion was redissolved, dialyzed, and lyophilized.

RESULTS
A comparison of a preparation from filtrate and one from mycelium is presented in Table 1. Both preparations were carried through \((\text{NH}_4)_2\text{SO}_4\) precipitation and not further purified. While the weight of material obtained from the crude filtrate was 10 times as great as that extracted from the mycelium, the latter preparation was 10–20 times more potent. Most of the inactive impurities contained in the preparation from the culture filtrate are undoubtedly residual constituents of the medium, since it has been found that Bacto casitone contains a large amount of \((\text{NH}_4)_2\text{SO}_4\)-precipitable material. Whether or not the active principles from the 2 parts of the fungus culture are identical in chemical nature has not yet been ascertained.

Data presented in Table 2 illustrate the effectiveness of an acetone precipitate from culture filtrate. The volumes of the tumors have been calculated on the assumption that the tumor is spherical in shape. It probably approximates more closely an ellipsoid (6), but in these experiments...
calculations on this basis are not possible, since the tumors were measured in living intact animals, and data on three dimensions could not be obtained. The intraperitoneal administration of 8.8 mg/kg in saline in divided doses over a period of 1 week was sufficient to cause approximately 90 per cent inhibition of tumor growth. Larger doses resulted in still greater inhibition, while as little as 4.4 mg/kg had an appreciable effect.

### Table 1

**Comparison of Tumor-Retarding Agents from Culture Filtrate and from Mycelium of Aspergillus fumigatus**

<table>
<thead>
<tr>
<th>Prep.</th>
<th>Yield per flask (mg)</th>
<th>Total dose (mg/kg)</th>
<th>Effect upon Sarcoma 180</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>160</td>
<td>225</td>
<td>+</td>
</tr>
<tr>
<td>Mycelium</td>
<td>18</td>
<td>12.5</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.8</td>
<td>−</td>
</tr>
</tbody>
</table>

* Administered intraperitoneally in divided doses for a period of 1 week only.

† + indicates good inhibition, ± slight inhibition. Explained in text under "Methods."

### Table 2

**Effect of Dose Upon Retarding of Sarcoma 180 by Tumor-Inhibiting Agent from Aspergillus fumigatus**

<table>
<thead>
<tr>
<th>Total dose (mg/kg)</th>
<th>Average size of tumor (cc.)</th>
<th>Retardation of tumor growth (per cent)</th>
<th>Average weight change in grams per mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.0</td>
<td>0.0091</td>
<td>90</td>
<td>−4.5</td>
</tr>
<tr>
<td>12.5</td>
<td>0.0056</td>
<td>90</td>
<td>−5.5</td>
</tr>
<tr>
<td>8.8</td>
<td>0.053</td>
<td>90</td>
<td>−2.0</td>
</tr>
<tr>
<td>4.4</td>
<td>0.10</td>
<td>70</td>
<td>−1.5</td>
</tr>
<tr>
<td>2.2</td>
<td>0.25</td>
<td>30</td>
<td>−0.5</td>
</tr>
<tr>
<td>Control</td>
<td>0.36</td>
<td></td>
<td>−0.5</td>
</tr>
</tbody>
</table>

* Administered intraperitoneally in divided doses for a period of 1 week only.

† Volume calculated on the assumption that the tumor is a sphere. V = 4.189 r³.

† Per cent retardation = \( \frac{\text{Vol. of control tumor} - \text{Vol. of treated tumor}}{\text{Vol. of control tumor}} \) × 100.

In Table 3 are shown tracings of tumors in mice that received 0.05 mg/day for 5 days, for a total dose of 12.5 mg/kg. On the eighth day after implantation, marked inhibition of tumor development was evident. As has been the case, however, with other agents used in the control of tumor growth such as the folic acid analogs (2), the tumor tissue was not completely destroyed. When treatment was stopped, the tumors proceeded to grow and after 15 days were approximately the same size as the tumors in the untreated control mice.

