ENDOTOXIN-INDUCED TOLERANCE TO TOXIC MANIFESTATIONS OF *CANDIDA ALBICANS*

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ABSTRACT

HASENCLEVER, H. F. (National Institutes of Health, Bethesda, Md.) AND WILLIAM O. MITCHELL. Endotoxin-induced tolerance to toxic manifestations of *Candida albicans*. J. Bacteriol. **85**:1088–1093. 1963.—Mice exposed to 425 r total body irradiation failed to become tolerant to *Candida albicans* toxicity after injection with lipopolysaccharide. Mice injected with lipopolysaccharide and then X-rayed did not demonstrate the tolerant state. An injection of thorium dioxide in mice that had previously received tolerance-inducing amounts of lipopolysaccharide rendered them as susceptible to acute *C. albicans* toxicity as control mice. A bimodal manifestation of tolerance was noted. Groups of mice given single injections of lipopolysaccharide at 6 or 1 days before challenge demonstrated high levels of tolerance, whereas the tolerance in mice given a single dose at 3 days was negligible. The bimodal effect was not observed in tolerant mice challenged with lipopolysaccharide. Injections of viable or nonviable pathogenic fungi known to produce tolerance to the toxicity of *C. albicans* in recipient mice did not produce tolerance to lipopolysaccharide. Serum from mice injected with lipopolysaccharide showed in vitro inhibitory activity for *C. albicans*.

Recent studies by Hasenclever and Mitchell (1962*a*, *b*, *d*) have shown that tolerance or resistance to the acute toxic manifestations of *Candida albicans* for mice could be achieved by a variety of methods. Sublethal intraperitoneal infections with *C. albicans* or a number of antigenically related or unrelated pathogenic fungi produced this response. Nonviable *Coccidioides immitis* spherule fragments and complete Freund's adjuvant were effective, whereas nonviable *C. albicans* cells were not. *Salmonella enteritidis* and *S. typhosa* lipopolysaccharides were quite active in producing the tolerant state. This paper presents additional experimental observations relating to this lipopolysaccharide-induced phenomenon.

MATERIALS AND METHODS

The animals used in this study were general purpose female mice weighing 15 to 19 g and were obtained from the Animal Production Section of the National Institutes of Health. They were placed into groups containing 26 to 31 animals and treated as described for the various experiments.

S. enteritidis endotoxin (Difco) was used for all experiments. It was dissolved in pyrogen-free 0.9% NaCl solution, and 30 μg were injected intraperitoneally 6 and 1 days before challenge, unless otherwise indicated. In each experiment, control groups were injected with the NaCl solution at the same time as those receiving endotoxin. For the results in Fig. 7, mice were challenged with 500 μg of endotoxin by the route indicated. The initial tolerance-inducing dose was 30 μg. Controls were injected with NaCl solution, by the route indicated in Fig. 7, 1 day before challenge.

C. albicans yeast cells for challenge were obtained from 48 hr 2% glucose-1% neopeptone stationary broth cultures incubated at 30 C. The yeast cell growth was harvested, washed, and suspended in pyrogen-free 0.9% NaCl solution. Yeast cell numbers were determined by direct count and by quantitative agar pour plates. Pour-plate determinations were accepted as the final count. Mice were challenged intravenously with 10^7–1.8×10^7 yeast cells.

For X-ray studies, a dual-tube Westinghouse machine operating at 200 kv and 15 ma with 0.25-mm Cu filters and 0.55-mm Al filters was used. The mice were placed 54 cm from the X-ray tubes and were exposed to 118.2 r for 3.6 min, for a total body irradiation of 425 r. They were X-rayed 1 day after the endotoxin injection and challenged 5 days later.

The effect of reticuloendothelial system blockade in tolerant and control mice with thorium dioxide (Thorotrast, Testagar & Co., Inc.) was studied. This colloidal preparation contained 24 to 26% thorium dioxide by volume, and 0.2 ml was injected intravenously into each mouse 2 hr before challenge.

After challenge, the experimental mice were observed frequently and the number of deaths was recorded. The average survivor time was determined from these observations.

