STUDIES ON THE ORIGIN OF HUMAN LEUKOCYTIC PYROGEN*

By JAMES J. NORDLUND,‡ M.D., RICHARD K. ROOT,§ M.D., AND SHELDON M. WOLFF,§ M.D.

(From the Department of Health, Education, and Welfare, National Institutes of Health, National Cancer Institute, Medicine Branch, and National Institute of Allergy and Infectious Diseases, Laboratory of Clinical Investigation, Bethesda, Maryland 20014)

Granulocytes and monocytes derived from inflammatory exudates or the peripheral circulation of several mammalian species (1–5) can be stimulated to release a pyrogen, leukocytic pyrogen (LP)[1], which induces a characteristic febrile response in rabbits. Chemical characterization of LP has shown it to be a protein molecule (6–8) and since the original discovery of its production by rabbit peritoneal exudate cells (9), many attempts have been made to determine the mechanism of its synthesis by leukocytes. With one exception (10), preformed pyrogen has not been obtained from disrupted rabbit cells (11, 12). Its release in vitro has been shown to take place over a several hour period (4, 11, 12), to be dependent upon the temperature of incubation (11), and inhibited by agents which bind sulfhydryl groups in enzymes (13). These findings suggest that LP is either synthesized *de novo* or activated from a precursor substance after contact with an appropriate stimulating agent.

In the present studies, the role of RNA and protein synthesis and the energy requirements for pyrogen release by human peripheral leukocytes activated by the ingestion of heat-killed bacteria have been investigated. The results reported here indicate that both transcription of messenger RNA and its translation by ribosomes into new protein synthesis are essential for pyrogen production by these cells in vitro. In addition, the energy derived from glycolysis appears to be important for the activation process.

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[1] *Abbreviations used in this paper:* HBSS, Hanks' balanced salt solution; HMS, hexose monophosphate shunt; LP, leukocytic pyrogen; MHS, modified Hanks' solution; NaF, sodium fluoride; PSB, polystyrene balls; PWBC, phagocytes (monocytes and granulocytes); m-RNA, messenger RNA; s-RNA, soluble RNA.

Materials and Methods

Materials.—All needles, syringes, media and solutions were sterile and pyrogen-free.

Leukocytes.—Whole, anticoagulated (ACD) human blood was obtained daily and centrifuged at 1500 g for 3 min. The buffy coat (approximately 75 ml per unit of whole blood) was aspirated with sterile precautions. Further separation of white and red blood cells was achieved by the addition of homologous plasma in a 2:1 ratio (by volume) to the buffy coat, and the cells were allowed to sediment for $1\frac{1}{2}$ hr at 37°C. The supernatant, containing mostly white and a few red blood cells, was aspirated and the white blood cells counted in an electronic particle counter.[2] 200 cell differential counts were made on smears prepared with Wright's stain to determine the number of phagocytes (monocytes and granulocytes, PWBC) in the preparation, since these have been shown to be the only cells capable of LP release (3, 4, 14).

Reagents.—*Escherichia coli* endotoxin[3] was diluted in modified Hanks' solution[4] (MHS) to a concentration of 0.3 μg/ml. To produce leukocytic pyrogen, 1 ml of endotoxin was added to 100 × 10^6 PWBC to give a final concentration of 0.03 μg endotoxin per ml of solution. *Staphylococcus albus* isolated from a patient was grown in a semisolid media and kept at room temperature as a stock supply. When needed, a loopful was incubated overnight at 37°C in trypticase soy broth. The culture was centrifuged at 1500 rpm for 20 min and washed once in MHS. The bacteria were boiled for 30 min in a water bath and cultured to insure sterility. The final concentration was determined by densitometry at 650 mμ.[5] To stimulate the release of LP, a bacteria to PWBC ratio of 40:1 was used.

Drugs employed were of analytic grade and obtained from commercial sources, except cycloheximide.[6] Solutions of actinomycin D (50 μg/ml), vincristine (2 × 10^{-4} M), and cycloheximide (25 μg/ml) were prepared daily. Puromycin (4 × 10^{-5} M) was prepared weekly, and sodium fluoride (NaF) (2 × 10^{-1} M) and potassium cyanide (KCN) (1 × 10^{-2} M) every 2–3 wk. All drugs were diluted in MHS and stored at 4°C. Drugs were added to the final cell suspensions to give a 10-fold dilution from the above concentrations.

Preparation of Leukocytic Pyrogen.—Volumes of white blood cell-rich plasma containing 100 × 10^6 phagocytic cells (granulocytes and monocytes) were pipetted into 40 ml test tubes. These were centrifuged at 800 rpm (150 g) for 8–10 min at 4°C and the plasma decanted. To each portion of 100 × 10^6 phagocytic cells was added 1.5 ml of plasma (15% of final volume) and various drugs, killed *S. albus*, or endotoxin as required by the experiment. The volume of each sample was then made up to 10 ml by the addition of MHS containing 10 units of heparin per ml. The test tubes were placed in a shaking incubator[7] at 37°C for an appropriate period of time. At the termination of the experiment, all solutions were centrifuged at 3000 rpm for 1 hr at 4°C to remove *S. albus*, and portions were cultured in thioglycollate broth for 72 hr before injection into rabbits. Contaminated material was discarded and sterile solutions were stored at 4°C until used.

