Febrile Responses of Rabbits to Bacterial Endotoxin Following Incubation in Homologous Serum and Plasma. (31720)

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It has been widely reported that gram-negative bacterial endotoxins, incubated in normal rabbit serum or plasma, are more pyrogenic for rabbits than similarly incubated endotoxin-saline mixtures(1-7). The possible role of the interaction of serum and endotoxin in the mechanism of endotoxin fever has been discussed in detail(8). However, failure to confirm the enhanced pyrogenicity of endotoxin incubated in serum has been reported in one study, suggesting that perhaps variations in technique, type of incubation media or endotoxin employed may be important determinants for the demonstration of altered febrile responses(9).

In the present study, the pyrogenicity of various endotoxins incubated in homologous serum has been determined. In addition, the extent to which febrile responses may be altered by changes in experimental design have been examined.

Materials and methods. Rabbits. Except where noted, all rabbits used in this study were male and female New Zealand albino rabbits from the same colony (National Institutes of Health) weighing between 1800 and 2200 g. On one occasion, New Zealand albino rabbits were purchased commercially from Rowmar Rabbitry, Inc., Sandy Spring, Md. Animals were housed in air-conditioned quarters, tested in the same room and used only once, to avoid the possibility of modified febrile reactivity.

Endotoxins. Purified bacterial endotoxins derived from *S. typhosa** (Lot 460628) and *Escherichia coli** (Lot 473142) and heat-killed *S. typhosa* organisms (58°C for 2 hours) were used in the present study. The endotoxins were prepared in a concentrated stock solution (100 μg/ml) with pyrogen-free sterile phosphate buffered (pH 7.4) saline and stored at —20°C. Immediately prior to use, the stock solution was defrosted and diluted to appropriate concentration with saline. *S. typhosa* vaccine was stored at 4°C. Sterile, pyrogen-free disposable needles and syringes were used throughout.

Incubation with serum and other blood products. Following aseptic technique, blood was collected by cardiac puncture from normal rabbits and serum or plasma separated and stored overnight at —20° prior to use. In experiments in which plasma was used, the anticoagulants were sodium citrate (0.38% concentration) or heparin (20 units/ml). On many occasions serum and plasma were cultured and shown to be sterile. One ml volumes of endotoxin were mixed and incubated for the appropriate period of time in a water bath at 37°C with either 4 or 8 ml of sera or plasma or pyrogen-free saline. During incubation, the solutions were intermittently shaken to ensure proper mixing. At the end of the incubation period the solutions were immediately injected into a marginal ear vein. In each individual experiment, endotoxin was incubated in serum (or other blood products) and in saline as a control.

Temperature recording. Temperatures (°C) were monitored with rectal thermistors connected to a recording telethermometer.† On the day prior to testing, the animals were trained for 6 hours in wooden stocks with loose fitting collars. On the day of study, temperatures were monitored for at least one hour prior to injection of test solutions. Animals were used only if baseline temperatures were between 38.5 and 39.9° and did not vary greater than 0.2°C for the 30 minutes prior to injection. Febrile responses were plotted on 1 × 1 inch graph paper where one hour and one degree each equaled one inch. The

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† Yellow Springs Instrument Co., Yellow Springs, Ohio.

TABLE I. Febrile Responses of Normal Rabbits to Endotoxins Following Incubation* in Saline or Normal Rabbit Sera.

Endotoxin	Dose	No. of rabbits	Incubation media NRS† (ml)	Saline (ml)	Temperature response F.I. (cm^2)	ΔT (°C)	Latent period (min)
S. typhosa	.005 μg	5	8	0	13.7 ± .97‡	1.1 ± .02	31.2 ± 1.53
		3	0	8	11.6 ± 2.37	1.0 ± .29	30.0 ± 6.00
	.01 "	5	4	0	14.7 ± .63	1.1 ± .04	24.0 ± 2.68
		8	0	4	16.1 ± 1.24	1.1 ± .05	20.6 ± 2.50
	.10 "	7	4	0	23.5 ± 2.31	1.5 ± .11	19.1 ± 1.90
		8	0	4	25.7 ± 3.06	1.4 ± .13	20.8 ± 1.91
E. coli	.01 "	8	8	0	16.6 ± 2.51	1.2 ± .10	29.6 ± 2.50
		4	0	8	15.0 ± 2.07	1.2 ± .14	31.0 ± .71
S. typhosa (vaccine)	1×10^7 organisms	4	4	0	19.1 ± 2.45	1.0 ± .03	20.3 ± 2.84
		5	0	4	27.4 ± 2.89	1.6 ± .17	25.8 ± 2.24
	1×10^8 organisms	5	4	0	38.5 ± 3.38	1.5 ± .15	21.0 ± 1.64
		5	0	4	39.5 ± 3.19	1.5 ± .08	17.2 ± .80