The in vitro inhibitory activity of serum was determined using 10% serum in 0.25% Trypticase Soy Broth. The tubes were incubated at 37 C and pour plates were made from 0.1-ml samples from each tube at the indicated time after inoculation. Chloramphenicol (50 μg per ml of serum and broth) was added. The final volume in each tube at the beginning of an experiment was 2.0 ml.

Results and Discussion

Studies with X-rayed and control mice are shown in Fig. 1. Mice irradiated 4 days before the first injection of lipopolysaccharide (group 1) did not become tolerant to the toxicity of *C. albicans* as did the unirradiated tolerant controls (group 3). The X-rayed mice (groups 1 and 2) died somewhat faster than the unirradiated controls (group 4). These results clearly show that X-rayed mice do not respond to injections of lipopolysaccharide and develop tolerance to the toxicity of *C. albicans*.

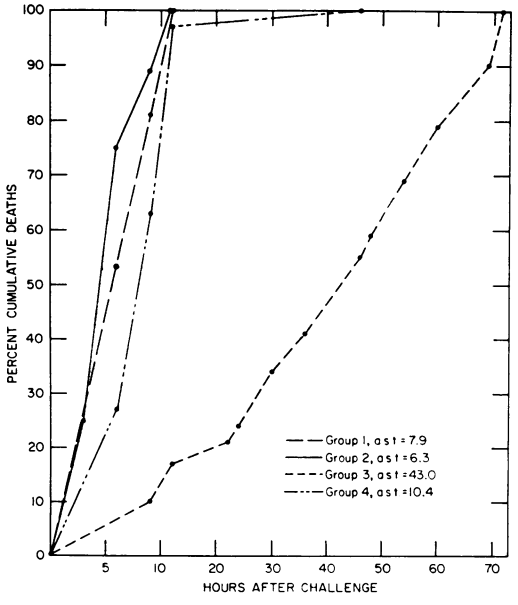

FIG. 1. *Production of tolerance to the toxicity of Candida albicans in X-rayed and non-X-rayed mice injected after irradiation with lipopolysaccharide. Group 1, X-rayed, endotoxin; group 2, X-rayed, saline; group 3, no X ray, endotoxin; group 4, no X-ray, saline. Challenge dose, 1.8 × 10⁷ yeast cells or aggregates.*

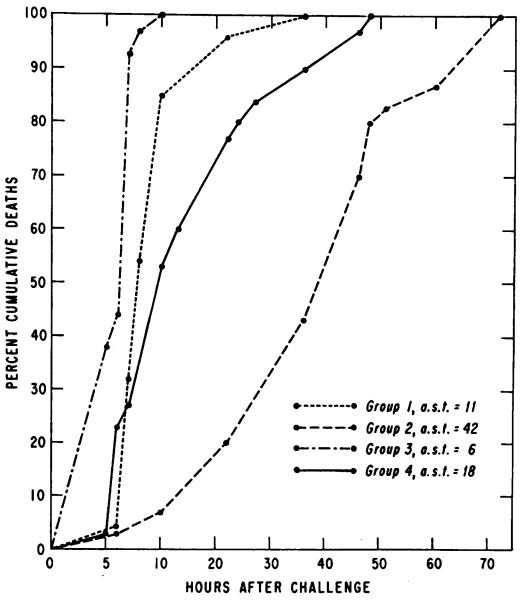

FIG. 2. *Production of tolerance to the toxicity of Candida albicans in X-rayed and non-X-rayed mice injected with lipopolysaccharide before X-ray exposure. Group 1, endotoxin, X-ray; group 2, endotoxin, no X-ray; group 3, saline, X ray; group 4, saline, no X-ray. Challenge dose, 1.8 × 10⁷ yeast cells or aggregates.*

The effect of injecting mice with lipopolysaccharide before X-ray exposure may be seen in Fig. 2. The mice that received lipopolysaccharide and were subsequently X-rayed were quite susceptible to the toxic activity of *C. albicans* (Fig. 2).

