MECHANISMS OF ENDOTOXIN TOLERANCE

IV. Specificity of the Pyrogenic Refractory State during Continuous Intravenous Infusions of Endotoxin*

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When a constant dose of bacterial endotoxin is administered to normal rabbits or healthy volunteers as a single intravenous injection at 24-hr intervals, approximately 1 wk is required for induction of maximum pyrogenic tolerance (1, 2). In contrast, when bacterial endotoxins are infused continuously at a constant rate, maximum febrile unresponsiveness can be achieved within hours; in man, the subjective toxic reactions wane rapidly in parallel with the febrile response. To distinguish such acute unresponsiveness to endotoxin from the tolerant state induced by repeated daily single intravenous injections, the term "pyrogenic refractory state" has been employed (3). Certain of the mechanisms underlying this pyrogenic refractory state have been explored in the previous studies (3) and it was concluded that (*a*) refractoriness resulted from inability of the host to continue to mobilize endogenous pyrogen during sustained endotoxemia, and (*b*) this in turn was consistent with specific cellular desensitization to endotoxin. The role of nonspecific mechanisms, however, including generalized depletion of "available" endogenous pyrogen, could not be excluded. The present studies further explore the concept that the pyrogenic refractory state is mediated by specific desensitization to endotoxin at the cellular level.

Materials and Methods

All syringes, needles, and glassware were either of the sterile, pyrogen-free disposable type (Burron Medical Products, Inc., Bethlehem, Pennsylvania), or were heated overnight in a dry-air oven at 180°C to eliminate extraneous pyrogens.

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Pyrogen Assay.—Polyethylene tubing (PE 90, Clay-Adams, Inc., New York), previously rinsed with pyrogen-free, sterile saline was inserted into the marginal ear vein of healthy male New Zealand rabbits (1.7 to 2.3 kg) and taped securely. Indwelling thermocouples were placed 6 in. into the rectum. The animals were loosely restrained by chain collars in fiberglass stalls and acclimatized for 18 to 24 hr in an air-conditioned room. *Escherichia coli* endotoxin (O127:B8),[1] diluted in physiologic sterile saline, was then administered using a constant infusion pump (Harvard Apparatus Co., Inc., Dover, Massachusetts) which ensured accurate and reproducible flow rates. Pyrogen-free, sterile tubing (Baxter Laboratories, Inc., Morton Grove, Illinois) conducted the endotoxin from the syringe to the ear vein catheter; total volumes of endotoxin preparations infused were less than 15 ml/kg. Temperatures were monitored for 1 hr before and every ½ hr during the endotoxin infusion by means of a telethermometer (Yellow Springs Instrument Company, Inc., Yellow Springs, Ohio). Animals with baseline temperatures above 104°F or varying more than 0.3°F in any 30 min preinfusion control period were discarded. Groups of 4 to 10 rabbits were used for each study, and mean fever curves were constructed by averaging the temperature increments at each 30 min period; fever indices were calculated from the area under the curve obtained by plotting these increments as a function of time on standard graph paper. A fever index of 100 reflected 1°F rise in mean rectal temperature sustained for 1 hr. Intravenous infusions of the *E. coli* endotoxin at 18×10^{-4} μg/min produced mean febrile responses within the sensitive dose-response range (3); this rate was therefore employed as the "standard" in each study unless otherwise specified.

Pyrogens Other than Endotoxin.—

Type A influenza virus, PR 8 strain:[2] Virus was harvested from allantoic fluids after the 16th chick embryo passage and stored at −70°C. The virus was thawed and diluted in pyrogen-free saline immediately prior to intravenous injection.

Old tuberculin, 4× United States standard (lot 2723-72).[3] The tuberculin was diluted in pyrogen-free saline to concentrations approximating 100 mg/ml. 100 mg of this preparation administered as a single intravenous bolus produced no significant febrile responses in nonsensitized rabbits. Sensitization to tuberculin was induced either by a single intravenous innoculation of 0.25 ml BCG. vaccine (Glaxo Laboratories, Ltd., Greenford, Middlesex, England) containing approximately 1×10^6 viable *Mycobacteria*, or by innoculation of 0.1 ml of complete Freund's adjuvant (Difco Laboratories) into each foot-pad concurrent with 6 subcutaneous innoculations of 0.1 ml each into the nape of the neck; 3 wk later, a 0.1 ml booster was administered in the latter site.[4] Pyrogen studies were carried out 4 to 6 wk after the initial sensitizing injections.

Staphylococcal enterotoxin, type B:[5] The pyrogenic activity of this toxin has been documented previously and is not dependent upon endotoxin contamination (6, 7). Toxin was diluted in pyrogen-free saline and stored at 4°C.

Pneumococcal suspensions: These were prepared by innoculation into trypicase soy broth of an isolate recovered from the cerebrospinal fluid of a patient with meningitis. The broth was incubated overnight, the pneumococci harvested, killed by heating to 60°C for 1 hr,

[1] A Boivin type preparation (4) supplied by Difco Laboratories, Detroit.

[2] Kindly supplied by Dr. Yasushi Togo, Department of Medicine, University of Maryland School of Medicine, Baltimore.

[3] Kindly supplied by Dr. Murray Cooper, Lederle Laboratories, Pearl River, New York.

[4] Indebtedness for this method of sensitization is expressed to Dr. Gardner Middlebrook, Department of International Medicine, University of Maryland School of Medicine, Baltimore.

