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Volunteers infected with *Salmonella typhosa* develop a remarkable hyperreactivity to the pyrogenic and subjective toxic activities of homologous (*S. typhos*) and heterologous (*Pseudomonas*) endotoxins. The present studies quantitate this augmented reactivity and demonstrate by three differing approaches that significant tolerance to these endotoxins can be readily induced within the framework of the hyperreactive state. Thus, (*a*) tolerance induced before illness by repeated daily intravenous injections of the endotoxins remained demonstrable during overt illness, (*b*) daily intravenous injections of the endotoxins begun during overt illness evoked progressively increasing tolerance, and (*c*) continuous intravenous infusions of *S. typhosa* endotoxin during illness rapidly induced a pyrogenic refractory state. Despite unequivocal activation of the endotoxin tolerance mechanisms by any of the above methods, the febrile and toxic course of typhoid fever proceeded unabated. Similarly, in other volunteers with *Pasteurella tularensis* infection, continuous intravenous infusions of *S. typhosa* endotoxin evoked initial hyperreactive febrile and subjective toxic responses followed by rapid appearance of a pyrogenic refractory state without modification of the underlying clinical illness. These observations suggest that *circulating* endotoxin plays no major role in pathogenesis of the*sustained* fever and toxemia during typhoid fever and tularemia in man.

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The Role of Endotoxin during Typhoid Fever and Tularemia in Man

IV. THE INTEGRITY OF THE ENDOTOXIN TOLERANCE MECHANISMS DURING INFECTION

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ABSTRACT Volunteers infected with *Salmonella typhosa* develop a remarkable hyperreactivity to the pyrogenic and subjective toxic activities of homologous (*S. typhosa*) and heterologous (*Pseudomonas*) endotoxins. The present studies quantitate this augmented reactivity and demonstrate by three differing approaches that significant tolerance to these endotoxins can be readily induced within the framework of the hyperreactive state. Thus, (*a*) tolerance induced before illness by repeated daily intravenous injections of the endotoxins remained demonstrable during overt illness, (*b*) daily intravenous injections of the endotoxins begun during overt illness evoked progressively increasing tolerance, and (*c*) continuous intravenous infusions of *S. typhosa* endotoxin during illness rapidly induced a pyrogenic refractory state. Despite unequivocal activation of the endotoxin tolerance mechanisms by any of the above methods, the febrile and toxic course of typhoid fever proceeded unabated. Similarly, in other volunteers with *Pasteurella tularensis* infection, continuous intravenous infusions of *S. typhosa* endotoxin evoked initial hyperreactive febrile and subjective toxic responses followed by rapid appearance of a pyrogenic refractory state without modification of the underlying clinical illness. These observations suggest that *circulating* endotoxin plays no major role in pathogenesis of the *sustained* fever and toxemia during typhoid fever and tularemia in man.

The mechanisms responsible for the systemic hyperreactivity to endotoxin during typhoid fever and tularemia were further investigated. Low grade endotoxemia, nonspecific effects of tissue injury, impaired ability of the reticuloendothelial system to clear circulating endotoxin, and production of cytophilic antibodies capable of sensitizing leukocytes to endotoxin did not appear responsible. Inflammatory reactions to intradermal *S. typhosa* endotoxin increased significantly during typhoid fever. However, since no such dermal hyperreactivity developed to *Pseudomonas* endotoxin during typhoid fever nor to *S. typhosa* endotoxin during tularemia, the systemic hyperreactivity to bacterial endotoxins during typhoid fever and tularemia could not presently be ascribed to enhanced levels of acquired hypersensitivity.

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INTRODUCTION

The importance of endotoxemia in the production of clinical illness by Gram-negative bacteria is unknown. It has long been recognized, however, that healthy man rapidly acquires tolerance to the pyrogenic and toxic activities of intravenous injections of bacterial endotoxins administered at 24-hr intervals (1). Based upon these observations in healthy man, the inference has been drawn that circulating endotoxin can play no major role in the pathogenesis of fever during sustained Gram-negative bacterial infections (2, 3). While this proposition appears reasonable, it rests upon two unproven assumptions: (*a*) that the mechanisms responsible for tolerance to daily intravenous injections of endotoxin will continue to operate effectively during the febrile

phase of infection, and (*b*) that tolerance to sustained endotoxemia can also develop during the febrile phase. Recent studies in volunteers with induced typhoid fever and tularemia demonstrated the appearance of a remarkable hyperreactivity to single intravenous injections of endotoxins, both homologous and heterologous, during overt illness. This hyperreactivity occurred regardless of whether a high degree of tolerance was preinduced by repeated daily intravenous injections of endotoxin before infection, or whether only a single control intravenous base line test was performed. It was emphasized that it was uncertain as to whether this hyperreactivity to endotoxin involved the inhibition of those mechanisms that participate in tolerance, or whether the tolerance mechanisms remained functional and offered some partial protection (4). The effect of sustained endotoxin infusions during illness was not determined. The present studies were designed to evaluate more critically the role of endotoxemia in the pathogenesis of the febrile and toxic course of typhoid fever and tularemia in man by quantitating the integrity of the endotoxin tolerance mechanisms during the febrile stage of illness, employing both intermittent and sustained endotoxin administration. In addition, further studies were conducted on the mechanisms underlying the remarkable hyperreactivity to endotoxin seen during these infections.

METHODS

Volunteers for these studies were male inmates of the Maryland House of Correction, Jessup, Md., whose ages ranged from 20 to 52 yr. Each volunteer was fully apprised of all aspects of the investigation and the attendant risk. They were free to cease participation at any time. Complete medical surveys were performed to verify the fitness of each participant. The preparation and administration of the inocula of *Salmonella typhosa* and *Pasteurella tularensis* and the clinical course and treatment of the resulting illnesses have been outlined previously (5, 6). All infected volunteers tested with endotoxin during the present studies were unvaccinated controls who were given the infectious agents for the primary purpose of evaluating the efficacy of existing vaccines. Critical appraisal of the clinical course of induced illness was carried out by daily evaluation and recording, by the same internist, of all symptoms and signs of disease. In addition, levels of bacteremia were quantitated serially. Comparisons of these detailed records, designed primarily to provide a sensitive assessment of disease modification by vaccines, also permitted reliable assessment of disease modification in the present volunteers treated with purified endotoxins.

Three preparations of bacterial endotoxins were employed: a highly purified endotoxin from *S. typhosa* (0-282),[1] previously described (7), *Pseudomonas* endotoxin[2] prepared by tryptic digestion (8), and *Escherichia coli* endotoxin (0127B8)[3] prepared by Boivin-type extraction (9). All endotoxin preparations were suspended in sterile, pyrogen-free physiologic saline and stored in rubber-stoppered vaccine bottles at 4°C. The *E. coli* endotoxin was heated at 100°C for 10 min before use. The preparations were bacteriologically sterile, and the same bottle of diluted endotoxin was always employed throughout any given study.

Pyrogen assay in man. Volunteers were hospitalized, and all responses to endotoxin were assayed with the subjects confined to bed and covered with a light blanket. Syringes were either of the sterile, pyrogen-free, disposable type[4] or were heated overnight in a dry air oven at 200°C to eliminate extraneous pyrogens. Each test was begun between 8 and 9 a.m. Flexible thermocouples were inserted 6 inches into the rectum and temperatures recorded by a telethermometer.[5] Temperatures were monitored immediately before and every half-hour after the intravenous injection of endotoxin. The results were plotted on standard graph paper, and fever responses quantitated by two methods: (*a*) the area under the 7 hr fever curve, with the initial temperature as the base line, was calculated by summing the number of enclosed small squares. This value, termed the fever index, represents a measure of the height and duration of the febrile response (10). The coordinates were plotted so that a fever index of 100 reflected a 1°F rise in rectal temperature for a 1 hr duration; (*b*) the maximum rate of temperature rise was calculated by dividing each temperature increment (°F) after endotoxin administration by the time (hours) required to attain this increment and selecting the highest quotient. The derived value reflects the steepest slope of the temperature rise from the origin (Fig. 1 A, slope a). This value, termed the maximum pyrogenic response rate, was consistently obtainable within the initial 3 hr. In addition to the febrile responses, subjective toxic reactions after endotoxin administration were graded as follows: 1+ = mild headache, anorexia; 2+ = moderate headache, anorexia, chills; 3+ = severe headache, shaking chills, myalgia; 4+ = extremely severe headache, shaking chills, myalgias, vomiting.

