STUDIES ON THE PATHOGENESIS OF FEVER*

VIII. Further Observations on the Role of Endogenous Pyrogen in Endotoxin Fever

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(Received for publication, June 19, 1961)

During the febrile response to intravenous endotoxin, there appears in the circulation an endogenous pyrogen, the properties of which are indistinguishable from those of the fever-producing substance contained in polymorphonuclear leucocytes (1, 2). Whereas most of the injected endotoxin is cleared from the blood stream during the latent period, the endogenous factor becomes demonstrable with the onset of fever and is present in a concentration which parallels the degree of pyrexia (2). The establishment of these facts has led to the concept that the hypothalamic stimulus which causes endotoxin fever is provided, not by the injected endotoxin itself, but rather by the endogenous pyrogen released from toxin-injured leucocytes (2, 3).

Despite the numerous experimental observations which support it (3, 4), the endogenous pyrogen hypothesis has been challenged (5, 6) on the grounds that during endotoxin fever endogenous pyrogen was not demonstrable in the sera of animals previously made leucopenic with nitrogen mustard. The apparent absence of the circulating endogenous factor under these circumstances has been interpreted as evidence against the thesis that it plays a central role in endotoxin fever (5–13).

Because of the importance which has been placed upon this evidence (5–13), it has been reexamined in the present study.

Methods

Elimination of Extraneous Pyrogens.—All glassware and needles were rendered pyrogen-free by exposure to 180°C for 2 hours. Normal saline was autoclaved and was demonstrated to be non-pyrogenic.

Bacterial Pyrogen.—Typhoid vaccine, obtained from Col. A. S. Benenson of the Walter Reed Army Institute for Research, Washington, D. C., was used as the source of bacterial endotoxin. The specifications of the vaccine have been described elsewhere (14).

* This work was supported by a grant from the Life Insurance Medical Research Fund and from The United States Public Health Service (E-3772).

Rabbits.—Male rabbits weighing 5 to 8 lbs were obtained from a single dealer and housed in air-conditioned quarters. The temperature studies were performed in a temperature-controlled room (68–70°F). The rabbits were placed in metal stalls fitted with curved restraining rods which locked loosely about their necks. Before being used as recipients they were trained to remain quietly in their stalls for several hours. During the course of each experiment, they received neither food nor water.

Recording of Temperatures.—Temperatures were measured by means of thermistor rectal probes and a telethermometer (Yellow Springs Instrument Company, Yellow Springs, Ohio). To avoid trauma of the large bowel, the probes were inserted to a distance of only 6 inches. A constant baseline temperature (maximum variation of 0.1°C in 30 minutes) was a prerequisite of each experiment. After the intravenous inoculations, done under local anesthesia (lidocaine hydrochloride), temperatures were recorded every 5 minutes for the 1st hour, every 10 minutes for the 2nd hour, and every 20 minutes during the 3rd hour.

Induction of Leucopenia.—Leucopenia was induced by intravenous injection of 5 to 7 mg of nitrogen mustard (mustargen hydrochloride, Merck Sharp & Dohme) freshly dissolved in 5 to 7 ml of normal saline. To suppress bacterial infections, all rabbits given nitrogen mustard were treated daily with 0.5 to 1.0 ml of crysdimycin (Squibb) which contained 200,000 units of procaine penicillin G and 167 mg each of streptomycin and dihydrostreptomycin per ml. No antibiotic was given on the day of injection of typhoid vaccine. White blood cell counts were done on the days of expected maximal leucopenia (3rd to 5th following nitrogen mustard, 15). The blood was obtained from the marginal ear vein. Rabbits which failed to develop a leucopenia of less than 1000 cells per mm^3 were given a second and occasionally a third injection of nitrogen mustard (3 to 7 mg) and were followed in the same manner.

