

SYNTHESIS OF ENDOGENOUS PYROGEN BY RABBIT LEUKOCYTES*

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It has been shown in at least three laboratories that when blood leukocytes are stimulated to secrete leukocyte pyrogen, a two-stage process is involved (1-3). During the first stage, protein synthesis occurs; and if this is prevented by any of several inhibitors, no leukocyte pyrogen subsequently appears. At the end of the first stage, very little pyrogen can be obtained by extraction of the cells with a variety of solvents, and it is presumed that pyrogen exists in the form of an inactive precursor (4). During the second stage, pyrogen release from the cells can be inhibited only by reagents that bind to sulfhydryl groups (5); neither protein synthesis nor a source of energy seem to be required (1-3). The first stage is referred to as "activation" of leukocytes, and the second, as "secretion" of pyrogen by leukocytes.

It seemed likely that pyrogen was itself synthesized during the activation of leukocytes (1, 2). However, several investigators (3, 6) had failed to demonstrate incorporation of radiolabeled amino acids into partially purified pyrogen samples. Since leukocyte pyrogen constitutes only about 0.01% of the protein present in crude pyrogen (7), it seemed likely that labeling of pyrogen was obscured by large amounts of other labeled proteins. If leukocytes were activated in the presence of radiolabeled amino acids and the pyrogen they secreted was then purified to homogeneity, it should be possible to demonstrate labeling of leukocyte pyrogen. A series of such labeling and purification experiments are here described.

Methods

The general conduct of the experiments and the precautions taken to prevent contamination by bacteria and bacterial pyrogens were as described previously (7). Peritoneal exudates were induced, using 200 mg/liter of shellfish glycogen in saline, and were harvested by killing the rabbits with pentobarbital. The cells were spun down at 250 g for 10 min at 4°C and were

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suspended at a concentration of 3.5×10^7 cells/ml in minimal essential medium (MEM)¹ for suspension cultures (Microbiological Associates, Inc., Bethesda, Md.), vitamins and amino acids being omitted. More than 90% of the cells were polymorphs.

Leukocytes were obtained from heparinized blood by centrifuging at 500 *g* for 20 min at 4°C and aspirating the buffy coats as single sheets. They were washed twice in MEM and suspended in MEM at a concentration of 3.5×10^7 cells/ml. Differential counts showed 40–50% polymorphs, 10% monocytes, and 40–50% lymphocytes.

The stimuli used to activate leukocytes were (a) *Escherichia coli* endotoxin (0111:B4 strain, Difco Laboratories, Detroit, Mich.), 0.05 $\mu\text{g}/10^8$ leukocytes; (b) heat-killed *Staphylococcus aureus* opsonized with normal human serum, 100 organisms/leukocyte, and (c) intermediate strength purified protein derivative (PPD) (Connaught Medical Research Laboratories, Toronto, Canada), 5 $\mu\text{g}/\text{ml}$. In the case of *S. aureus* activation, 5 meq/liter of calcium chloride was added to the MEM to enable adequate phagocytosis to occur. For tuberculin activation, exudates were produced in rabbits that had been sensitized 3 wk previously by an intravenous injection of 5 mg of heat-killed tubercle bacilli (BCG strain) in 0.5 ml of Bayol F and 0.5 ml of saline.

The stimulated cells were incubated in a shaking water bath for 2 h at 37°C to allow activation to occur. Radiolabeled amino acids (³H and ¹⁴C amino acid mixtures, ICN, Nutritional Biochemicals Div., International Chemical and Nuclear Corp., Waltham, Mass.) were added at a concentration of 0.5 $\mu\text{Ci}/\text{ml}$ at 0, 25, 50, and 75 min of incubation. The cells were spun down at 250 *g* for 10 min at 4°C and were resuspended in saline at a concentration of 3.5×10^7 cells/ml. They were again incubated for 2 h at 37°C to allow pyrogen release to occur. The supernates from this incubation contained crude pyrogen, and were stored at 4°C with 0.1% sodium azide.

The purification technique was as previously described (7). Samples containing soluble amino acids were counted on paper disks after washing with hot trichloroacetic acid. Column effluents were counted directly in Triton-toluene fluor. Acrylamide gel slices were digested in 30% hydrogen peroxide at 65°C for 18 h and were counted in Triton-toluene fluor.

RESULTS

It was discovered that if a peritoneal exudate was induced, using one-tenth the usual concentration of glycogen, the leukocytes obtained from it were not spontaneously activated. They could be activated by endotoxin or phagocytosis, and activation could be blocked by cycloheximide or puromycin. A typical experiment is shown in Fig. 1.