Fever Measurement.—New Zealand white rabbits of either sex weighing 1.5 to 2.5 kg from the same colony (National Institutes of Health, Bethesda, Md.) were used. Details of housing, feeding, training, and temperature recording were as previously reported (15). Only those rabbits with stable baseline temperatures for at least the preceding 30 min were injected. A rise in temperature of less than 0.3°C was not considered meaningful. Each solution to be tested was injected into at least three rabbits. No rabbit was used more than once each day,

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[2] Coulter Electronics, Hialeah, Fla.

[3] Difco Laboratories, Inc., Detroit, Mich.

[4] $K_2H_2PO_4$, 1.568 g/liter; NaCl, 8.124 g/liter; dextrose, 2 g/liter; distilled H_2O to 1000 ml.

[5] Coleman junior spectrophotometer.

[6] Cancer Chemotherapy National Service Center, Bethesda, Md.

[7] Model 5970, New Brunswick Scientific Co., New Brunswick, N. J.

and all were discarded after three injections or 3 days after their first injection to prevent the development of fevers due to hypersensitivity to human proteins. Those animals receiving leukocytic pyrogen obtained from endotoxin activated cells were given 1.5 μg endotoxin intravenously on the preceding day to induce febrile tolerance (16).

Phagocytosis.—To quantitate phagocytosis, cell suspensions were prepared in an identical manner to those used for pyrogen assays. Drugs were added as described, and *S. albus* was added in a 40:1 ratio to PWBC. The suspension was then placed in an agitator at 37°C and allowed to incubate for 1 hr. To concentrate the PWBC, the suspensions were centrifuged at 1500 rpm for 20 min, the supernatants discarded, and 1 ml of plasma added. Smears were stained with Wright's stain, and the per cent of 100–200 PWBC's containing intracellular *S. albus* were enumerated.

Hexose Monophosphate Shunt Activity.—To assay metabolic responsiveness of cells and their phagocytic capacity, the release of labeled CO_2 from the hexose monophosphate shunt (HMS) was measured (17). The method of Skeel (18) was duplicated with minor exceptions. ^{14}C-1-glucose[8] was diluted to 1.0 μCi per ml in Hanks' balanced salt solution (HBSS).[9] Polystyrene balls (PSB)[10] with a diameter of 1.099 μ ± 0.0059 were diluted 1:5 in HBSS. $\frac{1}{2}$ ml of ^{14}C-1-glucose was added to each reaction mixture and 0.5 ml of drug when required by the experiment. HBSS was added to bring the volume to 3 ml. 1 ml of plasma prepared to contain 10 × 10^6 PWBC was then added and the flasks immediately capped with rubber stoppers[11] to which a polyethylene cup[11] was attached. Into each cup, 0.2 ml of 10% potassium hydroxide, made fresh weekly, was injected with a tuberculin syringe. The mixtures were placed in a Dubnoff shaker-incubator at 37°C for 1 hr and the reaction terminated by the addition of 1 ml of 1 N hydrochloric acid to the cell suspension. The polyethylene cups were then gently removed and placed into counting vials which contained 20 ml of a scintillation solution. This latter mixture was prepared with 40 ml of Liquifluor[12], 660 ml of toluene, and 300 ml of absolute methanol. The vials were then counted in a liquid scintillation counter[13] to insure an error of less than 1.5%.

RESULTS

Production Rate of LP.—Prior to examining whether the agents employed affected the activation or release phases of pyrogen production, it was necessary to determine the rate and duration of LP release by stimulated cells. Dead *S. albus* were added to a series of tubes containing 100 × 10^6 PWBC, and supernatant fluid was removed from two tubes by centrifugation every 4 hr during a 24 hr period, and new supernatant containing only plasma and MHS was added. Each sample of supernatant was tested for its content of pyrogen. Beginning by 4 hr, leukocytic pyrogen was produced at a constant rate for about 12–16 hr after which production declined (Fig. 1) so that after 16 hr, no further LP release occurred. To study the effects of repeated centrifugation and manipulation on LP production by cells, other suspensions were allowed to incubate 8, 12, or 16 hr before subsequently replacing the supernatant every 4 or 8 hr (Fig. 1). Under these conditions, pyrogen release was detectable for up to 12–16 hr.

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[8] Specific activity 5.4–6.3 mCi/mM. New England Nuclear Corp., Boston, Mass.

[9] Flow Laboratories, Inc., Rockville, Md.

[10] Dow Chemical Co., Midland, Mich.

[11] Kontes Glass Co., Vineland, N. J.

[12] New England Nuclear Corp.

[13] Packard Model No. 3375.

