On the Demonstration of Circulating Human Endogenous Pyrogen[1] (36217)

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In 1953, Bennett and Beeson, employing techniques to eliminate extraneous bacterial pyrogens, described a rapidly acting pyrogen derived from rabbit exudate leukocytes (1). Subsequently, pyrogens with similar biologic properties have been prepared from stimulated blood leukocytes, lung macrophages, exudate monocytes, lymph nodes, spleen cells, and hepatic Kupffer cells of rabbits, and from stimulated circulating granulocytes and monocytes of man (2–7). These findings support the concept that fevers induced by a variety of stimuli, microbial and hypersensitivity, are mediated by release of such endogenous tissue pyrogens, which then enter the circulation and are carried to the target organ, the thermoregulatory centers in the hypothalamus (8). While endogenous pyrogens are readily demonstrable in plasma of various experimental animals during fever induced by antigenic and microbial agents, attempts to demonstrate a similar substance in plasma of febrile man have thus far proven unsuccessful (9–12). The present studies describe additional efforts to establish the existence of a circulating endogenous pyrogen in plasma of humans with fever induced by *Rickettsia rickettsi* and by *Salmonella typhosa* endotoxin.

Methods. All syringes, needles, and glassware were preheated at 200° overnight to eliminate extraneous pyrogen contamination. Two systems for assay of circulating endogenous pyrogen were employed: a) heterologous, based upon transfer to acclimatized rabbits of plasma obtained from febrile patients infected with *Rickettsia rickettsi;* b) homologous, based upon transfer to afebrile volunteers of blood or plasma obtained from febrile volunteers given *S. typhosa* endotoxin. Pyrogen assays in man and rabbit were carried out by standard techniques employing indwelling rectal thermistor probes (13).

Results. Heterologous system. 5 ml/kg aliquots of fresh, heparinized plasma collected from healthy afebrile volunteers with pyrogen-free precautions consistently induced febrile responses when injected intravenously into acclimatized healthy rabbits. Intravenous injection of 10 ml/kg aliquots of freshly collected normal human plasma often resulted in hypothermia and death, Fig. 1 (A). As shown in Fig. 1, the fever evoking property of normal human plasma could not be eliminated by heat inactivation at 56° for 30 min Fig. 1 (B), by a single overnight absorption with packed washed rabbit blood cells at 4° Fig. 1 (C), or by a combination of these latter procedures Fig. 1 (D). It is emphasized that the normal human plasma depicted in Fig. 1 contained heterophile agglutinins against rabbit erythrocytes which were not eliminated by the procedures employed. The general occurrence of such heterophile agglutinins was seen upon analysis of 42 samples of randomly selected normal human sera; all contained significant titers, Fig. 2. These heterophile agglutinins could be removed completely only by repeated overnight absorptions of human plasma with equal volumes of packed rabbit erythrocytes at 4°[2]. When all detectable heterophile agglutinins were removed from plasma collected

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[2] The low temperature was employed to minimize the extensive hemolysis that otherwise accompanied absorption of freshly collected human plasma with packed rabbit erythrocytes.

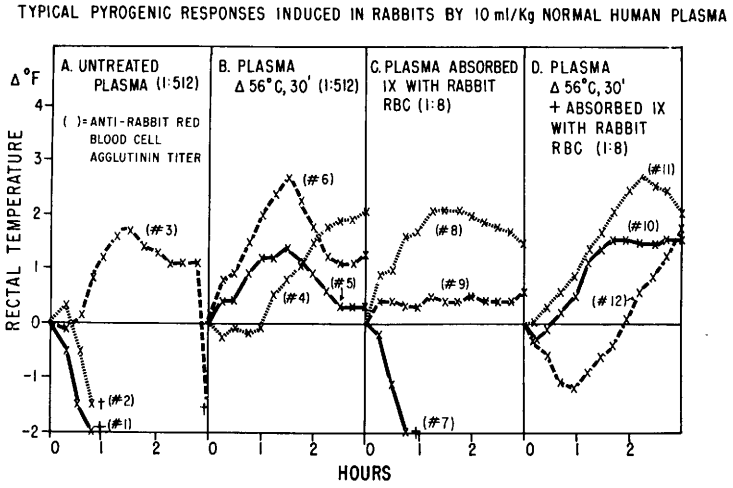

FIG. 1. Representative responses of acclimated healthy rabbits to intravenous administration of 10 me/kg heparinized fresh normal human plasma aliquots. All responses shown are to plasma aliquots from a single blood sample. Plasma pyrogenicity was not removed by heating at 56° for 30 min, overnight absorption at 4° with packed washed rabbit erythrocytes or by a combination of these procedures. Note the presence of heterophile agglutinins for rabbit erythrocytes which was not eliminated by the above procedures.