[5] A highly purified and homogenous protein prepared by Dr. Edward J. Schantz and coworkers (5) and supplied by The United States Army Medical Unit, Fort Detrick, Frederick, Maryland.

washed with pyrogen-free saline, and resuspended in pyrogen-free saline at a concentration of 3×10^8/ml. To minimize changes in microbial concentration during prolonged intravenous administration, 1.5% gelatin was added to produce a maximally viscous infusable preparation. Commercially available gelatin, as well as a specially prepared pyrogen-free, sterile gelatin preparation[6] was employed. 2 ml of an 8% solution of this latter gelatin preparation evoked no fever when injected intravenously into 4 normal acclimatized rabbits as a single bolus.

In vitro Detoxification of Endotoxin.—Groups of rabbits refractory to the standard rate of *E. coli* endotoxin infusion were exsanguinated by cardiac puncture into heparin-coated syringes utilizing aseptic and pyrogen-free precautions. The abdominal wall, which had been shaven and treated with iodine and alcohol, was immediately opened and the anemic liver removed, placed in iced pyrogen-free saline (1:1 w/v) and promptly homogenized for 1 min at high speed in a pyrogen-free micro-Waring blendor. Plasma and liver homogenates were similarly prepared from normal rabbits. All plasma samples were stored at −20°C until use; liver homogenates were tested immediately. *E. coli* endotoxin was added to comparable volumes of normal and refractory phase liver homogenates, and incubated at 39°C (normal rabbit core temperature). Aliquots were removed and assayed for pyrogenicity at 0 time, and after 15 and 60 min using groups of 4 acclimatized normal recipients for each determination. Mixtures of the *E. coli* endotoxin and normal or refractory phase plasma were similarly tested at 0 time and after 60 min incubation. The quantity of endotoxin added to the plasma and liver homogenates was selected to produce febrile responses for the 0 time assays near the top of the sensitive portion of the dose-response relationship; the incubation times were selected so that sufficient endotoxin activity remained to induce fever within the sensitive dose-response range.

Splenectomy.—Rabbits were anesthetized with sterile, pyrogen-free pentobarbital (Diabutal, Diamond Laboratories, Inc., Des Moines, Iowa). The shaven abdominal wall was treated with iodine and alcohol, covered with sterile drapes, and splenectomy performed using sterile gloves and instruments. Sham-operated animals were treated identically except that the spleen was merely brought out through the incision and replaced. Sutures were removed after 7 days. Endotoxin infusion studies were conducted after 3 to 4 wk of convalescence. Following each study, the animals were sacrificed to verify completeness of splenectomy; the occasional rabbit with a small accessory spleen was discarded.

RESULTS

Responsiveness of Endotoxin Refractory Rabbits to Endogenous Pyrogen.—Plasma obtained from rabbits during the febrile phase of endotoxin infusion induced immediate monophasic pyrogenic responses when reinfused during the refractory phase (3). Such observations form a cornerstone for the concept that the refractory state is attributable to inability of the host to continue to mobilize, rather than respond to, endogenous pyrogen. These findings, however, do not indicate that the refractory animal remains *fully* responsive to endogenous pyrogen, i.e. whether in addition to failure to mobilize endogenous pyrogen, impaired thermoregulatory responsiveness contributes to the refractory state. Standardized volumes of endogenous pyrogen were prepared from normal rabbit donors by pooling plasma drawn 2 hr after intravenous injection of 5 μg/kg *E. coli* endotoxin; 10 ml/kg aliquots of such plasma induced prompt monophasic fevers in normal recipients such that the 2 and 3 hr temperature

[6] Kindly supplied by Dr. Dee Tourtellotte, Knox Gelatine, Inc., Camden, N.J.

increments fell sharply as the dose of endogenous pyrogen was reduced. Animals refractory to the standard rate of *E. coli* endotoxin infusion exhibited no appreciable blunting of responsiveness to this endogenous pyrogen, Fig. 1.

Responsiveness of Endotoxin Refractory Rabbits to Endotoxin.—To evaluate further the specificity of the endotoxin pyrogenic refractory state, animals were rendered refractory to the standard rate of *E. coli* endotoxin infusion and the degree of impairment of febrile responsiveness to a single intravenous bolus of endotoxin then contrasted with that to heterologous pyrogens known to

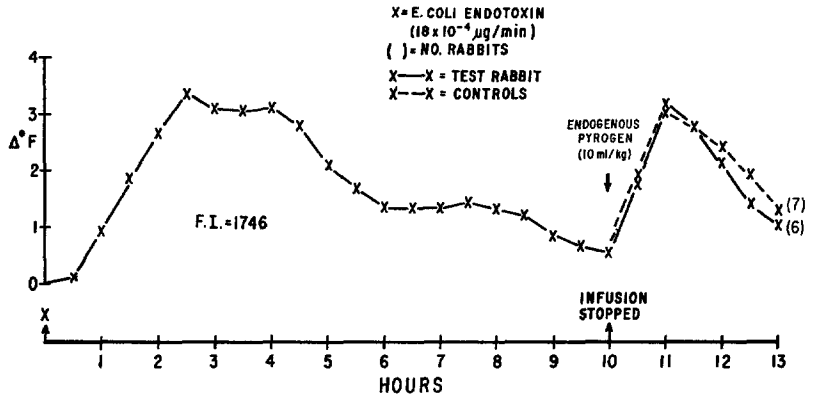

FIG. 1. Maintenance of febrile responsiveness to preformed endogenous pyrogen during the endotoxin-induced pyrogenic refractory state. The endogenous pyrogen was prepared from pooled rabbit plasma obtained 2 hr after an initial intravenous injection of 5 µg/kg *E. coli* endotoxin. (F.I., fever index).

liberate endogenous pyrogen. The single dose of *E. coli* endotoxin employed for testing, 0.05 µg/kg, induced mean 5-hr febrile responses in normal animals within the sensitive dose-response range, Fig. 2 (*control animals*). Refractory animals responded to this test bolus of endotoxin with fever indices less than those of normal animals receiving $\frac{1}{10}$ this dose, Fig. 2. Indeed, from extensive dose-response data, the response was equivalent to that of normal rabbits receiving $\frac{1}{50}$ the test dose, and was consistently monophasic, in contrast to biphasic fevers elicited in all control animals.

