Oncolysis by Clostridia. I. Activity of Clostridium butyricum (M-55) and Other Nonpathogenic Clostridia against the Ehrlich Carcinoma

J. R. Möse and G. Möse
(The Institute for Hygiene, University of Graz, Austria)

SUMMARY

Intravenous injections of suspensions of spores of Clostridium butyricum (Strain M-55) produced a more or less extensive lysis of tumor tissue. A number of other strains of nonpathogenic clostridia did not show substantially improved results. In general, strains which were able to ferment carbohydrate performed equally well, whereas primarily proteolytic strains gave inferior results. Combination of Strain M-55 with Clostridium felsineum, as well as a combination of Strain M-55 with culture filtrates of Cl. paraputrificum, did not yield improved results. All growth phases of Clostridium butyricum could at least in principle produce the phenomenon of oncolysis, but the results were most unfavorable if vegetative forms were used. To some extent an enhancement of oncolysis could be obtained by a combination of spores with certain tumor extracts.

In two previous papers (3, 4), we described the observation that an apathogenic species of clostridia was able to destroy tumor tissue in vivo. This strain, which had been isolated by us from a soil sample, was a Clostridium butyricum, which we designated as M-55. It fulfilled the conditions which we required for such an organism—namely, that it was apathogenic in any form of application, that it was able to lyse tumor tissue, and, finally, that it could be easily prepared as spores. The reasons for using suspensions of spores purified as far as possible were the following: (a) In this form large numbers of organisms could be injected intravenously if needed more than once, which procedure took advantage of the biological differences between the vegetative forms and the spores of the organisms; (b) after intravenous injection the spores were distributed passively throughout the body and were not nearly so rapidly killed by the normal defense mechanisms of the body as were the vegetative forms. This fact facilitated the localization and massive germination of the microorganisms in the tumor.

The test tumor in this work was first the Ehrlich carcinoma in the solid form, but later on we also worked with other tumors of the mouse, rat, and rabbit. The rapid germination of the rods occurred in the tumor-bearing animal nearly exclusively in the tumor, although, by culture methods, the presence of microorganisms could be shown for considerable lengths of time in normal organs (particularly in liver). In the normal organs, however, the decisive effect that occurred in the tumor was missing—namely, the massive germination with destruction of tissue. The destruction of tumor tissue occurred in the form of a more or less extensive lysis. Even if the lysis of the tumor was nearly complete, the process came to a halt rather sharply at the border of the healthy tissue.

This paper describes the basic phenomenon, its limitations and a number of experimental variations designed to improve the end-results.

MATERIALS AND METHODS

The spores were prepared in an iron nail broth (1). This is a meat broth with increased content of peptone (4 per cent), to which a small iron nail in each culture tube is added. The microorganisms were incubated at 37°C. in this broth until optimal sporulation had been obtained. The medium was filtered through paper, the spores were washed with physiological saline, resuspended, and again filtered through paper and washed twice more. The washed spores were thoroughly suspended in saline, and any precipitate which formed at this point was discarded. Suspensions of spores from various culture tubes in this supernatant were transferred to common containers after being diluted to a predetermined spore count. The combined suspension was then heated for 25 minutes in a water bath at 73°C. The spore suspension was checked for the absence of other microorganisms, subdivided in small aliquots into test tubes and/or ampules, and kept frozen at -25°C. until use.

Later on we also used freeze-dried spores prepared by Dr. D. Gericke. All the species and strains of clostridia listed in Table 1 were prepared as spore suspensions essentially in the manner described.

Received for publication June 16, 1963.
TABLE 1
METABOLIC CHARACTERISTICS OF DIFFERENT SPECIES OF CLOSTRIDIA AND COMPARISON OF THEIR ONCLYTIC BEHAVIOR WITH Cl. BUTYRICUM (M-55)

