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Research Article

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Mechanisms of Endotoxin Tolerance

THE ROLE OF THE SPLEEN

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ABSTRACT Splenectomy markedly impaired the pro-
duction of circulating anti-endotoxin antibodies during
the initial 10 days after i.v. administration of a Boivin
preparation of *Escherichia coli* endotoxin (ET) in both
rabbit and man. Increases in antibodies with secondary
(flocculating and bactericidal) activities were virtually
abolished, whereas increases in antibodies with primary
(binding) activity were significantly reduced. On the
basis of these findings, splenectomized rabbit and man
were employed to test the hypothesis that the early
phase (< 72 h) of pyrogenic tolerance to endotoxin is
independent of anti-endotoxin antibody but that such
antibody contributes significantly to the later phase
(≥ 72 h) of tolerance.

In the splenectomized rabbit, the initial pyrogenic
responses to ET and the subsequent tolerant responses
at 24 and 48 h were comparable to sham-operated con-
trols. Thereafter, splenectomized rabbits exhibited im-
paired ability to acquire further pyrogenic tolerance.
Such impairment was evidenced by significantly retarded
rates of tolerance commencing 72 h after daily i.v. in-
jections of ET and by significantly reduced tolerance
5 days after a single i.v. injection of ET. Similarly,
healthy splenectomized human volunteers exhibited py-
rogenic and subjective toxic responses to an initial i.v.
injection of ET comparable to volunteers with intact
spleens. Upon retesting 5 days later, however, the sple-
nectomized volunteers exhibited significantly reduced
levels of tolerance. When additional ET was subse-
quently administered, tolerance increased progressively
in all volunteers, but its rate of maturation was signifi-
cantly retarded in the splenectomized subjects. To re-
produce equivalent impairment of the late phase of
tolerance in volunteers with intact spleens, the immuniz-
ing doses of ET had to be decreased to levels extrapo-

lated to fall between 1/10 and 1/100 of those used in
the splenectomized subjects. The impairment of late-
phase pyrogenic tolerance after splenectomy appeared
to be the result of the concomitant suppression of anti-
endotoxin antibody production rather than the loss of
splenic reticuloendothelium required to clear and in-
activate the ET. This was evidenced by: (a) the sig-
nificant retardation in appearance of humoral factors
in the splenectomized rabbits capable of transferring
high levels of specific tolerance; (b) the inability to
impair the late phase of tolerance in human volunteers
with intact spleens until the immunizing doses of endo-
toxin were reduced to levels that virtually eliminated
circulating anti-endotoxin antibody production; (c) the
unimpaired development of tolerance in partially hepa-
tectomized rabbits lacking more functional reticuloendo-
thelium than splenectomized animals, but capable of
normal anti-endotoxin antibody synthesis.

It is concluded that in rabbit and man the spleen plays
no important role in the initial pyrogenic response to
i.v. ET, or in the early phase of pyrogenic tolerance.
The spleen, however, by virtue of its anti-endotoxin
antibody-producing capabilities, contributes significantly
to the late phase of tolerance after a single i.v. injec-
tion of ET and accelerates significantly its rate of ac-
quisition after repetitive i.v. injections of the toxin.

INTRODUCTION

The importance of antibody in the development of tol-
erance to the pyrogenic and toxic activities of gram-
negative bacterial endotoxin has been an area of con-
siderable controversy. Some investigators have con-
cluded that anti-endotoxin antibodies represent the pri-
mary underlying mechanism (1); others have concluded
that they play no demonstrable role (2–6). It is well
established that when small quantities of insoluble anti-

gens are administered i.v., the spleen constitutes the major site of antibody formation, particularly during the initial week of immunization (7–9). Since these are the conditions under which endotoxin tolerance is generally studied, splenectomy was employed in the present investigations as a means of suppressing anti-endotoxin antibody production. The findings, which quantitate the resulting impairment of tolerance, demonstrate the contribution of anti-endotoxin antibody and establish the role of the spleen during the development of pyrogenic tolerance to endotoxin in the rabbit and man.

METHODS

Syringes, needles, and glassware were either of the sterile, pyrogen-free disposable type or were heated overnight in a dry-air oven at 180°C to eliminate extraneous pyrogens. A stock preparation of a Boivin-extracted *Escherichia coli* endotoxin (ET),[1] serotype 0127B8 (Difco Laboratories, Detroit, Mich.) was employed throughout the study. This was diluted in sterile, pyrogen-free saline before use and stored at 4°C. The aliquots for human testing were preheated at 100°C for 10 min and shown to be bacteriologically sterile.

Volunteers for these studies were fully informed 26–51-yr-old male inmates of the Maryland House of Correction, Jessup, Md. Complete medical evaluations were performed to verify the fitness of each participant. The protocols for the studies were reviewed and approved by the University of Maryland Human Research Committee. In addition to man, healthy male New Zealand albino rabbits obtained from a uniform source and weighing approximately 2 kg were employed.

Splenectomy

Man. Splenectomized volunteers were healthy subjects whose spleens had been removed 7–11 yr previously after abdominal trauma. Endotoxin tolerance studies in these subjects were performed concomitantly with those in control subjects with intact spleens.

