STUDIES ON THE PATHOGENESIS OF FEVER

XV. The Production of Endogenous Pyrogen by Peritoneal Macrophages*

By HELMUT H. HAHN, M.D., DAVID C. CHAR,‡ M.D., WILFRED B. POSTEL,§ M.D., and W. BARRY WOOD, Jr., M.D.

(From the Department of Microbiology, the Johns Hopkins University School of Medicine, Baltimore, Maryland, 21205)

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Endogenous pyrogen produced by polymorphonuclear leukocytes is now known to be the principal "messenger" molecule in a variety of experimental fevers (1, 2). Its identification, however, has not provided a satisfactory explanation for natural fevers in patients with agranulocytosis or other diseases in which mononuclear rather than polymorphonuclear leukocytes dominate the pathological lesions (1, 2). Accordingly, many attempts have been made to obtain endogenous pyrogen from cells other than granulocytes (3-7). All, until very recently, have failed.

In one such study, "tissue pyrogens" were isolated which later proved to be contaminated with endotoxin (5, 6); in another this difficulty seems to have been avoided, but the quantities of tissue needed to yield detectable pyrogen were very large (several grams), and the authors concluded that the role of tissue pyrogens in the pathogenesis of fever had not been established (7).

The present studies deal with the production of endogenous pyrogen by rabbit peritoneal macrophages.

EXPERIMENTS

In 1962 two different types of experiments were performed with macrophages from oil-induced peritoneal exudates. Both yielded results suggesting that these cells are capable of generating endogenous pyrogen.

Experiment A.—

Rabbits anesthetized with intravenous pentobarbital were injected intraperitoneally with 50 ml of sterile mineral oil (Nujol, Plough, Inc., New York N. Y.) and were sacrificed 3 days later with an overdose of the same anesthetic. The cells were washed from the peritonea[1]

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‡ Present address: Department of Medicine, University of Washington School of Medicine, Seattle, Washington.

§ Present address: U. S. Army Medical Corps, Fort Bragg, North Carolina.

cavity of each rabbit with 200 ml of chilled, pyrogen-free,[1] modified Hanks' solution (11) containing 100 units of penicillin G, 50 μg of streptomycin, and 1 unit of heparin per ml (hereafter referred to as MH). The recovered cells were pooled, centrifuged at about 100 *g* for 15–20 min at 2-5°C, and washed twice in MH. Differential counts done on Wright-stained smears revealed 10–15% granulocytes; the rest of the cells were macrophages, with only an occasional small lymphocyte. Less than 10% of the total cells stained with eosin Y (12).

When aliquots of 25 million washed peritoneal cells (containing about 3 million contaminating granulocytes) were incubated in 2.5 ml of 0.15 M NaCl for 4 hr at 37°C, centrifuged at 100 *g* for 20 min in the cold, and reincubated for a second 4 hr in the NaCl medium, the pooled supernatants from the two incubated samples were consistently found to be free of detectable pyrogen. Earlier quantitative studies with acute granulocytic exudates had revealed that a minimum of about 10 million granulocytes was needed to generate detectable amounts of pyrogen under similar conditions (10).[2]

Shortly before these studies were begun, it had been learned in other experiments (13)[3] that acute exudate fluid harvested 4 hr after the intraperitoneal injection of glycogen-saline (14) is itself nonpyrogenic but contains a macromolecular, heat-labile "activator". The activator, when incubated with granulocytes, stimulates their release of pyrogen during subsequent incubation in 0.15 M NaCl. Granulocytes so treated release about twice as much pyrogen as granulocytes incubated only in saline.[3] On the assumption that the activator might have a similar effect on macrophages and that it might at times be present in mononuclear exudates in vivo, the experiment described in the previous paragraph was modified as follows.

The harvested cells were first incubated for 4 hr in acute (4 hr) exudate fluid (10^7 cells/ml) at 37°C and then incubated for a second 4 hr in the NaCl medium. The supernatants from both incubation steps were pooled and assayed for pyrogen. As shown in Table I (fourth column) each aliquot of 25×10^6 cells from the mononuclear exudates produced measurable quantities of pyrogen.