Responsiveness of Endotoxin Refractory Rabbits to Influenza Virus.—Groups of rabbits refractory to the standard rate of *E. coli* endotoxin infusion were injected intravenously with a single bolus of influenza virus. The dose of virus was chosen: (*a*) to induce mean 5-hr febrile responses in normal controls approximating that produced by the test bolus of endotoxin used in the previous study; and (*b*) to produce fever within the sensitive portion of the dose-response range, Fig. 3 (*control animals*). In contrast to endotoxin, febrile responses to

virus were not significantly blunted during the endotoxin refractory state, Fig. 3.

Responsiveness of Endotoxin Refractory Rabbits to Old Tuberculin.—To assess febrile responsiveness of specifically sensitized rabbits to old tuberculin during the endotoxin refractory state, a dose-response relationship for old tuberculin

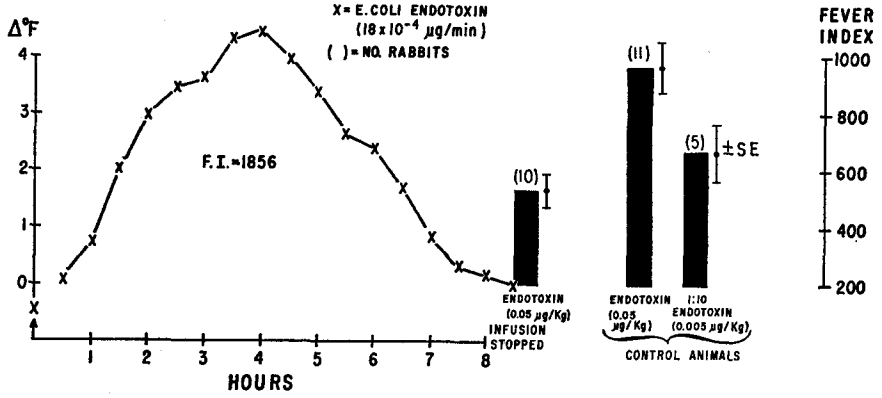

FIG. 2. Impairment of febrile responsiveness to a single intravenous test bolus of *E. coli* endotoxin during the endotoxin-induced pyrogenic refractory state. From additional dose-response data, the mean fever index of the refractory animals was equivalent to that of normal animals receiving 1/50 the test dose.

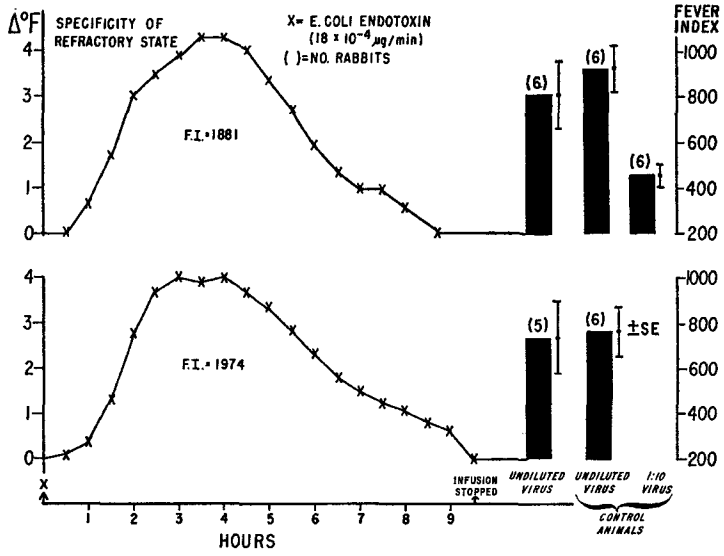

FIG. 3. Maintenance of febrile responsiveness to influenza virus during the endotoxin-induced pyrogenic refractory state.

was first established. This presented a difficult problem since great variability in individual febrile responses to an initial intravenous injection of old tuberculin was encountered despite identical tuberculin sensitization schedules.[7] Advantage was taken of observations by Moses and Atkins that the mean 5 hr febrile response of tuberculin-sensitized animals to a second intravenous dose of 100 mg of old tuberculin was but minimally depressed when this dose was readministered 3 days after the first (8). The present studies confirm this point, and also demonstrate that 10 mg of old tuberculin given 3 days after an initial 100 mg challenge evokes appreciably lower fever. Thus the second

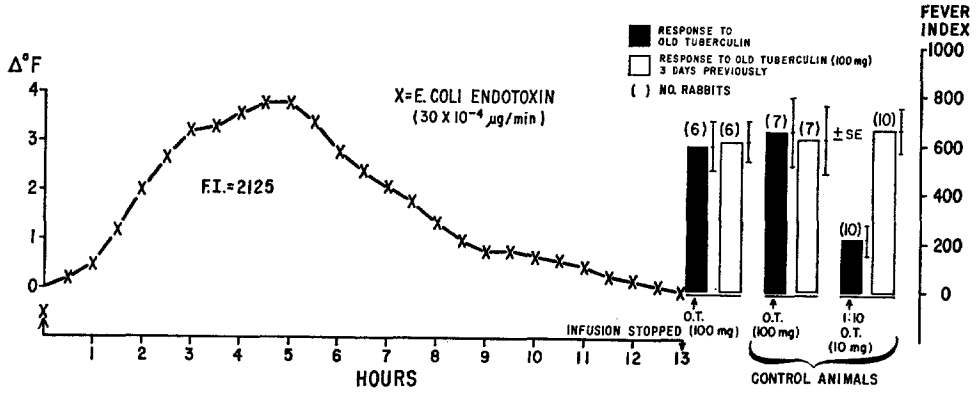

FIG. 4. Maintenance of febrile responsiveness to old tuberculin (O.T.) in specifically sensitized rabbits during the endotoxin-induced pyrogenic refractory state. (See text for method of selection of the tuberculin-sensitized animals).