The effect of intravenously administered thorium dioxide in lipopolysaccharide-induced tolerance may be seen in Fig. 3. It is quite apparent that treatment with this material effectively eliminated tolerance in endotoxin-treated mice (group 2). These mice reacted in a similar manner to *C. albicans* toxicity as did the saline-treated controls (group 3). Treatment of saline-injected control mice (group 4) with thorium

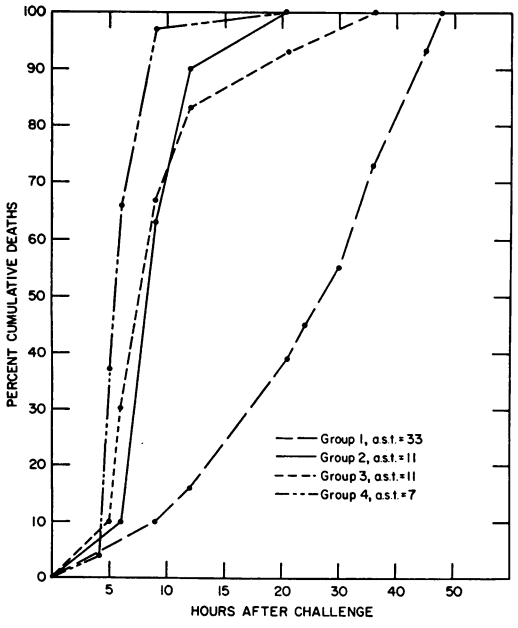

FIG. 3. *Effect of thorium dioxide upon tolerance to the toxicity of Candida albicans. Group 1, endotoxin; group 2, endotoxin, thorium dioxide; group 3, saline; group 4, saline, thorium dioxide. Challenge dose, 1.8 × 10⁷ yeast cells or aggregates.*

dioxide decreased their average survivor time to below that of group 3 (Fig. 3).

From the results of these three experiments, there is little doubt that the development of tolerance, or that functioning tolerance to the toxicity of *C. albicans*, is directly associated with the reticuloendothelial system. Sublethal irradiation, known to inhibit immunological processes initiated after exposure for a limited period of time, prevented the development of lipopolysaccharide-induced tolerance. Similarly, mice exposed to X rays after receiving injections of lipopolysaccharide failed to show tolerance. The intravenous injection of thorium dioxide, a material known to interfere with reticuloendothelial activity, into tolerant mice rendered them as susceptible as control animals. Control mice injected with thorium dioxide were more susceptible than nontreated normal animals. This suggests enhanced phagocytic activity or possible serum factors.

Experiments designed to determine the time before challenge for the administration of a single dose of endotoxin to elicit maximal tolerance indicated a bimodal response (Hasenclever and Mitchell, 1962*b*). That is, endotoxin given 6 or 1 days before *C. albicans* challenge extended the average survivor time of groups of mice (29 to 30 per group) that received a single injection of 30 μg of lipopolysaccharide before challenge at the time indicated on the abscissa or in the graph (Fig. 4). The mice that received the lipopolysaccharide at 0 hr were challenged intravenously immediately after the endotoxin injection. This group showed an increased susceptibility when compared with the control group. All other groups demonstrated some tolerance with maximal levels being observed at 1 and 6 days, lower levels at 2 and 4 days, and with the minimal amount observed at 3 and 10 days.

Earlier studies (Hasenclever and Mitchell, 1962*b*) indicated that at 10 to 14 days endotoxin-induced tolerance to *C. albicans* toxicity had almost disappeared. The low point on Fig. 4 at 10 days probably is the result of disappearance of the tolerant effect.