A total of 500 ml of ¹⁴C-labeled crude pyrogen was obtained by pooling material from five experiments in which exudate leukocytes had been activated by endotoxin in the presence of labeled amino acids. The total soluble protein counts amounted to 6.85×10^7 , or about 5% of counts supplied; and about 3,000° C units of pyrogen were obtained. A pool of unlabeled pyrogen produced in the same way and amounting to 7,000° C units was added to serve as carrier.

The pyrogen content and the radioactivity of the fractions from the gel filtration step are shown in Fig. 2. Four ¹⁴C-labeled peaks were seen, and the largest of these aligned precisely with the pyrogen. The pyrogen peak from gel filtration was subjected to ion exchange chromatography at pH 8. Assay of the effluent for radioactivity and pyrogen showed only one radioactive peak of consequence,

¹ Abbreviations used in this paper: MEM, minimal essential medium; PPD, purified protein derivative.

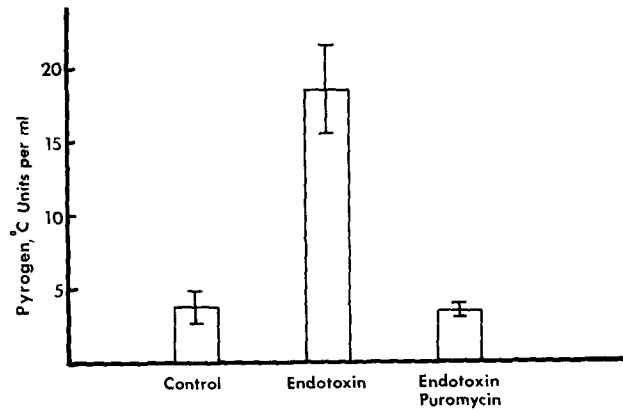


FIG. 1. Activation of peritoneal exudate cells by endotoxin, and suppression of activation by 10^{-4} M puromycin. The 95% confidence limits are indicated by vertical bars.

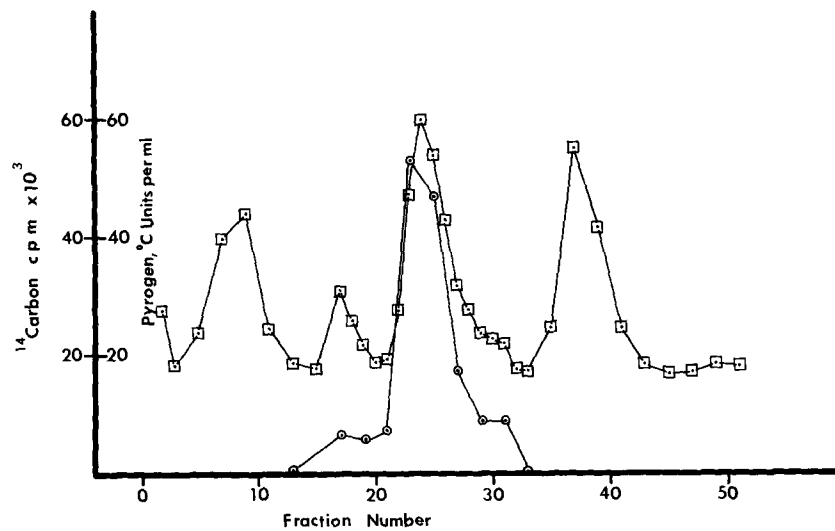


FIG. 2. Gel filtration of ^{14}C -labeled pyrogen from exudate cells stimulated with endotoxin. \circ — \circ , pyrogen content, °C units/ml; \square — \square ^{14}C cpm.

and that corresponded precisely with pyrogenic activity (Fig. 3). The data suggested that pyrogen might already be the only labeled protein present.

The pyrogen peak was then subjected to isoelectric focusing, and the effluent was tested for radioactivity, pyrogen content, and pH. Only one radioactive peak was seen, and, again, it coincided with pyrogenic activity (Fig. 4). The overall yield of pyrogen was 18%, and 1.1% of the original protein counts were present in the final sample. Thus about 6% of the protein counts originally found in the crude material must have been associated with pyrogen.

