DEMONSTRATION AND CHARACTERIZATION OF TWO DISTINCT HUMAN LEUKOCYTIC PYROGENS

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It is now widely accepted that the interaction of exogenous pyrogens, such as bacteria or bacterial products, with host phagocytic cells, activates these cells to release a protein which stimulates the hypothalamus and results in fever (1). This protein is called endogenous or leukocytic pyrogen and is considered to be the mediator of a wide variety of clinical and experimental fevers. Although it has not been convincingly demonstrated in vivo in human beings, leukocytic pyrogen can readily be produced in vitro from human blood leukocytes (2, 3). It is known that, in addition to being a protein, this pyrogen has a mol wt of approximately 13,000 (4). Both human and rabbit leukocytic pyrogen will result in febrile responses in rabbits and have similar characteristics. Considerably more is known about the molecular nature of rabbit leukocytic pyrogen (5–7) than about the human material (4, 8, 9).

During the course of purifying and characterizing human leukocytic pyrogen we observed that human blood leukocytes produce two distinct molecules with pyrogenic properties. One of these pyrogens is derived from peripheral blood monocytes and the other from neutrophils. The present report describes some of the biological and chemical properties of the two pyrogens.

Materials and Methods

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

Pyrogen Assay.—New Zealand albino rabbits of either sex from the National Institutes of Health colony and weighing 2–3 kg were used throughout. The details of housing, feeding, training, and temperature recording were as previously reported (3, 10). A rise of less than 0.3°C was not considered significant. Temperature data presented are derived from mean responses. Rabbits were occasionally injected twice in a day (morning and afternoon). All injections were given intravenously into a lateral ear vein.

Leukocytes.—Buffy coat concentrates from 450 ml of fresh ACD (Transfer Pack, Fenwall Laboratories, Monroe Park, Ill.) whole human blood were obtained after centrifugation at 1,500 *g* for 3 min. The plasma was removed and discarded. The upper 50–75 ml of red cells were either sedimented in 3% dextran (mol wt 200,000) (Sigma Chemical Co., St. Louis, Mo.) in physiologic saline or separated on a Ficoll-Hypaque (Ficoll, Pharmacia Fine Chemicals, Inc., Uppsala, Sweden; Hypaque, Winthrop Laboratories, New York) gradient (11) in 50-ml conical centrifuge tubes. Another source of leukocyte preparations was blood remaining in the

pump oxygenator apparatus following cardiopulmonary by-pass operations. Such blood was sedimented in 4.5% dextran and the red blood cells were removed by hypotonic lysis. Total white blood cell counts were done in an electronic particle counter (Coulter Electronics, Hialeah, Fla.). Differential counts (300 cells) were done on Wright's stained smears on slides prepared in a cytocentrifuge (Shandon Scientific, Sewicley, Pa.).

Preparation of Leukocytic Pyrogen.—20 million total white blood cells were suspended per ml of Hank's balanced salt solution (HBSS) with 100 U of penicillin G, 2 U of heparin and 8 μg of gentamicin per ml. AB serum which had been stored at −70°C was added to make a final concentration of 10%. Heat-killed *Staphylococcus albus* was added to make a final bacteria:leukocyte ratio of 30:1 (3). Suspensions were then placed in a shaking incubator (American Optical Corp., Richmond, Calif.) at 37°C for 30 min and centrifuged at 1,000 rpm for 10 min to remove *S. albus* and supernatant serum. Cell pellets were resuspended in fresh serum-free HBSS to make a final concentration of 5 million cells per ml and incubated 18 h at 37°C. Following incubation, suspensions were spun at 3,000 rpm for 30 min. The supernate was removed and stored at 4°C in 0.02% sodium azide.

Concentration Procedures.—Crude pyrogen lost significant activity when concentrated by filtration under positive pressure. Some preparations were dialyzed in cellulose tubing (Union Carbide Corp., Chicago, Ill.) against 20 volumes of cold water, lyophilized, and stored at −20°C until used. Other preparations were dialyzed and then evaporated using a high speed fan at room temperature. With the latter technique, the temperature of the liquid during evaporation was approximately 12°C, and it was subsequently noted that this method yielded the best recovery of pyrogenic activity.

