MECHANISMS OF ENDOTOXIN TOLERANCE

I. Relationship Between Tolerance and Reticuloendothelial System Phagocytic Activity in the Rabbit*

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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), evidence has accumulated suggesting that accelerated phagocytosis of endotoxin by the RES represents the major mechanism of tolerance.

Tolerant animals clear endotoxin more rapidly from the circulation as detected by bioassay of plasma samples (1–3) and by disappearance of lethal doses of radioactively tagged endotoxin (4); the latter studies also demonstrate increased hepatic localization. In the tolerant animal, RES phagocytic activity for colloids is increased (5), weight and histologic appearance indicate RES hypertrophy (5), and treatment with a variety of agents which blockade the RES abolishes tolerance (7). Tolerance cannot be correlated with specific antibody titer (8, 9) and can be induced readily in subjects with agammaglobulinemia (10). Serum from tolerant donors confers no detectable (8) or minimal (11) tolerance; moreover, after loss of tolerance following RES blockade, the *in vitro* effect of serum on endotoxin pyrogenicity remains unaltered (12).

Recently, Freedman reported the successful passive transfer of significant endotoxin tolerance in rabbits employing specific endotoxin dose schedules for inducing tolerance in donor animals (13). Although these data indicate unequivocally the participation of humoral factors in endotoxin tolerance, the importance of such factors and the mechanism by which they operate remain speculative. Freedman suggested that the humoral factors act in rabbits and mice by enhancing RES phagocytic capacity; *i.e.*, transfer of tolerance was correlated with increased ability of the recipient animal to clear colloidal carbon, and hence presumably endotoxin, from the blood (14). In contrast, studies in man indicate that tolerance to the pyrogenic

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activity of bacterial endotoxins, administered as single daily intravenous injections, develops in the absence of increased RES phagocytic activity as measured by clearance of intravenously injected I^{131}-tagged heat-denatured human serum albumin (15). That such endotoxin tolerance, characterized by dissociation from a generalized enhancement of RES phagocytic activity, is mediated by humoral mechanisms was indicated by passive transfer studies in volunteers employing citrated plasma (16).

The present investigation was carried out to test the concept that induced tolerance to the pyrogenic activity of endotoxin can be dissociated from enhanced RES ability to clear heterologous colloidal particles.

Methods and Materials

Glassware, syringes, and needles were preheated in a dry air oven at 180°C for a minimum of 2 hours to eliminate extraneous pyrogenic contamination. Physiologic saline employed as a diluent was sterile and pyrogen-free.

All pyrogen studies were conducted in albino rabbits loosely restrained by chain collars in wooden stalls after acclimatization for 2 days. Temperatures were monitored with thermistor rectal probes inserted 6 inches into the rectum and connected to a recording telethermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio). Temperatures were determined for 1 hour before each experiment; animals exhibiting initial temperatures higher than 104°F or varying more than 0.2°F in any ½ hour period were discarded. After endotoxin injection, temperatures were monitored every 30 minutes for 5 hours. The resulting fever index was calculated from the area under the curve obtained by plotting the change in temperature as a function of time on standard graph paper; a fever index of 100 indicated a rise of 1.0°F sustained for 1 hour.