Rabbit. Animals were anesthetized with sterile, pyrogen-free pentobarbital (Diabutal, Diamond Labs, Des Moines, Iowa). The shaven abdominal wall was prepared with 2% tincture iodine and then washed with 70% alcohol and covered with sterile drapes, and the spleen was removed using sterile gloves and autoclaved instruments. Sham-operated animals were treated identically, except that the spleen was exposed through the incision and then replaced. The sterile silk sutures employed for closing the abdomen were removed after 7 days. Endotoxin tolerance studies were conducted concomitantly on the splenectomized and sham-operated animals after 3–4 wk of convalescence. After each study, the rabbits were sacrificed to verify completeness of splenectomy; the occasional animal with a small accessory spleen was excluded.

Partial hepatectomy

Anesthetization of rabbits and general sterile operative procedures were similar to those employed for splenectomy. The main liver mass, comprising approximately 75% of the total liver tissue, was extirpated after placing a sterile liga-

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[1] *Abbreviations used in this paper:* ET, *E. coli* endotoxin; 6-MP, 6-mercaptopurine.

ture around the portal area close to the inferior vena cava as described by McMaster and Drury (10). Free blood in the peritoneal cavity was removed by sterile sponging. The subsequent immediate mortality was 60%. Sham-operated animals were treated identically, except that the main liver mass was not ligated but exposed through the incision and then replaced. Endotoxin tolerance studies were conducted concomitantly on the hepatectomized and sham-operated animals after 2–3 wk of convalescence. Since liver regeneration after partial hepatectomy was rapid, deficits in reticuloendothelial function were verified 24 h after completion of the endotoxin studies by assay of the blood clearance and hepatic and splenic uptake of i.v. colloidal [198]Au. For each study, [198]Au tracer (Squibb E. R. and Sons, Inc., Princeton, N. J.) was added to nonradioactive colloidal gold, and assays were performed after i.v. injection of 2.5 mg/kg total colloid.

Pyrogen assay

Man. Studies were conducted in an air-conditioned room (70°F) starting between 8 and 9 a.m. with the volunteers confined to bed and covered with a light blanket. Flexible thermocouples were inserted 6 inches into the rectum. Temperatures were monitored by a central telethermometer immediately before and every half h for 7 h after i.v. injection of the 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 paper; a fever index of 100 reflected a 1°F rise in rectal temperature sustained for 1 h. As a result of diurnal variations, temperatures seldom returned to preinjection values. In such instances, the fever curves were extrapolated to base line. In addition to the febrile responses, toxic subjective reactions after endotoxin administration were graded as follows: 0 = asymptomatic; 1+ = mild headache, anorexia; 2+ = moderate headache, anorexia, chills; 3+ = severe headache, shaking chills, myalgia, nausea.

Rabbit. Animals were loosely restrained in stalls after acclimatization for 18–24 h in an air-conditioned room. Thermistor rectal probes were inserted 6 inches and connected to a central telethermometer. Temperatures were monitored 1–2 h before each experiment; animals exhibiting initial temperatures greater than 104°F or varying more than 0.5°F in any 30-min control period were excluded. After endotoxin injection, temperatures were monitored every 30 min for 5 h. Both the 3-h temperature increment proposed by Watson and Kim (11) and the area under the fever curve (fever index) were calculated. The former paralleled the sensitivity of the latter as previously reported (12), was simpler to calculate, and yielded smaller standard deviations; the 3-h temperature increment was, therefore, selected as the parameter for assay of febrile responsiveness. As emphasized in previous studies, rectal temperature sometimes fell below the starting base line by the 4th or 5th h; downward extrapolation of the 3-h increments to a line drawn between the initial and the 5-h sub-base-line temperature consistently reduced the standard deviations (13), and such extrapolation was routinely incorporated into the present assay system. Fig. 1 depicts the dose-response relationships in previously untested rabbits challenged with the standard ET preparation. It is evident that the sensitive portion of the dose-response relationship applies when the mean 3-h temperature increments fall between 1° and 4°F; the subsequent studies in rabbits were, therefore, conducted with dosages of endotoxin selected to evoke febrile responses within this sensitive range.
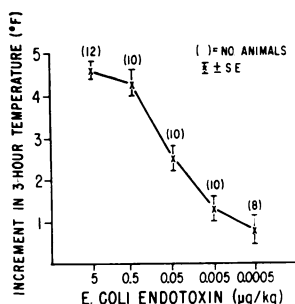
FIGURE 1 Relationship between ET dose and 3-h temperature increments in normal acclimatized rabbits.

Antibody determinations

Anti-endotoxin antibody titers, expressed as geometric means, were assayed by three methods: (a) the bentonite flocculation technique as described by Wolff et al. (14): (b) the bactericidal technique as outlined by Weidanz and Landy (15); and (c) a modification of the Farr technique. The latter was performed as follow: duplicate sets (1-ml aliquots each) of twofold dilutions of serum in saline were placed in plastic test tubes, starting at 1:20. 100 μg of unlabeled Boivin preparations of homologous (E. coli 0127B8) or of heterologous (Salmonella typhimurium) endotoxin suspended in 1 ml saline was added to each set of tubes, respectively. The homologous unlabeled endotoxin served to suppress both the nonspecific and the specific binding capacity of the serum; the heterologous unlabeled endotoxin served to suppress only the nonspecific binding capacity. The differences between the two sets of tubes, therefore, reflected the specific binding capacity. To determine this value, 10 min after addition of the unlabeled endotoxins, 1.0 μg ^{51}Cr-labeled homologous (E. coli 0127B8) endotoxin in 0.1 ml saline was added to all tubes with constant vortex stirring, and 10 min later the immune globulins (as well as all other proteins except for a trace of albumin as de-

