MECHANISMS OF ENDOTOXIN TOLERANCE

II. Relationship between Endotoxin Tolerance and Reticuloendothelial System Phagocytic Activity in Man*

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Plates 16 and 17

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Following the demonstration by Beeson that induced tolerance to the pyrogenic activity of bacterial endotoxins could be reversed by reticuloendothelial system (RES) "blockade" (1), emphasis has been placed on the importance of the rate of clearance of intravenously injected endotoxin by the RES. Most such studies have supported a positive correlation between tolerance and accelerated uptake of endotoxin by the RES.

Employing passive transfer techniques, Beeson observed that rabbits tolerant to the pyrogenic activity of typhoid vaccine cleared endotoxin more rapidly from the circulation than did normal controls. Moreover, after tolerance was "abolished" by RES blockade with thorotrast, endotoxin clearance rates declined sharply (1). Atkins and Wood (2, 3), employing similar techniques, confirmed the former findings. Howard *et al.* (4), using a variety of P^{32}-labeled endotoxin preparations in mice, found that endotoxin administered several days previously induced significant acceleration of endotoxin clearance and that RES blockade with thorotrast depressed the clearance rates. Carey *et al.* (5), employing Cr^{51}-labeled *Escherichia coli* endotoxin, demonstrated accelerated clearance of lethal quantities of endotoxin by tolerant rabbits and mice. Although increased clearance rates of sublethal quantities were also observed, this was not considered significant. However, subsequent studies by Chedid and Parant (6) and by Herring *et al.* (7), indicated that small quantities of Cr^{51}-labeled endotoxins are cleared significantly more rapidly by tolerant mice and rabbits. Koch-Weser *et al.* (8) also reported accelerated clearance of Cr^{51}-labeled *E. coli* endotoxin in tolerant rabbits, although the dosages employed were not given. Reasons for regarding such

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accelerated endotoxin clearance as a major factor in the mediation of tolerance have been extensively reviewed by Atkins (9).

The mechanisms whereby the RES of the tolerant animal clears endotoxin more rapidly from the circulation are not defined. Of especial interest is the specificity of such accelerated clearance. Recent studies in the rabbit indicate that *generalized* increases in RES phagocytic activity, as reflected by the ability to clear colloidal carbon, are not a requisite for tolerance to the pyrogenic activity of endotoxin. Moreover, plasma from tolerant donors could transfer significant pyrogenic tolerance without enhancing the ability of the RES to clear colloidal carbon. It was therefore suggested that tolerance involves the participation of opsonins which provide specificity for the accelerated clearance of endotoxin (10). The present studies in man, employing the clearance of I^{131}-labeled aggregated human serum albumin as one index of RES phagocytic activity, confirm the concept that generalized stimulation of RES phagocytic activity is not a requisite for the acquisition of high levels of tolerance to the pyrogenic activity of endotoxin. Moreover, the data support the importance of specific immunologic mechanisms in the mediation of such tolerance.

Materials and Methods

These studies were initiated as part of an overall program designed to evaluate the efficacy of typhoid vaccine in man. Volunteers for these studies were healthy male inmates of the Maryland House of Correction, Jessup, Maryland. Their ages ranged from 20 to 35. Each volunteer was fully apprised of all aspects of the investigation. Complete medical evaluation was performed to verify the fitness of each participant.

All syringes and needles were of the disposable type and were sterile and pyrogen-free. Two endotoxin preparations were employed to induce tolerance: *Salmonella typhosa* endotoxin (0-282),[1] diluted in pyrogen-free physiologic saline to 0.5 μg/ml, and *Pseudomonas* endotoxin,[2] diluted to 10 μg/ml. Both endotoxin preparations were bacteriologically sterile. The same endotoxin suspensions were employed throughout the study. Pyrogen assays of each preparation were performed in groups of acclimatized albino rabbits at two different dose levels for each endotoxin; no loss of potency was detectable during the course of these investigations. The antigenic properties of these endotoxins have been documented in previous studies (12, 13).

Pyrogen Assay in Man.—All assays were begun between 9 and 10 a.m. with the subjects confined to bed and covered with a light blanket. Flexible thermocouples were inserted into the rectum for fixed distances (6 inches) and temperatures recorded by means of a telethermometer (Yellow Springs Instrument Company, Yellow Springs, Ohio). Temperatures were monitored immediately prior to and every half hour for 7 hours following the intravenous injection of endotoxin. The resulting fever index was calculated from the area under the curve obtained by plotting the increment in temperature as a function of time on standard graph

[1] A highly purified material (11) kindly supplied by Dr. Maurice Landy, National Institutes of Health, Bethesda.

[2] Kindly supplied as Piromen® by Dr. Thomas A. Garrett, Baxter Laboratories, Morton Grove, Illinois.

paper; a fever index of 100 reflected a 1°F rise in rectal temperature sustained for 1 hour. As a result of diurnal variation, temperatures seldom returned to preinjection values. In such instances, the fever curves were extrapolated to the baseline. The subjective reactions to the endotoxins (headache, chills, myalgia, anorexia) were graded as follows: 1+, mild; 2+, moderate; 3+, severe; 4+, extremely severe.

Assay of RES Phagocytic Activity.—RES phagocytic activity was assessed with a colloidal preparation of heat-aggregated I^{131}-labeled human serum albumin. The method of preparation of this material and the evidence that its clearance in man after intravenous injection is a function of RES phagocytic activity have been detailed previously (14). Table I lists the characteristics of the aggregated albumin preparation. The distribution of the radioactivity in the liver can be seen in the scintillation scan of a normal subject following the administration of 0.025 mg/kg (Fig. 1). Fig. 2 shows the concentration of radioactivity in both the liver and spleen following a 50 mg/kg dose. Prior studies in animals have shown that the clearance of such aggregated albumin particles parallels that of colloidal carbon (15). Moreover, since the aggregated albumin is rapidly metabolized by the RES (16), repeated determinations of RES phagocytic activity can be performed with high levels of safety and reproducibility (14). Serial baseline control studies of RES phagocytic activity were obtained prior to endotoxin

TABLE I

Characteristics of I^{131}-Labeled Aggregated Human Serum Albumin

1. An aggregate of approximately 10 albumin molecules.
2. Distributed in a manner characteristic of RES phagocytized particles; trace doses concentrated in the liver, large doses in both liver and spleen; virtually no uptake by circulating buffy coat.
3. Rapidly metabolized after phagocytosis.
4. Not antigenic to man.
5. Not associated with excessive radiation.

