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Effect of Metabolic Inhibitors on Pyrogen Production by Rabbit Leukocytes[1] (38802)

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Conflicting data exist in the literature concerning the effects of metabolic inhibitors on the production of leukocytic pyrogen. Nordlund *et al.* (1) and Moore *et al.* (2) have shown that actinomycin D, puromycin, and cycloheximide block the production of pyrogen by leukocytes of the rabbit stimulated by phagocytosis of heat-killed Staphylococci. Gander *et al.* (3) have shown that, although puromycin inhibits protein synthesis in rabbit leukocytes, pyrogen production after stimulation with endotoxin is not affected.

The present studies were undertaken to investigate the effects of several metabolic inhibitors on the production of pyrogen after endotoxin stimuli to investigate if *de novo* synthesis occurs or if the mechanism of formation favors the theory of an inactive "propyrogen" which is converted to an active form at the time of release.

Materials and Methods. New Zealand white rabbits of both sexes weighing between 2 and 2½ kg and less than 1 yr of age were used throughout the study. The animals were housed and all pyrogen assays were performed in air-conditioned rooms maintained at 70°F. Rectal temperatures were measured with a scanning Yellow Springs Telethermometer. Animals with initial rectal temperatures of over 39.8°C were excluded from the experiment. The fever index was calculated by measuring the area in cm^2 under a 2-hr fever curve (4) and by measuring peak height of the fever curve (5). The leukocytic pyrogen used in these experiments was prepared from rabbit peritoneal exudate cells as previously described (4, 6). Several lots were pooled, diluted, if necessary, to provide a uniform preparation, and stored in sealed ampoules at −70°C. The bacterial endotoxin obtained from Difco Laboratories was prepared from *Salmonella abortus equi.* All glassware and equipment was made pyrogen-free by heating at 180°C for 2 hr in a hot-air oven. Double-distilled pyrogen-free water used for reagents was collected in a pyrogen-free carboy from an all-glass pyrogen-free still. All reagents and samples were cultured on blood agar plates. Any samples showing contamination were discarded.

Rabbit blood was obtained by heart puncture, anticoagulated with heparin (10 U/ml blood), and centrifuged at 250*g* at 4°C for 15 min; the buffy coat was removed by aspiration with needle and syringe. The buffy coat cells were washed in phosphate-buffered saline (PBS) solution twice. The cells were counted and resuspended in PBS to yield 1×10^8 cells/tube.

Actinomycin D (Merck Co) was used at 2.5 μg/ml; cycloheximide, *p*-fluorophenylalanine, puromycin dihydrochloride, and puromycin aminonucleoside were procured from Sigma Company and were used at 20 μg/ml, 50 μg/ml, 20 μg/ml, and 20 μg/ml, respectively. Cortisol (hydrocortisone sodium succinate) was purchased as Solu-Cortef from Upjohn and was used at a concentration of 250 μg/ml. The dose of cortisol was the same as previously used (7). The doses for all other inhibitors were based on the studies of Tan *et al.* (8). All solutions were filtered by Millipore filter (0.45 μm) and were tested for inherent pyrogenicity prior to use in experiments by injecting 2 ml of each solution iv into rabbits and observing their temperature curves.

Experimental Results. 1. *Endotoxin induction*—20-min preincubation with endotoxin. Endotoxin was used at a concentration of 0.1 μg to stimulate 1×10^8 buffy coat cells to release leukocytic pyrogen which gave an average change of temperature of 1.92°C.

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The endotoxin was added 20 min before the addition of the following metabolic inhibitors: (1) actinomycin D, (2) cortisol, (3) cycloheximide, (4) puromycin aminonucleoside, (5) parafluorophenylalanine, and (6) puromycin dihydrochloride. The pyrogen concentration was expressed as fever index and also by temperature elevation expressed as maximum peak height of the plotted fever curve. The peak height was plotted against fever index and a straight-line regression analysis using the least-squares method was performed. A correlation coefficient of 0.97 was obtained. Because of the high correlation of peak height method to the fever index method, results are reported as mean peak height ± SEM. The data in Table I indicate that none of the inhibitors used caused a reduction in the amount of pyrogen released when using endotoxin at a dose of 0.1 μg. When experiments were repeated using a dose of 0.05 μg of endotoxin, the only inhibitor which reduced the amount of pyrogen released was cortisol. The difference of the means was 1.07° and was statistically significant at a P value of <0.001.