tected electrophoretically) were precipitated by addition of 95% ethanol to yield a final concentration of 47%. Unlike the 50% ammonium sulfate employed in the usual Farr method, this concentration of ethanol precipitated minimal (10–15%) ^{51}Cr-labeled endotoxin from saline suspensions. The quantity of precipitated labeled endotoxin in serum was measured by centrifugation for 10 min at 1,500 g, removal of the upper half of the total volume (without disturbing the precipitate) and measuring radioactivity of the supranatant and corresponding infranatant in an automatic gamma well counter for sufficient time to permit reproducibility to within 5%. Subtraction of the value of the supranatant from the infranatant gave the radioactivity of the precipitate. The antibody titer was taken as that dilution of antiserum capable of specifically binding the ^{51}Cr-labeled endotoxin. Fig. 2 illustrates typical antibody titrations utilizing this modified Farr technique in the rabbit (panel A) and in man (panel B). Base-line preimmunization antibody titers were consistently less than 1:20.

RESULTS

Rabbit

Effect of splenectomy on the early and late phases of pyrogenic tolerance. To test the effect of splenectomy on late-phase pyrogenic tolerance, splenectomized and sham-operated rabbits were injected i.v. with sufficient ET to evoke fever within the sensitive dose-response range. Both groups responded comparably, Fig. 3A (day 0). Upon retesting 5 days later, both groups exhibited pyrogenic tolerance; splenectomized animals, however, exhibited significantly less tolerance ($P < 0.01$, t test). Indeed, the splenectomized animals developed more fever than did sham-operated controls given a

FIGURE 3 Effect of splenectomy on late phase pyrogenic tolerance. Panel A: note that splenectomy does not affect the initial pyrogenic responsiveness to endotoxin but does significantly impair anti-endotoxin antibody production and late phase tolerance 5 days after a single i.v. endotoxin injection in the rabbit. Panel B: a 10-fold increment in endotoxin dose (0.5 μg/kg) is required on day 5 to evoke febrile responses in animals with intact spleens comparable to the splenectomized group.
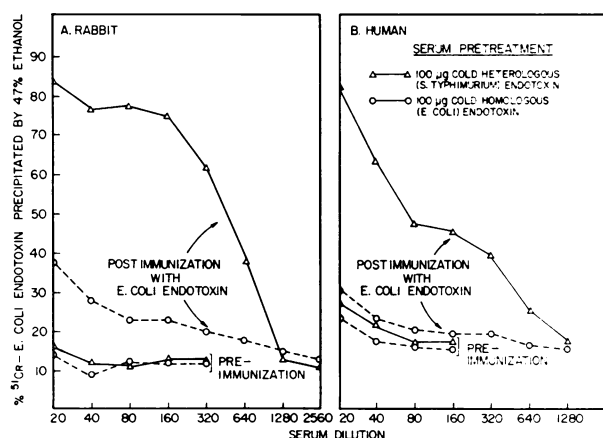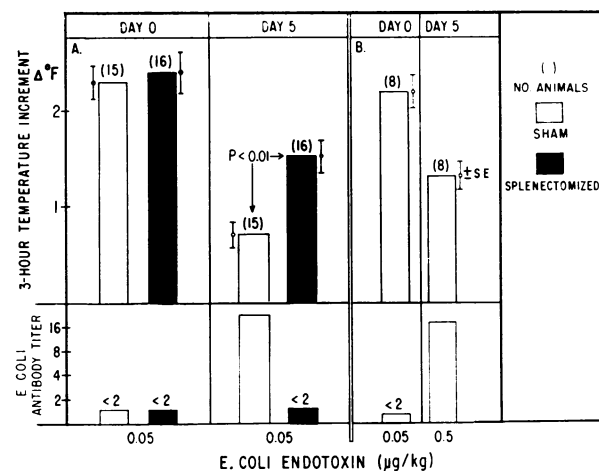

FIGURE 2 Typical anti-endotoxin antibody titrations utilizing the modified Farr technique. The endpoint for the postimmunization rabbit antiserum is 1:1,280 (panel A) and for the human antiserum 1:1,280 (panel B). Preimmunization titers in both species were consistently less than 1:20.

10-fold greater challenge dose of the endotoxin on day 5, Fig. 3B. As shown in the lower half of Fig. 3, an unequivocal elevation in bentonite-flocculating anti-endotoxin antibody was evident on day 5 in the sham-operated animals, whereas no detectable elevations developed in the splenectomized group.

The subsequent studies were performed to test the effect of splenectomy on the response to repetitive daily endotoxin injections. As in the previous studies, i.v. injections of ET sufficient to evoke fever within the sensitive dose-response range induced initial mean febrile responses of equivalent magnitude in splenectomized and sham-operated animals, Fig. 4 (day 0). Upon retesting 24 h later (day 1) with a 10-fold increment in endotoxin dosage, significant but equal tolerance was evident in both groups, the mean febrile responses being comparable to the day 0 reactions. Retesting with a further 10-fold increment in endotoxin dose on day 2 evoked enhanced responses but again revealed no differences in pyrogenic reactivity of the splenectomized and sham-operated animals. No effect of splenectomy was therefore apparent during the early phase of endotoxin tolerance. In contrast, at 72 h (day 3), when circulating anti-endotoxin antibodies initially became detectable in sham-operated but not in splenectomized animals, highly significant impairment of pyrogenic tolerance became discernible in the splenectomized group $(P < 0.001)$, Fig. 4. However, as the

daily i.v. endotoxin injections were continued, tolerance increased progressively and differences between the splenectomized and sham-operated groups decreased progressively despite the inability of splenectomized animals to develop circulating anti-endotoxin antibodies detectable by the bentonite flocculation technique, Fig. 4. Thus, splenectomy significantly retarded the rate of late-phase tolerance development after repetitive daily endotoxin administration but did not prevent its maturation.