Passive Transfer Test for Circulating Pyrogen.—Both normal and leucopenic rabbits (white counts below 1000 cells per mm^3) were given intravenous injections of typhoid vaccine (1 ml diluted with 4 ml of normal saline). Twenty out of twenty-four developed peak fevers of at least 1.2°C within 2 hours after injection of the vaccine. Each of these was exsanguinated by cardiac puncture 120 minutes after receiving the endotoxin. The techniques employed for bleeding the rabbits and separating the serum have already been described (16). The sera from the leucopenic animals were collected over a period of 3 weeks, and the individual samples were stored at −20°C. The sera from the normal animals were obtained over a period of 2 days, stored at 4°C, and used within a week after collection. Control aliquots of serum taken 2 hours after endotoxin injection were shown to exert the same febrile effect whether previously stored at 4°C or at −20°C.

After the individual 2 hour sera had been shown to be free of bacterial contamination,[1] separate serum pools were made of the samples from the normal donors and of those from the leucopenic donors. Aliquots of each pool were then tested for transferable pyrogen by injection into normal recipients.

Identification of Transferable Pyrogen.—The character of the transferable pyrogen in the "leucopenic" pool was determined by comparing the febrile responses which it induced in normal and endotoxin-tolerant recipients. The details of this test are given elsewhere (2, 17).

Calculation of Fever Index.—All fever curves were charted on 1 mm graph paper. Time in hours (3 cm = 1 hour) was plotted on the abscissa and fever in degrees centigrade (4 cm = 1°) on the ordinate. The area beneath each mean 2 hour fever curve (in square centimeters) is designated as the "fever index" (18).

RESULTS

Demonstration of Transferable Pyrogen in Serum of Leucopenic Rabbits Following Injection of Typhoid Vaccine.—Contrary to the findings of Bennett

[1] One was discarded because of a positive culture.

et al. (5, 6), transferable pyrogen was readily detected in the serum of leucopenic rabbits with endotoxin fever, provided a sufficient volume of serum was used in the passive transfer test (2). The febrile responses of normal recipients to intravenous injections of 25 ml aliquots are shown in Fig. 1. It will be noted that the resulting fever curves exhibit the relatively short latent period, the peak within 60 minutes, and the rapid defervescence characteristic of the febrile reaction to both leucocytic and serum endogeous pyrogen (3, 4, 16, 19).

Nature of Transferable Pyrogen.—In order to determine whether the pyrogen transferred in the above experiments consisted primarily of the originally injected endotoxin or of newly formed endogenous pyrogen, its fever-produc-

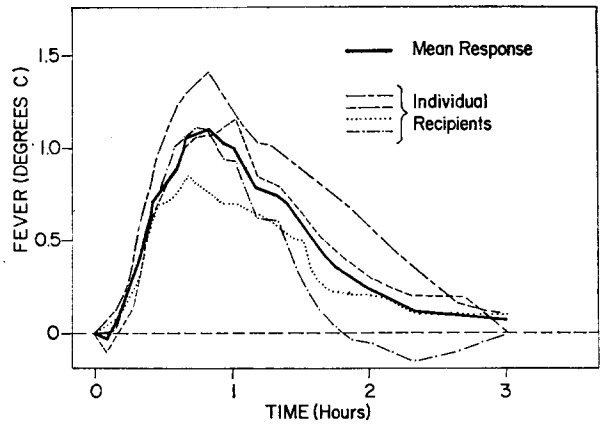

FIG. 1. Fever responses of four normal rabbits given 25 ml aliquots of serum obtained from leucopenic rabbits with endotoxin fever. The leucopenic donor rabbits were bled 2 hours after receiving intravenous typhoid vaccine (1 ml).

ing effect was compared in normal and endotoxin-tolerant recipients. Each of five 10 ml aliquots of the "leucopenic" serum pool was incubated with 5 ml of normal rabbit serum for 30 minutes and then injected intravenously into a normal recipient. Each of five additional aliquots was incubated for 30 minutes with 5 ml of "tolerant" serum and was injected into the same five rabbits after they had been made tolerant to typhoid vaccine.[2] Since endogenous pyrogen is equally active in normal and tolerant recipients, whereas the pyrogenicity of endotoxin is markedly depressed in tolerant animals, it is possible to differentiate endogenous pyrogen from endotoxin by this test (2). The reliability of the method is markedly enhanced by pre-incubating the test samples with normal and "tolerant" serum as described (2, 17, 20). The fact that the "leuco-

[2] "Tolerant" serum refers to serum obtained from rabbits previously made tolerant to typhoid vaccine. Tolerance was induced by repeated injections of typhoid vaccine (1 ml) given at least daily for 1 week or longer. All rabbits so treated failed to develop fever following test doses of 0.1 ml of typhoid vaccine.

penic" serum was equally active in the normal and tolerant recipients indicates that it contained no detectable endotoxin (see Fig. 2).