The association of pyrogenic activity and radioactivity was confirmed by

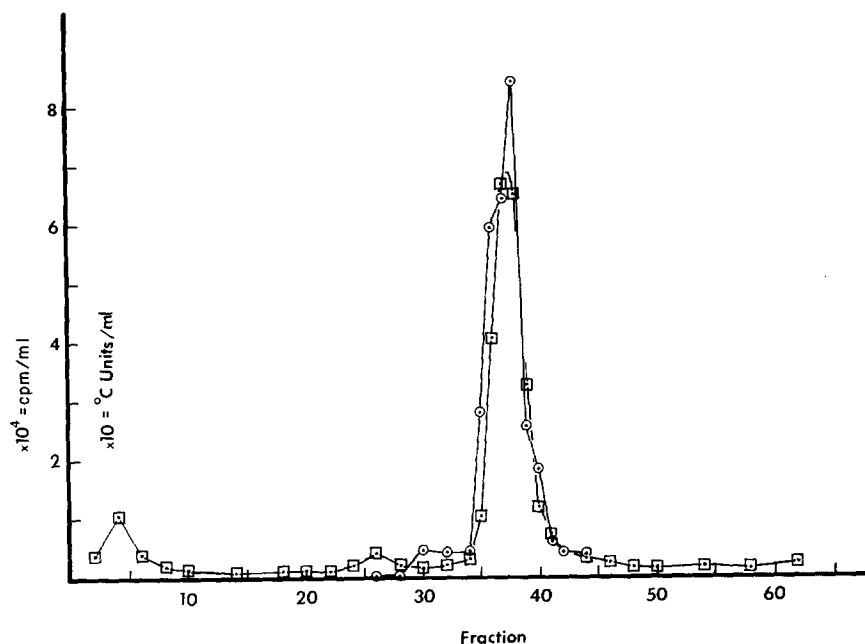


FIG. 3. Ion exchange chromatography at pH 8.1 of ^{14}C -labeled pyrogen from exudate cells stimulated with endotoxin. \circ — \circ , pyrogen content, $^{\circ}\text{C}/\text{ml}$; \square — \square , ^{14}C cpm.

electrophoresis and isoelectric focusing on acrylamide gels. Two types of experiment were performed. In the first, biological activity and ^{14}C counts were compared by cutting gels transversely into 2-mm slices. Each slice was bisected; one half was eluted and assayed for pyrogen, and the other half was digested and counted. The second type of experiment compared radioactivity with stained protein bands. In these experiments gels were first stained to reveal the protein band. After the band had been located and the gels photographed, the stained band was cut out, digested, and counted. The results of both types of experiment enabled a comparison of biological activity, radioactivity, and protein to be made. On isoelectric focusing and electrophoresis at pH 3.5, only one band was seen, and this contained both biological activity and counts. On the gel electrophoresed at pH 9, there was an artifact, also visible on a blank gel; but pyrogen and radioactivity coincided with the protein band (Fig. 5).

Another batch of ^{14}C -labeled pyrogen has since been prepared by the same technique and a further criterion of purity obtained. If the radioactivity was indeed associated with the pyrogen molecule, then it should be possible to make a point-for-point correlation of biological activity and radioactivity across the pyrogen peak in the isoelectric focusing column effluent. The results of such an experiment are shown in Table I.

An experiment was next designed to test whether exudate leukocytes acti-

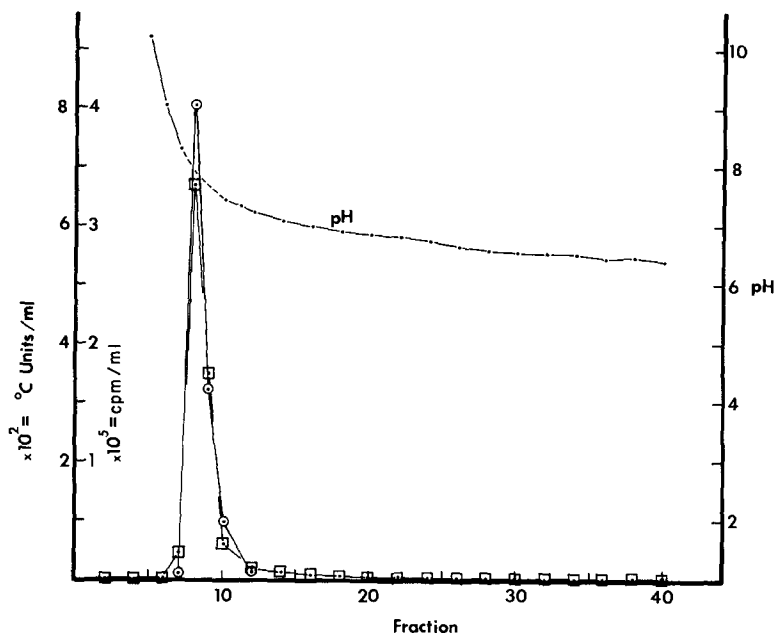


FIG. 4. Isoelectric focusing of ^{14}C -labeled pyrogen from exudate cells stimulated with endotoxin. \circ — \circ , pyrogen content, $^{\circ}\text{C}/\text{ml}$; \square — \square , ^{14}C cpm.

