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STUDIES ON THE MECHANISM OF ENDOGENOUS PYROGEN PRODUCTION I. INVESTIGATION OF NEW PROTEIN SYNTHESIS IN STIMULATED HUMAN BLOOD LEUCOCYTES**

In many experimental models of fever, the release of an endogenous pyrogen from cells of the host in response to the appropriate stimulus has been implicated in the pathogenesis of the fever.[1] This material, first identified in extracts of rabbit leucocytes,[2] is now known to be released from several different leucocyte types, of various species including man, and in response to a number of different stimuli.[1] Endogenous pyrogen from rabbit[3] and human cells[4] is a small molecule, of molecular weight between 10 and 20,000, containing an essential protein moiety.

Previous studies have identified some characteristics of the mechanism of release of endogenous pyrogen from rabbit exudate PMN leucocytes.[5-8] When such cells are suspended in a potassium-free medium such as saline, pyrogen begins to be released after 20 to 30 minutes, and continues to appear in the medium for a few hours. This release is not inhibited by fluoride, which is known to block anaerobic glycolysis, the main source of energy for PMN leucocytes. However, pyrogen does not appear in the medium in normal amounts when iodoacetate, parachloromercuribenzoate, or N-ethyl maleimide are present. The data suggest that some step during the process of production and release requires a protein containing an essential -SH group. Since very little pyrogen can be extracted from exudate cells at any time,[8] release of pyrogen into the medium presumably occurs immediately after it is produced in the cell. Puromycin does not interfere with release of endogenous pyrogen,[8] so that continuous synthesis of this protein probably does not occur in these cells, and conversion of pyrogen from a precursor, perhaps by a simple enzymatic step, seems a likely possibility.

Blood leucocytes, unlike exudate leucocytes, do not release pyrogen when suspended in saline.[9,10] Some stimulus, such as exposure to endotoxin or phagocytic particles, is required to "activate" the cell. In addition, potassium does not inhibit pyrogen release from activated blood cells, in contrast to exudate cells. It appears, then, that exudate cells have already been activated *in vivo,* perhaps by a macromolecular substance in the exudate fluid,[11] and

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that the mechanism of pyrogen release from these cells differs from that of blood cells.

In order to study in detail the early steps in production and release of pyrogen from non-stimulated cells, human blood leucocytes, primarily polymorphonuclear leucocytes, were activated by phagocytosis of heat-killed staphylococci. The action of various inhibitors was studied at intervals after activation, intracellular and extracellular pyrogen was assayed, and production of endogenous pyrogen in the presence of tritium-labelled phenylalanine was investigated.

MATERIALS AND METHODS

Leucocytes. Methods for preparation of leucocytes were as described previously.[10] All materials, glassware, and reagents used in this and subsequent procedures were sterile and pyrogen-free. Briefly, a three percent dextran solution was added to heparinized human blood obtained from male volunteers. After 30 to 40 minutes at room temperature, the supernatant was centrifuged. The cell button was washed with modified Krebs-Ringer phosphate (KRP) buffer and the remaining red cells removed by hypotonic lysis. After an additional wash, the leucocytes were suspended in buffer to a concentration of about 5×10^7 per ml. Counts were done on a Coulter particle counter.

Incubation. In the standard incubation procedure, leucocytes were incubated in a concentration of about 1×10^7 per ml. in 15% serum-buffer containing 150 mg. % glucose. After shaking on a Dubnoff shaker at 37°C. for two hours, penicillin (6000 units) was added, and the flasks were placed in a stationary incubator at the same temperature overnight. Alternatively, after two hours incubation the contents of the flask were centrifuged at 600 *g* for 15 minutes, the supernatant discarded, the cells resuspended in fresh buffer or serum-buffer with glucose and penicillin, and placed in the stationary incubator overnight. Heat-killed *Staphylococcus albus,* prepared as described previously,[10] were added initially in some experiments in a ratio of 10 to 20 bacteria per leucocyte.

Inhibitors, dissolved in sterile saline, filtered, and stored at −10°C., were added in some experiments as follows: Puromycin dihydrochloride (Nutritional Biochemicals Corp., Cleveland, Ohio) 5 μg/ml., Na iodoacetate (Eastman Organic Chemicals, Rochester, N.Y.) 2×10^{-4} M, Na fluoride, 10^{-2} M. Actinomycin D (Nutritional Biochemicals Corp.) was dissolved in acetone or sterile pyrogen-free alcohol with 10% acetone, filtered, and stored at −10°C. On the day of the experiment, an amount calculated to give a final concentration of 5 μg/ml. during incubation was added to an Erlen-

meyer flask, the solvent evaporated by compressed air blown through a sterile pipette, and the coated flask then used for the incubation. Solutions of all inhibitors were tested for pyrogenicity before use.

Preparation of medium and cell supernatants. After leucocyte incubation, to obtain medium supernatant, flask contents were centrifuged at 2,000 *g* for 30 minutes at 4°C. To obtain cell supernatants, centrifugation was first done at 600 *g* for 15 minutes. The medium supernatant was then removed, (and when needed re-centrifuged as above), the leucocytes were washed once with 40 ml. of buffer, centrifuged again at 600 *g*, and the cell button suspended in a few milliliters of buffer. It was then disrupted by five cycles of freeze-thawing in dry ice and acetone. In some experiments the cells were then stored at −10° or −120° for a few days. After thawing, 10 to 30 ml. of buffer was added, the mixture was shaken on a Dubnoff shaker at 37° for 30 minutes, and then centrifuged at 2,000 *g* for 30 minutes. Medium and cell supernatants were stored at 4°C. and assayed (see below) for extracellular and intracellular pyrogen, respectively.

Pyrogen assay. Rabbits were injected intravenously and temperatures measured at 15 minute intervals, as described previously.[13] In most experiments, the same rabbits received control and experimental solutions. Supernatant from 2 to 3 × 10^7 leucocytes provided the usual test dose for each rabbit.