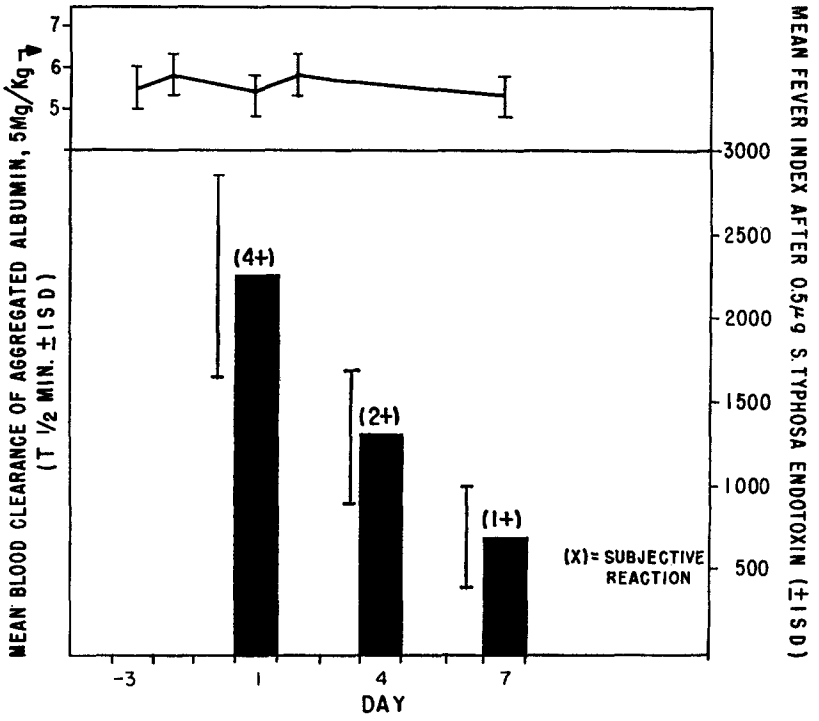
administration. For this purpose, 5 mg/kg of I^{131}-labeled aggregated albumin was injected intravenously, and the time required for plasma clearance ($T\frac{1}{2}$) was calculated by methods previously outlined (14). This value was employed as one index of RES phagocytic activity; increases reflect impaired phagocytosis of aggregated albumin whereas decreases reflect enhancement of phagocytosis. The $T\frac{1}{2}$ of the 5 mg/kg dose of the aggregated albumin was determined serially as tolerance to endotoxin pyrogenicity was established. Periodically, the phagocytic *capacity* of the RES for I^{131}-labeled aggregated albumin (V_{max}) was determined by measuring the clearance of both 0.03 and 5 mg/kg doses. For this purpose, the plasma clearance ($T\frac{1}{2}$) of 0.03 mg/kg and 5 mg/kg doses of I^{131}-labeled aggregated albumin were measured by methods outlined previously (14). The phagocytic capacity of the RES (V_{max}) was then calculated using the equation (see reference 25)

$$V_{max}\ (\text{mg/kg/min.}) = \frac{39.8}{12.0\ T'\frac{1}{2} - 11.5\ T''\frac{1}{2}}$$

where $T'\frac{1}{2}$ equals the halftime clearance (minutes) of the 5 mg/kg dose and $T''\frac{1}{2}$ equals that of the 0.03 mg/kg dose (25). In the studies with *S. typhosa* endotoxin, the clearance of aggregated albumin was always measured approximately 1 hour prior to the injection of endotoxin. In the *Pseudomonas* endotoxin study, the same relation was observed up to day 8. Thereafter, no endotoxin was administered on the day of the clearance determination. All subjects were afebrile and asymptomatic at the time each clearance study was performed.

RESULTS

Effect of Constant Daily Doses of Endotoxin on RES Phagocytic Activity.— Five normal subjects were given daily intravenous injections of 0.5 μg *S. typhosa* endotoxin for 7 days. The fever indices, toxic reactions, and plasma clearance rates of I^{131}-labeled aggregated albumin are shown in Text-fig. 1.

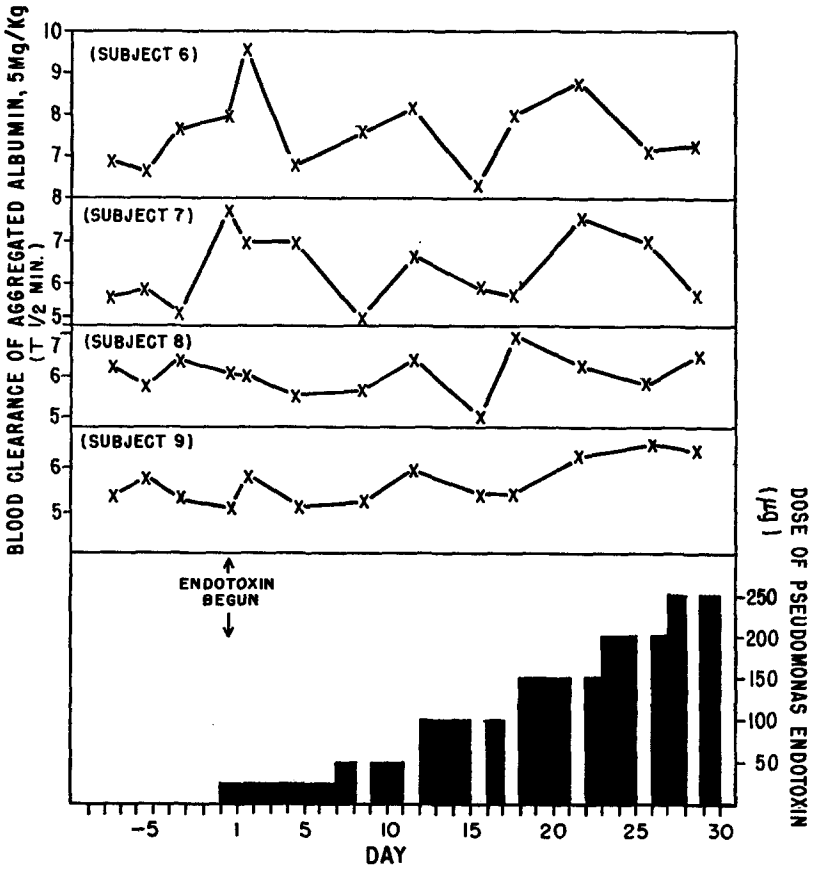


TEXT-FIG. 1. RES phagocytic activity in 5 normal subjects during the acquisition of tolerance to daily intravenous injections of 0.5 μg of *S. typhosa* endotoxin. The time (minutes) required for 50 per cent clearance of 5 mg/kg of I^{131}-labeled aggregated human serum albumin ($T\frac{1}{2}$) is employed as the index of RES phagocytic activity. ISD, 1 standard deviation.

Marked tolerance was established to the pyrogenic and toxic effects of the endotoxin in each subject. The ability of the RES to clear the aggregated albumin, as reflected by the $T\frac{1}{2}$ value, remained unaltered.

Effect of Progressive Increments in Dose of Endotoxin on RES Phagocytic Activity.—Four subjects were rendered tolerant to *Pseudomonas* endotoxin by daily intravenous injections of progressively larger quantities. The dose of endotoxin was increased when the toxic subjective reactions to the preceding injection reached a minimal level. The dosage schedule required to achieve such tolerance, and the concomitant plasma clearance rates of I^{131}-labeled

aggregated albumin for each subject are given in Text-fig. 2. Marked tolerance was established; the fever indices and subjective reactions in response to 25 μg *Pseudomonas* endotoxin on day 1 were greater than that following 250 μg on day 30 in each subject; the mean fever indices were 1049 and 798, and the mean



TEXT-FIG. 2. RES phagocytic activity, measured by clearance of I^{131}-labled aggregated human serum albumin, in four normal subjects during the acquisition of tolerance to daily intravenous injections of increasing quantities of *Pseudomonas* endotoxin. The mean fever index and subjective reactions to 25 μg of the endotoxin on day 1 were 1049 and 2+ respectively; the reactions to 250 μg on day 30 were 798 and 0.5+ respectively.

