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COMPARISON OF ENDOGENOUS PYROGENS FROM HUMAN AND RABBIT LEUCOCYTES UTILIZING SEPHADEX FILTRATION‡

A fever-producing substance derived from rabbit exudate leucocytes was first described in 1953 by Bennett and Beeson.[1] The subsequent demonstration of pyrogens with similar biologic properties derived from other variously stimulated leucocytes[2-5] or present in the blood of febrile rabbits[6,7] has led to the concept of a single pyrogenic substance, termed endogenous pyrogen. Most investigations of the nature of this material have been carried out with leucocyte pyrogen released from rabbit exudate polymorphonuclear leucocytes incubated in saline, and assayed for pyrogenic activity by injection into rabbits. These studies[8,9] have demonstrated that endogenous pyrogen is moderately heat-stable, precipitated by perchloric acid, not extractable in butanol, and destroyed by treatment with trypsin or pepsin. It is believed to be a small protein, with a molecular weight of 10,000 to 20,000, and with perhaps a lipid moiety.[10,11]

Recently a number of different types of cells have been found to release pyrogenic material following appropriate stimulation. From rabbits, such cells include blood leucocytes,[3,4] lung macrophages,[5] exudate monocytes,[12] cells from lymph node, spleen,[5] and liver Kupffer cells;[14] and from man, circulating polymorphonuclear leucocytes[12] and monocytes.[15,16] Initial studies on the pyrogenic material released from human blood leucocytes after phagocytosis indicated that pyrogenic activity was destroyed by incubation with trypsin.[13] Pyrogen derived from human cells produces fever in rabbits,[13] and recently biologic cross-reactivity has also been reported for pyrogens from several animal species.[17] It seemed of interest, therefore, to compare the molecular weight of pyrogens from human and rabbit cells, as well as from polymorphonuclear leucocytes and mononuclear cell types, by the technique of sephadex filtration.

MATERIALS AND METHODS

Leucocytes

Methods for preparation of leucocytes were as described previously.[16] All materials, glassware and reagents used for leucocyte preparation and incubation were sterile

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and pyrogen-free. Briefly, to obtain blood leucocytes, a three per cent dextran solution was added to heparinized human or rabbit blood. 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. The method for obtaining human blood monocytes was a modification of the method of Bennett and Cohn,[15] as described previously.[16] Rabbit lung macrophages were obtained as described previously.[5]

All cell suspensions were incubated in 15 per cent serum-KRP buffer with heat-killed staphylococci for 1½ hours, on a Dubnoff shaker. Autologous serum was used for human cells, and normal rabbit serum for rabbit cells. Bacteria were prepared as described previously,[13] except that autoclaving for 15 minutes at 120° C. was substituted for heating at 100° C. After an initial incubation, the cells were recovered by centrifugation at 300 *g* for 15 minutes, and resuspended in KRP buffer with 150 mg. per 100 ml. glucose and 6,000 units of penicillin. Incubation was then continued in a stationary incubator for about 16 hours. The flask contents were then centrifuged at 2,000 *g* for 30 minutes, and the supernatant stored at 4° C. for intervals up to several weeks. All supernatants were cultured in thioglycollate broth to confirm sterility. Contaminated supernatants were discarded.

Rabbit exudate pyrogen was obtained using the method of Kozak, *et al.*[11] for preparing "crude pyrogen w," with the exception that exudates were collected from live rabbits.

Preparation of pyrogen samples

Material from several incubations was pooled, tested for pyrogenic activity, and lyophilized. Pyrogen testing was carried out by intravenous injection of samples into rabbits, as described previously.[8] Each preparation was then suspended in a small volume of sterile distilled water, and dialyzed for 24 hours against at least 100 volumes of phosphate-buffered saline, pH 6.0 or 6.5. In one experiment, tris buffer, 0.1M, pH 9.2 was used for dialysis. Before use, it was filtered, cultured, and proven to be nonpyrogenic. A small aliquot was taken after dialysis to test for pyrogen and for culture.