Labeling and clearance assay of Pseudomonas-^{51}Cr endotoxin. *Pseudomonas* endotoxin was labeled with $^{51}CrCl_3$ or with $Na_2{}^{51}CrO_4$,[6] as described by Braude, Carey, Sutherland, and Zalesky (11). For use in man, the endotoxin was reconstituted to 25 μg/ml in pyrogen-free physiologic saline. After proof of bacteriologic sterility, 1-ml aliquots containing a maximum radioactivity of 150 μc were employed for each study. A 20 gauge needle was inserted into the antecubital vein of the arm opposite to that used for intravenous injection of the radioactively labeled endotoxin. A control blood sample was obtained and the needle left *in situ* for collection of serial timed blood samples after the endotoxin injection. Approximately 5-ml blood samples were collected at each time period, placed into plastic test tubes, and then weighed on a precision balance. The mean weight of 10 similar empty tubes was subtracted to give the net weight of each blood sample. Weighing error was under 5%. The samples were counted in a 3 inch scintillation crystal, and sufficient counts accumulated to produce less than 5% error. All counts were converted to net counts per minute per 5 g of blood. The initial blank sample count was also corrected to 5 g of blood and this value subtracted from each postendotoxin sample. The counts per minute per 5 g of blood were plotted against time on 2-cycle semilog paper and the time for

[1] Kindly supplied by Dr. Maurice Landy, National Institutes of Health.

[2] Kindly supplied as Piromen by Dr. T. A. Garrett, Baxter Laboratories, Inc., Morton Grove, Ill.

[3] Supplied by Difco Laboratories, Detroit, Mich.

[4] Plastipak, supplied by Becton-Dickinson & Co., Rutherford, N. J.

[5] Yellow Springs Instrument Company, Inc., Yellow Springs, Ohio.

[6] Supplied by E. R. Squibb & Sons, New Brunswick, N. J.

clearance of 50% of the endotoxin ($t_{\frac{1}{2}}$) extrapolated employing the 3 min value as the reference point.

Endotoxin clearance studies in rabbits were performed as above except that the timed serial blood samples were obtained by cardiac puncture with a 22 gauge needle after injection of the endotoxin into a marginal ear vein.

Since bacterial endotoxins are complex molecules, the toxic moiety of which has not yet been precisely defined, only those labeled preparations which exhibited blood clearance rates that could be related to their pyrogenic activity in rabbits were employed for studies in man. Thus, endotoxin preparations were considered acceptable only when rabbit assay demonstrated: (*a*) significant acceleration of blood clearance after pyrogenic tolerance was established by six daily intravenous injections of the unlabeled endotoxin; (*b*) significant retardation of blood clearance after reticuloendothelial "blockade" with 3 ml/kg of thorotrast; (*c*) no retardation of blood clearance after reticuloendothelial "blockade" with a colloid that failed to increase febrile responsiveness to the endotoxin (i.e., heat-aggregated human serum albumin); (*d*) blood clearance rates appreciably slower than that of aggregated $^{51}CrCl_3$.[7] Two batches of ^{51}Cr-labeled *Pseudomonas* endotoxin were prepared which met these criteria, one employing $^{51}CrCl_3$, the other $Na_2{}^{51}CrO_4$.

[7] This latter criterion was found essential when labeling with $^{51}CrCl_3$ because of spontaneous aggregation occurring in the pH 7 phosphate buffer; i.e., significant amounts of the $^{51}CrCl_3$ became nondialyzable during control labeling studies in which endotoxin was omitted from the procedure. When tested in four healthy volunteers, 25-μg aliquots of such aggregates of $^{51}CrCl_3$ were cleared from the blood at very rapid rates ($t_{\frac{1}{2}} = 2.9 \pm 0.25$ (SE) min), and these rates were not significantly accelerated after induction of endotoxin tolerance ($t_{\frac{1}{2}} = 2.7 \pm 0.29$ min). This spontaneous aggregation of $^{51}CrCl_3$ was only eliminated by keeping the reaction pH between 3 and 4 with acetate buffer, although this procedure generally resulted in several fold reductions in the pyrogenicity of the added endotoxin.

RESULTS

Quantitation of endotoxin tolerance during typhoid fever and tularemia

Concept of maximum pyrogenic response rate. The area under the fever curve, the fever index, is generally employed as the assay parameter for pyrogenic responsiveness to bacterial endotoxins. The validity of this index is based upon the assumption that the base line temperature will remain stable during the 5–7 hr period of measurement if pyrogen were not administered; it is not useful, therefore, for the assay of responsiveness to endotoxin during clinical infection in man where base line temperatures do not remain stable for these long periods. In the course of quantitative studies of pyrogenic reactivity of healthy man to purified endotoxins, it became apparent that the maximum rate of temperature rise represented an assay parameter which could effectively circumvent this unstable base line problem. The validity of the maximum pyrogenic response rate as an assay for reactivity to endotoxin was demonstrated by its highly positive correlation with the fever index under three differing sets of conditions: (*a*) as the intravenous dose of *E. coli, S. typhosa,* or *Pseudomonas* endotoxins

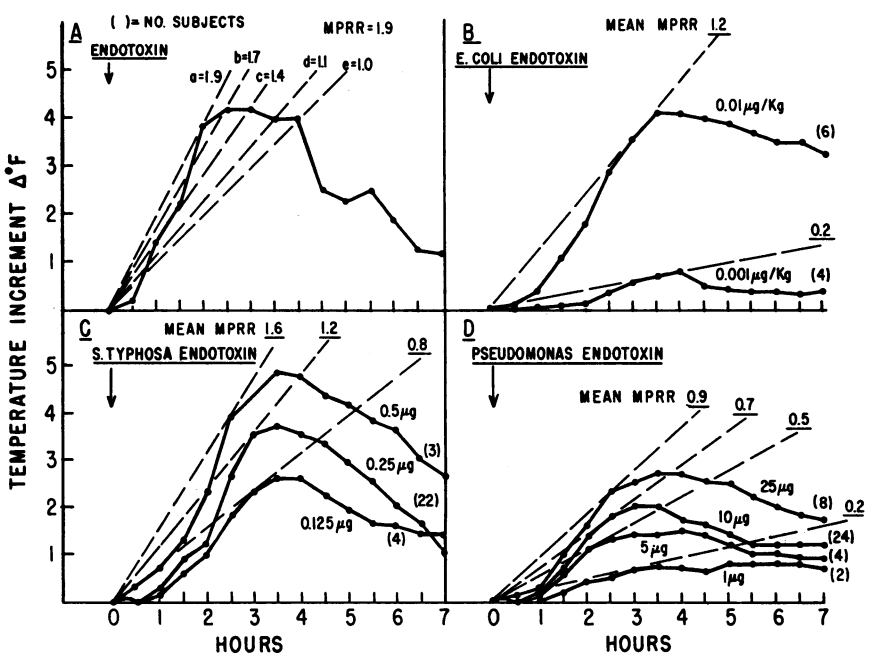

FIGURE 1 (A) Derivation of the maximum pyrogenic response rate (MPRR). The steepest slope from the origin (slope *a*) determines the MPRR. (B)–(D) Correlation between mean maxium pyrogenic response rates and mean 7-hr febrile responses with varying endotoxin dosages in healthy volunteers.

TABLE I

Comparative Changes of Fever Index (FI) and Maximum Pyrogenic Response Rate (MPRR) during Induced Alterations in Responsiveness to Endotoxin in Man

	Tolerance								
	Day 1		Day 7			Day 1		Day 6	
Subject*	FI	MPRR	FI	MPRR	Subject‡	FI	MPRR	FI	MPRR
1	1218	0.9	1038	0.5	8	1588	1.0	1024	0.8
2	913	0.6	769	0.5	9	2052	1.6	1577	1.1
3	814	0.5	771	0.5	10	1680	1.0	531	0.3
4	650	0.7	692	0.4	11	1736	1.2	953	0.7
5	948	0.8	317	0.1	12	1850	1.1	780	0.6
6	1127	1.0	960	0.7	13	2351	1.5	1367	0.8
7	1420	1.4	927	0.9	14	1200	1.0	1122	0.9
Mean	1013	0.8	782	0.5	15	1706	1.3	906	0.7
					16	726	0.7	474	0.5
					Mean	1654	1.2	970	0.7

	Hyperreactivity			
	Control		Typhoid fever	
Subject§	FI	MPRR	FI	MPRR
17	1012	1.1	1140	3.2
18	265	0.5	850	2.9
19	880	0.9	1049	2.5
20	652	0.4	962	1.9
21	379	0.2	858	2.6
Mean	638	0.6	972	2.6

* Challenged with single intravenous injections of 0.5 μg of *S. typhosa* endotoxin (lot no. 1).

‡ Challenged with single intravenous injections of 0.01 μg/kg of *E. coli* endotoxin.