from healthy volunteers (4 absorptions sufficed in most instances), intravenous injections of 10 ml/kg aliquots now failed to evoke any febrile or lethal reactions in acclimatized rabbits.[3]

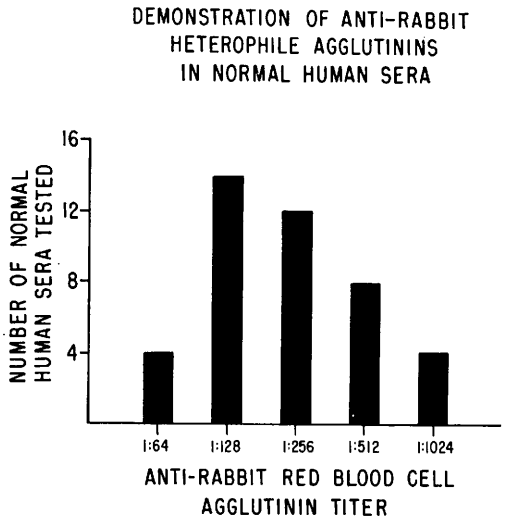

FIG. 2. Distribution of heterophile agglutinin titers for rabbit erythrocytes in 42 randomly tested normal human sera samples.

Heparinized plasma was subsequently obtained with pyrogen-free precautions from 11 patients with Rocky Mountain spotted fever exhibiting rectal temperatures sustained for at least 2 hr above 104°F. The blood was collected in iced tubes, and the plasma immediately separated in the cold and frozen at —20° until assay. Each plasma was processed prior to assay by 4 overnight absorptions at 4° with equal volumes of packed washed rabbit erythrocytes; as with normal plasma, this sufficed to remove all detectable heterophile agglutinins. Ten milliliters/kilogram aliquots of each plasma were then injected intravenously into acclimatized 1.5–2.0 kg rabbits.[3] Ten of 11 animals receiving such febrile phase human plasma failed to exhibit any untoward reaction or any significant elevation of rectal temperature, Fig. 3. To determine if such negative findings could be related to nonspecific depres-

[3] To ensure actual administration of 10 ml/kg of human plasma, 15 mg/kg volumes of the processed plasma were injected to correct for dilutional effects during absorption.

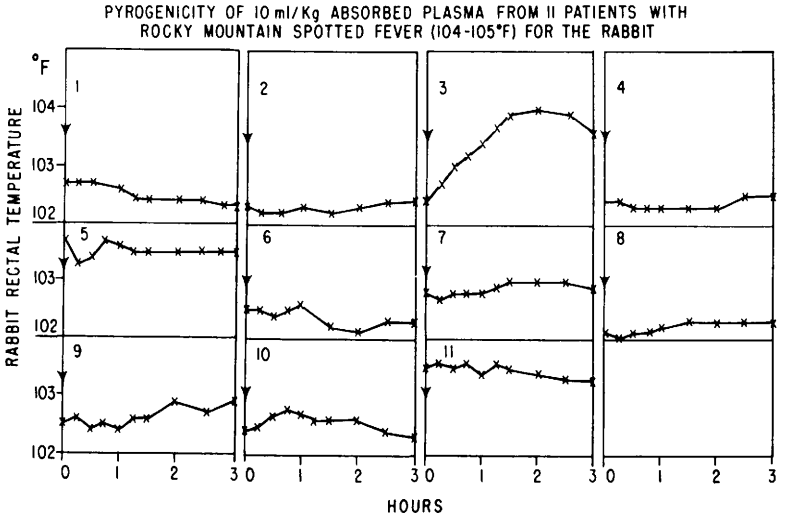

FIG. 3. Absence of endogenous pyrogen activity in 10 ml/kg human plasma aliquots in rabbits following removal of all detectable anti-rabbit erythrocyte agglutinins. Plasma was obtained during Rocky Mountain spotted fever and all patients exhibited rectal temperatures = 104–105°F. Temperature elevation in rabbit #3 may be related to emotional disturbances consequent to plasma injection.

