STUDIES ON THE PATHOGENESIS OF FEVER

XVIII. Activation of Leukocytes for Pyrogen Production*

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Rabbit blood leukocytes[1] contain no detectable preformed pyrogen (4) and produce much less pyrogen than exudate leukocytes (about 1/50) when incubated in 0.15 M NaCl (4).[2] After phagocytosis (5) or exposure to endotoxin (6),[3] on the other hand, they produce about the same or even greater amounts of pyrogen than exudate leukocytes. These findings suggest that blood leukocytes must be "activated" to release pyrogen in 0.15 M NaCl, and that activation of blood leukocytes automatically takes place after phagocytosis or exposure to endotoxin. The following experiments deal with the process of activation and the presence of an activating factor in acute peritoneal exudates.

Materials and Methods

The procedures used to exclude exogenous pyrogens (7), to prepare suspensions of leukocytes from both rabbit blood (4) and acute peritoneal exudates (7), to generate pyrogen from the cells (7), to assay the amount of pyrogen released (7, 8), and to measure the cellular contents and fluid concentrations of Na and K (5, 9) and the fluid concentration of Ca (5, 9) and of protein (7) have all been previously described. Endotoxin (lipopolysaccharide B from *Escherichia coli* 0111:B4) was purchased from Difco Laboratories, Detroit, Mich., and cycloheximide (Acti-dione), puromycin dihydrochloride, and actinomycin D were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

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[1] Since rabbit monocytes, as well as granulocytes, are capable of generating pyrogen (1–3), the term leukocyte will be used to include both types of cells, there having been no attempt to distinguish between them in these experiments.

[2] Berlin, R. D., and W. B. Wood, Jr. Unpublished observations.

[3] Moore, D. M., and W. B. Wood, Jr. Unpublished observations.

RESULTS

Evolution of Acute Peritoneal Exudate.—To study the in vivo conditions that accompany the accumulation of detectable amounts of pyrogen in acute peritoneal exudates, rabbits were injected intraperitoneally in the usual manner with 400 ml of glycogen-saline (7) and were sacrificed at intervals of 1, 4, 8, 12, and 18 hr. The results of the analyses performed at each interval are summarized in Table I. Pyrogen could not be detected in 1 ml doses of the peritoneal exudate until (*a*) considerable time had elapsed (>8 hr), (*b*) the protein concentration had become relatively high (about 0.5 g per 100 ml), and (*c*) the cell count had exceeded 1×10^6 per ml. As anticipated, the concentration of K in the fluid, after the first hour, was approximately that of plasma (about 5 meq per liter); whereas total Ca rose somewhat more slowly (presumably because of binding

TABLE I

Release of Endogenous Pyrogen during Evolution of Sterile Peritoneal Exudate Induced by Glycogen-Saline‡*

Time	Volume	Cell count	Protein	K	Ca	Pyrogen
hr	*ml*	*10^6 ml*	*g/100 ml*	*meq/liter*	*meq/liter*	*FI_{120}/ml*
1	405	<1.0	0.14	3.7	0.7	1.1
4	340	<1.0	0.14	4.8	1.6	1.4
8	260	<1.0	0.19	4.1	2.7	0.8
12	130	7.5	0.46	5.2	3.4	10.0
18	120	12.2	0.76	5.6	4.2	10.1

* The pyrogen released into the exudate fluid produced fevers characteristic of endogenous pyrogen and was not affected by endotoxin tolerance (6).

‡ 400 ml of 0.1% glycogen (w/v) in 0.15 M pyrogen-free NaCl injected intraperitoneally. All recorded values represent averages from fluids of 3 rabbits sacrificed at each time interval.

to protein) but eventually reached levels to be expected from equilibration with free plasma Ca^{++}. The release of pyrogen that occurred after 12 hr clearly indicated that the leukocytes, which had originally come from the blood, had somehow become activated in the exudate and, once activated, were able to release pyrogen in the presence of physiological concentrations of K^+ and Ca^{++}. Factors that influence the release of pyrogen under these conditions are discussed below (see section on Generation of Pyrogen in Exudate Fluid).

