STUDIES ON THE PATHOGENESIS OF FEVER*

X. THE EFFECT OF CERTAIN ENZYME INHIBITORS ON THE PRODUCTION AND ACTIVITY OF LEUCOCYTIC PYROGEN*

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Polymorphonuclear leucocytes obtained from acute peritoneal exudates in rabbits are capable, when appropriately stimulated, of producing large amounts of endogenous pyrogen (1–3). There is mounting evidence that this pyrogenic factor plays an important role in the pathogenesis of fever (4, 5). Exploratory studies relating to the production of leucocytic pyrogen *in vitro* have revealed that the process is temperature-dependent and that it results in a substantial net increase of active pyrogen per cell during incubation in saline (2). Since these findings are in keeping with the hypothesis that the active pyrogen is formed, either from an inactive precursor or by *de novo* synthesis within the cell, experiments were undertaken to investigate the possible effects of certain enzyme inhibitors upon the combined metabolic reactions involved in the formation and release of the pyrogen.

Methods

The technical procedures employed in obtaining appropriate quantities of rabbit granulocytes from acute peritoneal exudates and in assaying the endogenous pyrogen which they produce during incubation in saline were identical with those of the preceding study (2). The standard aliquot of granulocytes used in all experiments contained 3.5×10^8 polymorphonuclear leucocytes from 12 to 18 hour peritoneal exudates. All cell suspensions or extracts to which enzyme inhibitors or activators had been added were brought to neutrality before being incubated, unless otherwise stated. After incubation, the samples to be assayed for pyrogenicity were dialyzed in cellophane tubing against a minimum of 30 volumes of saline at 4°C for 20 hours. Three changes of saline were made during each dialysis.

RESULTS

Sulfhydryl Reagents.—

Each of four standard aliquots of granulocytes harvested from 18 hour exudates was washed in 30 ml of cold normal saline[1] and was resuspended in 11.7 ml (3×10^7 cells per ml) of saline

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[1] When unwashed cells were used, results were irregular.

containing a final concentration of 2×10^{-4} M of one of the four sulfhydryl reagents listed in Table I. The mixtures of cells and reagents were incubated at 37°C for 4 hours, following which the cells were separated by centrifugation at 4°C, and the supernatant fluids were dialyzed against cold saline and tested for pyrogenicity. The results are recorded in Table I. When exposure of the cells to the reagents was prolonged to 20 hours at 37°C, similar results were obtained.

To determine the possible effect of the sulfhydryl reagents upon the pyrogen molecule itself, analogous experiments were performed using pyrogen already released from granulocytes, rather than suspensions of the cells. Each aliquot of preformed pyrogen consisted of the supernatant from a saline-cell mixture containing one standard aliquot of cells (3.5×10^8) incubated at 37°C for 4 hours. As indicated in Table II, the reagents were added in concentrations of

TABLE I

Effect of Sulfhydryl Reagents on Production of Endogenous Pyrogen by Intact Granulocytes

Inhibitor	Concentration of inhibitor*	Pyrogen produced (mean fever index‡)		
None (control)	0	22.8	(2.53)	(4)
Arsenite	2×10^{-4} M	2.1	(0.56)	(6)
Iodoacetate	2×10^{-4} M	2.3	(2.12)	(2)
p-chloromercuribenzoate	2×10^{-4} M	3.1	(1.13)	(2)
N-ethylmaleimide	2×10^{-4} M	0.5	(0.71)	(4)

* Exposure of cells to inhibitor: 4 hours at 37°C.

‡ Fever index = measure of height and duration of febrile response as indicated by area beneath fever curve (6). Figures in parentheses = standard deviations (left) and number of observations on which mean value was based (right).

2×10^{-4} M and 1×10^{-2} M and were incubated with the pyrogen for a period of 4 hours. Additional experiments, in which the exposure time was prolonged to 20 hours, yielded essentially identical data.

The results summarized in Tables I and II indicate: (*a*) that relatively low concentrations of arsenite, iodoacetate, *p*-chloromercuribenzoate, and *N*-ethylmaleimide (*i.e.*, 2×10^{-4} M) inhibited the production of endogenous pyrogen by intact leucocytes, (*b*) that none of them grossly affected[2] the activity of the preformed pyrogen at these concentrations, and (*c*) that at higher concentrations (1×10^{-2} M) only the iodoacetate inactivated the preformed pyrogen, even when the exposure time was prolonged to 20 hours.

