Growth Retardation and Prevention of Ehrlich Solid Tumor by Clostridium perfringens Type A Spores and Culture Supernatant

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ABSTRACT

When given by direct s.c. injection into the Ehrlich solid carcinoma 1 week after s.c. tumor transfer, viable crude spores of Clostridium perfringens type A (attenuated mutant strain LNG11 ATCC 29348) inhibited tumor growth and significantly prolonged the life span of male outbred Swiss mice. Under these conditions a concentrated sterile supernatant of a C. perfringens culture proved to be slightly more effective than were viable crude spores. In contrast viable crude spores were ineffective in the treatment of female Swiss mice, but the sterile supernatant retained significant activity. When given at the time and site of s.c. grafting of Ehrlich tumor cells, a concentrated sterile supernatant of a C. perfringens culture prevented tumor growth in 80% of male outbred Swiss mice. Under these conditions viable crude spores prevented tumor growth in 70% of mice and significantly prolonged the life span in the other 30%. When given by i.p. injection and before i.p. grafting of tumor cells, viable crude spores of C. perfringens prevented Ehrlich ascites tumor in 5 of 12 Swiss mice and prolonged life span in the other 7. In contrast concentrated sterile supernatant and viable purified spores were ineffective in the prevention or delay of the growth of Ehrlich ascites tumor. These data indicate that C. perfringens can be a potent antitumor agent without producing the harmful acute anaerobic infection of solid tumors (clostridial oncolysis).

INTRODUCTION

Clostridial spores injected i.v. into tumor-bearing animals germinate selectively in the tumor tissue, thus inducing a local acute anaerobic infection which results in the early death of the host. In the course of such an infection, solid tumors soften, gradually become fluctuant, break open, and discharge a brown liquid in which vegetative cells are easily demonstrable; it then remains a large cavity instead of solid tumors (clostridial oncolysis).

This anaerobic infection of solid tumors or clostridial oncolysis was reproduced with Clostridium butyricum strain M-55 and with several other nonpathogenic spore-former anaerobes (1, 4, 10-12, 17); with pathogenic clostridia, it was reproduced only with Clostridium histolyticum (13). Clostridium tetani spores are also able to germinate in neoplastic tissue, but they do not reproduce the local infection because they kill the tumor-bearing animals by tetanus within 48 hr after i.v. injection, whereas non-tumor-bearing controls remain asymptomatic throughout the observation period (9).

In earlier investigations we have not succeeded in reproducing the acute anaerobic infection of solid tumors with Clostridium perfringens type A (7). However, we observed that crude spore preparations of C. perfringens could inhibit tumor growth, although they did not provoke clostridial oncolysis (5, 7). Because the strain used was an attenuated mutant (8), antibiotics and antitoxins were not administered and the tumor-bearing mice given injections of C. perfringens spores did not develop gas gangrene.

This paper describes further observations on the therapeutic value of C. perfringens culture products in outbred Swiss mice bearing Ehrlich solid tumor. It is shown that the antitumor activity of C. perfringens is not a clostridial oncolysis or an acute anaerobic infection of solid tumors and is best reproduced by the s.c. route into the tumor site and with the concentrated and fractionated culture supernatant. Preliminary investigation on the origin of activity of crude spores and on the mechanism of action of culture supernatant was carried out with purified spores and Ehrlich ascites tumor.

MATERIALS AND METHODS

C. perfringens. C. perfringens type A strain LNG11 ATCC 29348, a low-virulence mutant, was previously isolated from a fully virulent wild-type strain after nitrosoguanidine treatment (8).

Culture Method and Media. Crude spore preparations and culture supernatants were prepared from a culture produced in a dialysis tubing apparatus at 37° by a sequential culture method (6). Bacteria were first grown on Trypticase soy broth (Baltimore Biological Laboratories, Cockeysville, Md.) with 1.0% dextrose for 24 hr and then on broth composed of 7.0% Trypticase (Baltimore Biological Laboratories) and 1.5% peptone (Difco Laboratories, Inc., Detroit, Mich.) for 48 hr, the latter exhausted broth having been once renewed after 24 hr.