Activator in Exudate Fluid.—Although early peritoneal exudate fluid (4 hr) contains no detectable pyrogen in doses of 1 ml (Table I),[4] it does contain a factor that activates blood leukocytes and "superactivates"[5] exudate leukocytes (Table II). The activation factor is composed of a heat labile macromolecular

[4] FI_{120} values below 3.0 are not significant (8, 9).

[5] The term superactivate is used because exudate leukocytes are already activated as compared to blood leukocytes.

component and a heat stable dialyzable cofactor (Table III), which are readily separated by Sephadex G-50 sieve chromatography.[2] The dialyzable cofactor can be replaced by a suitable inorganic buffer (phosphate), as shown in Table IV. The nondialyzable macromolecular component behaves like a protein and is readily inactivated by treatment with mercaptoethanol, *p*-chloromercuribenzoate, or trypsin (Table V).

Activating Properties of Subpyrogenic Quantities of Endotoxin.—Incubation

TABLE II

Effect of Exudate Fluid Activator on Subsequent Release of Pyrogen from Blood and Exudate Leukocytes in 0.15 M NaCl

Source of cells	Prior treatment of cells	Pyrogen released in 0.15 M NaCl*
		%
Exudate	None	100
Blood‡	None	2
Blood‡	Incubated in exudate fluid§	98
Exudate	Incubated in exudate fluid§	300

* During incubation at 37°C for 1 hr.

‡ From buffy coat.

§ 3.5×10^8 cells in 10 ml at 37°C for 4 hr.

TABLE III

Cofactor Requirement of Activator in Exudate Fluid

Activation medium*	Pyrogen released (FI_{120})‡
Unheated exudate fluid + 0.15 M NaCl§	6.5 (±0.6)
Dialyzed exudate fluid + 0.15 M NaCl§	0.6 (±0.5)
Heated‖ exudate fluid + 0.15 M NaCl§	0.7 (±0.1)
Dialyzed exudate fluid + heated exudate fluid§	9.3 (±1.8)

* In which exudate cells were first incubated for 4 hr at 37°C.

‡ By 5×10^6 cells incubated for 1 hr at 37°C in 0.15 M NaCl; FI_{120} value, average febrile response in 3 rabbits ± standard error of mean.

§ 10 ml of each per 3.5×10^8 cells; final concentration of K^+ adjusted to 5 meq per liter.

‖ At 100°C for 30 min.

of exudate leukocytes with amounts of *E. coli* endotoxin that are themselves nonpyrogenic causes the cells to produce more than the usual amount of pyrogen when incubated in 0.15 M NaCl (Table VI). This superactivation effect is indistinguishable from that caused by the activator in acute exudate fluid (Tables II–V). Not only does it have approximately the same pH optimum (cf. Tables IV and VII), but it is also nullified by the same amount of heat and the same concentration of mercaptoethanol (see Tables III and V). Besides superactivating exudate leukocytes, subpyrogenic doses of endotoxin activate blood leukocytes, and the exudate fluid activator does the same.[3]

Kinetics of Superactivation Process.—As shown in Fig. 1, the superactivating effect of the exudate fluid activator on exudate leukocytes is readily detectable within 30 min but continues to increase for several hours. In contrast, heat-inactivated exudate fluid fails to superactivate the cells.

TABLE IV

pH-Dependence of Activator in Exudate Fluid

Treatment of exudate fluid*	pH	Pyrogen released (FI_{120})‡
None	8.0	10.9 (±0.6)
Dialyzed vs. unbuffered NaCl§	6.0	0.6 (±0.3)
Dialyzed vs. buffered NaCl‖	6.0	0.7 (±0.3)
" " " "	7.0	9.1 (±0.8)
" " " "	8.0	14.2 (±0.9)

* In which exudate cells were incubated as described in footnote § of Table II.

‡ By 1.7×10^7 cells incubated for 1 hr at 37°C in 0.15 M NaCl; average response of 3 rabbits ± standard error of mean.

§ 0.15 M NaCl.

‖ 0.15 M NaCl + 0.01 M phosphate buffer.

