STUDIES ON THE PATHOGENESIS OF FEVER

XIX. Localization of Pyrogen in Granulocytes*

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Relatively little intracellular pyrogen can be detected in rabbit granulocytes (1, 2). Even "activated" (2) exudate granulocytes contain only a fraction of the pyrogen they release when incubated for an hour in 0.15 M NaCl. "Unactivated" blood granulocytes (1, 2) contain even less. These findings suggest that either the active pyrogen is synthesized *de novo* during the release process, or is stored in the cell (bound or unbound) as an inactive "propyrogen" which is converted to an active form at the time of release.

A pyrogen has been reported to be demonstrable in the granules of rabbit neutrophils (3). The results of the present studies fail to confirm this finding, and indicate that what little active pyrogen there is in the rabbit granulocyte is in its cytoplasm. Furthermore, they exclude the possibility that active pyrogen is synthesized during release and hence support the concept that it is derived from an inactive propyrogen.

Methods

All methods and reagents have already been described (1, 2, 4–6) except for the following:

Cells were disrupted by lysis in 0.34 M sucrose as described by Cohn and Hirsch (7). The concentration of cells in the sucrose suspensions was 3.5–7.0 $\times$ 10^7 per ml. When the two exposures to sucrose, prescribed in this method, did not achieve complete lysis, the procedure was modified to include washing the cells in 0.15 M NaCl before exposure to sucrose.

Lactic acid was determined as described in (8), and aldolase was assayed according to reference 9. The method was modified as in Worthington Enzyme Assay Sheet 4-63, Worthington Biochemical Corp., Freehold, N. J. One unit of aldolase activity (Fig. 1) is defined as a change in absorbancy of 1.00 OD unit per min at 25°C under the conditions defined for the method.

Acid phosphatase was measured by a modification[1] of the assay method for alkaline phos-

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[1] Schellenberg, K. A. Unpublished observations.

phatase described in (10). Thymolphthalein phosphate was used as the substrate, and the reaction was carried out at 35°C in 0.1 M acetate buffer (pH 5) containing 0.005 M $MgCl_2$, the total reaction volume being 5 ml. When the pH was raised to 11 by the addition of 0.5 ml of 0.1 M K_3PO_4, the thymolphthalein formed a blue dianion, which was measured at 595 mμ in a Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). The molar extinction coefficient for the thymolphthalein dianion is 38,000 M^{-1} cm^{-1}. Units of activity (Fig. 1 and Table IV) are expressed in millimicromoles of dianion formed per min at 35°C.

Lysozyme was measured by the method of Jollès (11). Both the substrate (*Micrococcus lysodeikticus*) and the lysozyme were purchased from Mann Research Labs, Inc., New York. Units (Fig. 1) are expressed in micrograms of egg white lysozyme equivalents per 3.5×10^8 cells.

TABLE I

Comparative Pyrogen Yields from Intact Exudate Granulocytes and Cell-Free Extracts Incubated at 37°C for 1–2 Hr

State of cells	Incubation medium	Pyrogen yield*
		%
Intact	0.15 M NaCl	100
Pressed	0.15 M NaCl	10
Sonicated‡	0.15 M NaCl	10
Sucrose-lysed	0.34 M sucrose	10
Sucrose-lysed	Citrate-phosphate (pH 3.5)	20
Pressed	Citrate-phosphate (pH 3.5)	10

* To nearest 10%.

‡ Data from reference 1.

All suspensions of whole cells contained 3.5×10^7 cells per ml. Cell-free extracts were made from cell suspensions of twice this concentration and were then diluted with an equal volume of 0.1 M Na phosphate (pH 7.0) or citric acid–Na phosphate (pH 3.5) buffer to maintain the desired pH. In the case of the particulate fractions (G and MN), the particles from 3.5×10^8 cells were packed by centrifugation (400 *g* for 15 min), washed once in the desired buffer, and then resuspended in 10 ml of the buffer.

RESULTS AND DISCUSSION

Effect of Cell Integrity on Pyrogen Yield.—Once exudate granulocytes have been disrupted, they yield only about one-tenth to one-fifth the pyrogen released by intact cells incubated under the same conditions (Table I). Acidification (pH 3.5) of sucrose lysed cells seems to increase the yield by a factor of approximately 2 (cf. lines 4 and 5).

Localization of Pyrogen in Subcellular Fractions.—As shown in Table II, active pyrogen could be detected only in those preparations that contained the cytoplasmic (C) fraction of the cells (lines 1–3). Virtually no pyrogen was demonstrated in either the granular (G) or the membrane-nuclear (MN) fraction (lines 4 and 5).