Alcohol Precipitation.—Crude pyrogen preparations were precipitated by slowly adding 99% ethyl alcohol at −10°C under constant magnetic stirring. After 18 h at −20°C, the precipitates were spun at 5,000 rpm (−10°C) for 30 min. The supernatant solutions were discarded and the precipitate pellets resuspended in saline, distilled water, or buffers. The suspensions were vigorously pipetted until dissolved and then centrifuged at 10,000 rpm for 30 min. These supernates were pyrogen tested in rabbits. Using eight rabbits, a two-point dose-response curve was obtained before and after alcohol precipitation to determine loss of activity.

Gel Filtration.—All columns were siliconized and autoclaved. The various sizes of the columns are presented in the text. Sephadex (Pharmacia Fine Chemicals) was autoclaved in the buffers for 30 min before packing. Gel filtration was downward and the material was moved either by pump (Extracorpeal Medical Specialties, Inc., King of Prussia, Pa.) or Mariotte flask. The sterility and pyrogen-free nature of the columns, buffers, and Sephadex were verified by trial runs before application of pyrogen. Protein was determined either by optical density at 280 mμ or by the method of Lowry, et al. (12).

Isoelectric Focusing.—The techniques described by Radola (13) and Felgenhauer and Pak (14) were modified for semipreparative isoelectric focusing. 30–40 ml of salt-free pyrogen preparations (either concentrated or partially purified) were slowly mixed with 2.5–3 g of dry superfine Sephadex G-75. Ampholines (LKB Instruments, Bromma, Sweden) were added to make a final concentration (wt/vol) of 2% of the total swollen Sephadex-sample mixture. After 2-4 h at room temperature, the swollen gel was poured onto glass plates (20 cm × 10 cm) and air dried until the edges began to recede.

Electrode contact was made with wet filter paper applied to the 10 × 0.5 cm surface edge of the swollen gel. The plate was placed on a cooling block (Desaga, Heidelberg, Germany), maintained at 4°C and 400 V were applied through electrode baths (0.5% H_3PO_4 and 0.75% NaOH). After 24 h, the paste-like Sephadex was scraped into 10 cm × 1 cm strips and suspended in 2 ml of distilled water. 2 h later, pH was determined (Model 320, Fisher Scientific Co., Fair Lawn, N. J.). After pH measurement, the samples were further eluted in 10 ml of 0.85% saline, mixed thoroughly, and centrifuged at 1,000 rpm for 5 min. Supernatant fluids were injected into rabbits.

Neuraminidase.—A nonpyrogenic preparation of recombinant equine influenza neuraminidase was tested for its ability to liberate sialic acid during each experiment using the technique of Warren (15).

RESULTS

Dose-Response Assay of Monocyte and Neutrophil Preparations.—Buffy coat concentrates separated in 3% dextran contained a mixture of both neutrophils and monocytes. On the other hand, buffy coat concentrates separated from a Ficoll-Hypaque gradient had significantly greater numbers of monocytes and a marked decrease in the number of neutrophils. The latter preparations, although not pure, were labeled "monocytes." In contrast, leukocytes obtained after dextran sedimentation of blood obtained from pump oxygenators had significantly fewer monocytes ($P < 0.01$, Student's t test). These preparations were labeled "neutrophils." The differential counts for these preparations are given in Table I. In considering pyrogen producing cells, the lymphocyte can be discarded, since it has been shown that these cells do not produce pyrogen (3).

All cell preparations were stimulated under the same conditions and the supernatant fluids injected into rabbits. Fig. 1 depicts the dose-response curves for each of the three preparations. All fevers were monophasic with a peak between 48 and 60 min following injection. Following heating (80°C for 15 min), all preparations became nonpyrogenic. As can be seen, monocytes release approximately 20 times more pyrogen per cell than neutrophils. These data confirm other previously reported data on pyrogen release from human monocytes (16).

Gel Filtration of Crude Human Pyrogen.—Initially, pyrogen obtained from buffy coat concentrates (containing approximately 68% neutrophils and 5% monocytes) were lyophilized, then reconstituted in 0.85% phosphate-buffered saline (pH 7.4), and placed on a Sephadex G-50 column. The main protein peak was associated with the void volume. A single pyrogen peak was obtained,

TABLE I

Differential Counts of Blood Separated by Various Techniques

Separation technique	Neutrophils	Monocytes	Lymphocytes	Eosinophils
Dextran sedimentation (3%)*	68.3 ± 3.0‖	5.0 ± 0.4	26.0 ± 3.1	1.7 ± 0.4
Ficoll-Hypaque‡	15.3 ± 3.2	22.0 ± 0.5	62.6 ± 4.7	0.0
Dextran sedimentation (4.5%)§	71.1 ± 4.9	1.4 ± 0.5	25.2 ± 4.3	1.2 ± 0.4

* Cells obtained from whole blood and data derived from nine separate experiments.