Inhibition of Protein Synthesis.—Puromycin (19, 20) and cycloheximide (20, 21) are capable of inhibiting protein synthesis by interfering with translation of messenger RNA (m-RNA). In the first series of experiments, either of these agents were added to the cell suspensions at the beginning of the incubation period with the *S.*
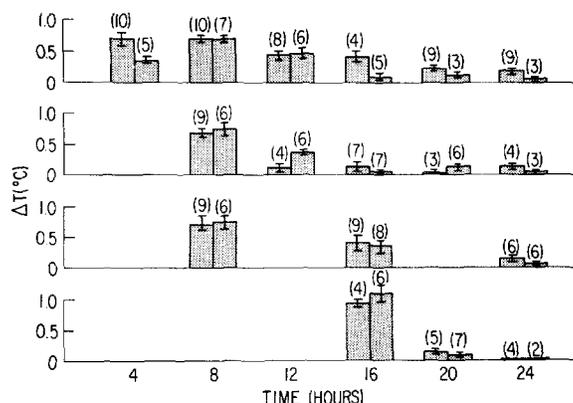

Fig. 1. Mean febrile responses of rabbits after the injection of LP derived from 15 × 10⁶ PWBC incubated with *S. albus* for the periods of time indicated. The number of rabbits injected is noted by the number in parentheses and the standard error of the mean indicated by the brackets. Experiments were done in duplicate as indicated by the pairs of bars. In the upper graph, the media was changed every 4 hr from the beginning, while in the middle two, the media was first changed at 8 hr and in the bottom at 16 hr.
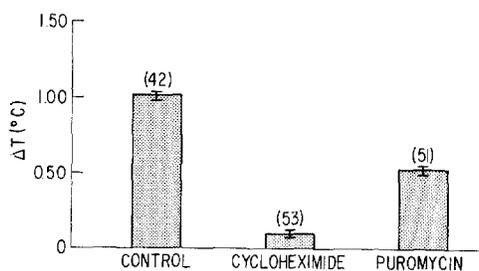

Fig. 2. Effect of cycloheximide and puromycin on LP production by human PWBC in vitro. Control cells (100 × 10⁶ PWBC) were incubated for 16 hr at 37°C with *S. albus* only. To the other suspensions, either cycloheximide (2.5 μg/ml) or puromycin (4 × 10⁻⁶ M) and *S. albus* were added together and incubated for 16 hr. Mean febrile response is indicated by the bar graph and the standard error of the mean by the brackets. The number of rabbits injected is denoted by the number in parentheses.

albus and the entire contents incubated for 16 hr. The supernatant fluids were then tested for pyrogenicity. Cycloheximide completely suppressed all LP production (Fig. 2). Puromycin also showed significant suppression of LP production (*P* < 0.005, *t* test), but it was not as complete as cycloheximide (Fig. 2).

To exclude inhibition of pyrogenicity of LP by the drugs, cycloheximide, puromycin, actinomycin D, sodium fluoride, and potassium cyanide were added to preformed leukocytic pyrogen in the same concentrations as in the cell suspensions and incubated for 1 to 7 days prior to injection into rabbits. Under these conditions, there was no diminution of pyrogenicity (Table I) by any of these agents.

Since it is possible that the drugs employed reduced LP production by killing the cells, a second series of experiments was performed. PWBC's were incubated at 37°C with plasma, MHS, and cycloheximide or puromycin in an appropriate concentration for 4 hr, but without the addition of $S.$ $albus$. After centrifugation, the supernatants were discarded, the cells washed twice in 10–15 ml of MHS, and divided into two equal parts. One part was resuspended in plasma (1.5 ml) and MHS (8.5 ml) and $S.$ $albus$ was added. To the other part, 1 ml of drug was added as well as plasma (1.5

TABLE I

*Pyrogenicity of Preformed LP Incubated with Drugs**

Experi-ment	Control			Cycloheximide			Puromycin			Actinomycin D			Sodium fluoride		
	No.‡	Mean	SE	No.	Mean	SE	No.	Mean	SE	No.	Mean	SE	No.	Mean	SE
		$\Delta T°C$§			$\Delta T°C$			$\Delta T°C$			$\Delta T°C$			$\Delta T°C$	
I	2	1.35	0.10	4	1.15	0.06	3	0.88‖	0.04						
II	4	0.95	0.06	5	1.27‖	0.02	3	1.23	0.22						
III	5	1.35	0.09	2	1.05	0.10	2	0.95	0.15						
IV	2	1.28	0.12	2	1.15	0.05	2	1.18	0.12						
V	7	1.11	0.06	4	1.31	0.15	3	1.22	0.19				3	1.22	0.12
VI	3	1.05	0.06							2	0.92	0.12			
VII	3	1.25	0.10							6	1.14	0.07			
Total	26	1.17	0.04	17	1.21	0.04	13	1.09	0.08	8	1.08	0.02	3	1.22	0.12

* LP derived from incubation of 15 × 10⁶ PWBC for 18 hr with $S.$ $albus$.

‡ Number of rabbits injected.

§ Maximum temperature (°C) change from baseline during the 1st hr after injection of LP.

‖ $P > 0.05$ compared to control; t test.

ml), MHS (7.5 ml), and $S.$ $albus$. All mixtures were then incubated with agitation for 16 hr at 37°C. Those cells exposed to drug for the second period of incubation did not produce LP, although cells washed free of drug after 4 hr could still produce amounts of LP comparable to controls (Fig. 3).

To study if both cycloheximide and puromycin exerted their effects on LP production by the inhibition of phagocytosis, smears of cell suspensions containing drugs and $S.$ $albus$ were stained with Wright's stain after 1 hr in a shaking incubator and the per cent of phagocytic cells containing $S.$ $albus$ counted. Table II shows that there was no inhibition of phagocytosis by cells exposed to the drugs employed.