Sephadex columns

Sephadex G-75 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) was allowed to swell for 24 hours, and then autoclaved for 20 minutes at 120° C. Glass columns, including teflon stopcock and sintered glass filter, were fully assembled and autoclaved for 1½ hours. The sephadex column was poured and kept at 4° C. It was equilibrated with phosphate-buffered saline, prepared by addition of .05 M KH_2PO_4 to saline to a final pH 6.5 or, in the last experiment, pH 6.0. A sample of effluent was obtained before each experiment, tested for sterility and pyrogenicity. Initially, a diluted sample of normal rabbit serum was added to the column, and the effluent determined to be nonpyrogenic. A sample of rabbit blood leucocyte pyrogen* was placed on the column, and testing of the effluent revealed good recovery of pyrogenic activity. After the column was washed with buffered saline, samples were added in total volumes of 3 to 6 ml. Flow rate was adjusted to 0.5 to 1.0 ml./minute, except as noted below. Fractions were collected with a Gilson fraction collector in sterile, pyrogen-free tubes, and transferred as soon as possible to other sterile tubes. All

* Although leucocyte products released during incubation obviously include many compounds, the preparations used in this study are simply termed pyrogen.

samples were then cultured. In only one case were positive cultures obtained. Six tubes collected from a column after addition of human monocyte pyrogen contained small numbers of gram-positive cocci. These were cultured from four tubes that contained nonpyrogenic samples, and two that contained pyrogenic materials. Since the two febrile responses resembled those produced by endogenous pyrogen and not bacteria,[19] these results have been included.

In the first experiment, a sephadex column 2.5 × 36 cm. was used; 3.8 ml. fractions were collected, and three tubes combined for assay. In the second experiment, the column was 2.1 × 68 cm., 3.8 ml. fractions were collected, and two tubes were combined for assay. In both experiments, buffer was pH 6.5. In the third experiment, a column 1.3 × 30 cm. was used, with a flow rate of about 0.2 ml. per minute. The buffered saline was pH 6.0. Samples of 2.2 ml. were collected and usually not combined, except for some samples in the first half of the experiment.

Protein determinations were carried out by the method of Lowry.[20] Samples of 0.1 to 1.0 ml. effluent were used for assay. Bovine serum albumin was used as the standard, and optical density measured using a Klett-Summerson colorimeter. Fifteen milligrams of bovine ribonuclease (Worthington Biochemical Corps., Freehold, N.J.) were dissolved in 2 ml. of buffered saline. One ml. samples of effluent were analyzed for protein.

RESULTS

In the first experiment, leucocyte products containing pyrogen were prepared from four different cell sources. These included human blood leucocytes (4.3×10^8), human blood mononuclear leucocytes (4.5×10^7), rabbit blood leucocytes (5.3×10^8), and rabbit lung macrophages (1×10^9). Each pyrogenic sample was placed in turn on a sephadex column (see METHODS) and eluted with buffered saline, pH 6.5. Three successive effluent fractions totaling 11 ml. were tested for pyrogenic activity in rabbits. Since this was a preliminary experiment, one rabbit was injected with each 11 ml. sample. The same rabbit received all the samples from any one column, except the first three samples, which were nonpyrogenic and were tested later. In the case of rabbit blood leucocyte pyrogen, the first six samples were tested in one rabbit, and the other samples in another recipient.

The results are shown in Figures 1a and 1b. Although the separation of pyrogenic activity was crude in this preliminary experiment, pyrogen from all four cell types appeared to behave similarly. Peak pyrogenic activity appeared at an effluent volume of about 100 ml. In several of the preparations, especially the rabbit blood leucocyte sample, an early peak of pyrogenic activity also seemed to be present at a volume of about 50 to 60 ml. of effluent.

