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Induced Sensitization of Normal Laboratory Animals to *Brucella abortus* Endotoxin[1]

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A Boivin preparation of *Brucella abortus*, unlike common enterobacterial endotoxins, failed to depress water intake or increase numbers of hemolysin-producing spleen cells in mice, or to cause delayed inflammatory reactions in rabbit skin. Reactivity to the *B. abortus* endotoxin was found only in animals which were previously given the endotoxin with, but not necessarily in, complete Freund's adjuvant. Previous treatment with the endotoxin in saline or with only the adjuvant was ineffective. Sensitization appeared within 10 days and waned after 5 weeks. Passive sensitization was obtained with sensitized donor spleen cells but not with serum. Serum antibody titers did not correlate with the appearance and disappearance of sensitization.

Normal laboratory animals are highly reactive to endotoxin preparations isolated from those gram-negative bacteria to which they are commonly exposed. In contrast, identically obtained preparations from *Brucella* species show lesser endotoxic activity in these same animals. Toxicity for normal laboratory animals of endotoxin preparations of *Brucella* has been described by a number of workers (1, 3, 7, 14, 16, 19). Comparisons made in laboratories using equivalent preparations (3, 7, 14) have shown clearly that *Brucella* preparations are not as active as those of common enterobacterial endotoxins. The *B. abortus* Boivin preparation described in this report has previously been found (H. H. Freedman, *unpublished data*) to be nonpyrogenic for rabbits at more than 1,000 times the minimal pyrogenic doses of equivalent prepartions of *Salmonella typhosa* or *Escherichia coli*, and to be nonlethal for mice at doses up to 10 mg whereas the *S. typhosa* and *E. coli* preparations had LD_{50} values of approximately 0.25 mg. Further, similar preparations have been found to be inactive in the urinary nitrogen assay (4) and not to be cytotoxic for macrophages in vitro (13).

Brucellosis increases susceptibility of mice (1) and sensitivity of humans (2) to *Brucella* endotoxin. However, determining reactivity as a lethal outcome in infected animals is a complicated and unsatisfactory model, especially since greater susceptibility to the common endotoxins is also found (1). Attempts to actively sensitize animals to *Brucella* endotoxin by infection other than with live organisms have not generally succeeded. Kessel, Braun, and Plescia (13) found that immunization with killed *B. abortus*, unlike infection, did not induce susceptibility of macrophages to *B. abortus* endotoxin. Wilson, Kolbye, and Baker (19) were able to sensitize mice to *Brucella* endotoxin passively but not actively. Previous efforts by Freedman (*unpublished data*) to induce reactivity in mice and rabbits by using the Boivin-type endotoxin of *B. abortus* were uniformly unsuccessful. We have recently described an induced hyperreactivity to common enterobacterial endotoxins, with the characteristics of delayed hypersensitivity and dependence upon the protein content of the endotoxin preparation (9, 10). We undertook, therefore, to determine reactivity to our ordinarily inactive Boivin endotoxin of *B. abortus* in animals which had previously been injected with this endotoxin, either incorporated into Freund's complete adjuvant or given simultaneously with the adjuvant but at separate sites.

MATERIALS AND METHODS

Female CD-1 mice weighing 18 to 20 g were given 10 μg of a Boivin *B. abortus* endotoxin (Difco) in saline, either incorporated (1:1) into complete Freund's adjuvant (Difco) and injected subcutaneously, or injected intraperitoneally and with saline-adjuvant injected subcutaneously. Control mice were given either endotoxin only or saline-adjuvant only,

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or were untreated. Female albino rabbits weighing 1.5 to 2 kg were similarly treated with 10 μg of *B. abortus* endotoxin in complete adjuvant injected subcutaneously, with control rabbits given either endotoxin or adjuvant, or untreated. At intervals of from 10 days to 5 weeks after these pretreatments, treated and control animals were challenged (vide infra) with appropriate doses of the endotoxin in saline.

Endotoxin caused inhibition of water intake by mice (6). This was measured during the 16-hr period following a 1-μg intraperitoneal challenge dose (8). After a 10-μg intraperitoneal challenge dose, increase in numbers of hemolysin-forming spleen cells in mice 48 hr after endotoxin (11) was determined by localized hemolysis in agar (9). Rabbits were skin-tested with 1- and 0.1-μg intradermal challenge doses, and delayed inflammatory reactions caused by endotoxin were graded at 24 and 48 hr.