‡ Cells obtained from whole blood and data derived from six separate experiments.

§ Cells obtained from blood from pump oxygenator and data derived from eight separate experiments.

‖ Data expressed as mean percentage ± SE.

which was separate from the protein (Fig. 2). This single pyrogen peak at the same elution volume was confirmed several times in different buffers (0.5 M Na acetate. pH 4.5; 0.85% NaCl, pH 6.8; 0.85% NaCl in 0.007 M KH_2PO_4,

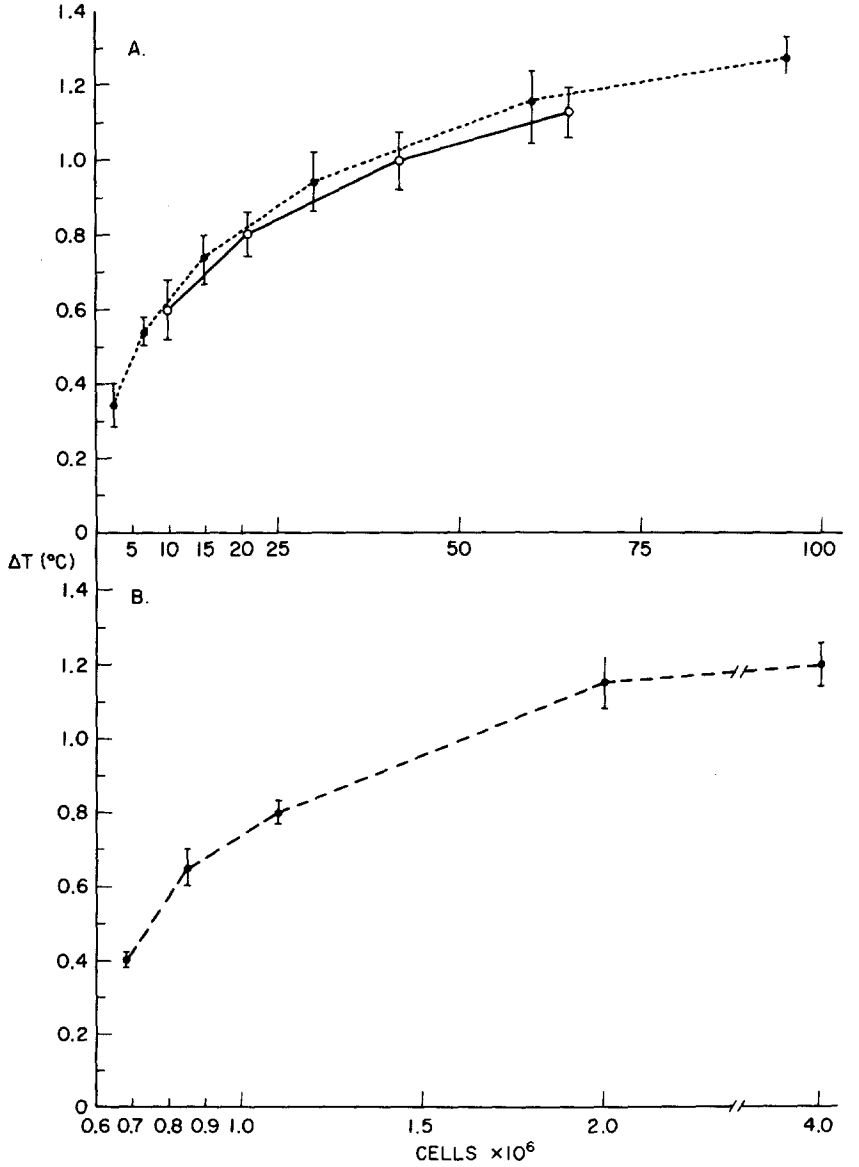

FIG. 1. (*A*) Dose-response curve of supernatant pyrogen from either neutrophil preparations (○—○) or neutrophils and monocytes (●-----●). See text. Each point represents the mean fever above baseline (ΔT) ± SEM of 6–12 separate injections. (*B*) Dose-response curve of supernatant pyrogen from monocyte preparations (●- - -●). Each point represents the mean fever above baseline (ΔT) ± SEM of 6–10 separate injections.