To test whether the cells were metabolically responsive during and after exposure to these drugs, the $^{14}CO_2$ production from the hexose monophosphate shunt was measured. Puromycin and, frequently, cycloheximide caused a slight but significant increase of $^{14}CO_2$ production in resting cells to which no polystyrene balls had been added (Table III). The addition of polystyrene balls to induce phagocytosis in other suspensions containing drugs showed equal increments in CO_2 release compared to

controls (Fig. 4). Moreover, even after 4 hr of preexposure to drugs, resting and stimulated (phagocytic) cells were capable of releasing CO_2 in amounts comparable to controls (Fig. 5). Thus, cell viability and metabolic responsiveness did not seem to be impaired by prior exposure to cycloheximide or puromycin as determined by the parameters of pyrogen release, phagocytosis, and hexose monophosphate shunt activity.
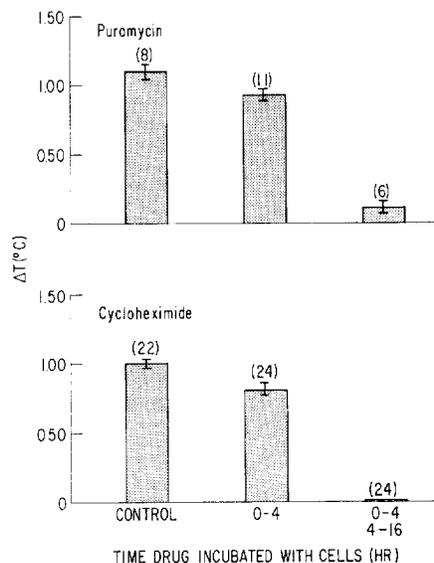
FIG. 3. PWBC were incubated for 4 hr at 37°C with cycloheximide, puromycin, or no protein inhibitor (control). After washing, *S. albus* was added to all suspensions, and either cycloheximide or puromycin was added to one-half of the suspensions previously exposed to one of these drugs (0–4, 4–16 hr). The remaining suspensions were not reexposed to cycloheximide or puromycin (0–4 hr). All suspensions were then incubated for 16 hr and supernatant derived from 15 × 10⁶ PWBC injected into rabbits. The mean temperature change and the standard error of the mean is recorded. The number in parentheses denotes the number of rabbits injected.

To ascertain whether the effects of these drugs were peculiar to *S. albus*-activated cells, similar experiments using endotoxin-stimulated cells were performed. The results show that cycloheximide also completely suppressed LP production by cells exposed to endotoxin (Table IV).

RNA Inhibitors.—In order to interrupt the transcription process of protein production, actinomycin D (22), and vincristine (23, 24) were used in experiments exactly as described above for the cycloheximide and puromycin. The addition of actinomycin D completely suppressed LP formation by cells incubated with *S. albus* (Fig. 6). No such effects were noted when vincristine was used (Fig. 6). When cells were preincubated with actinomycin D for 4 hr, and washed twice prior to the addition of *S. albus*, they were still unable to produce LP (Fig. 7). The addition of actinomycin D

to cells and *S. albus* did not impede phagocytosis as is shown in Table II. Moreover, the cells exposed to actinomycin D for 0–4 hr before the addition of polystyrene balls demonstrated hexose monophosphate shunt activity equivalent to control cells not exposed to actinomycin D (Fig. 8), indicating that there was no loss of cell viability.

Temporal Relationships.—To determine the duration of the requirements for m-RNA and protein synthesis for maximal pyrogen release, actinomycin D or cyclo-

TABLE II

*Effect of Metabolic Inhibitors on Phagocytosis**

Experiment	Control	Cycloheximide	Puromycin	Actinomycin D
	%‡	%	%	%
I	86	78	86	
II	73	82	78	79
III	96	96	98	99
IV	92	93	87	88
V	68	75	68	71
Total	83	85	83	84

* PWBC incubated with *S. albus* for 1 hr, were smeared on glass slides, and stained with Wright's stain.

‡ Per cent of granulocytes and monocytes containing bacteria.

TABLE III

Effect of Drugs on Hexose Monophosphate Shunt Activity in Resting Cells

Experi-ment	Control			Cycloheximide				Puromycin			
	No.	Mean	SE	No.	Mean	SE	P‡	No.	Mean	SE	P
		DPM§			DPM				DPM		
I	9	1766	71	8	2149	58	<0.001	9	1980	33	<0.02
II	9	1685	85	9	1488	34	>0.05	9	2120	74	<0.01
III	9	2218	68	9	2826	91	<0.001	9	2648	83	<0.001
IV	8	1893	39	9	1960	47	>0.20	9	2089	59	<0.01

* Number of individual vials prepared in each experiment.

‡ *t* test.

§ $^{14}CO_2$ release from cells incubated with ^{14}C-1-glucose without addition of polystyrene balls.

heximide were added to cells at either 1, 2, or 4 hr after the addition of *S. albus* and allowed to incubate for an additional time to cover a total incubation period of 16 hr. In the 4 hr experiments, the supernatant was replaced by fresh media, since previous experiments demonstrated LP generation within 4 hr (Fig. 1). When compared to control preparations incubated 18 hr without drugs, both cycloheximide and actinomycin D completely suppressed LP generation when added 1 hr after the addition of *S. albus* (Table V). Addition of drugs at 2 hr led to detectable but significantly reduced LP

release, indicating that some m-RNA had been formed and protein translated for LP production prior to this time. When added at 4 hr, larger quantities of LP were subsequently evolved, although less than in control solutions, suggesting that continuous m-RNA and protein synthesis were necessary for maximum LP generation even to this late period.