For passive transfer studies, male albino rabbits, 1.5 to 2.0 kg were rendered tolerant to either 2.0 μg/kg *Escherichia coli* endotoxin (Difco, lipopolysaccharide 0127B8) or 2.0 to 10.0 μg/kg *Salmonella enteriditis* endotoxin (Difco) by single daily intravenous injections of a fixed dose until maximum tolerance to the pyrogenic action of the endotoxin was established. Blood was drawn by cardiac puncture into heparinized tubes 24 hours after the last endotoxin injection and promptly centrifuged at 2000 RPM for 30 minutes. The plasma, 10 ml/kg, was injected into recipient rabbits weighing 0.8 to 1.2 kg. Control studies with plasma from nontolerant donors were performed concomitantly. The recipient animals were normal rabbits acclimatized for fever studies and prepared on the morning of study by intravenous injection of 3 ml/kg of thorotrast, a sterile colloidal suspension of 25 per cent thorium dioxide in dextrin (Testagar & Co., Detroit). After thorotrast, temperatures were monitored for 3 hours and the plasma then injected intravenously. 1 hour later, 0.05 μg/kg *Salmonella typhosa* endotoxin[1] was injected intravenously and the 5 hour fever index calculated, employing the immediate pre-endotoxin temperature as baseline. In most animals, the thorotrast *per se* produced febrile responses beginning after a 1.5 to 2 hour latent period (Fig. 1). In some groups of animals, the febrile response was uniformly steep and exceeded 104°F prior to the 4th hour; these were rejected. In other groups, the temperature rise was uniformly gradual, attaining a mean of 101.8°F by 4 hours.[2] These latter animals were subdivided so that one group received plasma from tolerant, the other from normal donors. As shown in Fig. 1, the base line temperatures

[1] A highly purified material (17) kindly supplied by Dr. Maurice Landy, National Institutes of Health.

[2] Whether this bimodal distribution of the febrile reaction was due to differences between rabbit groups or to differences between vials of thorotrast preparations was not determined.

immediately prior to endotoxin administration were comparable in these groups. This ability of thorotrast to induce fever has been noted previously in man (18) and did not appear attributable to endotoxin contamination since (*a*) the onset of fever was always delayed for over 1 hour, and (*b*) comparable fever developed in endotoxin-tolerant animals.

Studies of RES phagocytic activity were carried out employing the Gunther Wagner C-11/1431a carbon suspension prepared in gelatin by the method of Biozzi *et al.* (6). Control blood samples of 1 ml were drawn by cardiac puncture through a 22 gauge needle into heparinized syringes; 50 mg/kg of carbon was then injected into a marginal ear vein, and three timed cardiac bleedings of 1 ml each were collected during the subsequent 20 minutes. Each blood

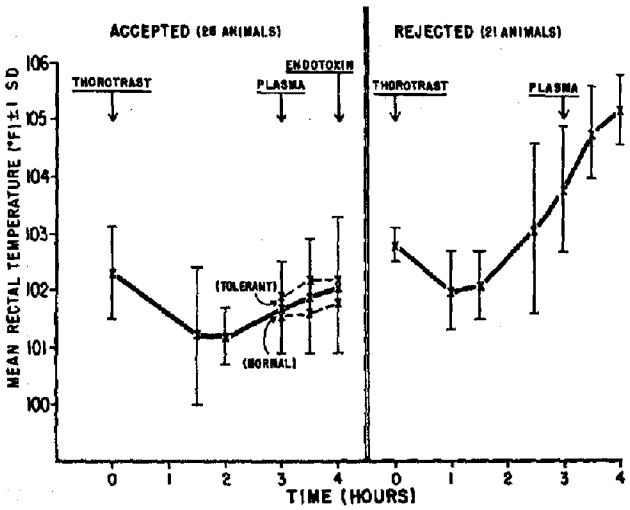

FIG. 1. Increase in rectal temperatures of acclimatized albino rabbits following intravenous administration of thorotrast (3 ml/kg).

sample was immediately diluted 1:10 in normal saline, centrifuged at 1000 RPM for 5 minutes to remove red blood cells and the optical density of the supernate immediately determined in a Beckman spectrophotometer at 1100 mμ. The carbon concentrations were calculated from known standards, and the logarithms of these values plotted as a function of time. The slope of the resulting straight line, K, is termed the "phagocytic index" and reflects the relative activity of the RES of each animal for the carbon particles according to the formula

$$K = \frac{\log C_1 - \log C_2}{T_2 - T_1}$$

in which C_1 and C_2 are carbon concentrations at time T_1 and T_2 (6).