§ Challenged with single intravenous injections of *Pseudomonas* endotoxin. Subject No. 17 received 10 μg; subjects 18–21 received 5 μg.

was increased in healthy subjects, the maximum rate of temperature rise increased concomitantly with the fever index (Fig. 1 [B]–[D]). Extrapolation of previously published data of the pyrogenic responses of man to *S. abortus equi* endotoxin (12) yields similar results; (*b*) induction of even minimal endotoxin tolerance by a single intravenous injection of endotoxin into healthy subjects, as judged by subsequent decreases in fever indices, also resulted in decreases in maximum pyrogenic response rates (Table I, Tolerance); (*c*) in selected volunteers with induced typhoid fever, both the maximum pyrogenic response rate and the fever index increased consistently in response to endotoxin (Table I. Hyperreactivity).[8]

In addition to the virtually consistent positive correlation between the maximum pyrogenic response rate and fever index, in any given volunteer tested in the preceding studies, the intensity of the subjective toxic reactions, i.e., headache, chills, anorexia, myalgia, and vomiting, always paralleled the rate of temperature rise more closely than the 7 hr fever index. From these data it is concluded that changes in maximum pyrogenic response rates represent a sensitive and reliable indicator of changes in pyrogenic responsiveness to bacterial endotoxins in man. Since the maximum pyrogenic response rate is determinable within the initial 3 hr after endotoxin administration, it is less subject than the fever index to distortion by the afternoon rises in temperature characteristic of typhoid fever. The maximum pyrogenic response rate was therefore selected for the assay of altered pyrogenic responsiveness to endotoxin during infection.

Evidence for continuing operation of preinduced tolerance during typhoid fever. The relationship of dosage

[8] Only subjects defervescing to their starting febrile base line level by the 7th hr after endotoxin administration were selected to ensure that the increased fever index was not an artifact based upon spontaneous afternoon rises in temperature induced by the underlying typhoid illness.

of *Pseudomonas* endotoxin to the maximum pyrogenic response rate in healthy subjects and those with typhoid fever is shown in Fig. 2. In both groups, plots of the maximum pyrogenic response rate against a logarithmic scale of the endotoxin dose yielded a linear relationship within the dose ranges tested. In those subjects with overt typhoid fever, hyperreactivity to the endotoxin was clearly evidenced by the heightened maximum pyrogenic response rates at each endotoxin dose level tested. For example, the mean maximum pyrogenic response rate after an intravenous injection of 0.6 μg of *Pseudomonas* endotoxin during typhoid illness became comparable to that produced by 25 μg in healthy subjects. Moreover, as shown in Fig. 2, the slope of the dose-response relationship during typhoid illness was considerably steeper than that in healthy subjects, and the severity of both the febrile and subjective toxic responses more rapidly approached extreme levels as the endotoxin dosage was increased.

The effect of preinduced endotoxin tolerance on the responsiveness to the *Pseudomonas* endotoxin during typhoid fever is superimposed on the above studies in Fig. 2. Tolerance was preinduced in five healthy volunteers by daily intravenous injections of 25 μg of *Pseudomonas* endotoxin for 30 days, continuing through the incubation period of typhoid illness. The initial mean maximum pyrogenic response rate was 0.8, decreasing to 0.1 after tolerance was induced. However, towards the latter portion of the typhoid incubation period, the maximum pyrogenic response rate increased progressively until during overt typhoid illness a mean value of 1.8 was reached (Fig. 2). Although hyperreactivity to the endotoxin thus appeared despite the preinduced tolerance, inspection of Fig. 2 reveals that the mechanisms of tolerance continue to function, i.e., the subjects behaved as did nontolerant volunteers with typhoid fever given only 2 μg of the endotoxin. Indeed, if tolerance had not been preinduced, the challenge with 25 μg of *Pseudomonas* endotoxin during illness would have been expected to evoke intolerable reactions. Despite this operation of the preinduced endotoxin tolerance mechanims, the incubation period, levels of bacteremia, febrile course, intensity of subjective toxic symptoms (headache, abdominal discomfort, apathy), and response to treatment remained indistinguishable from that seen in control subjects given comparable inocula of the viable *S. typhosa.*

As with *Pseudomonas* endotoxin, a plot of the maximum pyrogenic response rate against a logarithmic scale of *S. typhosa* endotoxin dosage for healthy subjects yielded a linear relationship within the range of endotoxin doses explored (Fig. 3). Not only was healthy man more reactive to the *S. typhosa* endotoxin preparation than to the *Pseudomonas* endotoxin at any given dose, but the slope of the *S. typhosa* endotoxin dose-response relationship was steeper (compare Figs. 2 and 3, normal controls). Because of this steeper dose-response relationship in healthy man, only two test doses of the *S. typhosa* endotoxin, 0.01 and 0.03 μg, were administered during overt typhoid fever. Hyperreactivity to the initial intravenous injection of *S. typhosa* endotoxin was evident during typhoid illness, 0.01 μg yielding responses equiva-

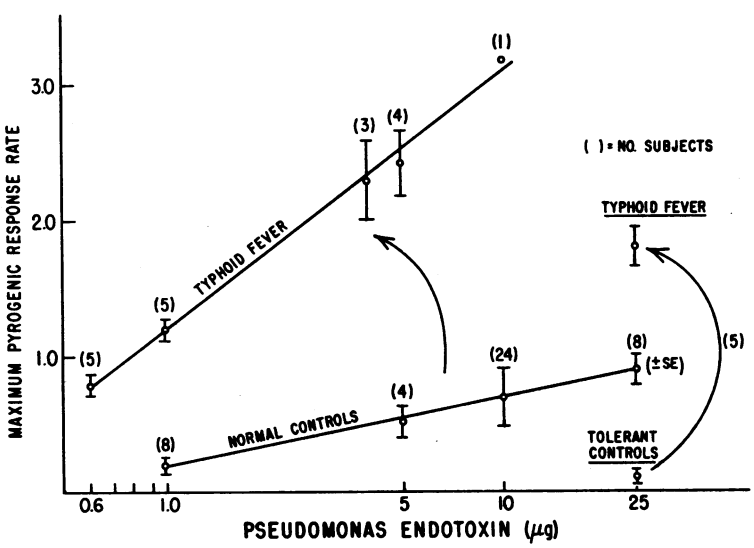

FIGURE 2 Relationship between dosage of *Pseudomonas* endotoxin and maximum pyrogenic response rate in healthy subjects, and the increment in this responsiveness in volunteers with induced typhoid fever. Note that preinduced tolerance in five subjects given typhoid fever remains demonstrable within the hyperreactive framework produced by illness.

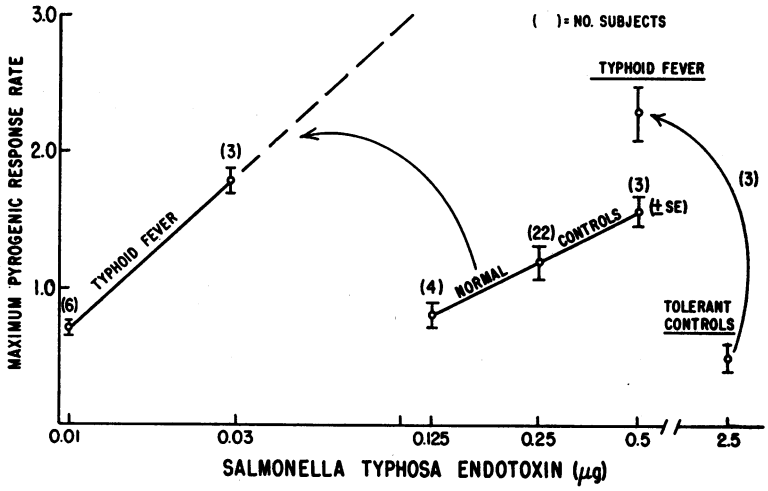

FIGURE 3 Relationship between dosage of *S. typhosa* endotoxin and maximum pyrogenic response rate in healthy subjects, and the increment in this responsiveness in volunteers with induced typhoid fever. Note that preinduced tolerance in three subjects given typhoid fever remains demonstrable within the hyperreactive framework produced by illness.

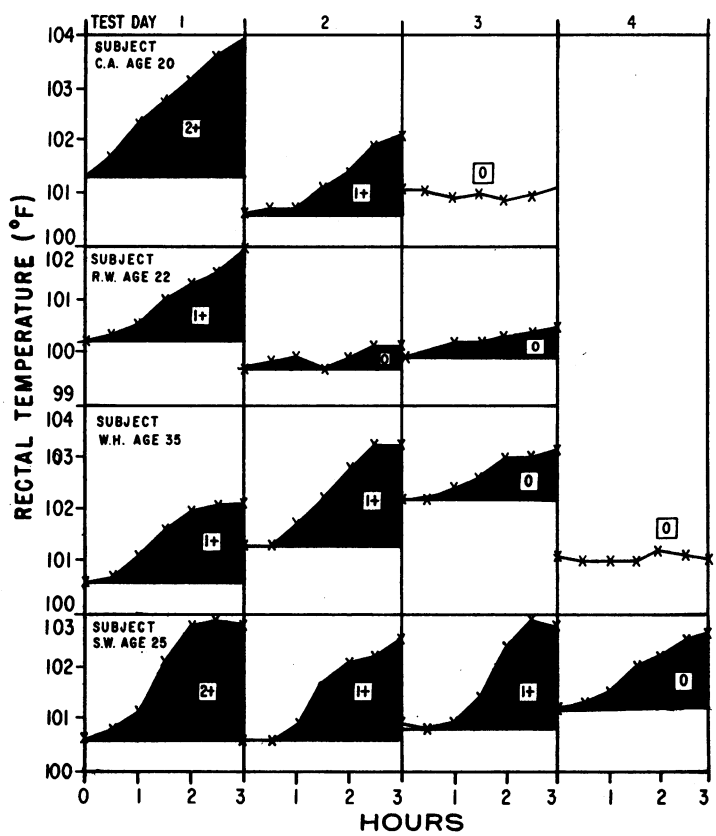

FIGURE 4 Ability of man to develop tolerance to the pyrogenic and subjective toxic activities of *Pseudomonas* endotoxin administered as single daily intravenous injections (0.6 μg) during typhoid fever. (See text for criteria of 1+ to 4+ grading of subjective toxic reactions.)

lent to those induced by 0.10 μg in control subjects. Moreover, as shown in Fig. 3 (and as previously demonstrated with *Pseudomonas* endotoxin), hyperreactivity during typhoid fever was also apparent from the steeper slope of the *S. typhosa* endotoxin dose-response relationships.