The data presented in Fig. 4 were results using 30-μg doses of lipopolysaccharide. Although we had shown before (Hasenclever and Mitchell, 1962*b*) that a 5-μg dose elicited some response, amounts of more than 30 μg had not been used. The results in Fig. 5 indicate the comparative observations, using single 100- or 30-μg doses administered intraperitoneally in groups of mice given endotoxin at 6, 3, 1, or 0 days before *C.*

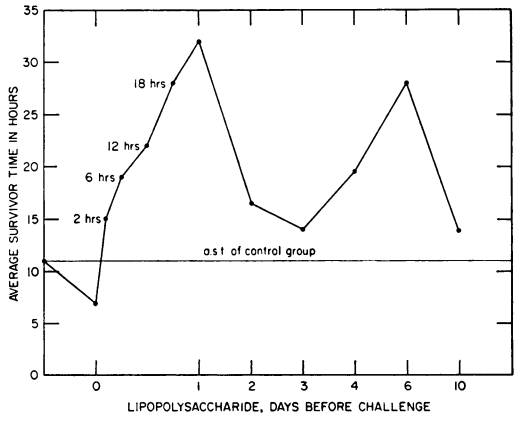

FIG. 4. *Bimodal manifestation of tolerance to the toxicity of Candida albicans as the result of time of administration of lipopolysaccharide. Each point on the graph represents the average survivor time of 29 to 30 mice given 30 μg of lipopolysaccharide intraperitoneally per animal at the time indicated. Challenge dose, 1.2 × 10⁷ yeast cells or aggregates.*

albicans challenge. There appear to be only slight differences in the results, suggesting that the bimodal or cyclic phenomenon was not dose-related.

It appears from the evidence just presented that, in a mouse injected intraperitoneally with a single dose of lipopolysaccharide, tolerance to *C. albicans* toxicity reaches a rather high level 1 day later, starts to recede, reaches a point at 3 days after injection where little resistance is manifested, and then returns at 6 days to approximately the level of tolerance observed 1 day after injection. After this, there is a gradual return to normal appearing 10 to 14 days after the initial injection (Hasenclever and Mitchell, 1962*b*). Previous work had shown that tolerance produced by endotoxin given in two doses at 6 and 1 days before challenge was additive when compared with single doses given at 6 or 1 days. We therefore questioned what might happen if doses were given groups of mice at 6 and 3 days, 6 and 0 days, 3 and 1 days, and 1 and 0 days before *C. albicans* challenge. Mice that received one 30-μg dose of lipopolysaccharide 6 days before challenge (group 1) were considerably more tolerant than group 2 mice which were injected with two 30-μg doses, one dose at 6 days and one dose at 3 days before challenge (Table 1). The mice in group 3 were given 30-μg doses 6 and 1 days before challenge and were highly tolerant. The results obtained from group 2 were quite comparable with those observed in group 5 (one 30-μg dose 3 days before challenge). The tolerance of mice receiving lipopolysaccharide at 6 and 0 days (group 4) was also reduced. Mice in group 6, which received lipopolysaccharide at 3 and 1 days before challenge

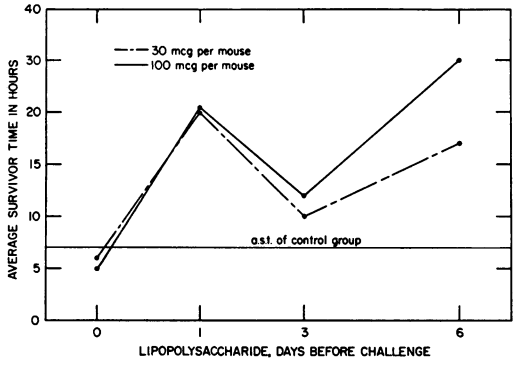

FIG. 5. *Effect of a larger dose of lipopolysaccharide on tolerance to the toxicity of Candida albicans. Each point on the graph and the controls represents the average survivor time (a.s.t.) for groups of mice containing 29 to 30 animals. The lipopolysaccharide was administered intraperitoneally in a single dose at the time indicated on the abscissa. The controls were untreated. Challenge dose, 10^7 yeast cells or aggregates.*

TABLE 1. *Effect of single or multiple injections of lipopolysaccharide on tolerance to the toxicity of Candida albicans when given at different time intervals before challenge*