Sephadex filtration. Sephadex G-75 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) was prepared as described previously,[4] and poured to a height of 47 cm. in a glass column with an internal diameter of 1.3 cm. It was equilibrated with phosphate-buffered saline pH 6.5. Samples of 2.8 ml. were collected in sterile tubes, at a flow rate of about 0.25 ml. per minute, with a Gilson fraction collector, as described previously.[4] Sterility of all samples was confirmed by culture in thioglycollate broth. Protein determinations were carried out by the method of Lowry,[18] as described previously,[4] using samples of 0.1 to 0.5 ml. for assay.

Electrofocusing. Supernatant from 18-hour incubations of leucocytes was obtained by centrifugation at 2,000 *g* for 30 minutes, and dialyzed for 24 hours against 20 volumes of 2M urea in distilled water. This dialysis was repeated two or three times. The pyrogenic solution was then focused using LKB 8100 ampholine electrofocusing equipment and carrier ampholytes pH 5-8 by techniques described in the instruction manual, except for the following modifications. The column was carefully washed with sterile pyrogen-free water and dried with sterile gauze before use, all tubing was rinsed with alcohol and sterile, pyrogen-free water, and all solutions were sterile and pyrogen-free. The dense solution employed in preparing the

sucrose gradient contained 2M urea. Initial watts were 1.5, and the column was run for 24 hours. Samples of 3 to 4 ml. were collected into sterile tubes using a peristaltic pump and a fraction collector. Since complete sterility could not be assured in this system, cultures were not routinely performed on all fractions. In early studies, all samples to be injected were filtered through millipore filters (Swinnex -25 filter unit, Millipore Corporation, Bedford, Mass.), and the filter then rinsed through with twice the volume of buffer. However, samples which were not filtered before injection were later determined to be similarly pyrogenic or non-pyrogenic when injected into rabbits. Estimates of total protein concentrations were made on 0.5 ml. aliquots by measuring absorption at 280 λ on a Gilford spectrophotometer. Absorption of 1.0 was estimated to be equivalent to a protein concentration of 1.0 mg/ml.

Preparation of leucocyte homogenates for incubation. To prepare cell homogenates, 2-hour stimulated leucocytes were centrifuged at 600 *g* for 15 minutes, resuspended in 2 to 6 ml. of .25 or .34 M sucrose, and homogenized in ice with a teflon plunger for 5 minutes. Aliquots containing 1 to 2 × 10^8 leucocytes were then incubated in a total volume of 3 ml. containing the following materials: 0.67 MKH_2PO_4–Na_2HPO_4 buffer at pH 5.1 to 7.4, 39 μmoles, glucose 30 μmoles, sucrose 400 to 545 μmoles, KCl 100 μmoles, $MgSO_4$ 20 μmoles, ATP 3 μmoles, nicotinamide 120 μmoles, DPN 6 μmoles, TPN 6 μmoles. In a few experiments Cytochrome C 25mμmoles was present. After incubation for 2 to 18 hours, the mixture was centrifuged at 2,000 *g* for 30 minutes, and the supernatant injected for pyrogen assay.

RESULTS

Initially, experiments were set up to investigate the time needed for activation and release of pyrogen in the experimental system. Similar studies have been done with rabbit leucocytes.[14] Human blood leucocytes were incubated with heat-killed staphylococci, flask contents were removed after 2, 4, 6, 8, and 22 hours, and after centrifugation, the medium supernatants were injected into rabbits for pyrogen assay. Control cells, not stimulated, were also incubated. As shown in Figure 1, pyrogen release is not significant until after two hours of incubation, even though, as shown previously,[15] over 90 percent of the added bacteria have been ingested during the first 40 minutes of incubation. Pyrogen release is rapid between 2 and 6 hours, but thereafter the level of extracellular pyrogen remains fairly constant.

Leucocytes from the same flasks were broken by freezing and thawing, and the amount of pyrogen present within the cell at each time interval assayed by injection of cell supernatant. As shown in Figure 2, very little

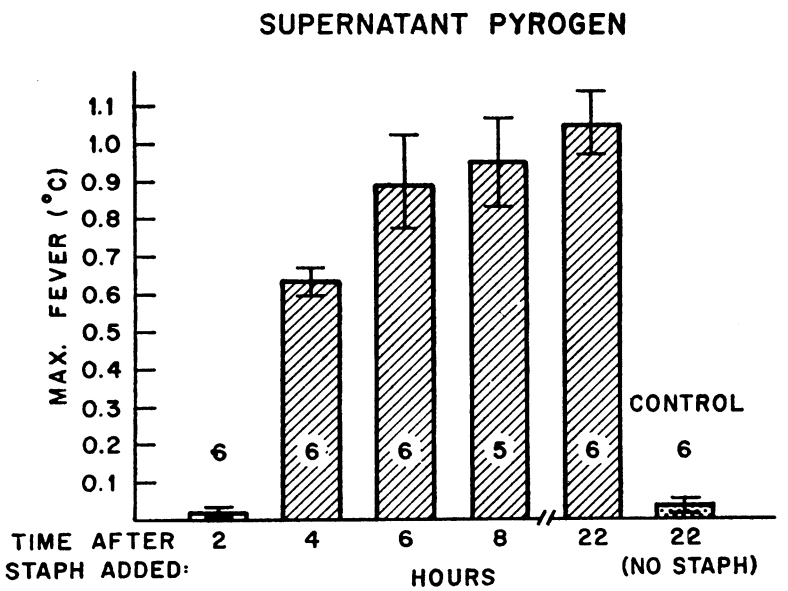

FIG. 1. Average maximum febrile responses of the same group of rabbits to supernatants from 3 × 10^7 leucocytes incubated with heat-killed staphylococci for various periods or for 22 hours without staphylococci (control). In this and subsequent Figures, S.E. of the mean is indicated, and the number of rabbits is given within the bars.

