Action of Bacterial Polysaccharide on Tumors

III. Repeated Response of Sarcoma 37, in Tolerant Mice, to Serratia marcescens Endotoxin

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SUMMARY

In the treatment of mice with Serratia marcescens polysaccharide, the pronounced tolerance induced by a single dose was surmounted when the subsequent doses were sufficiently increased. The overcoming of tolerance was observed in three phenomena: mortality of tumor-bearing mice; tumor-damaging potency of blood serum from treated mice; and repeated depression of tumor growth.

The tolerance which develops in various mammalian species following the administration of Gram-negative bacteria, or of the endotoxins prepared from them, has an analog in the failure of previously sensitive tumors to respond to repeated treatment with such materials. Despite the sensitivity of mouse Sarcoma 37 to a first injection of Serratia marcescens endotoxin, subsequent doses of larger size failed (8) to interrupt tumor growth again. In like fashion, this endotoxic polysaccharide induced tolerance in normal mice. A single injection conferred, upon their blood serum, potency in damaging 6-day-old implants of Sarcoma 37 in recipient mice; a second dose, even when increased several-fold, failed (9) to elicit tumor-damaging activity again.

The tolerance as reflected in the response of tumors in mice has several features different from that encountered in the febrile response (1, 2, 5, 7). The decrease in fever production usually is neither sudden nor complete. Much the same degree of fever may be evoked several times without increasing the dose of endotoxin. After a considerable degree of tolerance is established following repeated injection of a given dose, as seen from the gradual decline in fever production, doubling or tripling the dose is capable of restoring the previous response. On the other hand, a single dose of endotoxic polysaccharide blocked, rapidly and completely, tumor response to a second dose even when the latter was increased to 2.5 or 4 times the initial one.

This communication reports that the growth of Sarcoma 37 can be interrupted repeatedly by successive injections of the S. marcescens polysaccharide. The marked tolerance induced by the first dose was overcome by increasing the subsequent ones to higher levels than had been employed previously.

MATERIALS AND METHODS

CAF₁ mice and the tumor-necrotizing polysaccharide (Lot No. P45) from S. marcescens were employed as before (8, 9). The technics used were the same—viz., implantation of Sarcoma 37, measurement of tumor size, and bioassay in tumor-bearing mice of pooled blood serum. The polysaccharide was given by the intravenous route; serum was injected intraperitoneally. In all the experiments described in this paper the first dose of the polysaccharide was given on the 6th day after tumor implantation.

RESULTS

Lethality.—The first indication that the tolerance could be surmounted was provided by mortality data of tumor-bearing mice.

The minimum intravenous dose of this polysaccharide required to induce hemorrhage and necrosis in 6-day-old implants of Sarcoma 37 is about 2 μg/mouse. The mice withstand, in a single dose, amounts considerably above this, because deaths do not occur until a level ca. tenfold higher is reached.

Data on mortality were assembled from the many experiments which happened to include a
group of mice given a single dose of the polysaccharide on the 6th day of tumor growth. These findings, from more than 1,000 mice, are summarized in Table 1. At the 25-μg. dose level deaths were few; about half of the mice died (LD₅₀) when the dose was in the range of 50–100 μg/mouse.

A dose lethal as a first injection did not, however, yield the mortality shown in Table 1 when it followed by several days a prior dose of the polysaccharide. For example, 200 μg., which killed 73 per cent of the animals (see Table 1), killed none when preceded by a 10-μg. dose the previous day (see Table 2). Similarly, a single injection in the dose range 500–1,000 μg. killed almost all the mice (see Table 1), whereas a single injection in the range 500–2,000 μg. killed only 7 per cent of the animals when it followed a first small dose of 10 μg. (see Table 2).

This evidence of tolerance to the lethal effect of the endotoxin is even more strikingly exhibited in the experiments in which the initial dose (80 or 100 μg.) was approximately an LD₅₀. Here the second dose, greater than the first one by six- to tenfold, killed none (see Table 2).

These experiments, in addition to demonstrating the presence of tolerance, also indicated that a sufficiently high second dose was capable of overcoming the tolerance, to some extent, as shown by death of some of the animals after the second injection.

