Stimulation of Host Defense against Experimental Cancer

I. Zymosan and Sarcoma 180 in Mice*

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The yeast product zymosan was originally received by this laboratory in early 1955 and tested against Sarcoma 180 (S-180) in mice for possible tumor-inhibitory properties. Our interest in the material was fostered by the discovery by Pillemer and associates (10) of the interaction of zymosan with the properdin system. The fact that properdin may be an important component of host natural immunity raised the question of its possible involvement as a barrier against progressive neoplastic disease. Parenteral administration of zymosan to animals has been shown to affect markedly serum properdin levels. Thus, low doses of zymosan stimulate high levels, while large doses apparently remove properdin and maintain lowered serum levels (11). In addition, there was a historic rationale for examining high molecular weight polysaccharides with respect to tumor-inhibitory properties considering the activity of similar materials observed in the past (8, 13). Our studies with zymosan in mice bearing S-180 indicate that under certain conditions this material is capable of promoting tumor loss by a host defense reaction (9). Data supporting this assumption are presented herein.

MATERIALS AND METHODS

The zymosan used throughout these experiments was Fleischmann Lot #5-B-171.1 It was homogenized to a uniform suspension in 0.5 per cent carboxymethylcellulose-normal saline (CMC) and sterilized by being boiled in a water bath for 1 hour. Female Swiss albino mice 100 or more days old from the O'Grady Farm were used for all experiments. The Sarcoma 180 was implanted by trocar in the axillary region, and the mice were maintained according to procedures previously described (4). Tumor growth and regression were determined weekly or oftener by measurement of the tumor mass through the skin in two perpendicular diameters and by calculation of the average diameter. The intraperitoneal route of injection was used with doses made up in the same volume for injection: 0.5 ml/mouse.

RESULTS

Zymosan was tested initially in the S-180 screening program with the routine 7-day chronic dose therapy regimen employed for examining materials for antitumor activity (5). Toxicity was achieved in mice with S-180 at 500 mg/kg/day (total 3250 mg/kg) with 60 per cent mortality, though no tumor inhibition was noted at 8 days—the usual time for terminating negative screening experiments. There was weight loss and diarrhea noted in the surviving treated animals at the end of therapy but not in the controls. Since we were aware of the possible effects of zymosan on host defenses, the material was re-examined from the standpoint of bacterial pathology by challenging normal mice with 250, 500, and 1000 mg/kg/day (total 1500, 3000, and 6000 mg/kg) for 7 days. The animals that died and the survivors who were killed were frozen and later autopsied. Previous work has shown that post-mortem cultures are fairly reliable indices of bacteremia in the living mouse (2). Cultures from the heart indicated probable bacteremia of enteric origin involved in the death of the animals, since from ten out of ten mice receiving the 1000 mg/kg/day dose, gram-negative bacteria were recovered (mostly Proteus mirabilis). Only one animal had such a bacteremia in the group of ten receiving 500 mg/kg/day, and none among three similar groups receiving 250 mg/kg/day, CMC, or no injections; similar, more extensive observations have been made with rats by Cappuccino (4). Mortality data are shown in Table 1. With this

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evidence that the animals' defenses against endogenous infection may have been impaired by zymosan, we began testing the material at low levels on a variety of dose schedules in the hope that the converse phenomenon, stimulation of defense mechanisms, might occur and effects on tumor would ensue. A representative experiment is presented to show the effect of zymosan treatment. Two groups of 30 mice each were implanted with S-180. Twenty-four hours later one group received a single dose of 20 mg of zymosan/kg body weight given intraperitoneally, while the other group received CMC diluent only. Tumor measurements were taken through the skin 3 times weekly for 33 days, and the average diameter for each group was calculated. On the graph (Chart 1) the treated group is subdivided into two groups, so that separate lines indicate the tumor growth in the animals that died and the tumor trend in mice that survived. The tumors in all categories grew in identical fashion for 2 weeks. During and after the 3d week, many tumors in the treated mice became extensively necrotic, ulcerated through the surface of the skin, and were gradually reduced in size until complete extrusion was accomplished. It seemed identical to the process occasionally observed in the past among control animals. This similarity was also apparent on histological examination of the regressing tumors. \(^2\) We noted in particular that the response to the treatment was by no means universal; rather, it was quantal in nature, with the tumors that killed the host following closely the control path of growth. This was borne out by the fact that there was no significant difference between the mean survival times of the animals that died in the treated and control groups. Mice that survived have been held for periods of time up to 6 months. In no case has a tumor returned. Some survivors from this experiment and from many other similar experiments have been implanted with S-180 a second time. In all cases the second implant failed to grow, although each cutting was tested and found viable and lethal in animals that were primary hosts.

