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## Modification of Lethality of Endotoxin in Mice by Zymosan. (26381)

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Benacerraf *et al.*(1) have shown that pre-treatment of mice with the yeast extract, zymosan, increases susceptibility to lethality of *intravenously* administered endotoxin, despite the concomitant stimulation of phagocytic activity of the reticuloendothelial system commonly associated with refractoriness to endotoxic effects. We decided to determine whether plasma of such RES-stimulated but endotoxin-susceptible mice would protect normal recipients against lethality of endotoxin, as does plasma of RES-stimulated endotoxin-tolerant donors(2). Mice were treated with an available zymosan preparation, plasma taken from some, the others being challenged *intraperitoneally* with endotoxin. Surprisingly, these zymosan-treated donors showed increased resistance to the *i.p.* endotoxin challenge.

The present report confirms the finding of Benacerraf *et al.*(1), for the experimental model they used, and describes the modification of endotoxin susceptibility by zymosan pre-treatment, ranging from increased resistance to increased susceptibility, depending upon the zymosan employed, method of preparation for injection, and route of administration of the endotoxin challenge. Stimulation of phagocytic activity was observed uniformly and the protection seen was not attributable to contamination by bacterial endotoxin or to stimulation of antibody formation to the challenging endotoxin.

*Materials and methods.* For studies on modification of endotoxin lethality and phagocytic activity, mice of both sexes, weighing 16-20 g, were used. Experiments to determine possible endotoxin contamination of zymosan were performed on male rabbits weighing 2 to 2.5 kg.

The results obtained with 2 zymosan preparations, designated "A" and "B",* are reported herein. The suspension of zymosan was prepared either exactly as described by Benacerraf *et al.*(1) or by substituting vigorous boiling directly on a hot plate for the lesser degree of heating in a boiling water-bath. Precautions to avoid endotoxin contamination were observed: all vials, beads, syringes, and needles were heated in an oven at 175°-180°C for 2-3 hours before use. Saline for dilutions was demonstrated to be pyrogen-free by test in rabbits. Pre-treatment of mice was standardized at 1 mg in 0.2 ml,

* Zymosan "A" was obtained from Mann Res. Labs., lot #4393, and "B" from Fleischmann Labs., lot #9B551.

intravenously, on days 1 and 3, with testing for endotoxin susceptibility and phagocytic activity, in separate groups of mice, on day 4. The endotoxin used was the lipopolysaccharide of *S. typhosa* 0 901 (Difco), administered either *i.p.* or *i.v.* at varied doses as indicated below. Deaths were recorded for 72 hours, with most occurring within 24 hours.

Clearance of carbon from the blood for determination of phagocytic activity of the RES was measured by the method of Biozzi *et al.*(3), as modified in our laboratory. We have previously shown that daily doses of more than 1 μg of endotoxin are required to protect against an $LD_{75}$ challenge (0.6 mg) of the lipopolysaccharide used here(2). It is clear, then, that the 1 mg daily mouse-dose of zymosan would have to have an endotoxin contamination of not less than 0.1% for this to explain the increased resistance to the challenge. The normal rabbit responds to *i.v.* endotoxin, in μg doses, with a characteristic biphasic fever and an acute leucopenia. To test for contamination, zymosan preparations were injected *i.v.* at a 10 mg dose following which rectal temperatures and peripheral white cell counts were recorded. The preparation of rabbits for, and the handling during, the testing have been described(4). The sensitivity of the test is such that less than 0.01% contamination (<0.1 μg/mouse-dose zymosan) would be revealed.

Zymosan has been shown to be antigenic, capable of increasing bactericidal antibody in rabbits, and also to enhance hemolysin production in the rat(5,6). Therefore, the possibility existed that the zymosan administration used in these experiments stimulated production of antibody reactive to the challenging bacterial endotoxin employed. Thus sera pooled from 6 normal mice and from the same number of mice treated with representative zymosan preparations were collected on the 4th day of treatment and kept at −20°C until used. The pooled sera were then titrated for antibody to *S. typhosa* 0 901 lipopolysaccharide based on the method of Neter *et al.*(7), using sensitized sheep red blood cells.