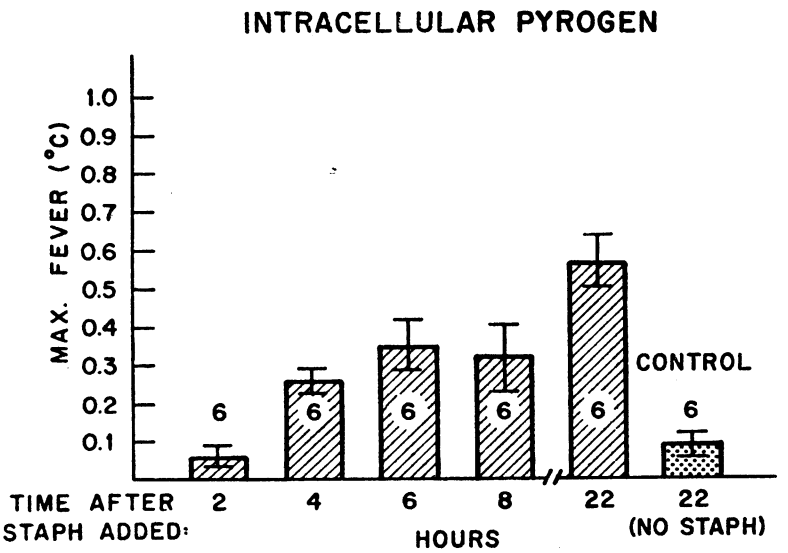

FIG. 2. Average maximum febrile responses in the same rabbits to cell supernatant from 3 × 10^7 leucocytes, disrupted after incubation with heat-killed staphylococci for various periods, or without staphylococci for 22 hours (control).

pyrogen is detectable within the cell at the early intervals after activation. A small amount is detectable at 4 and 6 hours, a time when rapid release is occurring (see Fig. 1), but at 22 hours there is significantly more intracellular pyrogen than in the earlier samples.

These results suggest that production of pyrogen is active during a limited number of hours, during which time little pyrogen remains within the cell, but that as release slows, intracellular levels of pyrogen rise.

In a group of experiments in which the bacteria used as stimulus were incubated with cells for 1, 2, or 18 hours, there was no significant difference in the amount of pyrogen released into a serum-buffer medium over the 18 hour period.

Effect of actinomycin D on pyrogen release. In order to investigate the role of new RNA synthesis in the production of pyrogen, actinomycin D, 5 μg/ml., was added to flasks (see METHODS). One aliquot of leucocytes in serum-buffer was placed in one of these flasks at the same time as bacteria (0 time). Other leucocytes were incubated in other flasks with bacteria for one or two hours, they were then centrifuged, resuspended in serum-buffer, and transferred to flasks containing atcinomycin. As shown in Figure 3, there is a marked difference in the effect of this inhibitor on subsequent pyrogen release depending on the time at which it is added. Pyrogen release

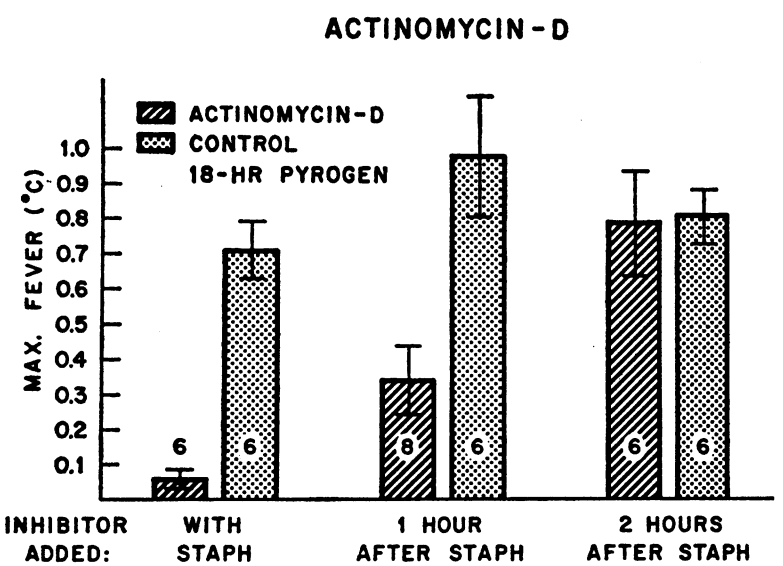

FIG. 3. Average maximum febrile responses in rabbits to supernatants from 18-hour incubations of leucocytes stimulated by heat-killed staphylococci (Staph). Actinomycin D, 5 μg/ml., was present at varying times in one group of flasks (actinomycin), but not the other (control).

is completely suppressed when the inhibitor is present early in the incubation, but there is no effect on pyrogen release when it is added two hours after the bacteria. Other studies,[18] including some from our laboratory, have shown that actinomycin has no inhibitory effect on phagocytosis of bacteria.

In two experiments, leucocytes incubated with actinomycin (at 0 time) and bacteria were assayed for intracellular pyrogen after 18 hours, in 6 rabbits. No significant amount of intracellular pyrogen was detected, (.03°C.), in contrast to cells incubated in the same experiment with bacteria but not actinomycin (.58°C.). Thus, actinomycin apparently prevents production as well as release of pyrogen under these conditions.