Crude Spore Suspensions. Vegetative cells contaminating sporulated cultures were killed and ruptured in a Ribi cell fractionator (Ivan Sorvall, Norwalk, Conn.) at 25,000 psi (Fig. 1A). Spores were then washed, suspended in distilled water, and frozen at −76° or freeze-dried. Colony-forming units (viable or heat-stable spores) were counted after heating of samples for 20 min at 75° and after serial dilutions and plating on sheep blood agar (6).
**Culture Supernatants.** Crude supernatants were prepared from cultures by centrifugation for 20 min at 17,680 \( \times g \); they were concentrated and fractionated by ultrafiltration through XM100A membranes (Amicon Corp., Lexington, Mass.), dialyzed against distilled water, and sterilized by filtration through 0.45-\( \mu \)m Millipore membranes (Millipore, Ltd., Mississauga, Ontario, Canada); they were then freeze-dried and kept at 4\(^\circ\).  

**Purified Spores.** Sporulated cultures were produced at 37\(^\circ\) by a sequential culture method in standard Pyrex tubes (150 x 25 mm). Bacteria were first grown on Trypticase soy broth with 0.25% glucose for 40 hr and then on broth composed of 7.0% Trypticase and 1.5% peptone for 48 hr. Spores were then purified (Fig. 1B) by centrifugation of the sporulated culture for 180 min at 17,680 \( \times g \) (Beckman J-21 refrigerated centrifuge, type JA-10 fixed-angle rotor; Beckman Instruments, Inc., Montréal, Québec, Canada) onto a supporting medium adjusted to their specific density (2.16), which was equal to that of a 55% solution of 76% Renografin (E. R. Squibb and Sons, Ltd., Montréal, Québec, Canada). Purified spores were suspended in distilled water and frozen at -76\(^\circ\).  

**Mice.** Experiments were carried out on outbred male or female Swiss albino mice [Charles River COBS CD-1(ICR) BR weighing 17 to 22 g and 4 weeks old, obtained from Canadian Breeding Farm and Laboratories, Ltd., St.-Constant, Québec, Canada]. They were given Purina Laboratory Chow 5001 (Raislon Purina of Canada, Ltd., Woodstock, Ontario, Canada) and tap water ad libitum.  

**Tumor.** The Ehrlich carcinoma was provided by Dr. R. Daoust of the Institut du Cancer de Montréal; it was maintained in male Swiss mice by 6 serial passages into the peritoneal cavity every 7 days, followed by 1 s.c. passage, this protocol being repeated periodically. For experiments Ehrlich tumor cells were withdrawn from the peritoneal cavity, counted by the dye exclusion test with the use of trypan blue, and adjusted to the desired concentration with a medium composed of Medium 199 with Hanks’ solution; 37\(^\circ\) by a sequential culture method in standard Pyrex tubes (150 x 25 mm). Bacteria were first grown on Trypticase soy broth with 0.25% glucose for 40 hr and then on broth composed of 7.0% Trypticase and 1.5% peptone for 48 hr. Spores were then purified (Fig. 1B) by centrifugation of the sporulated culture for 180 min at 17,680 \( \times g \) (Beckman J-21 refrigerated centrifuge, type JA-10 fixed-angle rotor; Beckman Instruments, Inc., Montréal, Québec, Canada) onto a supporting medium adjusted to their specific density (2.16), which was equal to that of a 55% solution of 76% Renografin (E. R. Squibb and Sons, Ltd., Montréal, Québec, Canada). Purified spores were suspended in distilled water and frozen at -76\(^\circ\).  