The effects of preinduced tolerance to *S. typhosa* endotoxin are superimposed on the control studies shown in Fig. 3. Three subjects were rendered tolerant to the *S. typhosa* endotoxin by daily intravenous administration of 0.5 μg increasing to 2.5 μg over a 16 day period. The mean maximum pyrogenic response rate to the initial 0.5 μg challenge was 1.4, decreasing to 0.5 in response to 2.5 μg of endotoxin after tolerance induction. The *S. typhosa* endotoxin was discontinued after oral administration of the viable *S. typhosa*, and 1 wk later, during the 1st–3rd day of overt typhoid illness, each subject was retested with 0.5 μg of *S. typhosa* endotoxin. Although hyperreactive febrile and subjective toxic responses were now encountered (mean maximum pyrogenic response rate 2.3), the continued activity of the tolerance mechanisms can be inferred from inspection of Fig. 3. As with the *Pseudomonas* endotoxin studies, despite the operation of the preinduced tolerance, the febrile and toxic course of typhoid illness was indistinguishable from that of nontolerant control subjects given comparable inocula of viable *S. typhosa*.

Four additional volunteers addicted to the use of intravenous narcotics for several years were also studied during overt typhoid fever. Prolonged exposure to intravenous endotoxins could be presumed, since nonsterile techniques were routinely employed for the narcotic administration. Each subject failed to manifest the slightest increment in febrile or subjective toxic responses after intravenous injections of 0.01 or 0.03 μg of *S. typhosa* endotoxin during typhoid fever. Nevertheless, as in those volunteers with tolerance preinduced by the repeated intravenous injections of purified bacterial endotoxins, the febrile and toxic clinical course of illness in these "naturally" tolerant subjects was indistinguishable from that in the control nontolerant subjects given comparable inocula of the viable *S. typhosa*.

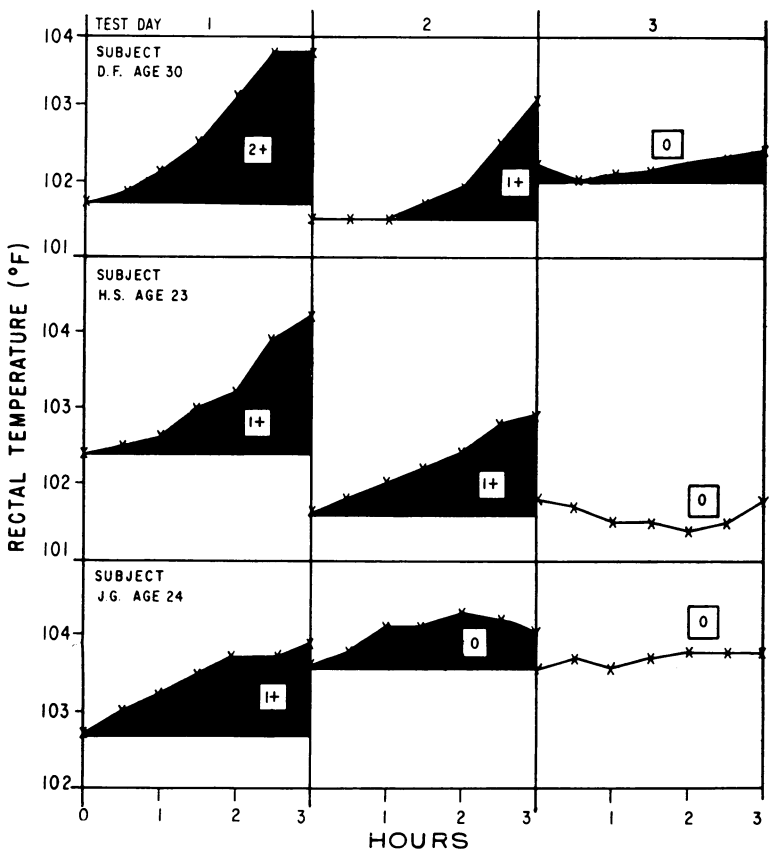

FIGURE 5 Ability of man to develop tolerance to the pyrogenic and subjective toxic activities of *S. typhosa* endotoxin administered as single daily intravenous injections (0.01 μg) during typhoid fever.

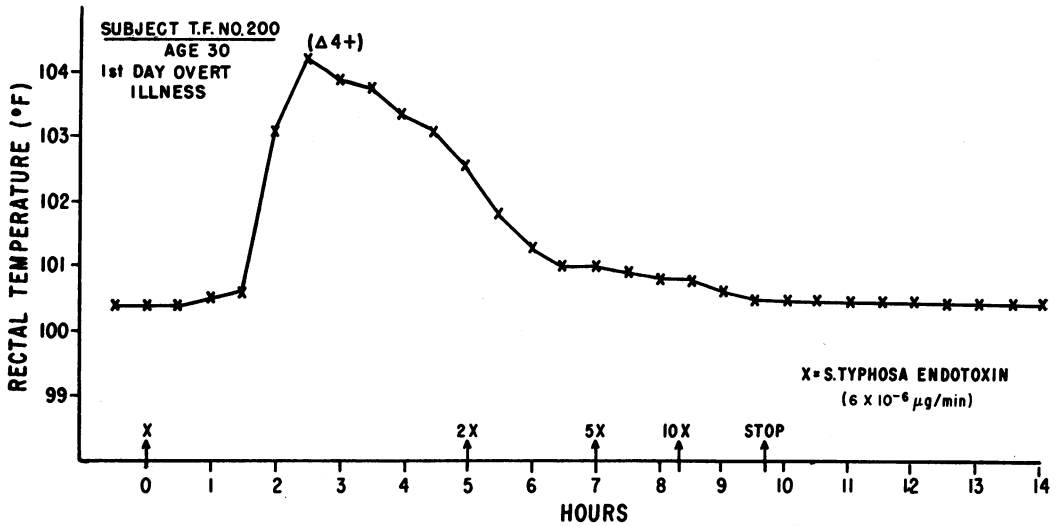

FIGURE 6 Ability of man to develop refractoriness to the pyrogenic activity of a sustained intravenous infusion of *S. typhosa* endotoxin during typhoid fever.

Development of tolerance to daily intravenous injections of endotoxin during typhoid fever. Another group of volunteers, not previously injected with bacterial endotoxins, were given a single intravenous injection of *Pseudomonas* or *S. typhosa* endotoxin commencing on the 1st–4th day of overt typhoid illness. The dose of endotoxin chosen was sufficient to induce clearly detectable increments in the existing febrile and toxic state. Repeated injections at daily intervals resulted in progressive diminution of the febrile and toxic responses to both the *Pseudomonas* endotoxin (Fig. 4) and to the *S. typhosa* endotoxin (Fig. 5). Despite such induced tolerance to the exogenous endotoxins, the febrile illness continued unabated.

Development of pyrogenic refractoriness to continuous intravenous infusions of endotoxin during typhoid fever. The administration of *S. typhosa* endotoxin by continuous infusion during the 1st–4th day of overt typhoid fever induced initial exacerbations of the febrile and toxic state when given at rates only 1/10–1/100 that which evoked comparable temperature increments in normal subjects. However, despite the maintenance of the endotoxin infusion, after 2–4 hr the increments in fever and in the subjective toxic reactions began to return progressively towards the control febrile base line state. One such example is shown in Fig. 6. In three of eight of the acutely ill subjects tested, the defervescence phase during the endotoxin infusion was interrupted by a second temperature rise which persisted after the infusion was discontinued (Fig. 7). The time of onset, the magnitude, and the duration of this second temperature rise in each subject was virtually superimposable upon the spontaneous afternoon rises in temperature observed in the absence of the endotoxin infusion during control studies performed on the following day of illness.