In the next experiment, a more accurate technique was employed. Pyrogen-containing material was obtained from three cell sources. These included human blood leucocytes (2×10^9), rabbit blood leucocytes

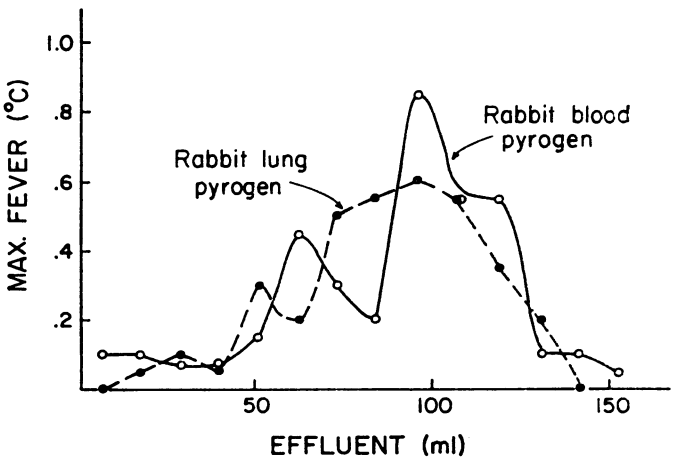

FIGURE 1a

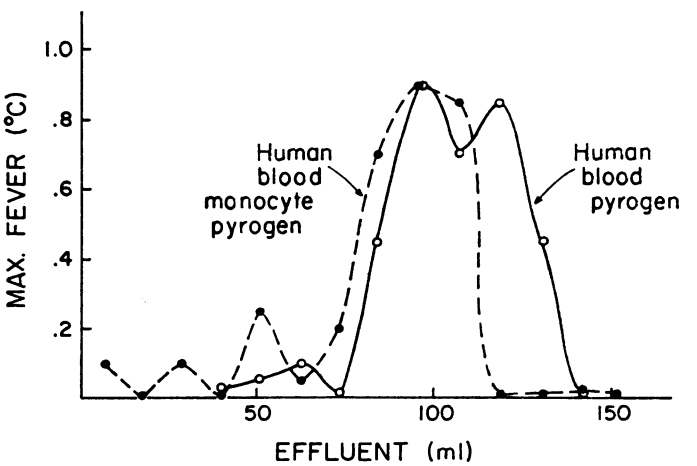

FIGURE 1b

FIG. 1. Maximum febrile responses in a rabbit to injection of 11 ml. samples of effluent from a sephadex column. Samples of four different leucocyte pyrogens were eluted, as labeled. All effluent samples from each material were injected into the same rabbit, except as noted in the text.

(1.8×10^9), and rabbit lung macrophages (4.3×10^9). Human blood mononuclear leucocytes were not included. A larger pool of pyrogenic material was obtained for this experiment, and two or three rabbits were used to assay pyrogenic activity from each column. Fractions of 7.5 ml. each were collected, and 2 ml. were injected for each pyrogen assay, except in the case of effluent from rabbit lung pyrogen, in which 1 ml. was used. Aliquots of all fractions from each column were tested in the same rabbits.