For passive transfer, donor mice were given 10 μg of *B. abortus* endotoxin in complete adjuvant, and spleen cells and serum were harvested 4 weeks later. Preparation and transfer of cells and serum to normal recipient mice were done as previously described (9). Recipient mice were challenged with 10 μg of the endotoxin in saline within 4 hr or at 4 days after transfer; control mice were similarly challenged. After treatment with endotoxin in adjuvant, serum antibody titer to *B. abortus* endotoxin in mice and rabbits was followed for 6 weeks by complement-fixation assay. Dilutions for injection were made in nonpyrogenic saline, and we observed rigorous precautions to avoid contamination with extraneous endotoxins.

RESULTS

The failure of the *B. abortus* endotoxin, when injected in saline, to influence either the numbers of hemolysin-forming spleen cells or the water intake in mice is described in Tables 1–5. This is in contrast to the marked influence in both experimental models of the typical enterobacterial endotoxins (6, 9–11) and confirms the atypical lesser host reactivity to *Brucella* endotoxins seen in other models (4, 13; H. H. Freedman, *unpublished data*). When mice were given the *B. abortus* endotoxin in complete adjuvant, they responded with increased numbers of hemolysin-forming spleen cells which were seen 3 weeks later (Table 1), but which appeared earlier in additional experiments. This stimulation had waned by the 4th week and normal background counts were found thereafter. Mice challenged with the ordinarily ineffective endotoxin in saline at intervals after treatment with the endotoxin in adjuvant responded to the *B. abortus* endotoxin as they do to common endotoxins. This reactivity was best seen at 4 weeks, when the pretreated control mice had normal background counts, and the reactivity appeared to wane by the 5th week.

Table 2 describes the response to challenge with the endotoxin in saline at the previously determined 4-week interval after treatment with endotoxin in complete adjuvant. Control mice previously treated with only adjuvant did not respond to the *B. abortus* endotoxin in saline. Additional experiments demonstrated that complete adjuvant alone, unlike common endotoxins, was almost

TABLE 1. *Influence of previous treatment on numbers of hemolysin-forming spleen cells in mice 48 hr after challenge with Brucella abortus endotoxin in saline*

Pretreatment[a]	Challenge[b]	Pretreatment-challenge interval (weeks)	No. of hemolysin-forming cells per ⅓ spleen[c]
Untreated controls		—	4 ± 3.9
—	+	—	1 ± 0.7
+	—	—	16 ± 1.1[d]
+	+	3	30 ± 3.2
+	—	—	1 ± 0.5[d]
+	+	4	22 ± 1.8
+	—	—	1 ± 0.5[d]
+	+	5	8 ± 2.4

[a] *B. abortus* endotoxin (10 μg) in complete adjuvant.
[b] *B. abortus* endotoxin (10 μg) in saline.
[c] Mean (±SE) for groups of five mice each.
[d] Assayed at 23, 30, and 37 days after pretreatment, corresponding to intervals to assay in pretreated groups challenged at 3, 4, and 5 weeks, respectively.

TABLE 2. *Influence of previous treatment on numbers of hemolysin-forming spleen cells in mice 48 hr after challenge 4 weeks later with Brucella abortus endotoxin or sheep red blood cells (SRBC)*

Pretreatment[a]	Challenge[b]	No. of hemolysin-forming cells per ⅓ spleen[c]
Untreated controls		1 ± 0.4
None	*B. abortus*	1 ± 0.7
Adjuvant only	*B. abortus*	3 ± 0.6
B. abortus in adjuvant	*B. abortus*	35 ± 2.4
None	SRBC	47 ± 1.2
None	SRBC and *B. abortus*	50 ± 2.4
Adjuvant only	SRBC and *B. abortus*	48 ± 1.9
B. abortus in adjuvant	SRBC and *B. abortus*	98 ± 7.8

[a] *B. abortus* endotoxin (10 μg) in adjuvant, or equivalent 1:1 saline-adjuvant.
[b] *B. abortus* endotoxin (10 μg) in saline, or 10^8 washed SRBC, intravenously.
[c] Means (±SE) for groups of five mice each.

completely without effect upon numbers of hemolysin-forming spleen cells, even within the few days after injection. Mice treated with the endotoxin in adjuvant followed 4 weeks later by endotoxin in saline responded with increased numbers of hemolysin-forming spleen cells typically induced by common endotoxins in previously untreated animals. The hemolysin-forming spleen-cell response to specific antigen, sheep red blood cells, is enhanced when the antigen is given with a common endotoxin (9, 11). Here too, the *B. abortus* endotoxin failed to influence the response to specific antigen when injected together either in untreated mice or in those previously treated with only complete adjuvant. Mice previously injected with the *B. abortus* endotoxin in adjuvant, however, showed enhanced responses 4 weeks later to the specific antigen injected with the *B. abortus* endotoxin.