Passive transfer of pyrogenic tolerance. To test the concept that the retarded late phase of pyrogenic tolerance in the splenectomized animal was attributable to depressed anti-endotoxin antibody synthesis rather than to loss of splenic reticuloendothelial cells required to clear and inactivate endotoxin, plasma transfer studies were performed. Pyrogen-free, pooled heparinized plasma (10 ml/kg) was infused i.v. into acclimatized healthy recipients 1 h before endotoxin challenge. Plasma from splenectomized donors obtained 24 h after the third endotoxin injection (given as outlined in Fig. 4) failed to transfer any detectable protection, whereas plasma from sham-operated controls transferred slight but definite protection $(P < 0.01)$, Fig. 5. By day 5, each pool of plasma (from another group of donors) transferred definite tolerance, although that from the splenectomized donors remained less effective $(P$
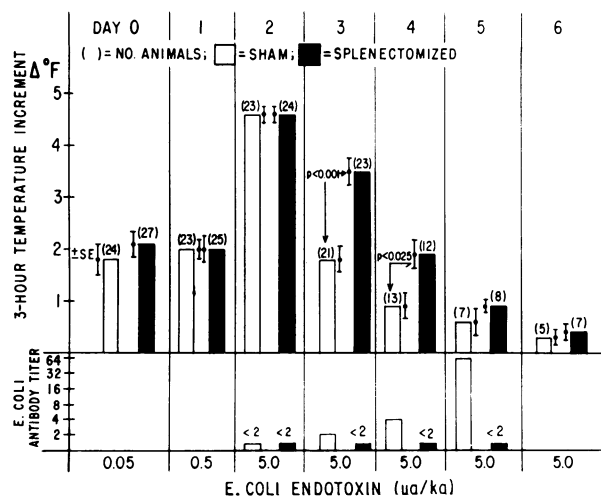

FIGURE 4   Effect of splenectomy on early and late phase pyrogenic tolerance. Note that splenectomy does not affect the initial pyrogenic responsiveness to endotoxin or early phase (days 1 and 2) tolerance but suppresses anti-endotoxin antibody production and significantly retards the rate of subsequent tolerance development after repetitive daily i.v. endotoxin injections in the rabbit. (The decrement in animals after day 3 reflects random removal for obtaining plasma for passive transfer studies).
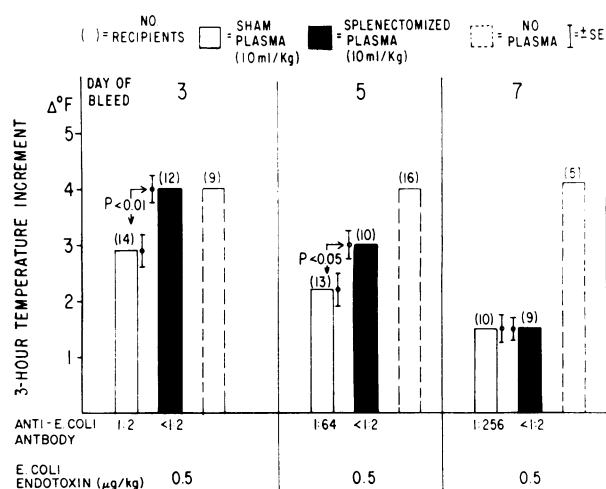
< 0.05). By day 7 (using a third donor group) differences were no longer discernible in the level of transferable tolerance, even though plasma from splenectomized animals exhibited no detectable increments in bentonite-flocculating anti-endotoxin antibodies. Although seemingly lacking anti-endotoxin antibody, when the day 7 plasma from splenectomized donors was tested for specificity of passive protection, high levels of specificity were found, i.e., 10 ml/kg plasma transferred minimal pyrogenic tolerance to a heterologous (*Salmonella typhimurium*) endotoxin despite careful testing within the sensitive dose-response range. These latter findings conform with recently reported studies on the high level of specificity of passively transferred pyrogenic tolerance to endotoxin (25). As a result of these findings, the day 7 pooled rabbit plasma from the splenectomized animals was reexamined for anti-endotoxin antibodies by use of the modified Farr technique. An antibody titer of 1:160 was found, compared to the preimmunization titer of < 1:20. The corresponding pooled plasma from the sham-operated animals exhibited a titer of 1:640 compared to the preimmunization titer of < 1:20. Thus, the splenectomized rabbit is not totally immunosuppressed and is capable of producing appreciable circulating anti-endotoxin antibodies by day 7 in response to i.v. endotoxin; these antibodies, however, are primarily of a type incapable of flocculating bentonite particles coated with the specific antigen.