sion of the rabbit thermoregulatory mechanisms, 3 hr after failing to respond to the febrile phase plasma, recipient animals were injected intravenously with 0.05 μg/kg *E. coli* endotoxin (Boivin preparation, Difco Laboratories). This quantity of toxin evoked febrile responses in control animals within the sensitive dose-response range. No impairment of responsiveness to this pyrogen was detected. To determine if the negative findings might be attributed to removal or inactivation of human endogenous pyrogen during the absorption of heterophile agglutinins, human leukocyte pyrogen was prepared by the technique of Bodel and Atkins (6). Quantities of such pyrogen which evoked fever in rabbits within the sensitive dose-response range were then added to aliquots of fresh human plasma obtained from three healthy volunteers. Absorption of heterophile agglutinins failed to induce any detectable loss of the added leukocytic pyrogen activity, Fig. 4.

Homologous system. Seven volunteers were employed. Subjects 1 and 2 were injected

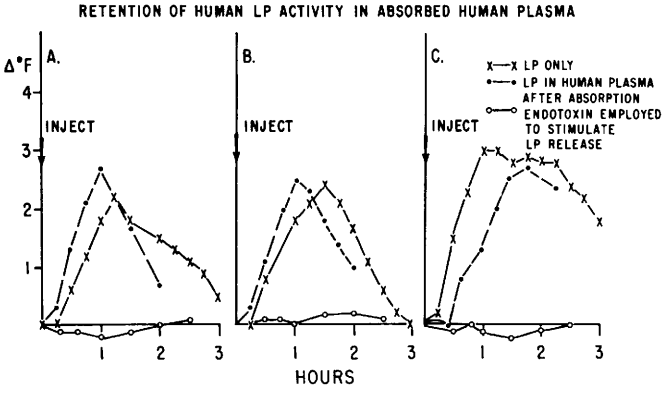

FIG. 4. Evidence that human leukocyte pyrogen (LP) is not removed from human plasma or inactivated during absorption of heterophile agglutinins.