**Spores.** As shown in Chart 1, the pattern of tumor growth in mice given s.c. injections of crude spores was similar to that in controls, whereas that in mice given s.c. injections was significantly different (\(p < 0.1\)) from that of the first 9 mice dead of tumor in the control group. The influence of route of injection was investigated once more in a second experiment (Chart 2). In both i.v. (Group B) and s.c. (Group C)-injected mice, the mean tumor area was different from that in the controls (\(p < 0.1\) on Days 12 and 17 in the i.v. group; \(p < 0.1\) on Days 17 and 27 in the s.c. group; and \(p < 0.05\) on Day 22). The mean survival time of mice that received s.c. spores was significantly different from that of the controls (\(p < 0.01\)), whereas that of the i.v.-

**RESULTS**

**Influence of Route of Injection of C. perfringens Crude Spores.** As shown in Chart 1, the pattern of tumor growth in male mice given i.v. injections of crude spores was similar to that in controls, whereas that in mice given s.c. injections was significantly different (\(p < 0.1\)) from that of the first 9 mice dead of tumor in the control group. The influence of route of injection was investigated once more in a second experiment (Chart 2). In both i.v. (Group B) and s.c. (Group C)-injected mice, the mean tumor area was different from that in the controls (\(p < 0.1\) on Days 12 and 17 in the i.v. group; \(p < 0.1\) on Days 17 and 27 in the s.c. group; and \(p < 0.05\) on Day 22). The mean survival time of mice that received s.c. spores was significantly different from that of the controls (\(p < 0.01\)), whereas that of the i.v.-
injected mice was similar to that of the controls. In the 2 experiments, the incidence of tumor regression in treated mice was not statistically different from that in controls.

Comparison of *C. perfringens* Culture Supernatant and Crude Spores. As shown in Chart 2 (Group D), the culture supernatant significantly inhibited the growth of Ehrlich's solid carcinoma; the mean tumor areas of this group of mice were significantly different from those of untreated mice in Group A (p < 0.02 on Days 17 and 32; p < 0.01 on Days 22 and 27). The mean survival time of the treated mice was also improved (p < 0.001). No significant differences were observed between the tumor sizes of mice treated with s.c. crude spores (Group C) and that of mice treated s.c. with the culture supernatant; no significant differences were detected between the mean survival times of these 2 groups.

Effect of *C. perfringens* Culture Supernatant and Crude Spores in Female Mice. Crude spores were ineffective against Ehrlich's solid carcinoma in female mice (Chart 3, Group B), whereas the culture supernatant was very effective (Group C). The mean tumor areas in mice treated with the culture supernatant were significantly different from those of untreated mice (p < 0.1 on Day 21; p < 0.02 on Day 26). The mean life span (62.0 ± 5.9) of all (10) mice dead of tumor in Experimental Group C was statistically different (p < 0.001) from that (32.0 ± 1.8) of the first 10 mice dead of tumor in the control group. At the same dosage of tumor cells, the incidence of spontaneous tumor regression in untreated mice was higher in female mice than that in male mice (Chart 2).

Prevention of Tumor Growth with *C. perfringens* Crude Spores and Culture Supernatant. Growth of 1 × 10⁶ Ehrlich tumor cells could be prevented completely when mixed, immediately before s.c. transplantation, with 7.0 × 10⁶ viable spores or with 0.5 ml of the fractionated and 3 times concentrated culture supernatant (Chart 4). A significant number of mice (14 of 20) treated with viable spores failed to develop a solid tumor (p < 0.005 when compared to the number of mice without tumor in control group); the mean survival time of the remaining mice (6 of 20) that developed a solid tumor was improved by 36 days over that of all mice dead of tumor in the control group (p < 0.01) and by 55 days over that of the first 6 mice dead of tumor in the control group (p < 0.005). Of the 20 mice treated with the culture supernatant, 16 failed to develop a solid tumor (p < 0.0005 when compared to untreated mice); the remaining 4 mice had a mean survival time (39.5 ± 9.0) statistically different (p < 0.1) from that (21.0 ± 2.4) of the first 4 control mice dead of tumor. No significant differences were observed between the results obtained in both treated groups.

Clostridial Oncolysis of Solid Tumors by *C. perfringens*. For all previous animals treated with *C. perfringens* products, the phenomenon of clostridial oncolysis described by Möse and Möse (12) was not observed either by daily inspections of solid growing tumors or by macroscopic autopsy in mice dead of solid tumors.