Mice injected with the *B. abortus* endotoxin and complete adjuvant simultaneously, but at separate sites, also responded subsequently to the ordinarily inactive *B. abortus* endotoxin in saline (Table 3). In comparison to the effect shown by the endotoxin incorporated into complete adjuvant (Table 1), these separately injected mice showed only normal background counts 12 days later. Previous treatment with adjuvant alone or, as previously found (H. H. Freedman, *unpublished data*), with the endotoxin in saline alone, did not result in reactivity to the *B. abortus* endotoxin in saline given 10 or 28 days later. Mice given both the endotoxin and the adjuvant, separately and simultaneously, and challenged with the endotoxin in saline 10 or 28 days later, showed marked increases in numbers of hemolysin-forming spleen cells 48 hr after challenge.

Spleen cells harvested from donors made reactive by prior treatment with the endotoxin in adjuvant served to transfer reactivity to normal mice (Table 4). Serum transfer was ineffective, whether challenge was given at 4 days (Table 4) or, as in other experiments, within 4 hr of challenge.

TABLE 3. *Influence of separate injection of Brucella abortus endotoxin and complete adjuvant on numbers of hemolysin-forming spleen cells in mice 48 hr after subsequent challenge with B. abortus endotoxin in saline*

Pretreatment[a]	Challenge[b]	Pretreatment-challenge interval (days)	No. of hemolysin-forming cells per ⅓ spleen[c]
Untreated controls		—	1 ± 0.5
None	+	—	4 ± 2.3
Adjuvant only	—	—	7 ± 3.3[d]
Adjuvant only	+	10	7 ± 1.9
B. abortus only	+	10	6 ± 1.7
B. abortus and adjuvant	—	—	2 ± 1.0[d]
B. abortus and adjuvant	+	10	56 ± 5.0
B. abortus and adjuvant	—	—	2 ± 0.9[d]
B. abortus and adjuvant	+	28	40 ± 2.8

[a] *B. abortus* endotoxin (10 μg) intraperitoneally, or 1:1 complete adjuvant-saline, subcutaneously or both.

[b] *B. abortus* endotoxin (10 μg) in saline, intraperitoneally.

[c] Means (±SE) for groups of five mice each.

[d] Assayed at 12 or 30 days after pretreatment, corresponding to intervals to assay in pretreated groups challenged at 10 or 28 days, respectively.

TABLE 4. *Influence of sensitized donor spleen cells or serum on numbers of hemolysin-forming spleen cells in mice 48 hr after challenge 4 days later with Brucella abortus endotoxin in saline*

Treatment of assayed recipients	No. of hemolysin-forming cells per ⅓ spleen[a]
Untreated controls	3 ± 1.1
B. abortus only	1 ± 0.7
Sensitized donor serum[b] only	0 ± 0.2
Sensitized donor serum and *B. abortus*	1 ± 0.7
Sensitized donor cells[c] only	1 ± 0.6
Sensitized donor cells and *B. abortus*	25 ± 2.4

[a] Means (±SE) for groups of five mice each.

[b] A 0.25-ml amount, intraperitoneally.

[c] Spleen cells (1.7×10^8), intraperitoneally.

TABLE 5. *Influence of previous treatment on water intake in mice during 16 hr after challenge 4 weeks later with Brucella abortus endotoxin in saline*

Pretreatment[a]	Challenge[b]	Water intake (ml per 5 mice per 16 hr)	
		Expt A	Expt B
Untreated controls		27	29
None	+	28	26
B. abortus in adjuvant	—	26	28
Adjuvant only	+	27	30
B. abortus only	+	26	28
B. abortus in adjuvant	+	15[c]	15[c]

[a] *B. abortus* endotoxin (10 μg) in adjuvant or in saline, or 1:1 adjuvant-saline, subcutaneously.

[b] *B. abortus* endotoxin (1 μg) in saline, intraperitoneally.

[c] From statistical analysis of water intake of over 600 control mice, values of 15 ml per five mice are significant at $P < 0.001$.

The *B. abortus* endotoxin in saline failed to inhibit water intake in normal mice or in those given either the endotoxin alone or the adjuvant alone 4 weeks earlier (Table 5). Only those mice previously given the endotoxin in adjuvant subsequently responded to the endotoxin in saline with marked decreases in water intake.