* Incubation for 15 min. † Normal rabbit serum. ‡ Mean ± S.E.

area in square centimeters (Fever Index, FI) under a 5-hour fever curve was measured with a compensating polar planimeter. For each fever curve the maximum temperature change from baseline was also measured and designated as ΔT. The latent period was defined as the time in minutes between injection to the point at which greater than a 0.2°C rise in temperature ensued.

Endotoxin tolerance. Febrile tolerance was established by 7 daily injections of either endotoxin or vaccine, and the animals tested on the eighth day.

Results. Febrile responses of normal rabbits to various endotoxins incubated with normal rabbit sera or saline. S. typhosa endotoxin in concentrations of 0.005 to 0.10 μg was incubated for 15 minutes with either 4 or 8 ml of normal rabbit sera or saline. No significant difference was demonstrated for either FI or ΔT (Table I). Likewise, there were no significant differences in the duration of the latent period between animals that received endotoxin incubated in serum or in saline (Table I). Studies were also performed with *S. typhosa* vaccine at doses of 10^7 and 10^8 organisms and with *E. coli* endotoxin at 0.01 μg and the results were similar to those observed with *S. typhosa* endotoxin (Table I). In one instance, the ΔT of rabbits that received 10^7 *S. typhosa* organisms incubated in saline was significantly higher than those which received vaccine incubated in normal rabbit serum ($p = .03$, t test).

As expected, febrile responses increased with increasing dosage of both saline and normal rabbit serum incubated endotoxins and were generally associated with a shortening of the latent period (Table I). With larger doses of endotoxins and vaccine, fever curves were often biphasic and were similar for both the saline and serum groups of animals.

Febrile responses of tolerant rabbits to various endotoxins incubated with normal rabbit sera or saline. Table II shows the results of 9 experiments in which the febrile responses of tolerant animals to the endotoxins and vaccine were measured after incubation for 15 minutes in either saline or normal rabbit serum. Pyrogenic tolerance was established by 7 daily intravenous injections of 3 μg of either *E. coli* or *S. typhosa* endotoxin or 10^8 *S. typhosa* organisms. Febrile responses of these tolerant animals were tested at minimal pyrogenic doses of endotoxin (*e.g.*, FI of 0 to 20 cm^2) as well as at doses which evoked moderate fevers (FI of 20-40 cm^2). In seven experiments, no significant differences were noted in either the febrile responses or the duration of the latent period in animals receiving pyrogens incubated in saline or normal sera. Following the administration of *S. typhosa* endotoxin in 0.10 μg doses, the FI and latent period of the rabbits which received endotoxin incubated in saline was higher and shorter than those receiving

TABLE II. Febrile Responses of Endotoxin Tolerant Rabbits to Endotoxins Following Incubation* in Saline or Normal Rabbit Sera.

Endotoxin	Dose	No. of rabbits	Incubation media NRS† (ml)	Incubation media Saline (ml)	Temperature response F.I. (cm²)	Temperature response ΔT (°C)	Temperature response Latent period (min)
S. typhosa	.10 μg	6	8	0	9.3 ± 1.17‡	0.8 ± .07	30.5 ± 1.96
		3	0	8	15.7 ± 2.05	0.8 ± .20	18.7 ± 5.70
	1.00 "	5	4	0	14.0 ± 1.00	1.5 ± .09	16.2 ± 1.20
		4	0	4	13.0 ± 2.12	1.3 ± .13	22.5 ± 3.77
	3.00 "	6	8	0	17.5 ± 1.16	1.3 ± .09	25.0 ± 2.14
		3	0	8	20.7 ± 1.53	1.5 ± .06	22.6 ± 2.40
	5.00 "	5	4	0	33.5 ± 2.80	1.9 ± .09	17.4 ± 2.20
		3	0	4	36.7 ± 6.52	1.8 ± .07	21.0 ± 1.41
E. coli	.50 "	5	4	0	11.2 ± 1.72	1.1 ± .12	21.6 ± 3.06
		4	0	4	13.9 ± .84	1.2 ± .07	33.0 ± 3.00
	3.00 "	9	4	0	23.4 ± 1.98	1.6 ± .84	22.3 ± 1.24
		10	0	4	21.7 ± 3.32	1.5 ± .12	28.5 ± 2.87
S. typhosa (vaccine)	1×10^6 organisms	3	4	0	0	0	0
		2	0	4	0	0	0
	1×10^7 organisms	4	4	0	3.3 ± 2.65	0.2 ± .08	—
		4	0	4	4.1 ± 1.58	0.2 ± .09	—
	1×10^8 organisms	4	4	0	10.3 ± 3.81	0.7 ± .17	30.0 ± 8.83
		4	0	4	9.8 ± 2.85	0.7 ± .18	40.0 ± 3.77