subjective reactions were 2+ and 0.5+, respectively. Despite this acquired tolerance, no increase in RES phagocytic activity for I^{181}-labeled aggregated albumin was demonstrable in any subject. To exclude the possibility that decreased RES blood flow occurred which masked increases in generalized RES phagocytic capacity at the cellular level, serial clearance studies were

performed in each subject employing trace quantities (0.03 mg/kg) of the I^{131}-labeled aggregated albumin. Alterations in the clearance of such trace quantities of colloid would primarily reflect alterations in RES blood flow (15). Moreover, since the clearance of the trace quantity and of the 5 mg/kg dose of aggregated albumin was measured, the maximum phagocytic capacity (V_{max}) of the RES could be calculated for this colloid (14). No evidence of decreased

TABLE II

Relationship between Tolerance Induced by Daily Intravenous Administration of Pseudomonas Endotoxin and Maximum Phagocytic Capacity of the RES (V_{max}) for I^{131}-Labeled Aggregated Human Serum Albumin

Subject	Control	Prior endotoxin injections*	
		8	17
6	0.77‡ (2.8) (7.0)	0.53 (2.0) (8.2)	—
7	0.95 (2.3) (5.7)	0.74 (2.3) (6.7)	0.90 (2.1) (5.7)
8	0.92 (2.8) (6.3)	0.77 (2.2) (6.4)	0.71 (2.4) (7.0)
9	1.07 (2.3) (5.3)	0.85 (2.1) (5.9)	0.88 (1.6) (5.3)
Mean ± SE	0.93 ± 0.06 (2.6 ± 0.15) (6.1 ± 0.15)	0.72 ± 0.07 (2.2 ± 0.07) (6.5 ± 0.15)	0.83 ± 0.05 (2.0 ± 0.25) (6.0 ± 0.18)

* See Text-fig. 2 for dose schedule of *Pseudomonas* endotoxin.

‡ Maximal phagocytic capacity of the RES for I^{131}-labeled aggregated albumin (mg/kg/min.) as calculated from the $T_{1/2}$ clearance values of 0.03 mg/kg (upper parenthesis) and 5 mg/kg (lower parenthesis), measured 20 minutes later.

RES perfusion or increase in maximum RES phagocytic capacity was observed as tolerance was acquired (Table II).

The preceding studies indicate that generalized increases in RES phagocytic capacity in man are not a requisite for the acquisition of marked tolerance to endotoxin pyrogenicity. Further observations support the concept that pyrogenic tolerance in man involves more specific mechanisms.

Pattern of Acquisition of Endotoxin Tolerance: Reaction to the Second Injection.—When a fixed quantity of endotoxin is repeatedly administered intra-

venously to man, tolerance rapidly develops to its pyrogenic and toxic activities (17). Certain aspects of the pattern of such tolerance have not been emphasized previously. Thus, when healthy volunteers were challenged at daily intervals with a constant dose of the *S. typhosa* or *Pseudomonas* endotoxin, the

TABLE III

Pyrogenic and Symptomatic Reactions to a Fixed Dose of Bacterial Endotoxin on Days 1 and 2

Subject	Day 1		Day 2		Change in fever index
	Fever index	Subjective reaction	Fever index	Subjective reaction	
Salmonella typhosa endotoxin (0.125 μg)					
					per cent
10	1250	0	1640	3+	+31
11	1160	1+	1580	3+	+36
12	450	1+	1400	3+	+211
13	240	0	1300	3+	+422
14	1086	2+	1248	3+	+15
15	127	0	366	1+	+188
16	1092	2+	951	3+	−13
17	376	2+	438	3+	+16
18	442	0	464	0	+5
19	637	1+	400	0	−37
20	716	0	727	1+	+2
21	645	1+	413	1+	−37
Mean	685	0.8+	911	2+	+70
Pseudomonas endotoxin (10 μg)					
22	652	1+	780	2+	+20
23	721	0	1012	2+	+40
24	510	1+	500	1+	−2
25	560	1+	640	1+	+14
		(25 μg)			
26	1607	3+	1780	4+	+11
27	853	1+	870	1+	+2
28	685	1+	770	1+	+13
29	1052	2+	731	2+	−30
Mean	830	1.2+	885	1.8+	+9

pyrogenic and toxic reactions associated with the 2nd day challenge were frequently greater than the initial reactions (Table III). Such increments in reactivity on day 2 were highly variable from one subject to the next and bore no apparent relation to their reactivity on day 1. This enhanced reaction to endotoxin on the 2nd day could not be attributed to a generalized toxic im-

pairment of RES phagocytic function; the ability of the RES of control subjects to clear aggregated albumin on day 2 remained within the range seen during subsequent tolerance (Text-figs. 1 and 2). In contrast, if the second injection of endotoxin was delayed for 1 week, tolerance rather than enhanced reactivity was apparent (Table IV).

Passive Transfer of Tolerance.—The frequent occurrence of exaggerated day 2 reactions with the endotoxin preparations employed in these studies permitted evaluation of the hypothesis (10, 18) that pyrogenic tolerance involves the participation of antibody. Pyrogenic reactivity was monitored on day 1, and the effect of tolerant plasma on the 2nd day response was then determined. Eleven healthy volunteers were given an initial intravenous injection of 10

TABLE IV

Pyrogenic and Symptomatic Reactions to a Fixed Dose of Bacterial Endotoxin (0.5 μg S. typhosa Endotoxin) on Days 1 and 7

Subject	Day 1		Day 7		Change in fever index
	Fever index	Subjective reaction	Fever index	Subjective reaction	
					per cent
30	1206	2+	1035	1+	−14
31	913	2+	769	1+	−16
32	814	1+	771	1+	−5
33	650	1+	692	1+	+6
34	948	2+	317	0	−67
35	1127	2+	960	1+	−15
36	1402	3+	927	2+	−34
Mean.....	1009	1.9+	781	1+	−21

μg *Pseudomonas* endotoxin. Seven hours later, when the pyrogenic and toxic reactions subsided, five subjects (mean weight 60 kg) were each infused intravenously with 400 to 500 ml of fresh citrated pyrogen-free plasma pooled from two to three normal donors. The six other subjects (mean weight 58 kg) each received similar volumes of pooled fresh citrated plasma obtained from tolerant donors drawn 24 hours after the seventh daily intravenous injection of 0.125 μg *S. typhosa* endotoxin; this quantity of *S. typhosa* endotoxin possessed pyrogenic potency comparable to the 10 μg *Pseudomonas* endotoxin. In one subject, the tolerant plasma evoked a shaking chill and a febrile reaction beginning 1 hour after infusion; the fever peaked at 3 hours and returned to baseline by 7 hours. The pyrogen was not identified. The following morning all subjects were afebrile and were again given 10 μg of *Pseudomonas* endotoxin intravenously. The results are shown in Table V. The pyrogenic reaction following the second injection of *Pseudomonas* endotoxin was increased a mean of 23 per cent in

the subjects receiving normal plasma, and the toxic reactions increased from a mean of 1.0 + to 1.8+. Thus, normal homologous citrated plasma did not inhibit the exaggerated 2nd day reaction. In contrast, a 25 per cent reduction