Energy Requirements.—In experiments using rabbit leukocytes, the presence of NaF in cell suspensions incubated with heat-killed pneumococci suppressed pyrogen
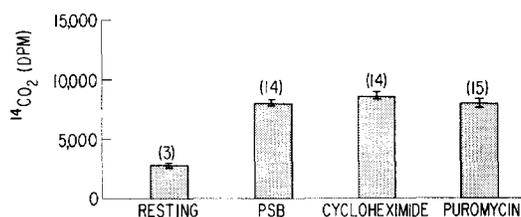

Fig. 4. The mean $^{14}CO_2$ release (dpm) from 10×10^6 PWBC incubated with ^{14}C-1-glucose for 1 hr at 37°C without stimulation (resting cells) or with stimulation by phagocytosis of polystyrene balls (PSB). Either cycloheximide (2.5 μg/ml) or puromycin (4×10^{-6} M) were added with polystyrene balls to some suspensions. The standard error of the mean is denoted by brackets and the number of suspensions by the number in parentheses.
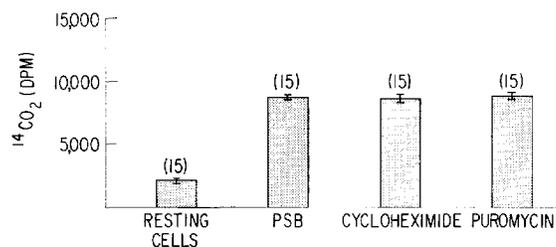

Fig. 5. 10×10^6 PWBC were incubated with either puromycin (4×10^{-6} M) or cycloheximide (2.5 μg/ml) for 4 hr at 37°C before the addition of polystyrene balls (PSB) to stimulate $^{14}CO_2$ release from ^{14}C-1-glucose by phagocytosis. Neither drugs nor PSB were added to resting cells, and only polystyrene balls were added to control stimulated cells. Bars indicate mean $^{14}CO_2$ release, and the brackets denote the standard error of the mean. Number of suspensions is indicated by the number in parentheses.

release only when this agent was added prior to phagocytosis (25), suggesting that the failure of pyrogen production was due to impaired phagocytosis, a process which requires energy derived from glycolysis (17). Similar experiments were performed with human leukocytes and in contrast to the above findings reported for rabbit cells, NaF added 1 hr after the initiation of phagocytosis also suppressed pyrogen output (Fig. 9). On the other hand, if the cells and bacteria were allowed to incubate for 2 hr prior to the addition of NaF, this suppressive effect was lost. In preparations incubated for 1 hr prior to the addition of NaF, the mean percentage of granulocytes and monocytes in three representative experiments which had ingested bacteria was 82% (range 77–

TABLE IV

Effect of Cycloheximide on LP Generation by Endotoxin Activated PWBC

Experiment	Controls			Cycloheximide			
	Endotoxin and PWBC*			Endotoxin and PWBC			
	No.‡	Mean	SE	No.	Mean	SE	P§
		$\Delta T°C\|$			$\Delta T°C$		
I	3	0.83	0.04	6	0	0	<0.001
II	6	0.87	0.10	5	0	0	<0.001
Total	9	0.86	0.06	11	0	0	<0.001

* *E. coli* endotoxin (0.03 µg/ml) incubated with 100 × 10⁶ PWBC in 10 ml solution and 1.5 ml supernatant injected into endotoxin tolerant animals.

‡ Number of rabbits injected.

§ *t* test.

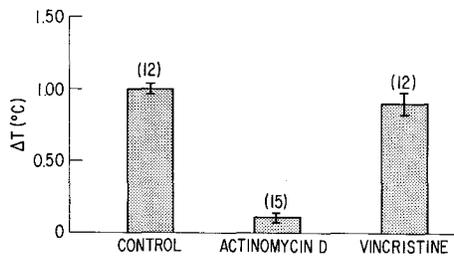‖ Maximum temperature (°C) change from baseline during the 1st hr after injection of supernatant.

Fig. 6. PWBC to which *S. albus* alone or *S. albus* and either actinomycin D (5.0 µg/ml) or vincristine (2 × 10⁻⁵ M) were incubated 16 hr at 37°C. Supernatant derived from 15 × 10⁶ PWBC was injected into rabbits and the mean maximum temperature change (°C) and standard error (brackets) graphed. The numbers in parentheses indicate the number of rabbits injected.
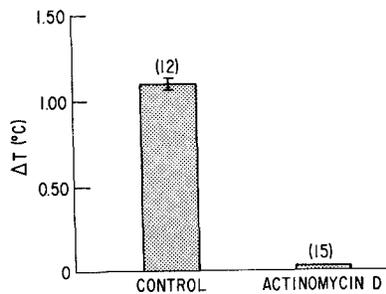
Fig. 7. PWBC previously exposed to actinomycin D (5 µg/ml) for 4 hr at 37°C were washed and reincubated with *S. albus*, only, for 16 hr. Control cells were similarly treated but were not exposed to actinomycin D at any time. The supernatant from the second incubation equivalent to 15 × 10⁶ PWBC was injected into rabbits and mean febrile responses and standard error recorded. The number in parentheses indicates the number of rabbits injected.

