Identification of Contaminating Clostridium Spores as the Oncolytic Agent in Some Chalone Preparations

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SUMMARY

Crude concentrates of epidermal chalone from pig skin in some cases have shown striking oncolytic activity in melanomata in hamsters and mice. Attempts to purify this antitumor factor by biochemical methods failed. A correlation of epidermal or melanocytic chalone activity in vitro and oncolytic activity in vivo was absent. From fluid collected from lysing tumors spore-forming bacteria could be isolated and classified as Clostridium. It could be proved that the oncolytic activity in the active pig skin fractions is caused by contaminating spores of these clostridia.

INTRODUCTION

The epidermis appears to contain mitotic inhibitors: chalones. These substances are tissue specific but are not species specific (4, 13, 15, 17). Similar observations have been made in other tissues, e.g., in the hemopoietic system (22, 23) and lung alveoli (25). Mitotic activity and cell differentiation are supposed to be dependent on the concentrations of the chalones in the tissue (6).

Reports on the significance of these substances in carcinogenesis, (3, 5, 14) have had only theoretical value until now, as experimental proof is lacking.

In earlier investigations, we tested appropriate pig skin extracts on mice and hamsters bearing transplanted melanomata; with some preparations we observed tumor lysis in all test animals, and in some animals we observed permanent cure (19). At that time we supposed that a chalone mechanism might be involved.

Subsequent studies have shown, however, that microorganisms, i.e., clostridia, contaminating the extracts, are the underlying cause of the therapeutic oncolysis. We report here the evidence that led us to this conclusion.

MATERIALS AND METHODS

One hundred eighty-six preparations obtained from pig skin extracts were tested on more than 3000 animals: Syrian golden hamsters, NMRI and C57BL mice, and Sprague-Dawley rats. The animals were kept in separate Makrolon cages and fed with RMH-B pellets (Hope Farms, Woerden, Holland) and water ad libitum.

The lyophilized fractions were administered s.c. (ventrally) for 5 consecutive days. The test animals bore (dorsally) cherry-sized transplanted tumors (specified in the text). Each group receiving the same treatment consisted of 5 animals. Substances were tested in doses increasing in a geometric series; if sufficient material was available, the total dose ranged from 25 to 400 mg/animal. Corresponding control groups were treated with the solvent (0.9% NaCl solution).

The main criteria for a possible tumor inhibition were the size of the tumor, estimated every 7 days (multiplication of diameters measured in 3 perpendicular planes); the tumor weight at death; and the survival times.

The results were evaluated with the Friedman or the Spearman-Kaerber test. Sections of tumors and organs were investigated histologically by means of hematoxylin and eosin, Giemsa, and Gram stains. Liquid scintillation measurements were carried out after the administration i.p. of 50 μCi thymidine-3H per kg body weight 2 hr before death. The radioactivity of 2 mg homogenized and lyophilized tumor material was measured in a Packard Tri-Carb apparatus after combustion, according to the method of Kalberer and Rutschmann (18). The data were evaluated with covariance analysis and the method of Scheffé (24). The inhibitory activity of the preparations has been recorded (for the most favorable dose, see "Results") according to the following scale: ++, total regression of the primary tumors in 50% or more of the treated animals (3 weeks after start of treatment); +, total regression in 1 or more treated animals (but less than 50% of the total number); ±, some inhibitory effect (partial or temporary regression or growth inhibition); 0, no significant effect.

RESULTS

The 1st preparation showing melanoma inhibition in vivo (19) was a fraction of a pig skin water extract. It consisted of an 81% (v/v) alcohol precipitate prepared as an intermediate in the isolation of epidermal chalone from pig rind (12).

During subsequent studies to develop a reproducible method for the purification of this antitumor factor, many alcohol fractionations were carried out, starting from water or 0.9% NaCl solution extracts of pig rind. The results are summarized in Table 1. Only in a total of 8 preparations could the strong (+++) activity, reported in a previous publication (19), be reproduced. Three of them were 55%, 2 were 72%, and 3 were 81% alcohol precipitates of pig rind water extracts. On the whole, the activity (+++, +, and ±) was distributed...
Table 1

Distribution of activity in alcohol fractionations

All preparations were tested on hamsters bearing amelanotic melanomata. The fractions were administered s.c. in 0.9% NaCl solution for 5 consecutive days. Effects were estimated 3 weeks after the start of treatment.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>++</th>
<th>+</th>
<th>±</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>55% alcohol precipitates</td>
<td>3</td>
<td>3</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>72% alcohol precipitates</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td>81% alcohol precipitates</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>81% alcohol supernatants</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 2

Distribution of activity in dialysis experiments

All preparations were tested according to the method described in Table 1. In the experiments included in A (below), the fraction retained in the dialysis tubing was tested as a whole, regardless of a possible precipitate. In the experiments of B, the dialysate was not tested. The precipitate formed during dialysis was isolated by centrifugation and tested separately from the soluble part of the retentate.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>++</th>
<th>+</th>
<th>±</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Dialysate and total retentate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialysate</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Retentate (total)</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>B. Soluble and insoluble fractions of retentate (tested separately)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retentate, soluble part</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Retentate, precipitate</td>
<td>1</td>
<td>6</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

Erratically over the different fractions (the 55, 72, and 81% precipitates and the 81% alcohol supernatant), and only a slight preference for the earlier precipitates could be observed. Total regression of primary tumors (++ or + activity) was never found in a preparation consisting of an 81% alcohol supernatant.