TABLE V

Inactivation of Macromolecular Component of Activator Factor in Exudate Fluid

Treatment of exudate fluid*	Pyrogen released FI_{120}‡
Control§	9.0 (±1.1)
2-Mercaptoethanol (0.6 M)§	3.6 (±0.4)
" (1.2 M)§	0.8 (±0.6)
p-Chloromercuribenzoate (0.02 M)§	0.1 (±0.1)
Trypsin (1 mg per ml)§	0.6 (±0.1)
Incubated at 37°C for 18 hr	11.9 (±1.0)

* In which exudate cells were incubated as in Table II.

‡ By 5×10^6 exudate cells incubated for 1 hr at 37°C in 0.15 M NaCl; average response ± standard error of mean.

§ Fluid was incubated for 1 hr at 37°C, dialyzed at 4°C against 0.15 M NaCl (to remove inactivating reagent when dialyzable) and was finally mixed with an equal volume of heated exudate fluid to replace the necessary dialyzable factor (see text).

Cellular Na and K.—Measurements of cellular Na and K were done before and after superactivation of exudate cells, and no correlation with pyrogen productivity in 0.15 M NaCl was demonstrated.

Generation of Pyrogen in Exudate Fluid.—When exudate leukocytes are incubated in early (4 hr) exudate fluid in vitro, only small amounts of pyrogen are gradually released from the cells (cf. Table VIII and Fig. 1 in reference 9). Indeed, if the exudate fluid is first heated to destroy the activator, no pyrogen

is released at all (Table VIII). This is presumably due to the inhibitory effect of the K^+ and Ca^{++} (10) in the exudate fluid. If, on the other hand, the virtually anaerobic conditions of the in vivo exudate are simulated in an in vitro experiment, the release of pyrogen is significantly enhanced (Table IX). Thus it

TABLE VI

Comparison of Primary Pyrogenicity and Activating Effect of Endotoxin

Endotoxin	Primary pyrogenicity	Activating effect
μg	FI_{120}*	FI_{120}‡
0.1	17.0	13.2
0.03	4.2	11.4
0.01	2.0	9.6
0.005	0.6	7.3
0.001	0.2	4.7
0.0005	0.3	4.2
0.0	0.0	1.3

* Endotoxin injected in 10 ml of heated exudate fluid; FI_{120}, average febrile response of 2–4 rabbits.

‡ Represents average pyrogen released from 20×10^6 exudate granulocytes incubated for 1 hr in 0.15 M NaCl after having been incubated for 4 hr in heated exudate fluid (10 ml) containing endotoxin; FI_{120}, average response of 3 rabbits.

TABLE VII

pH-Dependence of Endotoxin Activation

pH of activation medium*		Pyrogen released (FI_{120}) §
At start	After 4 hr. incubation with cells‡	
6.0	6.0	1.3 (±0.4)
6.5	6.4	8.5 (±0.4)
7.0	6.7	11.9 (±0.9)

* 0.15 M NaCl buffered with 0.01 M phosphate containing 5 meq of K^+ and 0.01 μg of endotoxin/ml.

‡ As in Table II.

§ By 20×10^6 exudate cells after incubation for 1 hr at 37°C in 0.15 M NaCl; FI_{120}, average response of 4 rabbits ± standard error of mean.

seems probable that both the presence of the activator and the anaerobic character of the exudate contribute to the in vivo release of pyrogen demonstrated in Table I.

Evidence that Activation Involves Synthesis of Protein.—The nature and kinetics of the activation process suggest that it may involve the synthesis of new cellular proteins. In keeping with this possibility are the observations that both puromycin (1×10^{-4} M) and cycloheximide (8.9×10^{-5} M) block the

activation of blood leukocytes, whether induced by endotoxin (Table X) or phagocytosis (Table XI). In addition, endotoxin-induced activation is blocked by actinomycin D (1×10^{-5} M) (Table XII).[6] It will be recalled from the