A third dose was also capable of partially overcoming the tolerance induced by two preceding injections. In these experiments, summarized in Table 3, the second dose was greater than the first by an order of magnitude or more. The third dose was still greater in all cases but one. The injections were spaced several days apart, as shown in the footnotes to the table. Percentage mortality was calculated on the basis of those which had survived the preceding dose.

In the first set of experiments summarized in Table 3 the initial dose was 25 μg. in all cases; no deaths followed. A second dose of 500 μg. was given 4 or 2 days later; induction of tolerance was evident from the low mortality (10–17 per cent) after this large second dose. Two days later a third dose was given, of different magnitude in each of the three groups. Again the presence of tolerance was indicated by the low death rate (11 or 4 per cent) produced by a third dose (600 or 1,000 μg.). However, a much larger third injection, of 4,000 μg., overcame the tolerance sufficiently to kill 45 per cent of the animals.

Analogous findings were obtained in the second
set of experiments shown in Table 8. Here, the first dose (60 μg.) was considerably greater than in the previous set; it was approximately LD₅₀. The few deaths following the second and third doses showed partial overcoming of the tolerance by these large doses.

Potency of blood serum.—We had found (9) that a single dose of this polysaccharide, in normal mice, conferred upon blood serum a potency in damaging tumors; this was shown by injection of their serum into mice bearing S-37. The conditions of dosage, and of interval between endotoxin injection and drawing of blood, required for demonstration of this potency have been described. This was accomplished when the subsequent serum from these mice failed to display any tumor-damaging potency. With a second dose 2.5 times greater, tolerance was still strong. However, when a more than sixfold second dose was given, the tolerance was overcome with a dose of 0.6 ml. of serum; even smaller amounts of serum showed considerable potency.

The clues from the observations on mortality suggested further attempts to overcome the tolerance. This was accomplished when the subsequent doses were elevated suitably. The results are summarized in Table 4.

The upper portion of the table gives the findings in two kinds of controls. Serum from untreated normal mice was injected intraperitoneally into recipients bearing 6-day-old implants of Sarcoma 37. To obtain enough serum for a dose of 0.6 ml. for each of ten mice, blood from 30 untreated controls was pooled. Normal mouse serum did not induce hemorrhagic necrosis in any of the tumors. Each of 30 normal mice was then given a single intravenous dose of 80 μg. of the polysaccharide; blood was drawn 45–60 minutes later. Serum from this blood produced damage in the tumors of all the recipients.

The set of experiments in the middle portion of this table summarizes the effects of a second dose. An initial dose of 80 μg. was given to each of 130 mice, which were then divided into three groups for the three different dose levels (80, 200, 500 μg.) of the second injection, given 4 days after the first. The lowest dose (80 μg.) failed to break through the tolerance induced by the prior injection; serum from these mice failed to display any tumor-damaging potency. With a second dose 2.5 times greater, tolerance was still strong. However, when a more than sixfold second dose was given, the tolerance was overcome with a dose of 0.6 ml. of serum; even smaller amounts of serum showed considerable potency.

The bottom third of Table 4 summarizes a set of experiments in which three doses were given. The initial dose of 60 μg. was given to each of 105 normal mice; a second, of 600 μg., was given 5 days later. Seven days after that, a third dose of 200, 800, or 2400 μg. was given. Tolerance was completely overcome, even at 0.1 ml. of serum, in the

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**Table 3**

<table>
<thead>
<tr>
<th>Dose* (μg./mouse)</th>
<th>Mice (initial no.)</th>
<th>Mortality after:</th>
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<td>First dose† (per cent)</td>
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<td>48</td>
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<td>60, 600, 2400‡</td>
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<td>47</td>
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</table>

* First dose given, in all cases, on 6th day of tumor growth.
† Calculated on basis of no. of survivors of preceding dose.
‡ Second and third doses, given, respectively, 2 and 4 days later.
§ Second and third doses, given, respectively, 6 and 10 days later.

