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Mechanisms of Endotoxin Tolerance with Special Reference to Man

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In this review of the mechanisms underlying pyrogenic tolerance to bacterial endotoxins, two earlier concepts require modification. (*1*) Granulocytes are the major source of endogenous pyrogen release by endotoxin. (*2*) Pyrogenic tolerance results simply from accelerated clearance of endotoxin by the reticuloendothelial system (RES), diverting the toxin from the granulocytes. Rather, it now appears that hepatic Kupffer cells are the major source of endogenous pyrogen release by endotoxin, and that pyrogenic tolerance results primarily from refractoriness of these cells to further release of endogenous pyrogen. Accelerated clearance by the RES appears to be an ancillary protective mechanism that brings toxin more rapidly to the refractory cells. Refractoriness to the release of endogenous pyrogen can be achieved both by direct cellular interaction with endotoxin and by antibodies to endotoxin. The direct cellular effect is transient and requires closely spaced injections of endotoxin for maximal maintenance; antibody-mediated protection is delayed but more enduring. The diverse, often conflicting observations on pyrogenic tolerance are explicable by the interplay of these protective mechanisms.

Assessment of the role of endotoxin in the pathogenesis of gram-negative bacterial infection of man requires an understanding of the mechanisms used by man to develop resistance to this toxin. In contrast to studies with animal models, however, few critical studies exist on human mechanisms of acquisition of tolerance to endotoxin. Moreover, such studies in man are by necessity restricted to parameters (e.g., pyrogenicity) that pose minimal risk to the subjects. The following report presents a review, drawn from investigations conducted in this and other laboratories, of the tolerant responses of man to bacterial endotoxin. Our experience is based upon studies in several hundred volunteers given repetitive intravenous (iv) or intradermal (id) inoculations of bacterial endotoxins from various sources. These studies were conducted for the prime purpose of developing improved prophylactic and therapeutic approaches to gram-negative bacterial infections. All volunteers were fully informed of the nature of these studies and were free to withdraw at any time. Experimental protocols were reviewed and approved by the University of Maryland Human Research Committee. Where pertinent, results of animal studies are included to supplement the human data.

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General Considerations

Quantitation of reactivity. Man is one of the species most reactive to gram-negative bacterial endotoxin. When highly purified preparations, including those derived from rough mutant strains, are administered id to healthy adults, gross inflammatory responses are readily elicited with 10^{-3}–10^{-4} μg/0.1 ml. These responses become visibly greater than those at saline control sites after approximately 1 hr and are well developed by 3 hr. Histologically, the 3-hr lesions are characterized

by predominance of mononuclear cells. As the concentration of endotoxin is increased logarithmically, dermal inflammation increases in intensity, and the 3-hr infiltrate becomes progressively more polymorphonuclear leukocytic in nature. Polymorphonuclear predominance generally appears with concentrations of endotoxin approximately 100-fold above the minimal inflammation-inducing dose. When endotoxin is administered daily into the same site for one week, tolerance does not develop to its local inflammatory activity. Rather, the inflammatory lesions persist and become intensely infiltrated with lymphocytes and macrophages. The implications of the fact that tolerance to the local inflammatory activity of endotoxin fails to develop, at least in some tissue sites, will be considered later.

When endotoxin is administered to him iv, healthy man responds in a characteristic fashion. On a per-kg basis, rabbit and man are almost equally reactive to threshold pyrogenic quantities of endotoxin. In both species, unequivocal elevations of temperature can be evoked with less than 0.001 μg/kg of purified preparations. However, as the quantity of endotoxin is increased, the dose-response relationship becomes considerably steeper for man, and the intensity of the subjective human toxic responses increases in parallel with the pyrogenic response [1]. When endotoxin is administered to man in single, daily, iv injections, tolerance to both febrile and subjective toxic reactions of endotoxin becomes evident within 48 hr. It is of interest that, with certain endotoxins, the febrile and subjective toxic responses are actually increased 24 hr after the initial iv administration [2]. Such enhanced febrile responses at 24 hr are never seen in the rabbit. Thereafter, however, progressive tolerance ensues and is virtually maximal within the week. Tolerance in man, as in the rabbit, is relative, not absolute, and fever and subjective toxic responses can be elicited again by an increased dose of endotoxin [3].