Comparative Concentrations of Transferable Pyrogen in Sera of Normal and Leucopenic Rabbits.—Four normal rabbits were injected on each of 4 successive days with 2, 5, 10, and 25 ml aliquots, respectively, of serum from the "leucopenic" pool. These same recipients, plus one additional normal rabbit, were then injected with 2, 5, and 10 ml aliquots from the normal serum pool. As indicated by the asterisk in Fig. 3, only one of the recipients was given a 25 ml aliquot from the normal serum pool. Comparison of the mean fever curves and mean fever indices of the recipients receiving the various aliquots of the

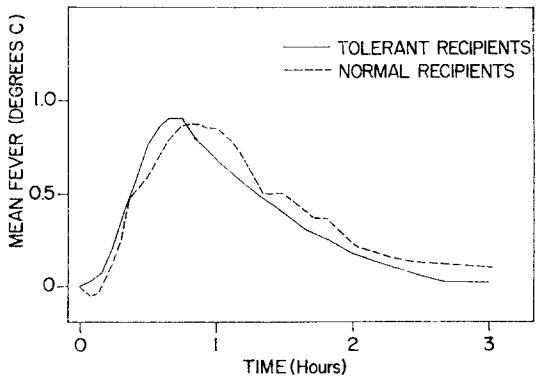

FIG. 2. Comparison of mean fever responses of normal and tolerant rabbits to 10 ml aliquots of serum obtained from leucopenic donor rabbits with endotoxin fever. The same five rabbits were used as recipients, before and after being made endotoxin-tolerant by daily injections of typhoid vaccine. Tests performed with four other tolerant rabbits (data not plotted) yielded the same results.

two kinds of serum reveals: (*a*) that each mean temperature response was roughly proportional to the amount of serum transferred, and (*b*) that the serum from the normal donors was consistently more potent than that from the leucopenic donors. The fact that the amount of pyrogen in the serum from the leucopenic donors was less than that contained in the serum of the normal donors strongly suggests that much, if not all, of the endogenous pyrogen, which is present in the circulation during endotoxin fever, is derived from polymorphonuclear leucocytes.

DISCUSSION

The hypothesis that a pyrogenic factor, apparently derived from polymorphonuclear leucocytes, plays a central role in the pathogenesis of fever (2–4) has been supported by the repeated demonstration of endogenous pyrogen in the sera of animals with experimental fevers caused by bacterial endotoxins

(2), influenzal viruses (21–23), tuberculin hypersensitivity (24), and infections due to *Diplococcus pneumoniae* (25) and *Streptococcus pyogenes* (25). Consistent with this hypothesis in the widely studied endotoxin model are the long latent period, which correlates with the lag between endotoxin administration and the appearance of endogenous pyrogen in the circulation (2), and the post-