* Incubation for 15 min. † Normal rabbit serum. ‡ Mean ± S.E.

TABLE III. Effect of Varying Age and Source of Recipient Rabbits on Febrile Responses to *S. typhosa* Endotoxin Incubated* in Saline or Normal Rabbit Sera.

Dose of endotoxin (μg)	No. of rabbits	Wt or origin of recipient rabbits	Incubation media NRS† (ml)	Incubation media Saline (ml)	Temperature response F.I. (cm²)	Temperature response ΔT (°C)	Temperature response Latent period (min)
.01	5	>2500 g	8	0	12.0 ± 1.13‡	1.0 ± .06	21.2 ± 3.80
	5	1600-2000 g	4	0	14.7 ± .63	1.1 ± .04	24.0 ± 2.68
.01	4	Commercial	4§	0	16.3 ± 1.19	1.1 ± .04	22.5 ± 1.50
	3	"	4‖	0	15.9 ± .61	1.0 ± .06	30.0 ± 1.73
	4	NIH	4§	0	16.5 ± 2.26	1.2 ± .13	21.0 ± 2.12
	3	Commercial	0	4	16.4 ± .78	1.3 ± .10	25.3 ± .90

* Incubation for 15 min. † Normal rabbit serum. ‡ Mean ± S.E. § Sera from Commercial rabbits. ‖ Sera from NIH rabbits.

the endotoxin serum mixture ($p<.05$, t test). On the other hand, the latent period was longer in the animals that received 0.50 μg *E. coli* incubated in saline ($p<.05$, t test), although there were no differences in FI or ΔT at this dose.

Effect of age, source of animals, type of incubation media and duration of incubation on febrile responses. Since enhancement of febrile responses with serum-incubated endotoxin was not demonstrated in either normal or tolerant animals, experiments were performed to assess the possible importance of other experimental variables.

Two groups of normal rabbits, one weighing 1600 to 2000 g (prepubertal), the other in excess of 2500 g (postpubertal) were challenged with 0.01 μg *S. typhosa* incubated for 15 minutes in normal serum obtained from 2000 g rabbits (Table III). No significant differences in febrile responses or duration of latent periods were observed. In a second experiment, normal rabbits weighing 1800-2200 g were obtained from commercial sources and tested with serum and saline-incubated endotoxin. Regardless of the origin of the animals or the source of the normal sera used for incubation, results were similar: no en-

TABLE IV. Effects of Various Incubation* Media on Febrile Responses of Normal Rabbits to *S. typhosa* Endotoxin.

Dose of endotoxin (μg)	No. of rabbits	Incubation media: Blood product (ml)	Incubation media: Saline (ml)	Temperature response: F.I. (cm²)	Temperature response: ΔT (°C)	Temperature response: Latent period (min)
.01	5	8 CP†	0	14.5 ± 2.18‡	1.1 ± .15	21.0 ± 1.34
	7	4 CP	0	15.8 ± 3.41	1.1 ± .15	22.0 ± 2.25
	2	0	4	13.0 ± .65	1.2 ± .25	19.5 ± 1.58
.10	5	4 HP§	0	17.9 ± 2.71	1.4 ± .14	24.0 ± 3.15
	4	4 WB‖	0	27.5 ± 3.93	1.5 ± .07	15.7 ± 3.75
	4	0	4	27.3 ± 4.54	1.3 ± .15	24.0 ± 2.74
.01	4	4 FS¶	0	18.3 ± 1.64	1.3 ± .12	26.2 ± 1.89
	4	0	4	15.6 ± 1.33	1.4 ± .06	31.5 ± .87

* Incubation for 15 min. † Citrated plasma. ‡ Mean ± S.E. § Heparinized plasma. ‖ Whole blood. ¶ Sera stored for 5 mo at −20°C.