Development of pyrogenic refractoriness to continuous intravenous infusions of endotoxin during tularemia. As with typhoid fever, the administration of *S. typhosa* endotoxin by continuous intravenous infusion during tularemia induced exacerbations of the febrile and toxic state when administered at rates only 1/10–1/100 those which evoked comparable toxicity and temperature increments in normal subjects. Similarly, despite the maintenance of the endotoxin infusion, after 2–4 hr the increments in fever and subjective toxic reactions returned progressively towards the control febrile base line state. One example is shown in Fig. 8. In four of eight of the acutely ill subjects tested, the defervescence phase during the endotoxin infusion was interrupted by a second temperature rise that persisted after the endotoxin infusion was discontinued; one such response is depicted in Fig. 9. As with typhoid fever, the time of onset, the magnitude, and the duration of this second temperature rise in each subject was virtually superimposable upon the spontaneous afternoon rises in temperature observed in the absence of the endotoxin infusion on the subsequent day of illness.

Mechanisms of hyperreactivity to endotoxin during typhoid fever and tularemia

Nonspecific effects of tissue injury. To determine if the hyperreactivity to endotoxin observed during typhoid fever and tularemia might be secondary to nonspecific effects of cell injury, varying degrees of gross tissue necrosis were induced in normal rabbits by inoculations of turpentine or formalin, intraperitoneally, intramuscu-

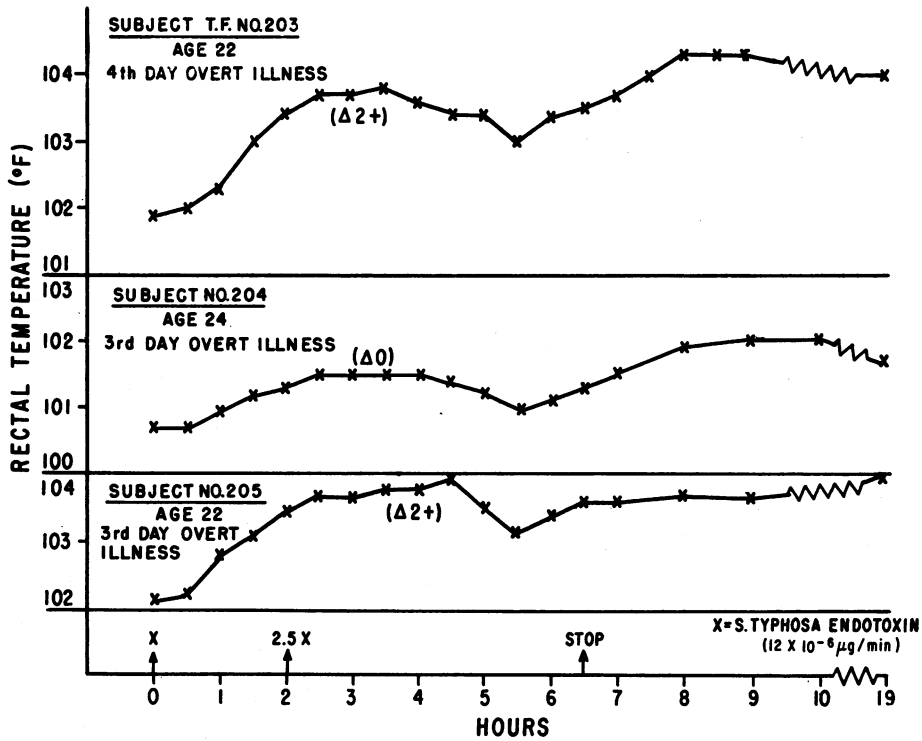

FIGURE 7 Biphasic fever pattern observed in three of eight volunteers given sustained intravenous infusions of *S. typhosa* endotoxin during typhoid fever. The time of onset, magnitude, and duration of the second phase of fever in each subject was virtually superimposable upon the spontaneous rise in late afternoon temperature observed in the absence of the endotoxin infusion on the following day of illness.

larly, or intrathoracically. The effects on responsiveness to bacterial endotoxins are shown in Table II. No increase in pyrogenic reactivity was detectable by any of these procedures; indeed, febrile responses diminished.

Effect of sustained "low grade" endotoxemia. While continuous infusions of pyrogenic quantities of bacterial endotoxins lead to progressive febrile unresponsiveness in healthy rabbits and man (13), the possibility that

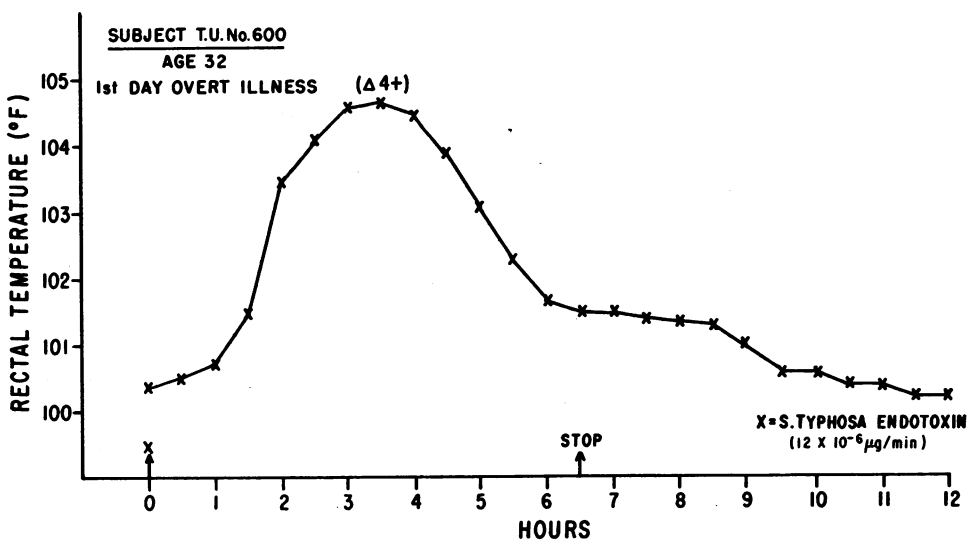

FIGURE 8 Ability of man to develop refractoriness to the pyrogenic activity of a sustained intravenous infusion of *S. typhosa* endotoxin during tularemia.

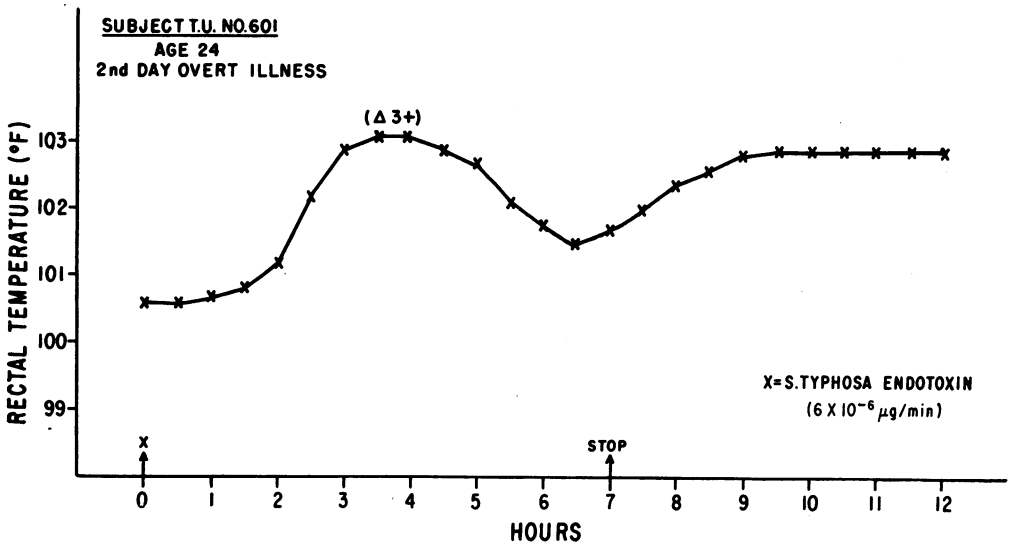

FIGURE 9. Biphasic fever pattern observed in a volunteer given a sustained intravenous infusion of *S. typhosa* endotoxin during tularemia. The time of onset, magnitude, and duration of the second phase of fever was virtually superimposable upon the spontaneous rise in late afternoon temperature observed in the absence of the endotoxin infusion on the following day of illness.

TABLE II

Effect of Chemically Induced Tissue Injury on Pyrogenic Responsiveness to Endotoxin in the Rabbit

Site of inoculation	No. animals	Irritant	5-hr fever index*
		Turpentine, in olive oil	
Intraperitoneal	10	4%, 5 ml/kg	931
	4	4%, 25 ml/kg	927
	7	20%, 5 ml/kg	1300
		Controls	
	6	—	1294
	5	Olive oil	1250
Intramuscular	5	Turpentine, 100%, 0.5 ml in 4 sites	322
	5	Formalin, 0.4%, 0.25 ml in 4 sites	830
	5	Controls	1108
Intrathoracic	6	Turpentine, 100%, 0.5 ml	278
		Formalin	
	5	0.4%, 0.5 ml	869
	6	3.8%, 0.5 ml	889
	12	Controls	991

* 0.50 μg/kg of *E. coli* endotoxin given intravenously 24 hr after administration of irritant.

continuous infusions of endotoxin at low rates, insufficient to evoke fever, might augment the responsiveness to larger febrile dosages was explored. Continuous intravenous infusions of *Escherichia coli* endotoxin were administered to 13 rabbits for 18–40 hr at constant rates which just failed to elicit any febrile responses (18×10^{-6} μg/kg per min). At the completion of the infusion, all animals were challenged intravenously with single dosages of the endotoxin sufficient to evoke fevers within the sensitive portion of the dose-response range (0.05 μg/kg). No significant augmented (or diminished) febrile responses resulted; 5-hr fever indices of 720 (±80 SE) occurred in the control group compared with 750 (±88 SE) after infusion.