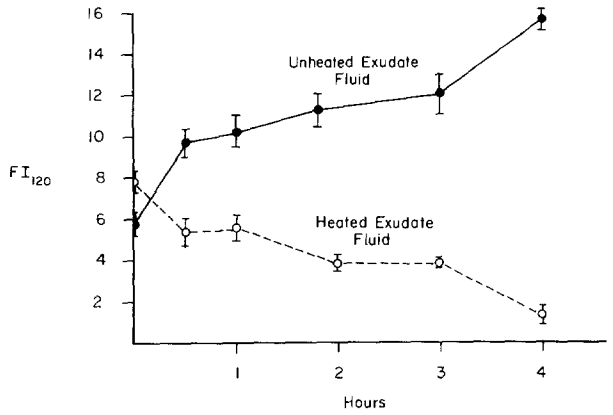

FIG. 1. Kinetics of superactivation process. The average amount of pyrogen produced by 3.5×10^7 exudate cells incubated (37°C) in 0.15 M NaCl for 1 hr is plotted on the ordinate, and the time the cells (3.5×10^8) were preincubated in unheated (●——●) or heated (O----O) exudate fluid (10 ml) is plotted on the abscissa. The latter fluid was heated at 100°C for 30 min. The bars indicate ± standard error of the mean.

TABLE VIII

Slow Generation of Pyrogen by Exudate Leukocytes during Incubation in 4 Hr Exudate Fluid

State of 4 hr exudate fluid	Incubation (37°C) time	Pyrogen released
	hr	FI_{120}*
Unheated	4	7.3 (±2.1)
Unheated	8	9.7 (±1.7)
Unheated	12	15.6 (±3.3)
Heated‡	4	0.6 (±0.3)
Heated‡	8	0.7 (±0.4)
Heated‡	12	0.3 (±0.3)

* From 3.5×10^8 cells in 10 ml of fluid; average response of 3 rabbits ± standard error of mean.

‡ 100°C for 30 min.

preceding paper (9) that inhibition of protein synthesis by puromycin does *not* affect the release of pyrogen from exudate cells that are already activated.

[6] The increased yield resulting from the presence of actinomycin in the NaCl medium (line 4) is, at present, unexplained.

TABLE IX

More Rapid Release of Pyrogen in 4 Hr Exudate Fluid during Anaerobic Incubation

State of exudate fluid	Conditions of incubation*	Pyrogen released FI_{120}‡
Unheated	Aerobic	1.4 (±0.6)
Unheated	5% CO_2–95% N_2	17.0 (±1.4)
Heated§	5% CO_2–95% N_2	1.4 (±0.8)

* For 2 hr at 37°C with continuous aeration.

‡ From 3.5×10^8 cells in 10 ml of fluid ± standard error of mean.

§ 100°C for 30 min.

TABLE X

Blocking Effects of Puromycin and Cycloheximide on Activation of Blood Leukocytes by Endotoxin

Incubation* media		ΔT° (± SE)‡
Activation period (0–2 hr)	Release period (2–4 hr)	
A. *Puromycin*		
Blood-endotoxin§	0.15 M NaCl	1.13 (±0.20)
Blood-endotoxin§ + puromycin‖	0.15 M NaCl	0.01 (±0.01)
(Controls)		
Blood alone (no activation)	0.15 M NaCl	0.04 (±0.02)
Blood-endotoxin§	0.15 M NaCl + puromycin‖	1.04 (±0.16)
B. *Cycloheximide*		
Blood-endotoxin¶	0.15 M NaCl	0.78 (±0.10)
Blood-endotoxin¶ + cycloheximide**	0.15 M NaCl	0.12 (±0.04)
(Controls)		
Blood alone (no activation)	0.15 M NaCl	0.05 (±0.02)
Blood-endotoxin¶	0.15 M NaCl + cycloheximide**	0.84 (±0.08)

* 37°C.

‡ Average response (C°) of 4 rabbits in A and of 9–11 rabbits in B to supernatant (of release period) from 3.5×10^7 leukocytes.

§ 0.1 μg per 10^8 leukocytes in whole blood (heparinized).

‖ 10^{-4} M.

¶ 0.025 μg per 10^8 leukocytes (new lot of endotoxin about 4 times as potent as lot used in all of preceding experiments).