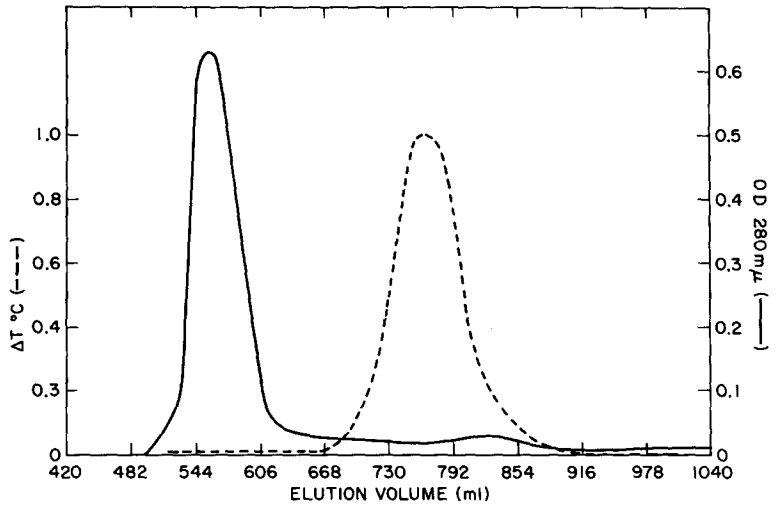

FIG. 2. Median pyrogen peak of 13 separate chromatography experiments using concentrated leukocytic pyrogen derived from whole buffy coat leukocytes. Sephadex G-50 (coarse); column, 105 × 3.8 cm; solvent, 0.85% NaCl at pH 6.8; flow rate, 60 ml/h; each fraction, 6.2 ml; sample size, 13.0 ml. Each fraction was injected into three rabbits. (——), protein; (-----), change in temperature (ΔT) above baseline in °C.

pH 7.4; 1.6% NaCl, 1% casein acid hydrolysate, pH 6.5) (Sigma Chemical Co.). Losses of approximately 50–60% occurred regularly during gel filtration; however, the addition of casein acid hydrolysate (1%) reduced the loss to 20% (7). Using ovalbumin, chymotrypsinogen, and cytochrome *c* as known standards, the mol wt of the pyrogen peak was repeatedly in the 15,000 range.

Isoelectric Focusing of Crude and Column-Purified Pyrogen.—Crude pyrogen from buffy coat concentrates was dialyzed, lyophilized and then focused for 18–24 h. A double pyrogen peak was occasionally seen. The main peak occurred at pH 6.8 and a minor one at pH 5.1 (Fig. 3). When crude pyrogen prepared from monocytes obtained from Hypaque-Ficoll gradients was focused, the main peak shifted to pH 5.1 and the minor peak was now at pH 6.8. However, when pyrogen effluents from gel filtration on Sephadex G-50 were focused, only a single peak occurred at pH 6.9 (Fig. 3).

Gel Filtration of Crude Monocyte Pyrogen.—Since crude pyrogen obtained from suspensions of primarily monocytes seemed to increase the peak focused at pH 5.1, crude monocyte pyrogen was chromatographed on Sephadex G-50. Large quantities of monocytic pyrogen were prepared and concentrated by dialysis and evaporation. A single run through the Sephadex G-50 column showed a broad peak immediately after the void volume. In order to determine the molecular weight of this peak, crude monocyte pyrogen was applied to a G-75 (fine) Sephadex column (60 cm × 2.6 cm) and a double peak was present in 0.85% phosphate-buffered saline at pH 7.4. In addition, a similar run in 6 M urea, 0.03 M KH_2PO_4, pH 7.8, did not change the elution volume of the double

peak in relation to known standards. Fig. 4 shows the approximate mol wt for crude monocyte pyrogen chromatographed on G-75 Sephadex. The larger pyrogen is about 38,000 while the smaller pyrogen is approximately 15,000.

