Stimulation of Host Defense against Experimental Cancer
II. Temporal and Reversal Studies of the Zymosan Effect*

WILLIAM T. BRADNER† AND DONALD A. CLARKE
(Experimental Chemotherapy Division, Sloan-Kettering Institute for Cancer Research, and Sloan-Kettering Division of Cornell University Medical College, New York, N.Y.)

It has now been amply confirmed that the injection of zymosan can stimulate (6, 24) or depress (13, 27) host defenses against certain transplanted tumors, depending upon doses used and test circumstances. Initial experiments in our laboratory demonstrated that, if high chronic doses of zymosan were administered to mice, the animals frequently died with bacteremia of enteric origin. Indeed, these studies led to the discovery of Salmonellosis in the mouse colony (5). Based on this evidence of effect on host defense mechanisms, tests of low doses of zymosan were initiated to ascertain whether postulated defense stimulation (31) would occur which, in this case, would be active against tumors. For the past 3 years repeated experiments have been conducted in which the injection of 20 mg/kg zymosan intraperitoneally within several days of tumor implantation in 100+-day-old Swiss mice bearing Sarcoma 180 has caused 50 per cent or more regression of tumor in treated animals, as compared with 10 per cent or less regression among controls. Explorations into any possible role played by properdin in this phenomenon have been delayed by difficulties encountered in properdin assay procedures. In the meantime, information has been gathered regarding materials which can reverse zymosan activity, i.e., block the tumor regression phenomenon so that the tumors in treated animals grow and kill their hosts just like those in untreated controls.

It is the purpose of this paper to present the results of some of these studies on the reversal of the effect of zymosan on S-180 in Swiss mice and to explore further various theories which have been entertained with respect to zymosan-tumor interaction. Sarcoma 180 is recognized to be sensitive to regression; but it is this very property which makes S-180 a useful tool with which to study the process of host defense stimulation. If efforts to elucidate mechanisms meet with eventual success, the application of such findings to isologous and spontaneous tumor systems would be a logical next step.

MATERIALS AND METHODS
Female, Swiss Webster mice from the Flora O’Grady farm were used. These animals were always 100 or more days old, since it has been established that young mice respond poorly to zymosan. (This observation has now been confirmed with Millerton Farms ICR mice.) Sarcoma 180 was implanted by the usual trocar procedures (8) and tumor growth determined weekly by external caliper measurement. In all experiments the ultimate survival of the animal was the endpoint. This has been taken as 3 weeks after complete disappearance of the tumor. Experience in our laboratory has shown that S-180 has never returned after this time and that mice cured of this tumor are still immune to a second implant of it 11 months later. As previously alluded to (7) and discussed at a recent meeting (4), problems were encountered with the use of carboxymethylcellulose-saline (CMC) as a vehicle for zymosan injection. Since that time the zymosan† has been prepared in normal saline and sterilized by being boiled in a water bath for 1 hour. The injections were made intraperitoneally with the material so diluted that the volume was always 0.5 ml/injection. Since it is well known that stress factors may influence defense mechanisms, strict attention has been addressed to control procedures; e.g., mice treated with a single dose of zymosan were given multiple doses of saline like the controls if another animal group in the experiment was receiving multiple-dose treatment.

† The zymosan used, Fleischmann Lot 85-B-171, was received through the courtesy of Mr. Robert F. Light, Technical Director, Special Products Division, Standard Brands, Incorporated.

Received for publication February 10, 1959.
RESULTS

We have previously reported that a high single dose of zymosan (320 mg/kg) injected 2 days post-tumor implantation into mice treated on day 1 with 20 mg/kg of zymosan can reduce the survival rate from 50+ per cent, characteristic of the low dose, to the ca. 25 per cent characteristic of the high dose. In the following experiment multiple doses of zymosan were injected to determine whether still higher total quantities could obliterate the low-dose curative effect completely. Administration of 1000 mg/kg (200 mg/kg/day 5 X) could block the 20-mg. effect whether started 2 or 9 days after tumor implantation (1 or 8 days after low-dose treatment). These high doses were completely ineffective by themselves in promoting tumor regression (Table 1). Toxicity (many early deaths) was noted in the group receiving 20 plus 200 (5 X) mg/kg beginning at day 9, but not in any of the other groups. Whether this represents, for example, a toxic manifestation from the possible formation of large amounts of properdin-zymosan complex or some other type of sensitization remains to be determined.