To determine how much of the detected pyrogen had come from the "activated" contaminating granulocytes it was necessary to perform extensive control experiments with cells from granulocytic exudates. The cells from these exudates, which were induced by intraperitoneal injection of 0.1% glycogen in 0.15 M NaCl (400 ml/rabbit), were more than 90% granulocytes.[4] The amounts of pyrogen released by 1.25, 2.5, and 5.0×10^6 granulocytes, which were activated and then incubated in 0.15 M NaCl (exactly as in the monocytic exudate experiment), are graphed in Fig. 1. By subtracting the estimated yield

[1] The procedures routinely followed to exclude extraneous pyrogens from all reagents and glassware have been previously described (8–10).

[2] Details of the pyrogen assay method are given in this reference.

[3] Morton, J. D. R. D. Berlin, S. F. Cheuk, and W. B. Wood, Jr. Unpublished observations.

[4] For details of method see reference 10.

of the contaminating granulocytes (Table I, columns 3 and 5) from the total pyrogen generated in each mononuclear exudate experiment (Table I, column 4), it was possible to estimate the amount of pyrogen coming from the macrophages. The figures thus obtained in five out of six experiments (see Table I, last column, and Fig. 1) suggest that some of the pyrogen was derived from the macrophages.

TABLE I

Pyrogen Production by "Activated" Cells from Oil-Induced Peritoneal Exudates

Expt. No.	Total cells ($\times 10^6$)	Granulocytes ($\times 10^6$)	Mean + ΔT* (°C)	Expected + ΔT from granulocytes‡	Apparent + ΔT from macrophages
1	25.0	2.7	0.60	0.28	+0.32
2	25.0	3.7	0.32	0.39	−0.07
3	25.0	2.8	0.40	0.29	+0.11
4	25.0	4.2	0.67	0.44	+0.23
5	25.0	3.2	0.60	0.33	+0.27
6	25.0	3.1	0.80	0.33	+0.47

* Average response in five rabbits.

‡ Determined in control experiments with "activated" granulocytes from peritoneal exudates induced by injection of glycogen-saline.

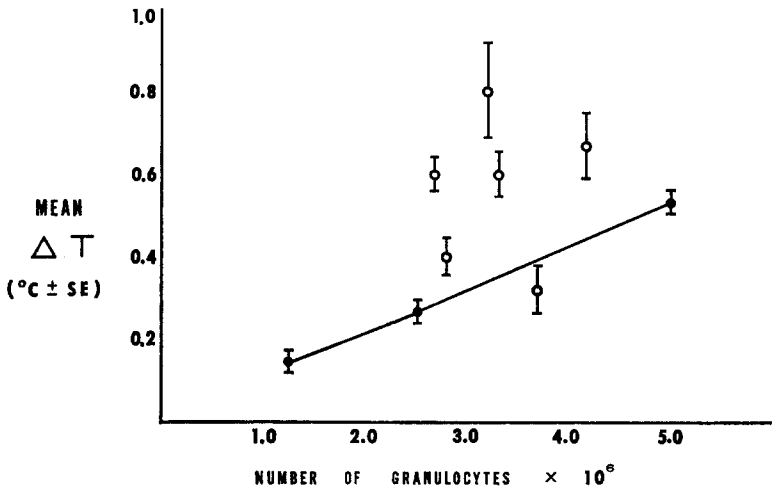

FIG. 1. Comparison of pyrogen production in saline by activated cells of macrophage exudates (○) with that of activated cells of granulocytic exudates (•). The mean temperature rises caused by injection of the saline supernatants from the cells are plotted in degrees centigrade (± SE) against the number of granulocytes × 10^6 in each of the exudates. (For details of incubation in saline, see "Materials and Methods".)

Experiment B.—

To eliminate as many of the contaminating granulocytes as possible from the macrophage suspensions, cells from the original mononuclear exudates were washed in MH and then incubated in 33% rabbit plasma and 67% TC 199 medium (Cappel Laboratories, West Chester Pa.). The plasma–TC 199 medium contained the same concentrations of penicillin, streptomycin, and heparin as the MH (see Experiment A). Aliquots of 100 ml of the medium containing 10^6 cells/ml were incubated without agitation for 3 days at 37°C in 2-liter Erlenmeyer flasks sealed with Parafilm®. After incubation the flasks were decanted and the medium centrifuged to recover any suspended cells. The cells that remained on the bottom of each flask were removed by adding 10 mg of trypsin[5] in 100 ml MH and incubating for 20 min at room temperature. The cells thus recovered were combined with those from the originally centrifuged medium and were washed twice in cold MH. Differential counts made in Wright-stained smears revealed less than 1% granulocytes in each 200–500 cells examined (Table II, column 3), and more than 90% of the cells in each sample were shown to be "viable" by the Eosin Y test (12). Thioglycollate broth cultures of the 3-day-culture supernatants were all