challenge with 100 mg of old tuberculin after a 3 day interval not only evokes a predictable mean 5 hr febrile response, but also one which falls within a sensitive dose-response range, Fig. 4 (*control animals*). A group of tuberculin-sensitized animals was now selected which reacted to an initial intravenous injection of 100 mg old tuberculin comparably to the control groups described above. 3 days later these animals were rendered refractory to a continuous *E. coli* endotoxin infusion (a somewhat higher than the standard rate of the endotoxin infusion was employed) and rechallenged with 100 mg of old tuberculin; no significant blunting of febrile responsiveness was detected, Fig. 4.

Responsiveness of Endotoxin Refractory Rabbits to Staphylococcal Enterotoxin.— As with the tuberculin studies, animals were preselected to permit quantitation of pyrogenic responsiveness to the staphylococcal toxin during the endotoxin refractory state. Preliminary studies indicated that on day 1 some normal rabbits responded with high 5-hr fever indices to an initial intravenous injec-

[7] Sensitization induced with complete Freund's adjuvant.

tion of 1 μg/kg enterotoxin whereas others reacted minimally. The former animals were rejected and the latter tested on day 4 with 10 μg/kg enterotoxin. Animals with 5-hr fever responses to 10 μg/kg appreciably greater than to 1 μg/kg (i.e. those reacting in a sensitive dose-response range) were retested on day 7 with 10 μg/kg enterotoxin; the mean 5 hr fever response was found to be reproducible, Fig. 5 (*control animals*). Groups of animals, comparably selected for their febrile responses to 1 and 10 μg/kg enterotoxin on days 1 and 4 respectively, were then rendered refractory to the standard rate of *E. coli* endotoxin infusion on day 7 and retested with 10 μg/kg enterotoxin. No blunting of febrile responsiveness to enterotoxin was detectable; indeed, responsiveness appeared enhanced, Fig. 5.

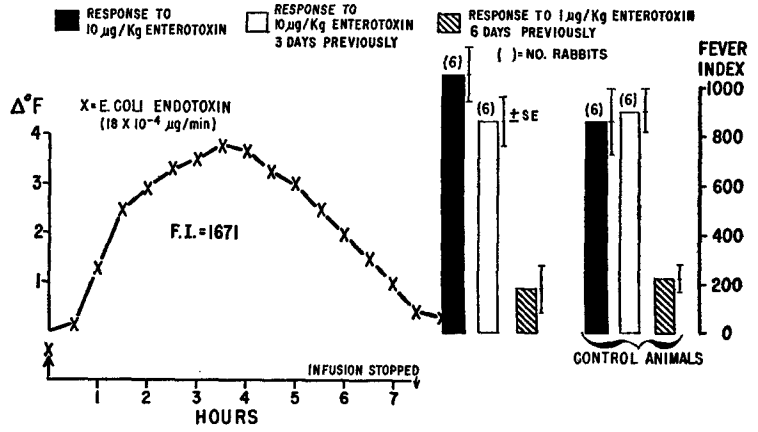

FIG. 5. Maintenance of febrile responsiveness to staphylococcal enterotoxin B during the endotoxin-induced pyrogenic refractory state. (See text for method of selection of animals).

Febrile Responses to Continuous Infusions of Old Tuberculin.—The febrile responses of control and tuberculin-sensitized rabbits to a continuous intravenous infusion of old tuberculin at a constant rate, 0.4 mg/min, are shown in Fig. 6. The old tuberculin, in the dosage employed, possessed minimal but sustained pyrogenic activity for nonsensitized control rabbits which became apparent only after several hours of infusion. The cause of such fever is at present unknown, but because of its delay and persistence, it cannot be attributed simply to endotoxin contamination. The febrile responses of the specifically sensitized rabbits were significantly greater, and animals sensitized with both BCG vaccine and complete Freund's adjuvant were more responsive than those sensitized only with the latter. In all sensitized animals, a pyrogenic refractory state developed to the continuous infusion of old tuberculin in a fashion resembling that produced by endotoxin. Moreover, as with