Qualitative aspects of reactivity. When healthy man is given an iv injection of purified endotoxin, a latent period of 45–90 min generally elapses before any clinical reaction is seen. The shortest latent period encountered in this laboratory was 35 min and was observed in a patient during the hyperreactive phase of typhoid illness [3]. The febrile response characteristically peaks in about 3 hr. Unless the dose of endotoxin is very large, the temperature then begins to decline rapidly. During the phase of ascending temperature, accompanying subjective symptoms are chills, rigor, headache, myalgia, anorexia, nausea, and (at times) vomiting. For unknown reasons, despite equipyrogenic doses, nausea and emesis are consistently more pronounced with some endotoxins (e.g., *Escherichia coli* 0127B8) than with others (e.g., *Pseudomonas* sp., Piromen®).

The pyrogenic response of man differs from that of the rabbit in one important respect. The rabbit exhibits a rapidly appearing first-fever peak with a latent period of approximately 15 min and a summit at about 90 min. This early response is absent in man. With larger doses of endotoxin, a second phase of fever develops in the rabbit, whose peak and onset (as seen when the first phase is blocked by perfusion of the endotoxin directly through the liver [5]) closely parallel the time course of the human response. Morover, as tolerance is induced by constant, single, daily iv doses of endotoxin, the second febrile peak in the rabbit again parallels the human response in that it declines progressively, whereas the first peak is not reduced appreciably [4, 5].

Evidence for circulating endogenous pyrogen. If we assumed that man behaves as does the rabbit, tolerance to the pyrogenic activity of endotoxin would be based upon reduced generation of endogenous pyrogen in response to the toxin; the thermoregulatory centers (presumably the anterior hypothalamus) would remain fully responsive to this protein [6]. A major criterion for the activity of endogenous pyrogen in man is the onset of an unequivocal rise in temperature that commences within 40 min of administration of the pyrogen [7]. In addition, in contrast to endotoxin, tolerance to repetitive doses of endogenous pyrogen, or to its continuous iv infusion, does not develop [6]. Considerable effort has been devoted to proving the existence of a circulating intermediate responsible for the pyrogenic effects of endotoxin in man. Circulating endogenous pyrogen is extremely difficult to detect in humans, although this is not the case in experimental animals. Currently, there exist only two instances in which circulating endogenous pyrogen has been presumptively demonstrated in volunteers after administration of endotoxin [8, 9]. Large quantities of plasma must

be transferred, and the resulting febrile response is small [9].

Cells responsible for generation of endogenous pyrogen. In 1948, Beeson extracted a pyrogenic substance from polymorphonuclear leukocytes [10]. For years thereafter, the polymorphonuclear leukocyte was regarded by most investigators as the major source of endogenous pyrogen release by endotoxins [6]. Indeed, studies by Herion et al. with nitrogen mustard in rabbits led to the conclusion that the granulocyte was the sole source of endogenous pyrogen after administration of endotoxin [11]. The general poisonous effects of mustard, however, make such studies difficult to interpret.

The important studies of Page and Good with respect to man, although carried out before many of the studies with rabbits, were largely overlooked and deserve great emphasis. These workers observed that some patients with cyclic neutropenia and others with agranulocytosis in the absence of circulating neutrophils developed a febrile response to endotoxin-containing preparations (typhoid vaccine) "at least as high as that produced when normal numbers of neutrophils were present in the blood" [12]. The authors stated: "It must be concluded from this study that injury to neutrophils with liberation of endogenous pyrogen plays no important role in the development of fever following the intravenous injection of pyrogens in man." Moreover, when tolerance was tested, it was concluded that ". . . refractoriness to endotoxin develops in the complete absence of neutrophils in the circulating blood or in the blood forming tissues and consequently demonstrates that these cells are not essential to this defense reaction" [12].

Later studies by Bodel and Atkins demonstrated that human monocytes could elaborate endogenous pyrogen when stimulated by pyrogens [13]. Moreover, a series of classic studies carried out by Dinarello et al. indicated that, in the rabbit, isolated hepatic Kupffer cells could be stimulated in vitro by endotoxin to generate endogenous pyrogen. Furthermore, the isolated Kupffer cells (but not blood leukocytes) from rabbits tolerant to endotoxin were found to be refractory to the release of endogenous pyrogen by this toxin [14]. Subsequent in-vivo studies in our laboratory, using direct hepatic perfusion with endotoxin via chronic, indwelling, portal-vein cannulae, indicated that the second phase of the biphasic febrile response in the rabbit (the one paralleling the human response) could be related primarily to hepatic release of endogenous pyrogen, and tolerance to refractoriness of such release [5]. If these studies can be extrapolated to man, the findings of Page and Good in their agranulocytic patients become readily explicable.