Pursuant to the original observations of Andervont (1), the rejection of a second implant of S-180 in a mouse previously conditioned by growth of this tumor has been assumed to indicate acquired immunity to S-180. Chart 2 shows the change in weight of the zymosan-treated and control mice during the same 33-day period. All groups were similar in this respect for 2 weeks. Then, during the 3d week, the treated mice destined to survive maintained nearly normal weight, while those fated to succumb lost weight as did the controls. Apparently, reversal of tumor toxicity for the animal as revealed by weight change accompanied the necrotic process.

**Single-dose response.**—The dose was titrated in twofold steps from 10 to 320 mg/kg. All injections were given intraperitoneally 24 hours after tumor implantations. Survival data from this and all other experiments to date, in which the dose regimen conditions were identical, were included (Chart 3). There was a broad spectrum of response between 10 and 320 mg., with no suggestion of a straight line function. The increase in survival over that of the controls (av., 6 per cent) was significant (P < .001) for the doses from 10 to 160 mg/kg. Limited experiments with doses above 320

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**TABLE 1**

**TOXICITY OF MULTIPLE ZYMOSAN DOSES**

Normal mice treated for 7 days with divided daily doses for a maximum of thirteen injections

<table>
<thead>
<tr>
<th>Dose (Mg/kg/day)</th>
<th>Mortality</th>
<th>Days 3</th>
<th>Days 4</th>
<th>Days 5</th>
<th>Days 6</th>
<th>Days 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>9/10</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>1/10</td>
<td>1</td>
<td>2</td>
<td>2/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>2/10</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
mg. and below 10 mg. yielded survival results which were essentially the same as those for the controls.

In a separate single-dose toxicity study with tumor-bearing mice, doses from 250 to 8000 mg. were given, and the LD$_{50}$ was calculated at 1900 mg/kg (7). Although the general curative effects obtained with low doses (e.g., 20 mg/kg) were not apparent, a regression rate comparable with the controls continued as high as 1500 mg. These recovered mice were also immune to reimplantation.

**Multiple-dose response.**—In tumor-free animals given multiple doses, deaths occurred following injections of amounts totaling from 1500 mg/kg to 6500 mg/kg. It is noteworthy that the modal dose was 2000 mg/kg (Table 1). In an experiment to ascertain tumor effects, mice implanted with S-180 were given injections of daily doses ranging from 5 to 160 mg/kg, given divided twice daily starting 24 hours after tumor implantation and extending for 7 days for a total of thirteen injections (Table 2). An unusually large number of spontaneous regressions occurred among control animals (normal rate, 6–10 per cent), an event which detracted from the meaningfulness of the curative effects of the treatment. Nevertheless, the per cent surviving at the two lowest doses was significantly higher than among the controls. With respect to the total amount of zymosan given, these curative responses were in the range noted for single doses. Thus, it would seem that the effect was cumulative. Regarding the control regression, two explanations are feasible: either these mice were rather poor tumor hosts to start with or they may have responded in some way to multiple CMC injections. The observation that the 160-mg. dose group manifested the lowest survival suggests that this dose might actually have improved tumor growth by depressing host resistance. Evidence making such an idea plausible is presented below.

**Reversal of curative effects.**—Considering the lack of curative effect by high doses of zymosan and reports that such doses increase susceptibility to bacterial infection (12), we examined the possibility that a high dose might therefore antagonize the curative effects of a low dose. Four groups of mice bearing S-180 were treated in the following manner: the first groups was given 20 mg of zymosan/kg on day 1 and the CMC diluent on day 2. The second group was given CMC on day 1 and 320 mg. on day 2; the third, 20 mg. on day 1 followed by 320 mg. on day 2; and the fourth group was given injections twice, with CMC as a control. Sixty-seven per cent of the mice were cured of their tumors in the 20-mg. group and 25 per cent in the 320-mg. group (Table 3). Although the
same in all cases. A single test with mouse mammary Adenocarcinoma 755 grown in B16BC mice
gave a response of 9/23 tumor-free survivors and 14/23 dead of tumor at the termination of the
experiment 8 weeks after treatment with 20 mg/kg zymosan, compared with 17/21 dead of tumor and
4/21 large growing tumors in controls (control regression rate <0.5 per cent). The nine survivors
were reimplanted: four with Ca-755 and five with S-160. The Ca-755 would not grow at all, since
even the implant pieces were impalpable by 3 weeks; the S-160 grew and killed the remaining
animals in typical fashion. Experiments have been performed which suggest that zymosan treatment
may significantly alter the growth of the Cloudman S-91 Melanoma in DBA/2 mice.
Zymosan has been
tested against S-180 and normal mouse tissues (connective tissue and epithelium) in tissue culture.
It had no direct cytotoxic effect as assessed by mitotic counts and numbers of degenerate
nuclei.