vated by phagocytosis or tuberculin synthesized pyrogen. Leukocytes were activated by phagocytosis in the presence of tritium-labeled amino acids, and leukocytes from sensitized rabbits were activated with tuberculin in the presence of ^{14}C -labeled amino acids. Both leukocytes stimulated with tuberculin and leukocytes stimulated by phagocytosis secreted about three times as much pyrogen as unstimulated cells. The phagocytic pyrogen contained about 6,000 $^{\circ}\text{C}$ units of pyrogen and 1.5×10^7 tritium cpm. The PPD pyrogen contained 2,400 $^{\circ}\text{C}$ units of pyrogen and 2.89×10^7 carbon cpm.

The two types of pyrogen were then pooled and purified together. At all stages of purification there was a definite peak of ^{14}C and tritium radioactivity that aligned with the pyrogen. The purified pyrogen was subjected to electrophoresis at pH 3.5 and at pH 9 and to isoelectric focusing in acrylamide gel. In all three systems of biological activity, ^{14}C and tritium coincided, and a single protein band was seen on the stained gels that contained all the radioactivity (Fig. 6).

Finally, it was demonstrated that blood leukocytes synthesized pyrogen when stimulated with endotoxin. 400 ml of crude pyrogen was prepared from blood cells activated by endotoxin in the presence of ^{14}C -labeled amino acids. The pool contained about 1,250 $^{\circ}\text{C}$ units of pyrogen and 1.58×10^7 protein cpm. It was purified as usual; the pyrogen peak in the effluent from the IEF column