<table>
<thead>
<tr>
<th>Species of clostridia</th>
<th>Strain*</th>
<th>Fermentation of carbohydrate</th>
<th>Proteolysis</th>
<th>Hemolysis on blood agar</th>
<th>Optimal growth temp., °C.</th>
<th>Extent of lysis</th>
<th>Start of lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrobutyricum</td>
<td>T-9</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>32-37</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acetobutyricum</td>
<td>M-10, T-10a, T-10b</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>37</td>
<td>—</td>
<td>- to +</td>
</tr>
<tr>
<td>Felsenium</td>
<td>M-13</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>37</td>
<td>= to +</td>
<td>—</td>
</tr>
<tr>
<td>Sporogenes</td>
<td>M-35, T-35</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>37</td>
<td>=</td>
<td>—</td>
</tr>
<tr>
<td>Cochlearum</td>
<td>T-50</td>
<td>—</td>
<td>±</td>
<td>+</td>
<td>30-35</td>
<td>— to =</td>
<td>— to =</td>
</tr>
<tr>
<td>Leptotutocrescens</td>
<td>M-63</td>
<td>—</td>
<td>+</td>
<td>—</td>
<td>37</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>Pasteurianum</td>
<td>M-70, T-70</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>25</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Roseum</td>
<td>T-71</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>37</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Nigrificans</td>
<td>M-79</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>55</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Butyricum</td>
<td>M-82</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>30-37</td>
<td>— to =</td>
<td>=</td>
</tr>
<tr>
<td>Paraputrificum</td>
<td>M-91</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>37</td>
<td>+</td>
<td>= to +</td>
</tr>
<tr>
<td>Tetnum</td>
<td>T-92</td>
<td>+</td>
<td>—</td>
<td>+</td>
<td>30-35</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>Multifermentans</td>
<td>M-333, T-353</td>
<td>+</td>
<td>—</td>
<td>+</td>
<td>37</td>
<td>—</td>
<td>= to +</td>
</tr>
<tr>
<td>Tetanomorphism</td>
<td>M-36</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>30-37</td>
<td>— to =</td>
<td>= to =</td>
</tr>
</tbody>
</table>

* The authors are indebted to Drs. E. H. Thiele and G. E. Boxer, Merck Institute, Rahway, New Jersey, for the strains designated by the letter "T".
† Extent of lysis: 0, no lysis; ——, substantially less than M-55; —, less than M-55; =, same as M-55; +, better than M-55.
‡ Start of lysis: ——, substantially slower than M-55; —, slower than M-55; =, same as M-55; +, faster than M-55.

For animal experiments the spore suspension was made up to 200 million spores/ml in all instances, and the injection was done intravenously in volumes of 0.1-0.2 ml. (i.e., 20-40 million spores/mouse). The tumor was the Ehrlich ascites solid tumor of the mouse, which was transplanted from a standardized number of fresh ascites cells intramuscularly into the hind leg of the mouse. For each experiment a series of tumors of about equal size was chosen. At the beginning of the experiments the tumors were ca. 14 days old, felt hard and elastic to the touch, did not show any necrosis on the outside, and had dimensions of approximately 23 × 17 mm.

RESULTS

General description of the oncolysis.—A few days after the injection of the spores, the tumor softened noticeably and shortly thereafter fluctuated on palpation. At this time it usually broke through to the outside with spontaneous discharge of brownish liquid necrotic masses, which had the consistency of thin pus. The afflicted leg frequently died off, and a large cavity remained which sometimes reached close to the peritoneum. The animals usually did not survive this stage of oncolysis for any length of time. At this point, the tumor appeared macroscopically to have disappeared completely; nevertheless histologically in many cases one could find at the inner edge of the cavity more or less abundant tumor tissue which was covered by a layer of necrotic material. Permanent survival of the animals occurred only rarely and then only if the tissue defect had not been too large.

Of the surviving animals which after formation of scars gave the impression of a clean amputation at the hip joint, about 65 per cent had a recurrence of the tumor at the same place. If the treatment was later repeated, lysis occurred again as soon as the tumor had reached the corresponding size, but the animals generally died a short time later. No more than two treatments were done in this series of experiments.

Another form of macroscopically total lysis occurred so that only a relatively small tissue defect was produced after discharge of the necrotic masses. In these instances, the whole leg rapidly dried off, mummified, and after a short time could be taken off easily at the hip joint. The further course was similar to the one just described. Frequently, however, the lysis was already macroscopically only incomplete, so that after the spontaneous discharge of the necrotic masses, a more or less deep hole was formed which was surrounded by a solid residue of tumor. In these instances further therapeutic effects could only be obtained infrequently, even if intravenous injections of spores were done repeatedly. It seemed probable that at this stage the unavoidable secondary infection with other microorganisms was of considerable importance.

In the ascitic form of the Ehrlich tumor a single intravenous injection of the spore suspension produced an arrest of further weight gain and sometimes a slight decrease in weight for a short time which was followed by a renewed rise in the weight curve and did not lead to a prolongation of life. After repeated injections, the arrest and slight lowering of the weight curve could be maintained until the death of the animal. The optimal time for the first injection of the spores was the beginning of the logarithmic growth phase of the tumor. In the ascites fluid of the tumor, vegetative forms were found 24 hours after the intravenous injection of the spores. In contrast to the behavior in the case of solid tumors, the
number of the organisms which were visible in the ascites remained always relatively small.