Inhibition of Ehrlich Ascites Tumor with *C. perfringens* Crude Spores. The growth of Ehrlich ascites tumor was significantly inhibited by crude spores injected i.p. before tumor graft (Table 1); of the 12 treated mice, 5 failed to develop tumor as compared to 0 of 12 in the untreated group (p < 0.05); the mean survival time of the 7 remaining mice was improved by 9 days over that of all control mice dead of tumor (p < 0.1) and by 13 days over that of the first 7 dead control mice (p = 0.05). A number of mice (2 of 12) died early after i.p. injections carried out after tumor graft, whereas the 10 other mice presented transient side effects (breakdown, breath shortness, and shivering); no significant inhibition of Ehrlich ascites tumor was observed with spores injected after tumor graft, although 3 treated mice failed to develop tumor.

Inhibition of Ehrlich Ascites Tumor with *C. perfringens* Purified Spores. Purified spores injected i.p. before or after tumor graft had neither toxicity nor side effects, but they did not modify the incidence of Ehrlich ascites tumor (Table 2). The mean survival time of mice treated after tumor graft was improved by 5 days (p < 0.1), and that of mice treated before tumor graft was improved by 9 days (p > 0.1, not significant).

Effect of *C. perfringens* Culture Supernatant against Ehrlich Ascites Tumor. The fractionated and twice concentrated culture supernatant did not modify the incidence of...
Antitumor activity of crude spore suspensions against Ehrlich ascites tumor

Each male mouse was given an i.p. injection of $1.0 \times 10^5$ Ehrlich tumor cells. Group A received i.p. injections of distilled water, 0.2 ml/dose, on Days 1, 3, 5, and 7 after tumor graft. Group B received i.p. injections of spores, $5.7 \times 10^5$/dose (0.2 ml), on Days 1, 3, 5, and 7 after tumor graft. Group C received i.p. injections of spores, $5.7 \times 10^5$/dose (0.2 ml) on Days 7, 5, 3, and 1 before tumor graft.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice with tumors/no. of mice inoculated with tumor cells</th>
<th>Survival time (days) of mice dead of tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Control (distilled water)</td>
<td>12/12 (100.0)$^a$</td>
<td>$22.5 \pm 1.7^b$</td>
</tr>
<tr>
<td>B. $2.3 \times 10^6$ spores inoculated after tumor graft</td>
<td>7/10 (70.0)</td>
<td>$20.0 \pm 1.6$</td>
</tr>
<tr>
<td>C. $2.3 \times 10^6$ spores inoculated before tumor graft</td>
<td>7/12 (58.3)</td>
<td>$31.6 \pm 5.9$</td>
</tr>
</tbody>
</table>

$^a$ Numbers in parentheses, percentage of mice with tumors.  
$^b$ Mean ± S.E.

Antitumor activity of purified spores against Ehrlich ascites tumor

Each male mouse was given an i.p. injection of $1.0 \times 10^5$ Ehrlich tumor cells. Ten mice of Group A received i.p. injections of distilled water, 0.2 ml/dose, on Days 1, 3, 5, and 7 after tumor graft; the other 10 received distilled water on Days 7, 5, 3, and 1 before tumor graft. Group B received i.p. injections of spores, $9.0 \times 10^5$/dose (0.2 ml), on Days 1, 3, 5, and 7 after tumor graft. Group C received i.p. injections of spores, $9.0 \times 10^5$/dose, on Days 7, 5, 3, and 1 before tumor graft.

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</tr>
</thead>
<tbody>
<tr>
<td>A. Control (distilled water)</td>
<td>17/20 (85.0)$^a$</td>
<td>$19.4 \pm 1.0^b$</td>
</tr>
<tr>
<td>B. $3.6 \times 10^5$ spores inoculated after tumor graft</td>
<td>16/19 (84.2)</td>
<td>$24.9 \pm 3.0$</td>
</tr>
<tr>
<td>C. $3.6 \times 10^5$ spores inoculated before tumor graft</td>
<td>17/20 (85.0)</td>
<td>$28.2 \pm 6.2$</td>
</tr>
</tbody>
</table>

$^a$ Numbers in parentheses, percentage of mice with tumors.  
$^b$ Mean ± S.E.