TABLE V

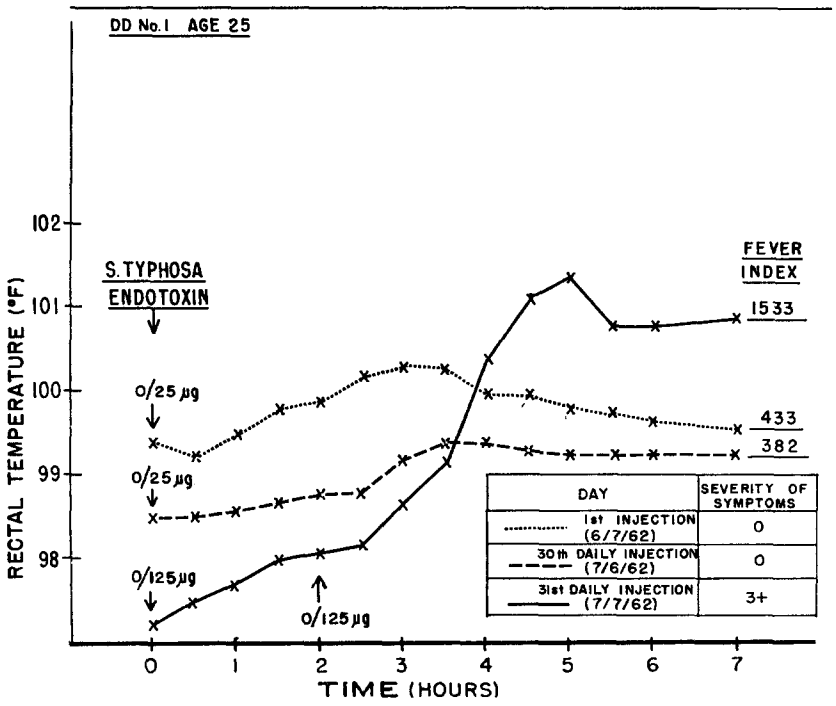
*Effect of Passive Transfer of Normal and Tolerant Plasma on Responsiveness to 10 μg Pseudomonas Endotoxin**

Subject	Day 1		Day 2		Change in fever index
	Fever index	Subjective reaction	Fever index	Subjective reaction	
Recipients of normal plasma					
					per cent
37	1063	1+	1038	2+	−2
38	882	1+	1202	2+	+36
39	465	0	552	0	+19
40	995	2+	1080	3+	+10
41	570	1+	875	2+	+54
Mean.....	795	1+	950	1.8+	+23‡ (SE ± 9.8)
Recipients of tolerant plasma					
42	1114	3+	834	2+	−25
43	1005	2+	687	1+	−32
44	581	1+	434	0	−25
45	813	2+	719	1+	−12
46	765	2+	513	0	−33
Mean.....	856	2+	637	0.8+	−25‡ (SE ± 3.9)
Recipient of tolerant plasma + pyrogen					
47	811	2+	1480	3+	+81

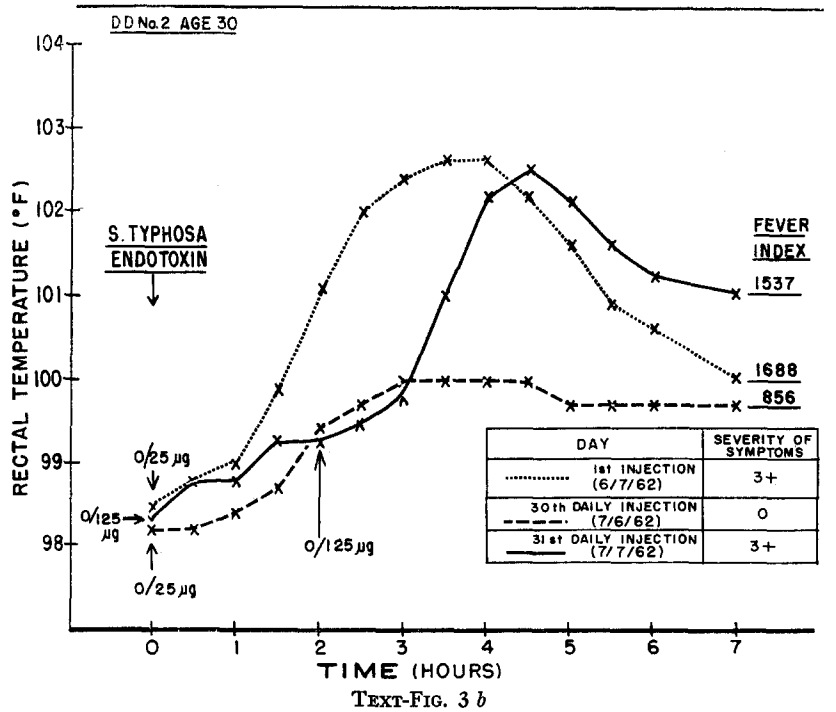
* Baseline responses to the *Pseudomonas* endotoxin were obtained on day 1, and 400 to 500 ml of fresh citrated plasma was infused immediately after these control studies. Testing with the *Pseudomonas* endotoxin was then repeated on day 2. Plasma from tolerant donors was obtained 24 hours after the 7th daily intravenous injection of 0.125 μg *S. typhosa* endotoxin.

‡ $P < 0.005$.

occurred in the pyrogenic reaction to the second injection of *Pseudomonas* endotoxin in the subjects given plasma from donors tolerant to *S. typhosa* endotoxin. This inhibition of fever by the plasma from tolerant donors was highly significant ($p < 0.005$); in addition, the toxic subjective reactions were also



TEXT-FIG. 3 *a*

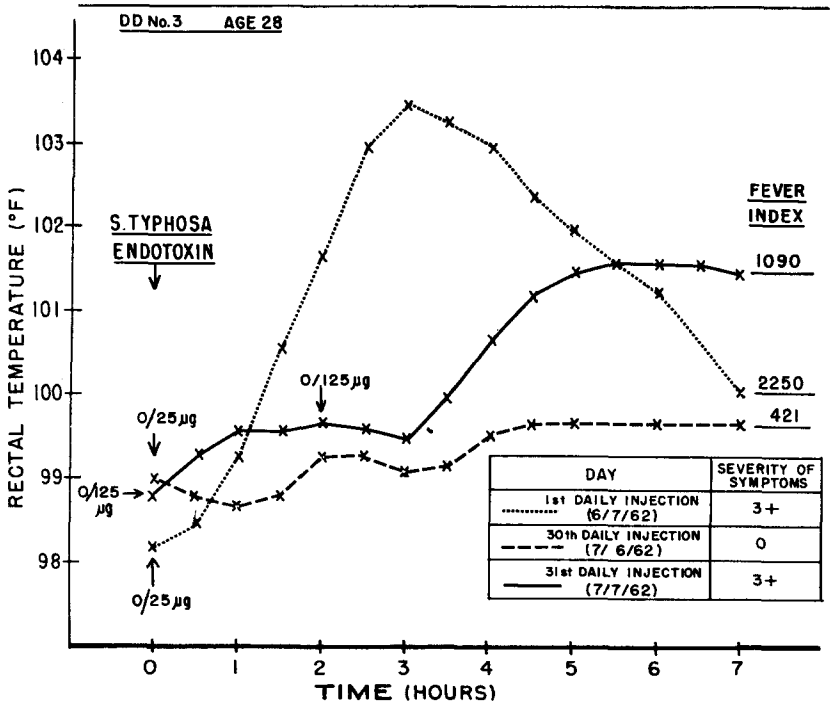


TEXT-FIG. 3 *b*

TEXT-FIGS. 3 *a*, 3 *b*, and 3 *c*. Patterns of tolerance reversal produced by dividing the endotoxin dose. Tolerance was induced by 30 daily intravenous injections of 0.25 µg *S. typhosa* endotoxin; on day 31 half the dose was given and the second half dose was injected 2 hours later. DD, divided dose.

mitigated. The one subject that received tolerant plasma with pyrogen was not protected on day 2 (Table V).