RESULTS

RES Phagocytic Activity during Endotoxin Tolerance.—The ability to clear intravenously injected colloidal carbon particles was compared in normal and tolerant rabbits. Clearance studies were performed on the 8th day in animals rendered tolerant to 2.0 μg/kg *E. coli* endotoxin by 7 single daily intravenous

injections. The results are shown in Fig. 2. The mean fever index during tolerance was reduced 70 per cent. The mean phagocytic index during tolerance was 0.038 (SE ± 0.003), significantly greater ($p < 0.001$) than the non-tolerant mean index of 0.025 (SE ± 0.002). This conforms with previous reports of enhanced RES phagocytic activity in tolerant animals (5).

Effect of RES Blockade on Endotoxin Tolerance.—It has been assumed that tolerance to endotoxin and accelerated carbon clearance both result primarily from RES stimulation at the cellular level and therefore develop in parallel

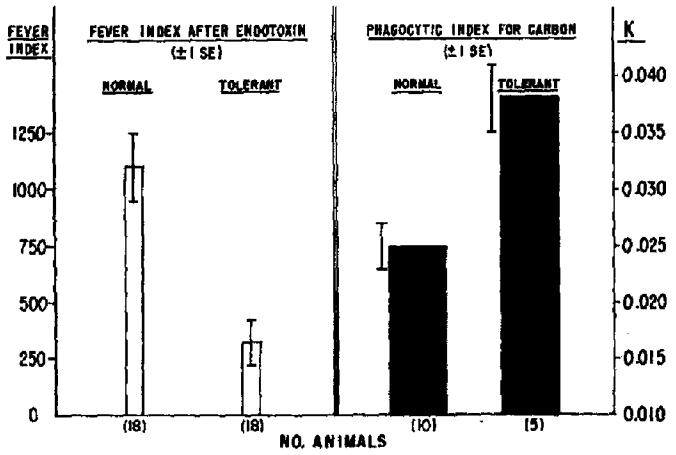

FIG. 2. Relationship between febrile responses to endotoxin and RES phagocytic activity of albino rabbits as measured on day 1 and day 8 following daily intravenous challenge with 2.0 μg/kg *E. coli* endotoxin.

(14). If endotoxin tolerance is indeed based upon enhanced RES phagocytic activity, then tolerant animals should react to endotoxin as do normals when both are subjected to equivalent RES blockade. Nineteen rabbits were given daily intravenous injections of 2.0 μg/kg *E. coli* endotoxin. After 7 days, tolerance was established as in the previous group (Fig. 2); on day 8 all animals received 3 ml/kg thorotrast intravenously. 4 hours later, when the effect of RES blockade is maximal (19), 9 animals received 0.05 μg/kg *S. typhosa* endotoxin intravenously while the remainder were subjected to carbon clearances; control groups of normal rabbits were treated similarly. The febrile reactions to thorotrast were comparable in the tolerant and control groups, and the base line mean rectal temperatures immediately prior to endotoxin were 102.1°F, and 102.3°F, respectively. The subsequent results are shown in Fig. 3. Carbon clearances were depressed consistently after RES blockade and animals previously tolerant to *E. coli* endotoxin now cleared carbon as did normals; the mean fever index following *S. typhosa* endotoxin, however, was 57

per cent lower in the tolerant group, a significant reduction ($p < 0.005$). The tolerant animal thus possesses some defense against endotoxin which is independent of enhanced RES phagocytic activity for carbon and is not specific for the endotoxin employed to induce tolerance.

One additional conclusion can be derived from comparison of Figs. 2 and 3. Normal rabbits given 2.0 μg/kg *E. coli* endotoxin responded comparably to those given 0.05 μg/kg *S. typhosa* endotoxin after RES blockade. Since the *E. coli* and *S. typhosa* endotoxins possessed equivalent pyrogenic potency