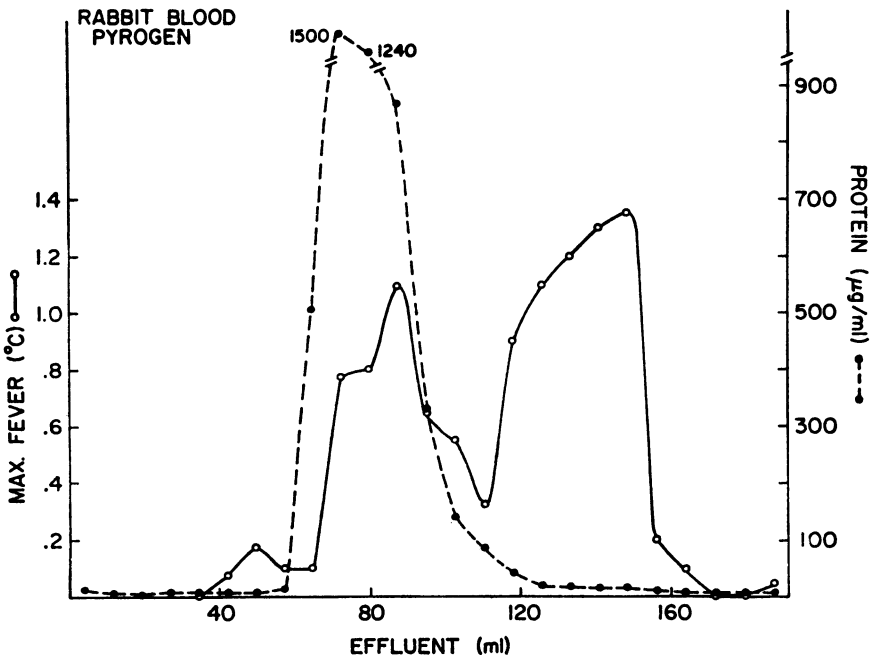

FIGURE 2a

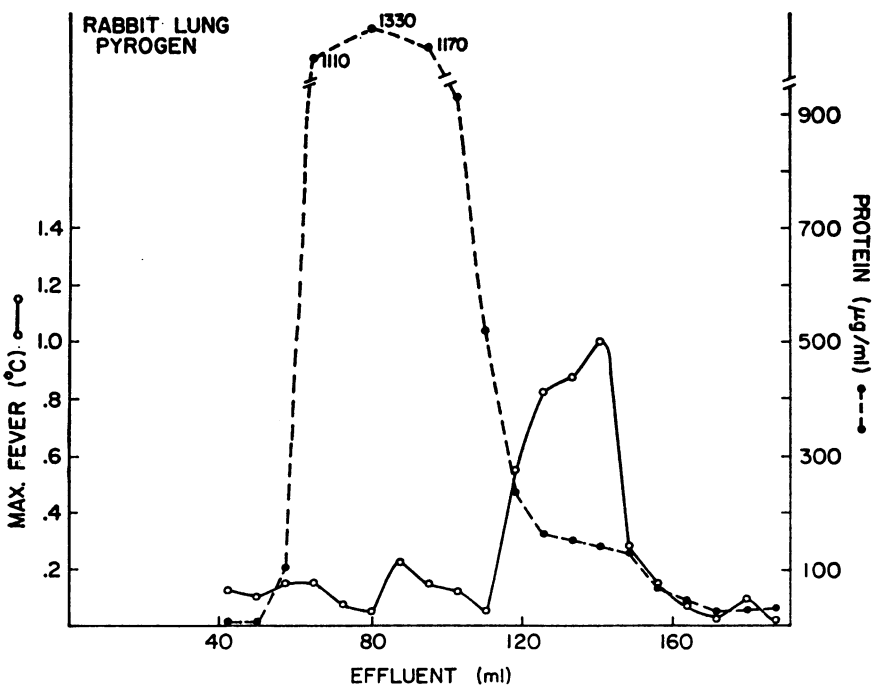

FIGURE 2b

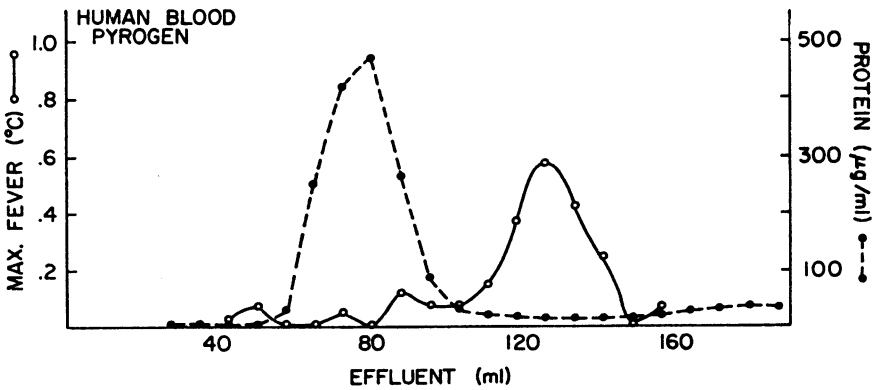

FIGURE 2c

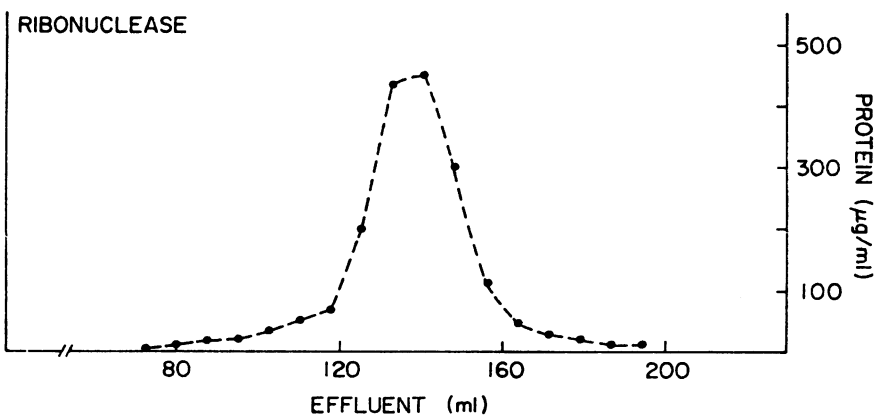

FIGURE 2d

FIG. 2. (a - c) Left ordinate, average maximum febrile responses in two rabbits to 1 or 2 ml. successive samples of effluent from a sephadex column; right ordinate, total protein of these same samples. (d) Total protein determinations of effluent from the same sephadex column to which 15 mg. of ribonuclease were added.

Total protein determinations were carried out on all fractions. After the three pyrogens had been eluted, 15 mg. of ribonuclease was placed on the column, and effluent collected for determination of total protein.

The results of the second experiment are shown in Fig. 2, a - d. With this column, the peak fever-inducing activity of all three pyrogens appeared at an effluent volume of 130 to 140 ml. Ribonuclease protein appeared at the same effluent volume. In addition, an early peak of pyrogenic activity, which had been thought to be present in the first experiment, was clearly evident with the rabbit blood pyrogen, with maximal activity appearing

at an effluent volume of about 90 ml. A similar early peak appeared to be present in similar fractions from the two other pyrogens although only in minimal amounts.

The volume of effluent chosen for pyrogen testing of the rabbit blood pyrogen proved to exceed the sensitive range of the assay[21] at the peak of pyrogenic activity, so that it was not possible to determine accurately the relative pyrogenic activity of the early and late peaks, although there was clearly more in the late peak. Subsequent testing with one-third the amount of material was carried out in two other rabbits. This showed that there was about half as much activity in the first peak as in the second (average maximum fever 0.38° C. compared to 0.67° C.).

Total protein determinations of all effluent fractions from each pyrogen showed that the largest amount of protein appeared at about 80 ml. of effluent (Fig. 2, a - c). With all pyrogens, very little protein was present in the fractions containing the most pyrogenic activity. This was most clearly evident with rabbit blood leucocyte pyrogen, in which about 10 μg. of protein appeared to contain two to three pyrogenic doses.*

Kozak, *et al.*[11] have reported that pyrogen from rabbit exudate leucocytes is inactivated at alkaline pH, but that activity can be restored by treatment with mercaptoethanol or dithiothreitol (DTT). Attempts were made to repeat these observations with a preparation of human blood leucocyte pyrogen.