TABLE I. Modification of Endotoxin Lethality by Zymosan "A," Prepared on Hot Plate.

| Group | Route of challenge | No. of exp. | Dead/ Total | % dead |
|---|---|---|---|---|
| Controls | i.p.* | 3 | 21/30 | 70 |
| Zymosan-treated | " | 3 | 9/30 | 30 |
| Controls | i.v.† | 3 | 11/30 | 37 |
| Zymosan-treated | " | 3 | 11/30 | 37 |

* .4 to .6 mg endotoxin in individual exp.
† .1 to .2 " *idem*

*Results. Susceptibility to endotoxin challenge.* In Table I are summarized the data demonstrating that pre-treatment with zymosan "A", prepared for injection by boiling directly on a hot plate, resulted in increased resistance to intraperitoneal endotoxin challenge, without alteration of normal susceptibility to intravenous challenge. Treatment with zymosan "A" prepared in the water-bath resulted in increased susceptibility to *i.v.* challenge (controls 2/10 dead, zymosan 6/10).

With the hot plate treatment, zymosan "B" (Table II) did not alter the susceptibility to *i.p.* challenge and increased susceptibility to *i.v.* challenge. When this zymosan was prepared in the water-bath, increased susceptibility to both routes of challenge was found, with much greater sensitivity to the *i.v.* route. These results confirm those of Benacerraf *et al.*(1).

In an attempt to determine whether the increased susceptibility to endotoxin seen 24 hours after the last dose of zymosan "B" was a transitory effect, mice were given 1 mg of the water-bath suspension *i.v.* on days 1, 3, and 6, and groups of 10 mice each were challenged with 0.6 mg endotoxin *i.p.* on

TABLE II. Modification of Endotoxin Lethality by Zymosan "B" Pre-treatment; Boiling Water-Bath *vs* Hot Plate Preparations.

| Group | Route of challenge | No. of exp. | Dead/ Total | % dead |
|---|---|---|---|---|
| Controls | i.p.* | 4 | 15/40 | 38 |
| Zymosan, water-bath | " | 3 | 20/30 | 67 |
| " , hot plate | " | 3 | 13/30 | 43 |
| Controls | i.v.† | 4 | 5/40 | 13 |
| Zymosan, water-bath | " | 5 | 22/50 | 44 |
| " , hot plate | " | 3 | 8/30 | 27 |

* .4 mg endotoxin. † .1 mg endotoxin.

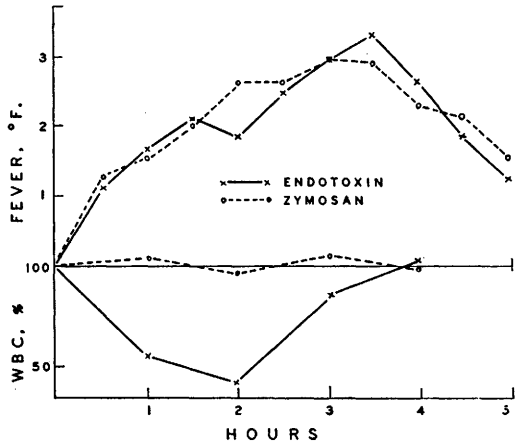


FIG. 1. Febrile and leucocyte responses of rabbits given 10 μg *S. typhosa* endotoxin or 10 mg zymosan "A," i.v.

days 7, 8, 9, and 10. No evidence for development of a resistant state was obtained.

*Stimulation of RES.* Both zymosan preparations produced equivalent stimulation of phagocytic activity as measured by rate of clearance of carbon from the blood 24 hours after the second injection, the time of testing for endotoxin susceptibility. At a dose of 12 mg/100 g body weight, the half-times for carbon clearance were: controls, 11 minutes; zymosan "A", 6 minutes; zymosan "B", 6.5 minutes.

*Test for endotoxin contamination.* The distinction between endotoxin and zymosan "A" as regards course of fever induced and peripheral leucocyte response is described in Fig. 1. Both "A" and "B" zymosans were equally pyrogenic, producing monophasic fevers differing qualitatively from the typical biphasic endotoxin fever shown. The minimum endotoxin contamination to account for the protective effect of zymosan "A" in the mouse would require that the 10 mg dose given these 7 rabbits contain 10 μg, the dose used in the 5 endotoxin-treated rabbits. It may be seen that the total magnitude of fever (area under the curve) is the same for both substances, lending emphasis to the qualitative difference in pattern at this level of fever. The acute leucopenia characteristic of endotoxin(8) was not elicited by either zymosan.