To confirm the observations that splenectomy does not ablate anti-endotoxin antibody responses but rather primarily removes the source of flocculating type antibodies, normal, splenectomized, and 6-mercaptopurine-treated rabbits were immunized i.v. with 3 µg ET daily for 7 days and bled on day 8. Anti-endotoxin antibody titers were assayed by both the bentonite flocculation technique and by the modified Farr method (Table I). While antibody titers were equally suppressed in the splenectomized and 6-mercaptopurine-treated animals when measured by the bentonite flocculation method, the modified Farr method detected significantly greater rises in the splenectomized animals ($P < 0.005$). These findings militate against the possibility that the differences observed in antibody titers obtained by the bentonite flocculation and modified Farr techniques are attributable simply to differences in relative sensitivity of these two methods for the same antibody population. Additional evidence that splenectomy does not ablate anti-endotoxin antibody responses but rather selectively suppresses the production of antibodies with secondary activities was reflected in the ability of the modified Farr technique to detect rises in anti-ET antibody titers in splenectomized man despite no significant increments measurable by the extremely sensitive bactericidal method (see below).

TABLE I

Effect of Splenectomy and of 6-MP (18 mg/kg i.v. daily) on Anti-Endotoxin Antibody Titers 24 h after seven Daily i.v. Injections of 3 µg E. coli Endotoxin in the Rabbit*

Group	Bentonite flocculation titer	Modified Farr titer
Splenectomized		
S1	4	320
S2	4	320
S3	4	160
S4	4	320
S5	4	320
Geometric mean:	4	278
Controls		
C1	512	1,280
C2	64	640
C3	512	1,280
C4	2,048	2,560
C5	256	1,280
Geometric mean:	386	1,280
6-MP‡		
M1	8	320
M2	16	160
M3	8	20
M4	8	40
M5	16	80
M6	2	80
M7	4	80
M8	2	20
Geometric mean:	6	67
Controls‡		
C1	512	1,280
C2	256	640
C3	256	640
C4	256	320
C5	128	320
C6	256	640
Geometric mean:	256	562

* Preimmunization base-line titers were less than 2 by the bentonite flocculation technique and less than 20 by the modified Farr method.
‡ Sera kindly provided by Dr. Sheldon M. Wolff, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Effect of partial hepatectomy on pyrogenic tolerance. To further test the concept that the retardation of the late phase of pyrogenic tolerance in the splenectomized rabbit was mediated by the reduced anti-endotoxin anti-

Endotoxin Tolerance 1601

body synthesis and not by a loss of a portion of the reticuloendothelial system required to clear and inactivate the endotoxin, tolerance acquisition was quantitated 2–3 wk after partial hepatectomy. The partially hepatectomized animals, in contrast to the splenectomized group, exhibited no suppression of anti-endotoxin antibody synthesis and acquired pyrogenic tolerance at rates entirely comparable to sham-operated controls, Fig. 6. That reticuloendothelial phagocytic function was impaired in these partially hepatectomized rabbits was confirmed by measuring uptake of radioactive colloidal gold after administration i.v. Although approximately 75% of the total liver was initially ablated, restoration of phagocytically active reticuloendothelium occurred such that the hepatic depletion was now evident only by slight retardation in blood clearance of the colloidal [198]Au and by only a 5% decrease in the hepatic contribution to the total uptake of injected colloid, Fig. 7. This latter difference, however, was significant ($P <$ 0.005 at 3 h and $P < 0.02$ at 24 h). Since the splenic contribution to total uptake of injected colloid at 24 h amounted to only 1%, it is apparent that despite regeneration, the partial hepatectomy accomplished the removal of more functional reticuloendothelial tissue than did splenectomy. Moreover, since these data were obtained *after* completion of the endotoxin tolerance studies, even greater deficits of functional hepatic reticuloendothelium presumably existed during the actual period of endotoxin testing.

Man

The development of pyrogenic tolerance and of anti-endotoxin antibodies after i.v. *E. coli* endotoxin was compared in healthy volunteers with and without spleens. Since marked initial subjective toxic responses
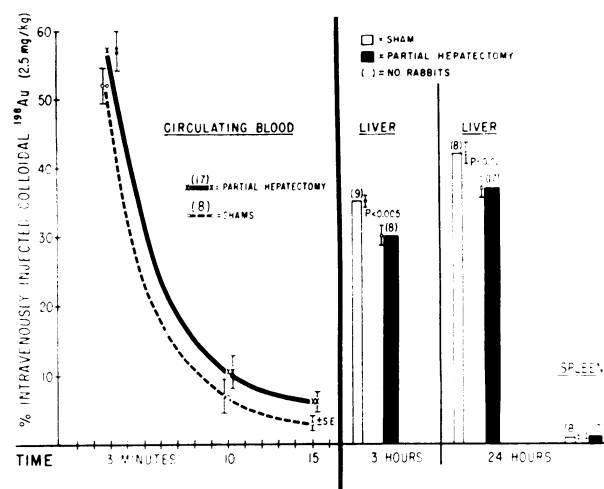
FIGURE 7 Confirmation of impaired hepatic reticuloendothelial phagocytic activity in the partially hepatectomized rabbits depicted in Fig. 6. Note that the reduction in the hepatic contribution to uptake of colloidal gold after partial hepatectomy exceeds the entire splenic contribution.

developed in all volunteers given the standard test dose of ET (0.01 μg/kg), and since augmented toxic responses rather than tolerance may occur in man when endotoxin is re-administered 24 h later (5, 16–19), retesting was delayed for 5 days, i.e., only the late phase of tolerance was studied. The results are outlined in Fig. 8 and can be summarized as follows:

(*a*) The spleen plays no important role in the initial response of man to submicrogram quantities of i.v. endotoxin. The initial (day 0) pyrogenic and subjective toxic responses of splenectomized volunteers were comparable to those of the controls (group A).