Group*	Number of deaths after challenge†																		Average survivor time (hr)
	Time (hr) after challenge																		
	5	8	10	12	21	24	27	30	33	36	45	48	51	54	60	68	72	75	
1		2		3	3	1	1		1	1	6		2	2	3	3	1	1	41
2		8		10	4	1	2		1		2	1		1					19
3							1		2	1	4	5	1	1	4	4		7	57
4	2	13		4	5				1		1			1				3	21
5		5		11	9	1					1				1	1			19
6				4	4	1	2	2	1	1	2	2	1	3	3	3		1	36
7				3	5	1				1	2	2		1	3	4	1	6	49
8	2	11		4				1				1			2	1		7	33
9	7	17		5		1													8
10		17		9	4														12

* Group 1, one injection at 6 days; group 2, two injections at 6 and 3 days; group 3, two injections at 6 and 1 days; group 4, two injections at 6 and 0 days; group 5, one injection at 3 days; group 6, two injections at 3 and 1 days; group 7, one injection at 1 day; group 8, two injections at 1 and 0 days; group 9, one injection at 0 days; group 10, untreated controls.

† Challenge dose = 1.5×10^7 viable particles.

demonstrated less tolerance than group 7, which was given one dose of endotoxin 1 day before challenge. Tolerance was reduced more in mice injected 6 and 3 days than in those treated 3 and 1 days before challenge. The results observed of mice in group 8 deserve special consideration. These animals received lipopolysaccharide 1 and 0 days before challenge. Of 29 mice, 17 died within 12 hr, but the majority of those remaining survived for 75 hr at which time the experiment was terminated. It appears that the protective mechanism developed rapidly enough in most of those animals surviving the acute toxic reaction to protect them for the entire experimental period.

Tolerance induced by lipopolysaccharide to subsequent challenge with lipopolysaccharide is shown in Fig. 6. The purpose of this experiment was to determine whether, by the methods utilized to show endotoxin-induced tolerance to *C. albicans* toxicity, a bimodal manifestation would be observed with lipopolysaccharide as the challenging agent. Such an effect was not observed, and a high level of tolerance was produced when the initial dose of endotoxin was given 3 days before challenge.

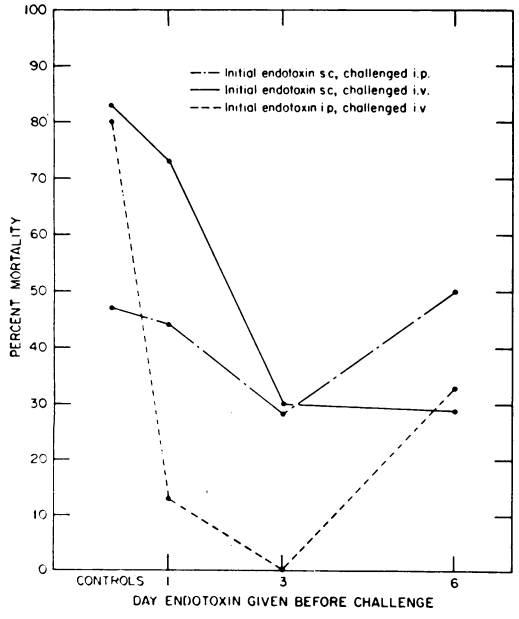

FIG. 6. *Tolerance to lipopolysaccharide produced by injection of lipopolysaccharide prior to challenge. Each point on the graph represents values obtained from 29 to 31 mice.*

TABLE 2. *Tolerance to lipopolysaccharide produced by Candida albicans or Coccidioides immitis*

Treatment	Mortality	Per cent
Two injections of 1.5×10^7 viable *C. albicans* cells intraperitoneally, 6 and 4 days before challenge*.	30/31†	97
Nonviable *C. immitis* spherule fragments (1 mg) subcutaneously, 6 days before challenge*	21/33	64
Saline (0.1 ml) intraperitoneally, 6 days before challenge*	20/29	69

* Challenge was 500 μg of lipopolysaccharide injected intraperitoneally.

† Number dead/number injected.