*Antibody titer to S. typhosa endotoxin.* The sera taken from normal mice demonstrated no detectable antibodies to *S. typhosa* 0 901 lipopolysaccharide as measured by the sensitive hemolysis technic. The sera from mice pre-treated with zymosan "A" or "B" likewise gave no evidence for the existence of any detectable antibodies to the challenging endotoxin.

*Discussion.* Zymosan is a yeast cell wall fraction, principally polysaccharide, but known to contain some lipid and protein. There is no primary chemical standard, standardization being based upon immunological properties(9). With the demonstration that one zymosan preparation may protect against endotoxin lethality while another, producing equally great stimulation of phagocytic activity, increases susceptibility, one is led to look for a "contaminant," defined in terms of other than RES-stimulating activity. Since available zymosan preparations are not sterile, the possibility of endotoxin contamination immediately suggests itself, and such contamination could explain increased resistance. This has been shown not to be the case: no evidence for endotoxin contamination was found. The pyrogenicity of zymosan, equally present in protective and sensitizing zymosans, is similar to that of other polysaccharides and distinctly different from that of bacterial endotoxin at doses producing equivalent fevers. The absence of an acute leucopenic response provides a further distinction from endotoxic activity.

On the supposition that an anamnestic reaction might occur in our mice which might have normal antibody cross-reactive with the challenging endotoxin, or more remotely that *de novo* antibody might appear stimulated by zymosan treatment, the hemolysis tests were conducted. However, since no detectable circulating antibody was found either in normal or treated mouse sera under our experimental conditions, it is clear that such an immunological mechanism is not operable here.

It is clear that preparations of zymosan equivalent in one activity may be quite different in another. Zymosan administered *i.v.* is not without toxicity and it would seem

reasonable to consider a heat labile toxic contaminant variably present which may mask the protective effect afforded by RES stimulation. Benacerraf's findings(1) suggest a deleterious effect upon adrenal cortical function, which doubtless plays a permissive role in resistance to endotoxin. The relatively greater sensitivity of zymosan-treated mice to *i.v.* than to *i.p.* endotoxin challenge, compared to normal controls challenged by the same routes, was found regardless of the type or preparation of zymosan. The slower absorption of the *i.p.* endotoxin challenge may allow a more effective detoxification by the RES in a pre-existing state of susceptibility.

Passive transfer experiments require further study; protection was afforded normal recipients by plasma of resistant zymosan "A"-treated donors, but, peculiarly, plasma of donors given zymosan "B" conferred neither protection nor the donors' own susceptibility to endotoxin challenge.

*Summary.* Pre-treatment of mice with different preparations of zymosan modified the lethal effect of a subsequent endotoxin challenge, ranging from increased resistance to increased susceptibility, despite equivalent RES stimulation. Source and degree of heating in preparation for injection were determinants. Greater relative sensitivity to *i.v.* than to *i.p.* challenge was found regardless of the zymosan used. Protection was not attributable to endotoxin contamination or to stimulation of serum antibody titer to the challenge. Zymosan produced a monophasic fever in the rabbit, differing from the biphasic endotoxin fever, and did not elicit an acute leucopenia.

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## Apparatus for Simultaneous Infusion and Multiple Blood Sampling. (26382)

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Continuous withdrawal of simultaneous arterial and venous blood samples is required in experimental procedures such as determination of regional blood flow(1,2). This can be done with some commercially available pumps. Rose and Pfaff(3) have described a mechanism designed specifically for this purpose. The design of these pumps, however, requires syringes to be placed at a distance from the experimental animal. This necessitates a long cannula with consequently a considerable volume of "dead space." Also, simultaneous infusion and withdrawal are possible with available commercial pumps only by using 2 separate units.

A description follows of an apparatus designed specifically for use in regional blood flow studies. Two objectives were kept in mind, (a) to place the syringes as close as possible to the experimental animal and thus reduce cannula length to a minimum, (b) to

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