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Effect of Inhibitors of Protein Synthesis on Pyrogen Production by Granulocytes.* (32515)

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Polymorphonuclear leukocytes from inflammatory exudates of rabbits release a pyrogen when incubated in saline(1-3). This leukocytic pyrogen (LP) is a protein(4-6) which may play an important role in the pathogenesis of fever in rabbits.

There is little specific information available concerning the process of pyrogen formation by leukocytes. Fessler and co-workers(7) and Kaiser and Wood(3) found that mechanical disruption of granulocytes did not release significant amounts of LP and that subcellular fragments of leukocytes were not pyrogenic. On the other hand, Herion and his colleagues showed that intact granulocyte lysosomes or their lysates induced fever when injected intravenously into rabbits, suggesting that there may be a relationship between some constituent of lysosomes and leukocytic pyrogen(8). These studies permit the hypothesis that, during incubation, granulocytes either synthesize pyrogen *de novo* or release it from an inactive precursor. Gander and Goodale favored the precursor theory because puromycin failed to abolish the production of pyrogen by granulocytes *in vitro*(9). The experiments to follow were undertaken to assess further the effect of inhibitors of protein synthesis on production of pyrogen.

Materials and methods. Rabbits weighing 2 to 3 kg were used in all experiments. Glassware was sterilized and rendered pyrogen-free by heating at 170°C for 2½ hours. Commercially available sterile, nonpyrogenic distilled water was used to prepare all solutions.

Leukocytes, 80 to 90% of which were granulocytes, were obtained by procedures outlined by others(5). Leukocyte-saline suspensions containing 3.5×10^7 cells/ml were incubated for 20 hours at 37°C unless otherwise specified, and the cells were then removed by centrifugation. Extracts were passed through a bacterial filter, frozen and stored at 0°C until used.

The procedures employed for assaying the pyrogenicity of leukocyte extracts by intravenous injection into rabbits were essentially the same as those previously described (10). Febrile responses were quantitated by measurement of the fever index ($F.I._{120}$), the area in cm^2 under a 2-hour fever curve (10 cm on the ordinate equaled 1°C and 4 cm on the abscissa represented 60 minutes).

Rabbits used for test purposes were obtained in groups which were treated as units and kept intact as long as they were suitable for pyrogen assays. Only results obtained within a given group of test animals were used to compare the pyrogenicity of various

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extracts(11). The animals were trained to the test situation while tolerance was induced and maintained by daily intravenous injection of 0.1 μg of *E. coli* 026:B6 lipopolysaccharide. Animals were not used in pyrogen assay until the $F.I._{120}$ induced by 0.05 μg of endotoxin was less than 20 cm^2.

Effect of chloramphenicol treatment on pyrogen production. Rabbits were housed in pairs and allowed food and water *ad libitum.* Daily weights were recorded, and on 2 successive days prior to treatment and on the second day of treatment, the predominant bacterial flora in the stool was identified and quantitated.

Sixteen rabbits received four intramuscular injections of 750 mg chloramphenicol succinate at 12-hour intervals. In 4 animals, the levels of chloramphenicol in the serum at 1, 2, 4, 6 and 12 hours were estimated by the serial 2-fold dilution technique. The animals treated with chloramphenicol were processed in groups of 4 and alternated with control groups of untreated animals or animals which had received equivalent doses of ampicillin. Peritoneal exudates were induced 32 hours after the first injection of antibiotic and were harvested 16 hours later. The total number of leukocytes recovered from each exudate was determined, and individual extracts were prepared in the usual manner. The protein concentration of extracts was measured by a modification of the method of Lowry(12). Each extract was tested in 5 tolerant recipients and a mean $F.I._{120}$ was calculated by averaging the febrile responses observed. The extracts from 8 animals treated with chloramphenicol and 8 untreated animals were compared in one group of tolerant recipients (Group A), while in a second group (Group B), extracts from the remainder of the chloramphenicol treated animals, 7 untreated controls and 4 animals which had received ampicillin were compared. The significance of the difference between the febrile response induced by various extracts was estimated from the ratio of the difference in means to the standard error of that difference (13).