Lowering the pH of the extraction medium to 3.5 consistently improved the yield from those cell fractions that contained detectable pyrogen. Since incubation of the preparations for 60–90 min caused no further increase in yield, the effect of the acid was assumed to be due to improved extraction rather than generation of pyrogen in the acidified cell-free preparations. A similar improvement of yield from cells disrupted in a French press was achieved by merely increasing the volume of 0.15 M NaCl with which the disintegrated cells were extracted (Table III).[2]

TABLE II

Pyrogen Yields in Neutral and Acid Extracts of Various Cell Fractions of Sucrose-Lysed Exudate Granulocytes

Cell fraction*	Yield of pyrogen from 9×10^7 cells ΔT‡	
	Neutral medium	Acid medium pH 3.5
Whole sucrose-lysate	0.47 (±0.07) (14)	0.61 (±0.08) (12)
C + G	0.33 (±0.05) (36)	0.60 (±0.06) (41)
C	0.56 (±0.09) (16)	0.87 (±0.11) (15)
G	0.00 (6)	0.04 (±0.02) (9)
MN	0.18 (±0.14) (3)	0.00 (3)

* Abbreviations: C, cytoplasmic supernatant after removal of G and MN; G, granular fraction sedimented at 9000 *g* for 15 min; MN, membrane-nuclear fraction sedimented at 400 *g* for 15 min.

‡ Mean temperature rise (°C) caused by intravenous injection of fraction ± standard reror of mean; numbers of assays indicated in last parentheses. All fractions were centrifuged at 1000 *g* for 15 min to remove gross particles before being injected.

TABLE III

Effect of Volume of Extraction Fluid on Pyrogenicity of Cell Pressate

Volume*	Mean Δ T‡
ml	
1	0.45 (±0.13)
5	0.63 (±0.08)
10	1.05 (±0.05)

* Per 3.5×10^8 exudate cells.

‡ Average responses of six rabbits given extract from 3.5×10^8 cells ± standard error of mean.

[2] Improved extraction of pyrogen from sonicated cells with an ethanol-ammonium sulfate-saline (1:1:1) solvent has also been reported (12). This procedure, however, did not increase the yield from sucrose-lysed cells (see section on Whole cell lysates). Nor did acidification increase the yield from pressed cells (Table I). Clearly, the efficiency of extraction is influenced by the manner in which the cells are disrupted.

Absence of Detectable Pyrogen in Granules.—Because of the report of Herion et al. (3) that acid extracts of the granules of rabbit neutrophils are pyrogenic, repeated attempts were made to demonstrate pyrogen in granular lysates. As shown in Table IV, pyrogen was never detected, no matter how the granules were lysed or disrupted, even though their numbers exceeded those extracted by Herion et al., and the yields of acid phosphatase (last column) left no doubt that the granular contents had been recovered from the cells. In addition, as already stated, incubation of the combined cytoplasmic and granular fractions (C + G), at a pH low enough to lyse the granules (3.5), failed to increase the amount of pyrogen already in the cytoplasmic fraction.

TABLE IV

Failure to Detect Pyrogen in Granules of Rabbit Neutrophils from Peritoneal Exudates

Treatment of granules	No. of cells from which granules were separated	Fever response	Yield of acid phosphatase*
None: whole granules	3.5×10^8	None	—
Acid lysis:			
Citric acid (0.2%)	1.4×10^9	None	7.37
0.005 N HCl	7.0×10^7	None	8.30
French press:			
0.15 M NaCl	8.7×10^7	None	7.13
Citrate-phosphate buffer (pH 3.5)	7.0×10^8	None	8.11
Acid lysis (0.2 N H_2SO_4) + ethanol (20%) extraction‡	3.5×10^9	None	—

* In units (see Methods) per 3.5×10^8 cells.

‡ Procedure of Herion et al. (3).

Attempts to Extract Pyrogen from Whole-Cell Lysates.—Numerous attempts were also made to extract pyrogen from sucrose-lysates with such reagents as NH_4SO_4-alcohol-saline (12), Triton X-100 (13), sodium dodecyl sulfate (14), deoxycholate (15), ethylenediaminetetraacetic acid (EDTA) (16), and trypsin (17). None of these procedures significantly increased the yield.