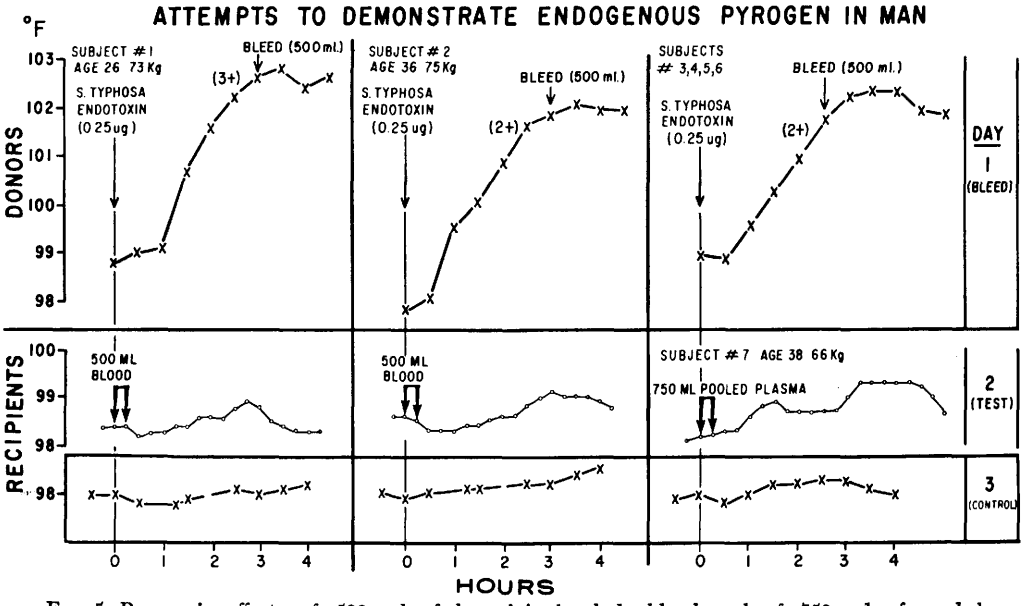

FIG. 5. Pyrogenic effects of 500 ml of heparinized whole blood and of 750 ml of pooled heparinized plasma obtained from volunteers with fever induced by intravenous administration of *S. typhosa* endotoxin. Shown are the febrile responses of the donors and the bleeding times (day 1), the pyrogenic responses to the blood or plasma reinfusions (day 2), and the control responses in the absence of any infusion (day 3). The increase in temperature in subject 1 and 2 following blood reinfusions on day 2 are gradual and compatible with responses to residual circulating endotoxin. In contrast, recipient 7, given 750 ml of febrile phase plasma exhibited both an early and a delayed pyrogenic response compatible with the presence of endogenous pyrogen and residual bacterial endotoxin, respectively.

intravenously with 0.25 μg of a highly purified sterile *S. typhosa* endotoxin.[4] Approximately 30 min before the maximum febrile response, 500 ml blood was drawn into pyrogen-free, heparinized bottles, Fig. 5 (day 1). The blood was chilled immediately and held at 4° until the following morning, warmed to 37° and infused intravenously over a 15 min period into the respective subjects. As seen in Fig. 5 (day 2), the hallmark of a human endogenous pyrogen response failed to appear, *i.e.*, no rise in rectal temperature occurred within 40 min (10). Rather, a delayed temperature elevation resulted, peaking near 3 hr and then declining, entirely characteristic of man's response to small intravenous quantities of bacterial endotoxin (13). The latter febrile pattern was not seen when rectal temperatures were monitored the following morning in the absence of any infusion, Fig. 5 (day 3).

Volunteers 3–6 were selected for blood donors on the basis of a) same blood group as the recipient volunteer 7, b) negative histories for hepatitis, drug addiction, jaundice, or receipt of previous blood transfusions, and c) the presence of normal liver function studies.[5] Each volunteer was given 0.25 μg *S. typhosa* endotoxin intravenously, and 500 ml bleedings performed approximately 1 hr before the peak of fever; the mean febrile response and the bleeding time is shown in Fig. 5 (day 1, end panel). The blood was collected in pyrogen-free, heparinized bottles and chilled immediately. The plasma was separated by centrifugation in the cold employing pyrogen-free precautions and held at 4° until the following morning. Then, 750 ml of the pooled plasma was warmed to 37° and infused intravenously over a 15 min period into volunteer 7. The latter was selected on the basis

[4] Kindly supplied by Dr. Maurice Landy, N.I.H., Bethesda.

[5] Australia antigen had not been described at the time of these studies.

of a predicted small plasma volume (subject weight = 66 kg). No temperature elevation was observed for 30 min after completion of the infusion; then an unequivocal rise commenced (exact onset 37 min after infusion) reaching a peak increment of 0.7°F at 75 min. A second febrile peak appeared at 3 hr. Both a rapidly acting and a slower acting pyrogen thus appeared demonstrable in the pooled plasma, consistent with a response to endogenous pyrogen and to residual bacterial endotoxin, respectively. The sole other observable response to the plasma infusion consisted of two transient urticarial lesions 30 min after completion of the infusion.