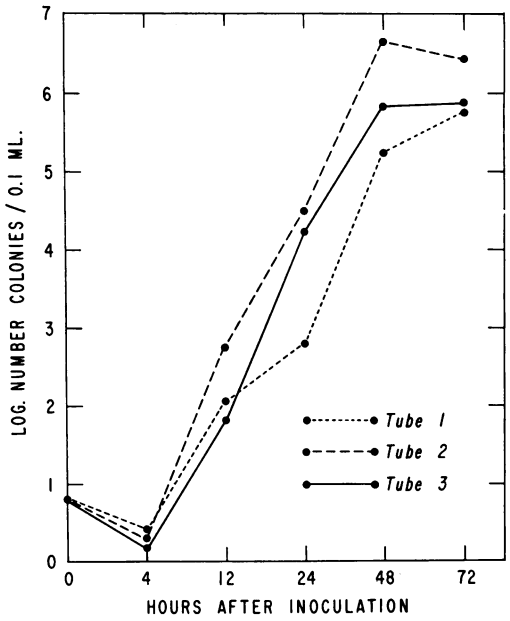

FIG. 7. *Fungistatic effect for Candida albicans of serum from mice injected with lipopolysaccharide. Tube 1, 10% serum from tolerant mice; tube 2, 10% serum from control mice; tube 3, growth control.*

Table 2 shows the effect of two methods known to produce tolerance to the toxicity of *C. albicans* in producing tolerance to lipopolysaccharide. It is quite apparent from these results that tolerance to endotoxin was not observed. Mice preinfected

intraperitoneally with viable *C. albicans* cells were somewhat more susceptible than the control group. Hypersusceptibility to endotoxin of mice infected with *Histoplasma capsulatum* has been reported by Box and Briggs (1961) and with other pathogenic microorganisms by Suter (1962).

The fungistatic activity for *C. albicans* of pooled serum from mice injected with lipopolysaccharide is shown in Fig. 7. The greatest effect was observed after 24 hr, but some effect was apparent 72 hr after inoculation. Normal serum, at the concentration used, enhanced growth.

Two of the methods utilizing fungus cells, or products known to produce tolerance in mice to the toxicity of *C. albicans* (Hasenclever and Mitchell 1962*a*), were ineffectual in producing tolerance to *S. enteritidis* lipopolysaccharide in mice. Conversely, this endotoxin is highly effective in producing tolerance to *C. albicans* toxins as well as to itself. Although the end results appear to be the same, i.e., the extension of survivor time, there are other observable differences between the resistance produced by lipopolysaccharides and that produced by viable or nonviable fungi. No bimodal manifestation of tolerance to *C. albicans* toxicity was noted when viable or nonviable fungal preparations were employed (Hasenclever and Mitchell, 1962*b*). Injections of viable *C. albicans* cells and nonviable *C. immitis* spherule fragments produced some protection in mice to chronic candidiasis, and, although lipopolysaccharide was quite effective in extending the survivor time in acute toxicity studies, it did not protect mice against death due to chronic infection (Hasenclever and Mitchell, 1962*c*).

Although the toxic factor of *C. albicans* would probably be classed as an endotoxin, it appears to be more heat-susceptible than the classical ones. Heating whole cells at 65 C for several hours destroys viability and toxicity. Mourad and Friedman (1961) found toxic activity in the supernatant fluid and sediment of sonically disrupted cells. We have had some success with separation from whole viable cells but reproducibility has been a problem. Salvin (1952) reported endotoxins from nonviable pathogenic fungi, including *C. albicans*, but adjuvants were necessary to elicit their lethal properties.

Whitby et al. (1961) have shown increased levels of lytic antibodies to gram-negative bacteria in the sera of mice injected with lipopolysaccharides. We have presented evidence that inhibitory substances for *C. albicans* were found in similarly treated mice. The observations that X-ray exposed mice do not develop tolerance and that thorium dioxide effectively blocks or eliminates the enhanced resistance otherwise seen in tolerant mice provide evidence that this protective phenomenon resides at the cellular level. The role of the fungistatic serum factors in relation to tolerance to *C. albicans* toxicity is not clear but is under study.

ACKNOWLEDGMENT

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