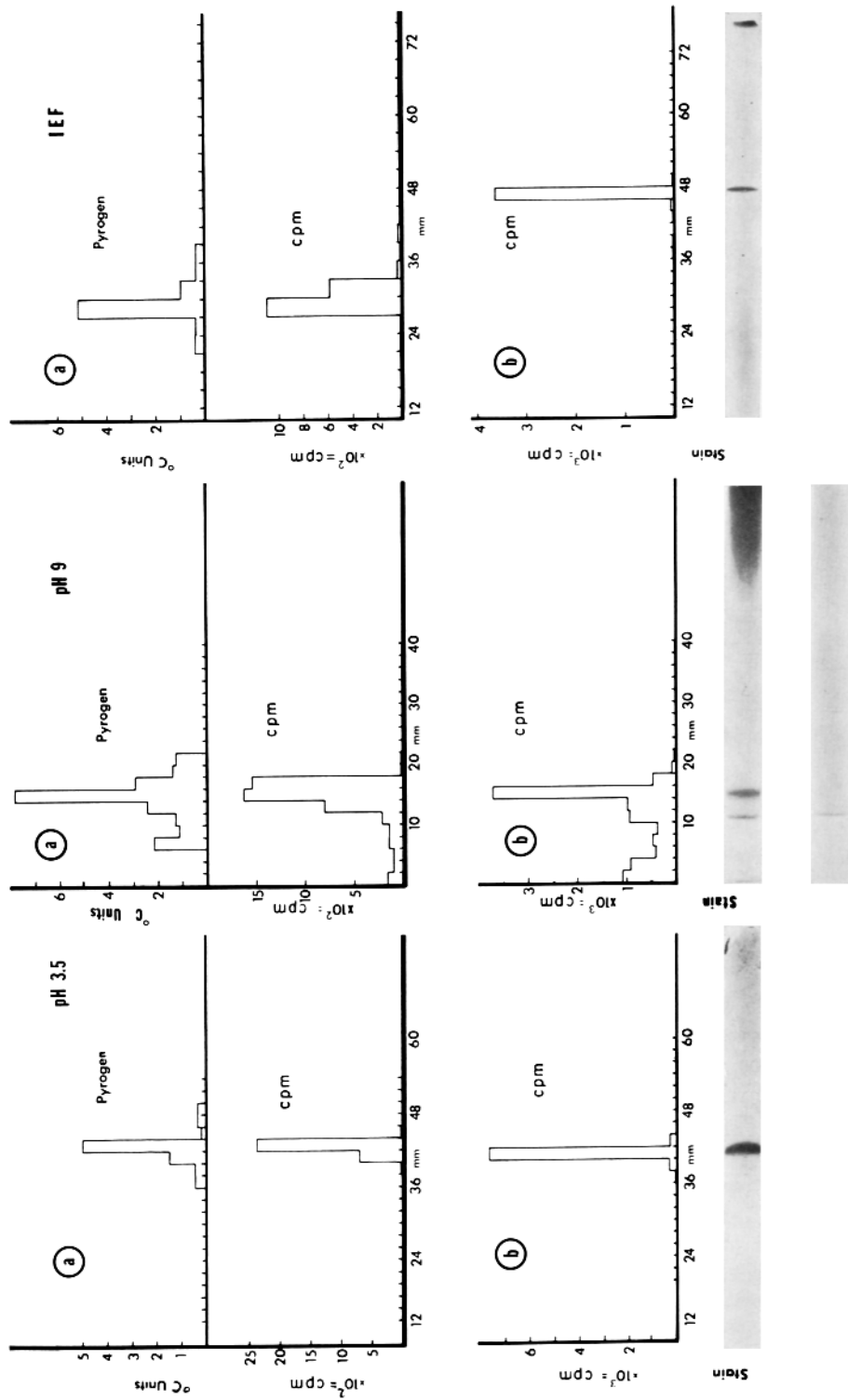


FIG. 5. (a) Correlation of pyrogenic activity and radioactivity of ¹⁴C-labeled pyrogen from exudate cells stimulated with endotoxin. Acrylamide gel electrophoresis at pH 3.5 and at pH 9, and isoelectric focusing in acrylamide gel. (b) Correlation of stained band and radioactivity for the same pyrogen in analytical gels.

TABLE I
Correlation of Radioactivity and Pyrogen Content for the Second Batch of ¹⁴Carbon-Labeled Pyrogen from Exudate Cells

Fraction no.	¹⁴ C	Pyrogen concentration	Ratio of cpm to pyrogen concentration
	<i>cpm</i>	<i>°C/ml</i>	
14	33	15.5	2.13
15	150	68	2.21
16	565	340	1.66
17	2,306	1,140	2.02
18	2,488	1,320	1.88
19	975	550	1.77
20	400	190	2.11
21	167	94	1.78

The pyrogen concentrations were derived graphically from the febrile responses (7).

contained 3.25×10^4 cpm, or 0.16% of the protein counts in the crude material. Since the overall pyrogen yield was 25%, only about 0.6% of the original protein counts were associated with pyrogen. Electrophoresis at pH 9 and at pH 3.5 and isoelectric focusing in acrylamide gel showed clearly that the pyrogen was not pure; however, pyrogen was the only radioactive protein present (Fig. 7).

It was of great interest to determine whether the pyrogens produced by blood leukocytes and exudate leukocytes were the same. A small batch of tritium-labeled pyrogen was prepared from exudate cells. It was purified up to and including the ion exchange chromatography stage, at which point it was clear that pyrogen was the only radioactive protein present. The tritiated exudate pyrogen was then mixed with the ¹⁴C-labeled blood leukocyte pyrogen and subjected to electrophoresis and isoelectric focusing in acrylamide gel. There was complete coincidence of ¹⁴C and tritium in all three systems (Fig. 8).

DISCUSSION

The results of these experiments seem to be unequivocal. We have three times isolated radioactive leukocyte pyrogen from peritoneal exudate cells stimulated with endotoxin, once each from exudate cells stimulated with phagocytosis and tuberculin, and once from blood cells stimulated with endotoxin. On all six occasions, pyrogenic activity, radioactivity, and stained protein band coincided in three different analytical gel systems. The conclusion that the leukocytes used the radiolabeled amino acids supplied to synthesize leukocyte pyrogen seems inescapable.

The results with these radioactive pyrogens confirm and extend the data about the purification of unlabeled radioactive pyrogen (7). With radioactive pyrogen, there was no doubt about the coincidence of stained band and radio-