In order to separate the two pyrogen peaks, a longer (165 × 5.6 cm) column

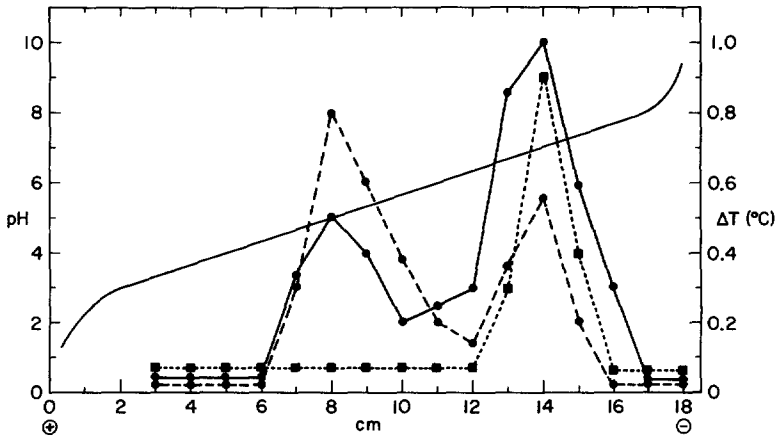

FIG. 3. Median pyrogen peaks from different pyrogen preparations after isoelectric focusing. The solid straight line represents the pH gradient. Febrile responses: (●—●), crude pyrogen from buffy coat concentrates; (●- - -●), crude pyrogen from monocyte preparations; (■-----■), G-50 pyrogen peak from buffy coat supernates. All points are the mean of 3–4 injections and the median curves are taken from not less than three experiments. Voltage, 400 × 24 h. See text.

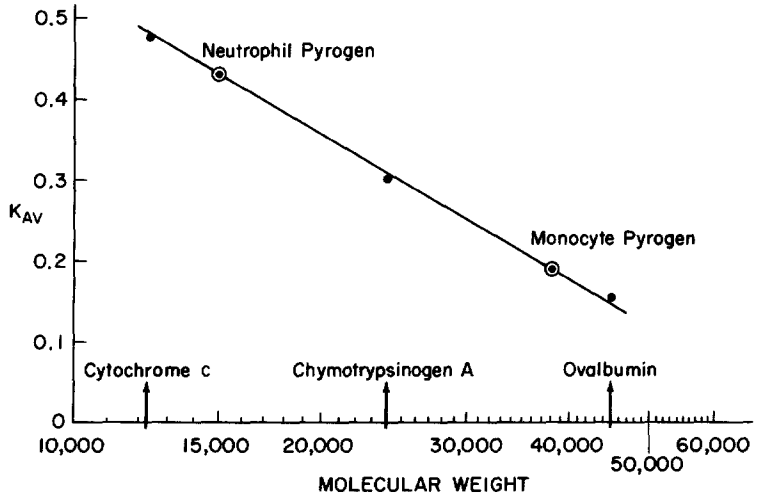

FIG. 4. K_{AV} values and approximate molecular weights for known standards and the two pyrogen peaks. Sephadex G-75 (fine); column, 60 × 2.6 cm; solvent, 0.85% phosphate-buffered NaCl at pH 7.4; flow rate, 10 ml/h; each fraction, 3.2 ml; sample size, 3.0 ml.

was made (Glass Shop, NIH). A typical run of concentrated crude monocytic pyrogen through Sephadex G-50 is shown in Fig. 5. Although the two peaks were clearly separated, up to 80% of the biologic activity of the crude monocytic pyrogen was lost. Both peaks produced monophasic fevers and after heating for 15 min at 80°C they lost their pyrogenicity. However, the large molecular weight pyrogen produced a mean fever peak at approximately 57 min after injection into rabbits while the smaller molecule attained a mean peak at 41 min (Fig. 6). Using paired samples in the same rabbit, this difference