** 8.9×10^{-5} M.

DISCUSSION

Blood leukocytes of rabbits differ from exudate leukocytes in two important respects: (*a*) they contain virtually no detectable pyrogen in their cytoplasm (4), and (*b*) they release relatively little pyrogen when incubated in 0.15 M

NaCl (4).[2] Thus, as far as pyrogen production is concerned, they appear to be in a dormant, or unactivated, state. For such cells to produce pyrogen in saline, two distinct steps are necessary: first, they must become activated; and, secondly, they must be stimulated to release pyrogen. The saline release process, which is highly sensitive to cationic control, requires energy, and involves an

TABLE XI

Blocking Effects of Puromycin and Cycloheximide on Activation of Blood Leukocytes by Phagocytosis

Treatment of cells during incubation* (activation and release)	ΔT° (± SE)‡
A. Puromycin	
Phagocytosis§	1.00 (±0.06)
Phagocytosis§ + puromycin‖	0.07 (±0.02)
None (control)	0.05 (±0.03)
B. Cycloheximide	
Phagocytosis§	1.03 (±0.06)
Phagocytosis§, + cycloheximide¶	0.09 (±0.04)
None (control)	0.04 (±0.02)

* 4 hr at 37°C.

‡ Average response (C°) of 12–13 rabbits for treatments (and 4–6 rabbits for controls) to supernatant from 3.5×10^7 blood leukocytes.

§ Of heat-killed rough pneumococci (strain R36NC) in whole blood (heparinized); multiplicity of organisms to leukocytes was 50:1 (5).

‖ 10^{-4} M.

¶ 8.9×10^{-5} M.

TABLE XII

Blocking Effect of Actinomycin D on Activation of Blood Leukocytes by Endotoxin

Incubation* media		ΔT° (± SE)‡
Activation period (0–2 hr)	Release period (2–4 hr)	
Blood-endotoxin§	0.15 M NaCl	0.64 (±0.05)
Blood-endotoxin§ + Actinomycin D‖	0.15 M NaCl	0.20 (±0.04)
(Controls)		
Blood alone (no activation)	0.15 M NaCl	0.03 (±0.03)
Blood-endotoxin§	0.15 M NaCl + actinomycin D‖	1.09 (±0.07)

* 37°C.

‡ Average response (C°) of 7–15 rabbits to supernatant (of release period) from 3.5×10^7 leukocytes.

§ 0.025 μg per 10^8 leukocytes in whole blood (heparinized). See footnote ¶, Table X.

‖ 10^{-5} M.

increase in membrane permeability, has already been discussed in the preceding report (9).

The activation step requires the synthesis of protein. The critical protein(s) synthesized may include an inactive precursor of the pyrogen molecule (propyrogen) or an enzyme needed to convert it to active pyrogen (11). Evidence is presented elsewhere that neither is synthesized during the release process (9).[7]

Only when pyrogen production is induced by incubation in K-free saline, can the activation and release steps be clearly separated. Since blood leukocytes produce just as much pyrogen after phagocytosis as do exudate leukocytes (5), it is clear that both activation and release are stimulated by the ingestion of foreign particles. Similarly, in the endotoxin system, both processes are stimulated (6)[3] and hence are difficult to distinguish, unless the experiment is done in two stages (as in Table VI).

When unactivated blood granulocytes participate in phagocytosis or are exposed to endotoxin, pyrogen production is inhibited by puromycin and by cycloheximide, since activation must occur before the pyrogen can be released. Actinomycin D also inhibits activation of blood leukocytes stimulated by endotoxin. Similar findings have been obtained by Bodel, et al. (11) with activation by phagocytosis and by etiocholanolone, and by Nordland et al. (12) with activation by phagocytosis and by endotoxin. When, on the other hand, exudate leukocytes or activated blood leukocytes (see Tables X and XII) are incubated in 0.15 M NaCl, pyrogen production is not influenced by inhibitors of protein synthesis (9, 11), since the cells have already been activated.

The presence in exudate fluid of the activator factor, which (like endotoxin) induces both activation and release, accounts for the production of pyrogen in acute exudates, despite the presence of K^+ and Ca^{++}. The anaerobic conditions in the exudate also enhance the release process, perhaps by causing a rise in cationic content (particularly H^+) of the cells (9).