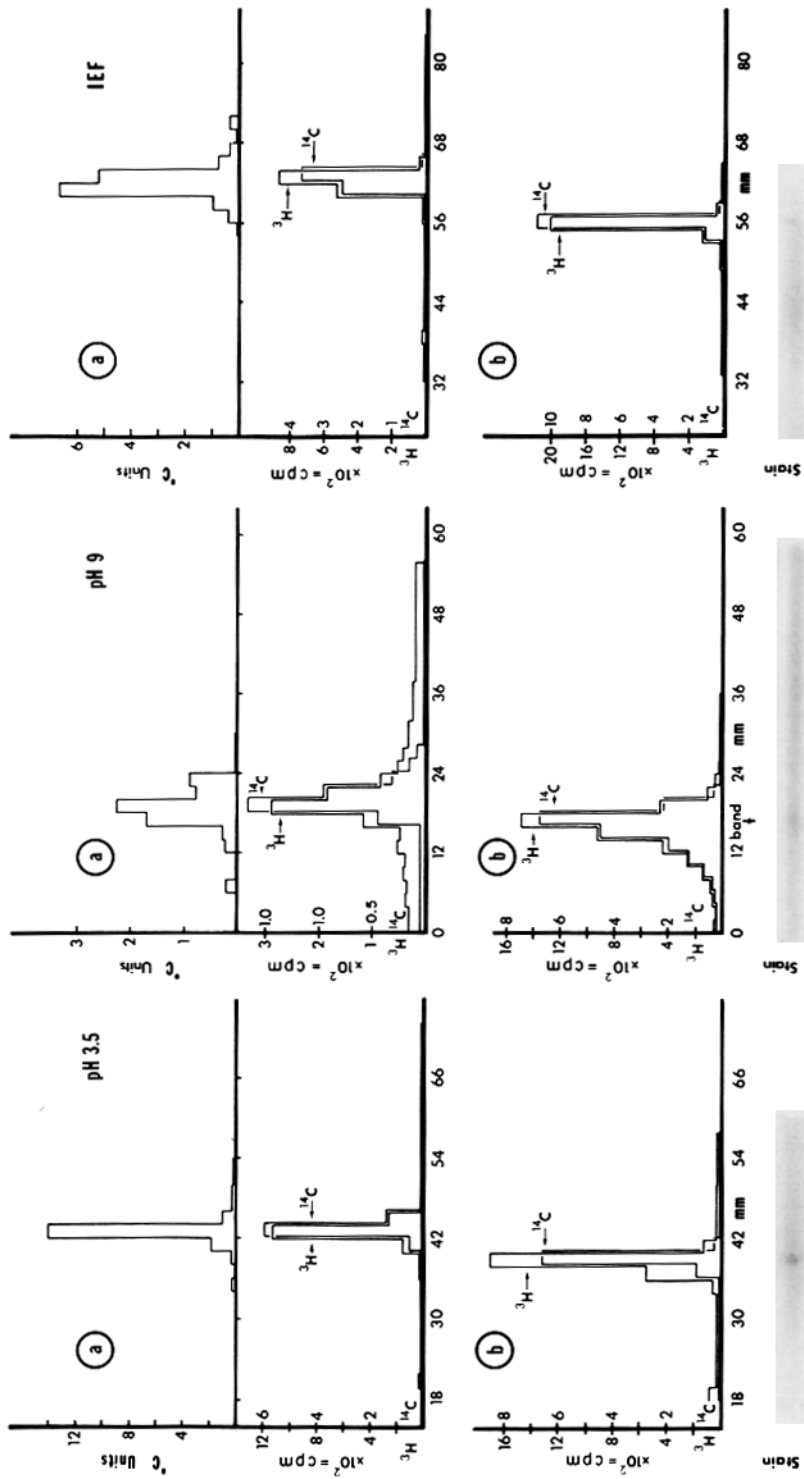


FIG. 6. (a) Correlation of pyrogenic activity and radioactivity for purified mixture of ^{14}C -labeled pyrogen from exudate cells stimulated with tuberculin and tritium-labeled pyrogen from exudate cells stimulated by phagocytosis. Acrylamide gel electrophoresis at pH 3.5 and pH 9, and isoelectric focusing in acrylamide gel. The graphs of ^{14}C and tritium counts have been displaced slightly for clarity. (b) Correlation of stained band and radioactivity for the same pyrogen in analytical gels.