A supply of 5.5×10^8 human blood leucocytes was used to prepare a pool of pyrogen. Initial testing revealed over 50 doses of pyrogen. After lyophilization, the pyrogen was dissolved in a small volume of water, and a sample divided into three aliquots. The first was dialyzed at 4° C. against phosphate-buffered saline pH 6.0, the second and third against tris buffer pH 9.2. The third sample was then dialyzed for an additional 24 hours against phosphate-buffered saline pH 6.0 with .01M DTT added. After 24 hours of dialysis, each aliquot was tested for pyrogenicity. Samples at high pH were diluted with saline before injection. All three aliquots were found to be equally pyrogenic. The failure of dialysis at alkaline pH to inactivate pyrogen from human blood leucocytes was confirmed in two other experiments, one of which is shown in Figure 3, a. In this experiment dialysis at pH 9.2 was carried out for 48 hours. When a sample of lyophilized rabbit exudate pyrogen was used, however, the results reported by Kozak, *et al.*[11] were confirmed (see Fig. 3, b).

Aliquots of human leucocyte pyrogen, dialyzed either at pH 6.0 or 9.2, and containing equal pyrogenic activity, were then placed on a small sephadex column (see METHODS, third column), and effluent collected and

* One pyrogenic dose is an amount which causes a fever of about 0.7°C. in a rabbit.

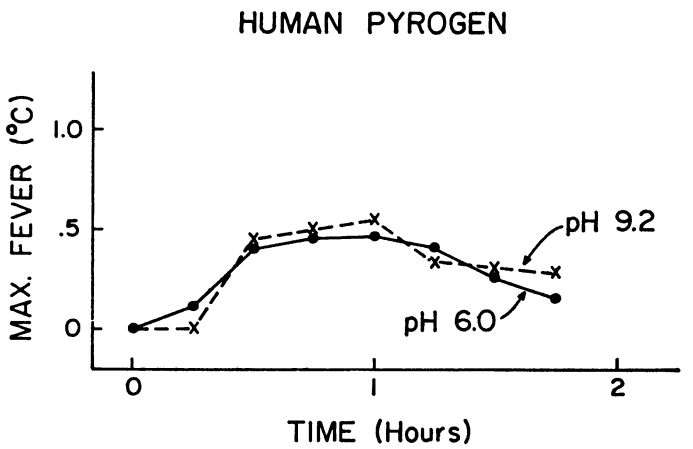

FIGURE 3a

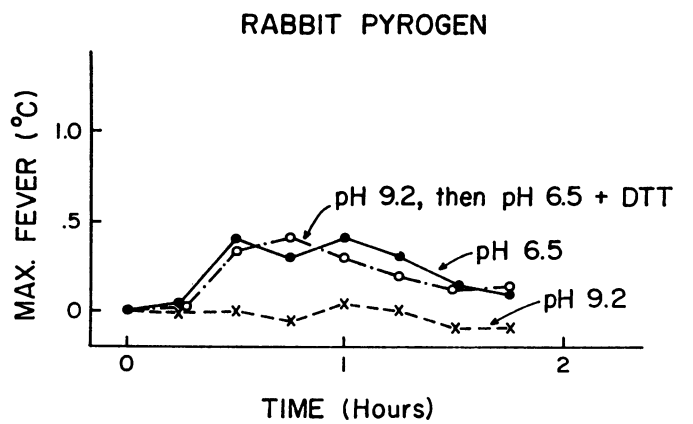

FIGURE 3b

FIG. 3. Febrile responses of one rabbit to (a) two samples of lyophilized human blood leucocyte pyrogen dialyzed at pH 6.0 or 9.2; (b) three samples of rabbit exudate pyrogen, dialyzed at pH 6.5, pH 9.2, or pH 9.2 followed by pH 6.5 with .01M dithiothreitol (DTT).