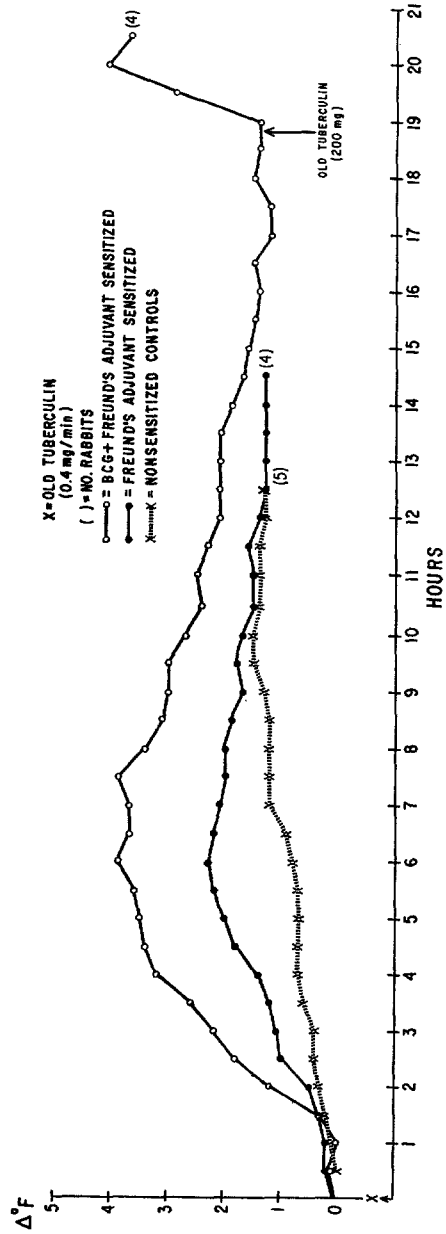

FIG. 6. Pyrogenic response patterns to continuous intravenous infusions of old tuberculin in specifically sensitized and nonsensitized rabbits. Note the slight but sustained pyrogenic activity of the old tuberculin for the nonsensitized animals and the development of the pyrogenic refractory state in the sensitized animals.

endotoxin, this refractory state was relative since the introduction of a large (200 mg) bolus of old tuberculin reevoked a brisk febrile response, Fig. 6. There were, however, certain definite differences from the endotoxin response: (*a*) the latent period before the onset of fever was longer with comparably pyrogenic quantities of old tuberculin (1.5 hr compared to 0.5 hr for endotoxin); and (*b*) the onset of defervescence was more delayed (6 to 8 hr compared to 3 to 5 hr for endotoxin).

Febrile Responses to Continuous Infusions of Heat-Killed Pneumococci.—In contrast to endotoxin and old tuberculin, continuous intravenous infusions of heat-killed pneumococci stabilized in gelatin evoked sustained febrile responses.

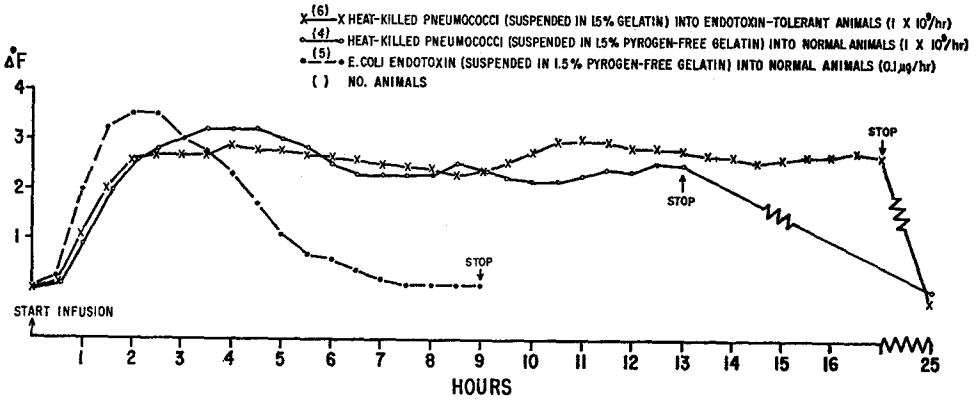

FIG. 7. Maintenance of the pyrogenic response during continuous intravenous infusions of heat-killed, gelatin-stabilized pneumococcal suspensions in the rabbit. Note the contrasting rapid development of the pyrogenic refractory state to endotoxin in gelatin, identical to that of endotoxin in saline.

The initial gelatin preparation employed, obtained commercially, was pyrogenic per se and the pneumococcal suspensions were therefore infused into endotoxin-tolerant recipients. When the heat-killed washed pneumococci were suspended in specially prepared pyrogen-free, sterile gelatin and infused into normal recipients, comparably sustained febrile responses were again observed, Fig. 7. In further contrast to endotoxin (3), restart of the pneumococcal infusions on day 2 reelicited the initial temperature elevations for the duration of the infusion (6 hr); restart of the infusion on day 3 evoked identically sustained responses. Finally, to demonstrate that endotoxin contamination of the gelatin did not account for the sustained fever, control studies were performed with standard rates of infusion of *E. coli* endotoxin suspended in the pyrogen-free gelatin; the refractory state developed as if the endotoxin were suspended in pyrogen-free saline, Fig. 7.

Effect of Fresh Whole Blood during the Endotoxin Refractory State.—Hepa-

rinized rabbit blood (40 to 75 ml) drawn 5 to 10 min previously from normal donors was infused rapidly during the pyrogenic refractory state. Despite the transfer of an average of 4×10^8 leukocytes (1.6×10^8 granulocytes) and continuance of the *E. coli* endotoxin infusion at the original rate, no restoration of febrile responsiveness was detectable. The immediate fall in rectal temperature, followed by the return to baseline, Fig. 8, was identical to that observed in groups of normal control animals receiving comparable blood transfusions.