Reversal by hydrocortisone.2—The depressive effects of the corticoids on host defense mechanisms have been extensively investigated (20). These substances can alter host defenses sufficiently to permit take and growth of heterologous tumors (39). A single injection of 150 mg/kg of hydrocortisone 2 days after implantation of the human epidermoid carcinoma #3 (H.Ep. #3) into Swiss mice will effectively condition the animals for growth of this heterologous tumor.3 To see whether hydrocortisone could block the zymosan-stimulated host defense reaction a series of experiments were performed in which single blocking doses were injected either on day 2, 9, or 13 post-tumor implantation, with zymosan treatment given day 1. The total survival data are presented in Table 2. Zymosan therapy followed by hydrocortisone on day 2 was essentially as effective as zymosan alone with respect to tumor regression but was rendered ineffective if the steroid was administered on day 9 and partially so if the steroid was given on day 18. Hydrocortisone did not promote tumor regression by itself.

Influence of time of zymosan injection.—Earlier studies showed that zymosan could be injected at 4, 3, 2, or 1 day before tumor implantation or at 1 or 3 days after tumor implantation and be just as effective in causing tumor regression. The following experiment was designed to explore this time relationship still further. Groups of mice were treated separately with 20 mg/kg zymosan either 14, 7, or 3 days before or 1, 3, 7, or 14 days after tumor implantation. The results were unexpected in that nearly all groups appeared to respond to the treatment to some degree, regardless of the time given (Table 3). The treatment on days 1 and 7 post-tumor transplantation yielded the highest percentage of survivors.

Effect of zymosan and of hydrocortisone on second set phenomena.—Since it was apparent that the primary host defense could be blocked we were interested in learning whether the established immunity could be altered in any way by the treatments used. Several large groups of animals were implanted with S-180 and treated with 20 mg/kg of zymosan. After 8 weeks the animals that had experienced complete regression of their tumors were segregated and reimplanted with S-180. A small group of normal animals was also implanted with pieces from the same tumor as a control on viability. The five groups of immune mice were treated with one of the following schedules: 150 mg/kg hydrocortisone once on either day 1 or 9, 150 mg/kg hydrocortisone once on day 1 followed by 200 mg/kg/day zymosan (5 X) on days 2–6, the 200-mg. zymosan regimen alone, or saline (control) at all injection times. In all groups only an occasional tumor grew significantly above tumor piece size, and within 3–4 weeks nearly all the tumors had disappeared completely. Thus these treatments could not alter the acquired immunity to S-180 once it was established.

### Table 1

<table>
<thead>
<tr>
<th>Treatment in mg/kg/day</th>
<th>Survivors/Per cent</th>
<th>Days following tumor implantation</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-Z</td>
<td>S</td>
<td>14/17</td>
</tr>
<tr>
<td>200-Z</td>
<td>S</td>
<td>1/18</td>
</tr>
<tr>
<td>200-Z</td>
<td>S</td>
<td>5/18</td>
</tr>
<tr>
<td>S</td>
<td>S</td>
<td>2/16</td>
</tr>
<tr>
<td>S</td>
<td>S</td>
<td>5/19</td>
</tr>
</tbody>
</table>

This table comprises a summary of two separate experiments.

20-Z = 20 mg zymosan per kg, given intraperitoneally in 0.5 ml saline. The 200-mg/kg dose (200-Z) was given 5 times for a cumulative total of 1000 mg/kg body weight.

S = Saline, 0.5 ml intraperitoneally.

1 Hydrocortone acetate, Merck, Sharp & Dohme.

2 Unpublished experiments conducted by Dr. P. C. Merker.
Additional observations.—Because the chemical properties of zymosan are reminiscent of those of the bacterial endotoxins which have received so much attention in experimental cancer therapy, we believed it would be of interest to see whether zymosan possessed some of the characteristics ascribed to endotoxins. One activity, the Shwartzman phenomenon, has been examined in some detail. It has been associated with tumor necrotic activity since the observations of Gratia and Linz (10). Shwartzman and Michailovsky (36), in a like manner, described the action of bacterial filtrates on S-180 in mice. Therefore, it seemed desirable to determine whether zymosan would function in the Shwartzman phenomenon as a test of its conformity to the general properties of endotoxins. Recently, Kelly and associates (22) described a Shwartzman-like activity in mice which was strain-dependent. Zymosan has been examined as both a preparatory and provocative agent in Swiss-O’Grady mice, in BSVS-Rockefeller mice, and in rabbits (1). It was found that zymosan in a dose of 20 mg/kg could neither prepare nor provoke the Shwartzman skin reaction of hemorrhagic necrosis in susceptible mice and rabbits (Escherichia coli 0117 B Lipopolysaccharide, Difco was used as a positive control). Furthermore, this reaction could not be elicited at all from the Swiss-O’Grady mice with E. coli endotoxin, even though a Shwartzman-negative lignosulfonate capable of necrotic activity against Sarcoma 37 has been described by Perrault et al. (90).