Generally, it can be said that the clostridial oncolysis is not due to intracellular growth of the microorganisms. It is, therefore, likely that the phenomenon of oncolysis is connected with the metabolic requirements of the anaerobic strain of Cl. butyricum (M-55). Aerobic spore-forming organisms—e.g., Bacillus mesentericus, Bacillus subtilis, which were prepared in a similar manner, did not show any oncolysis under these conditions.

Behavior of different species and strains of clostridia.—The following series of experiments was concerned with determining whether other species and strains of clostridia produced oncolysis to a smaller or larger extent than the standard strain M-55. A comparison of the metabolic properties (Table 1) indicates that all strains which are not able to ferment carbohydrate yield results inferior to Strain M-55—that is, Cl. cochlearium, lentoputrescens, and nigrificans. The unfavorable results with Cl. nigrificans may also be explained by its thermophilic behavior. The proteolytic capacity of these strains was, therefore, undoubtedly not of primary importance for the phenomenon of oncolysis. Capacity for proteolysis in the presence of the capacity to ferment carbohydrate, however, did not prevent lysis from occurring, as indicated by the behavior of the strains of Cl. acetobutylicum, felsineum, sporogenes, and roseum. Of these four species, Cl. roseum gave the least favorable results.

The remaining strains, according to Table 1, gave results approximately comparable to those of Strain M-55. The relatively unfavorable result with Cl. pasteurianum was probably due to its optimal growth temperature, which did not correspond to the physiological conditions found in the mouse. Cl. multi fermentans had to be excluded from consideration because of its comparative pathogenicity in tumor-bearing animals. Whether the capacity of this strain to hemolyze is of importance for its pathogenicity cannot be stated, since other strains that are capable of hemolysis do not show any particular pathogenicity.

The differences observed with Cl. tyrobutyricum and Cl. tertium were probably due to differences in virulence of the strains as compared with the control strain M-55. In particular, Cl. tertium required rather large doses of spores to obtain marked lysis. Cl. paraputricum was the only strain in this series which was clearly superior to the control strain M-55 as far as its oncolytic effect was concerned. Its great disadvantage, however, was the fact that it was not clearly nonpathogenic. Metabolically, it is different from the standard strain M-55 in its capacity to reduce nitrate (Cl. paraputricum, positive; M-55, negative).

It should be mentioned that there were some quantitative differences within different strains of the same species. Qualitatively, however, the trend of the effect was the same within a species, and it would be difficult indeed to make a choice which of the different strains within a given species would be of better practical use.

Combinations of different strains of clostridia.—Since investigations of the oncolytic property of different strains of clostridia had shown that in some respects more favorable results were obtained with Cl. felsineum and Cl. paraputricum than with original strain M-55, combinations of these strains with M-55 were tested which were designed to take advantage of the specific properties of these strains.

A spore suspension of Strain M-55 was mixed just prior to intravenous injection with an equal part of a spore suspension of Cl. felsineum. The doses per animal were 40 million spores of each type of organism. For comparative purposes, Strain M-55, as well as the felsineum strain, was used alone in doses of 40 and 80 million spores per animal. The results can be summarized as follows: The lytic effect of the combination was qualitatively and quantitatively better than injection of each strain separately. The start of lysis was precisely the same as that observed with Strain M-55 alone, but in the following days the percentage of macroscopically complete lyses was more favorable. In instances of only partial lysis, no further lysis was observed later on, even though the strain of felsineum should start its effect later owing to its slower course of action. Repeated injection of the mixture of strains, or even of the strain M-55 alone, resulted in more frequent deaths of the animals. This effect was never observed if Strain M-55 was used exclusively. Since this behavior of the combined strains would be of great disadvantage in any practical application, the results of this combination experiment had to be considered as negative.