(*b*) The human spleen plays an important role in the production of anti-endotoxin antibodies to i.v. endotoxin, at least during the initial 10 days. Control volunteers given 0.01 μg/kg of the ET (group A) developed marked rises in bentonite flocculating and bactericidal antibody titers, whereas no detectable titers developed in the splenectomized group. The consistency of this pattern is especially striking when the antibody responses for individuals within each group are analyzed, Table II. In contrast to flocculating and bactericidal antibody, unequivocal although blunted increases in anti-endotoxin antibodies could be detected in the splenectomized volunteers by use of the modified Farr technique.

(*c*) The spleen plays an important role in the development of the late phase of tolerance after a single i.v. injection of endotoxin in man. Tolerance to ET developed 5 days after the initial injection in both control (group A) and splenectomized volunteers, but its development was unequivocally impaired in the splenectomized group. Thus, compared to their respective initial
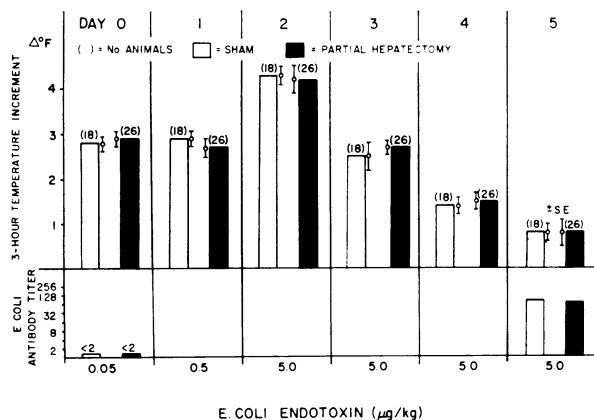
FIGURE 6 Effect of partial hepatectomy on pyrogenic tolerance. Note that partial hepatectomy does not suppress anti-endotoxin antibody production or retard late-phase pyrogenic tolerance in the rabbit.

TABLE II

Effect of Splenectomy on Bentonite Flocculating and Bactericidal Antibodies to i.v. E. coli Endotoxin (0.01 µg/kg i.v.) in Healthy Human Volunteers

Subject	Day...0*	1	2	3	4	5*	7*	1C*
Splenectomized								
B. C.	<2‡ (900)§	<2 (1,600)	<2 (1,420)	<2	<2 (800)	<2	<2 (50)	<2
J. O.	<2 (95)	<2 (58)	<2 (100)	<2	<2 (95)	<2	<2 (<32)	<2
J. P.	<2 (98)	<2 (58)	<2 (88)	<2 (74)	<2 (70)	<2 (76)	<2 (210)	<2
Controls								
L. G.	<2 (32)	<2	<2	<2	<2	32	64 (900)	128
A. M.	<2 (2,800)	<2 (3,000)	<2 (4,000)	<2	<2 (4,700)	64	512 (11,000)	512
R. B.	<2 (35)	<2	<2	<2	<2	128	256 (35,000)	512
E. W.	<2 (200)	<2	<2	<2	<2	128	256 (>100,000)	512
N. H.	<2 (275)	<2 (240)	<2 (255)	<2 (135)	<2 (940)	64 (1,120)	128 (5,200)	512 (12,000)
D. D.	<2 (350)	<2 (420)	<2 (440)	<2 (610)	<2 (1,300)	64 (12,000)	128 (96,000)	256 (>100,000)

* Serum for antibody obtained immediately before endotoxin injections. The endotoxin was given on days 0, 5, 7, and 10.

‡ Reciprocal of titer determined by bentonite flocculation technique.

§ Reciprocal of titer determined by bactericidal technique.

responses, the fever indices decreased by 35, 42, 45, 47, 58 and 68% in the control subjects whereas in the splenectomized group the decline was only 7, 23, and 31% ($P < 0.02$). When additional i.v. endotoxin injections were administered after the 5th day, tolerance increased in all volunteers, but its maturation was significantly retarded in the splenectomized group. Thus, compared to their respective initial responses, the fever indices decreased by 47, 56, 61, 71, 73, and 73% on day 7 and by 66, 66, 68, 75, 77, and 99% on day 10 in the control subjects whereas in the splenectomized group the decline was only 24, 38, and 48% on day 7 and 44, 47, and 69% on day 10 ($P < 0.02$ for day 7 and $P < 0.05$ for day 10). Fig. 8 depicts the mean rates of tolerance retardation (splenectomized vs. control group A).

(d) Quantitative studies further emphasize the importance of the human spleen for anti-endotoxin antibody formation and late-phase tolerance. The impairment of both these latter parameters seen in the splenectomized volunteers on day 7 could be reproduced in volunteers with intact spleens only by reducing the immunizing doses of ET to levels extrapolated to fall between 1/10 and 1/100 of those used in the splenectomized group (groups B and C, respectively).

(e) Late-phase tolerance in man bears no direct relationship to the initial pyrogenic or toxic responses to endotoxin. In volunteers with intact spleens, pyrogenic tolerance on day 7 was comparable regardless of whether immunization was carried out with 0.01 µg/kg (group A) or with 0.001 µg/kg (group B) ET. The former dose evoked severe pyrogenic and subjective toxic responses, while the latter evoked no detectable toxic reactions. The common denominator underlying these differing immunizing doses of endotoxin was their elicitation of substantial increments in anti-endotoxin antibodies at the time of tolerance testing. Only when the immunizing dose of the ET was reduced to 0.0001 µg/kg (group C), a quantity that elicited minimal increments in anti-endotoxin antibody, was day 7 tolerance significantly impaired.