Effect of puromycin on pyrogen release. A similar group of experiments was carried out with puromycin, 10^{-5}M, added simultaneously, or one and two hours after the bacteria. In some of these studies, the inhibitor was added directly to flasks at the appropriate time, instead of to stimulated leucocytes resuspended in fresh serum-buffer. Since the results of both types of experiment were the same, the data were combined, and are shown in Figure 4. As in the results with actinomycin, marked inhibition of subsequent release occurred when the inhibitor was present at 0 or 1 hour, but little or none when it was added 2 hours after the bacteria. Similarly, sig-

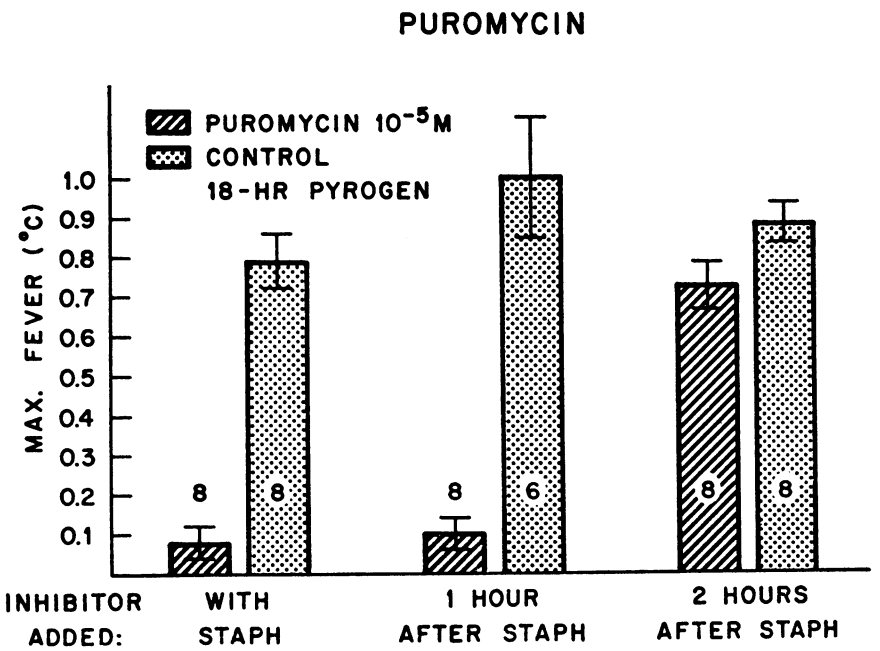

FIG. 4. See Figure 3. Puromycin, 10^{-5}M, was added at varying times.

nificant amounts of intracellular pyrogen were not detected in cells incubated for 18 hours with puromycin and bacteria. Puromycin, like actinomycin, has been shown not to interfere with phagocytosis.[16]

The results with these two inhibitors suggest that in order for normal production and release of endogenous pyrogen to occur, synthesis of new RNA and protein is required within 1 to 2 hours after leucocyte activation. Little or no pyrogen is detectable within or outside the cell by 2 hours, however. Therefore, after this critical time, final steps in production as well as release of endogenous pyrogen can apparently proceed in the absence of new protein synthesis.

Effect of fluoride on pyrogen release. Fluoride, 2×10^{-2}M, was added to leucocytes 1 or 2 hours after bacteria. Since fluoride inhibits phagocytosis, experiments at 0 time were not done. The results of these experiments are shown in Figure 5. When fluoride is added one hour after bacteria, it effectively blocks pyrogen release, but when added at 2 hours it has very little effect. Thus, this inhibitor also appears to influence primarily the early

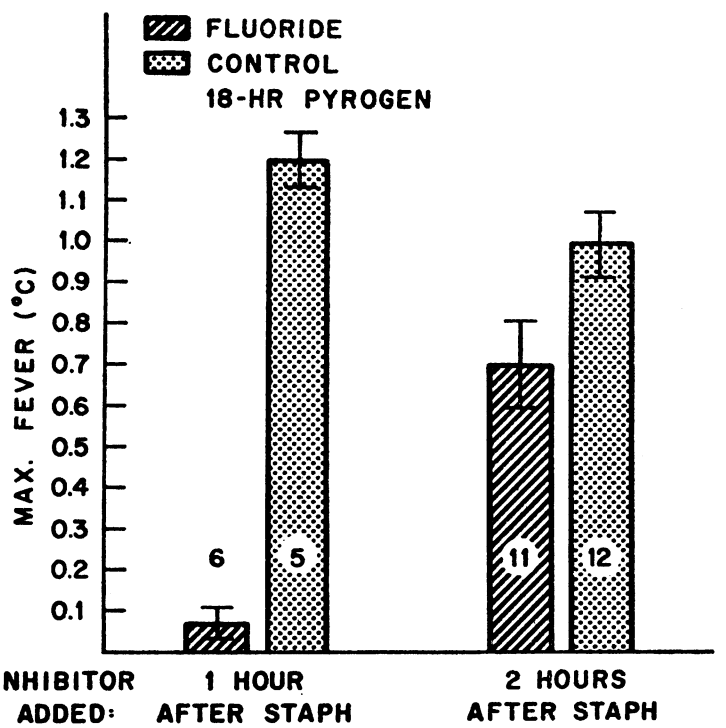

FIG. 5. See Figure 3. Sodium fluoride, 2×10^{-2}M, was added one or two hours after heat-killed staphylococci.

events in cell activation. Since in addition to inhibiting glycolysis, fluoride has been shown to interfere with protein synthesis in reticulocytes,[17] a similar mechanism could be operating here.

Effect of iodoacetate on pyrogen release. Iodoacetate, 2×10^{-4}M, was added to leucocytes at 0, 1, and 2 hours after bacteria. Although studies in other systems[18] have indicated that iodoacetate interferes with phagocytosis, no such inhibition was observed under the conditions presented here. As shown in Figure 6, this compound effectively prevents subsequent pyrogen release when added at 0 time or 1 hour, and it is still quite inhibitory when added at 2 hours. Thus, unlike the other inhibitors, iodoacetate appears to influence both early and late steps in pyrogen production and release.

Control experiments were carried out to determine whether any of the inhibitors altered the pyrogenic activity of endogenous pyrogen. As shown in Table 1, the activity of endogenous pyrogen was slightly reduced by incubation at 37°C. for 18-hours, but none of the inhibitors had any significant effect.