Clearance of ^{51}Cr-labeled Pseudomonas endotoxin. The criteria for acceptable preparations of labeled endotoxin have been considered in the Methods section. The blood clearance patterns of an acceptably labeled preparation in the rabbit are illustrated in Fig. 10; rates of clearance in the normal, tolerant, thorotrast-blockaded normal, and thorotrast-blockaded tolerant animals paralleled in inverse fashion the documented changes in febrile responsiveness to the endotoxin (14–16). As further controls, endotoxin clearance studies were performed in 10 rabbits given 200 mg/kg of heat-aggregated human serum albumin intravenously 1 hr previously. In contrast to thorotrast, this reticuloendothelial ingested colloid (17) did not alter febrile responsiveness to quantities of endotoxin within the sensitive portion of the dose-response range (three groups of six rabbits each tested); moreover, no alterations in *Pseudomonas*-^{51}Cr endotoxin

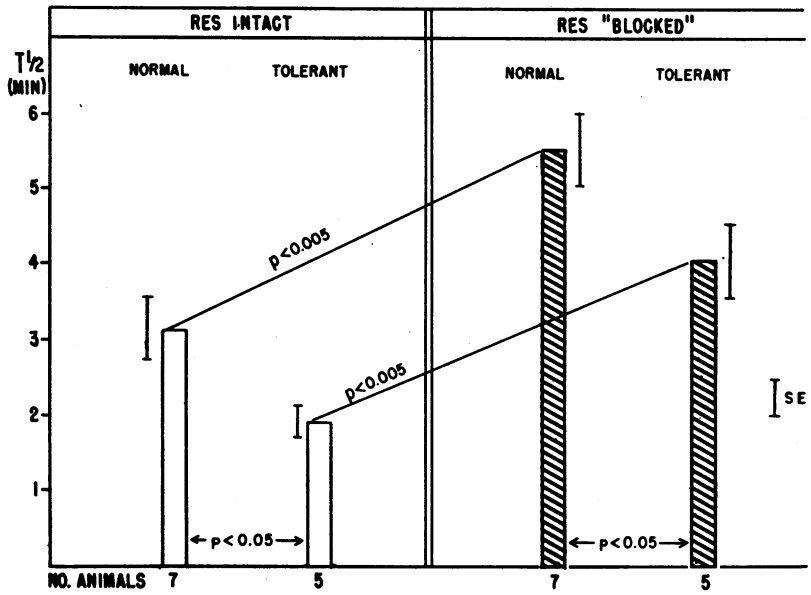

FIGURE 10 Blood clearance rates of 25 μg of *Pseudomonas*-^{51}Cr endotoxin in normal, tolerant, thorotrast-blockaded normal, and thorotrast-blockaded tolerant rabbits. Tolerance was induced by seven daily intravenous injections of 25 μg of unlabeled *Pseudomonas* endotoxin and "blockade" by 3 ml/kg of thorotrast intravenously 3 hr beforehand. Note that the differences in rates of endotoxin clearance in normal and tolerant animals remain fully demonstrable after thorotrast "blockade."

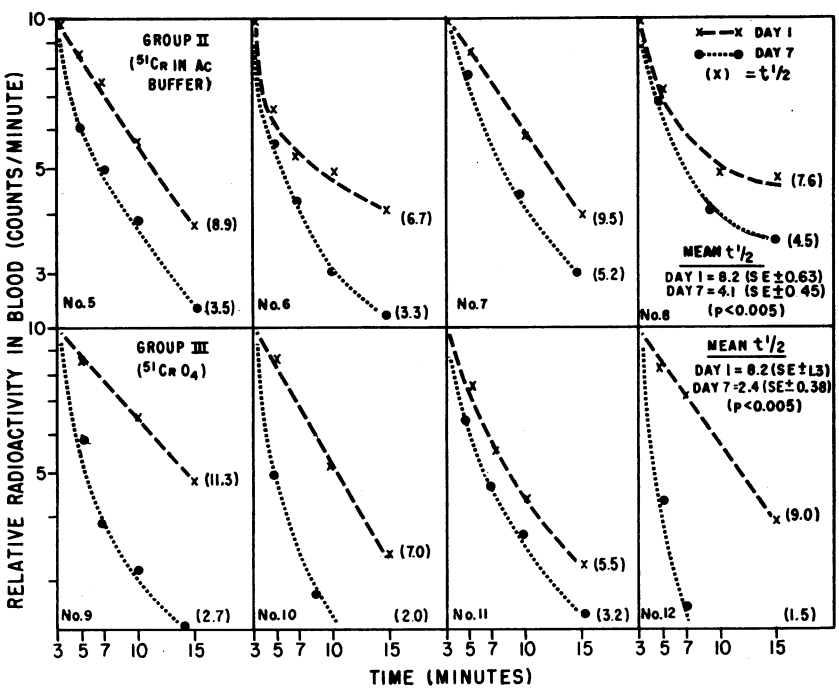

FIGURE 11 Acceleration of clearance of 25 μg of *Pseudomonas*-^{51}Cr endotoxin (labeled with $^{51}CrCl_3$, group II and with $Na_2{}^{51}CrO_4$, group III) in healthy volunteers after six daily intravenous injections of the endotoxins.

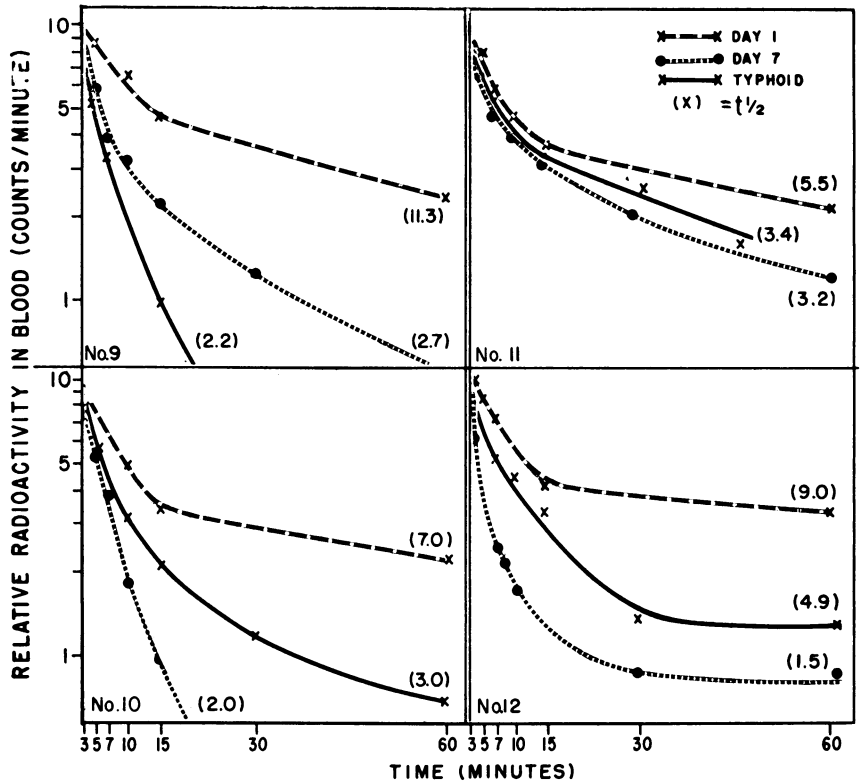

FIGURE 12 Evidence that hyperreactivity to endotoxin during typhoid fever is not attributable to impaired reticuloendothelial clearance of circulating endotoxin. Four volunteers tolerant to *Pseudomonas* endotoxin (group III shown in Fig. 11) were retested with 25 μg of *Pseudomonas*-^{51}Cr endotoxin during induced typhoid fever; despite systemic hyperreactivity (see text), endotoxin clearance rates remained accelerated.

clearances were detectable ($t_{\frac{1}{2}}$ 2.7 ±0.5 (SE) compared to control values of 2.8 ±0.4 (SE) min). The blood clearance rates of such acceptable preparations of ^{51}Cr-labeled *Pseudomonas* endotoxin in eight healthy volunteers and the effect of tolerance induction is shown in Fig. 11; as in the rabbit, repeated daily intravenous injections led to significantly enhanced rates of blood clearance ($P < 0.005$). The effect of induced typhoid fever on the rates of endotoxin clearance in four of these tolerant volunteers 1 wk later is superimposed upon their initial and tolerant clearance curves in Fig. 12. Despite the hyperreactivity to the *Pseudomonas* endotoxin during illness (mean maximum pyrogenic response rate of 1.8 compared with the initial control value of 0.7 and the tolerant value of 0.1), the blood clearance rates of the labeled endotoxin remained accelerated.