The above discussion is not meant to imply that endotoxin stimulates the release of endogenous pyrogen only from hepatic Kupffer cells. Certainly, endogenous pyrogen is also released in vitro from granulocytes, lung and peritoneal macrophages, blood monocytes, and spleen cells [13–16]; endotoxin also appears to be capable of acting directly upon the thermoregulatory centers to evoke fever without the intermediate of endogenous pyrogen [17, 18]. However, since the hepatic Kupffer cells comprise the major segment of the reticuloendothelial system, and since this segment sequesters the major portion of iv-injected endotoxin [19], it is this segment that would be expected to play the major role in the generation of endogenous pyrogen. In this respect, it is of interest that the first phase of the biphasic febrile response in the rabbit, in contrast to the second phase, can be reduced or eliminated by direct perfusion of the liver with endotoxin; presumably, therefore, the first febrile phase in the rabbit (for which a human counterpart does not appear to exist) is not mediated by hepatic release of endogenous pyrogen [5] and may reflect release of neutrophil endogenous pyrogen or a direct cerebral effect, as postulated by Bennett et al. [17].

Mechanisms of Tolerance to Endotoxin

Based on the above considerations, and in complete agreement with the concept of Dinarello et al. [14], our current working hypothesis for the acquisition of pyrogenic tolerance to iv administration of endotoxin entails the development of refractoriness of hepatic Kupffer cells to the generation and release of endogenous pyrogen. The mechanisms whereby such refractoriness operates will now be considered.

Early refractory state to continuous iv infusion of endotoxin. In both rabbit and man, continuous iv infusions of endotoxin lead to pyrogenic

refractoriness within hours. Volunteers express disbelief that the infusion of endotoxin is continuing during this refractory state, since the subjective toxic responses also subside. This early refractory state is relative and can be overcome by increasing the rate of infusion. It is not associated with increments in titers of antibody to endotoxin and is not transferable with plasma. Furthermore, it develops readily in splenectomized rabbits that exhibit depressed synthesis of antibody to endotoxin. This state is not associated with enhanced ability of the plasma to inactivate endotoxin. It is specific for endotoxins as a class (at least at low rates of infusion) but exhibits no interendotoxin specificity. It is associated with inability to generate endogenous pyrogen, even while endotoxin is demonstrable in the bloodstream.

Full responsiveness persists to preformed endogenous pyrogen. It appears, therefore, that a cellular state refractory to the generation of endogenous pyrogen in response to endotoxemia can develop rapidly in both rabbit and man [20, 21]. Since this refractory state is not reversible by infusion of large quantities of fresh whole blood and occurs despite an increased number of circulating granulocytes, the findings remain consistent with the concept of the hepatic Kupffer cell as the primary target cell responsible for endotoxin fever. Moreover, the data suggest that it is a direct interaction of these target cells with endotoxin, rather than depletion or production of humoral factors, that rapidly renders them refractory to the release of endogenous pyrogen. The cellular events leading to the refractory state, however, are unknown. There is no enhancement in the potency of liver homogenates to detoxify endotoxin [21]. The hypothesis proposed by Janoff et al. [22] that depletion of the more susceptible intracellular lysosomes may be responsible for resistance to endotoxin appears pertinent in this regard and may ultimately prove to be relatable to this form of early tolerance.

Specificity of tolerance. Initial classic studies in man by Morgan demonstrated that tolerance produced by repetitive, daily iv injections of one endotoxin evoked cross-tolerance to endotoxins from heterologous bacterial species, that tolerance waned within four to five weeks after discontinuance of the injections, and that O antibody titers did not correlate with tolerance [23]. It should be emphasized that, while tolerance to heterologous endotoxins was unequivocally demonstrated, the relative effectiveness of such "heterologous" tolerance as compared with "homologous" tolerance was not quantitated. Careful quantitative studies of this nature have yet to be carried out in man. When such studies have been performed in animals, tolerance has been found to be significantly greater to the homologous endotoxin preparation [24].

In any case, it became clear by 1948 that man behaved similarly to the experimental animal in that tolerance to one endotoxin conferred definite tolerance to all others tested. More recent studies demonstrated that this problem of specificity of tolerance is complex. By study of the induction of pyrogenic tolerance after a single iv injection of endotoxin at various intervals, it could be shown in the rabbit that two temporally distinct mechanisms were involved. An early phase of pyrogenic tolerance, i.e., that appearing within hours, was probably identical with that elicited by the continuous iv infusion of endotoxin. This tolerance was specific for endotoxins as a class (provided massive doses of toxin were not used) but exhibited no interendotoxin specificity. This early phase was not associated with increments in antibody to endotoxin and could be readily induced by endotoxins lacking O-specific polysaccharide side chains (i.e., endotoxins from rough mutants). The degree of early tolerance was directly proportional to the intensity of the initial response. This early tolerance after one injection of endotoxin waned rapidly and became minimal by 48 hr. However, tolerance reappeared by 72 hr and increased over the next several days. In contrast to early tolerance, this later phase was unrelated to the initial intensity of the febrile response but was related rather to the antigenicity of the immunizing endotoxin. Moreover, it was largely, though not completely, specific for the homologous endotoxin used for the initial injection when endotoxins from smooth bacteria were employed for immunization.