The ability of preparations from the fungus to retard the growth of Sarcoma 180 has been accompanied by marked general toxicity for the animal, as indicated by the significantly high loss of weight in mice even at dose levels necessary to cause approximately 90 per cent retardation of tumor growth (Table 2). That the inhibitory effects obtained with this agent are not merely the result of malnutrition of the animals has been shown by starvation experiments. Mice bearing Sarcoma 180 were placed on a low caloric diet 1 day after tumor implantation. After 1 week on such a diet the animals lost an average of 4–5 gm. in body weight, but only a slight inhibition of tumor growth occurred. Numerous chemical compounds that have been tested have caused similar losses in weight without having any effect upon tumor growth.

### Table 3

**Effect of 943–375–IBI Upon Growth of Sarcoma 180 in Mice**

<table>
<thead>
<tr>
<th>Treated with 943–375–IBI (mg)</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Died on 15th day</td>
</tr>
<tr>
<td></td>
<td>Died on 19th day</td>
</tr>
<tr>
<td></td>
<td>Died on 25th day</td>
</tr>
<tr>
<td></td>
<td>Died on 35th day</td>
</tr>
<tr>
<td></td>
<td>Died on 45th day</td>
</tr>
</tbody>
</table>

* Prepared by \((\text{NH}_4)_2\text{SO}_4\) precipitation followed by precipitation with 50 per cent acetone. Each mouse received 0.05 mg/day for the first 5 days after tumor implantation, resulting in a total dose of 12.5 mg/kg.

† Grams change in weight during experiment.

Thus far, efforts to detoxify preparations have resulted in complete loss of ability to inhibit tumor growth.

Detailed pharmacological and pathological studies will be reported elsewhere.

**Properties of the tumor-retarding agent.**—Unless otherwise indicated, the following studies were made on material extracted from the culture filtrate.

The tumor-retarding agent is soluble in water but not in ether. It is not precipitated from crude culture filtrate by 50 per cent saturation with \((\text{NH}_4)_2\text{SO}_4\). Although the active principle is readily adsorbed from the culture filtrate by Darco G-

* K. Sugiuura, personal communication.

† J. J. Buckley, S. S. Sternberg, S. Buckley, and F. S. Philips, to be published.
60, attempts to elute it quantitatively from the carbon have been unsuccessful.

Preparations partially purified, i.e., through the precipitation with 50 per cent acetone, have given positive biuret, Millon’s, Molisch, ninhydrin, and xanthoproteic reactions.

The tumor-retarding capacities of several preparations have decreased appreciably after treatment with nitrous acid, quinone, and trypsin.

The activity of crude culture filtrates at pH 4 or 7 was completely destroyed by heating in a boiling water bath for 10 minutes. On the other hand, a sterile crude culture filtrate stored at 6° C. for over 15 months appeared to have lost none of its activity. Sterile solutions of an ammonium sulfate precipitate, adjusted to pH's 2, 4, 5.8, and 7.8, were stored at 6°, 25°, and 37° C. After 1 and 7 days, the preparations were tested for activity. The results indicate the following conclusions: (a) Storage for 1 week at 6° and at 25° C. at all pH's tested did not appear to destroy the active agent. (b) At 37° C. complete inactivation of the active agent occurred at pH 2 after only 1 day and at pH 4 after 1 week, while preparations at pH's 5.8 and 7.8 showed no appreciable loss in potency after 1 week's storage.

In electrophoretic studies the activity originating both from culture filtrate and from the fungus mycelium has been found in a group of highly basic protein components. Although Stedman et al. (3) have found that the basic proteins, protamine and histone, retard the development of Carcinoma 141 in the mouse, protamine sulfate (Armour), when tested as outlined above, brought about no appreciable inhibition of Sarcoma 180, even at a dose that caused the death of 40 per cent of the mice.

In vitro activity.—As had been found with crude culture filtrate (4), the (NH₄)₂SO₄ precipitate (2 mg/ml) and the more purified acetone precipitate (0.2 mg/ml) from A. fumigatus exhibited no demonstrable effect upon Sarcoma 180 in vitro. On the other hand, it has been possible in tissue culture to show considerable damage to cells of mouse sarcoma T241 at concentrations that are not toxic to normal tissue. This latter effect has been obtained by the addition of a fresh supply of the active agent to the cultures each day for 4 consecutive days.

REFERENCES

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