Glutathione.—Because the above compounds suppressed the production of leucocytic pyrogen, experiments were performed to determine whether reduced

[2] Since the fever index on the untreated sample was > 20, *i.e.*, in the relatively insensitive range of the assay procedure (2), partial inactivation of the pyrogen could have occurred without being detected (6).

glutathione would enhance it. On the contrary as shown in Table III, the glutathione exerted a striking inactivating effect of its own. Since this effect did not occur at 4°C and required several hours to reach completion, it was tentatively attributed to a degradation of the pyrogen by an enzymatic process activated

TABLE II

Effect of Sulfhydryl Reagents on Thermogenic Activity of Preformed Granulocytic Pyrogen

Inhibitor	Concentration of inhibitor*	Activity of pyrogen (mean fever index‡)		
None (control)	0	22.8	(2.53)	(4)
Arsenite	2×10^{-4} M	20.8	(3.46)	(2)
	1×10^{-2} M	18.1	(0.35)	(2)
Iodoacetate	2×10^{-4} M	20.8	(0.28)	(2)
	1×10^{-2} M	3.4	(0.85)	(2)
p-chlormercuribenzoate	2×10^{-4} M	20.1	(3.94)	(3)
	1×10^{-2} M	20.2	(4.65)	(6)
N-ethylmaleimide	2×10^{-4} M	23.8	(3.70)	(2)
	1×10^{-2} M	21.4	(5.93)	(2)

* Exposure of pyrogen to inhibitor: 4 hours at 37°C.
‡ As in Table I.

TABLE III

Gross Inactivating Effect of Glutathione on Crude Preparations of Leucocytic Pyrogen

Concentration of glutathione	Incubation temperature*	Activity of pyrogen‡
	°C	
2×10^{-4} M	37	++
2×10^{-3} M	37	+
2×10^{-2} M	37	0
2×10^{-2} M	0	++

* Incubation time, 20 hours.
‡ ++ = full pyrogenic activity, + = depressed activity, 0 = no activity.

by the presence of the glutathione. When the experiments were performed with partially purified leucocytic pyrogen (7) from which much of the non-pyrogenic protein had been removed, the gross degradation did not occur. If, however, crude pyrogen was incubated with purified pyrogen in the presence of glutathione, the mixture was inactivated (see Table IV). It was concluded, therefore, that proteases, present in the crude leucocytic extracts (8, 9) and activated by reduced sulfhydryl groups (10), were probably destroying the active pyrogen.

To test this hypothesis further, four standard aliquots of preformed leucocytic pyrogen (*vide supra*) were handled in the following manner.

Aliquot 1 was stored at 4°C, while aliquot 2 was incubated at 37°C for 20 hours. Glutathione, in a final concentration of 2×10^{-2} M, was added to aliquots 3 and 4, which thereafter were treated like 1 and 2, respectively. To determine the degree of proteolysis occurring at 37°C in both the presence and absence of added glutathione, 0.5 ml of 60 per cent perchloric acid was added to a 5.8 ml sample of each aliquot, which was then stored at 0°C for 2 hours, centrifuged in the cold to remove the precipitated protein, and measured for optical density at 280 mμ in a Beckman DU spectrophotometer.

TABLE IV

Comparative Inactivating Effects of Glutathione on Crude and Partially Purified Preparations of Leucocytic Pyrogen

Preparation of pyrogen	Concentration of glutathione	Pyrogenic activity* (mean fever index‡)		
Crude	0	17.2	(2.20)	(3)
	2×10^{-2} M	0.7	(0.30)	(3)
"Purified"	0	18.0	(2.46)	(3)
	2×10^{-2} M	15.8	(0.39)	(2)
"Purified" plus crude	0	24.3	(5.16)	(3)
	2×10^{-2} M	3.9	(1.33)	(3)

* After incubation for 20 hours at 37°C.

‡ As in Table I.

TABLE V

Concentrations of 280 mμ Absorbing Acid-Soluble, Proteolytic Products in Preparations of Crude Leucocytic Pyrogen Incubated with and without Added Glutathione

Time of incubation	Concentration glutathione added	Optical density (280 mμ)*
hrs. at 37°C		
0	0	0.501
20	0	0.526
0	2×10^{-2} M	0.593‡
20	2×10^{-2} M	0.936

* Measured on supernatant fluids of perchloric acid precipitate.