In the only studies in man currently available, this late phase of tolerance after a single iv injection of endotoxin was found, as in the rabbit, to be independent of the initial pyrogenic response and to be related to the O antigenicity of the endotoxin preparation [24]. These findings led to the conclusion that tolerance to endotoxin in-

volved at least two distinct mechanisms: an early, transient, cellular, refractory state that extended to all endotoxins, probably mediated by a direct cellular effect of the toxin; and a later, antibody-mediated phase that was, at least in part, directed against O-related antigens. The concept was developed that the importance of antibody in the mediation of pyrogenic tolerance was dependent on the interplay of these mechanisms [21, 24]. Closely spaced injections or continuous infusions would provide optimal conditions for the direct cellular refractory mechanism, and pyrogenic unresponsiveness could thus be induced rapidly and maintained without the requirement for antibody. However, as the interval between challenges was lengthened, the direct cellular effect waned, and tolerance became increasingly dependent upon antibodies to endotoxin. The former tolerant state would exhibit no O specificity; the latter, a significant level. After repetitive daily injections, both states would coexist, and either O specificity or its absence could be emphasized, depending upon the prejudice of the investigator.

Passive transfer of tolerance to endotoxin. Although earlier investigators reported failure in transferral of tolerance to endotoxin with serum, there is no longer any question that this can be accomplished in the experimental animal (see review in [24]) and in man [2]. The importance of using serum and serum fractions collected with pyrogenfree precautions cannot be overemphasized, since contamination with endotoxin will itself evoke the characteristic early tolerant state, which will be proportional to the degree of contamination and which will possess no O specificity. Recent studies have been done in our laboratory on quantitation of the specificity of transfer of pyrogenic tolerance with serum and with pyrogenfree serum fractions (prepared by ammonium sulfate or by Sephadex G-200 fractionation) from rabbits immunized daily for one week with smooth gram-negative bacteria or endotoxins derived therefrom. These studies demonstrated that the humoral tolerance factors appear to be directed primarily, though not completely, towards O-specific antigens of the toxin. This O-specific protection was divided between the antibody fractions sensitive to 2-mercaptoethanol and those resistant to it; this finding suggests that both IgM and IgG antibodies are important.

Earlier studies on the specificity of tolerance transferable with spleen cells from rabbits immunized with smooth endotoxins support this concept by demonstrating that an "anamnestic" tolerant response could be transferred, but only with viable cells, and that this was largely (though not entirely) O-specific [25]. Protective antibodies in the rabbit have also been demonstrated in other laboratories with use of passive protection against lethality and against the Shwartzman reaction. Here, again, when pyrogen-free sera from animals immunized with smooth gram-negative bacteria or their endotoxins were used for transfer, protection was always significantly greater to the homologous toxin; indeed, at times protection extended only to the homologous toxin [26–29].

Of special importance is the fact that, when certain rough-mutant, gram-negative bacteria are used for immunization, the resulting antisera, carefully shown to be pyrogenfree, give high levels of cross-protection to heterologous endotoxins. The latter findings by Braude and Douglas were interpreted to indicate that, in the absence of the O-antigenic, polysaccharide side chains of endotoxin, common-core antigenic configurations are more readily exposed; these configurations allow the induction of high titers of protective antibody to these antigens [29]. These findings have recently been fully confirmed in our laboratory and are of potential therapeutic significance, since a method now appears available for development of an effective antiserum to endotoxin with broad specificity.

Minimal dose of endotoxin required to evoke tolerance. Studies in this laboratory have quantitated the relationship of tolerance of man toward endotoxin to the immunizing dose. Tolerance on day 7 was found to be similar in healthy volunteers, regardless of whether they were injected iv with 0.01 μg/kg of *E. coli* endotoxin on days 0 and 5 or with 0.001 μg/kg. Since the former immunizing doses evoked marked febrile and subjective toxic responses, whereas the latter elicited no clinical reactions, it is clear that the later phase of tolerance in man, as in the rabbit, is not dependent upon the initial reactivity to endotoxin. Rather, the factor common to both doses of endotoxin was their ability to evoke elevated titers of O antibody by the time of the

test for tolerance. Only when the immunizing dose of endotoxin was reduced to 0.0001 μg/kg and antibody titers were minimal did tolerance become minimal.