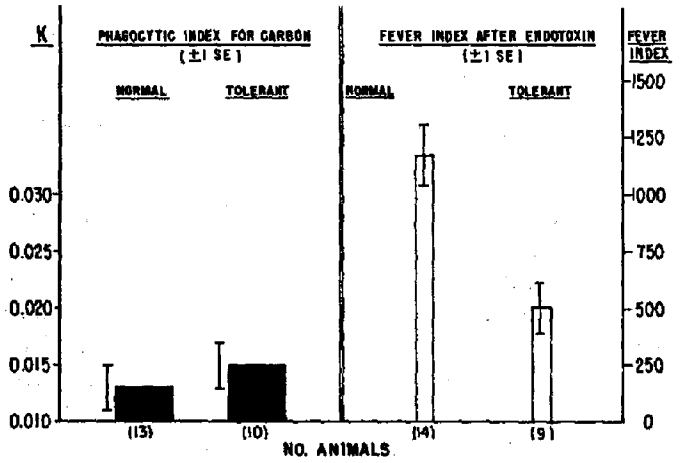

FIG. 3. Relationship between RES phagocytic activity and febrile responses to 0.05 μg/kg *S. typhosa* endotoxin in normal and in tolerant rabbits 4 hours after intravenous thorotrast (3 ml/kg).

(determined by assay in normal rabbits), RES blockade virtually abolishes the resistance of the normal animal to endotoxin. Therefore, to assess the influence of RES blockade during tolerance, this effect in the normal animal must be considered and the data evaluated as follows:—

(1) RES intact:

$$\frac{\text{Mean fever index (Normal)} - \text{Mean fever index (Tolerant)}}{\text{Mean fever index (Normal)}} \times 100 \text{ per cent} = \text{Per cent reduction in mean fever index attributable to tolerance mechanisms in the presence of an intact RES.}$$

(2) RES blockaded:

$$\frac{\text{Mean fever index (Normal-blockaded)} - \text{Mean fever index (Tolerant-blockaded)}}{\text{Mean fever index (Normal-blockaded)}} \times 100$$

per cent = Per cent reduction in mean fever index attributable to tolerance mechanisms in the presence of RES blockade.

Comparison of (1) and (2) relates the ability of RES blockade to affect

tolerance mechanisms. If (1) = (2), RES blockade does not affect the ability of tolerance mechanisms to inhibit fever. Two precautions must be observed: (*a*) RES "blockade" in the normal and tolerant animals in equation (2) must be equivalent; (*b*) the endotoxin test dose employed for equation (2) should be adjusted so that the mean fever indices of the control groups (Normal and Normal-blockaded) remain comparable.[3]

Substituting the present data, the effect of RES blockade on tolerance to endotoxin pyrogenicity can be expressed in quantitative terms:

(1) RES intact: $\frac{1110 - 330}{1110} \times 100$ per cent = 70 per cent reduction in mean fever index attributable to tolerance mechanisms.

(2) RES blockaded: $\frac{1171 - 500}{1171} \times 100$ per cent = 57 per cent reduction in mean fever index attributable to tolerance mechanisms.

RES blockade does not abolish the mechanisms of tolerance. Rather it resets the level of endotoxin reactivity in the normal and tolerant animal, rendering both exquisitely reactive, but permitting retention of the major portion (81 per cent in this case) of tolerance. Although the mild inhibition (19 per cent) of tolerance observed following RES "blockade" is probably physiologically significant, further studies are required for statistical confirmation. Moreover, some of the inhibition may not be related to RES "blockade" but rather to the testing of animals in (2) with an endotoxin heterologous to that employed to induce tolerance.

Passive Transfer of Endotoxin Tolerance to RES Blockaded Animals.—Plasma from rabbits tolerant to *E. coli* or *S. enteriditis* endotoxin was injected into normal recipients prepared with thorotrast as described under Methods. 1 hour later, some recipients received 0.05 μg/kg *S. typhosa* endotoxin while others were subjected to carbon clearances. Control groups of recipients received plasma from non-tolerant donors. The effects of plasma from donors tolerant to *E. coli* and *S. enteriditis* endotoxins were comparable and the results are summarized in Fig. 4. RES blockaded animals receiving 10 ml/kg of plasma from *E. coli*- or *S. enteriditis*-tolerant donors, although unable to clear carbon more rapidly than animals receiving normal plasma, resisted the *S. typhosa*