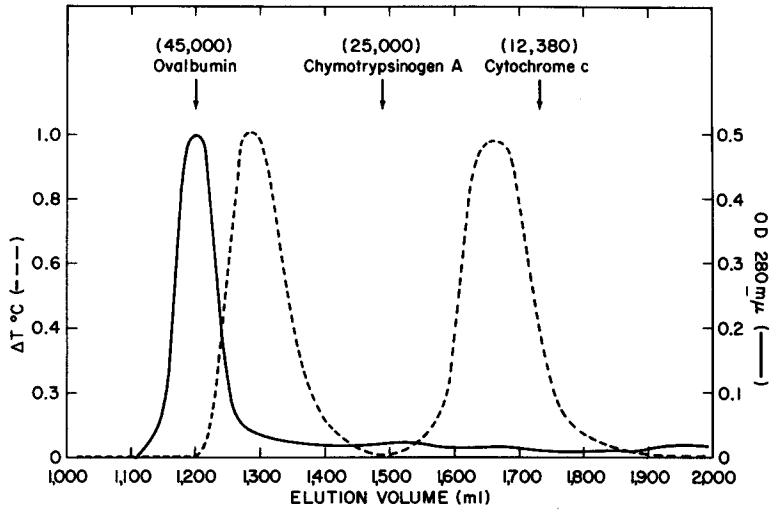

FIG. 5. Median pyrogen peaks of four chromatography experiments using concentrated crude monocyte pyrogen. Sephadex G-50 (fine); column, 165 × 5.6 cm; solvent, 0.85% phosphate-buffered NaCl in 1% casein hydrolysate at pH 7.4; flow rate, 120 ml/h; each fraction, 6.5 ml; sample size, 33 ml. (——), protein; (----), change in temperature (ΔT) above baseline in °C.

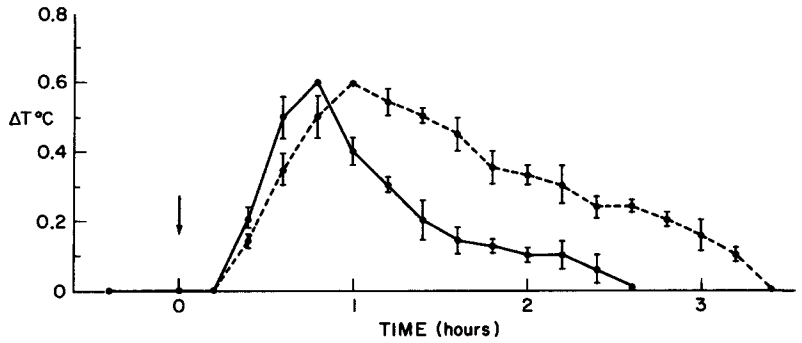

FIG. 6. Mean temperature elevation (± SEM) of 0.6°C from baselines of six rabbits injected with the 38,000 mol wt pyrogen (----) and six rabbits injected with the 15,000 mol wt pyrogen (——) purified by gel filtration.

was significant ($P < 0.05$, t test). In addition, the return of temperature to normal following the 38,000 mol wt pyrogen was slower than with the 15,000 mol wt pyrogen (Fig. 6). Following repeated daily doses of the 38,000 mol wt pyrogen, the febrile responses remained unchanged.

After treatment with neuraminidase, both peaks retained their biological activity. In addition, crude monocytic pyrogen treated with neuraminidase for 2 h eluted at the same volume after gel filtration as untreated preparations.

Isoelectric Focusing of Monocytic Pyrogen.—Crude monocytic pyrogen was concentrated and chromatographed on a G-50 Sephadex column (165 × 5.6 cm) in the presence of 1% casein hydrolysate (1.6% NaCl, pH 6.5). The large mol wt pyrogen peak (38,000) was isolated, concentrated by evaporation, and dialyzed. This material was then focused on thin layer Sephadex G-75 for 24 h. This column-purified pyrogen molecule focused at pH 5.21 and corresponds to the crude pyrogen with pI = 5.15. A column-purified preparation focused at the same pH even after treatment with neuraminidase.

Other Characteristics of the Two Pyrogens.—Crude pyrogen preparations were precipitated in various concentrations of ethyl alcohol at −10°C. 75% of neutrophil or buffy coat pyrogen activity was recovered from the precipitates which formed at a final alcohol concentration of 50–70% (vol/vol). Monocyte pyrogen preparations, on the other hand, lost 70–75% of the activity at these same concentrations of alcohol and no activity was recovered at other concentrations. Furthermore, no monocyte pyrogen activity was present in the alcohol soluble compartment. The alcohol was removed by dialysis before injection into rabbits.

Alkaline dialysis for 24–48 h in Tris-HCl 0.05 M, pH 9.0, 4°C did not reduce the pyrogenic activity of any of the three crude preparations. Occasionally, crude monocytic pyrogen lost approximately 50% of its pyrogenic activity after dialysis against 0.05 M PO_4, pH 5.5, 4°C or Na acetate 0.5 M, pH 4.5, 4°C. Although crude pyrogen preparations from monocytes were occasionally acid labile, the large molecular weight pyrogen isolated after G-50 Sephadex gel filtration was always stable in acid. Crude neutrophil or buffy coat pyrogen as well as isolated small molecular weight pyrogen never lost activity with acid exposure at 4°C or 37°C.

All three crude preparations as well as both pyrogen peaks isolated from the Sephadex G-50 columns were nonpyrogenic after heating at 80°C for 15 min or incubating with trypsin at pH 7.8 for 1 h at 37°C. The characteristics of the two pyrogens are summarized in Table II.