It may be concluded that antigenicity, not toxicity, constitutes the major factor in evoking the late phase (i.e., day 7) of endotoxin tolerance in man, when the refractory state that follows each endotoxin dose is permitted to wane by appropriate spacing of injections; the pattern is similar in the rabbit [24].

Tolerance and reticuloendothelial system (RES) phagocytic activity. In 1947, Beeson reported that pyrogenic tolerance in rabbits was associated with accelerated clearance of endotoxin from the blood, and that the RES "blockade" retarded clearance and abolished tolerance. Since tolerance did not appear to be transferable with serum and extended to heterologous endotoxins, it was postulated that resistance was based on accelerated RES clearance of toxin from the blood and its consequent diversion from other target organs [30]. Subsequent studies in our laboratory [31], confirmed by Wolff et al. [32], demonstrated that tolerance to endotoxin was not actually abolished by the RES blockade. Rather, the reactivity of both normal and tolerant animals was increased markedly, while striking differences in reactivity between the normal and the tolerant states persisted.

The mechanism whereby the RES blockade enhances pyrogenic reactivity of both normal and tolerant animals, while leaving the tolerance mechanisms intact, is not entirely clear. However, data now available permit a reasonable working hypothesis. Since, as discussed previously, pyrogenic tolerance appears to be based primarily upon refractoriness of hepatic Kupffer cells to release of endogenous pyrogen by endotoxin, this refractoriness presumably persists after the RES blockade. Instead, the diversion of endotoxin to less refractory tissues after after blockade (as evidenced from studies on comparative rates of clearance of Cr^{51}-labeled endotoxin in normal, tolerant, blockaded normal, and blockaded tolerant rabbits [33] and on reduced total endotoxin uptake by the liver of blockaded animals [34, 35]), could account for the observed responses.

This hypothesis is identical to that proposed by Dinarello et al. [14] and is extended to include the normal RES-blockaded animal (figure 1). According to this concept, the tolerant blockaded animal remains tolerant when compared with the normal blockaded control, because the former still (*1*) possesses refractory hepatic reticuloendothelial cells and (*2*) clears the endotoxin into these refractory cells at a faster rate (compare panels B and D, figure 1). That antibodies to endotoxin continue to contribute to these tolerant mechanisms after the RES blockade is suggested by the findings that sera from blockaded tolerant rabbits retain the ability to transfer protection to normal recipients [36] and that sera from tolerant rabbits transfer protection to blockaded normal rabbits [31]. The antiserum appears capable of contributing to both tolerant mechanisms (see below). The hypothesis outlined in figure 1 is also compatible with earlier observations that the RES blockade fails to enhance the pyrogenic reactivity of either the normal or the tolerant animal when endotoxin is administered as a slow iv infusion; this method of administration would not tax the phagocytic capacity of a blockaded RES as would sudden injection of the entire bolus of toxin [20].

The hypothesis presented in figure 1 states that the enhanced fever evoked in normal animals by the RES blockade results from diversion of endotoxin from hepatic to extrahepatic tissues (panel A versus panel B). The converse of this postulate is that enhanced uptake of toxin by normal Kupffer cells should result in tolerance. Such tolerance, if it occurred, could never be as impressive as that achieved in the tolerant animal whose Kupffer cells are simultaneously refractory to the generation of endogenous pyrogen. In the absence of such refractoriness, no reduction in pyrogenic response was observed after diversion of endotoxin to the Kupffer cells by means of injection of the toxin via portal-vein cannulae [5]. Studies in this and other laboratories indicate that RES clearance of endotoxin can be accelerated within 24 hr after an initial injection of endotoxin in the absence of humoral factors that transfer such acceleration (presumably a direct stimulating effect), as well as after passive transfer of tolerant-phase serum (presumably an opsonizing effect of antibodies to endotoxin [29, 37]. Thus, the same stimuli that evoke refractoriness of Kupffer cells to the release of endogenous pyro-