Endotoxin Inactivation by Refractory Phase Plasma and Liver Homogenates.—Plasma and liver homogenates were prepared from normal and from endotoxin

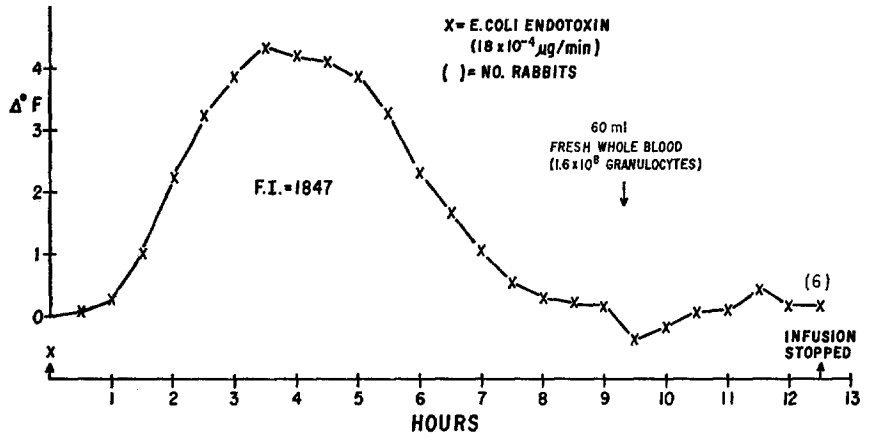

FIG. 8. Inability of normal fresh whole blood containing an average of 1.6×10^8 granulocytes to restore febrile responsiveness during the endotoxin-induced pyrogenic refractory state. The immediate fall in rectal temperature followed by return to baseline was identical to the responses in normal control rabbits receiving comparable blood transfusions.

refractory donors and incubated with *E. coli* endotoxin for various periods in vitro (see Materials and Methods). Tissues from the endotoxin refractory donors exhibited no enhanced ability to inactivate the pyrogenic activity of the endotoxin, Fig. 9.

Influence of Splenectomy on the Endotoxin Refractory State.—Specific antibody responses to single intravenous injections of *E. coli* endotoxin can be drastically suppressed in both rabbit and man by prior splenectomy (9). To test the concept that such specific antibody production is not concerned in the pathogenesis of the endotoxin refractory state, continuous infusions of *E. coli* endotoxin were administered at standard rates to splenectomized and sham-operated rabbits. Splenectomy failed to inhibit the development of the pyrogenic refractory state, Fig. 10.

The above studies permitted evaluation of the role of antibody in the pro-

duction of the intensified dermal inflammatory responses to endotoxin previously observed during the pyrogenic refractory state in the rabbit (3). Duplicate intradermal injections of 100 mg *E. coli* endotoxin in 0.2 ml saline were placed into the shaven anterior abdominal wall of the endotoxin refractory splenectomized and sham-operated rabbits. Gross inflammatory

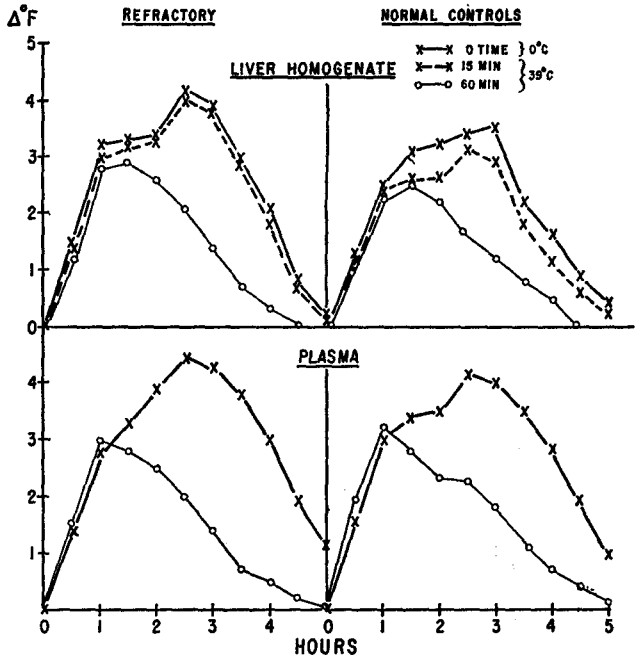

FIG. 9. Demonstration of the comparable capacities of plasma and of liver homogenates from normal and from *E. coli* endotoxin refractory donors to inactivate *E. coli* endotoxin during incubation at 39°C. Each curve represents the mean febrile response of 4 normal acclimatized recipient rabbits.

reactions 24 hr later fell into two distinct groups: large erythematous and edematous lesions in the sham-operated animals and suppressed responses in the splenectomized group, Fig. 11. Gross dermal inflammatory responses of control splenectomized and sham-operated animals, not infused intravenously with endotoxin, were intermediate and comparable to those reported previously in normal control animals (3).

DISCUSSION

When bacterial endotoxins are infused intravenously into healthy volunteers or normal rabbits at constant rates, rectal temperatures increase progressively during the initial several hours, plateau, then decline toward baseline.

This rapidly developing pyrogenic refractory state appears to be based upon progressive inability of the host to continue to mobilize endogenous pyrogen despite the continuing exogenous pyrogenic stimulus (3). The present studies demonstrate the specificity of such paralysis of endogenous pyrogen mobiliza-

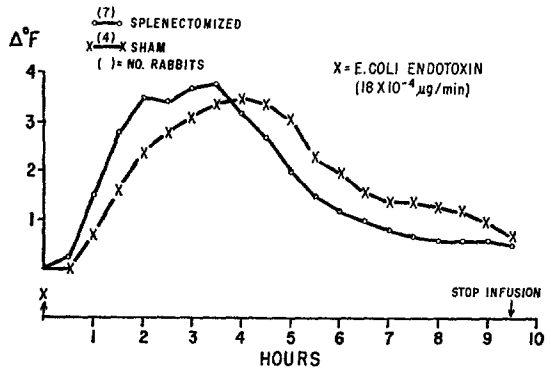

FIG. 10. Inability of splenectomy to inhibit the development of the endotoxin-induced pyrogenic refractory state.