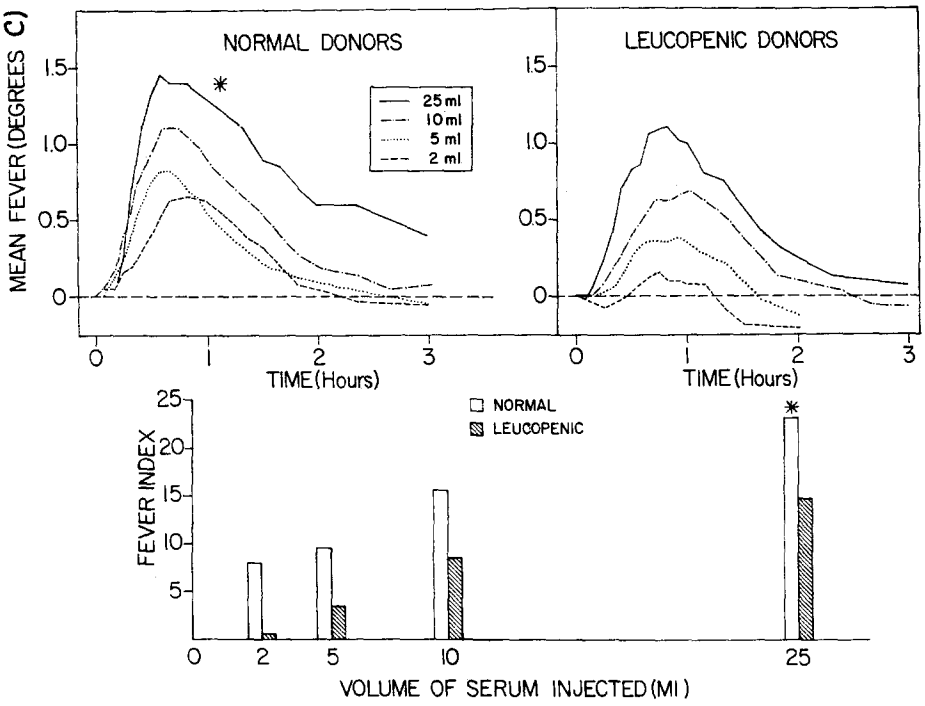

FIG. 3. (Upper left). Comparison of the mean fever responses of normal rabbits to aliquots of 2, 5, and 10 ml of serum obtained from *normal* rabbits with endotoxin fever. The response of one recipient to 25 ml of this serum is also shown (asterisk).

(Upper right). Comparison of the mean fever responses of four of the same recipients to aliquots of 2, 5, 10, and 25 ml of serum obtained from *leucopenic* rabbits with endotoxin fever.

(Lower). Comparison of the 2 hour fever indices, measured from the above temperature curves.

injection leucopenia, which appears to result from leucocytic injury. Furthermore, endotoxin is known to have both an affinity for (26), and a toxic effect upon (27, 28), polymorphonuclear leucocytes. When injected intravenously, endotoxin causes leucocytes to stick to the endothelium of small blood vessels (29), thus giving rise to the characteristic post-injection leucopenia. In addition, when bacterial endotoxin is incubated with polymorphonuclear leucocytes *in vitro*, the endotoxin is inactivated, and the endogenous pyrogen is released

from the leucocytes (17). Finally, there is indirect evidence that leucocytic and endogenous pyrogens act upon the thermoregulatory centers of the brain (14).

Despite the foregoing evidence, the observations of Bennett, Petersdorf, and Keene (5) are frequently cited as contradicting the endogenous pyrogen hypothesis (5–13). These workers found: (*a*) that the febrile responses of both rabbits and dogs to typhoid vaccine were not significantly affected when the animals were made leucopenic with nitrogen mustard, (*b*) that endogenous pyrogen failed to appear in detectable quantities in 20 ml samples of sera obtained from the leucopenic dogs during the apparently unaltered febrile responses to the vaccine, (*c*) that the intrathecal injections of minute amounts of endotoxin caused rabbits to develop high fevers during which endogenous pyrogen was not demonstrable in the serum, and (*d*) that the cerebrospinal fluid of normal dogs contained endotoxin following intravenous injections of both purified *Shigella* endotoxin and typhoid vaccine. Accordingly, they drew two conclusions: first, that endotoxin is capable of causing fever by acting directly upon the central nervous system, and second, that endogenous serum pyrogen probably does not play an important role in the pathogenesis of endotoxin fever.