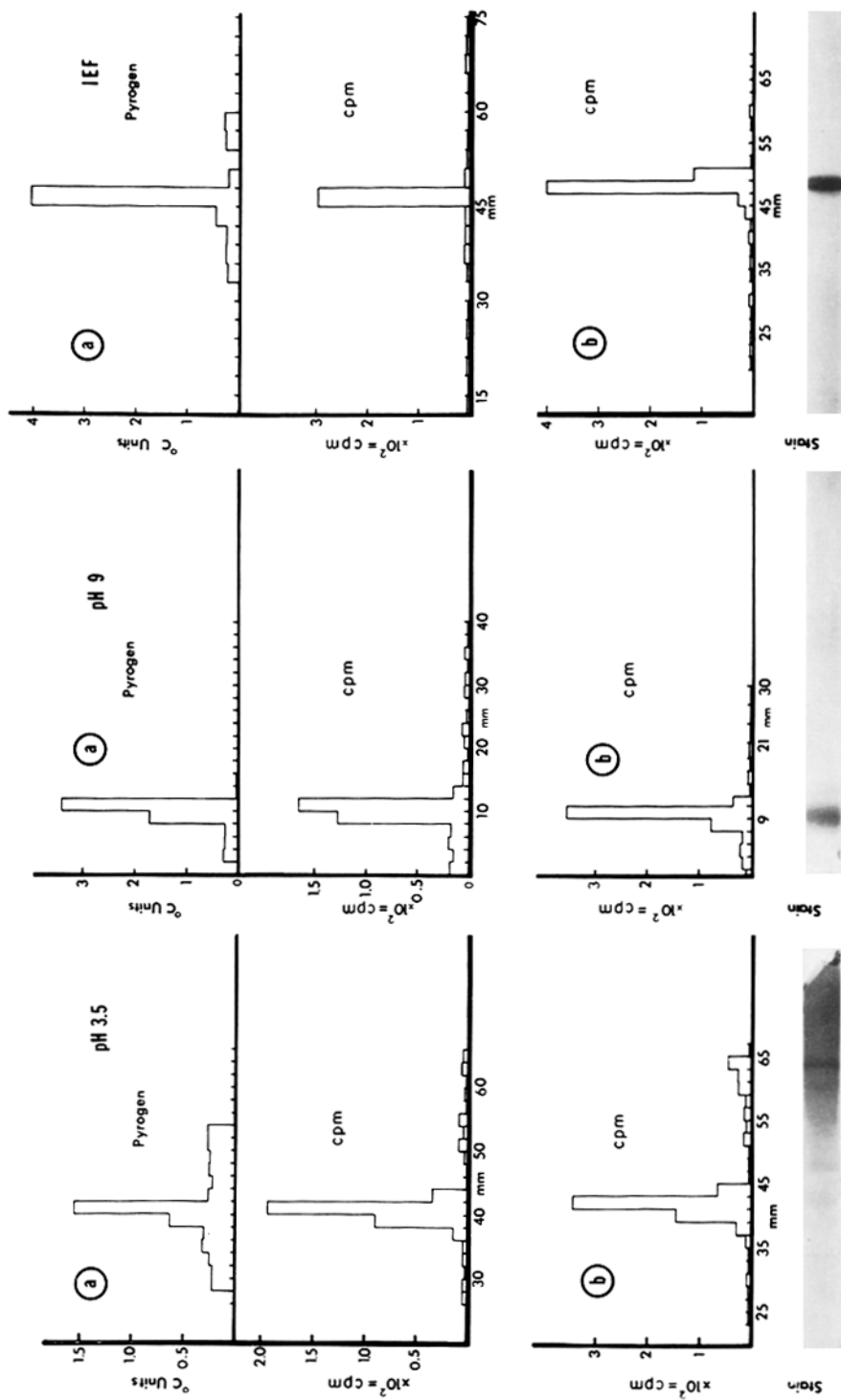


FIG. 7. (a) Correlation of pyrogenic activity and radioactivity of purified ^{14}C -labeled pyrogen from blood cells stimulated with endotoxin. Acrylamide gel electrophoresis at pH 3.5 and pH 9, and isoelectric focusing in acrylamide gel. (b) Correlation of stained band and radioactivity for the same pyrogen in analytical gels.