These results indicate that new RNA and protein synthesis is required within two hours for subsequent production and release of endogenous pyrogen. During this time no significant amount of pyrogen can be detected either inside or outside the cell. The critical new protein synthesized, there-

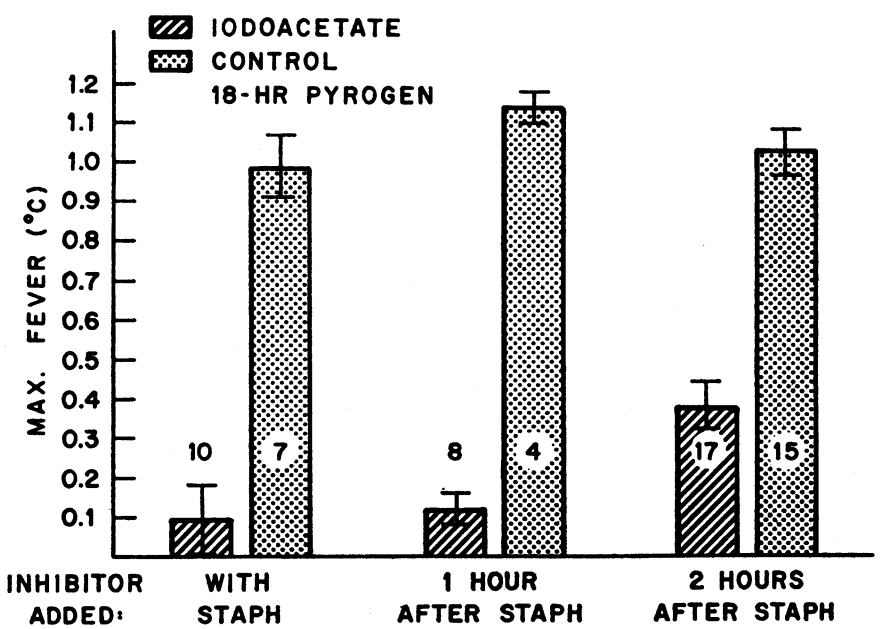

FIG. 6. See Figure 3. Iodoacetate, 2×10^{-4}M, was added at varying times.

TABLE 1. EFFECT OF 18-HOUR INCUBATION OF LEUCOCYTE PYROGEN (EP) WITH METABOLIC INHIBITORS

	Δ °C.†	
	No incubation	*37°C. for 18 hrs.*
EP	.80 ± ‡.19	.60 ± .19
EP + Actinomycin 10^{-5}M	—	.61 ± .12
EP + Puromycin 10^{-5}M	—	.56 ± .16
EP + NaF 2×10^{-2}M	—	.56 ± .12
EP + NaIAA 2×10^{-4}M	—	.64 ± .20

† In this and subsequent tables, average maximum febrile response in rabbits to injection of supernatants is given. The same 4 rabbits received all injections.

‡ S.D.

fore, could include a non-pyrogenic precursor of endogenous pyrogen, an enzyme or enzymes required to convert a precursor to a pyrogenic molecule, or both. In any case, a non-pyrogenic molecule which is later converted to endogenous pyrogen is presumably present in the cell two hours after stimulation. This molecule could either be synthesized during the first two hours after activation, or it might have been synthesized in the cell previously, during maturation of the leucocyte. Two types of experiments were designed to look for evidence of the first of these possibilities.

Blood leucocytes were prepared as usual, and aliquots each totaling 1.75×10^9 cells were incubated in serum-buffer either alone, with heat-killed bacteria, or with bacteria in the presence of actinomycin. After two hours, the cells were centrifuged, resuspended in plain buffer, and incubated for 16 hours. This buffer supernatant was then collected by centrifugation, lyophilized, and dialyzed against pH 6.5 phosphate-buffered saline. After a small aliquot was removed for culture and pyrogen assay, each material was placed in turn on a column of sephadex G-75, eluted with buffered saline, and fractions tested for pyrogen and total protein.

As shown in Figure 7, pyrogen was released, as expected, only from cells incubated with staphylococci without actinomycin (triangles). Solid lines show the results of pyrogen assays, dotted lines indicate total protein determinations. Although there was considerably more total protein released into the supernatant from cells that phagocyted bacteria than from those that did not, there was no significant difference in the amount or distribution of proteins from phagocyting cells treated with actinomycin compared to untreated cells. Identical results (not shown here) were obtained in a similar experiment in which rabbit blood cells were stimulated with and without actinomycin. Results of these two experiments suggest that most of the pro-

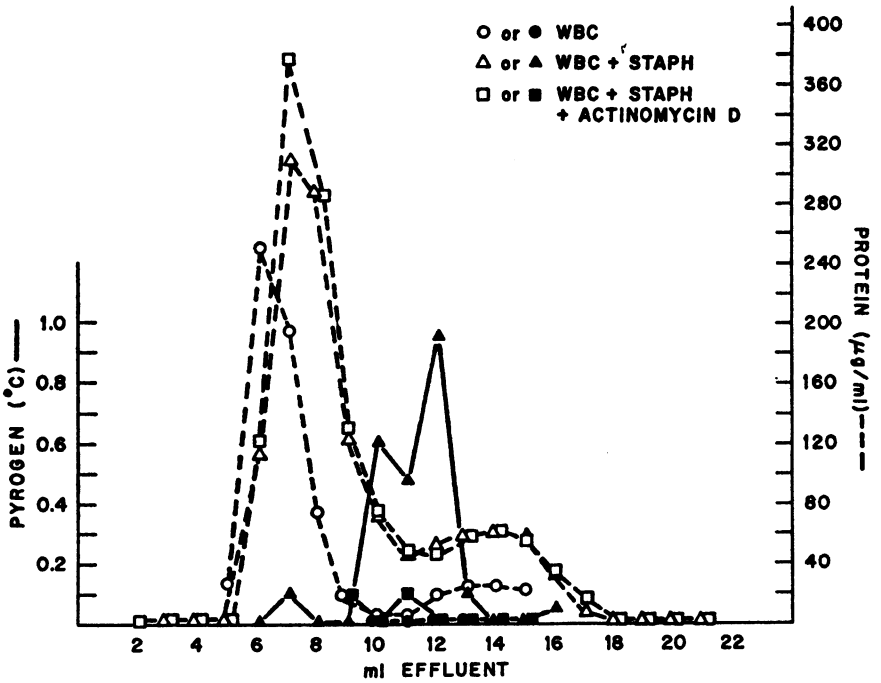

FIG. 7. Total protein concentration and average maximum febrile responses (2 rabbits) to injection of fractions from sephadex G-75 filtration of 18-hour buffer medium from three preparations of leucocytes. White cells were incubated either alone (WBC), previously incubated for two hours with heat-killed staphylococci (WBC and Staph), or with heat-killed staphylococci in the presence of actinomycin D 5 μg/ml. (WBC + Staph + Actinomycin D).