TABLE V. Effect of Varying Incubation Time on Febrile Responses of Normal Rabbits to *S. typhosa* Endotoxin.

Dose of endotoxin (μg)	No. of rabbits	Time of incubation (min)	Incubation media: NRS* (ml)	Incubation media: Saline (ml)	Temperature response: F.I. (cm²)	Temperature response: ΔT (°C)	Temperature response: Latent period (min)
.01	5	5	4	0	18.0 ± 2.49†	1.2 ± .09	31.8 ± 3.87
	5	30	4	0	14.1 ± 1.75	1.1 ± .16	26.4 ± 2.20
	6	60	4	0	12.8 ± 1.08	1.0 ± .13	31.0 ± 4.15
	5	60	0	4	20.0 ± 1.35	1.3 ± .09	30.6 ± 5.23

* Normal rabbit serum. † Mean ± S.E.

hancement of febrile responses was demonstrated (Table III).

Citrated plasma in both 4 and 8 ml quantities was incubated for 15 minutes with 0.01 μg *S. typhosa* endotoxin and the pyrogenicity of these mixtures was compared in normal rabbits with *S. typhosa* endotoxin incubated in saline (Table IV). No augmentation of fever was noted. In another study, heparinized plasma or whole blood was used as incubation media for 0.10 μg *S. typhosa* endotoxin and again no significant differences were observed (Table IV). When serum, following storage at −20°C for a 5-month period was incubated for 15 minutes with 0.01 μg *S. typhosa* endotoxin, there were no significant differences in febrile responses compared to the saline controls (Table IV).

Finally, the period of incubation was varied from 5 minutes to 60 minutes for 0.01 μg *S. typhosa* endotoxin incubated in normal serum or saline (Table V). No enhancement in febrile responses was noted. On the contrary, serum incubated endotoxin was less pyrogenic (as measured by FI) than saline controls when the incubation period was either 30 or 60 minutes ($p<.05$, t test). Latent periods did not significantly change.

Discussion. Enhancement of febrile responses to endotoxin incubated in homologous serum has been reported to be readily observed when minimal pyrogenic doses of endotoxin are employed, when the period of incubation is brief (generally less than 30 minutes) and when endotoxin tolerant recipient animals are used(1-7). In the present studies, both minimal and moderate pyrogenic doses of 2 different endotoxins and a vaccine, incubated 15 minutes with 4 or 8 ml of normal serum, were given to both normal and tolerant recipients. Despite the fact that febrile responses were measured by two different methods, the results failed to confirm previous reports with the exception of that of Rall *et al,* who found no enhancement of pyrogenic responses following incubation of *S marcescens* endotoxin with fresh rabbit serum(9). It has also been reported that a reduction in the latent period is associated with augmented febrile responses of serum

endotoxin mixtures(1,2,4); however, careful measurement of latent periods in the present work failed to demonstrate any significant differences.

Additional experiments were performed to evaluate the effects of varying the age and source of test animals, the type of sera and other blood products utilized as incubation media (fresh and frozen sera, heparinized and citrated plasma, heparinized whole blood), and the period of incubation (5 to 60 minutes). In none of these studies were serum- or plasma-incubated pyrogens found to elicit significantly enhanced febrile responses or shortened latent periods when compared to saline controls.

In all, 22 controlled experiments (endotoxin in serum and in saline) were performed. In 14 of 22 studies, the fever indices of saline recipients were greater than serum; in 8 of 15, the ΔT was greater with saline; and in 8 of 19, the saline control animals had shorter latent periods (ties excluded). None of these findings differs significantly from that which might be expected by chance alone ($p > .30$, sign test). In the individual studies, on the other hand, differences significant at the 5% level of confidence were occasionally found. However, in view of the large number of experiments performed and the numerous tests of significance carried out, the findings of 6 results (out of a possible 81) with differences significant at the 5% level can also be expected by chance alone.

The present work does not offer an explanation for the discrepant results of our studies and those of Rall *et al*(9) with previous reports(1-7). In view of these extensive negative results, however, it would seem to us worthwhile to reexamine what role, if any, postulated serum factors play in the pathogenesis of endotoxin fever.

Summary. Quantitative febrile responses of normal and tolerant rabbits to 2 purified endotoxins and a heat-killed vaccine were examined after prior incubation in normal rabbit serum and saline. In addition, the effects on febrile responses of varying the age and source of test animals, the type of incubation media and the period of incubation were studied. No evidence of enhanced pyrogenicity of endotoxin incubated in serum was found.

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