Cellular Enzymes Released with Pyrogen.—Although out-right degranulation does not occur when pyrogen is being released from rabbit granulocytes,[3] it seemed possible that the granular membranes might become abnormally permeable during the release process and "leak" granular proteins into the cytoplasm and thence into the extracellular medium. To test this possibility the intracellular and extracellular concentrations of two granulocytic enzymes

[3] Cheuk, S. F., J. J. Marr, and W. B. Wood, Jr. Unpublished observations.

were studied during pyrogen release: one a granular enzyme (acid phosphatase), the other a cytoplasmic enzyme (aldolase). The data plotted in Fig. 1 show that the aldolase rapidly leaves the cell (upper panel), while the pyrogen is being released; whereas the acid phosphatase tends to be retained (middle panel). Further evidence that aldolase (an enzyme involved in the conversion of

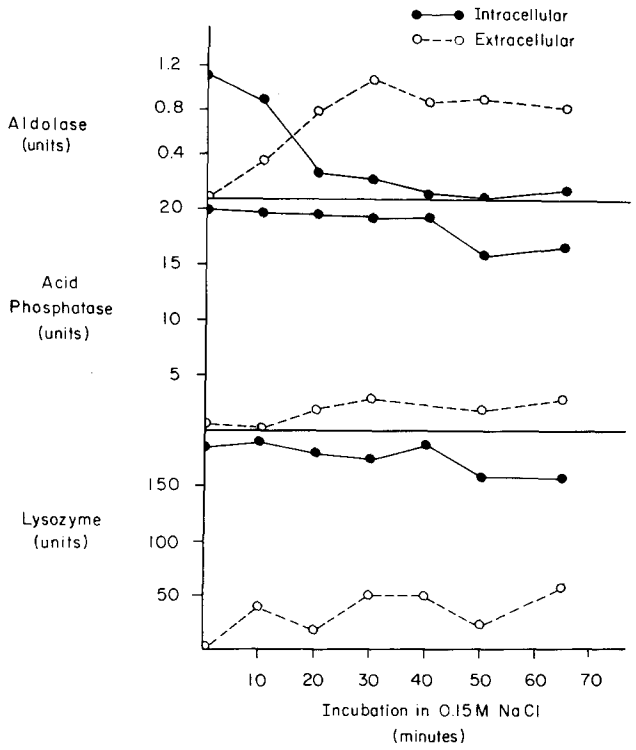

FIG. 1. Shifts of intracellular enzymes to extracellular medium during incubation (37°C) of cells (3.5×10^8) in 0.15 M NaCl (10 ml). Enzyme units are as described in Methods and are plotted per 3.5×10^8 cells. Intracellular enzyme measurements were done on sucrose lysate of the cells (see Methods). Extracellular enzymes were measured in the incubation medium.

glucose to lactic acid) leaves the cell with the pyrogen is provided by the data in Table V, which indicate that cells releasing pyrogen and aldolase in Na medium produce less lactic acid than cells not doing so in sodium-potassium medium (4).

A similar study was done with lysozyme, a protein of approximately the same molecular weight as pyrogen (14,000). A large fraction of this enzyme (circa 50%) is stored in the granules (7). It too tended to remain within the cells during pyrogen release, though some (probably from the cytoplasm) did enter the extracellular medium (Fig. 1, lower panel).

Also colchicine, in a concentration reported to block postphagocytic degranulation of human neutrophils without interfering with phagocytosis (18), had no

TABLE V

Generation of Lactic Acid by Exudate Granulocytes Incubated in Na Medium and in Na-K Medium

Incubation time	Increase in cellular lactic acid *	
	Na medium	Na-K medium
min	%	%
20	80 (±44)	127 (±22)
40	70 (±23)	160 (±18)
60	100 (±37)	224 (±50)
120	120 (±32)	193 (±38)

* Average value from four experiments ± standard error of mean.

TABLE VI

Pyrogen Release during Phagocytosis in Presence and Absence of Colchicine*

Colchicine	Pyrogen released from 1.2×10^8 exudate granulocytes, ΔT‡
M	
0	0.45 (±0.07). (11)
2.5×10^{-4}	0.46 (±0.08) (13)

* 3.5×10^8 exudate granulocytes were incubated for 4 hr at 37°C with 1.9×10^9 unencapsulated pneumococci in 10 ml of modified Hank's solution as described in reference 19.

‡ Average temperature rise in number of rabbits indicated in last parentheses ± standard error of mean.

TABLE VII

Failure of Puromycin to Inhibit Release of Pyrogen from Rabbit Exudate Granulocytes Incubated in 0.15 M NaCl*

Concentration of puromycin	Pyrogen release (FI_{129})‡
M	
0	8.2 (±1.1) (9)
2×10^{-4}	10.4 (±1.1) (6)
2×10^{-3}	9.8 (±0.8) (6)

* At 37°C for 1 hr.