Concerning the first of these conclusions, there seems to be little doubt that endotoxin is capable of eliciting fever when injected intrathecally through an indwelling catheter. Whether, following the intravenous injection of moderate doses of endotoxin, enough "spills over" into the spinal fluid to act directly is not known. It should be noted in this regard, however, that the intravenous dose of typhoid vaccine used by Bennett *et al.* to cause the "spill over" phenomenon was about 1000 times the minimum amount needed to produce a biphasic febrile response (30). Although this discrepancy may be accounted for in part by the relative insensitiveness of the passive transfer method (*vide infra*) used to detect the endotoxin in the spinal fluid, the magnitude of the dosage difference suggests that "spill over" may not occur following ordinary pyrogenic doses of endotoxin. Even if, under certain circumstances, endotoxin does act directly on the central nervous system, this mechanism must be considered a special case as far as the pathogenesis of fever in general is concerned. Certainly it can play no role in those forms of fever in which bacterial endotoxins are not involved (*e.g.*, hypersensitivity (24) and viral (21–23) and pneumococcal (25) fevers).

The conclusion, on the other hand, that endogenous serum pyrogen is not an essential factor in the febrile reaction to endotoxin is based primarily on the "failure of endogenous pyrogen to appear in the serum" of leucopenic animals[3] with endotoxin fever. That this failure was due to the use of an insuffi-

[3] White blood counts ranged from 550 to 2000 per mm.^{3} in comparison to the even more pronounced leucopenias of the present study (all < 1000 per mm^3).

cient volume of serum in the passive transfer test is clearly indicated by the results of the present study. Thus, the principal basis for the second conclusion is invalid.[4] In fact, as reviewed elsewhere, there are several reasons for concluding that the endogenous pyrogen, which is present in the "leucopenic" serum, is the primary factor responsible for the fever (3, 4).

The question may still be raised, however, as to why leucopenic animals, which have less transferable pyrogen in their sera during endotoxin fever than normal animals, exhibit undiminished febrile responses to the endotoxin. How is such a pyrogen-fever dissociation accounted for, if the pyrogen is responsible for the fever? The answer involves the following quantitative relationships.

The first relates to the comparative insensitiveness of the passive transfer method (25). Since only a small fraction of the donor animal's serum is transferred in the test, a recipient may fail to react to the sample injected even though the donor's total circulating serum contains enough endogenous pyrogen to cause a high fever. This apparent discrepancy is due to the marked dilution of the donor pyrogen in the recipient's blood stream. Naturally, the smaller the volume of serum transferred, the more likely is the recipient to remain afebrile. In other words, a negative passive transfer test cannot be construed as indicating the *absence* of endogenous pyrogen in the donor serum (25). The test is useful only for detecting relatively large amounts of circulating pyrogen.

The second quantitative relationship concerns the amount of endogenous pyrogen, which may be present in the donor's circulation, over and above that needed for the production of maximum fever. That the maximum febrile response in rabbits is limited by a definite hyperthermic "ceiling" is indicated by the comprehensive dose-response measurements recently reported by Keene, Silberman, and Landy (13). When their data are plotted arithmetically, as shown in Fig. 4, the existence of the response ceiling is clearly demonstrated.[5] Since the amount of endogenous pyrogen generated is proportional to the dosage of endotoxin (6), it follows that an animal given a relatively large dose of endotoxin (*e.g.*, 50 μg of *Serratia marcescens* endotoxin, lot P-45) will generate considerably more endogenous pyrogen than one given a smaller dose (*e.g.*, 20 μg), despite the fact that both will have about the same amount of fever

[4] In analogous experiments relating to endotoxin tolerance, cited by Petersdorf and Bennett (10), no circulating endogenous pyrogen was found: (*a*) during the febrile response on the 3rd day of successive daily injections of endotoxin (7), (*b*) during the "remnant of fever" which occurs when large doses of endotoxin are given to tolerant animals (5), and (*c*) during the more pronounced fevers which result from still larger doses of endotoxin (7). The failure to demonstrate the circulating factor in the sera of these tolerant animals has been shown (31) to be due to the same dosage factor stressed in this study and in a preceding one relating to the fever of experimental bacterial infection (25). This factor also appears to apply to the studies of Göing (32) relating to endotoxin tolerance.