DISCUSSION

The present experiments establish the existence of a new, previously unrecognized, pyrogen which is released from human blood monocytes. The monocyte pyrogen molecule is a larger and more acidic protein than neutrophil pyrogen. As demonstrated, there are other chemical and biological charac-

TABLE II

Characteristics of Neutrophil and Monocyte Pyrogen

	Neutrophil pyrogen	Monocyte pyrogen
μg protein/ml (crude)*	Crude‡ = 248 ± 18 (8)	Crude = 51 ± 17 (5)
μg protein/ΔT 0.6°C§ (crude)	Crude = 1,783 ± 200 (8)	Crude = 41 ± 16 (5)
Mol wt (crude)	15,000	38,000
Isoelectric point (chromatographed or crude)	6.92 ± 0.09 (14)	5.14 ± 0.05 (8)
Precipitation and recovery from 50% alcohol (crude)	Yes (18)	No (7)
Mean fever peak in minutes (chromatographed pyrogen)	41.0 ± 2.0 (12)	57.0 ± 4.0 (12)
Inactivation by trypsin (crude or chromatographed)	Yes (9)	Yes (4)
Inactivation at 80°C × 15 min (crude)	Yes (10)	Yes (6)
Inactivation by neuraminidase (chromatographed pyrogen)	No (5)	No (5)
Change in pI or mol wt after treatment by neuraminidase (chromatographed pyrogen)	No (2)	No (2)
Inactivation after alkaline dialysis (crude)	No (4)	No (3)
Decreased activity during storage at 4°C (crude or chromatographed)	No (after 4 mo)	Yes (after 24–48 h)

* The number in parenthesis indicates the number of observations; the words indicate the particular preparations.

‡ All crude preparations were made from 5×10^6 total white blood cells/ml of buffer.

§ μg of protein required to elicit a temperature rise of 0.6°C.

teristics which distinguish it from neutrophil pyrogen. In a previous report, no distinction was made between the elution peak of monocyte or whole white blood cell preparations following chromatography on Sephadex G-75 (4). In the present study, a distinct separate peak was not observed until a longer column was employed for gel filtration, which may in part explain why this molecule was not recognized earlier.

Some of the other reasons which might explain why the monocyte pyrogen molecule was not previously detected are: (*a*) the molecule is unstable and undergoes rapid degradation; (*b*) the molecule might be a dimer or other combination of neutrophil pyrogen with carrier protein; and (*c*) monocyte pyrogen production is either suppressed or destroyed during incubation with neutrophils. It is clear from these investigations that crude monocyte pyrogen as well as that purified by gel filtration loses activity during storage at 4°C, while comparable neutrophil pyrogen preparations do not. It is highly unlikely that

the monocyte pyrogen molecule is a dimer of neutrophil pyrogen since the pI's of the two pyrogens are almost two pH units apart and the size of such a dimer would be smaller than the size of monocyte pyrogen. Furthermore, the elution volume of the monocyte pyrogen did not change during gel filtration in 6 M urea, which should have separated a dimer. There is the possibility that a pyrogenic moiety similar to neutrophil pyrogen is attached to a nonpyrogenic parent protein from monocytes. Attempts to separate these two proteins with high solute concentrations and isoelectric focusing failed. Yet we cannot rule out the possibility that the monocyte pyrogen molecule requires an enzyme to separate it into smaller units, i.e., similar to the size of neutrophil pyrogen. Although unlikely, we have not excluded the latter possibility.

Although the monocyte releases at least 20 times more pyrogen than the neutrophil, it is unlikely that it contributes this order of magnitude of pyrogen in the buffy coat incubations since the 38,000 mol wt pyrogen peak was absent from repeated chromatographed crude pyrogen preparations In fact, it is possible that 5% of monocytes in the buffy coat incubations do not contribute to the pyrogenic activity of the crude pyrogen derived from such an incubation The presence of such large numbers of neutrophils in these buffy coat preparations may have a suppressive effect on monocytes to release endogenous pyrogen. In support of this concept, is the fact that human buffy coat cells release more pyrogen when the supernatant medium is changed (3). The possibility that the presence of large numbers of neutrophils suppresses monocyte pyrogen release may help clarify an unexplained observation in the pathogenesis of fever induced by antigen-antibody complexes. Despite the fact that antigen-antibody complexes infused in vivo cause fever (17), release of endogenous pyrogen from buffy coat cells stimulated with such complexes has not been demonstrated in vitro.[1] Since the monocyte may play a key role in the pathogenesis of this immune fever, the large number of neutrophils present in the preparations tested in vitro may account for the seeming failure to demonstrate the pyrogen in vitro.