Just how the activator factor affects the cell is not known. Of interest is the fact that it is inactive in the pH range (<6.5) that inhibits phagocytosis (13, 14). One possibility that is difficult to exclude is that the macromolecular component of the exudate activator contains subpyrogenic quantities of endotoxin. Although the activator factor in acute exudate fluid evidently contains an essential protein moiety, further studies are needed to define its precise nature and mode of action.

SUMMARY

Blood leukocytes, in contrast to exudate leukocytes, release little or no pyrogen when incubated in 0.15 M NaCl unless previously activated by exposure

[7] Hahn, H. H., S. F. Cheuk, D. S. Elfenbein, and W. B. Wood, Jr. Studies on the pathogenesis of fever. XIX. Localization of pyrogen within granulocytes. To be published.

to endotoxin or to a protein activator that is present in acute exudate fluid. The activation process, which also occurs during phagocytosis, involves the synthesis of cellular protein, presumably related to the pyrogen molecule. Evidence is presented that generation of pyrogen in sterile inflammatory lesions depends on both the activator and the anaerobic conditions in the exudate fluid.

BIBLIOGRAPHY

1. Atkins, E., P. Bodel, and L. Francis. 1967. Release of an endogenous pyrogen from rabbit mononuclear cells. *J. Exp. Med.* **126**:357.
2. Bodel, P., and E. Atkins. 1967. Release of endogenous pyrogen by human monocytes. *N. Engl. J. Med.* **276**:1002.
3. Hahn, H. H., D. C. Char, W. B. Postel, and W. B. Wood, Jr. 1967. Studies on the pathogenesis of fever. XV. The production of endogenous pyrogen by peritoneal macrophages. *J. Exp. Med.* **126**:385.
4. Kaiser, H. K., and W. B. Wood, Jr. 1962. Studies on the pathogenesis of fever. IX. The production of endogenous pyrogen by polymorphonuclear leucocytes. *J. Exp. Med.* **115**:27.
5. Berlin, R. D., and W. B. Wood, Jr. 1964. Studies on the pathogenesis of fever. XIII. The effect of phagocytosis on the release of endogenous pyrogen by polymorphonuclear leucocytes. *J. Exp. Med.* **119**:715.
6. Collins, R. D., and W. B. Wood, Jr. 1959. Studies on the pathogenesis of fever. VI. The interaction of leucocytes and endotoxin *in vitro*. *J. Exp. Med.* **110**:1005.
7. Kozak, M. S., H. H. Hahn, W. J. Lennarz, and W. B. Wood, Jr. 1968. Studies on the pathogenesis of fever. XVI. Purification and further chemical characterization of granulocytic pyrogen. *J. Exp. Med.* **127**:341.
8. Bornstein, D. L., C. Bredenberg, and W. B. Wood, Jr. 1963. Studies on the pathogenesis of fever. XI. Quantitative features of the febrile response to leucocytic pyrogen. *J. Exp. Med.* **117**:349.
9. Hahn, H. H., S. F. Cheuk, D. M. Moore, and W. B. Wood, Jr. 1970. Studies on the pathogenesis of fever. XVII. The cationic control of pyrogen release from exudate granulocytes *in vitro*. *J. Exp. Med.* **131**:165.
10. Berlin, R. D., and W. B. Wood, Jr. 1964. Studies on the pathogenesis of fever. XII. Electrolytic factors influencing the release of endogenous pyrogen from polymorphonuclear leucocytes. *J. Exp. Med.* **119**:697.
11. Bodel, P. T., M. Dillard, and P. K. Bondy. 1968. The mechanism of steroid-induced fever. *Ann. Intern. Med.* **69**:875.
12. Nordland, J. J., R. K. Root, and S. M. Wolff. 1969. Studies on the origin of human leucocytic pyrogen (LP). *Clin. Res.* **17**:373.
13. Smith, M. R., and W. B. Wood, Jr. 1969. The reaction of heat labile opsonins and their role in antibacterial defense. I. Opsonins to avirulent (unencapsulated) pneumococci. *J. Exp. Med.* **130**:1209.
14. Mudd, S., M. McCutcheon, and B. Lucke. 1934. Phagocytosis. *Physiol. Rev.* **14**:210.