DISCUSSION

Many theories have been entertained regarding the mode of action of zymosan and of other high molecular weight polysaccharide substances with respect to anti-tumor properties (e.g., see Donnelly et al. [9]).

TABLE 2

Reversal of Low-Dose Zymosan Effect with Hydrocortisone. Survival of Swiss Mice Following S-180 Growth

<table>
<thead>
<tr>
<th>Treatment in mg/kg</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 9</th>
<th>Day 13</th>
<th>Survivors/No. Mice</th>
<th>Percent Survival</th>
<th>S. E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-Z</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td></td>
<td>20/35</td>
<td>53</td>
<td>±8.1</td>
</tr>
<tr>
<td>20-Z + 150-F</td>
<td>S</td>
<td>150-F</td>
<td>S</td>
<td></td>
<td>16/35</td>
<td>46</td>
<td>±8.4</td>
</tr>
<tr>
<td>20-Z</td>
<td>S</td>
<td>S</td>
<td>150-F</td>
<td></td>
<td>2/28</td>
<td>7</td>
<td>±2.7</td>
</tr>
<tr>
<td>S</td>
<td>S</td>
<td>S</td>
<td>150-F</td>
<td></td>
<td>8/36</td>
<td>22</td>
<td>±6.9</td>
</tr>
<tr>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td></td>
<td>0/27</td>
<td>0</td>
<td>±6.5</td>
</tr>
<tr>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td></td>
<td>2/21</td>
<td>10</td>
<td>±1.9</td>
</tr>
<tr>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td></td>
<td>1/28</td>
<td>4</td>
<td>±2.1</td>
</tr>
</tbody>
</table>

This table comprises a summary of five separate experiments.

20-Z = 20 mg. zymosan/kg given intraperitoneally in 0.5 ml. saline.
150-F = 150 mg. hydrocortisone acetate/kg given subcutaneously in 0.2 ml. saline.
S = Saline; route and quantity in accordance with treatment.

Values significantly different from control are: 53% (P < 0.01), 46% (P < 0.01), and 22% (P < 0.05).

TABLE 3

Treatment with Zymosan at Different Times with Respect to Tumor Implantation. Survival of Swiss Mice Following S-180 Growth

<table>
<thead>
<tr>
<th>Treatment schedule</th>
<th>Days before (-) or after (+) tumor implantation</th>
<th>Survivors/No. Mice at 0 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-14 -7 -3 +1 +5 +7 +14</td>
<td></td>
</tr>
</tbody>
</table>
Recently some investigators have suggested the possible relationship between the properdin system (30) and tumor growth or regression (e.g., 6, 9, 12). The remarkable correlation between the in vivo properdin system activities of zymosan (31) and endotoxins (9) and their oncoytic properties was a principal reason for this suggestion. Furthermore a fall in serum properdin levels with progressive tumorous growth has been reported in studies with humans (17, 34, 37) and with laboratory animals (5, 14). Isliker (18) has cited studies which show that neoplastic but not normal tissue binds considerable amounts of properdin which, under favorable conditions, can be released again in vitro. A small quantity of highly concentrated human properdin (400 μg/ml) was recently made available to us. Injection of 0.2 ml/mouse intravenously once 24 hours after tumor implantation resulted in 5/10 complete regressions among treated animals compared with 1/9 in saline controls. However, even if this observation can be reproduced, it will be necessary to ascertain whether the effect was due to the fact that the material injected was properdin or just a large molecule. Also, further investigation in tumor systems of some of the humoral factors which have properties suggestive of properdin has shown that they can be distinguished from properdin (15, 23). As observations accumulate it seems difficult to deny that the properdin system, by whatever definition (25), is in some way involved in the biology of certain mammalian neoplasms, even if only as a secondary indicator of the disease state. Its possible relationship to the in vivo tumor effects of zymosan remains unestablished in any system thus far investigated.