TABLE I. EFFECT OF INHIBITORS ON PYROGEN PRODUCTION BY BUFFY COAT CELLS (PREINCUBATION WITH ENDOTOXIN FOR 20 MIN).[a]

	No. of samples	T^0 Mean peak height ± SEM
Endotoxin alone	9	1.93 ± 0.27
Endotoxin + actinomycin D	6	1.52 ± 0.20
Endotoxin + cortisol	7	1.62 ± 0.16
Endotoxin + cortisol[b]	10	1.12 ± 0.13
Endotoxin + cycloheximide	7	1.99 ± 0.39
Endotoxin + parafluorophenylalanine	6	1.88 ± 0.23
Endotoxin + puromycin aminonucleoside	8	2.02 ± 0.26
Endotoxin + puromycin dihydrochloride	9	1.93 ± 0.32
Saline control	17	0.14 ± 0.05

[a] Endotoxin (0.1 μg) was incubated with buffy coat cells at 37°C for 20 min before the addition of the inhibitors. The tubes were then incubated for 18 hr at 37°C.

[b] The dose of endotoxin was 0.05 μg in these experiments.

TABLE II. EFFECT OF INHIBITORS ON PYROGEN PRODUCTION BY BUFFY COAT CELLS (PREINCUBATION WITH INHIBITOR FOR 1 HR)[a]

	No. of samples	T^0 Mean peak height ± SEM
Endotoxin alone	12	2.03 ± 0.19
Endotoxin + actinomycin D	6	2.01 ± 0.31
Endotoxin + cortisol	8	1.04 ± 0.28
Endotoxin + cortisol[b]	11	0.80 ± 0.16
Endotoxin + cycloheximide	5	1.62 ± 0.43
Endotoxin + para-fluorophenylalanine	5	2.41 ± 0.29
Endotoxin + puromycin aminonucleoside	13	1.61 ± 0.27
Endotoxin + puromycin dihydrochloride	9	2.32 ± 0.15
Saline control	16	0.15 ± 0.04

[a] Inhibitors were incubated with buffy coat cells for 1 hr before the addition of endotoxin (0.1 μg). The tubes were then incubated for 18 hr at 37°C.

[b] The dose of endotoxin was 0.05 μg in these experiments.

2. *Endotoxin induction*—1 hr preincubation with metabolic inhibitor. Endotoxin was used at a concentration of 0.1 μg to stimulate 1×10^8 buffy coat cells to release leukocytic pyrogen. The endotoxin was added 1 hr after the cells had been incubated with the aforementioned metabolic inhibitors. The results appear in Table II. None of the inhibitors reduced the production of pyrogen in statistically significant amounts when the dose was 0.1 μg of endotoxin. When experiments were repeated using 0.05 μg of endotoxin, only cortisol reduced the amount of pyrogen significantly. The difference of the means was 0.89° and was significant statistically at a level of $P < 0.005$.

3. *Poly(I):poly(C) induction.* Poly(I):poly(C) was used to stimulate buffy coat cells at varying concentrations from 0.02 μg/ml to 100 μg/ml. The production of an endogenous pyrogen from leukocytes in the buffy coat was not detected regardless of the concentration of poly(I):poly(C) used.

Discussion. Relatively little intracellular

pyrogen can be detected in rabbit granulocytes (9). Activated exudate granulocytes contain only a fraction of the pyrogen that they are capable of releasing at any one time (2). These findings suggested that either the active pyrogen is synthesized *de novo* during the release process or is stored in the cell (bound or unbound) as an inactive "propyrogen" (10) which is converted to an active form at the time of release.

The data from this study indicate that the metabolic inhibitors used did not block the production of pyrogen at the level of *m*-RNA formation, (actinomycin D), at the level of incorporation of amino acids into proteins (puromycin dihydrochloride, cycloheximide), or at the level of ribosomal RNA formation (puromycin aminonucleoside). An inactive pyrogen molecule was not formed due to the presence of *p*-fluorophenylalanine. Only cortisol showed an inhibitory effect. The mechanism of action remains to be elucidated. Weissman (11, 12) has shown that glucocorticoids stabilize leukocyte lysosomal membranes whereas endotoxin labilizes these membranes. Petersdorf and Shulman (13) have suggested that the leukocyte lysosome or granule is implicated in the formation of leukocytic pyrogen. It has been proposed by Gander *et al.* (7) that the antipyretic effect of glucocorticoids on leukocytes is primarily due to stabilization of their lysosomal membranes. All of these findings are consistent with the release of a preformed substance.