analyzed for pyrogen and protein. In contrast to fractions from the sample dialyzed at pH 6.0, effluent fractions collected from the sample which had been previously dialyzed against pH 9.2 contained almost no pyrogenic activity (see Fig. 4, a and b). In addition, a peak of protein concentration, occurring beyond the only fraction with pyrogenic activity, suggested the presence of smaller molecular weight proteins not present in the other sample. These were considered to be possible breakdown products of the

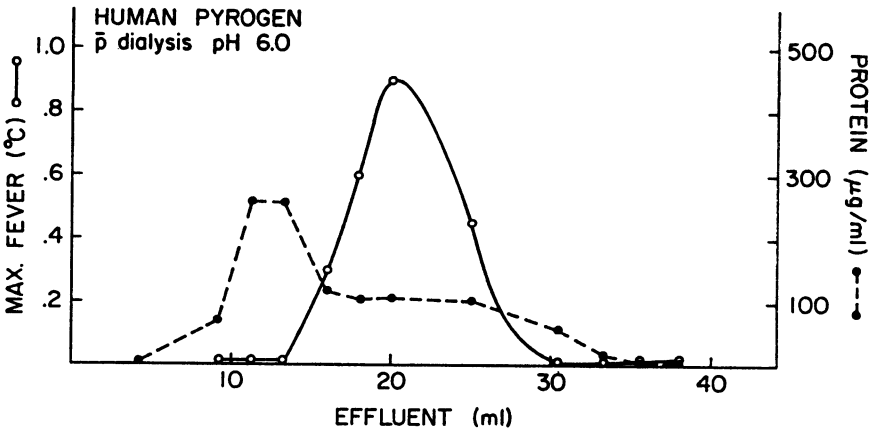

FIGURE 4a

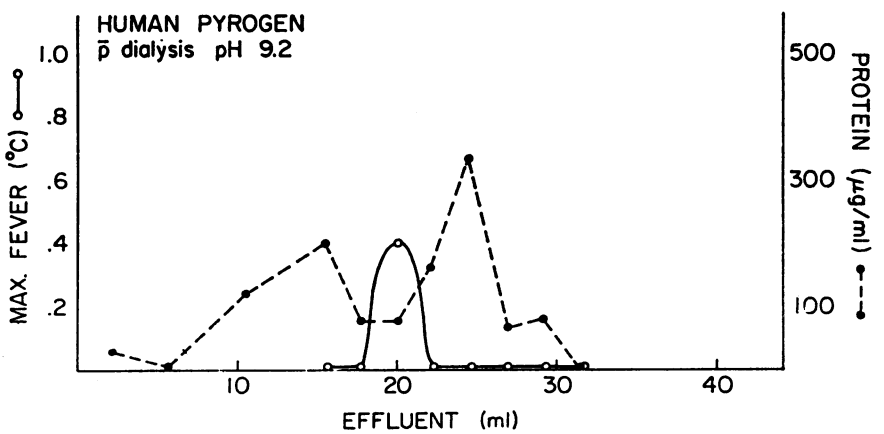

FIGURE 4b

FIG. 4. Febrile responses of single rabbits to 1 ml. aliquots of successive effluent samples from a sephadex column to which was added human blood leucocyte pyrogen after dialysis at pH 6.0 or 9.2. All effluent samples from each material were injected into the same rabbit, except for some early and late samples from pyrogen dialyzed at pH 6.0.

pyrogen molecule. Attempts to reconstitute these presumed fragments by dialysis with DTT, however, were unsuccessful, both in this and a similar experiment.