Antiendotoxin antibody cytophilic for human leukocytes. Cytophilic antibody capable of sensitizing human leukocytes to the injurious activity of bacterial endotoxin, such as reported for chicken leukocytes during *S. gallinarum* infection (18), was sought during typhoid fever. Fresh buffy coats from normal human heparinized blood samples were suspended in fresh serum obtained during the 1st wk of overt typhoid fever; varying concentrations of *Pseudomonas* endotoxin (1–100 μg) and of *S. typhosa* endotoxin (0.01–5 μg) were then added per milliliter and the buffy coat cells incubated for 30 min at 37°C. Eosin Y (1%) was added and the per cent of injured granulocytes, indicated by inability to exclude the vital dye, counted. No differences in the injurious effects of typhoidal and normal serum on human leukocytes exposed to the endotoxin were detectable.

Comparison of dermal and systemic reactivity to endotoxin. Volunteers were tested intradermally with *S. typhosa* or *Pseudomonas* endotoxins before and during typhoid fever and tularemia. Inflammatory responses were measured at 1, 2, 4, and 24 hr. Differences between the control dermal responses and those during illness, when present, were always most marked at 24 hr; therefore only these latter reactions are presented. In the six subjects tested during overt typhoid fever, dermal reactivity to *S. typhosa* endotoxin consistently increased

TABLE III

Inflammatory Responses to Intradermal S. typhosa and Pseudomonas Endotoxins during Typhoid Fever

Subject	Endotoxin	Control		Febrile illness		Convalescence	
L. E.	S*	0,	0‡	400, (3+)	420 (3+)		
J. S.	S	100, (2+)	110 (2+)	400, (3+)	440 (3+)	64, (1+)	64 (1+)
G. Y.	S	36, (1+)	64 (1+)	225, (2+)	156 (2+)	25, (1+)	36 (1+)
D. W.	S	340, (3+)	323 (3+)	840, (3+)	728 (3+)	324, (3+)	255 (3+)
F. G.	S	360, (3+)	300 (2+)	1280, (4+)	1050 (4+)		
J. G.	S	174, (2+)	144 (2+)	560, (2+)	360 (2+)	120, (2+)	120 (2+)
M. L.	P§	360, (2+)	360 (2+)	210, (2+)	210 (2+)		
O. P.	P	890, (4+)	1215 (4+)	1050, (3+)	900 (3+)		
E. M.	P	130, (2+)	120 (2+)	144, (3+)	110 (3+)		

* S = *S. typhosa* endotoxin, 0.01 μg in 0.10 ml of saline, injected intradermally into the forearm in duplicate sites.

‡ Area index obtained by multiplying largest cross diameters of erythema (mm^2) at 24 hr. Numbers in parenthesis indicate relative intensity of erythema.

§ P = *Pseudomonas* endotoxin, 1.0 μg in 0.10 ml of saline, injected intradermally into the forearm in duplicate sites.

during illness (Table III). No relationship to the hyperreactive systemic response to the endotoxin could be inferred, however, since no increases in dermal reactivity were detected to *Pseudomonas* endotoxin during typhoid illness (Table III) nor to *S. typhosa* endotoxin during tularemia (Table IV) despite comparable systemic hyperreactivity to these endotoxins.

DISCUSSION

While healthy man possesses potent mechanisms for developing pyrogenic unresponsiveness to Gram-negative bacterial endotoxins, whether administered by single intravenous injections or by continuous intravenous infusions (1, 13), the efficacy of these mechanisms during the febrile phase of Gram-negative bacterial infections is unknown. In 1963, McCabe reported significant endotoxin tolerance in patients with chronic pyelonephritis and Gram-negative bacteriuria; at the time of testing, however, the subjects were ambulatory and essentially afebrile (19). The present studies in volunteers with induced typhoid fever and tularemia demonstrate that the endotoxin tolerance mechanisms remain effective during overtly febrile Gram-negative bacterial illness. This demonstration was complicated by the remarkable enhancement of pyrogenic and subjective toxic reactivity to homologous and heterologous (*S. typhosa* and *Pseudomonas*) endotoxins induced by the infectious diseases. Such augmented responsiveness was probably first encountered in man during attempts to treat typhoid fever with *S. typhosa* and *E. coli* vaccines in the preantibiotic era (20–22), but controlled studies proving hyperreactivity have only recently been carried out (4). To evaluate the endotoxin tolerance mechanisms in the presence of this hyperreactivity, the maximum rate of temperature rise was selected as the assay parameter. That the maximum pyrogenic response rate represents a sensitive and reliable indicator of man's reactivity to endotoxin has been demonstrated in the Result section by comparisons with the more generally employed parameter, the fever index. The distinct advantage of the maximum pyrogenic response rate during infection, indeed its indispensability, is its determinability within the initial 3 hr after endotoxin administration, thus minimizing distortions induced by afternoon rises in temperature from the underlying illness. It could thereby be shown not only that hyperreactive responses are demonstrable during typhoid fever

TABLE IV

*Inflammatory Responses to Intradermal S. typhosa Endotoxin during Tularemia**

Subject	Control		Febrile illness		
R. S.	376, (3+)	380‡ (3+)	180, (1+)	200 (1+)	
R. M.	440, (3+)	528 (3+)	144, (2+)	225 (2+)	
W. P.	100, (1+)	120 (1+)	36, (1+)	9 (1+)	
W. B.	0,	0	0,	0	
E. C.	320, (3+)	210 (3+)	300, (3+)	266 (3+)	
			280, (3+)	280 (3+)	(repeat on following day)
J. B.	300, (3+)	440 (3+)	25, (1+)	25 (1+)	
L. H.	360, (2+)	144 (3+)	100, (1+)	100 (1+)	
W. D.	144, (3+)	210 (3+)	81, (2+)	81 (2+)	

* *S. typhosa* endotoxin, 0.01 μg in 0.10 ml of saline, injected intradermally into the forearm in duplicate sites.

‡ Area index obtained by multiplying largest cross diameters of erythema (mm²) at 24 hr. Numbers in parenthesis indicate relative intensity of erythema.

for all doses of endotoxins tested, but that the dose-response relationships also become appreciably steeper during illness. In those volunteers rendered tolerant to the pyrogenic activity of the endotoxins before induction of typhoid fever, hyperreactivity during illness though present was detectably blunted, i.e., the pyrogenic tolerance mechanisms, activated before illness, continued to operate within the framework of the hyperreactive state. Additional studies demonstrated that the tolerance mechanisms could also be readily activated during overt illness. Volunteers acutely ill with typhoid fever, not previously injected with endotoxin, exhibited progressive decreases in pyrogenic and subjective toxic responses to daily intravenous injections of single constant doses of *S. typhosa* or *Pseudomonas* endotoxins. In addition, intravenous infusions of *S. typhosa* endotoxin at constant rates during typhoid fever or during tularemia resulted in an initial intensification of the existing fever and subjective toxic reactions, but as in healthy subjects (13), this was rapidly followed by an endotoxin refractory state, i.e., a progressive return toward the base line febrile and subjective toxic levels despite the continuing infusion.

Although tolerance to the pyrogenic and subjective toxic activities of exogenous endotoxin could be induced readily during typhoid fever and tularemia as indicated above, no clinical evidence was obtained to suggest that the febrile or toxic course of illness was mitigated by such stimulated resistance. Indeed, in certain of the acutely ill volunteers, the expected rise in late afternoon temperature was not detectably blunted at the very time refractoriness was developing to the continuous intravenous infusion of the exogenous endotoxin. In addition, in one group of subjects either naturally failing to exhibit hyperreactivity to endotoxin during typhoid fever or, as seems more likely, tolerant to endotoxin as a result of prolonged use of intravenous narcotics without sterile precautions, unmitigated clinical typhoid illness was observed. The above observations, considered collectively, suggest that the sustained febrile and toxic course of typhoid fever and tularemia in man cannot be attributable primarily to sustained or to intermittent endotoxemia. Nevertheless, it could be argued that since tolerance to endotoxin is relative and can be overcome with increased endotoxin dosage (10), the induced or natural tolerance to the exogenous endotoxin could be overcome during illness by liberation of greater quantities of endogenous circulating endotoxin. Several lines of evidence do not support this possibility: (*a*) the degree of induced or of naturally occurring tolerance in the subjects described above was sufficient to protect against quantities of endotoxin capable of evoking marked pyrogenic and subjective toxic reactions. Indeed, the volunteers with preinduced tolerance were protected against quantities of endotoxin capable of eliciting febrile and toxic responses of greater severity than those observed during any stage of typhoid illness. Since the tolerance mechanisms remain functional during illness, some lengthening of the incubation period and (or) reduced severity of the early phase of clinical illness in these tolerant subjects would be expected if endotoxemia were critical. This did not occur; (*b*) release of sufficient circulating endotoxin during illness to overwhelm continuously the functional tolerance mechanisms and yet sustain a submaximal fever which can fluctuate for days within a narrow range (often less than 2°F) appears unlikely; any minute overrelease of circulating endotoxin in such hyperreactive subjects, judged by responses to exogenous endotoxin, would evoke steep increments in temperature; (*c*) finally, if it were postulated that the levels of endogenous endotoxemia during illness were continuously maintained slightly ahead of the functional capacity of the tolerance mechanisms, increasing levels of endotoxin tolerance would be expected as illness progressed. Instead, the hyperreactive febrile and toxic responses to exogenous endotoxin observed on the first day of illness were not detectably diminished during the early afebrile period of convalescence, i.e., progressively increasing tolerance was not apparent during the overt

phase of illness (4). Additional support for the concept that endotoxemia is not critical for the sustained fever during Gram-negative bacterial infection is provided by the well controlled studies of Bennett employing *Escherichia coli*–induced peritonitis in rabbits (23).