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**Table 4**

<table>
<thead>
<tr>
<th>Dose* (μg.)</th>
<th>Donor serum injected (ml.)</th>
<th>Recipient Mice with induced tumor damage (per cent)</th>
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<tr>
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<td>First (μg.)</td>
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* The normal mice, which served as serum donors, received 1, 2, or 3 injections of the polysaccharide. When the first dose was 80 μg., the second dose was given 4 days later. When the first dose was 60 μg., the second dose was given 5 days later, and the third dose 7 days after that.
† Ten mice in each group. Serum was given to all recipients, on the 6th day of tumor growth.
Growth curves of tumors.—The first report (8) in this series described the interruption of tumor growth by an initial dose of polysaccharide and the failure of subsequent doses to repeat this effect. The successful overriding of induced tolerance, as revealed in mortality data and in potency of blood serum, prompted investigation of the effect on tumor growth of such treatment schedules.

Repeated depression of tumor growth was obtained in eleven experiments, under the conditions summarized in Table 5. Two of these experiments are shown graphically. In Chart 1, Curve A gives the rate of growth of S-37 in untreated mice from the 6th day after implantation. Curve B shows the depression of tumor growth produced by a single injection (80 μg. on the 6th day), with resumption of growth about 4 days later. On the 11th day a second dose (of 600 μg.) was given to about half the animals. This produced a second interruption of growth (Curve C); the conditions of this experiment, in which two doses were given, are listed in the first line of Table 5.

In other experiments, in which three doses were given, the amounts injected and the time intervals between doses are also listed in this table. As was shown previously, the initial dose in all such experiments produces interruption of tumor growth and also induces tolerance. Under the conditions summarized in Table 5, the second dose in all cases produced a second depression of tumor growth. The third dose produced a third interruption in all instances in which it was larger than the preceding one. The conditions of one such experiment are given in line 5 of Table 5 and the results

<table>
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<tr>
<th>First dose (μg.)</th>
<th>Interval to next dose (days)</th>
<th>Second dose (μg.)</th>
<th>Interval to next dose (days)</th>
<th>Third dose (μg.)</th>
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* Each of the treated groups consisted of 40—102 tumor-bearing mice.
† In all cases the first dose was given on the 6th day of tumor growth.

CHART 1.—Second response of Sarcoma 37 in mice made tolerant to S. marcescens polysaccharide. Curve A gives the growth of tumors in untreated controls. Curves B and C show the tumor growth in the mice of two treated groups; both received, intravenously, 80 μg. on the 6th day after implantation. Curve C shows the growth in one of these groups given a second dose (600 μg.) on the 11th day.

CHART 2.—Repeated response of Sarcoma 37 to successive doses of S. marcescens polysaccharide. A shows the growth of the tumors in untreated control mice. The mice in B received, intravenously, an initial dose (60 μg.) on the 6th day of tumor growth, a second injection (600 μg.) on the 12th day, a third one (800 μg.) on the 16th day, and a fourth (1000 μg.) on the 20th day.

These experiments, in which some 2,500 treated
and untreated tumor-bearing mice were employed, showed that tolerance could be surmounted repeatedly. The cost in mortality, particularly from the first dose, and a countermeasure are discussed in the next section.

DISCUSSION

The damage, both microscopic and gross, produced with endotoxic polysaccharides in responsive tumors is often dramatic. However, no additional effect was ordinarily observed after subsequent doses, even when the amounts injected were increased several-fold. Although it is possible that a sensitive tumor may become refractory after a first response, there is as yet no proof that organized tumor tissue becomes resistant. At any rate, treatment with endotoxin does not result in the selection and proliferation of resistant sublines of tumor cells (8, p. 1167).

Tolerance to endotoxin is evident from the reduction or abolition, of responses such as fever, local Shwartzman reaction, leukopenia, etc. It is now clear that host tolerance also plays an important role in the failure of the polysaccharide to affect tumor growth repeatedly. When the tolerance induced by a first dose was overwhelmed by a sufficiently great increase in the size of the second one, tumor growth was interrupted a second time. A third and fourth interruption in the rate of tumor growth was obtained only when each dose was greater than the preceding one. Curiously enough, the ratio of increase for the latter doses did not have to be as great as for the second one.