DISCUSSION

Previous reports have indicated that samples of endogenous pyrogen obtained from various species and cell types, and elicited by various stimuli,

exhibit similar biologic activity.[2-5,12-17] For example, all endogenous pyrogens cause fever with an early time of onset (15 to 30 minutes in the rabbit), with prompt defervescence. In addition, pyrogens from man, cat, and rabbit all cause similar fevers when injected into rabbits.[12,17] The chemical nature of the pyrogen also appears to be similar regardless of species, cell source, or the particular stimulus applied. For example, endogenous pyrogens derived from both rabbit exudate leucocytes and human blood leucocytes are inactivated by heating above 56° C. for 30 minutes,[8,22] and by incubation with trypsin,[8,13,16] pepsin,[8] or pronase.[16]

The studies reported here on the molecular weights of pyrogens appear to confirm the similar nature of a number of endogenous pyrogens. In combination with previous reports on the behavior of rabbit exudate endogenous pyrogen on sephadex,[10,11] our results indicate that products released from several different cell types derived from two different species contain a pyrogenic material with a molecular weight approximating that of ribonuclease, 13,700. Thus polymorphonuclear leucocytes and mononuclear cells from both humans and rabbits apparently released similar pyrogens.

The pyrogen studied here from blood leucocytes after stimulation by phagocytosis is probably derived in large part from blood polymorphonuclear leucocytes. Previous studies have shown that human blood monocytes may release two to three times as much pyrogen as do polymorphonuclear leucocytes after a phagocytic stimulus.[15] However, monocytes probably contribute less than one-fourth of the endogenous pyrogen derived from blood leucocytes since sample differentials have indicated that they usually represent 10 per cent or less of these cells.

In addition to the major pyrogenic activity, which appeared at a molecular weight of 10,000 to 20,000, some activity was present in fractions containing larger molecular weight compounds. This early peak of pyrogenic activity was especially prominent in the pyrogen derived from rabbit blood leucocytes. Other studies, now in progress, suggest that this early peak appears at a molecular weight of about 60,000. This material may represent adsorption of compounds with smaller molecular weight on larger proteins. However, if this were the case, there should be a correlation between large amounts of "early" pyrogen and large amounts of high molecular weight proteins. This association was not always observed, cf., Figure 2, a and b. In addition, elution of a sample of rabbit blood pyrogen with a buffer of high ionic strength (OSM) did not remove the early peak.[22] Alternatively, this early peak might represent an entirely different pyrogen, or an intermediate form of pyrogen between a relatively large inactive precursor and a smaller, highly active molecule.

Previous reports on purification of endogenous pyrogen from rabbit exudate leucocytes have indicated that after butanol-methanol treatment 5 μg. of protein may contain significant pyrogenic activity.[11] Similar purification appears to be possible with this more simplified technique. Apparently, only small amounts of proteins with low molecular weight are normally released from cells during incubation in buffer.

Some apparent differences were noted in the susceptibility of human and rabbit endogenous pyrogen to alkaline pH. Thus, although rabbit exudate endogenous pyrogen is unstable in alkaline solution, presumably due to oxidation of —SH groupings,[11] similar findings were not observed with human endogenous pyrogen. In addition to a possible species difference, the degree of purification and composition of the surrounding media may alter the stability of these proteins. Human endogenous pyrogen frequently appeared to lose activity during processing, whereas this was less common with rabbit pyrogen. Activity could not be restored by treatment with dithiothreitol.

SUMMARY

Endogenous pyrogens were prepared from four different cell sources by incubation of leucocytes with heat-killed staphylococci in KRP buffer for 18 hours. The pyrogenic supernatants were compared using sephadex filtration. Pyrogens from human and rabbit blood leucocytes, human mononuclear leucocytes, and rabbit lung macrophages were studied.

The major pyrogenic fraction from each of the four cell sources was recovered at the same elution volume as that of ribonuclease, (MW 13,700). Another pyrogenic fraction was also observed in some samples, especially those derived from rabbit blood leucocytes. This pyrogen was of larger molecular weight, approximately 60,000, and did not appear to represent adsorption of pyrogen to proteins.

Unlike rabbit exudate leucocyte pyrogen, human blood leucocyte pyrogen was not inactivated by dialysis at pH 9.2. However, samples previously exposed to alkaline pH lost activity after sephadex filtration.

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