Reversal of Tolerance with Divided Doses of Endotoxin.—To further test the concept that tolerance involves the participation of antibody, ten normal subjects were rendered highly tolerant by 30 daily intravenous injections of 0.25 μg of *S. typhosa* endotoxin. On day 31, half the dose of endotoxin was administered and 2 hours later the remainder. If antibody were concerned with tolerance, the initial injection should reduce the titer and the second portion might now be free to induce toxic reactions (Danysz phenomenon). Text-figs. 3 *a*, 3 *b*, and 3 *c* represent the various patterns found in nine of ten subjects. Tolerance was generally reversed by this technique. In the subject depicted in Text-fig. 3 *a*, the reaction to the divided dose on day 31 was actually greater than to the initial single dose. To verify that such reversal of tolerance was not based upon general depression of RES phagocytic activity, studies were performed in six control subjects. No depression of RES phagocytic activity, measured by I^{131}-labeled aggregated albumin clearances, was detectable 2 hours after the initial injection of endotoxin. Thus the mean control clearance of 5 mg/kg I^{131}-labeled aggregated albumin ($T\frac{1}{2}$) was 5.6 (SE ±0.20) and 5.8 (SE ±0.30) minutes on days 3 and 2 respectively prior to endotoxin; 2 hours



TEXT-FIG. 3 *c*

TABLE VI

Reversal of Tolerance by Substitution of a Comparably Pyrogenic Quantity of a Heterologous Bacterial Endotoxin

Subject	Day	Endotoxin*		Fever index	Subjective reaction
			μg		
24	1	P	10	510	1+
	2		10	500	1+
	3		20	438	0
	4		50	608	0
	5		100	675	0
	6		100	610	0
	7		200	837	1+
	8		200	677	0
	9	S	2.5	320	0
	10	P	200	1440	4+
25	1	P	10	560	1+
	2		10	640	1+
	3		20	461	0
	4		50	608	0
	5		100	838	0
	6		100	765	0
	7		200	1021	1+
	8		200	493	0
	9	S	2.5	339	0
	10	P	200	1177	4+
48	1	P	10	1010	2+
	2		10	—	
	3		10	480	0
	4		10	485	0
	5	S	0.125	300	0
	6	P	10	720	1+
49	1	P	10	1120	3+
	2		10	—	
	3		10	770	0
	4		10	450	0
	5	S	0.125	310	0
	6	P	10	1030	3+
50	1	P	10	590	1+
	2		10	—	
	3		10	680	2+
	4		10	360	0
	5	S	0.125	280	0
	6	P	10	690	2+

*P, *Pseudomonas* endotoxin; S, *Salmonella typhosa* endotoxin.

TABLE VI—*Concluded*

Subject	Day	Endotoxin*		Fever index	Subjective reaction
			μg		
51	1	P	10	473	0
	2		40	1024	2+
	3		50	780	0
	4		200	974	0
	5, 6		—		
	7	S	1.0	1591	3+
	8	S	0.5	2172	4+
52	1	P	10	327	0
	2		40	863	2+
	3		50	80	0
	4		200	311	0
	5, 6		—		
	7	S	1.0	838	2+
	8	S	0.5	1698	4+

after an initial intravenous injection of 0.5 μg *S. typhosa* endotoxin, the T½ was 5.4 (SE ±0.16) minutes. The reversal of tolerance by the divided dose also could not be attributed to alterations in baseline temperatures. Although some rise following the initial half dose of endotoxin is seen in these subjects, other subjects with acquired tolerance during convalescence from typhoid fever (to be reported subsequently) exhibited reversals of tolerance without the slightest rise in baseline temperature after the initial half dose of endotoxin.

Specificity of Endotoxin Tolerance.—Seven subjects were rendered tolerant to *Pseudomonas* endotoxin by single daily intravenous injections for 4 to 8 days. Each subject was then given *S. typhosa* endotoxin in a dose comparably pyrogenic to that of the preceding dose of *Pseudomonas* endotoxin. As expected from the cross-tolerance known to occur in man (17), each subject exhibited tolerance to this heterologous endotoxin. However, upon testing on the following day with either the original or the heterologous endotoxin, tolerance was definitely impaired (Table VI). This ability of the heterologous *S. typhosa* endotoxin to reverse tolerance is highly significant when compared with the consistent absence of such enhanced reactions in groups of control subjects given comparable series of injections of the homologous endotoxin only ($p < 0.001$). Similar attempts were made to reverse tolerance in five other subjects rendered tolerant to 0.25 μg *S. typhosa* endotoxin for 25 days. A comparably pyrogenic quantity of *Pseudomonas* endotoxin (20 μg) was administered on day 26, and testing was resumed with the *S. typhosa* endotoxin on day 27. With this procedure, tolerance could not be impaired in any subject. The mean fever index on day 25 following the *S. typhosa* endotoxin was 320 (SE ±35), the

response to the *Pseudomonas* endotoxin on day 26 was 350 (SE ±63), and the reaction to the *S. typhosa* endotoxin on day 27 was 312 (SE ±46).

Dermal Reactivity to Endotoxin.—The intradermal injection of varying quantities of *S. typhosa* endotoxin into fifteen normal volunteers consistently resulted in a gross inflammatory reaction. The response became visible after

TABLE VII

*Effect of Induced Tolerance to S. Typhosa Endotoxin on Dermal Reactivity to this Endotoxin**

0.1 ml intradermal *S. typhosa* endotoxin	Time after intradermal inoculation					
	4 hrs.		15 to 24 hrs.		36 to 48 hrs.	
	Control	Tolerant	Control	Tolerant	Control	Tolerant
			Subject 53			
μg						
0.005	100 (1+)‡	81 (1+)	484 (3+) I	400 (3+) I	900 (2+) I	900 (2+) I
0.0005	25 (±)	36 (1+)	36 (2+) I	400 (3+) I	100 (1+) I	525 (1+) I
0.00005	4 (±)	4 (1+)	9 (1+)	400 (3+) I	36 (1+) I	900 (1+) I
Saline	0	0	0	0	0	0
			Subject 54			
0.005	225 (1+)	144 (1+)	225 (2+) I	225 (3+) I	100 (1+) I	100 (2+)I
0.0005	169 (1+)	25 (1+)	25 (1+) I	100 (3+) I	25 (1+) I	—
0.00005	9 (±)	25 (1+)	9 (±)	64 (3+) I	4 (±)	25 (2+) I
Saline	0	0	0	0	0	0

* Tolerance induced by 13 daily intravenous injections of increasing dosages of *S. typhosa* endotoxin and dermal testing performed on day −1 (control) and day 14. The fever index following 0.5 μg endotoxin on day 1 was 1860 and 1680 in subjects 53 and 54 respectively; on day 13 the response to 2.0 μg endotoxin was 200 and 220 respectively.