DISCUSSION

It is now recognized that several mechanisms underlie the development of tolerance to the pyrogenic activity of gram-negative bacterial endotoxins. One of these mechanisms appears to be related to a direct effect of endotoxin upon the target cells that synthesize and release endogenous pyrogens. This mechanism, which has been termed the "refractory state" develops within hours,
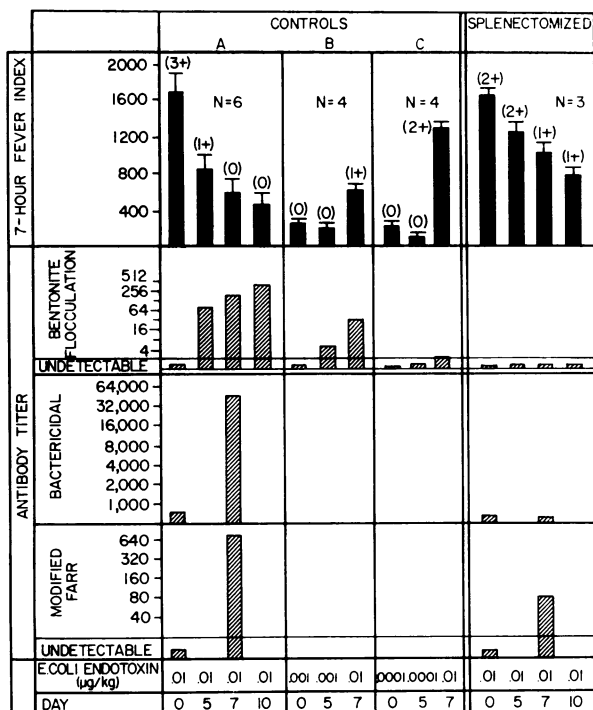
FIGURE 8 Effect of splenectomy on endotoxin tolerance in man. Comparison of responses of control subjects (group A) with the splenectomized group demonstrates that splenectomy does not affect the initial responsiveness of man to i.v. endotoxin but markedly impairs anti-endotoxin antibody production and significantly retards late phase pyrogenic tolerance acquisition. Responses of additional control volunteers immunized with 1/10 and 1/100 of the endotoxin dosages (groups B and C, respectively) permit quantitation of the high degree of anti-endotoxin antibody suppression and tolerance retardation in the splenectomized volunteers. (Numbers in parentheses above black bars represent subjective toxic responses; see text for grading. Brackets above black bars indicate 1 SE of the mean).

is specific for endotoxins as a class although lacking "O" specificity, is not transferable with serum, and wanes within several days after one i.v. endotoxin injection (13, 19–23). A second mechanism appears to be related to the activity of anti-endotoxin antibodies. This mechanism, which can be termed "immunity," develops only after several days (or more rapidly during an anamnestic response), is transferable with serum, possesses appreciable "O" specificity, and is relatively persistent (13, 23–25). When a single bolus of endotoxin is administered i.v. and pyrogenic tolerance is assessed at serial intervals thereafter, the refractory and immune mechanisms become apparent as temporally distinct early and late phases of tolerance (13, 23). When endotoxin is administered repetitively at daily (or more closely spaced) intervals, pyrogenic tolerance increases as the refractory and immune states appear to become

superimposed (26). When the daily injections of endotoxin are discontinued, pyrogenic tolerance decreases markedly during the subsequent several days but then persists at a significant residual level for at least several months in association with elevated specific anti-endotoxin antibody titers. These latter observations, documented by Mulholland et al. (21), have been postulated to reflect the rapid waning of the refractory state, exposing the more persistent underlying immune state (26). The present studies, utilizing the splenectomized host, were designed to test certain facets of the above hypothesis.

Although the splenic contribution to the total reticuloendothelial system (RES) clearance of circulating endotoxin is minor—less than 3% of i.v. endotoxin is fixed within the spleen (27)—the spleen plays a major role in the production of anti-endotoxin antibodies (28). If the concepts outlined above are valid, then splenectomy should not impair the development of the early (refractory) phase of pyrogenic tolerance but should significantly impair the later (immune) phase. Moreover, it would be predicted that the degree of impairment of tolerance would be dependent upon the schedule of endotoxin administration. Thus, if the late phase of pyrogenic tolerance after a single i.v. injection of endotoxin is indeed based primarily upon anti-endotoxin antibodies, it should be significantly diminished in the splenectomized host. In contrast, during repetitive daily i.v. injections of endotoxin not only do nonantibody tolerance mechanisms appear to be operative (e.g. the refractory state), but, in addition, during such hyperimmunization increasing amounts of anti-endotoxin antibodies would be derived from extrasplenic sites. Landy et al. have demonstrated that although the spleen is the principal organ involved in antibody responses to i.v. endotoxin, other lymphoid tissues participate, including bone marrow, peripheral blood leukocytes, and thymus (28). The present studies confirm the participation of extrasplenic sites in anti-endotoxin antibody production and indicate that in both rabbit and man such extrasplenic antibody possesses minimal secondary (flocculating and bactericidal) activities. Since such anti-endotoxin antibodies are nevertheless capable of transferring pyrogenic tolerance (25, 29) and since alternative protective mechanisms appear to be activated during closely spaced endotoxin injections, splenectomy would be expected only to retard, rather than prevent the development of pyrogenic tolerance during repetivitive daily i.v. administration of endotoxin. The present studies, employing doses of endotoxin carefully selected to evoke febrile responses within the sensitive dose-response range, support these concepts. Thus, whereas splenectomy failed to affect the initial pyrogenic responses or the development of pyrogenic tolerance during the subsequent 48 h after i.v. ET administra-