The reticulo-endothelial (R.E.) system has been under consideration for a long period of time with respect to a possible role in neoplastic disease (28). In fact, impaired R.E. function as reflected by phagocytic activity has been suggested as a diagnostic test for advanced neoplastic disease (38). Of direct interest here are the reports by Benacerraf and Sebestyen (2) and by Kelly (21) which reveal that moderate doses of zymosan injected into mice can cause marked stimulation of R.E. phagocytosis and presumably liver R.E. cellular hyperplasia. It is tempting to suggest that some form of R.E. stimulation or activity may comprise an initial step in zymosan-caused oncolysis; but some conflicts remain to be resolved (see below), and more meaningful ways of studying its function need to be applied. Investigations of the R.E. system are proceeding in this laboratory.

5 We wish to thank Dr. B. E. Sanders of Merck, Sharp & Dohme for the properdin sample.

Considering the inconclusiveness of the above outlined possibilities, we believe it would be unwise to postulate a specific mechanism for the reversal studies reported here. However, information available on in vivo corticoid activities suggests at least one interpretation. Thus, cortisone has been reported to inhibit antibody synthesis (20), block stimulation or restoration of R.E. phagocytosis but not affect normal R.E. activity (8, 33), depress R.E. phagocytosis (11, 26), block "immunological enhancement" (19), alter response of Sarcoma 37 to endotoxin (32), and depress host defenses against heterologous tumor transplantation (39). Hinz has reported that serum properdin levels in humans were not affected by adrenal replacement therapy or steroid administration (16). In view of this information the temporal aspect of the hydrocortisone reversal of zymosan activity becomes significant. Thus, at the very time when a similar dose of corticoids can markedly block R.E. hyperfunction or defenses against heterologous tumor transplantation, hydrocortisone fails to block zymosan activity effectively. It does, however, block completely at a time which would be consistent with new antibody synthesis. The block begins to diminish at 13 days—a time when antibody synthesis may be well under way. Toolan (40) has shown that humoral and cellular cytotoxins against heterologous tumors appear in a time sequence consistent with that noted above. She also reported, as has Sachs (32), that these cytotoxins were associated solely with the lymphoid tissues. The corticoid sensitivity of these tissues is common knowledge. Thus it would seem, in the S-180 zymosan experiments at least, that hydrocortisone may act directly upon the production of factors representative of acquired immunity, rather than what has been suggested (7) as an early, nonspecific phase in this host defense process. The fact that a discrepancy exists between the capacity of hydrocortisone and large single or multiple doses of zymosan to block the low-dose zymosan activity in the early stages adds further evidence to the hypothesis that at least two steps are involved in this host defense process.

Although mechanisms of the corticoid-sensitive phase of zymosan-induced tumor regression can be speculated upon, nothing is known of the mechanisms of the corticoid-resistant phase. Perhaps this is the phase during which the recognition of a foreign antigen as such occurs. Stimulation to make this process stronger and less specific while concurrently causing slight but antigenically competent changes in tumor tissue may represent a worthwhile approach to cancer therapy.
SUMMARY

1. Further studies have been conducted on the capacity of low doses of zymosan to stimulate host defenses against Sarcoma 180 in mice.

2. It was found that a single dose of 150 mg/kg hydrocortisone acetate injected at various times after tumor implantation could completely block at 9 days, partially block at 13 days, and not affect at 2 days the tumor regression which normally occurs following zymosan therapy.

3. Injection of multiple doses of zymosan totaling 1000 mg/kg starting either 2 or 9 days after tumor implantation could block the low-dose effect completely.

4. Treatment with zymosan at various times from 2 weeks before to 2 weeks after tumor implantation revealed a diversity of response, but the greatest number of regressions occurred among animals treated at 1 to 7 days after implantation.

5. Various theories regarding the zymosan effects are discussed. The existence of an early (non-specific?) phase and a later acquired immunity phase in this host defense process is suggested. Exact mechanisms remain to be established.

REFERENCES


33. RAWLINGS B. W.; GODMEREY, J.; TICHENER, J. B.; and BAKER, E. The Effect of (1) Hypophysectomy Alone and


Stimulation of Host Defense against Experimental Cancer II. Temporal and Reversal Studies of the Zymosan Effect

William T. Bradner and Donald A. Clarke