In order to test for sialic acid residues, which might have accounted for the acidic nature or large size of the monocyte pyrogen, the molecule was treated with neuraminidase. Following this treatment, there was no loss of activity or change in molecular size or isoelectric point.

The fact that both human as well as rabbit endogenous pyrogen produce the same type of fever pattern in rabbits does not necessarily mean that both molecules are similar. Until the present studies, both human and rabbit pyrogens were thought to be the same size (4). Most of the chemical characterization and purification of rabbit endogenous pyrogen has been done on material derived from peritoneal exudate granulocytes (5–7). On the basis of the present experiments, it would appear that there are significant differences between

[1] Root, R. K., and S. M. Wolff, unpublished observations.

rabbit and human leukocyte pyrogen when one compares the rabbit with the neutrophil pyrogen of man. Human neutrophil pyrogen has a slightly larger molecular weight than the rabbit. The human pyrogen is a more acidic protein than the rabbit and is alkaline resistant while the rabbit is not. Human and rabbit pyrogen has been compared and it was found that human pyrogen is alkaline resistant (4).

The fact that human monocyte and neutrophil pyrogens differ biologically may have important implications. The monophasic fevers that were produced by the 38,000 mol wt pyrogen had a peak elevation at about 60 min, while the 15,000 mol wt pyrogen had its peak temperature elevation at 36–48 min. The time of onset of both pyrogens was approximately 12–18 min following injection. Defervescence following the partially purified monocyte pyrogen was also slower than that of neutrophil pyrogen. It is possible that two different hypothalamic sites act as receptors for the two molecules and may explain the difference in fever curves. Furthermore, the larger molecule may exert its effect or be present in the circulation for a longer period of time. The cause of these differences, however, awaits further investigation.

The demonstration of two distinct pyrogens from human white blood cells should stimulate further investigations into the pathogenesis of fever in man. With improved techniques of isolation, we now may be able to begin to characterize and investigate the biological and physiological properties of these potent and potentially important materials.

SUMMARY

Human monocytes and neutrophils were separated from buffy coats of blood obtained from normal donors. Following incubation with heat-killed staphylococci, monocyte preparations contained 20 times more pyrogenic activity in the supernatant media than did supernates from an equal number of neutrophils. During purification of these pyrogens it was discovered that these cell preparations each produced a distinct and different pyrogen. The pyrogen obtained from neutrophils had a mol wt of 15,000 following Sephadex G-75 gel filtration, an isoelectric point of 6.9, and could be precipitated and recovered from 50% ethanol at −10°C. In contrast, the pyrogen derived from monocyte preparations had a mol wt of 38,000, an isoelectric point of 5.1, and was destroyed in cold ethanol. Both molecules were unaffected by viral neuraminidase but biologically destroyed at 80°C for 20 min and with trypsin at pH 8.0. The febrile peak produced by partially purified neutrophil pyrogen occurred at 40 min while that from monocytes was at 60 min. In addition, monocyte pyrogen produced more sustained fevers for the same peak elevation as neutrophil pyrogen. These studies demonstrate for the first time two chemically and biologically distinctive pyrogens derived from circulating human white blood cells and have important implications for our understanding of the pathogenesis of fever in man.

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Addendum.—Since there had been no attempt to separate lymphocytes from monocytes when obtaining pyrogen from the "monocyte preparations," the possibility exists that lymphocytes might play an essential role in the release of pyrogen from monocytes or that lymphocytes themselves release the larger pyrogenic molecule. Therefore, further experiments were performed to investigate these possibilities. Monocytes were prepared by allowing them to adhere to plastic surfaces and after 45 min were washed twice. These plastic adherent cells were then stimulated in the usual fashion with heat-killed *S. albus* and the supernatant fluid harvested after 18 h of incubation. This supernate was concentrated and passed through a Sephadex G-50 column. A peak of pyrogenic activity at the same elution volume as the 38,000 mol wt monocyte pyrogen was found. Thus, these experiments suggest that the lymphocyte is not the source of pyrogen and that they are not playing a role in the production or release of pyrogen from mixed monocyte-lymphocyte preparations in the present investigations.

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