Discussion. The immediate stimulus for development of fever during human infection is as yet unknown. Currently, a resetting of the central thermoregulatory mechanisms is believed to occur, mediated by liberation of endogenous pyrogens from activated granulocytic and macrophage populations (7, 8). While such endogenous pyrogens are readily demonstrable in plasma during fevers induced in experimental animals by a variety of microbial and hypersensitivity reactions (8), attempts to demonstrate its presence in man have presented serious problems. In one study (9), etiocholanilone was the agent employed to induce fever, and plasma volumes of 1 liter or greater obtained during the febrile phase (average 103°F) failed to evoke fever when reinfused into the same volunteers during the afebrile period. In another study, plasma, whole blood, or blood cells were obtained from patients febrile with various illnesses (generally infectious) and subsequently reinfused. 3 of 35 preparations (2 plasma and 1 whole blood) evoked early febrile responses, but the author stated that these could be explained on a basis other than the presence of circulating endogenous pyrogen (10). In a third report (11), 500 ml blood drawn during the febrile phase (103°F) from a patient with "periodic fever" failed to evoke fever when reinfused into the same subject 7 days later. In a fourth report, the cellular blood elements but not the plasma obtained from a patient acutely febrile with malaria evoked an early fever upon reinfusion, as did whole blood from a second malarial donor. Reinfusion of blood from 2 other such donors yielded negative results (12). Since only the cellular fraction evoked febrile effects, the plasma being devoid of detectable pyrogen, and since malarial parasites and altered erythrocytic components were undoubtedly transferred, the effects of which are unknown, interpretation of these data presently remain unclear. In a fifth study (14), 170 ml heparinized whole blood drawn from a patient following intravenous injection of 50 million killed *E. coli* evoked a definite rise in rectal and oral temperature when infused into a second recipient of the same blood group. The temperature increment commenced 20 min after injection. This early response represents the only convincing demonstration of the existence of a circulating human endogenous pyrogen thus far reported. Even here, however, the possibility that the early acting pyrogen was generated *in vitro* as a consequence of residual endotoxin acting on buffy coat leukocytes during the time lapse between blood collection and reinfusion (27 min) cannot be entirely excluded. Indeed, this possibility is strengthened by the fact that only 170 ml of blood evoked such an unequivocal febrile response. Moreover, that appreciable residual endotoxin was present to act upon the blood leukocytes *in vitro* is suggested from extrapolation of data presented in the text. The gentle febrile slope depicted during the initial 90 min following the blood infusion increased abruptly and markedly during the subsequent 30 min. Such an abrupt change in febrile slope after 90 min points to the action of two pyrogens in the transferred blood, one acting rapidly (compatible with either buffy coat leukocyte pyrogen or endogenous pyrogen), the other acting later, compatible with residual endotoxin. For critical proof that a human endogenous pyrogen is circulating *in vivo*, it would be preferable that blood be drawn into iced containers and the plasma prepared immediately in the cold and assayed separately to minimize *in vitro* interactions between exogenous pyrogen and buffy coat leukocytes.

The present studies represent further attempts to demonstrate a circulating endogenous pyrogen in febrile man. In initial studies, advantage was taken of observations by Bodel and Atkins, that a pyrogen derived

from human blood leukocytes can be detected by rabbit assay (6). In such a system, no fever is evoked unless the human leukocytes are stimulated by agents such as bacterial endotoxin or whole microbes (6). Febrile phase human plasma cannot, however, be readily tested for its content of such a pyrogen since serum or plasma from healthy afebrile subjects induce febrile responses and death when injected intravenously into rabbits. Indeed, such fever evoking activity of normal human plasma has been reported previously and appeared to exclude the rabbit as a meaningful recipient for bioassay of circulating human endogenous pyrogen (15). Nevertheless, from earlier studies in our laboratory on the toxicity of normal human plasma for heterologous species (16), the presence of anti-rabbit erythrocyte agglutinins was observed consistently in normal human plasma and is herein documented. When such heterophile agglutinins were completely removed by repeated overnight absorptions of human plasma with equal volumes of packed washed rabbit erythrocytes in the cold, as much as 10 ml/kg normal human plasma could be administered intravenously to healthy rabbits without evoking fever or any untoward reaction. Surprisingly, similar large volumes of plasma obtained from human patients febrile with Rocky Mountain spotted fever and absorbed for heterophile agglutinins also failed to evoke any significant temperature elevation in 10 of 11 recipient acclimatized rabbits. This illness was selected both because of the high fever, and to circumvent possible plasma contamination with bacterial endotoxins that might be associated with gram-negative bacterial infections. It is emphasized that no fever was transferred despite the fact that all human donors exhibited rectal temperatures sustained between 104 and 105°F. These negative findings could not be attributed to nonspecific depression of the rabbit thermoregulatory system by the absorbed human plasma, since febrile responsiveness to subsequently administered bacterial endotoxin remained intact. The negative findings also did not appear related to removal or inactivation of circulating endogenous pyrogen by the absorption of heterophile agglutinins, since added quantities of preformed human leukocytic pyrogen were not depleted by this procedure. It would thus appear either that insufficient human plasma was transferred (although the human plasma transferred approximated 25% of the rabbit plasma volume (17)), or that the rabbit is relatively insensitive to human endogenous pyrogen. Comparative studies on the sensitivity of rabbit and man to human leukocyte pyrogen are not yet available to decide this important point.