DISCUSSION

It would seem that zymosan promotes tumor loss in mice bearing S-180 through the medium
of host defense mechanisms rather than by any direct inhibitory action on the tumor. Evidence
which can be cited includes the facts that: (a) the effect is considerably delayed beyond the time the
treatment is given; (b) the response is quantal in nature, as opposed to the over-all tumour-suppressive
action seen with many chemical agents; (c) zymosan is more effective at low doses than at
high doses. This failure to reveal a typical ascending dose-response reaction in dose ranges relatively
innocuous to the host militates against a direct tumor toxic effect; (d) S-180 in tissue culture is
not affected by zymosan. It is important, however, that immediate direct stimulation of antitumor
antibody by zymosan appears to be ruled out by the available evidence (although acquired immuni-
ty is certainly the terminal phenomenon). The negative facts are: (a) the treatment can be given
before tumor implantation, i.e. before the animal has experience with the antigen—yet from one ex-
periment, at least, the terminal immunity formed seemed specific for the tumor present; (b) the
tumor loss effect can be created and largely destroyed within 48 hours (reversal experiment)—
far too fast for acquired antibody reactions; (c)

2 Properdin assays were performed in the laboratory of Dr.
Louis Pillemer. One of the problems encountered is the observa-
tion that CMC interferes with the assay procedure (Dr.
Pillemer, personal communication).

3 The B16BC mouse was developed by Dr. John J. Bittner.
It is derived from the C57BL/6.

4 Unpublished experiments by V. T. Riley.

5 Tissue culture experiments with zymosan were performed
for us through the courtesy of Dr. John J. Biesele.
animals receiving up to 1500 mg/kg single dose of zymosan could still lose tumors at the control rate and be immune thereafter to reimplantation of homologous tumor. Thus, high doses of zymosan, though capable of reversing low-dose effects, did not seem to interfere in any way with the innate ability of the animal to acquire immunity. These discrepancies might be explained on the basis of at least a two-step phenomenon. Low doses of zymosan (10–160 mg/kg) may stimulate some intermediate factor which in turn affects the tumor and/or the host in such a way that the tumor-host relationship is changed in favor of the host. As a consequence, the specific immune mechanisms of the host "recognize" the tumor as a foreign entity and participate in the final rejection of the tumor. Large doses (320+ mg/kg) appear to remove or block the intermediate factor without interfering with the capacity of the mouse to respond immunologically.

In appraising this hypothesis three reports become salient. These are (a) the experiments of Pillemer and Ross (11) showing that serum properdin levels can be raised by low doses and lowered by high doses of zymosan; (b) the demonstration by Palm (9) and by Herbut and Kraemer (6) that zymosan injection can alter host defense mechanisms sufficiently to permit heterologous tumor transplantation; and (c) the discovery by Southam and Pillemer (14) of a remarkable correlation between human serum properdin levels and host defense reactions against human cancer. These observations make recognition of the intermediate factor as some function of the properdin system a tempting thesis. However, it must be emphasized that only correlations relating to such an assumption are available. Possible identification of the intermediate factor discussed here with the properdin system must await more direct evidence.

SUMMARY
1. Treatment of mice bearing Sarcoma 180 with small doses of the yeast product zymosan has been shown to promote a significant degree of tumor loss.
2. The results of various experiments indicate that this phenomenon is entirely an immune reaction mediated by the host.
3. Involvement of some function of the properdin system is suggested by correlative evidence, but remains to be proved or disproved by more direct means.

ADDITION
After this manuscript was submitted for publication, Man-kowski et al. reported the activity of zymosan in promoting recovery from tumor in mice bearing Sarcoma 37 (Proc. Soc. Exper. Biol. & Med., 96:79–80, 1957).

REFERENCES
Stimulation of Host Defense against Experimental Cancer: I. Zymosan and Sarcoma 180 in Mice

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