[3] The rationale for adjustment of endotoxin dose so as to maintain comparable febrile reactions in the control animals of equations (1) and (2) is as follows: RES blockade permits a given quantity of intravenously injected endotoxin to achieve higher physiologically active plasma concentrations (1). It is therefore reasonable to assume that when the dose of endotoxin is adjusted so that the normal animals and RES blockaded controls respond with equal temperature elevations, they have comparable plasma levels of physiologically active endotoxin. By employing such paired controls, the effectiveness of mechanisms that reduce the physiologic activity of injected endotoxin and thus induce tolerance can now be compared in tolerant animals with and without blockade as indicated above.

endotoxin as evidenced by a 32 per cent lower mean fever index (significant at $p < 0.001$). Moreover, the shape of the fever curve was altered in a manner characteristic of tolerance (13), *i.e.* the second fever peak was depressed to

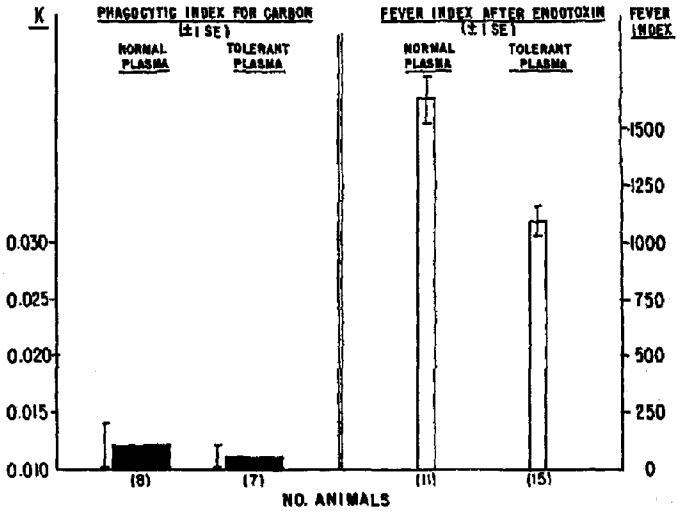

FIG. 4. Effect of plasma transfer (10 ml/kg) from normal and from tolerant rabbits on RES phagocytic activity and febrile responses to 0.05 μg/kg *S. typhosa* endotoxin in thorotrast-prepared recipients.

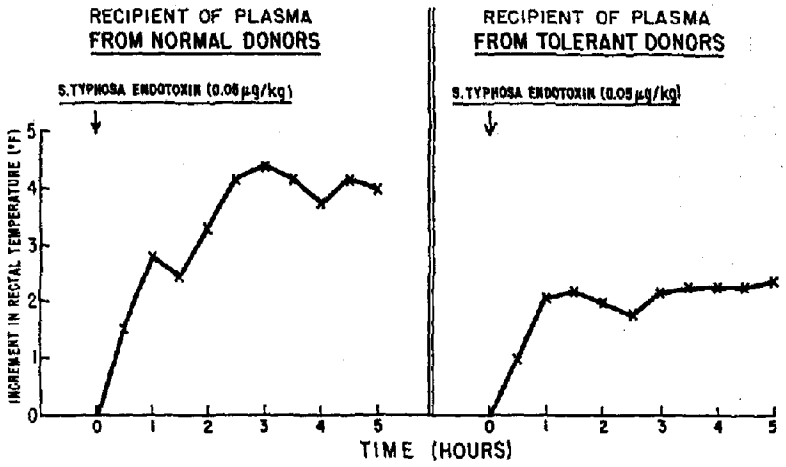

FIG. 5. Representative fever curves after 0.05 μg/kg *S. typhosa* endotoxin in thorotrast-prepared rabbits given plasma from normal and tolerant donors 1 hour prior to endotoxin.