Effect of inhibitors of protein synthesis on pyrogen production in vitro. Peritoneal exudates from several untreated rabbits were pooled before the leukocytes were washed. The final saline-leukocyte suspension was divided into 5 equal parts, and actinomycin D 10 μg/ml, puromycin 10 μg/ml, chloramphenicol 25 μg/ml or ampicillin 25 μg/ml was added to four aliquots while the fifth served as a control. The leukocyte suspensions were then incubated at 37°C for 20 hours and the cells removed by centrifugation. The extracts were dialyzed overnight at 4°C against 0.9% saline, pH 7.0, and their pyrogenicity was compared by quantitating the febrile response induced by each incubation mixture in at least 5 tolerant rabbits.

TABLE I. Effect of Chloramphenicol Treatment on Pyrogen Production.

	Mean $F.I._{120}$		
Tolerant group	Control animals	Chloro-treated animals	Amp-treated animals
A	42.4 (3.0)	26.4 (3.5)*	—
B	24.9 (.7)	12.6 (1.8)*	26.8 (3.0)

Numbers in parentheses = S.E. * $P < .001$

In some experiments, the concentration of actinomycin or puromycin in the incubation mixtures was increased to 25 μg/ml, or the time of incubation of the leukocyte suspensions was shortened to 4 hours.

Results. Treatment with chloramphenicol. Blood levels of chloramphenicol were 6 to 12 μg/ml 1 hour after intramuscular injection, and 0.5 μg/ml at 12 hours. Treated rabbits had no weight loss or apparent illness, but the number of bacteria in the stool, particularly Gram-negative organisms, was reduced. The number of leukocytes and the proportion of granulocytes recovered from peritoneal exudates was the same in treated and untreated animals. The mean protein concentration of extracts from animals treated with chloramphenicol was 383 μg/ml and 494 μg/ml in extracts from control animals. This difference was not statistically significant.

When tested in Group A tolerant rabbits leukocyte extracts from untreated controls and chloramphenicol-treated animals had mean fever indices of 42.4 and 26.4 cm^2, respectively (Table I). In the tolerant recipients of Group B, the febrile responses

TABLE II. The Effect of Inhibitors of Protein Synthesis on Pyrogen Production *in vitro*.

Inhibitor	Time of incubation (hr)	Conc of inhibitor (μg/ml)	Mean $F.I._{120}$
Control	20	—	31.6 (3.3)*
Actinomycin D	20	10	29.6 (3.7)
Chloramphenicol	20	25	29.9 (5.0)
Ampicillin	20	25	27.8 (1.8)
Puromycin	20	10	24.8 (2.7)†

* Numbers in parentheses = S.E. † P = 0.1

observed were somewhat lower than in the rabbits of Group A, but the pyrogenicity of extracts from chloramphenicol-treated animals was again low. In both instances, differences in pyrogenicity were statistically significant.

Effects of inhibitors of protein synthesis in vitro. The extracts of leukocytes incubated 20 hours with 10 μg/ml of actinomycin D, 25 μg/ml of chloramphenicol or 25 μg/ml of ampicillin were no less pyrogenic than control extracts (Table II). The $F.I._{120}$ of aliquots of leukocyte suspensions incubated with 10 μg/ml of puromycin, however, was 15 to 20% lower than the others. This difference was of borderline statistical significance and might be explained by the inherent variability in the biological assay for pyrogen. However, when the suspensions of leukocytes were incubated only 4 hours with inhibitors, all the extracts were equally pyrogenic.

When the concentration of actinomycin D or puromycin was increased to 25 μg/ml, the results were qualitatively and quantitatively similar to those observed with the lower concentrations.

Discussion. These studies demonstrate two points. First, leukocytes from animals treated with large doses of chloramphenicol produce reduced quantities of pyrogen when they are incubated *in vitro*. Second, pyrogen production by leukocytes is unaffected by incubation with certain inhibitors of protein synthesis.