tein released from stimulated cells is not newly formed following new RNA synthesis. In addition, it appears that the amount of endogenous pyrogen protein responsible for fever in a rabbit is, at the most, a few μg. Thus, although no conclusive evidence was obtained from these experiments, there was no suggestion that inhibition of new RNA-directed protein synthesis by actinomycin, including inhibition of endogenous pyrogen production, altered the amount or characteristics of protein released from stimulated cells as determined by sephadex filtration.

In the second type of experiment, human blood leucocytes were incubated in 30 ml. serum-buffer with tritiated phenylalanine* for two hours. Bacteria were then added to the leucocytes, and after an additional two hours of incubation the cells were centrifuged and resuspended in plain buffer for 16 hours. This supernatant was prepared for focusing in an electrofocusing

* L-Phenylalanine-H^3 (New England Nuclear) 1.0 m curie, .019 mg. in .01N NCl.

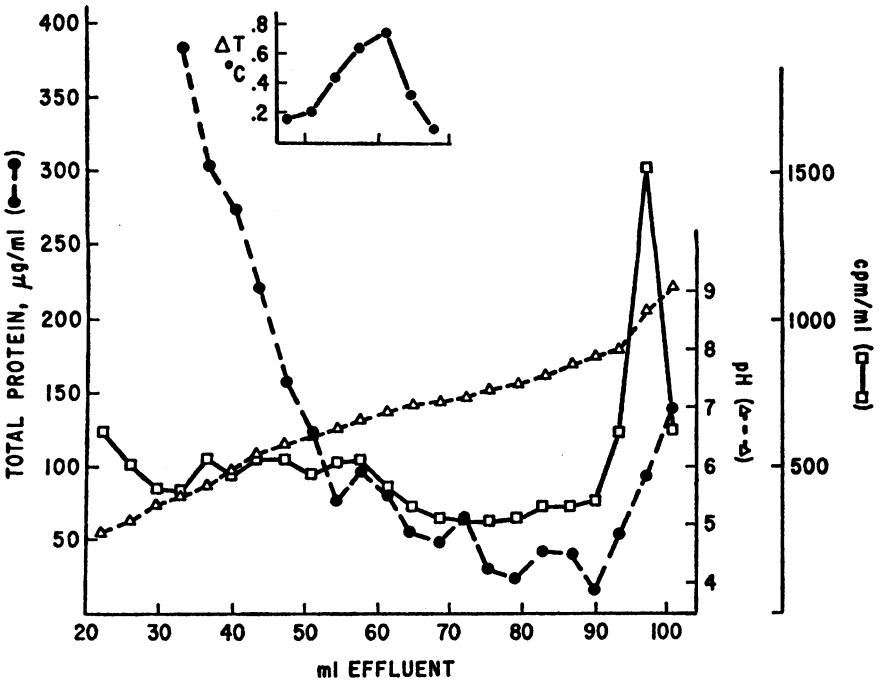

FIG. 8a

apparatus (see METHODS). The results of a representative experiment are shown in Figure 8a. Pyrogenic activity was consistently found in the fractions with pH between 6.6 and 6.9. There was no increase in specific activity of the proteins in this region, however, in any experiment, even though a small peak in total protein concentration was observed.

Analysis of one supernatant from stimulated leucocytes, prepared for focusing as described above, indicated that 60 percent of the tritium present was recovered in a washed protein fraction.** It was calculated that between 5 and 10 $\times$ 10^{-6} μmoles of phenylalanine were incorporated per milligram of released protein. This figure indicates that about 100 times less amino acid was incorporated into released proteins in these experiments than was incorporated into intracellular proteins in similar experiments by others.[20]

For comparison, results of a similar experiment in which non-stimulated cells were incubated are presented in Figure 8b. The reason for the higher

** Methods for preparing the protein fraction, as well as calculation of phenylalanine incorporation, were modifications of the methods of Winzler, Williams, and Best.[19] Serum phenylalanine was assumed to be .05 μmoles/ml.

specific activities of the samples at low pH in Figure 8b compared to 8a is probably due to altered distribution of proteins. Total counts present in prepared supernatants from stimulated and non-stimulated cells were comparable, although more total protein was released by stimulated cells. Samples below pH 5 contained precipitated protein in all experiments.

No evidence was provided, then, from these two types of experiments for the hypothesis that an endogenous pyrogen precursor molecule is newly synthesized by polymorphonuclear leucocytes that have been activated.

Attempts were therefore made to find evidence for a newly synthesized enzyme that could convert a pre-formed non-pyrogenic molecule to active pyrogen. Three types of experiments were tried.