86%). That this amount of phagocytosis was sufficient to fully activate the cells for pyrogen release was demonstrated in control preparations in which the leukocytes were washed free of uningested bacteria after 1 hr of incubation and allowed to incubate in fresh medium for the subsequent 15 hr (Fig. 9). Under these conditions, pyrogen production was equivalent to control suspensions in which cells were left in contact with bacteria for the entire 16 hr period (Fig. 9). These findings agree with earlier
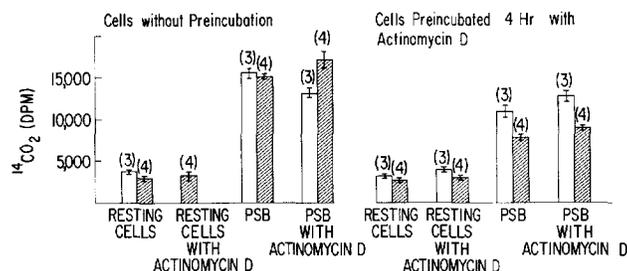

FIG. 8. The left set of bar graphs depicts the effect of actinomycin D (5.0 µg/ml) on the release of $^{14}CO_2$ from ^{14}C-1-glucose by nonphagocytizing (resting) cells and cells phagocytizing polystyrene balls (PSB). Identical comparisons are made in the right set of bar graphs after preincubation of the leukocytes for 4 hr at 37°C with actinomycin D (5.0 µg/ml) and washing before further exposure to actinomycin D. The experiments were each performed twice (shown by the double bars) and the results given as the mean and standard error for triplicate or quadruplicate determinations (numbers in parentheses).

TABLE V

The Effect on LP Generation of Addition of Actinomycin D or Cycloheximide 1, 2, or 4 Hr after S. albus

Ex- peri- ment	Control		Actinomycin D						Cycloheximide					
			1 hr		2 hr		4 hr		1 hr		2 hr		4 hr	
	Mean* ΔT	SE	Mean ΔT	SE	Mean ΔT	SE	Mean ΔT	SE	Mean ΔT	SE	Mean ΔT	SE	Mean ΔT	SE
I	0.93	0.09	0.12‡	0.08	0.44‡	0.10	0.70‡	0.05	0.05‡	0.05	0.28‡	0.07	0.62‡	0.06
II	1.18	0.05	0.31‡	0.14	0.73‡	0.09	0.78‡	0.05	0.25‡	0.07	0.75‡	0.05	0.87‡	0.09
III	1.02	0.15	0.42‡	0.12	1.01	0.16	0.84‡	0.04	—	—	0.52‡	0.03	1.06	0.06

* Temperature (°C) change from baseline during the 1 hr after the injection of supernatant
‡ $P < 0.05$ compared to control (student t test).

data (14). Finally, in two experiments, the increment in per cent phagocytizing cells between 1 and 2 hr incubation was only 3–9% (83–92%, and 63–66%), an amount not sufficient to explain the difference in LP production by preparations to which sodium fluoride had been added at 1 and 2 hr, respectively. These findings indicate that the suppression of LP production by NaF added at 1 hr is not due to inhibition of phagocytosis. Furthermore, the failure of NaF added at 2 hr to inhibit LP release suggests that blockade of glycolysis prevents the initial phase of cell activation, rather than release of the LP molecule itself.

In other experiments, the cytochrome oxidase inhibitor, potassium cyanide, was added to phagocytizing cells to study the role of aerobic glycolysis in supplying energy for LP generation. LP output in these preparations was equivalent to controls incubated without potassium cyanide, indicating that aerobic glycolysis had no significant role in energy production for LP generation.
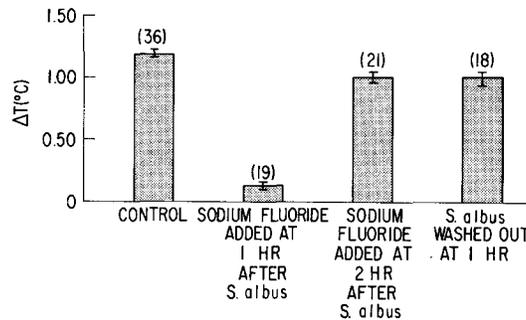

Fig. 9. *S. albus* were added to all suspensions containing 100×10^6 PWBC. To some suspensions, sodium fluoride $(2 \times 10^{-2}$ M$)$ was added at 1 or 2 hr after the *S. albus* and the cells incubated for 16 hr. To remove nonphagocytized bacteria, other preparations were washed 1 hr after adding *S. albus* and reincubated for 16 hr in new media. Supernatant from 15×10^6 PWBC was injected into rabbits, and the mean temperatures were recorded and the standard error calculated. The number of rabbits injected is indicated by the number in parentheses.

DISCUSSION

In contrast to cells derived from inflammatory exudates, rabbit and human blood leukocytes will not release pyrogen spontaneously unless activated by a specific stimulus such as phagocytosis (2, 12, 14, 25) or exposure to various agents, including endotoxin (2, 14, 26, 27) and etiocholanolone (28, 29). Two phases appear to be involved in the elaboration of pyrogen by stimulated cells. During the first, or "activation" phase, cellular metabolism becomes altered in such a way that pyrogen release can subsequently take place in the absence of any further stimulus (14, 25, 29). The second phase, that of pyrogen release, occurs only after a latent period which varies with the type of agent used to activate the cells. The present data provide information on mechanisms involved in both phases, using phagocytosis of heat-killed bacteria as the primary mode of stimulation.