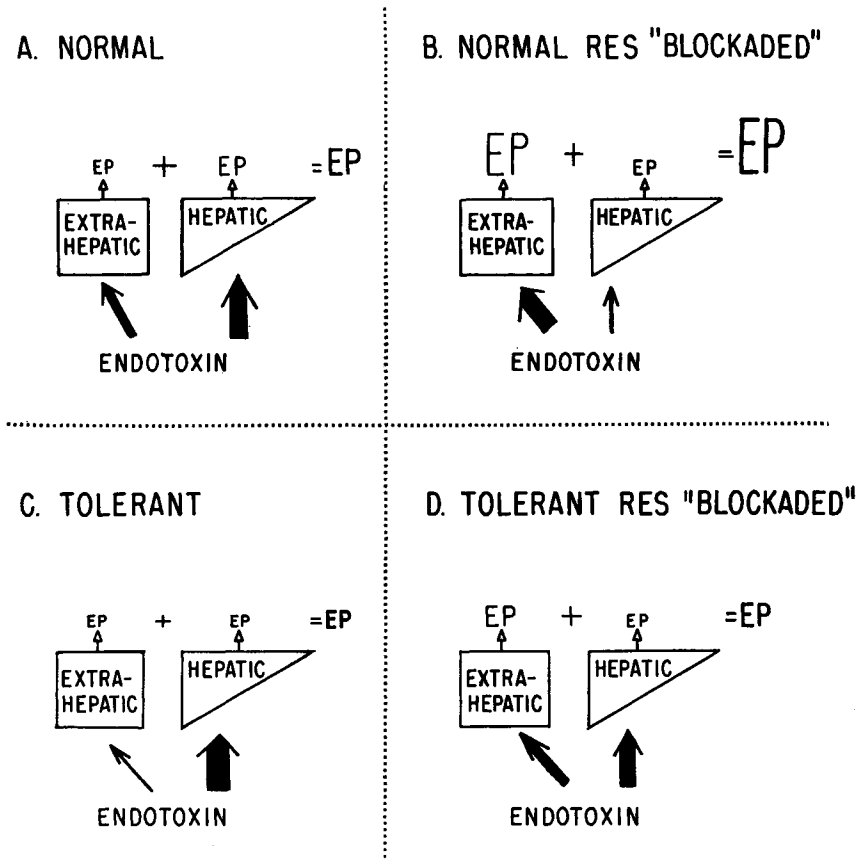

Figure 1. Hypothesis to account for the effect of blockade of the reticuloendothelial system (RES) on pyrogenic reactions to endotoxin in the normal and tolerant host. Concept is based on studies of rates of clearance from blood [33], hepatic uptake [34, 35] and pyrogenic reactions to ear vein [31] and portal vein [5] perfusion of endotoxin after RES blockade. This hypothesis is entirely comparable to that proposed by Dinarello et al. [14] based upon in-vitro studies of the release of endogenous pyrogen by endotoxin.

gen by endotoxin (direct cellular interaction with endotoxin and antibodies to endotoxin) are also those that evoke accelerated clearance of endotoxin. It is the former effect that permits the latter to become a significant protective mechanism.

The above concept implies that antibodies to endotoxin do not confer tolerance simply by accelerating RES clearance of endotoxin. This is strongly supported by the observations of Dinarello et al. [14] that tolerant-phase serum protects isolated hepatic Kupffer and spleen cells against the endogenous pyrogen-releasing activity of endotoxin. Since such protection did not extend to blood leukocytes or lung macrophages, it appears unlikely that the antiserum merely agglutinated the toxin. Rather, the findings suggest that antiserum to endotoxin directly protects the RES against the endogenous pyrogen-releasing activity of endotoxin, perhaps by enhancing its intracellular detoxification or by interacting with specific receptors on RES membranes (cytophilic antibody). Studies in our laboratory support the latter possibility, since absorption of tolerant-phase rabbit serum at 4 C with packed normal rabbit spleen cells (in contrast to rabbit erythrocytes) removed significant protective activity. That accelerated RES clearance of endotoxin is not a requisite for induction of tolerance by antibodies to endotoxin was recently demonstrated by Braude and Douglas [29]. These investigators used an antiserum prepared against a heterologous endotoxin to circumvent the potent opsonizing activity of antibody to O antigen and demonstrated excellent

protection against the local Shwartzman reaction in the absence of enhanced clearance of the toxin from the blood.

The concept that accelerated RES clearance of endotoxin confers minimal, if any, protection unless the RES is also refractory to the toxin would acount for the following observations.

(*1*) In 1955, Atkins and Wood reported that rabbits sensitized by one or two iv injections of typhoid vaccine three to five weeks previously cleared this pyrogen significantly more rapidly from the blood but exhibited febrile responses similar to those of nonsensitized controls [38].