‡ The first figure represents the area index of the inflammatory reaction obtained by multiplying the largest cross diameters (mm). The values in parenthesis indicate the gross intensity of the erythematous reaction: ±, minimal; 1+, mild; 2+, moderate; 3+, marked. I, induration.

1 hour and generally attained maximum size by approximately 5 hours, although in three subjects the maximum reaction was not seen until approximately 15 hours. This sequence of events is comparable to that reported with *Escherichia coli* endotoxin (19). The effect of induced tolerance on the dermal inflammatory response is illustrated in Table VII. Marked tolerance to the pyrogenic activity of intravenously administered endotoxin was not associated with impairment of the local inflammatory response. On the contrary, enhanced

local reactivity developed which was especially evident upon testing with the smaller doses of endotoxin.

DISCUSSION

Whenever bacterial endotoxins are administered intravenously at daily intervals, tolerance is acquired rapidly to their toxic and pyrogenic effects. While the mechanism for this tolerance in man is unknown, in the experimental animal it has been presumed to be mediated by enhanced ability of the RES to clear injected endotoxin from the circulation (see introductory statements). Several lines of evidence have supported the concept that the enhanced rate of endotoxin clearance, and hence tolerance, could be attributed to a non-specific stimulation of RES phagocytic activity: (*a*) Tolerant animals also clear various inert colloidal particles at enhanced rates (10, 20); (*b*) RES blockade with a variety of inert colloids appeared to abolish tolerance (1); (*c*) Immunologic mechanisms did not appear responsible since tolerance extended to endotoxins derived from heterologous bacterial species (17, 21), could not be readily transferred with serum (21–23), and lapsed after cessation of endotoxin administration despite elevated specific O agglutinin titers (17, 21). In more recent studies in which transfer of significant tolerance was accomplished with serum, it was correlated with an increased ability of the RES to clear colloidal carbon (24). In contrast, the present observations in man indicate that tolerance is not dependent upon generalized increases in RES phagocytic activity. Despite the induction of high levels of pyrogenic tolerance to constant doses of *S. typhosa* endotoxin or to progressively larger quantities of *Pseudomonas* endotoxin, no overt increases were detectable in the ability of the RES to clear an unrelated colloidal material, I^{131}-labeled heat-aggregated human serum albumin. The rate of uptake of such colloidal particles provides one index of non-specific or generalized RES phagocytic activity since the colloidal particles are immunologically inert in man and the kinetics of their clearance parallel those of other inert colloids such as carbon (14, 15). The failure to demonstrate accelerated clearance of aggregated albumin could not be attributed to inability of this material to detect alterations of RES phagocytic activity in man; significant enhancement and depression of its clearance have been observed during various infectious illnesses (25). Moreover, studies with trace quantities of the I^{131}-labeled aggregated albumin, in conjunction with larger quantities, permitted precise calculation of the maximum phagocytic capacity of the RES for this colloid (25); no increases were found during the acquisition of pyrogenic tolerance. This precludes the possibility that decreased RES blood flow during tolerance masked a generalized increase in RES phagocytic capacity at the cellular level.

The absence of generalized increases in RES phagocytic activity in man contrasts with that observed in rabbits rendered comparably tolerant to the

pyrogenic activity of endotoxin (10). It seems likely that this discrepancy stems in part from the relatively greater responsiveness of man to the pyrogenic effects of endotoxin.[3] Therefore, during the administration of comparably pyrogenic quantities of endotoxin, considerably lower doses of endotoxin in terms of mg/kg are given to man. It would be desirable to confirm the concept that quantities of endotoxin of a magnitude (mg/kg) that stimulate the generalized phagocytic activity of the RES in animal species would also do so in man, but such studies cannot be safely undertaken.

Certain other observations relate to the mechanism of endotoxin tolerance in man. In contrast to the development of tolerance in the rabbit, the pyrogenic and toxic reactions were enhanced in some subjects when the same quantity of endotoxin was administered 24 hours after the initial dose. Although the nature of the endotoxin preparation may influence its occurrence,—exaggerated day 2 reactions were not reported with *S. abortus equi* (pyrexal) (27),—it is unlikely that this hyperreactivity was peculiar to the endotoxin preparations employed in the present studies; exaggerated day 2 pyrogenic reactions in man have been documented graphically with other endotoxin-containing preparations (28–30). This hyperreactivity on day 2 bore no apparent relation to the intensity of the preceding initial reaction. It could not be attributed to a generalized toxic depression of RES phagocytic activity since the clearance rates of I^{131}-labeled aggregated albumin at this time consistently remained within the range seen during subsequent tolerance. An alternative hypothesis for the hyperreactive day 2 response and its relationship to endotoxin tolerance in man may be inferred from the available data:

The toxic activities of endotoxin depend, in part at least, upon acquired hypersensitivity of the host. This has been emphasized by Stetson (31) and is consistent with the observations of Schaedler and Dubos (32), and Watson and Kim (18). These latter investigators recently presented evidence supporting the concept that pyrogenic tolerance in the rabbit is based upon the induction of protective antibodies capable of assisting the RES in clearance and destruction of endotoxin; this presumably reduces the pyrogen response by preventing interaction of the endotoxin with more vulnerable sensitized cells (18). It is equally conceivable that the protective antibody acts by competition with other antibodies which form more toxic complexes with endotoxin (33). The findings in man are consistent with these theses. Thus it seems reasonable that the pyrogenic reaction following intravenous administration of endotoxin to man

[3] For example, 0.5 μg of an *S. typhosa* endotoxin preparation produced moderate to severe toxic reactions and a mean fever index of 920 in 23 normal volunteers weighing an average of 68 kg. This represents 0.007 μg/kg of endotoxin. In contrast, extrapolation from dose-response data obtained in three groups of normal rabbits (five animals per group) indicated that 0.7 μg/kg of this same endotoxin would be required to produce comparable fever indices in normal rabbits weighing 1 kg. Such enhanced responsiveness of man as compared with rabbits to dosages of endotoxin exceeding minimum pyrogenic levels has been documented previously (26).

may also be mediated in part by hypersensitivity reactions.[4] The severity of the pyrogenic reaction following an initial injection of endotoxin would depend upon the degree of hypersensitivity in relation to the titer of protective antibody. Following this initial injection, the protective antibody titer may fall. Significant reductions of specific antibody titers after an initial intravenous injection of endotoxin have been demonstrated in mice (38). Repletion of protective antibody may be incomplete by 24 hours, and hyperreactivity now ensues. Since the rate of antibody repletion would be influenced by prior immunologic experience of the host and by antigenic potency of the endotoxin, variability of the hyperreactive response might be anticipated between individuals and endotoxin preparations. In contrast, if the second injection of endotoxin be delayed for 1 week, sufficient time is permitted for augmented titers of protective antibody, and tolerance rather than hyperreactivity is now evident.