As is seen from the tables on mortality, an initial injection of 60 or 80 μg., such as was employed in the experiments shown in the charts, constitutes an LD₅₀ or more. However, the initial dose need not be a lethal one to obtain repeated depression of tumor growth with succeeding ones; this can be achieved when the first dose is so small that no mice are killed. For example, a safe initial dose of 10 μg. depressed the rate of tumor growth below that in untreated controls (see text—fig. 3 in Ref. 8), but the depression was not sufficient to prevent the tumors from continuing to increase in size. Under such conditions, although their growth was retarded with each subsequent injection, the tumors nevertheless grew progressively larger.

Dosage levels and intervals between injections necessary to restrain the tumors from continuing to enlarge were ascertained. Preliminary experiments indicated that close spacing of the doses was not sufficiently effective. Intervals longer than 4-6 days afforded the shrunken tumors time not only to recover but also to exceed their size at the start of treatment. To keep the tumors from becoming larger during the selected intervals, a dosage was required that was lethal to a high percentage of the mice. These conditions are not considered as definitive therapeutic schedules. They rather constitute a demonstration that endotoxic polysaccharide is capable of repeatedly interrupting tumor growth. Furthermore, it is clear that it can, with appropriate dosage, even prevent these mouse tumors from growing bigger. As yet this has been accomplished at a high mortality cost; it remains to be seen whether this price can be reduced.

Transplanted tumors such as Sarcoma S7 grow very rapidly. It is a common experience that repeated transplantation of even a slow-growing primary tumor usually selects out the fast-growing cells which then become predominant. In consequence, S-37 and many other widely used transplanted tumors kill their hosts in less than a month. For certain types of experimental work a transplanted tumor which would permit its host to survive for months instead of only a few weeks would be useful indeed. Regrettably, a slow-growing transplanted sarcoma of this kind has not been available; such a tumor might be kept from growing larger by nonlethal doses.

Because of the high death rate from the large dose required for the first injection to produce a reduction in tumor size, we tried various measures to lower this cost in mortality. In current experiments we are finding that salt solution is effective. For example, a first dose of 500 μg. of the polysaccharide, given on the 6th day of tumor growth, killed almost all the mice (see Table 1); salt solution, given after a suitable interval, protected all the mice from dying, and yet the tumors decreased in size. Attempts to prevent mortality from later doses are in progress.

Other ways of circumventing tolerance have also been under investigation. Various agents are being examined for capacity to enable tumors to respond repeatedly without necessitating a large increase in polysaccharide dosage. Among the materials under trial is pertussis vaccine which elicits hypersensitivity (4, 6). Treatment with this vaccine, interposed between injections of polysaccharide, has resulted in repeated interruption of tumor growth without requiring progressive increase in the dose of endotoxin. High mortality also accompanies this method of counteracting tolerance.

The striking difference, as regards the response to a second dose of endotoxin, between tumor damage and fever production (1, 2, 5, 7) was mentioned earlier. The pyrogenic response to a second
injection of the same dose may be as great as to the first, whereas the tumors completely failed to respond to a second dose even when it was double the first. With multiple doses of the same size, fever production may decrease gradually, whereas a single dose established complete tolerance with respect to tumor response. These differences may be a consequence of the disparity between the minute amounts required to elicit fever and the much greater quantities necessary to evoke tumor response.

In the course of these studies, complete regression of the tumor occurred in a few cases following multiple doses of the polysaccharide. Complete destruction of animal tumors has been reported from time to time by various workers, with crude bacterial preparations or with endotoxic products obtained from them. For example, in 1935 F. Duran-Reynals (3) obtained regression of a few mammary carcinomas of mice after injection of material from S. typhosa cultures. Since then, other workers have occasionally reported regressions of tumors with endotoxic materials, usually at the cost of considerable mortality.

The results obtained to date with S. marcescens polysaccharide have not led us to regard it as a therapeutic agent. It continues, however, to be useful in investigations of phenomena evoked by endotoxins, including the mechanisms by which sensitive tumor tissue can be destroyed selectively.

REFERENCES
9. ———. Action of Bacterial Polysaccharide on Tumors. II. Damage of S37 by Serum of Mice Treated with S. marcescens Polysaccharide, and Induced Tolerance. Ibid., pp. 1169—75.
# Action of Bacterial Polysaccharide on Tumors: III. Repeated Response of Sarcoma 37, in Tolerant Mice, to Serratia marcescens Endotoxin

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