In the first group of experiments, leucocytes were incubated with bacteria for 1½ to 2 hours, centrifuged, homogenized in a small amount of .25M

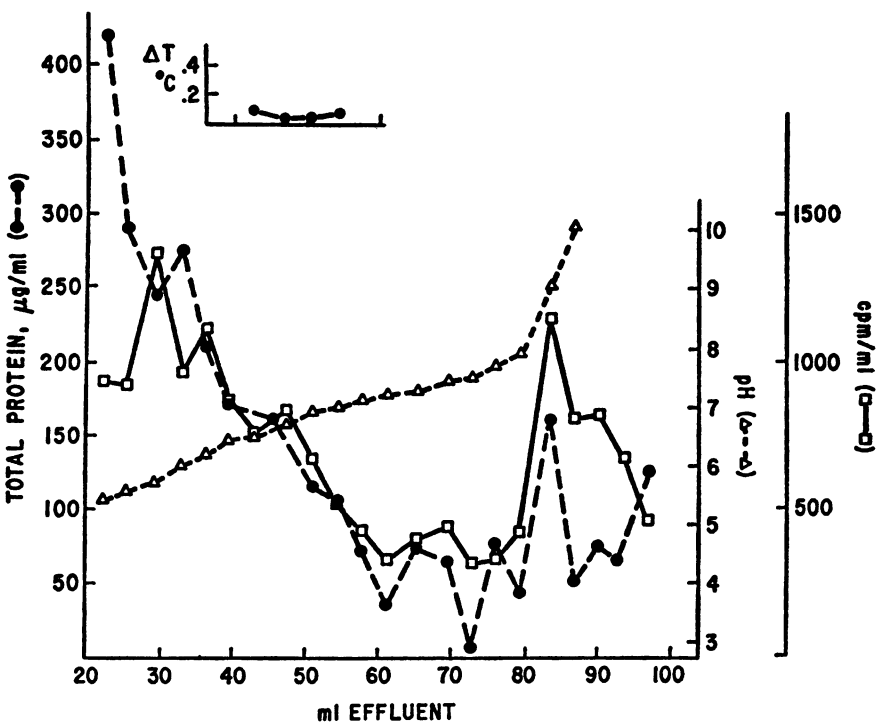

FIG. 8b

FIG. 8. Total protein concentration, pH, counts per minute per milliliter, and average maximum febrile responses (2 rabbits) to injection of fractions from electrofocusing of 18-hour medium from leucocytes previously incubated with H^3-phenylalanine. Fig. 8a) Leucocytes incubated with heat-killed staphylococci. Fig. 8b) Leucocytes incubated alone. For details see text.

or .34M sucrose, and incubated in an enriched medium (see METHODS) for 2 to 18 hours. Control leucocytes were resuspended in serum-buffer after centrifugation and incubated for the same time periods. Samples of homogenate were tested for pyrogenic activity before and after incubation. Supernatant from control leucocytes was also tested. As shown in Table 2, no pyrogen was detected at any subsequent time after leucocytes were homogenized, although the cells were activated, as evidenced by pyrogen release from control cells (WBC).

In the next group of experiments, an attempt was made to reverse the inhibition of pyrogen release by fluoride, added one hour after bacteria (see Fig. 5). If fluoride prevented synthesis of an enzyme, but not its subsequent function, it was reasoned that addition of supernatant from stimulated leucocytes to cells treated with fluoride might cause pyrogen production by these cells and hence demonstrate the presence of such an enzyme. Medium supernatant was used for convenience, and because many cell enzymes enter the incubation medium after phagocytosis.[21] Therefore, a preparation of leucocytes was divided into several aliquots. One of these was incubated with bacteria for 1½ to 2 hours, and the medium supernatant, as well as a control serum-buffer medium incubated with bacteria alone, was then centrifuged at 5,000 *g* for 20 minutes. A second aliquot of leucocytes was incubated with bacteria for one hour. These activated cells were then centrifuged and resuspended in either medium from stimulated cells or control medium, both containing 2×10^{-2}M sodium fluoride, or in control medium without fluoride. The results of these experiments are presented in Table 3. There was no evidence that the inhibition of pyrogen production by fluoride could be overcome by addition of products released from white cells shortly after activation, before the appearance of endogenous pyrogen.

TABLE 2. PYROGEN PRODUCTION BY ACTIVATED LEUCOCYTES (WBC) AND LEUCOCYTE HOMOGENATES

	WBC		*WBC Homogenates*	
Incubation	*Average WBC* ($\times 10^6$)†	Δ°C	*Average WBC* ($\times 10^6$)†	Δ°C
0	—	—	6.7	.14 ± .11*(10)
2 hrs.	4.2	.73 ± .17(5)‡	5.6	.14 ± .07 (7)
6 hrs.	5.2	1.05 ± .19(3)	7.3	.21 ± .17 (6)
18 hrs.	2.6	1.19 ± .19(6)	7.4	.17 ± .06 (5)

† Supernatant derived from this number of leucocytes was injected into each rabbit.
* S.D.
‡ In this and subsequent tables, the number of rabbits injected is shown in parentheses.

In the final group of experiments, an attempt was made to demonstrate an intracellular enzyme in stimulated leucocytes that could transfer the capacity for pyrogen production to normal cells. Leucocytes were incubated with or without bacteria for two hours. Bacteria were then added to the control cells at 0°C., and both stimulated and non-stimulated leucocyte preparations were centrifuged and disrupted by five cycles of freeze-thawing in dry-ice and acetone. These broken cell preparations were then centrifuged at 8,000 *g* for 30 minutes. In some experiments the supernatants of these preparations were also filtered through an UF sintered glass filter before use. After storage at −10°C. or −120°C. for a few days, they were then added to a fresh preparation of normal leucocytes, obtained from the same donor, and incubated for 18 hours.

The results are presented in Table 4. Pyrogen release from intact normal leucocytes was moderately stimulated by addition of cell supernatant from both normal and stimulated leucocytes, but the difference between them was not significant ($p = > .3$). No evidence was therefore obtained for a specific pyrogen-producing enzyme in stimulated leucocytes.

TABLE 3. EFFECT OF MEDIUM FROM ACTIVATED LEUCOCYTES ON PRODUCTION OF PYROGEN BY ACTIVATED LEUCOCYTES TREATED WITH FLUORIDE

	Δ °C.	
	Control medium	*Activated WBC medium*
—	.01 ± .04†(4)	.16 ± .15(8)
Activated WBC	.99 ± .13 (8)	—
Activated WBC + NaF	.22 ± .17 (10)‡	.32 ± .28(10)‡ p = > .3*

† S.D.
‡ The same 10 rabbits received the paired supernatants.
* Calculated by paired t-test.