The disadvantage of strain Cl. paraputricum, which by itself has excellent lytic properties, was its uncertain nonpathogenicity. It was, therefore, attempted to mix culture filtrates of this strain with the spores of the standard strain of Cl. butyricum (M-55). Previous results had shown that, in general, sterile culture filtrates of any of the strains investigated were not capable of producing oncolysis, either when they were injected intravenously or when they were injected directly into the tumor. Nevertheless, it seemed possible that the spores of M-55 combined with the metabolic products of strain Cl. paraputricum could produce an improved result. A sterile culture filtrate of a 5-day-old iron nail broth culture of Cl. paraputricum, therefore, was mixed with a spore suspension of Strain M-55 and was given intravenously to the tumor-bearing animals at a dose of 40 million spores. The results obtained with this mixture, and a number of variations of this experiment, can be summarized by saying that these mixtures did not show any particular pathogenicity in the animals but that the extent of lysis was not improved over that obtained with Strain M-55 alone.

Tests with Strain M-55 in different growth phases.—As already indicated in the introduction, even in the earliest experiment it was attempted to work with as pure suspensions of spores as possible. Nevertheless, it was of theoretical interest to determine whether other growth phases of the microorganism besides spores in the resting stage could be used.

For these purposes, the organisms were again grown in iron nail broth, and as soon as they had reached the various growth stages a suspension was prepared in the usual
PREPARATION
A. Resting spores
B. Pregermination spores
C. Sporangia
D. Rods

Resting lysis was observed than after intravenous injections. therefore, attempted to obtain an improved result by to those obtained after intravenous application. It was, intratumoral application usually gave results inferior was produced as with preparation A. intratumoral injection of preparation D, but no better tumor were already quantitatively less favorable. This number of microorganisms in these forms that entered the vegetative forms were rapidly removed by the normal resistance mechanisms of the body and that, therefore, the number of microorganisms in these forms that entered the tumor were already quantitatively less favorable. This was particularly noticeable with respect to the percentage of lysis obtained. This difference can probably be explained by the fact that the vegetative forms were rapidly removed by the normal resistance mechanisms of the body and that, therefore, the number of microorganisms in these forms that entered the tumor were already quantitatively less favorable. This assumption was borne out by the fact that, if five times the doses of preparation D were used about the same oncolysis was produced as with preparation A.

It had been noted previously in experiments with spores that intratumoral application usually gave results inferior to those obtained after intravenous application. It was, therefore, attempted to obtain an improved result by intratumoral injection of preparation D, but no better lysis was observed than after intravenous injections.

Combination of spores with tumor extracts.—In the search for conditions that might increase the number and extent of lysis to as close to 100 per cent as possible, experiments with tumor extracts were also performed. The early experiments were done with crude sterile filtrates of Ehrlich tumors that had been previously treated with spores; later on, filtrates from untreated tumors were mostly used. Since preliminary experiments gave encouraging results, extracts of human tumors were then drawn into the study. In particular, a filtrate obtained from a human stomach carcinoma seemed to produce decidedly enhanced effects. Since the results with extracts of different tumors were not easily reproducible, the

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Methylen blue*</th>
<th>Sporangia (per cent)</th>
<th>Rods (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stainable (per cent)</td>
<td>Non-stainable (per cent)</td>
<td></td>
</tr>
<tr>
<td>A. Resting spores</td>
<td>3</td>
<td>90</td>
<td>4</td>
</tr>
<tr>
<td>B. Pregermination spores</td>
<td>75</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>C. Sporangia</td>
<td>15</td>
<td>4</td>
<td>68</td>
</tr>
<tr>
<td>D. Rods</td>
<td>2</td>
<td>2</td>
<td>10</td>
</tr>
</tbody>
</table>

* Spores that are nonstainable with methylene blue are resting and heat-stable, whereas stainable spores are heat-labile and presumably in the process of germination.

fashion and washed as described. Heating of the suspension was, of course, omitted, since the effectiveness of heat-sensitive forms was also to be tested. As indicated in Table 2, four different preparations were tested. In these experiments, number of organisms did not refer to the number of spores injected but to the total number of microorganisms—i.e., a total of 40 million microorganisms was injected intravenously per animal.

At least in principle oncolysis could be elicited with all the growth phases of the microorganisms, although there were qualitative and quantitative differences. There were only minor differences in the results obtained with preparations A, B, and C, but the experiments using sample D, which contained primarily vegetative forms, yielded less favorable results. This was particularly noticeable with respect to the percentage of lysis obtained. This difference can probably be explained by the fact that the vegetative forms were rapidly removed by the normal resistance mechanisms of the body and that, therefore, the number of microorganisms in these forms that entered the tumor were already quantitatively less favorable. This assumption was borne out by the fact that, if five times the doses of preparation D were used about the same oncolysis was produced as with preparation A.