In some modified alcohol fractionations, the effect of various additives was studied. A concentration of 0.1 M NaCl was added in an attempt to decrease a possible association of the active component with high-molecular-weight contaminants and to increase the solubility of the factor. Lysozyme was added once, in an effort to improve the yield of the extraction by degradation of cell wall material. In separate experiments, the effects of RNase and bentonite (as an inhibitor of this enzyme) were investigated with the possibility in mind that the tumor inhibitor may consist of RNA [compare the reports of Askenova et al. (1) and Srebro et al. (26) and others]. The addition of a metal-complexing agent, i.e., 2-quinoline carboxylic acid, was studied, since the metal ions known to be present in pig rind extracts could affect the purification of the factor in question. However, none of these additives had any detectable influence on the distribution of activity over the fractions.

In the course of many experiments, active fractions or extracts were dialyzed. From the results (Table 2), it can be seen that the activity was usually retained within the dialysis bag. Often a precipitate appeared in the retentate in which the activity was concentrated.

In all other experiments aimed at further purification of active fractions (including gel filtration, column electrophoresis, and fractionations with different organic solvents at various pH values), the activity was lost. An epidermal chalone preparation, purified by alcohol fractionation and subjected to column electrophoresis at pH 3 and to dialysis, as described previously (12), also proved to be inactive. All efforts to characterize the active component, e.g., by using organic solvents or trypsin to classify the factor as a lipid or a protein, respectively, failed. In extracts of cod skin and pig lung, inhibitory activity could not be detected. Extracts of human lung tumors and melanomata of hamsters and mice gave the same negative results.

Extensive studies have been made of the antitumor activity of the most potent fractions. With higher doses, the growth of the tumors was correspondingly more inhibited. This is illustrated in Chart 1 for 2 different preparations in melanoma-bearing hamsters. Usually, about 1 week after the 1st injection, the tumor softened; after ulceration of the skin covering the tumors, necrotic material was discharged. In numerous animals, the transplanted tumors were completely

Chart 1. The effects of different doses of a chalone preparation on tumor growth in hamsters bearing amelanotic melanomata. A, preparation: a 72% alcohol precipitate of a pig rind water extract (12) coded OC 90-2; B, preparation: a similar 55% alcohol precipitate coded OC 89; n, tumor size in (cm)^3 (product of measurements in 3 planes); weeks, after start of treatment; C, control.

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destroyed 1 week after the 5-day-treatment. Thereafter the ulcers, often up to 4 cm in diameter, healed. The subsequent death of these animals was caused as a rule by metastases appearing mostly in the lungs or was caused, in some cases, by local recurrence.

The effect on survival time was also dose related. The graphs in Chart 2 show this for 2 experiments with NMRI mice bearing Harding-Passey melanomata. It can be seen that the survival time increases up to a certain dose level. With still higher doses, it decreases again because of the toxicity of the preparations, also indicated by a weight loss in the animals and the occurrence of skin necroses at the site of injection.

During these investigations, 2 mice were apparently cured, as they survived for more than a year with no signs of recurrence or metastases. Survival times of several months were observed regularly in hamsters and mice. No spontaneous regression of any sort occurred with the tumors in question during our experiments with more than 3000 animals.

All cases in which enough data had been collected for statistical treatment and in which significant effects on tumor inhibition criteria could be established have been compiled in Table 3. For those cases in which sufficient dose levels were studied, the most favorable total dose is included in Table 3, last column.

In an elaborate experiment, the incorporation of $^3$H-labeled thymidine into the tumor tissue was studied during and after treatment. Golden hamsters with amelanotic melanomata were treated in the usual way, with a total dose of 150 mg of a potent 81% alcohol precipitate over 5 days. The results, plotted in Chart 3, show that DNA synthesis is inhibited in comparison with that of control animals.