levels approximating the first in 13 of 15 animals, whereas all 11 animals receiving normal plasma exhibited high second fever spikes (Fig. 5). These observations indicate the presence of circulating endotoxin inhibitors in tolerant rabbits that act independently of increased RES phagocytic activity for col-

loidal carbon. To estimate the potency of these inhibitors, 6 normal rabbits were acclimatized for fever studies and then pretreated with 3 ml/kg thorotrast intravenously. Three animals were given 0.05 μg/kg S. *typhosa* endotoxin; the others received one tenth this dose. The mean fever index was reduced 24 per cent in the latter animals. This compares with the 32 per cent reduction induced by 10 ml/kg of plasma from tolerant donors and indicates that in the system employed, 10 ml/kg of plasma from tolerant donors inhibited approximately 90 per cent of the injected *S. typhosa* endotoxin.

DISCUSSION

The present studies confirm two basic observations pertaining to the mechanisms of endotoxin tolerance: (*a*) Tolerance is associated with enhanced ability to clear colloidal carbon from the circulation, and (*b*) tolerance can be transferred with plasma. However, additional data necessitate re-evaluation of the common mechanism proposed to underly these reactions; *i.e.*, generalized enhancement of RES phagocytic activity at the cellular level (14). By employing thorotrast, RES phagocytic activity of tolerant and of normal rabbits could be reduced comparably as determined by carbon clearance, yet tolerant blockaded rabbits retained tolerance. Moreover, tolerance could be transferred with plasma to normal rabbits previously blockaded with thorotrast without increasing RES phagocytic activity for colloidal carbon. These observations indicate that tolerant rabbits possess mechanisms which inhibit endotoxin pyrogenicity independently of any overt increase in RES ability to clear heterologous colloids.

The present data also necessitate reinterpretation of the concept that RES blockade abolishes tolerance. Thus RES blockade was shown to reset the level of endotoxin reactivity in the normal and the tolerant animal, rendering both exquisitely reactive, but permitting retention of the major portion of tolerance. That the bulk of the original tolerance mechanisms remain functional could be demonstrated by analysis of the febrile response of blockaded tolerant rabbits in relation to proper controls; *i.e.*, comparably blockaded normal rabbits. The precautions required and justification for such analysis have been described under Results.

The mechanisms which account for the above findings are not entirely clear. The present data indicate that the tolerant animal possesses a dual defense against endotoxin: one also possessed by the normal animal and abolished by thorotrast, the other primarily responsible for tolerance and thorotrast-resistant. Part of this latter system is humoral as shown by retention of the ability of serum drawn from tolerant animals after RES blockade to transfer pyrogenic tolerance to normal recipients (13). The present observations that significant pyrogenic tolerance can also be transferred to RES-blockaded recipients prove that the humoral inhibitors act effectively in the RES-blockaded

animal. Previous studies (cited under Introduction) indicate that the tolerant animal consistently clears endotoxin from the circulation at enhanced rates. More recent studies by Herring *et al.* (20) confirm these observations for small quantities of Cr^{51}-labeled *E. coli* endotoxin. When the above evidence is considered in conjunction with the low efficiency of serum from tolerant donors in neutralizing endotoxin toxicity despite *in vitro* incubation at 37° C for 1 hour (21), it appears likely that the humoral factors responsible for tolerance act, in part at least, indirectly by preparing endotoxin for more rapid phagocytosis by the RES; *i.e.*, they behave as opsonins. This thesis is consistent with the hypothesis of Jenkin and Rowley (22) that opsonins are required for RES phagocytosis of colloidal particles. It seems peculiar that such "opsonins" are resistant to thorotrast, since RES blockade with colloidal agents is accompanied by opsonin depletion (22). Recent findings by Murray (23) resolve this problem. It was shown that opsonins possess specificity determined by the surface properties of the colloidal particle. This suggests that the opsonins elaborated following exposure to endotoxin which participate in tolerance possess high endotoxin specificity and are therefore not depleted by thorotrast.