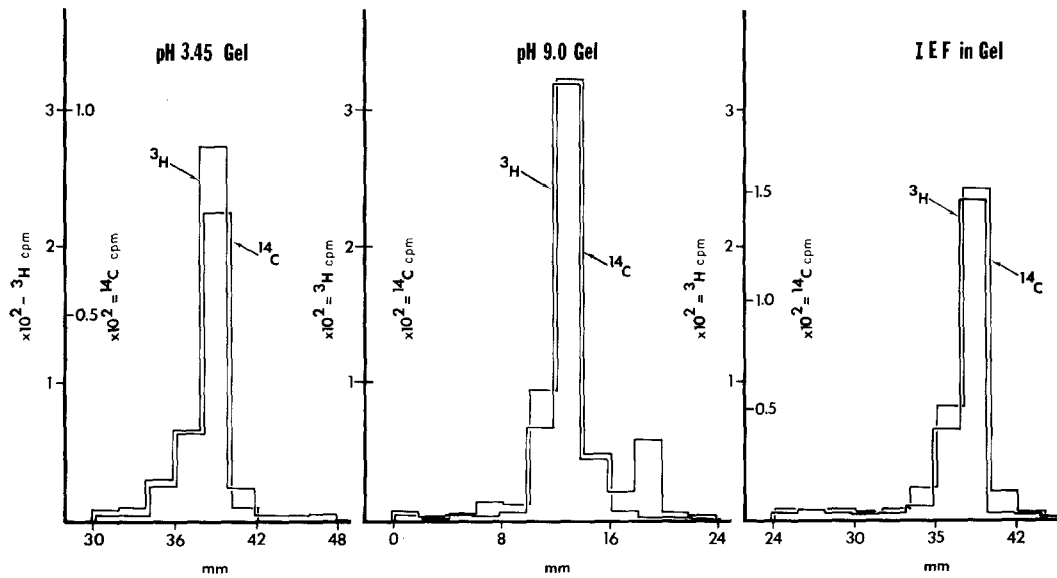


FIG. 8. Electrophoresis and isoelectric focusing in acrylamide gel of mixed purified ^{14}C -labeled pyrogen from blood cells stimulated with endotoxin and purified tritium-labeled pyrogen from exudate cells stimulated with endotoxin. The graphs of ^{14}C and tritium counts have been displaced slightly for clarity.

activity, since the band could be cut out and counted. Similarly, there was no doubt about the coincidence of pyrogenic activity and radioactivity since these were measured in the same gel slices. It was therefore possible to say with assurance that the visible bands on the various gels contained the pyrogenic activity. The results also confirmed that the purity of the final product is greatly affected by the specific activity of the starting material. The pyrogens isolated from exudate cells seem to have been rendered homogeneous by a four-stage purification process. The final blood leukocyte pyrogen peak was clearly impure, which reflected the fact that its starting specific activity was about 10 times less than that of pyrogen from exudate cells.

Several considerations seem to explain our success in obtaining radiolabeled pyrogen. Exudate cells were available in large quantity, with relatively little contamination by other cell types, and took up, incorporated into protein, and secreted into the medium fully 5% of the counts supplied. About 6% of this was radioactive pyrogen with a specific radioactivity of 1,550 cpm/ $^{\circ}\text{C}$ unit. Previous investigators used blood leukocytes; these were difficult to obtain in quantity, contained less than 50% polymorphs, and secreted into the medium as protein only 1.4% of the counts supplied. Only 0.6% of this was pyrogen, with a specific radioactivity of 108 cpm/ $^{\circ}\text{C}$ unit. Many of the radioactive proteins found in supernates of blood cells were not present in supernates of exudate cells, and

presumably were made by lymphocytes and monocytes. Thus the small quantities of radioactive pyrogen secreted by blood leukocytes were obscured by other proteins until the final stage of purification.

The evidence that pyrogen is synthesized *de novo* from amino acid precursors is difficult to reconcile with the idea that it is somehow derived from bacterial endotoxin (8). Leukocytes made the same pyrogen whatever the stimulus, and it seems unwarranted to presume that they broke down endotoxin to a biologically active fragment and also derived the same fragment from tuberculin and staphylococci.

The polymorph is an unlikely candidate for protein synthesis because it contains very few ribosomes and little endoplasmic reticulum (9). There is, in fact, no direct evidence that the neutrophil polymorph is the cell that synthesizes leukocyte pyrogen. The only available evidence is that pyrogen yields and neutrophil counts show a strong positive correlation (10). It would be surprising if the polymorph had nothing to do with leukocyte pyrogen formation; but it will be possible to prove the association conclusively only when pure populations of cells are available (which seems highly improbable) or when pyrogen can be identified in individual cells.

SUMMARY

Rabbit leukocytes from peritoneal exudates and from blood were stimulated to form leukocyte pyrogen in the presence of radiolabeled amino acids. The stimuli used were endotoxin, phagocytosis, and tuberculin. The crude leukocyte pyrogen samples were purified; pyrogen from exudate cells was rendered homogeneous; pyrogen from blood cells was still contaminated with other proteins. All the purified pyrogens were radioactive; and for all it was shown that radioactivity and pyrogenic activity coincided on electrophoresis at pH 3.5 and pH 9 in acrylamide and on isoelectric focusing in acrylamide. Furthermore, pyrogens obtained from exudate cells stimulated in different ways, or from blood cells and exudate cells stimulated with endotoxin, appeared to be identical. These results suggest that leukocyte pyrogen was synthesized *de novo* from amino acid precursors and that leukocytes made the same pyrogen whatever the stimulus used to activate them.

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