DISCUSSION

The results of this study indicate that the low-virulence mutant LNG11 ATCC 29348 of C. perfringens type A has a tumor-inhibitory effect that is different from that described with many other clostridia (4, 10–12, 17) since: (a) an antitumor activity was observed without local anaerobic infection; (b) survival time of treated tumor-bearing mice was constantly improved; (c) the antitumor activity was best reproduced with the sterile supernatant of culture. Tumor growth inhibition or suppression observed here and local tumor infection or clostridial oncolysis observed elsewhere (4, 10–12, 17) are then 2 distinct phenomena (3, 5, 7, 14); the latter phenomenon seems to us to be a harmful event in the course of tumor treatment with clostridial products; it appears to be a specific infectious process of tumor tissue (7) which could be provoked by some clostridial species seen as apathogenic in the past (12) and could be reproduced only with viable bacteria (4).

The results of our previous preliminary works (5, 7) and the present data prove that Ehrlich's solid carcinoma can be significantly inhibited by C. perfringens culture products. Moreover, suspensions containing live spores of an attenuated strain or sublethal doses of undetoxified culture supernatant improved survival time of tumor-bearing mice without administration of antibiotics or antitoxins. Tumor growth was completely suppressed when crude spore suspensions or culture supernatant were mixed with Ehrlich tumor cells immediately before s.c. inoculation. The s.c. route into the tumor site was more effective than was the i.v. route, and male mice were more sensitive than were female mice, whereas culture supernatant was more effective than were spore suspensions.

Spores must be preferred to vegetative cells if live bacteria are used in biological preparations of anaerobic species.
the former being less sensitive to freezing, to freeze-drying, and to the defense mechanisms of the animal host. The treatment of sporulated cultures in the Ribí apparatus ruptured the contaminant vegetative cells without altering spores, but residual bacillary fragments and toxic materials remain (Fig. 1A). Contrary to the sporulated cultures of the sequential culture method in dialysis tubing, those of the sequential culture method in standard Pyrex tubes have a low ratio of bacilli to spores which allowed us to purify spores (Fig. 1B) by centrifugation of sporulated cultures onto a supporting medium adjusted to the specific density of spores.

Crude viable spores, contaminated with ruptured bacilli, were effective against Ehrlich ascites tumor, whereas purified viable spores were practically ineffective. Contrary to what was expected (5), the activity of the former spore preparations could not thus be ascribed to the spores themselves but to contaminant ruptured bacilli or to soluble substance(s) released during the disintegration process and possibly identical with active soluble substance(s) present in the culture supernatant.

We have previously obtained transitory inhibition of Ehrlich's solid carcinoma with a crude culture supernatant injected i.v. (5), but the present results are much more satisfactory. In this study the culture supernatant was injected s.c. into the tumor site and was obtained after cultivation of the bacteria by an improved sequential culture method in dialysis tubing (6). This supernatant represented the concentrated fraction of the crude culture filtrate retained by a XM100A ultrafiltration membrane (M.W., approximately 100,000). In a recent and preliminary report, Sasaki et al. (15), testing several Clostridium toxoids for their antitumor activities, found that only commercial C. perfringens toxoid was active and detected its activity in a fraction of low molecular weight (M.W., approximately 30,000 to 50,000).

Although very effective against the solid form of Ehrlich's carcinoma, the culture supernatant was ineffective against the ascitic form. On the one hand, inasmuch as mice presented a low tolerance to the i.p.-injected culture supernatant, the decrease of dosage could explain the ineffectiveness of the culture supernatant against the ascitic form. On the other hand, the culture supernatant may act directly upon vascularization or connective tissue of the solid form but not upon free tumor cells of the ascitic form. The active substance(s) of the culture supernatant and the active substance(s) of the crude spore suspensions could then be different.

REFERENCES
Fig. 1. a, retractile spores and disrupted vegetative cells of crude spore suspensions; b, vegetative cell-free retractile spores of purified spore suspensions. Phase-contrast, × 1000.
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