The mechanism by which chloramphenicol reduces production of pyrogen might be related to alterations in bacterial flora(10), to an effect of the drug on development or function of leukocytes, or to interference with synthesis or release of pyrogen. Moderate changes in stool flora were observed but it is unlikely that they had a significant effect on pyrogen formation, because preliminary studies showed that large doses of chloramphenicol orally altered the bacterial flora of the gut without affecting the production of pyrogen by granulocytes, and leukocytes from animals treated with ampicillin yielded extracts with the same fever producing capabilities as controls.

The effect of chloramphenicol on leukocytes has not been as well characterized as its effect on erythroid elements. Scott *et al* found that bone marrow aspirations in one-third of patients receiving large doses of chloramphenicol showed vacuolation of the granulocytic series, but leukopenia was uncommon(14). Two bits of evidence indicate that chloramphenicol does not significantly alter the function of leukocytes in inflammation. Firstly, animals receiving massive doses of chloramphenicol have a normal response to inflammatory stimuli(15), and secondly, in this study, the number of leukocytes and the proportion of granulocytes recovered in acute inflammatory exudates was the same in chloramphenicol-treated animals and controls.

While the possibility that chloramphenicol interferes with the synthesis of enzymes necessary for the activation or release of pyrogen cannot be excluded, there is no evidence to support this hypothesis. The postulate that chloramphenicol interferes with the synthesis of leukocyte pyrogen is more attractive. This drug has long been recognized as an inhibitor of protein synthesis in bacterial systems, and recently, 600 mg/kg/d of chloramphenicol has been shown to inhibit protein synthesis in mammals(16,17). The studies reported here show that leukocytes from animals receiving high doses of this drug yield extracts which tend to have low protein concentrations, but their pyrogenicity is altered much more significantly. LP constitutes only a small fraction of the protein in leukocyte extracts and this apparent discrepancy might be related to a disproportionate inhibition of pyrogen synthesis. Daniel *et al*(18) suggested that chloram-

phenicol may be a particularly effective inhibitor of newly directed protein synthesis, and it is possible that at some stage early in the development of granulocytes or in the evolution of the inflammatory response, synthesis of LP is particularly active.

The results obtained by incubating leukocytes with actinomycin D, chloramphenicol, and puromycin indicate that the bulk of protein synthesis required for pyrogen production has been completed before the leukocytes are harvested from inflammatory exudates. Actinomycin D interferes with DNA-directed RNA synthesis(19), chloramphenicol probably acts by blocking the attachment of messenger RNA to its ribosomal binding site(17), and puromycin interrupts protein synthesis by causing the release of incomplete peptide chains from the ribosome-template RNA complex(20). Of these agents, only puromycin had even a minimal inhibitory effect on pyrogen production, and then only after prolonged incubation. These data indicate that only a small amount of pyrogen is synthesized during the incubation of leukocytes *in vitro,* but because actinomycin and chloramphenicol fail to interfere with the reaction, this protein synthesis probably does not depend upon the rapid turnover of RNA.

In conjunction with previous observations that leukocytes harvested from inflammatory exudates contain only a small fraction of the active pyrogen they are capable of producing(3,7), these studies permit some speculation regarding the production of leukocyte pyrogen. *In vivo,* granulocytes synthesize an inactive protein precursor of LP. This process probably begins during some stage of granulocyte maturation, but may well be accelerated when the cells participate in an inflammatory reaction. The rate of accumulation of pyrogen precursor exceeds its rate of conversion to active pyrogen, because leukocytes contain little active LP, but during incubation *in vitro* they can produce large quantities of pyrogen in the absence of active protein synthesis.

Summary. The formation of pyrogen by rabbit leukocytes can be inhibited significantly by treating animals with high doses of chloramphenicol. The mechanism of this effect probably involves inhibition of protein synthesis at some point during the development of granulocytes. On the basis of experiments with inhibitors of protein synthesis it appears that leukocytes from inflammatory exudates do not synthesize much additional pyrogen during incubation *in vitro.*

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