Since circulating human endogenous pyrogen could not be demonstrated employing the heterologous plasma transfer system, homologous transfer was attempted. Two volunteers rendered febrile by intravenous injection of *S. typhosa* endotoxin exhibited a substance in their blood capable of elevating their rectal temperature when reinfused on the following day. However, in both recipients, the temperature increment was delayed in onset and reached a maximum only after 3 hr, consistent with responses to small quantities of residual circulating bacterial endotoxin rather than to endogenous pyrogen (10, 13). The detection of such trace amounts of endotoxin may have been facilitated by the enhanced responsiveness to endotoxin known to develop in man 24 hr after its initial intravenous administration (18). A third recipient was then given 750 ml of pooled plasma obtained from four volunteers with fever induced by *S. typhosa* endotoxin. An early and unequivocal temperature elevation (0.7°F) now ensued, consistent with the presence of endogenous pyrogen (10). A subsequent 3 hr temperature peak paralleled that seen in the previous two recipients and probably reflects a response to residual circulating bacterial endotoxin. That such large quantities of febrile phase plasma had to be transferred to detect the early reacting human endogenous pyrogen, and that the resulting febrile response was small, is entirely consistent with the recently reported inability to transfer any febrile response with 220 g plasma aliquots obtained from volunteers rendered febrile with preformed human leukocytic pyrogen (19). Two alternative possibilities for the early febrile response demonstrated in our third recipient, other than the presence of endogenous pyrogen, must be considered: 1) residual circulating bacterial endotoxin might have liberated a rapidly acting leuko-

cytic pyrogen from buffy coat *after* the blood was withdrawn, and 2) a transfusion reaction to buffy coat or minor red cell antigens might have induced the response. Neither of these possibilities can be absolutely excluded. However, the first possibility was minimized by immediately chilling the blood and separating the plasma in the cold before reinfusion. While the second possibility is strengthened by two urticarial lesions in the recipient, in carefully timed studies of febrile responses during deliberately induced mild incompatible transfusion reactions, fever was consistently delayed for 60 min and resembled the response to bacterial endotoxin rather than to endogenous pyrogen (20). It is concluded, therefore, that *in vivo* endogenous pyrogen generation constitutes the most likely explanation for the early febrile response.

That a circulating endogenous pyrogen is the immediate stimulus for resetting the hypothalamus and evoking fever during human infection appears highly likely from experimental models. The present investigation yields a strongly presumptive demonstration of the presence of this substance in febrile phase human plasma and emphasizes the problems inherent in this accomplishment.

Summary. Acclimatized rabbits were injected intravenously with 10 ml/kg plasma aliquots from patients febrile with Rocky Mountain spotted fever (104–105°F). Prior to assay, the febrile phase human plasma was processed by repeated absorptions with packed washed rabbit erythrocytes at 4° to remove all detectable heterophile agglutinins. The latter were found in all human plasma samples tested and accounted for their febrile and lethal activity in the rabbit. Ten of 11 absorbed febrile phase human plasma aliquots failed to evoke any febrile response. These negative findings could not be attributed to depression of rabbit thermoregulatory mechanisms or to absorption of human endogenous pyrogen during heterophile agglutinin removal. Since the negative findings might be related to relative insensitivity of the rabbit to human endogenous pyrogen, additional attempts to demonstrate circulating human endogenous pyrogen were performed with human recipients. Five hundred milliliters whole heparinized blood drawn from each of 2 volunteers 30 min before their peak febrile response to *S. typhosa* endotoxin failed, when reinfused the following morning, to induce an early febrile reaction compatible with an endogenous pyrogen response. Rather, a delayed rise in rectal temperature occurred compatible with residual circulating bacterial endotoxin. A third recipient was given 750 ml of pooled heparinized plasma drawn from 4 other volunteers 1 hr before their peak febrile reaction to *S. typhosa* endotoxin. Both an early and a delayed febrile response now ensued, compatible with responses to endogenous pyrogen and residual circulating endotoxin, respectively. Reasons for relating the early febrile response to circulating endogenous pyrogen are considered.

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