‡ Glutathione itself raises OD (280 mμ) slightly when added in concentration of 2×10^{-2} M (7).

The results recorded in Table V indicate that the concentration of acid-soluble peptides and amino acids increased substantially during the incubation with added glutathione. This finding is in keeping with the postulated increase in proteolytic activity which might be expected to destroy the pyrogen (7).

Finally, it is of interest that concentrations of glutathione (2×10^{-1} M), which will often reduce disulfide bonds (11) at 37°C, had no demonstrable inactivating effect upon the purified pyrogen even after 6 hours. This finding is in agreement with the observation that exposure to 1.6×10^{-1} M bisulfite at 4°C for 4 hours also does not inactivate the pyrogen molecule (12).

Diisopropyl Fluorophosphate.—In an attempt to block the proteolytic enzymes activated by the glutathione, preliminary experiments were performed in which diisopropyl fluorophosphate (DFP) was introduced into the system. This compound, however, was found to have a definite inhibitory effect upon the activity of preformed leucocytic pyrogen (see Table VI).

TABLE VI

Effect of Diisopropyl Fluorophosphate (DFP) on Activity of Leucocytic Pyrogen

Concentration of DFP	Pyrogen preparation	Duration of Incubation	Incubation temperature	Activity of pyrogen (mean fever index*)		
		hrs.	°*C*			
1×10^{-4} M	Crude	6	37	15.8	(2.40)	(3)
1×10^{-3} M	"	6	37	16.0	(4.40)	(3)
3×10^{-3} M	"	6	37	2.4	(1.51)	(4)
5×10^{-3} M	"	6	37	0.3	(0.32)	(4)
1×10^{-2} M	"	6	37	0.3	(0.35)	(2)
1×10^{-2} M	Crude	20	4	15.7	(—)	(1)
1×10^{-4} M	"Purified"	6	37	13.9	(3.27)	(4)
5×10^{-4} M	"	6	37	4.2	(1.75)	(3)
1×10^{-3} M	"	6	37	2.6	(1.81)	(3)

* As in Table I.

Each aliquot of preformed pyrogen was prepared by incubating 3.5×10^{8} exudate leucocytes in saline at 37°C for 4 hours, removing the cells by centrifugation, and diluting the supernatant to a volume of 11.7 ml with additional saline and the appropriate amount of DFP in propylene glycol. The stock solution of DFP was prepared at a concentration of 0.05 M in propylene glycol. The latter solvent was shown to have no effect by itself in concentrations of 20 per cent when incubated with the pyrogen at 37°C for 20 hours.

It will be noted from the data recorded in Table VI that the inactivation of pyrogen by DFP did not occur at 4°C and that the partially purified pyrogen was more sensitive to inactivation than the crude pyrogen. Raising the pH of the incubation mixture to 8 did not increase the sensitivity of crude pyrogen to DFP (13).

Attempts to protect the pyrogen from the inactivating effect of DFP by first adding the known antagonist, 2-pyridine aldoxime methiodide (2-PAM) (14), to the incubation mixture were unsuccessful (see Table VII). Indeed the 2-PAM appeared to potentiate the DFP rather than block its effect (15). Nor did the

2-PAM reactivate DFP-treated pyrogen when added after the excess DFP had been removed by dialysis. Likewise, hydroxylamine failed to reactivate DFP-treated pyrogen even when added in concentrations as high as 1 M and kept in contact with the dialyzed DFP-pyrogen complex for as long as 48 hours at 37°C, or 3 weeks at 4°C.