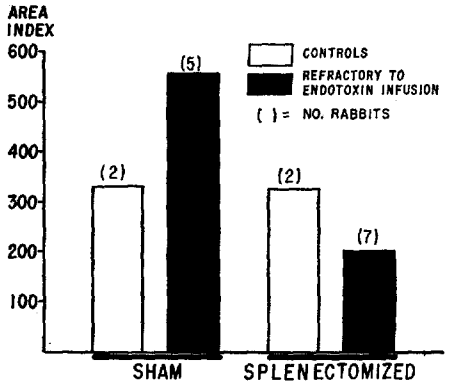

FIG. 11. Effect of splenectomy on inflammatory responses to intradermal injections of *E. coli* endotoxin (100 μg/0.2 ml saline) during the endotoxin-induced pyrogenic refractory state. Skin tests were performed in duplicate after the refractory state was fully developed; the endotoxin infusion was discontinued and reactions read at 24 hr. The area index is expressed as the product of the largest cross diameters of erythema (mm^2).

tion. Following induction of the pyrogenic refractory state in normal rabbits by a standard rate of intravenous *E. coli* endotoxin infusion (18×10^{-4} μg/min), responsiveness to a single intravenous test bolus of *E. coli* endotoxin was significantly depressed; indeed, the mean febrile response was reduced to levels observed in normal control animals receiving $\frac{1}{50}$ this test dose. In contrast,

rabbits rendered comparably refractory to the continuous *E. coli* endotoxin infusion exhibited no reduction in pyrogenic responsiveness to a single bolus of preformed endogenous pyrogen or to agents known to liberate endogenous pyrogen, i.e. influenza virus (10), old tuberculin in specifically sensitized animals (11), or staphylococcal enterotoxin (6, 12). It is emphasized that the dosages of all test pyrogens were carefully selected to evoke febrile responses within the sensitive portions of their respective dose-response regions. In addition, continuous intravenous infusions of heat-killed pneumococcal suspensions resulted in maintained febrile responses, observations comparable to those of Atkins and Freedman employing autoclaved staphylococci (13). Such responses presumably are mediated by sustained mobilization of endogenous pyrogen (13, 14). Considered collectively, the data demonstrate that the rapid development of the pyrogenic refractory state during sustained infusions of endotoxin cannot be attributed to nonspecific depletion of "available" endogenous pyrogen and that impaired thermoregulatory responsiveness to endogenous pyrogen makes no significant contribution to this syndrome.

The granulocyte appears to be the major source of endogenous pyrogen during endotoxin-induced fever (15–18). It has already been shown that destruction of this cell population does not account for the absence of circulating endogenous pyrogen during the endotoxin-induced pyrogenic refractory state (3). In the present studies, infusion of fresh whole blood from normal donors containing an average of 4×10^8 leukocytes (1.6×10^8 granulocytes) failed to restore any febrile responsiveness to endotoxin during the refractory state. In contrast, as few as 2×10^7 blood leukocytes can elaborate detectable amounts of leukocyte pyrogen in vitro (19). The pyrogenic refractory state, therefore, must represent a more complex reaction than direct interaction of the infused endotoxin with "exhausted" granulocytes. That certain endotoxin effects upon rabbit granulocytes are mediated in vivo predominantly by indirect actions has already been demonstrated by the observations of Berthrong and Cluff (20) and of Stetson (21), i.e. normal granulocytes suspended in plasma and exposed to endotoxin in vitro fail to exhibit alterations characteristic of their in vitro behavior when removed immediately after endotoxin injection (decreased migration and clumping). Moreover, Herring and coworkers (22) failed to detect direct interaction in vivo between small doses of radioactively tagged endotoxin and circulating leukocytes. In addition, Collins and Wood observed that a quantity of endotoxin capable of eliciting a brisk febrile response in vivo acted as a relatively weak stimulus for in vitro release of pyrogen from leukocytes suspended in fresh homologous serum (23). It would appear that the release of endogenous pyrogen in vivo may be mediated primarily by an indirect action of endotoxin upon granulocytes, a concept already proposed by Herring and coworkers (22, 24) and that it is this indirect mechanism which rapidly becomes refractory to the infused endotoxin. In any case, the blood transfer data further demonstrate that the endotoxin-induced pyrogenic refractory state cannot be attributable simply to generalized depletion of "available" supplies of endogenous pyrogen. The blood transfer data

also support the earlier conclusion that the pyrogenic refractory state is not attributable to depletion of humoral factors, including natural antibody, which interact with the infused endotoxin (3).

The previous study (3) demonstrated that the endotoxin-induced pyrogenic refractory state was not transferable with refractory phase plasma, could not be prevented nor reversed by reticuloendothelial system blockade with thorotrast, and developed despite pyrogenic quantities of circulating endotoxin demonstrable by bioassay. It appeared, therefore, that the failure of endogenous pyrogen mobilization during the refractory state was not based simply upon enhanced host ability to inactivate endotoxin. The present studies reinforce this conclusion. When endotoxin was incubated for various periods at 39°C with plasma and liver homogenates from control and refractory donors, no appreciable differences in rates of pyrogen inactivation were detectable. In addition, splenectomized rabbits were observed to develop the pyrogenic refractory state to endotoxin as readily as sham-operated controls, despite the loss of one segment of their reticuloendothelial system and despite suppressed ability to elaborate either anti–endotoxin antibody or plasma factors which transfer pyrogenic tolerance (9).