[5] A similar response ceiling to leucocytic pyrogen has also been defined by analogous titration studies (33).

(see Fig. 4). Although the existence of the excess of endogenous pyrogen in the serum of the animal receiving the larger dose of endotoxin may be demonstrable by the passive transfer test, it will not be indicated by a comparison of the fever responses to the two different doses of endotoxin.

From these considerations, it is evident that a quantitative dissociation between the febrile response to endotoxin and the amount of endogenous pyrogen in the serum may frequently exist without invalidating the thesis that serum endogenous pyrogen is directly involved in the pathogenesis of the fever. Furthermore, it follows that the quantitative relationship between the inten-

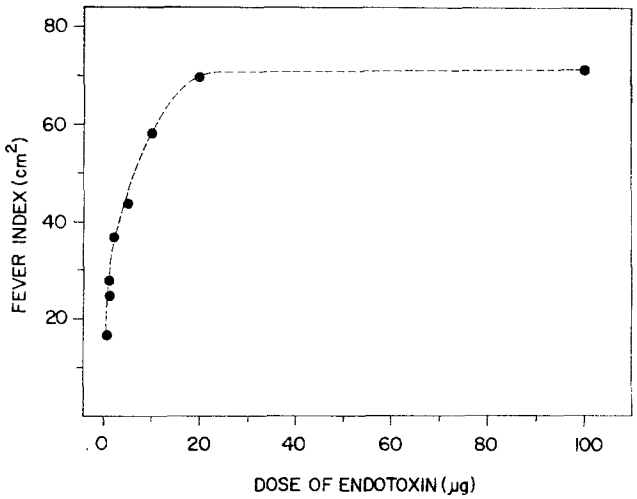

FIG. 4. Dose-fever response curve of normal rabbits to *Serratia marcescens* endotoxin, lot P-45, replotted arithmetically from data reported by Keene, Silberman, and Landy (13). Fever index units are smaller than those employed in present study. (conversion factor = 1.86, see Methods and reference 13).

sity of the fever and the amount of endogenous pyrogen in the circulation can be demonstrated only with relatively small doses of endotoxin which fall on the steep part of the dose-response curve.

More recent studies relating to the role of endogenous pyrogen in endotoxin fever have been reported by Herion, Walker, and Palmer (34) and by Freedman (12). The latter author failed to observe a relationship between the severity of the leucopenia and the amount of fever which resulted from the intravenous injection of endotoxin in normal and tolerant rabbits. Post-injection white counts, however, were made at only one interval—namely, 1 hour after the administration of the endotoxin. Herion *et al.* (34), on the other hand, by studying the entire leucocyte response curve, have demonstrated a direct relationship between the intensity of the fever and the *duration* of the leucopenia. Since the

duration of the depression in white cell count undoubtedly reflects the degree to which the leucocytes are affected by the endotoxin, the finding adds further support to the endogenous pyrogen hypothesis.

Freedman also reported that rabbits made leucopenic with nitrogen mustard develop more fever than normal rabbits when injected with endotoxin (12). Precisely the opposite result has been observed by Herion *et al.* (35). Because of the general toxicity of the drug, animals treated with large doses of nitrogen mustard frequently respond abnormally to pyrogens, including leucocytic pyrogen (36). Depression of the febrile response, particularly, may be due to toxic vasomotor disturbances rather than to an effect on pyrogen production. This difficulty was circumvented in the present study by use of the passive transfer method, in which the definitive temperature measurements were made in healthy recipient rabbits rather than in the nitrogen mustard–treated donors.

SUMMARY

Rabbits made granulocytopenic with nitrogen mustard have been shown to generate serum endogenous pyrogen when given a fever-producing dose of bacterial endotoxin. This finding is in accord with the hypothesis that endogenous pyrogen plays a central role in the pathogenesis of endotoxin fever. The fact that leucopenic animals produce less serum-endogenous pyrogen than normal animals given the same dose of endotoxin has also been confirmed and suggests that polymorphonuclear leucocytes constitute a major source of the endogenous pyrogen which is demonstrable in the circulation during endotoxin fever.

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