Histological investigation of the transplanted tumors during and after treatment yielded evidence of mixed infections, in particular in the ulcerated tumors. Our first attempts to identify Clostridium-like organisms in the sections did not meet with success. In the starting material (lyophilized pig rind powder) and various derivatives, only a very few of these bacteria could be detected microbiologically. The discovery that solutions of active preparations lost their activity, not only by heating in an autoclave but also by sterile filtration, constituted the first evidence that microorganisms might yet play an essential part. Some preparations incubated at 37° for 3 days under anaerobic conditions in 5% glucose solutions or

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Table 3

<table>
<thead>
<tr>
<th>Pig skin alcohol fraction</th>
<th>Test animal</th>
<th>Tumor</th>
<th>Criterion studied</th>
<th>$p$</th>
<th>Most favorable total dose (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>55% precipitate</td>
<td>NMRI mice</td>
<td>HPM</td>
<td>STI</td>
<td>0.05</td>
<td>50</td>
</tr>
<tr>
<td>(coded OC 89-2)</td>
<td>Hamsters</td>
<td>AM</td>
<td>TR</td>
<td>0.001</td>
<td>80</td>
</tr>
<tr>
<td>72% precipitate</td>
<td>NMRI mice</td>
<td>HPM</td>
<td>STI</td>
<td>0.05</td>
<td>50</td>
</tr>
<tr>
<td>(coded OC 90-2)</td>
<td>Hamsters</td>
<td>AM</td>
<td>TR</td>
<td>0.05</td>
<td>100</td>
</tr>
<tr>
<td>81% precipitate</td>
<td>Hamsters</td>
<td>AM</td>
<td>TR</td>
<td>0.05</td>
<td>200</td>
</tr>
<tr>
<td>(coded OC 28-3)</td>
<td></td>
<td>Fortner III melanoma</td>
<td>TR</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NMRI mice</td>
<td>HPM</td>
<td>STI</td>
<td>0.01</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>C57BL mice</td>
<td>Lung tumor</td>
<td>TR</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LAF, mice</td>
<td>Mastocytoma</td>
<td>STI</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Furth)</td>
<td>TR</td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The abbreviations used in the table are: HPM, Harding-Passey melanoma; AM, amelanotic melanoma (Green); STI, survival time increase; TR, tumor regression.
Preparation: an 81% alcohol precipitate of a pig rind water extract (12) coded OC 28-3; treatment: 5 daily doses (s.c.) of 30 mg/animal, Days 1 to 5.

in 0.9% NaCl solution proved to contain many spore-forming rods. These incubated solutions, when made of strongly active fractions, were still highly active but were more toxic than they were initially.

Fluid collected at the right moment from lysing tumors of treated animals also contained many spore-forming bacteria. From a sample of tumor punctate, a pure culture of the microorganisms contained therein could be isolated and characterized as Clostridium. As yet, this microorganism has not been fully identified, but it has been established among other things that pathogenicity for guinea pigs is absent.

Tumor punctate as well as microbiologically pure spore suspensions of the microorganism caused oncolysis in several animal-tumor combinations exactly as did our original, most active pig skin fractions. Quantitative data on this are presented in Paper 2 of this series (20).

**CONCLUSIONS AND DISCUSSION**

According to the definition by Bullough and Laurence (7, 8), a chalone is “a product of internal secretion which inhibits the mitotic activity of the tissue of origin.” The investigations described here were part of a study on the possible therapeutic value of chalones for cancer treatment.

In the 1st experiments, various pig skin fractions containing epidermal chalone prepared according to methods described previously (12) were tested on squamous cell tumors induced on the skin of NMRI mice with benzpyrene (19). The negative results were disappointing, since we would have expected this type of tumor to be a likely target for a specific action of the epidermal chalone. However, treatment of animals bearing transplanted melanomata with some crude pig skin fractions showed the striking oncolytic effects described before (19). A specific action could be expected in this case from the melanocytic chalone. According to the findings of Bullough and Lawrence (9, 10), this factor occurs not only in pig skin fractions but also in melanoma extracts. However, extracts of such tumors and fractions thereof, showing in vitro chalone activity, did not destroy or inhibit the tumors when tested in the same experimental models.

The existence of a tumor-inhibiting factor in our active preparations was beyond doubt, as various kinds of tumors could be lysed reproducibly. The effects on tumor growth and survival times proved to be dose dependent and statistically significant.

Attempts to purify this agent by the usual biochemical methods failed. The only clues about its character were the findings that it seemed to be nondialyzable and apparently insoluble. The isolation of spore-forming bacteria that could be classified as clostridia from the punctate of lysing tumors explained the purification difficulties. The loss of activity during fractionations was not caused by instability of an antitumor substance but by destruction of the Clostridium spores in our preparations by the biochemical procedures.

It is known that clostridia can usually be found on and in pig skin, and in pig rind (2). Möse (11, 21) has described a strain of Clostridium butyricum which, when administered i.v., can destroy various kinds of transplanted tumors. Evidently, we isolated a different type of Clostridium, with similar oncolytic properties.

Until now, pure chalones have not been available in sufficient amounts. Therefore, the crucial experiments to prove a possible therapeutic value for cancer treatment could not yet be performed. According to theoretical considerations of Iversen (16), a positive result is not very likely. In the investigations reported here, it could be proved that the striking oncolytic activity of some of our pig skin fractions is caused by contaminating Clostridium spores and not by possible chalone components.

Investigations on the specificity, mechanism of action, and therapeutic usefulness of the new Clostridium strain are reported in extenso (20).

**ACKNOWLEDGMENTS**

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**REFERENCES**


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