(*2*) In 1962, Stuart and Cooper elicited enhanced RES clearance of endotoxin in mice by treatment with simple lipids and found that the animals became more susceptible to endotoxin. They concluded that "phagocytosis by itself is of relatively little importance in resistance to endotoxin" [39]. Di Luzio and Crafton confirmed these conclusions, using other reagents to modify RES clearance of endotoxin [40].

(*3*) In 1966, Chedid et al. reported that, in contrast to endotoxins from smooth gram-negative bacteria, toxic rough (R) endotoxins were cleared equally rapidly from the blood of nontolerant and tolerant mice. These investigators concluded: "It is, therefore, difficult to explain the increased resistance of the tolerant mouse against R endotoxin solely on the basis of its greater disappearance from the blood" [37].

(*4*) Recent studies in our laboratory have shown that endotoxin lethality cannot be prevented by an early exchange transfusion of normal rabbits 20 min after an iv LD_{80} dose of endotoxin, even though circulating levels of toxin are thereby rapidly reduced to levels equivalent to those seen in tolerant animals that survive this dose. It appeared probable from these studies that tolerance to endotoxin could not be based simply upon enhanced uptake by the RES of endotoxin but rather rested upon enhanced resistance of the RES to the injurious effects of the toxin [41]. The marked susceptibility of the normal RES to injury caused by endotoxin has already been well demonstrated by Heilman [42]. While man exhibits accelerated blood clearance of Cr^{51}-labeled endotoxin as pyrogenic tolerance is induced [33], it now appears likely from the above considerations that such tolerance is not achieved by this accelerated clearance per se, but by the concomitant development of refractoriness of the hepatic reticuloendothelial cells to the generation of endogenous pyrogen.

Duration of tolerance. Once tolerance to endotoxin has been actively induced by repetitive daily iv injections, its duration after discontinuance is a complex response. It was concluded during early studies in man that "the resistant state had disappeared in from four to five weeks. . . ." [23]. Careful analysis of the data presented, however, indicates that tolerance had declined, not disappeared, at this time. We are unaware of any systematic studies in man that define quantitatively the rate of tolerance loss. Studies in a few volunteers in our laboratory parallel the findings of Mulholland et al. in the rabbit [43], wherein the rate of tolerance decline is initially rapid after discontinuance of injections of endotoxin but then slows so that residual tolerance remains evident for several months. We postulate that the initial rapid rate of decline in tolerance reflects the transient nature of the cellular refractory state and that the subsequent slower-decline phase reflects the decline in protective antibody. The early, rapid-decline phase of pyrogenic tolerance was well recognized by Beeson, who initially emphasized that maximal pyrogenic tolerance requires repetitive, closely spaced injections of endotoxin [4].

Split-dose effect. Of great interest is the ability partially to overcome established tolerance to endotoxin in man by dividing the endotoxin dose in half and administering the second half-dose 2 hr after the first [2]. This maneuver was employed years ago to facilitate fever therapy with typhoid vaccines. The second dose was described as having the effect of "exploding the charge" supplied by the first [44]. The mechanism underlying this phenomenon is unknown but has been likened to the Danysz reaction, wherein the initial half-dose of endotoxin binds a high proportion of protective antibody, allowing the second half to act without inhibition by antibody [2]. Further tests of this hypothesis are required, but it conforms with observations in our laboratory that the accelerated blood clearance of endotoxin is slowed in tolerant volunteers 2 hr after the initial dose of toxin. This phenomenon of overcoming pyrogen tolerance unfortunately cannot be dupli-

cated in the rabbit; the initial phase of the biphasic fever response to endotoxin is not abolished during tolerance in this animal, and division and administration of the endotoxin dose at intervals of 2 (or 3, 4, or 5) hr generally result simply in duplication of the initial febrile response. It should be stressed that, when a constant dose of endotoxin continues to be administered to tolerant man every 2 hr, tolerance is again observed by the fourth or fifth dose; thus, fever cannot be sustained by this method.

Effect of illness on tolerance. The tolerance mechanisms in man continue to function during febrile illness. In two such illnesses, typhoid fever and tularemia, it could be demonstrated that while the baseline reactivity to endotoxin was sharply increased, the tolerance mechanisms could still be activated within this hyperreactive framework. Tolerance could be readily induced during overt illness both by daily single injections of endotoxin and by continuous iv infusions. Tolerance induced before illness could also be demonstrated during illness [33]. Accelerated blood clearance of endotoxin, evident during induction of tolerance before illness, remained evident during typhoid fever [33]. This ability of man to acquire tolerance to endotoxin during typhoidal and tularemic illness and the inability to mitigate these illnesses by deliberate induction of tolerance permitted the conclusion that circulating endotoxin could not constitute the major cause of the sustained pyrexia and toxemia during these illnesses [33].