TABLE 4. EFFECT OF CELL SUPERNATANT FROM NORMAL AND ACTIVATED LEUCOCYTES ON PYROGEN RELEASE BY INTACT NORMAL LEUCOCYTES

	Δ °C.		
	Normal WBC supernatant	*Activated WBC supernatant*	*Serum buffer*
—	.04 ± .08†(5)	.13 ± .14(6)	—
WBC	.45 ± .16 (7)‡	.67 ± .43(7)‡	.20 ± .13(7)‡

‡ The same 7 rabbits received all injections.
† S.D.

DISCUSSION

The mechanism by which cells produce endogenous pyrogen, the mediator of many experimental, and presumably clinical, fevers is still poorly understood. Phagocytic cells of several types liberate endogenous pyrogen in response to various microbial and antigenic stimuli. Although studies of the production of endogenous pyrogen by rabbit exudate polymorphonuclear leucocytes[5-8] have provided much information, these cells appear to be already activated, unlike blood[7] and other tissue cells.[23] Endogenous pyrogens from rabbit and human cells of different types have similar molecular weights and chemical characteristics,[4,23] and the biologic cross-reactivity of endogenous pyrogens from several different species has been demonstrated.[10,24] It seems likely that production of this molecule is a basic feature of cellular response to an inflammatory stimulus.

Previous reports that pyrogen release was not inhibited by puromycin[25] or actinomycin[26]* have been clarified by subsequent work in this and other laboratories.[11] Exudate leucocytes, which are already activated cells, are not sensitive to these inhibitors, whereas blood leucocytes from the rabbit, like human blood leucocytes, can be inhibited by these agents if they are added early after activation.

The results of the studies reported here indicate that there are several distinct steps in the production and release of endogenous pyrogen: 1) The initial event, activation of the cell, is of brief duration, and in the case of phagocytosis is presumably related to ingestion of the organism; 2) During the next interval, lasting ½ to 1 hour, there is new RNA and protein synthesis which appears to be necessary for the subsequent production and release of endogenous pyrogen; 3) During both these periods, very little intracellular pyrogen can be detected. Pyrogen does not begin to appear in significant amounts in the cell until it is released into the medium. Since it does not accumulate in the cell until late in the incubation period, it appears to be released as soon as it is produced in pyrogenic form. Since this step is not inhibited by fluoride, the release of endogenous pyrogen does not require energy from the usual glycolytic pathway; 4) After the initial two-hour period, new RNA and protein synthesis is not required for subsequent normal production and release of pyrogen. Since at two hours intracellular pyrogen is not present in significant amounts, this new protein cannot be endogenous pyrogen in its active form.

These data are most consistent with one or the other of the following hypotheses: 1) A precursor of endogenous pyrogen is newly synthesized in

* While this manuscript was in preparation, a completed report of this work has been published (*J. exp. Med.*, 1970, *131*, 727).

the cell during the first two hours, and then enzymatically converted to a pyrogenically active form; 2) An enzyme is induced which acts on a previously existent, inactive precursor molecule and converts it to endogenous pyrogen.

The results reported here do not provide support for the first of these two hypotheses. When new protein synthesis was inhibited, there were no significant alterations in the proteins released from activated cells. Further, there was no significant labeling of proteins in the fractions containing endogenous pyrogen when tritiated phenylalanine was present during cell activation. Since the number of molecules of endogenous pyrogen necessary to produce fever in a rabbit may be very small,[23] their synthesis might be inapparent. Since there was very little total incorporation of phenylalanine into released proteins from leucocytes, however, even a small amount of newly formed, released protein might have been detected in these experiments. Mature polymorphonuclear leucocytes have little endoplasmic reticulum[27] and most proteins released by phagocyting cells presumably come from lysosomes.[28]

In regard to the second hypothesis, no evidence could be found by three different methods for the existence of an enzyme capable of changing a precursor to an active pyrogen. Presumably, intact cell structure is required for pyrogen production and release, since disruption of cells by homogenization or freeze-thawing virtually destroyed this capacity, whereas fluoride, which is known to cause severe morphologic alterations in granulocytes,[15] did not. Cell supernatants, derived from either activated or normal leucocytes after a two-hour incubation, equally stimulated pyrogen production from intact cells. Production of pyrogen in this instance apparently is due to activation of cells rather than an enzymatic alteration of a pyrogen precursor, since preliminary results indicate that it can be blocked by puromycin. The nature of this activator is unknown.

The inability to demonstrate the presence of an enzyme for production of pyrogen in these studies may have been due to many factors, including lack of purification of cell components and inappropriate conditions for the reaction. Future efforts to identify a precursor molecule or to purify enzymatically active cell fractions may lead to experiments that can provide conclusive results.

SUMMARY

The early steps leading to production and release of endogenous pyrogen were investigated using human blood leucocytes stimulated by phagocytosis of heat-killed staphylococci. During the first two hours, significant amounts

of endogenous pyrogen were not detected either in the incubation medium or in cell extracts. Addition of actinomycin D, puromycin, fluoride, or iodoacetate during this time prevented subsequent production and release of endogenous pyrogen. However, only iodoacetate was inhibitory when these agents were added two hours after stimulation.

Attempts to demonstrate new synthesis of endogenous pyrogen after stimulation by phagocytosis were unsuccessful. When cells were activated in the presence of H^3-phenylalanine, and the products released were fractionated by electrofocusing, no increased specific activity was detected in the fractions containing endogenous pyrogen. Similarly, no evidence was obtained for the existence of an enzyme system in activated leucocytes capable of converting a precursor to active endogenous pyrogen.

These results suggest that formation of new RNA and protein are essential early steps in the production and release of endogenous pyrogen. However, the identity of the critical protein or proteins and the nature of the subsequent steps by which this agent is converted into endogenous pyrogen remains unknown.

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