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tion, unequivocal impairment of the later phase of pyrogenic tolerance was observed in both splenectomized rabbits and man. This was evidenced by significantly reduced levels of tolerance 5 days after a single i.v. injection of endotoxin and by significantly retarded rates of tolerance development commencing 72 h after repetitive daily i.v. administration of endotoxin. The defect in the late phase of tolerance in man was such that to produce equivalent impairment in volunteers with intact spleens the immunizing doses of endotoxin had to be reduced to levels extrapolated to fall between 1/10 and 1/100 of those used in the splenectomized volunteers. The impairment of late-phase tolerance developed concomitantly with the impaired capacity to produce circulating anti-endotoxin antibodies. In addition to this temporal relationship, several other observations indicated that such suppression of antibody production was causally related to the impairment of late phase tolerance. Thus, (a) the appearance of humoral factors capable of passively transferring high levels of specific pyrogenic tolerance was significantly retarded after splenectomy in the rabbit; (b) to produce impairment of the late phase of pyrogenic tolerance in human volunteers with intact spleens the immunizing doses of endotoxin had to be reduced to levels that virtually eliminated production of circulating anti-endotoxin antibodies; and (c) the late phase of pyrogenic tolerance was not impaired in partially hepatectomized rabbits lacking significantly more functional RES than splenectomized animals but capable of normal anti-endotoxin antibody production. The findings, considered collectively, indicate that the impairment of the late phase of pyrogenic tolerance after splenectomy is based primarily upon suppression of anti-endotoxin antibody production rather than upon loss of splenic reticuloendothelium required to clear and inactivate endotoxin.

In earlier studies in which antibody synthesis was suppressed in rabbits by 6-mercaptopurine (6-MP), no impairment of pyrogenic tolerance to endotoxin was observed (6). This probably can be attributed to the fact that with the dosages employed (18 mg/kg) 6-MP does not eliminate anti-endotoxin antibody synthesis and that the tolerant responses were not quantitated until 24 h after seven successive daily i.v. endotoxin injections. The small but nevertheless unequivocal increments in circulating anti-endotoxin antibodies in the 6-MP-treated animals, together with the additional tolerance mechanisms activated by the repetitive daily endotoxin injections, could preclude detection of tolerance impairment late in the course of such toxin administration. Studies in our laboratory in rabbits given seven successive daily i.v. injections of 6-MP and ET confirm those of Wolff and co-workers (6) in that a high level of pyrogenic tolerance developed by day 8,

comparable to that in non-6-MP-treated controls. When each of the preceding daily responses was examined, however, it was observed that the 6-MP-treated animals exhibited unaltered early phase pyrogenic tolerance but significant retardation of the late phase, commencing at 72 h, coincident with the onset of impaired circulating anti-endotoxin antibody production and identical with that seen in the splenectomized rabbits. Moreover, utilizing quantitative plasma transfer studies, this delay in tolerance maturation was found to parallel the delayed production of humoral factors capable of transferring high levels of specific tolerance. Pyrogenic tolerance to endotoxin has also been reported to be unimpaired in patients with "agammaglobulinemia" (4, 5). However, in those patients studied for tolerance only the absence of antibody responses to the H antigen of the typhoid vaccine was recorded; the absence of anti-endotoxin antibody responses was not documented, nor were direct binding-assay techniques, the importance of which is evidenced from the present data, utilized to exclude such antibody responses. Moreover, since the endotoxin was administered repetitively at daily intervals, suppression of anti-endotoxin antibody-synthesizing capability would be expected primarily to retard rather than prevent the development of late pyrogenic tolerance. In this regard, it is of interest that in the one illustration that depicts in detail the daily febrile responses of a patient with "agammaglobulinemia" and of a normal control subject, impressive retardation of pyrogenic tolerance is demonstrated in the former (5). Thus, the earlier data do not exclude a significant contribution of anti-endotoxin antibodies to the late phase of pyrogenic tolerance. Rather, recent reports from a number of laboratories all provide additional evidence supporting the concept of a significant contribution of such antibodies, especially those possessing "0" specificity, to this phase of endotoxin tolerance (13, 23–25, 30–35, 35a).

Based upon the above considerations the spleen in both rabbit and man can be regarded as an organ that is not important for protection against the initial pyrogenic and subjective toxic responses to microgram quantities of i.v. endotoxin, or essential for tolerance acquisition to this toxin but that by virtue of its anti-endotoxin antibody-forming potential contributes significantly to the late phase of pyrogenic tolerance after a single i.v. endotoxin injection and enhances significantly the rate of its development after repetitive daily i.v. administration of the toxin. These conclusions are consistent with and extend the findings from other laboratories indicating that the spleen is not important for protection of the guinea pig (36) or mouse (37) against the lethal activity of endotoxin, or for tolerance to this activity when assessed 24 h after five-daily sublethal injections (37). It is stressed that the present conclusions are re-

stricted to pyrogenic tolerance that develops to an endotoxin derived from a smooth (wild type) gram-negative bacterial strain. The contribution of the spleen to late-phase tolerance against endotoxins from rough gram-negative bacterial mutant strains or the purified lipid A, wherein antibodies to common core and to lipid A rather than to "0" specific antigens appear to be primarily involved (25, 31, 38, 39), remains to be defined.

ACKNOWLEDGMENTS

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