As in typhoid fever and tularemia, so in brucellosis; man hyperreacts to endotoxin and readily acquires tolerance to its iv administration [45]. Tolerance to endotoxin has been demonstrated in man after convalescence from tularemia, typhoid and paratyphoid fevers, and during chronic pyelonephritis secondary to gram-negative but not gram-positive bacteria [46–48]; presumably, tolerance results from exposure to the endotoxin component of the infecting microbe. Pyrogenic tolerance is also seen in man after malaria [49], but its basis is unknown.

Local vs. systemic tolerance. When man is rendered tolerant to repetitive daily iv injections of endotoxin, tolerance does not develop to its dermal inflammatory reactivity. Indeed, dermal inflammation is enhanced, probably as a result of elevated titers of antibody to endotoxin [2]. Thus, it is clear that systemic tolerance to endotoxin cannot be equated with resistance to the local inflammation-evoking activity of the toxin. It has been proposed that endotoxin contributes to the sustained pyrexia and toxemia of typhoid fever primarily by virtue of this local inflammatory (rather than systemic) activity [33]. This concept would be consonant with the inability to mitigate this illness by deliberate induction of systemic tolerance to iv-injected endotoxin [33].

Effect of immunosuppression on endotoxin tolerance in man. Two types of studies have been performed in man as tests of the effect of impaired production of antibody on tolerance to endotoxin. Good et al. [50, 51] studied patients with agammaglobulinemia and reported that such patients acquired tolerance to the pyrogenic activity of typhoid vaccine, as did healthy controls. However, proof that titers of antibody to endotoxin were impaired was not documented. Wolff has studied three such patients and confirmed normal acquisition of tolerance in the absence of detectable increments in antibodies to endotoxin (S. M. Wolff, personal communication). In all of these studies, the endotoxin was administered iv at daily intervals. Since cellular refractoriness can rapidly lead to pyrogenic tolerance as discussed above, deficits in tolerance based upon deficits in protective antibody formation might not be apparent by such a schedule of challenge.

In our laboratory, a second model of impaired immunoglobulin production was studied (i.e., splenectomy), and tolerance was tested at intervals spaced sufficiently to allow waning of the cellular refractory state (five, seven, and 10 days after initial immunization). Three healthy volunteers who were splenectomized seven to 11 years previously after abdominal trauma responded to an initial iv injection of endotoxin in a manner identical to six healthy controls. These splenectomized subjects failed to develop increments in antibodies to endotoxin as measured by bentonite-flocculation and bactericidal-antibody techniques on days 5–10 after initial challenge and acquired tolerance at a rate significantly slower than that of the control group. From these findings, it would appear that while the spleen is not important in man's defense against (or reactivity to) initial challenge with small doses of endotoxin,

loss of the antibody-producing activity of the spleen does constitute a significant impairment to subsequently acquired tolerance to endotoxin. It should again be emphasized, however, that for achievement of these results, tests for tolerance must be conducted with appropriate spacing of the toxin to reduce the contribution of the cellular refractory state, thus amplifying the protective role of antibody.

Discussion

The mechanisms by which man acquires tolerance to bacterial endotoxins are complex and still incompletely defined. Certain of the required extrapolations from animal studies to man permit only a tentative hypothesis. Nevertheless, a concept emerges that can now be critically tested. This concept holds (*1*) that pyrogenic tolerance to endotoxin is based primarily on enhanced resistance of hepatic Kupffer cells to the release of endogenous pyrogen and (*2*) that such resistance can be achieved either by direct cellular interaction with endotoxin or by antibodies to endotoxin, which protect these cells against injury due to the toxin. The direct cellular effect responsible for tolerance is transient and requires closely spaced or continuous exposures to endotoxin for maintenance; antibody-mediated protection is delayed but more enduring. The protective antibodies comprise those that react with antigens associated with O-specific groupings as well as those that react with common core antigens. When intact smooth endotoxins are used for immunization, the former antibodies appear to be the more prominent humoral protective component; when rough mutants are administered, the latter antibodies appear to comprise the most important humoral protective component. Accelerated clearance by the RES appears to be an ancillary protective mechanism that brings the endotoxin more rapidly to the refractory Kupffer cells. The host thus has at its disposal several mechanisms for acquiring resistance to endotoxin, and the diverse, often conflicting observations on pyrogenic tolerance can be explained by analysis of the interplay of these protective mechanisms.

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