TABLE VII

Gross Effect of Diisopropyl Fluorophosphate (DFP) on Activity of Crude Leucocytic Pyrogen in Presence of DFP Antagonists: 2-Pyridine aldoxime methiodide (2-PAM) and Hydroxylamine (HA)

Concentration of DFP	Protection*	Reactivation‡	Duration of incubation	Incubation temperature	Pyrogenic activity§
				°C	
0	2-PAM, 10^{-2} M	—	20 hrs.	37	++
1×10^{-3} M	—	—	6 hrs.	37	++
1×10^{-3} M	2-PAM, 10^{-5} M	—	6 hrs.	37	+
1×10^{-3} M	2-PAM, 10^{-4} M	—	6 "	37	0
1×10^{-3} M	2-PAM, 10^{-3} M	—	6 "	37	0
3×10^{-3} M	—	—	6 hrs.	37	0
3×10^{-3} M	—	2-PAM, 10^{-2} M	6 hrs.	37	0
3×10^{-3} M	—	2-PAM, 10^{-2} M	30 "	37	0
0	HA, 1 M	—	6 hrs.	37	++
3×10^{-3} M	—	—	6 hrs.	37	0
3×10^{-3} M	—	HA, 1 M	6 hrs.	37	0
3×10^{-3} M	—	HA, 1 M	48 "	37	0
3×10^{-3} M	—	HA, 1 M	3 wks.	4	0

* Added before incubation with DFP.

‡ Added after incubation with DFP and removal of excess DFP by dialysis.

§ As in Table III.

Dinitrofluorobenzene.—Inasmuch as 2,4-dinitrofluorobenzene (DNFB) is known to inactivate certain DFP-sensitive enzymes (16, 17), its action on leucocytic pyrogen was also tested.

Aliquots of leucocytic pyrogen, each of which contained the amount produced by 3.5×10^8 exudate granulocytes during incubation at 37°C for 4 hours in 7 ml of saline, were diluted with 3.5 ml of 0.05 M phosphate buffer adjusted to pH 8. To each aliquot was added 1.16 ml of a solution of DNFB in propylene glycol. The final concentration of DNFB in the pyrogen samples varied from 10^{-4} to 10^{-2} M. Each sample was incubated at 37°C, dialyzed against

normal saline, and tested for pyrogenicity. The incubation times varied between 15 minutes and 6 hours. Control experiments with 20 per cent propylene glycol and phosphate buffer showed that neither of these reagents affected the activity of the pyrogen.

It will be seen from the results recorded in Table VIII, that DNFB inactivates leucocytic pyrogen at concentrations even lower than DFP. Decreasing the pH of the medium from 8 to 6.5 suppresses the inactivating effect of the DNFB. This finding is in agreement with similar experiments relating to the action of DNFB on chymotrypsin (17). The characteristic yellow chromogens, which result from the reaction of DNFB with free amino groups, regularly ap-

TABLE VIII

Gross Effect of 2,4-Dinitrofluorobenzene (DNFB) on Activity of Leucocytic Pyrogen

Concentration of DNFB	pH	Duration of incubation*	Activity of pyrogen‡
—	8	20 hrs.	++
10^{-4} M	8	6 hrs.	++
5×10^{-4} M	8	6 "	0
10^{-3} M	8	6 "	0
10^{-3} M	8	1 hr.	+
10^{-2} M	8	15 min.	++
10^{-2} M	8	6 hrs.	0
10^{-2} M	6.5	6 hrs.	++

* At 37°C.

‡ As in Table III.

peared after about 20 minutes of incubation (18). The proportion of chromogen formed by the interaction of DNFB with the pyrogen could not, of course, be distinguished from that resulting from the reaction of DNFB with other proteins in the crude preparation.

DISCUSSION

That the production of leucocytic pyrogen involves metabolic reactions of the cell, rather than a mere "leakage" of a preformed intracellular constituent through an altered cell membrane, is indicated by the observations recorded in the preceding report (2). In keeping with these observations is the fact that the sulfhydryl-reactive enzyme inhibitors used in the present experiments blocked the production of leucocytic pyrogen, when incubated with the cells in a con-

centration of 2×10^{-4} M.[3] It should be noted, however, that one of the inhibitors, iodoacetate, also inactivated the pyrogen molecule itself, at a concentration of 1×10^{-2} M. This finding complicates the interpretation of the first observation in the case of iodoacetate, since it may be argued that the apparent interference with the production process may have been due to an intracellular inactivation of the preformed pyrogen by iodoacetate concentrated in the cell. Such an explanation, however, seems less reasonable in the case of the other sulfhydryl reagents (arsenite, *p*-chloromercuribenzoate, and *N*-ethylmaleimide), since none of them inactivated the pyrogen at a concentration 50 times that which blocks production of pyrogen by the intact cells. Thus it may be tentatively concluded that the latter compounds exert their blocking effects by interfering with cellular reactions essential to pyrogen production.