As endotoxin is administered repeatedly to man, the rate of protective antibody production is stimulated progressively, and augmented titers develop within 24 hours. When endotoxin is injected at such intervals, the resulting antigen-antibody aggregates are cleared by the RES at accelerated rates, thus reducing the formation of more toxic antibody-antigen complexes and/or protecting more vulnerable sensitized host cells against injury. This is supported by current studies in ten volunteers which indicate significant acceleration of clearance of Cr^{51}-labeled *Pseudomonas* endotoxin in association with the acquisition of pyrogenic tolerance (70). The protection observed by passive transfer of plasma drawn 24 hours after repeated daily injections of endotoxin is also consistent with this thesis. Moreover, if half the dose of endotoxin is given after tolerance has been established by daily intravenous injections, and 2 hours later the second half is administered, tolerance may be reversed. This phenomenon parallels the effect described with two closely spaced full doses of endotoxin (39–41) and indicates that tolerance is not based upon desensitization of sensitized cells or exhaustion of factors required for pyrogenic reactivity. Although the mechanism for this response is unknown, it may be related in part to transient depletion of the protective antibody following the initial dose of endotoxin; indeed, slowing of clearance of Cr^{51}-labeled *Pseudomonas* endotoxin has been observed in association with the hyperreactive response at 2 hours (70). Certainly, the reversal of tolerance by the second half dose cannot be attributed simply to shifts in baseline temperature or to generalized RES depression induced by the first dose.

One additional series of observations is consonant with the immunologic interpretation of tolerance. When after 4 to 8 days of daily intravenous injections of *Pseudomonas* endotoxin, a comparably pyrogenic dose of *S. typhosa* endotoxin was injected, cross-tolerance to this heterologous preparation was observed. As suggested by the present passive transfer studies and by observations in animals (10, 18, 42), such tolerance

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[4] The inflammatory and indurative responses in normal man following intradermal inoculation with *E. coli* endotoxin (19) and with *S. typhosa* endotoxin are compatible with a delayed hypersensitivity reaction accelerated by the presence of circulating antibody (34). Moreover, febrile responses comparable to those elicited by endotoxin can be produced in man during systemic hypersensitivity reactions to tuberculin (35) and during immune hemolysis (36). Finally, *Brucella* endotoxin induces little fever in normal man, whereas once sensitized with brucellosis, tremendously enhanced reactivity develops (37).

can be explained by the ability of protective antibody to cross-react with heterologous endotoxins. However, on the following day, tolerance was significantly impaired either to the original or to the heterologous endotoxin. While the mechanism for this effect is currently under study, it may reflect depletion of cross-reactive protective antibody by the heterologous endotoxin, associated with inadequate repletion of such antibody within 24 hours. In any case, the findings point to the high specificity of tolerance. Certainly, if pyrogenic tolerance were based simply upon non-specific RES stimulation by endotoxin, it should make little difference whether the homologous or heterologous endotoxin were injected daily provided comparably pyrogenic doses were administered. It should be emphasized that the conditions employed to demonstrate impairment of tolerance by heterologous endotoxins are critical. After several weeks of daily intravenous injections of *S. typhosa* endotoxin, a comparably pyrogenic quantity of *Pseudomonas* endotoxin consistently failed to reverse tolerance 24 hours later.

The observation that the dermal inflammatory response to endotoxin is not mitigated during tolerance lends additional support to the concept that pyrogenic tolerance is not based upon exhaustion of host reactivity. Rather such data are consistent with the concept that the specific antibody which participates in tolerance may actually intensify cutaneous responsiveness to this material (34).

The present evidence that tolerance to bacterial endotoxins in man is mediated by specific mechanisms rather than by generalized stimulation of RES phagocytic activity is in accord with other clinical and experimental observations. Thus Westphal observed that tolerance is difficult to induce in man when highly purified preparations are employed (43). These findings, together with the observation that heat-inactivated endotoxin retains both antigenicity and the ability to induce tolerance (42), suggest that antigenicity, not toxicity, is the major determinant of pyrogenic tolerance in man. Similar conclusions were reached in the mouse and rabbit employing chemically inactivated endotoxin (44). Other studies utilizing fatty acids (45), zymosan (46), graft *versus* host reactions (47), and infection with *Mycobacterium tuberculosis* (48) indicate that generalized increases in RES phagocytic activity, as reflected by enhanced ability to clear colloidal carbon, do not *per se* confer tolerance to endotoxin. Indeed, despite increases in generalized RES phagocytic activity in man during typhoid fever and tularemia, tolerance is not observed; rather preinduced tolerance is completely suppressed (49). Conversely, depression of generalized RES phagocytic activity in man during sandfly fever infection does not impair tolerance (49). Finally, depression of generalized RES phagocytic activity in the rabbit by thorotrast does not abolish tolerance; the persistence of tolerance can be attributed to circulating opsonins capable of cross-reacting with endotoxins from heterologous bacterial species (10). The temporal correlation between the appearance and disappearance of pyrogenic tolerance and circulating protective factors (50–52) supports this hypothesis.

An immunologic basis of tolerance in man is also in accord with observations

in animals by Boivin and Mesrobeanu (53–55), Zahl and Hutner (56), Dubos and Schaedler (57), Mergenhagen and Jensen (58), and Wharton and Creech (42). The initial four groups of investigators showed that tolerance was most marked against the specific endotoxin preparation employed to induce the tolerant state. The latter investigators demonstrated that tolerance could be transferred with the gamma globulin fraction of serum from tolerant donors. Such passive protection was dependent upon the globulin concentrations transferred in relation to the quantity of endotoxin administered, and was maximum for homologous endotoxins; only with higher concentrations of gamma globulin could cross-protection against heterologous endotoxins be demonstrated. Moreover, using single injections of endotoxin and passive transfer techniques, protective humoral factors could be dissociated from O agglutinating antibody; the titer of the former increased and decreased more rapidly than the latter. It was postulated that endotoxin contains two separate antigenic factors, one the toxic factor which evoked protective antibody, the other responsible for eliciting non-protective O-agglutinating antibody. The appearance of a variety of antibodies in response to endotoxin administration, including an early occurring non-precipitating type, has been verified (34, 59, 60) and Braude *et al.* (61) recently demonstrated the ability of non-precipitating antibody to inhibit endotoxin pyrogenicity. These observations probably account for the lapse of tolerance in man and rabbits after discontinuing administration of endotoxin despite elevated specific O agglutinin titers (17, 21). Current evidence indicates that macroglobulin (19S) antibody meets the requirements for the protective antibody since it characterizes the response to particulate antigens with high carbohydrate content and appears and disappears more rapidly than 7S globulin antibodies (62, 63). Moreover, 19S antibody can be produced in subjects with hypogammaglobulinemia (64) and could contribute to the reported ability of such persons to acquire tolerance (30).

While the present studies in man support the concept that pyrogenic tolerance is mediated by specific antibody acting to facilitate the uptake and destruction of endotoxin by the RES, it is not meant to imply that antibody induction constitutes the sole alteration responsible for tolerance. There is now evidence that the RES of man, as in animals, consists of heterogeneous cell populations with highly varied preferences for the phagocytosis of particulate and colloidal substances (65–69). The importance of selective stimulation of those cellular components of the RES which preferentially phagocytize or inactivate endotoxin during tolerance remains to be defined.