In a previous study, it was noted that after the ingestion of heat-killed *S. albus*, pyrogen release was not readily detectable until 3–4 hr incubation time, although trace amounts could be detected earlier, using huge test doses for assay (14, 30). As shown in the present study, pyrogen release then occurs at a steady rate until 12–16 hr after the start of incubation. This differs from reported studies on rabbit peritoneal exudate cells which demonstrated failure

of granulocytes and monocytes to produce pyrogen beyond 3 and 7 hr, respectively (4). Such differences are probably due to species variability and the conditions used to obtain and stimulate the cells. Activation was found to relate directly to the per cent of phagocytizing cells and to require approximately 60 min after the addition of bacteria to become maximal (14).

Several previous studies have been undertaken to delineate the role of RNA and protein synthesis in the formation of LP by stimulated cells. When rabbit exudate granulocytes were employed, little or no suppression of LP release was found when puromycin or actinomycin D were added to the incubation medium (30, 31). On the other hand, when the rabbits were pretreated with chloramphenicol for several days before the exudates were harvested, some depression of LP release was noted, leading to the conclusion that the molecule might be preformed in vivo and bound in an inactive state within the cells until release in vitro by incubation of the leukocytes in saline (31). In contrast to the findings with peritoneal cells, studies on blood leukocytes have suggested that protein synthesis might be important in the activation process in vitro. For instance, puromycin has been found to inhibit LP generation by specifically sensitized rabbit cells exposed to tuberculin (32), and actinomycin D and puromycin suppressed release by human leukocytes incubated with etiocholanolone (33). These agents have also been reported to block pyrogen production by human cells activated by phagocytosis, although no data were given (34).

The results of the present study indicate that synthesis of both m-RNA and protein are involved in the production of LP by phagocytizing cells derived from the peripheral circulation. Actinomycin D, an inhibitor of m-RNA transcription, and the inhibitors of translation, puromycin and cycloheximide, blocked LP production without altering cell viability, interfering with phagocytosis, or inactivating the LP molecule itself. That these processes seem to be common to all modes of stimulation of human peripheral leukocytes for pyrogen release is suggested by the ability of cycloheximide to inhibit LP production by cells exposed to endotoxin, noted in the present investigation, and the ability of puromycin and actinomycin D to block release from cells incubated with etiocholanolone (33). Furthermore, similar findings have been observed for rabbit peripheral leukocytes stimulated by phagocytosis or endotoxin, using cycloheximide and puromycin as the inhibiting agents (35).

The failure of these drugs to block LP release in earlier studies on peritoneal cells suggests that they act at the level of activation rather than on the release phase itself, since these cells appear to be activated in vivo prior to removal from the donor (25). Further support for this concept was obtained when the effects of adding the inhibitory drugs at various times after the initiation of phagocytosis were studied. Complete suppression of pyrogen production occurred when cycloheximide or actinomycin D were added simultaneously with,

or 1 hr after, the start of incubation with *S. albus*. Progressively less inhibition was seen in preparations to which these agents had been added at 2 and 4 hr incubation, respectively, times at which the cells were already significantly activated for LP release. This indicates that ongoing m-RNA and protein synthesis are required for maximal LP production throughout the time period studied, but similar to the time required for maximal activation, the major requirements are early in the course of incubation. Two conclusions can be arrived at from these findings: (*a*) m-RNA and new protein synthesis are more essential to cell activation than to pyrogen release per se; (*b*) that the activation phase might not consist simply of the formation of a store of pyrogen which is then released over the subsequent 12–16 hr. As will be discussed below, the effects of inhibition of glycolysis on pyrogen formation might have bearing on this point.

Several points regarding the results found with the individual inhibitors of m-RNA and protein synthesis should be noted. Actinomycin D is known to inhibit m-RNA production by the formation of a strong bond with guanine on DNA molecules (22), thereby blocking RNA polymerase-mediated m-RNA production and prohibiting further protein synthesis. The tight bond between actinomycin D and DNA is reversible only under rather vigorous chemical conditions (22), which would explain the observation that LP generation was irreversibly blocked by this drug. In contrast, the inhibition of pyrogen release by cycloheximide and puromycin was reversible, consistent with the weak chemical bonding of these agents (20, 21).

The failure of vincristine to duplicate the inhibitory effect of actinomycin D on pyrogen formation is interesting. In investigations utilizing Ehrlich ascites carcinoma cells, it has been observed that vincristine was most suppressive of soluble RNA (s-RNA) production and less so against m-RNA or nuclear RNA (23). Minimal depression of protein synthesis was noted at 7 hr (20%), which became more marked by 16 hr incubation (50%) (23). In contrast, when neoplastic human leukocytes were studied, vincristine was found to depress synthesis in all classes of RNA as determined by density gradient ultracentrifugation (24). The incorporation of labeled amino acids into protein by the leukocytes was also suppressed by vincristine, although the mechanism of action was unclear (24). On the other hand, when mature normal granulocytes were employed, there was no significant inhibition of tritiated uridine incorporation into RNA nor labeled amino acids into protein. The failure of vincristine to suppress LP production in the present investigation is consistent with these observations.