However, the present data are not consistent with the results of Nordlund *et al.* (1) who have evidence that cycloheximide, puromycin, and actinomycin D markedly inhibit pyrogen production in human leukocytes stimulated by phagocytosis of heat-killed gram-positive bacteria. Others (2) have reported that rabbit peripheral leukocytes stimulated by phagocytosis and by endotoxin were inhibited from producing pyrogen by cycloheximide and puromycin.

Moore *et al.* (2) have proposed that blood leukocytes must be "activated" before the release of pyrogen. They contended that metabolic inhibitors block the "activation" process but not the release process. One possible explanation for the data from the present study is that the leukocytes are somehow "activated" *in vivo* and we are really dealing with the release phase. In the same paper, Moore *et al.* described the presence of an "activator" substance, supposedly protein in nature which is present in the exudate fluid of rabbits used for the harvest of peritoneal granulocytes. If this theory proves to be true, perhaps the data could be explained by the "activator" also exerting its influence on the blood leukocytes. All of the experiments in this study were done on buffy coat cells obtained from rabbits used for harvest of peritoneal exudate cells. Previous work with puromycin's effect on buffy coat cells from normal rabbits having no other prior treatment also showed the same results—no inhibition of pyrogen production (3). The control samples from the present studies also indicate no prior activation of the cells.

Other explanations for the lack of inhibition observed might be that all of the drugs used were inactive—a very unlikely possibility since precautions were taken as to proper storage and the periodic preparation of new stock solutions to avoid decay. In addition, before interferon assays could be made, samples had to be dialyzed for several days to rid them of drug-induced toxicity. This provides evidence that the drugs were still quite active.

One might also consider the possibility that the inhibitors did not enter the cells at all and, thus, had no effect. To solve this problem incorporation studies using radioactively labeled amino acids, i.e., [^{14}C]leucine, would have to be done. Previous incorporation studies using puromycin were done on the buffy coat system and it was found that the inhibitor entered the cell and decreased protein synthesis while pyrogen release was unaffected (3). Tan *et al.* (8) have done incorporation studies with similar dosage levels of puromycin, actinomycin D, and cycloheximide used in this study. They achieved 93% inhibition of protein synthesis with puromycin and 97% inhibition of RNA synthesis with actinomycin D as well as 90% inhibition of incorporation of ^{14}C-labeled amino acids with cycloheximide. All incorporation studies were performed on rabbit kidney cell cultures. Because of this support-

ing evidence, the nonentry of inhibitors into the cells is not felt to be playing a role.

Since we can exclude the possibility that our cells are "activated" before addition of the inhibitor, we are left with results supporting the theory that an inactive "propyrogen" exists preformed in the cell and is converted to an active pyrogen molecule after proper stimulation, i.e., bacterial endotoxin or phagocytosis of heat-killed bacteria. This conversion may not require the synthesis of new proteins. Berlin and Wood (14) have shown that this "conversion" is inhibited by sodium fluoride. The fact that cortisol inhibits the production is consistent with cortisol stabilizing membranes and that this would inhibit the second stage or the release of the pyrogen. It has been shown that membrane permeability increases with pyrogen release because some cytoplasmic enzymes, i.e., aldolase, appear in the media along with pyrogen (10).

Summary. The metabolic inhibitors, actinomycin D, cycloheximide, puromycin dihydrochloride, puromycin aminonucleoside, and *p*-fluorophenylalanine did not inhibit the release of leukocytic pyrogen whether endotoxin was preincubated with cells for 20 min at 37°C before addition of inhibitor or inhibitor was preincubated with cells for 1 hr before addition of endotoxin. On the other hand, cortisol inhibited release of pyrogen under both experimental conditions. Poly(I): poly(C) was not effective in inducing rabbit leukocytes to produce an endogenous pyrogen.

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