The thesis that humoral inhibitors with high endotoxin specificity contribute significantly to endotoxin tolerance permits interpretation of previous inexplicable observations: (*a*) Marked tolerance to daily intravenous administration of quantities of endotoxin sufficient to evoke severe pyrogenic and toxic reactions in man was not accompanied by any generalized increase in RES phagocytic activity as measured with I^{131}-labeled aggregated human serum albumin (15). Moreover, tolerance could be transferred with plasma to normal volunteers (16). (*b*) Induced tolerance is suppressed in man during typhoid fever and tularemia at a time when RES phagocytic activity as measured with I^{131}-labeled aggregated albumin is enhanced significantly (15). These results, considered together with the dissociation of endotoxin resistance and colloidal carbon clearance following zymosan (24), fatty acids (25), graft *versus* host reactions (26), and infection with *Mycobacterium tuberculosis* (27) indicate that generalized increases in RES phagocytic activity do not *per se* confer tolerance. (*c*) The pyrogenic tolerance established by 30 consecutive daily intravenous injections of fixed quantities of *S. typhosa* endotoxin in man could be "abolished" on day 31 by administering half the dose followed in 2 hours by the remainder. During this initial 2 hour period, the blockading dose of endotoxin produced no significant febrile or toxic reaction, and did not affect RES clearance of I^{131}-labeled aggregated human albumin in control subjects. To suppress pyrogenic tolerance in man with colloidal particles different from endotoxin (*i.e.* aggregated human albumin), several thousand-fold larger quantities are required (15). (*d*) The administration of endotoxin suspended in normal serum leads to partial or complete reversal of pyrogenic tolerance and addition of serum from tolerant donors inhibits this reaction (11, 28).

Endotoxin suspended in tolerant donor serum fails to reverse tolerance and this failure is not altered by pretreatment of the donor with thorotrast (12). Such behavior of tolerant serum is consistent with the presence of a specific (*i.e.* thorotrast-resistant) endotoxin inhibitor.

The precise nature and mechanism of action of the humoral factors that participate in endotoxin tolerance are under study. The failure of tolerant rabbit serum to transfer high levels of pyrogenic tolerance to normal recipients (8, 11, 29) does not exclude the importance of specific humoral endotoxin inhibitors; such factors, behaving as opsonins, may induce more intense inhibition in conjunction with the generalized RES stimulation that accompanies pyrogenic tolerance in this species. Moreover, quantitative data are not yet available to evaluate the effects of dilution following passive transfer to normal recipients. The spectrum of pyrogenic tolerance conferred by the humoral inhibitors indicates cross-reactivity with endotoxins from heterologous bacterial species. However, the alternative possibility remains to be explored that the humoral factors are highly specific but appear to cross-react because one endotoxin stimulates the production of inhibitors directed at heterologous endotoxins. Such a situation would parallel the general elevation of specific antibody titers following injection of any one endotoxin (30).

SUMMARY

Pyrogenic tolerance following 7 daily intravenous injections of 2.0 μg/kg *E. coli* endotoxin in albino rabbits was associated with significant increases in RES phagocytic activity as measured with colloidal carbon. Nevertheless, 4 hours after RES blockade with thorotrast (3 ml/kg), the tolerant rabbits exhibited significantly lower fever indices following intravenous endotoxin challenge than did non-tolerant control animals despite comparably depressed capacities to clear carbon from the blood. Moreover, plasma from rabbits tolerant to endotoxin induced significant tolerance in normal rabbits prepared by thorotrast blockade without enhancing the depressed carbon clearance. This passive protection extended to heterologous endotoxins.

Analysis of the data indicates that RES blockade does not abolish tolerance; rather blockade resets the reactivity to endotoxin in the normal and tolerant animal, rendering both exquisitely reactive, but permitting retention of the major portion of tolerance. Apparently the tolerant animal possesses a dual endotoxin defense system. One system is abolished by thorotrast; the other is in part humoral, accounts for the greater portion of tolerance, and is thorotrast-resistant. The nature of the humoral component is not defined but is consistent with that of an opsonin with high endotoxin specificity.

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