‡ Average response of number of rabbits indicated in last parentheses ± standard error of mean.

demonstrable effect upon the pyrogen yield of rabbit granulocytes engaging in the phagocytosis of heat-killed pneumococci (Table VI).

Collectively, these results add further support to the conclusion that the

pyrogen released from rabbit granulocytes is not derived from their granules.

Failure of Puromycin to Affect Release of Pyrogen.—As already reported (4) and further confirmed in Table VII, puromycin in concentrations known to block the incorporation of radiolabelled amino acids into rabbit granulocytic proteins[4] has no effect on the amount of pyrogen released from exudate granulocytes during incubation in K-free saline. In addition, it does not significantly modify the

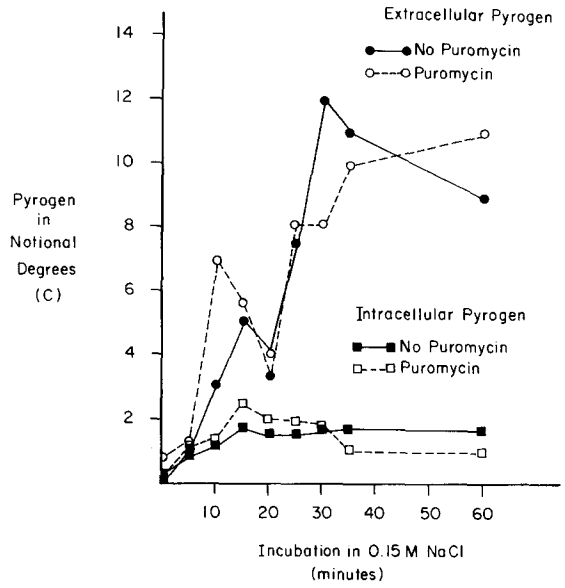

FIG. 2. Failure of puromycin (10^{-4} M) to affect either intracellular production or extracellular release of pyrogen during incubation (37°C) in 0.15 M NaCl. Intracellular measurements were done on sucrose lysates of cells (see Methods), and extracellular pyrogen was measured in the incubation medium. The yield of pyrogen is expressed in notional degrees centigrade of fever produced by the injection of the lysate (intracellular) or medium (extracellular) from 3.5×10^8 incubated cells.

slight intracellular buildup of pyrogen that precedes the release of pyrogen from the cells (4) (Fig. 2). These findings are not compatible with the hypothesis that pyrogen, a known protein (5), is synthesized *de novo* during the release process. Nor do they support the suggestion that its conversion from an inactive to an active form during release involves the synthesis of new protein, e.g., enzyme(s) (20). Rather they favor the concept that pyrogen is stored in the cells as an inactive precursor (propyrogen), which is converted to active pyrogen just prior to its release from the cells.

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

SUMMARY

Only *intact* exudate granulocytes from rabbits generated large amounts of endogenous pyrogen when incubated in 0.15 M NaCl. No matter how whole-cell lysates or combinations of subcellular fractions were incubated, their yields of pyrogen never approached those of whole cells; at most, only minimal amounts of pyrogen were formed, once the integrity of the cells had been destroyed.

Some pyrogen could be extracted from disrupted cells, but never more than a fraction (<25%) of that released from incubated whole cells. The yield could be slightly improved by lowering the pH (to 3.5) and by increasing the volume of extraction fluid.

Virtually all of the preformed pyrogen that could be extracted from sucrose-lysed cells was found in their cytoplasmic fraction. Contrary to the results of Herion et al. (3), none could be detected in the granular (or lysosomal) fraction. Likewise, all efforts to recover pyrogen from the membrane-nuclear fraction were unsuccessful.

In keeping with the finding that preformed pyrogen is contained in the cytoplasmic fraction were the observations that practically all of the aldolase, a cytoplasmic enzyme, and very little of the acid phosphatase, a granular enzyme, were lost from the cells during the release of pyrogen. Lysozyme, an enzyme stored in both the granules and the cytoplasm, was partially released from the cells under the same circumstances.

Neither the release of pyrogen nor its slight intracellular buildup that precedes release (4) were affected by concentrations of puromycin that block protein synthesis in the cells and prevent their activation. Hence, it is concluded that the release process, which also involves the formation of active pyrogen (4), does not require protein synthesis, whereas activation of the cells, which may involve the synthesis of an inactive precursor (2), does.

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