TABLE II

Pyrogen Production by Cultured Mononuclear Exudate Cells

Expt. No.	Total cells ($\times 10^6$)	Granulocytes ($\times 10^6$)	Mean + ΔT* (°C)	Expected + ΔT from granulocytes	Apparent + ΔT from macrophages
1	25.0	<0.25	0.60	0.0	+0.60
2	25.0	<0.25	0.65	0.0	+0.65
3	25.0	<0.25	0.80	0.0	+0.80
4	25.0	<0.25	0.38	0.0	+0.38

* Average response in five rabbits.

sterile, and 5 ml samples of the culture medium taken (*a*) prior to addition of the cells, (*b*) after incubation with the cells for 3 days, and (*c*) after incubation without cells for 3 days, contained no detectable pyrogen.

Samples of the cultured cells obtained in this manner[6] were then incubated for 4 hr at 37°C in each of two successive lots of pyrogen-free 0.15 M NaCl (10^7 cells/ml), and the supernatants from both incubations were pooled and assayed for pyrogen. As shown in Table II, supernatants from 25 × 10^6 cells, containing less than a quarter of a million contaminating granulocytes (column 3), produced significant rises in temperature (column 4). Since no detectable pyrogen could ever be obtained from a quarter million activated granulocytes in appropriate control experiments (see Fig. 1), it appeared that all of the demonstrable pyrogen had come from the cultured macrophages.

[5] Twice-crystallized salt-free trypsin (Mann Research Laboratories, New York, N. Y.) containing 10,000 BAEE units/mg. 1 mg samples suspended in MH were nonpyrogenic.

[6] In preliminary experiments, cells thus treated were found to release pyrogen when incubated in potassium-free saline, i.e., they were already activated either by the trypsin treatment or by some constituent of the plasma–TC 199 medium. Therefore, it was not necessary to incubate them in 4 hr exudate fluid, before incubating them in saline, as in Experiment A.

When the shapes of the fever curves caused by the macrophage and the granulocyte pyrogens were compared, they were found to be identical (Fig. 2). It will be noted, however, that the number of granulocytes required to produce a fever equal to that caused by 25×10^6 mononuclear exudate cells was 5×10^6, i.e., more than 20 times the number of granulocytes ($<0.25 \times 10^6$) contaminating the mononuclear exudates. This relationship also indicates that the pyrogen produced by the cells from the mononuclear exudates did not come solely from the contaminating granulocytes.

Finally, to make certain that the macrophage supernatants were not contaminated with endotoxin, their pyrogenicities were compared in normal and endotoxin-tolerant rabbits.[7] Even when preincubated in tolerant serum (16–18), the macrophage pyrogen was just as active in tolerant rabbits as

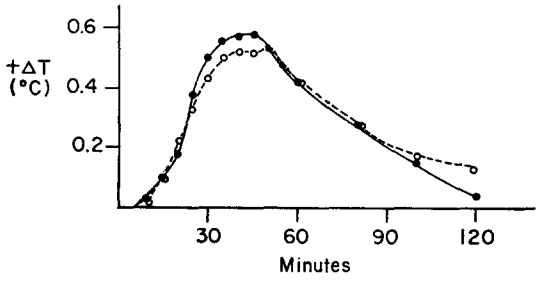

FIG. 2. Mean febrile response (five rabbits) to pyrogen from 25×10^6 cultured macrophages (•—•), compared to mean response (10 rabbits) to pyrogen from 5×10^6 granulocytes (o---o).

it was in nontolerant rabbits (Fig. 3), indicating that its thermogenic activity was not due to endotoxin (1, 2).

Because of numerous negative results obtained in other experiments with both peritoneal and alveolar macrophages,[8] and because cultured macrophages undoubtedly contain products of the leukocytes they ingest in the culture (19, 20), the results of Experiments A and B were withheld from publication until confirmed by a more convincing type of experiment.

Experiment C.—The design of the most conclusive experiment was based on the recent observation that granulocytes incubated in 0.15 M NaCl at 37°C release most of their pyrogen during the first hour of incubation (Fig. 4). This observation suggested that activated granulocytes should become rapidly depleted of pyrogen if repeatedly incubated in fresh saline for periods of 1 hr. On the assumption that the more durable macrophages might continue to produce pyrogen through a greater number of transfers, the amounts of pyrogen

[7] The source of the endotoxin used in these experiments was typhoid vaccine, the specifications of which are described in reference 15.