Considered collectively, the present observations provide additional support for the concept that the inability to elaborate endogenous pyrogen during the pyrogenic refractory state reflects the systemic component of specific cellular desensitization to endotoxin. It should be emphasized that cellular desensitization can entail both specific and nonspecific mechanisms (25, 26), and that the specificity of pyrogenic unresponsiveness demonstrated here with the standard rate of endotoxin infusion (total endotoxin dosages under 1 μg) may no longer hold if higher rates of infusion were employed. Indeed, this seems likely in light of the demonstration by Atkins and coworkers that intravenous injections of large single doses of endotoxin (i.e. 2 ml undiluted typhoid vaccine) or of other endogenous pyrogen–mobilizing agents results in a transient, nonspecific refractory state to heterologous endogenous pyrogen–mobilizing agents (8, 27). The seemingly paradoxical observation that dermal inflammatory responses to endotoxin are intensified during the endotoxin refractory state in the rabbit (3) was resolved in the present studies by the demonstration that inflammatory responses to intradermal endotoxin were decreased in those refractory animals that had been previously splenectomized, while refractory sham-operated controls exhibited the expected increased responses. Thus the pyrogenic refractory state is associated with decreased dermal reactivity to endotoxin both in man (3) and rabbit, but in the latter species rapid elaboration of some splenic factor, presumably antibody capable of evoking the Arthus-like reactions described by Lee and Stetson (28), appears to mask such decreased responsiveness.

Finally, the observation that a continuous intravenous infusion of old tuberculin into specifically sensitized animals results in a pyrogenic refractory response pattern generally similar to that produced by endotoxin further supports the desensitization thesis. The differences observed between old tuberculin and endotoxin, i.e. delayed onset of fever and longer time to defervescence with old tuberculin, need not represent basically different pathogenetic mechanisms, but rather may reflect differences related to antigenic structure, particle size, and host sensitivity. As with endotoxin (3), the tuberculin induced pyrogenic refractory state is relative since the intravenous introduction of a large bolus of tuberculin reevoked a brisk febrile response.

In 1947, Beeson reported that the least febrile responses to repeated small single intravenous doses of endotoxin were obtained when the pyrogen was injected at daily intervals (1). Subsequently, when this interval was shortened, higher degrees of febrile unresponsiveness were readily obtained, as observed by Petersdorf, Keene, and Bennett in tolerant animals (29), and by Braude, Zalesky, and Douglas in the normal host (30); in fact, the animals could thereby rapidly be rendered completely unresponsive. Moreover, such refractory animals remained responsive to both endogenous pyrogen and to peritoneal exudates, and in contrast to animals rendered tolerant by repeated daily injections of the endotoxin, did not clear radioactively-tagged endotoxin more rapidly than normal controls (29, 30). It appears likely that the mechanisms underlying the pyrogenic refractory state after such closely spaced single intravenous injections of endotoxin may be similar, if not identical, to those responsible for the pyrogenic refractory state induced by continuous intravenous infusions of endotoxin, i.e. specific cellular desensitization. Indeed, Stetson has previously emphasized the analogy between induction of tolerance to endotoxin and desensitization of a tuberculous animal by repeated injections of tuberculin (31, 32). In light of these observations, the importance of antibody in the mediation of pyrogenic tolerance would be relative and would depend upon the degree of cellular desensitization. Closely spaced injections, or continuous infusions, would provide optimal conditions for cellular desensitization and pyrogenic unresponsiveness to a given quantity of endotoxin can be induced rapidly and maintained without the requirement for antibody. However, as the interval between endotoxin challenge is lengthened, cellular desensitization wanes and tolerance becomes increasingly dependent upon those antibodies directed against the common toxophore groupings responsible for endotoxin pyrogenicity which assist the reticuloendothelial system in the clearance and destruction of this molecule (9, 33).

SUMMARY

The mechanisms underlying the pyrogenic refractory state which develops rapidly during a continuous intravenous infusion of bacterial endotoxin have been further explored. The findings demonstrate that: (*a*) rabbits rendered refractory to a continuous intravenous infusion of *E. coli* endotoxin at a standard rate (18×10^{-4} μg/min) become highly refractory to a single intravenous test bolus of endotoxin, but remain fully responsive to preformed endogenous pyrogen and to substances known to release endogenous pyrogen, i.e. influenza virus, old tuberculin in specifically sensitized rabbits, and staphylococcal enterotoxin; (*b*) administration of fresh whole blood from normal donors containing an average of 1.6×10^8 granulocytes fails to restore febrile responsiveness to the continuing *E. coli* endotoxin infusion; (*c*) refractory phase plasma and liver homogenates exhibit no enhanced capacity to inactivate *E. coli* endotoxin pyrogenicity; (*d*) splenectomized animals readily develop the pyrogenic refractory state during *E. coli* endotoxin infusions and exhibit diminished, rather than the increased inflammatory responses to intradermal endotoxin seen in sham-operated controls; (*e*) continuous intravenous infusions of gelatin-stabilized, heat-killed pneumococci produce sustained fevers; and (*f*) continu-

ous intravenous infusions of old tuberculin into specifically sensitized animals rapidly elicit a pyrogenic refractory state.

The present observations, considered together with those of other investigators, support the hypothesis that pyrogenic unresponsiveness to endotoxin involves two distinct immunologic mechanisms. In terms of this hypothesis, the rapid reduction in febrile responsiveness to endotoxin is mediated by desensitization at the cellular level. With small doses of endotoxin, such as those employed in the present studies, this desensitization is primarily specific; with larger doses, nonspecific mechanisms are superimposed. So long as the subsequent doses of endotoxin are closely spaced or continuously infused, optimal conditions are provided for cellular desensitization and pyrogenic unresponsiveness to a given quantity of endotoxin can be induced rapidly and maintained without the requirement for antibody. However, as the interval between endotoxin challenge is lengthened, cellular desensitization wanes and tolerance becomes increasingly dependent upon those antibodies directed against the common toxophore groupings responsible for endotoxin pyrogenicity which assist the reticuloendothelial system in the clearance and destruction of this molecule.

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