The finding that preformed leucocytic pyrogen is relatively sensitive to the inactivating effects of diisopropyl fluorophosphate and dinitrofluorobenzene raises interesting questions concerning the chemistry of the pyrogen molecule. Since there is already evidence that the pyrogen is either itself a protein or else is a macromolecule containing an essential protein constituent (7, 20), and since both DFP and DNFB inactivate certain enzymes by combining with specific reactive groups of the enzyme molecules (14, 17, 21, 22) and DNFB partially inactivates placental gonadotropic hormone (23), the possibility must be entertained that leucocytic pyrogen functions either as an enzyme or as a hormone and that it possesses specific reactive groups which are blocked by these inhibitors. The failure to reverse or block the inactivating effect of DFP with its antagonists, 2-pyridine aldoxime methiodide and hydroxylamine, indicates that the action of DFP on leucocytic pyrogen differs from its action on acetylcholinesterase (14, 24). Nevertheless, the demonstrations of interactions of leucocytic pyrogen with DFP and DNFB provide provocative leads which warrant further investigation.

The manner in which the higher concentrations of iodoacetate (1×10^{-2} M) inactivate the pyrogen molecule is not clear, in view of the failure of equal concentrations of arsenite, *p*-chloromercuribenzoate, and *N*-ethylmaleimide to exert the same effect. The fact that the pyrogen is capable of withstanding exposure to 1×10^{-2} M concentrations of the latter inhibitors at 37°C for 20 hours, makes it unlikely that the thermogenic action of the pyrogen molecule is dependent upon the presence of free sulfhydryl groups (25). It is possible, therefore, that the inactivating effect of the iodoacetate is due to its known influence upon reactive protein groups other than sulfhydryl radicals (26).

The experiments dealing with glutathione are of interest in two regards. First, the failure of glutathione to inactivate partially purified leucocytic pyrogen, even at concentrations as high as 2×10^{-1} M, suggests that the pyrogen mole-

[3] These same reagents are also known to affect the carbohydrate metabolism of polymorphonuclear leucocytes at similar concentrations (19).

cule does not possess disulfide bonds essential for activity (11). This suggestion is supported by the results of analogous experiments performed with bisulfite (12). Neither observation, however, can be considered to be conclusive. Second, the demonstration that leucocytes incubated in saline release proteolytic enzymes, which are apparently capable of degrading the pyrogen molecule when activated by glutathione, reveals a potential mechanism whereby the pyrogen may be destroyed *in vivo*. Such a reaction might thus contribute to defervescence. Although there is at present no evidence that the proteolytic mechanism actually operates under the conditions which pertain in inflammatory exudates, it is of interest that polymorphonuclear leucocytes contain detectable amounts of reduced glutathione (27, 28).

SUMMARY

The production of endogenous pyrogen by intact granulocytes obtained from acute peritoneal exudates is blocked by arsenite, iodoacetate, *p*-chloromercuribenzoate, and *N*-ethylmaleimide in concentrations of 2×10^{-4} M.

When the concentration of these sulfhydryl-reactive enzyme inhibitors is increased to 2×10^{-2} M, only the iodoacetate inactivates the pyrogen molecule, whereas the arsenite, the *p*-chloromercuribenzoate, and the *N*-ethylmaleimide have no gross effect upon its thermogenic activity.

Both diisopropyl fluorophosphate and dinitrofluorobenzene are even more potent inactivators of the pyrogen molecule than iodoacetate, although the action of the DFP cannot be blocked or reversed by known antagonists such as 2-pyridine aldoxime methiodide and hydroxylamine.

Proteolytic enzymes, potentially capable of degrading leucocytic pyrogen, are released from polymorphonuclear leucocytes, along with the pyrogen, when the cells are incubated in normal salt solution. These enzymes are readily activated by a sufficient concentration of glutathione (2×10^{-2} M). They are not present in preparations of partially purified leucocytic pyrogen from which much of the non-pyrogenic protein has been removed.

Glutathione by itself, even at concentrations as high as 2×10^{-1} M, does not affect in the gross the thermogenic activity of the purified pyrogen.

The implications of these findings in relation to both the production and the chemical characteristics of leucocytic pyrogen are discussed.

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