It had been noted previously in experiments with spores that intratumoral application usually gave results inferior to those obtained after intravenous application. It was, therefore, attempted to obtain an improved result by intratumoral injection of preparation D, but no better lysis was observed than after intravenous injections.

Combination of spores with tumor extracts.—In the search for conditions that might increase the number and extent of lysis to as close to 100 per cent as possible, experiments with tumor extracts were also performed. The early experiments were done with crude sterile filtrates of Ehrlich tumors that had been previously treated with spores; later on, filtrates from untreated tumors were mostly used. Since preliminary experiments gave encouraging results, extracts of human tumors were then drawn into the study. In particular, a filtrate obtained from a human stomach carcinoma seemed to produce decidedly enhanced effects. Since the results with extracts of different tumors were not easily reproducible, the

filtrate of one tumor was subjected to further fractionation, and the data in Table 3 refer to this extract.

A human stomach carcinoma was minced, lyophilized, freed of fat by extraction, and aliquots of the dried residue were then extracted with water and with acetone-water (1:7). The suspensions were centrifuged at 0–5°C. and both the residue and the supernatant were tested. As indicated in Table 3, addition of the extracts, as well as of the residue, produced an improvement in the lytic process which was most clearly evident in a more rapid onset of the lysis. Since these results indicated that the active substance was present in the aqueous extract, this aqueous extract was also used in a small series of mice bearing a methylcholanthrene-induced sarcoma. In this case the start of lysis was not particularly speeded up (7.7 days in the controls, 6.0 days in the treated group), but the extent of lysis was substantially improved. In the control animals treated with spores alone, three showed no lysis at all, seven showed partial lysis, whereas only one animal showed a macroscopically complete lysis. In the 23 animals which were treated with spores combined with the aqueous tumor extract, all animals showed macroscopically complete lysis. Further attempts at fractionation of the aqueous extract indicated that the material could be adsorbed on charcoal, and spectrophotometric examinations of the eluates from the charcoal showed a pronounced absorption maximum at 405 mÅ. These observations led Dr. Gericke (Farbwerke Hoechst, A.G.) to an investigation of porphyrins on the growth cycle of clostridia which are reported in a subsequent paper (2).

**DISCUSSION**

In principle, it appears that the spores find in the tumor conditions that permit germination and rapid growth which is accompanied by an extensive lytic process in the tumor. This phenomenon was strikingly observed in larger tumors in which the germination and growth of the microorganism occurred massively and correspondingly produced rapid and extensive lysis. If one considers the fact that such older and larger tumors already before treatment regularly contained small areas of necrosis, then it becomes very likely that the naturally occurring areas of necrosis are the primary point where the germination of the spores occurred. However, only in the tumor do the spores find a milieu which permits not only the
outgrowth of a few rods but the massive germination and continued growth of the organisms. Only in the tumor the abundant metabolic products of the microorganisms that are thus formed lead to the tissue destruction which has been described. In none of the tumors tested did this destruction go beyond the limits of the tumor, and the organs of the animals did not show any pathological changes. The observations also emphasize the distinctive properties of the tumor milieu for the growth requirement of the microorganisms.

Even after the microorganisms had produced extensive lysis, it seemed that secondary infections led to difficulty with further lysis of the remaining tumor. This was probably owing to changes in the milieu that accompanied the secondary infection. A direct antibacterial action of secondary infections on the strain M-55 could not be observed in vitro. All the experiments indicated that the tumor contained metabolic products that were favorable to the germination and growth of clostridia. One of the essential points of these studies was the observation that germination and growth of clostridia occurred in different transplanted and induced tumors.

The experiments with a number of different strains of clostridia indicated that none of the strains tested showed any substantial improvement over that obtained with Strain M-55. In general, strains which were capable of proteolysis only but showed only minimal carbohydrate fermentation were decidedly inferior. Combination of different strains of clostridia or their metabolic products did not seem to yield improved results. The experiments with different growth stages of clostridia indicated that there was no advantage in using other growth stages of clostridia but the heat-resistant spores.

ACKNOWLEDGMENTS

Appreciation is expressed to Dr. D. Gericke, Head of the Laboratory for Cancer Research, Farbwerke Hoechst, A. G., Frankfurt M, Germany, for freeze-dried spores.

REFERENCES

Oncolysis by Clostridia. I. Activity of Clostridium Butyricum (M-55) and Other Nonpathogenic Clostridia against the Ehrlich Carcinoma

J. R. Möse and G. Möse

Cancer Res 1964;24:212-216.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/24/2_Part_1/212

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.