[8] Hsu H. S., J. Bourland, and W. B. Wood, Jr. Unpublished observations.

produced under these conditions, by predominantly macrophage and predominantly granulocytic suspensions, were compared.

As expected, the activated macrophage suspensions (Fig. 5, upper chart), which contained only about 10% granulocytes, continued to generate large

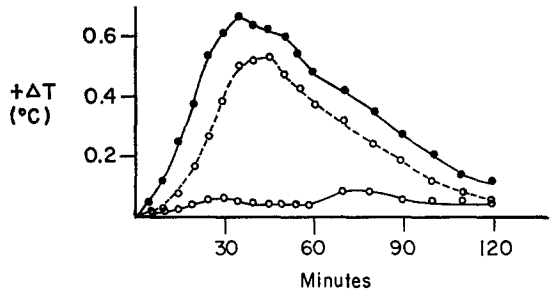

FIG. 3. Mean febrile responses of four rabbits to macrophage pyrogen before and after induction of tolerance to endotoxin. The broken curve (o---o) shows the mean response before tolerance, and the closed circle solid curve (•—•) shows the mean response after tolerance. The open circle solid curve (o—o) shows the failure of the rabbits to respond to a pyrogenic dose of the endotoxin when tolerant. All assays were done on the same four rabbits; and in the tolerance experiments both the macrophage pyrogen and the endotoxin were preincubated in tolerant serum to increase the sensitivity of the test (16–18).

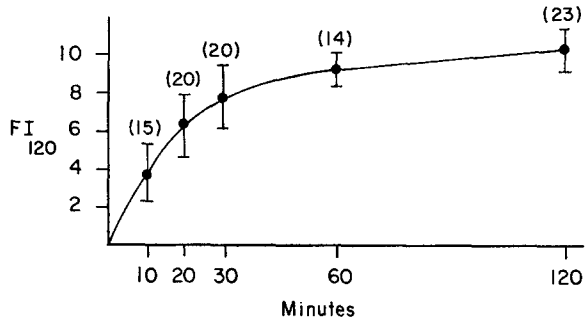

FIG. 4. Kinetics of pyrogen release from 17.5×10^6 granulocytes incubated in 0.15 M NaCl (10^6 cells/ml) at 37°C. Note that mean febrile responses (•) are expressed in FI_{120} units rather than in $+ \Delta T$ (°C)—see ref. (10). Numbers in parentheses = numbers of assays, and brackets indicate ± SE of means.

amounts of pyrogen during the first six incubation periods. In contrast, granulocyte suspensions[9] containing twice, and even 10 times, the number of granulocytes in the macrophage preparations (middle and lower charts) ceased to generate significant amounts of pyrogen after the third incubation period.

[9] 90% granulocytes (10).

From these results it was concluded that a large fraction of the pyrogen recovered from the activated macrophage suspensions, particularly that released after the first three transfers, must have come from the macrophages.