Also of interest was the normal hexose monophosphate shunt (HMS) activity exhibited by resting and phagocytizing leukocytes incubated for 1 hr with actinomycin D. This finding is in contrast to the report that incubation of normal human leukocytes with actinomycin D resulted in depression

of $^{14}CO_2$ release from the HMS (36, 37). No obvious explanation for this discrepancy is evident; it does not seem reasonable that the longer incubation times employed in the present study could cause the difference. In contrast to actinomycin D, both cycloheximide and puromycin caused a significant increase in HMS activity of resting cells.

Finally, the data concerning the energy requirements for pyrogen release is intriguing. Earlier investigations on rabbit peritoneal leukocytes demonstrated that pyrogen release could be inhibited by sodium fluoride only when this agent was added to the incubation medium prior to phagocytosis, but not after phagocytosis had occurred (25). It was concluded that because of the interference with glycolysis, no significant phagocytosis, and therefore, no activation of the leukocytes for pyrogen release had taken place. In contrast to these findings with rabbit peritoneal leukocytes, in the present studies employing human cells, the addition of sodium fluoride 1 hr after the initiation of phagocytosis completely suppressed pyrogen production. This inhibitory effect was lost if the cells were allowed to incubate with bacteria for 2 hr before adding sodium fluoride. The action of sodium fluoride did not appear to be mediated through interference with phagocytosis, since there was no significant difference in the per cent phagocytizing cells at 1 and 2 hr. Furthermore, in other preparations not containing sodium fluoride, halting phagocytosis at 1 hr by removing the cells from the bacteria did not lead to a suppression of LP output. Potassium cyanide-induced blockade of aerobic metabolism did not affect pyrogen production. These findings suggest that for human leukocytes, the energy derived from glycolysis is important not only for phagocytosis itself, but also for the initial intracellular metabolic activation that leads to subsequent pyrogen formation. Once the initial activation has occurred, leukocytes apparently contain enough stored utilizable energy for pyrogen release, most likely as high energy phosphate bonds, to bypass the blockade introduced by sodium fluoride and potassium cyanide. A similar situation has been noted during mitochondrial protein synthesis by liver cells in vitro (38, 39).

The finding that activation appears to be maximal during the 1st hr of incubation of cells with bacteria, whereas the requirements for m-RNA and protein synthesis for optimal LP release extend beyond this time, suggests that induction of a genome for LP generation may take place during this initial period. If this is true, inhibition of pyrogen production by sodium fluoride added prior to 2 hr raises the possibility that in mature human neutrophils and monocytes, the energy provided by anaerobic glycolysis might be essential for induction of this genome, after which the cells are then capable of utilizing stored energy both for m-RNA transcription and translation to new protein. An alternative explanation is derived from studies on rabbit reticulocytes in which addition of sodium fluoride caused a reversible degradation of polysomes into single ribosomes, thereby halting protein synthesis (40). Human leukocytes may be similar in that glycolytic energy is required for the integrity of poly-

somes during protein synthesis. Whether a portion of the new protein synthesized during the activation phase after phagocytosis is the LP molecule itself or an enzyme involved in freeing it from an inactive precursor state awaits further investigation.

SUMMARY

Release of the protein molecule, leukocytic pyrogen, is one of the many reactions exhibited by leukocytes after phagocytosis. After the ingestion of heat-killed *S. albus*, a 3-4 hr latent period exists, during which human peripheral leukocytes release no pyrogen, yet cellular metabolism is altered in such a way that pyrogen output may subsequently occur in the absence of further phagocytosis. Transcription of messenger RNA and translation of new protein are initial events in the activation process, since addition of the inhibitors, actinomycin D, and cycloheximide or puromycin, during this period markedly depressed or abolished subsequent pyrogen release. These effects were noted to be dependent upon the time of addition of the inhibitors. None of the inhibitor drugs interfered with cell viability as measured by phagocytosis and hexose monophosphate shunt activity, nor did they alter the pyrogenicity of preformed leukocytic pyrogen. Vincristine did not inhibit pyrogen formation, consistent with its reported failure to alter RNA synthesis in mature human granulocytes. The glycolytic inhibitor, sodium fluoride, blocked pyrogen release both when added prior to particle ingestion or 1 hr after the initiation of phagocytosis. Whereas inhibition of phagocytosis would explain the sodium fluoride effect prior to 1 hr, this was not observed in leukocyte preparations incubated for 1 hr with *S. albus* before adding sodium fluoride. When sodium fluoride was added to preparations 2 hr after the start of incubation, the LP production was unimpaired. Potassium cyanide had no effect on cell activation or pyrogen release. These findings suggest that the primary energy supply for the activation process is derived from high energy phosphate bonds provided by anaerobic glycolysis. Since the major amount of cell activation appears to occur in the 1st hr after phagocytosis, this energy might be involved in the induction of a genome leading to the transcription of m-RNA and its translation into new protein or is required for polysome integrity during protein synthesis. It is suggested that this new protein may be leukocytic pyrogen itself, or an enzyme responsible for cleaving it from an inactive precursor.

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