It is emphasized that the conclusion presently drawn is that *endotoxemia* plays no major role in pathogenesis of the *sustained* fever and toxemia of typhoid fever and tularemia. The above conclusion requires careful qualification in three respects: (*a*) it does not preclude the presence of endotoxemia during infection; indeed, serologic evidence exists for endotoxemia during the initial 7–10 days of typhoid fever (24). Rather, it is the response of man to endotoxemia, i.e., the rapid ability to develop tolerance, and the lack of clinical protection afforded by high levels of induced tolerance, that forms the basis for the inference that such endotoxemia cannot primarily account for the sustained fever and toxemia during typhoid fever; (*b*) the possibility is not precluded that abrupt increments in circulating endotoxin levels during typhoid fever or tularemia mediate acute exacerbations of fever and toxemia and shock in the hyperreactive host. Rather, it is postulated from the present data that these exacerbations would be superimposed upon the more basic mechanisms responsible for the sustained fever and toxemia, and that their frequency and intensity would be limited by the development of systemic tolerance; (*c*) the present conclusion does not preclude an important role of endotoxin acting directly at the sites of bacterial lodgement to enhance the local inflammatory response and the release of endogenous pyrogen from the surrounding leukocyte and macrophage populations. Indeed, hyperreactive inflammatory responses were observed after local (i.e., intradermal) injections of homologous endotoxin during typhoid fever. Such ability of *S. typhosa* endotoxin to potentiate *local* tissue injury during typhoid fever actually may contribute significantly to the pathogenesis of sustained fever and toxemia of illness.[9]

In light of the above considerations, the significance of the pyrogenic tolerance to endotoxin observed during late convalescence from typhoid fever and tularemia (26, 27), and the vascular hyperreactivity to catecholamines observed during typhoid fever (28), previously regarded as evidence for a major role of circulating endotoxin in pathogenesis of these illnesses, requires reevaluation. These findings are simply compatible with the presence of endotoxemia; they in no way define its relative importance in the production of illness. The present studies, on the other hand, now permit the interpretation that sufficient endotoxemia occurs during illness to produce or contribute to tolerance and vascular hyperreactivity without being primarily responsible for the sustained clinical disease. Additional support for this interpretation is presently available. The pyrogenic tolerance after recovery from typhoid fever and tularemia was evident by 50–70% reductions in mean fever indices (26, 27). Comparable degrees of tolerance recently have been induced in normal volunteers by intravenous injections of subfebrile doses of Boivin-extracted endotoxins, i.e., induction of significant pyrogenic tolerance to endotoxin in man does not require clinically demonstrable symptoms.[10] Exposure of man to such small but nevertheless tolerance-evoking quantities of endotoxin incidental to the prolonged typhoid and tularemia illness would not be unexpected. As for the vascular hyperreactivity to catecholamines observed during typhoid fever, this persisted for several weeks into the afebrile convalescent phase, was not demonstrable during the other comparably febrile illness induced by the endotoxin-containing Gram-negative microbe *Pasteurella tularensis*, and could not be reproduced in normal man by intravenous injections of toxic quantities of purified bacterial endotoxins. It was therefore emphasized that mechanisms other than endotoxemia, such as release of serotonin from the inflammed intestinal mucosa, might primarily account for this phenomenon (28). The recent demonstration that a variety of inflammatory diseases of the intestine evoke vascular hyperreactivity to catecholamines in man (29) renders this latter explanation more plausible.

The mechanisms underlying the hyperreactivity to endotoxin during typhoid fever and tularemia were further explored in the present studies. It has already been demonstrated that the phenomenon is not a nonspecific reaction to fever per se; the hyperreactivity was detectable during the afebrile incubation period and continued into the afebrile convalescent phase; moreover, volunteers febrile with the virus of Sandfly fever exhibited no detectable hyperreactivity (4). Nonspecific effects of tissue injury also do not appear responsible. The present findings demonstrate that rabbits with varying degrees of gross tissue necrosis induced by chemical irritants (turpentine and formalin) administered intraperitoneally, intramuscularly, or intrathoracically did not exhibit hyperreactive febrile responses to endotoxin; indeed, reduced responses were observed, confirming the findings of Raskova (30). Removal of natural protective circulating factors or enhancement of cellular reactivity by subfebrile levels of endotoxemia also appears an unlikely mechanism of hyperreactivity, since infusions of endotoxin for up to 40 hr at rates that just failed to elicit

[9] This latter possibility is not invalidated by the unmitigated clinical illness in volunteers rendered tolerant to the systemic effects of endotoxin. Systemic tolerance is not synonymous with local tolerance. At least one site in man, the skin, exhibits enhanced inflammatory responses to the endotoxin employed to induce systemic tolerance (25).

[10] Unpublished observations.

fever did not render rabbits more responsive to subsequent challenge with pyrogenic doses. Nor could evidence be obtained that the hyperreactive febrile responses during typhoid fever were mediated by inability of the reticuloendothelial system to clear the blood of the injected endotoxin labeled with ^{51}Cr; indeed, endotoxin blood clearance rates, accelerated as a result of induction of endotoxin tolerance before typhoid fever, remained accelerated during infection. Such findings further support the thesis that the mechanisms responsible for endotoxin tolerance continue to operate during infection. Moreover, the present endotoxin blood clearance data in man parallel those in mice infected with *Mycobacterium tuberculosis* (BCG strain) wherein hyperreactivity to *E. coli* endotoxin developed during infection, but clearance of the radioactively labeled endotoxin was accelerated (31).

An alternative hypothesis for the systemic hyperreactivity to endotoxin during typhoid fever and tularemia would be that suggested by Abernathy and Spink for the hyperreactivity to *Brucella* endotoxin during brucellosis, i.e., acquired hypersensitivity (32, 33). Important support for this hypothesis was derived from the apparent correlation between the intensity of the dermal inflammatory responses elicited by brucella skin test antigens and the intensity of the pyrogenic and toxic systemic responses to *Brucella* endotoxin (32). However, the *Brucella* endotoxin preparation employed by Abernathy and Spink possessed pyrogenic activities quite different from those of classical purified preparations.[11] As already emphasized by Beeson (2), it is therefore uncertain as to whether the observed systemic hyperreactivity during brucellosis reflects acquired hypersensitivity to the endotoxin per se or to other antigenic components in this preparation. In the present studies, significant increments in dermal inflammatory responses to highly purified *S. typhosa* endotoxin were detectable during overt typhoid fever; however, unlike brucellosis, the dermal hyperreactivity could not be correlated with the systemic hyperreactivity to the endotoxin. Thus, no dermal hyperreactivity was found to *Pseudomonas* endotoxin during typhoid fever nor to *S. typhosa* endotoxin during tularemia, although comparably hyperreactive pyrogenic and toxic responses followed the intravenous administration of these endotoxins. Moreover, whereas systemic hyperreactivity persisted for years in the subjects with brucellosis tested with the *Brucella* preparation (32, 33), systemic hyperreactivity waned rapidly during convalescence from typhoid fever and tularemia with the purified endotoxin preparations employed in the present studies (4). Alternative attempts to demonstrate increases in cytophilic antibody capable of sensitizing leukocytes to the injurious activity of bacterial endotoxin, as has been reported in the chicken during *S. gallinarum* infection (18), were also unsuccessful. It must be concluded, therefore, that the systemic hyperreactivity to bacterial endotoxins during typhoid fever and tularemia cannot be ascribed at present to enhanced levels of acquired hypersensitivity, and further studies to define the responsible mechanisms are currently in progress.

[11] The pyrogenic activity of the *Brucella* endotoxin was not demonstrable in normal subjects until dosages of 250 μg were administered intravenously (compared to less than 5 μg for *Pseudomonas* or 0.1 μg for *S. typhosa* or *E. coli* endotoxins). Moreover, the latent period before fever onset averaged more than 3 hr (even in the highly reactive group of subjects with brucellosis) compared with approximately 1 hr for the classical endotoxin preparations.

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