Normal untreated rabbits did not develop delayed inflammatory reactions to intradermal doses of 1 μg or less of our *B. abortus* endotoxin (Table 6). Foster and Ribi (7) found reactivity to submicrogram doses of a cell-wall preparation in normal rabbits by use of the assay of Larson et al. (14). Besides the difference in *Brucella* preparations used, different skin sites are used in the Larson procedure (14). In our procedure (10), we see only occasional positive delayed reactions with 1-μg doses of common endotoxins. Similarly, rabbits failed to respond to skin testing 3 weeks after they were given the endotoxin only or the adjuvant only. Rabbits which had been given the endotoxin in adjuvant, however, developed positive reactions at both dose levels when tested intradermally 3 and 4 weeks later. Here, as in the previous experiments, the induced reactivity appeared to wane by the 5th week. No Arthus-type reactivity was seen in these animals.

As found by Wilson et al. (19), our normal mice and rabbits had no detectable serum antibody titers to the *B. abortus* endotoxin as measured by complement-fixation with twofold serial dilutions starting with a 1:2 serum dilution. Rabbits given the endotoxin in complete adjuvant developed detectable titers, 1:64 to 1:256, by the 2nd week. For individual animals, these titers did not change significantly through the 6th week. Thus, there was no correlation between the serum antibody titers and the appearance of skin reactivity at the 3rd and 4th weeks and its disappearance at the 5th week. Serum antibody determinations in treated mice yielded similar results. Detectable titers, 1:128 to 1:512, appeared in mice given the endotoxin in adjuvant, with no significant fluctuations which could be correlated with the appearance and disappearance of reactivity to the *B. abortus* endotoxin injected in saline.

TABLE 6. *Influence of previous treatment on delayed inflammatory reactions in rabbits 48 hr after skin testing with Brucella abortus endotoxin in saline*

Pretreatment[a]	Pretreatment-challenge interval (weeks)	Delayed inflammatory reaction[b]	
		1.0-μg intradermal dose	0.1-μg intradermal dose
None	–	0, 0	0, 0
Adjuvant only	3	0, 0	0, 0
B. abortus only	3	0, 0	0, 0
B. abortus in adjuvant	3	++, +	+, 0
B. abortus in adjuvant	4	++, ++	+, +
B. abortus in adjuvant	5	0, 0	0, 0

[a] *B. abortus* endotoxin (10 μg) in adjuvant or in saline, or 1:1 adjuvant-saline, subcutaneously.

[b] Individual responses of two rabbits for each pretreatment, tested at both doses. Positive reactions were erythematous and indurated and at least 5 mm in diameter. Reactions of increased severity were graded accordingly.

DISCUSSION

Our previous studies on modification of host reactivity to subsequent endotoxin challenge (9, 10) have dealt with induced hyperractivity to common enterobacterial endotoxins in normally reactive animals. In the present work, we took advantage of the natural lack of reactivity to a Boivin endotoxin of *B. abortus*, an organism to which normal laboratory animals are unlikely to have been exposed. Our findings clearly support the hypothesis that host reactivity to endotoxins is conditioned by appropriate previous exposure.

From work with common ubiquitous endotoxins (15, 18), the hypothesis has evolved that normal adult reactivity has a basis in an acquired hypersensitivity of the delayed type. Parallel studies with *Brucella* endotoxin or killed cells (1, 2, 5, 12, 13, 16, 19) have emphasized the lack of host "preparation" for reactivity, assayed in vivo or in vitro. Reactivity to *B. abortus* endotoxic activity in the urinary nitrogen assay (4) has been correlated with low levels of serum antibody in passively sensitized mice (19). Sensitization to cytotoxicity for host macrophages in vitro has been shown to require infection of guinea pigs with *B. abortus*, with evidence for mediation by cell-associated antibody (12, 13). In the present experiments, reactivity to the *B. abortus* endotoxin was induced in uniformly unreactive animals only when the endotoxin was given with, but not necessarily in, complete Freund's adjuvant. The use of complete adjuvant to encourage development of delayed-type hypersensitivity has recently been critically discussed (17). The endotoxin used is a Boivin protein-containing preparation (12.5% N) which satisfies the previously established requirement for presence of the protein moiety of the endotoxin macromolecule in experimentally induced hyperreactivity (9, 10). The finding that reactivity to the *B. abortus* endotoxin could be transferred to unreactive recipients by spleen cells, but not by serum of sensitized donors, is also relevant. In the experimental models reported here, the low levels of serum antibody which

appeared after treatment with the endotoxin and complete adjuvant could not be correlated with the appearance and disappearance of sensitization.

With this means of inducing reactivity in uniformly unreactive animals, the *Brucella* endotoxin becomes a unique tool for studies on host reactivity to the macromolecular endotoxins, uncomplicated by the uncontrolled and widespread preexistent sensitivity of normal laboratory animals to common enterobacterial endotoxins, and free from the difficulties inherent in studies on animals suffering experimentally induced brucellosis.

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