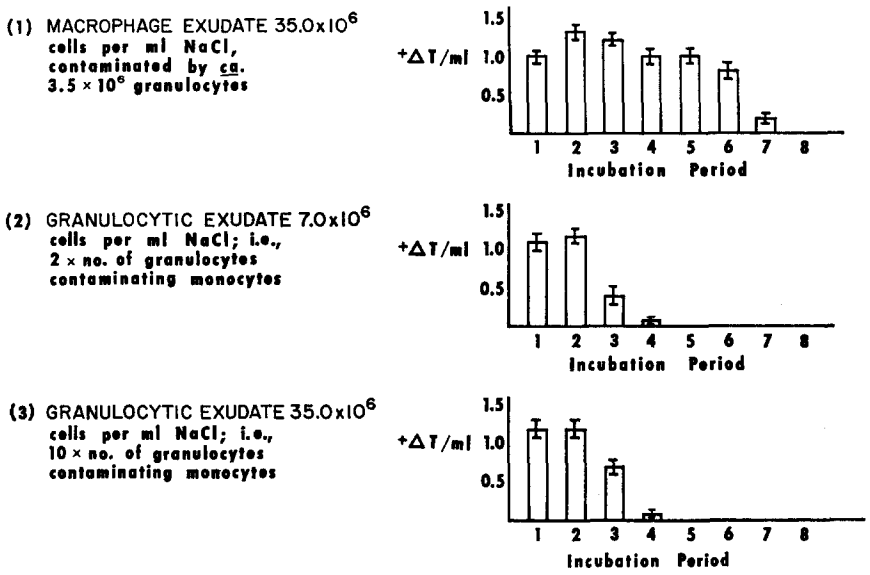

FIG. 5. Comparative kinetics of pyrogen release from activated macrophages and granulocytes incubated for successive 1 hr periods in 0.15 M NaCl at 37°C. Brackets in charts indicate ± SE of means. The upper chart summarizes the results of three separate experiments, the middle chart three experiments, and the lower chart two experiments. All samples in each experiment were assayed in duplicate. It should be noted that only the duration of the release process was studied; i.e., no attempt was made to measure the precise amount of pyrogen in each sample (10).

DISCUSSION

In their original studies of leukocytic pyrogen, Bennett and Beeson (21) demonstrated a pyrogenic factor in peritoneal exudates of rabbits made agranulocytic with nitrogen mustard.[10] Because of the sparsity of granulocytes in these exudates, they concluded that cells other than granulocytes must be capable of generating endogenous pyrogen. In no other tissue cells, however, including macrophages from oil-induced peritoneal exudates, were they able to detect a pyrogen (3). Similar results were later reported by Bennett (4) in an extensive study of tissues from rabbits treated with nitrogen mustard.

[10] Recent attempts to repeat this experiment have yielded much less pyrogen than originally reported (R. D. Collins, personal communication) and suggest that some of the original exudates may also have contained exogenous pyrogen.

The failure of Bennett and Beeson to detect pyrogen in peritoneal macrophages was undoubtedly due to the manner in which they handled the cells. In contrast to the present experiments, in which the cells were incubated for 4 hr at 37°C in isotonic NaCl, their macrophages were either injected directly in a saline suspension or were extracted with saline by mechanical shaking or freezing and thawing (3). When these methods (which did not include incubation at 37°C) were used to obtain pyrogen from granulocytes, approximately 400×10^6 cells were needed to produce a fever of 1°C (3). In contrast, when the saline-incubation method is used, only $20–40 \times 10^6$ cells are needed (10). Moreover, as previously stated, prior activation of the cells by incubation in 4 hr exudate fluid, as carried out in the present experiments, increases still further the yield of granulocytic pyrogen. It would appear, therefore, that Bennett and Beeson did not detect pyrogen in extracts of 500×10^6 macrophages (3) because (*a*) they did not incubate the cells at 37°C and (*b*) they did not activate them by preincubation at 37°C in 4 hr exudate fluid.

The results of Atkins et al., on the other hand, reported in the preceding paper (22),[11] are in agreement with those of the present studies. In their experiments they employed the phagocytosis of heat-killed staphylococci (11, 23) and tuberculin hypersensitivity (24) to stimulate rabbit mononuclear cells to release pyrogen. In neither of these systems is preactivation of the cells necessary to obtain satisfactory yields of pyrogen (11, 23, 24). In each of the experiments performed, the bulk of the pyrogen certainly appeared to have come from the mononuclear cells. Similarly, Bodel and Atkins (25) have reported release of endogenous pyrogen from human blood monocytes following phagocytosis (11, 23). The monocytes were obtained from bloods of normal subjects and of patients with agranulocytosis or monocytic leukemia. Here again, the numbers of contaminating granulocytes seemed to have been too small to account for the pyrogen released.[12]

All of this evidence, including that of the present study, indicates that mononuclear cells, as well as granulocytes, are capable of generating endogenous pyrogen (33). This capability not only provides a plausible explanation for the fevers observed in diseases where mononuclear cells dominate the pathological lesions, but it also raises the question as to whether "tissue pyrogen" (7) may not come from tissue macrophages rather than from parenchymal cells.

SUMMARY

Macrophages from oil-induced peritoneal exudates in rabbits produce endogenous pyrogen when first activated by incubation in 4 hr exudate fluid

[11] Preliminary results of the work, personally communicated to us by Atkins, prompted us to re-examine the evidence provided by Experiments A and B and eventually perform Experiment C.

[12] The claims of Johanovský (26–30) and Allen (31) to have obtained endogenous pyrogen from lymph node cells of sensitized rabbits has not been substantiated (22, 32).

and then stimulated by incubation in potassium-free isotonic sodium chloride solution. The failure of earlier investigators to obtain pyrogen from macrophages is explained, and the relevance of macrophage pyrogen to fevers of agranulocytosis and other diseases, in which mononuclear rather than granulocytic exudates predominate, is discussed.

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