SUMMARY

Healthy male volunteers were rendered tolerant to the pyrogenic and toxic activities of bacterial endotoxin by daily intravenous injections. Five subjects were given 0.5 μg *Salmonella typhosa* endotoxin for 7 days; four subjects were

given *Pseudomonas* endotoxin, increasing over a period of 30 days from 25 to 250 μg. Reticuloendothelial system (RES) phagocytic activity was assessed by serial measurements of the clearance of I^{131}-labeled aggregated human serum albumin. In no subject was an increase in RES phagocytic activity detectable. Such negative findings could not be attributed to decreased RES blood flow. —Additional studies on the pyrogenic responses of man to various schedules of endotoxin administration revealed: (*a*) Hyperreactivity of some subjects to a second injection of endotoxin administered 24 hours after the initial dose; (*b*) prevention of such hyperreactivity by plasma from donors tolerant to a heterologous endotoxin, but not from normal donors; (*c*) reduced reactivity to a second injection of endotoxin given 7 days after the initial dose; (*d*) reversal of induced tolerance by administration of half the dose of endotoxin followed 2 hours later by the second half; (*e*) reversal of induced tolerance 24 hours after administration of a heterologous endotoxin; (*f*) enhanced dermal reactivity to endotoxin induced inflammation during tolerance.

The observations are consistent with the hypothesis that tolerance to the pyrogenic activity of endotoxin in man is not based upon generalized enhancement of RES phagocytic activity or exhaustion of host reactivity but rather involves the participation of specific antibody which assists the RES in the clearance and inactivation of the endotoxin molecule.

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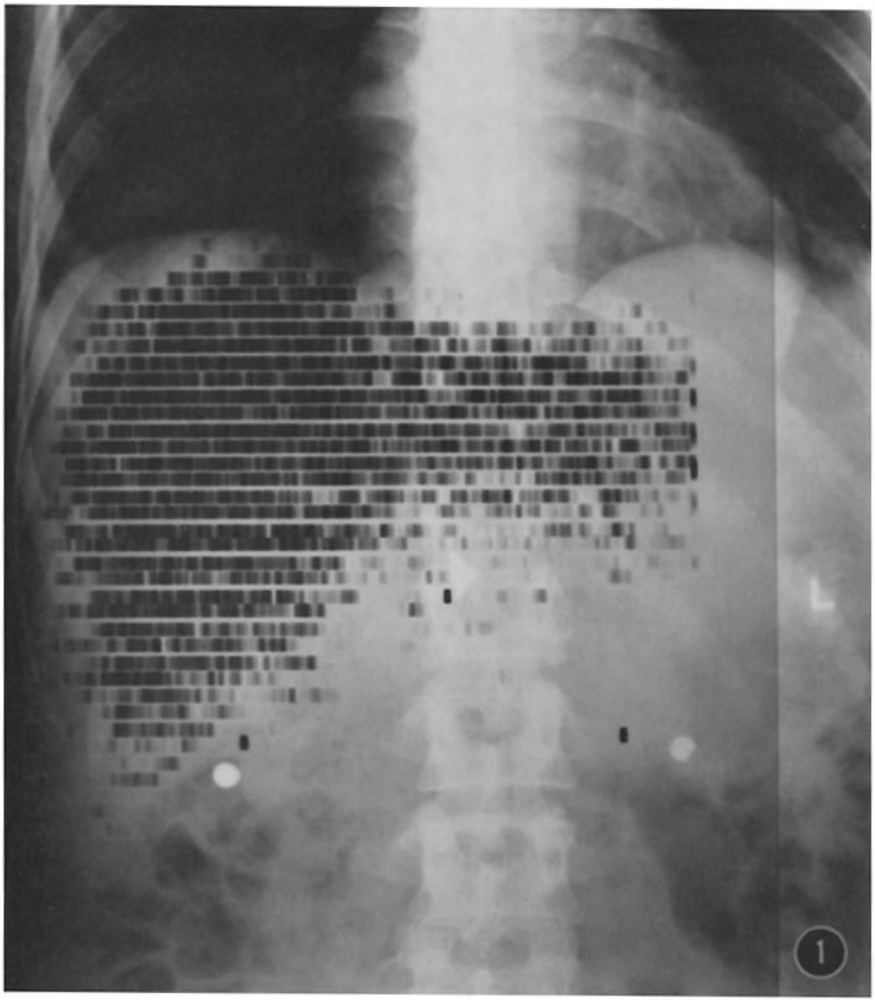
EXPLANATION OF PLATES

PLATE 16

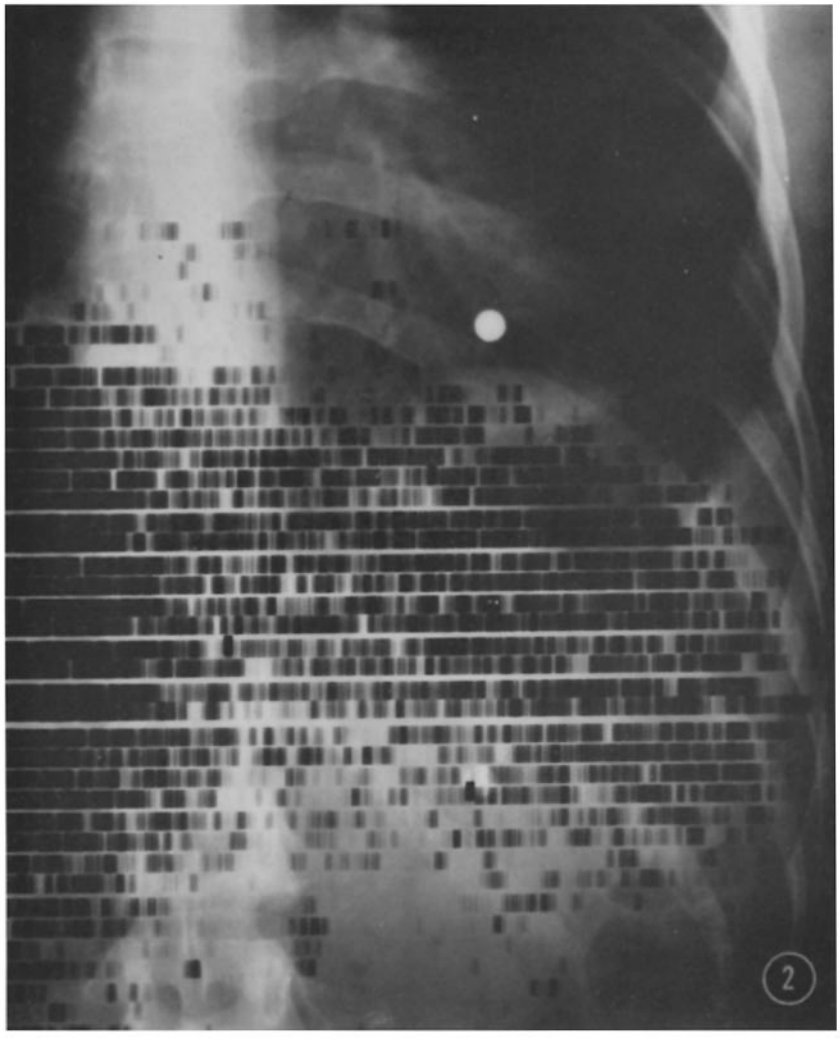
FIG. 1. Scintillation scan of a normal subject illustrating the concentration of radioactivity in the liver following intravenous administration of a trace dose (0.025 mg/kg) of I^{131}-labeled aggregated human serum albumin.



(Greisman *et al.*: Mechanisms of endotoxin tolerance. II)

PLATE 17

FIG. 2. Scintillation scan of a normal subject illustrating the concentration of radioactivity in both the liver and spleen following intravenous administration of a large dose (50 mg/kg) of I^{131}-labeled aggregated human serum albumin.



(Greisman *et al.*: Mechanisms of endotoxin tolerance. II)