SPONTANEOUS PYROGEN PRODUCTION BY MOUSE HISTIOCYTIC AND MYELOMONOCYTIC TUMOR CELL LINES IN VITRO*

By PHYLLIS BODEL with the technical assistance of KAREN WENC

(From the Department of Medicine, Yale University School of Medicine, New Haven, Connecticut 06510)

Fevers associated with neoplastic growth occur commonly in patients with acute leukemias (1), Hodgkin's disease (2), and other lymphomas (3), and less frequently in patients with carcinomas such as hypernephromas (4), hepatomas (5), or tumors metastatic to the liver (6). Since often no infectious agent can be implicated in such fevers, it is likely that many are caused by the malignant disease itself. Such tumor-associated fevers, like fevers studied in animal models, presumably result from the action of endogenous pyrogen (EP)[1], a small protein which is synthesized and released from leukocytes. When this protein enters the blood, it alters in some way the "set-point" of temperature-regulating neurons in the hypothalamus, inducing responses of heat production and conservation, and thus causing fever in the host (7). Experiments to date have shown that EP is only produced by granulocytes, monocytes, or tissue macrophages. In addition, these cells require stimulation by inflammatory agents such as endotoxin or phagocytosis before EP production and release occurs.

Fever associated with malignancies might be the result of EP production by neoplastic cells themselves. Alternatively, host mononuclear or polymorphonuclear leukocytes may be stimulated to release EP by factors released by tumor cells, or generated by other host cells in response to tumor growth. In previous studies, abnormal, spontaneous EP production was demonstrated in cell suspensions prepared from spleen and lymph nodes of patients with Hodgkin's disease (8), but the cell type(s) responsible for this production were not identified. We have now investigated release of EP by neoplastic cell lines in vitro, including six mouse tumor lines having the characteristics of leukemia or lymphoma cells, three carcinoma lines of human origin, and one viral-transformed mouse fibroblast line. Spontaneous EP release has been documented from five of the six mouse tumor cell lines.

Materials and Methods

All materials, glassware, and reagents were obtained or made sterile and pyrogen-free by methods described previously (9). All test supernates were centrifuged at 800–2,000 *g* for 10–15

* Supported by research grants CA-14655 from the National Cancer Institute and AI-01564 from the National Institute of Allergy and Infectious Diseases.

[1] *Abbreviations used in this paper:* BCG, bacillus Calmette-Guérin; CSA, colony-stimulating activity; EP, endogenous pyrogen; LAF, lymphocyte-activating factor; LPS, lipopolysaccharide; MEM, Eagle's minimal essential medium; PF, pyrogen-free.

min and cultured in thioglycollate broth before injection; any contaminated samples were discarded.

Pyrogen Assay. Culture supernates were tested for pyrogenicity using a mouse bioassay which has been described previously (10, 11). Briefly, 7–8-wk-old Swiss-Webster mice (Yale colony) were kept at 35°C for 1–2 h until stable temperatures were achieved. They were then injected intravenously with 0.3 ml of a test solution, usually a dilution of supernate from incubated cells, and rectal temperatures were monitored every 10 min for 50 min. Maximum temperature changes from baseline 20 min after injection were used to determine the pyrogen content of the sample.

Tissue Culture Cell Lines. All cell lines were maintained in 75 cm^2 plastic flasks (Falcon Plastics, Div. BioQuest, Oxnard, Calif.), and incubated in 5% CO_2, 95% air. Fresh medium was supplied every 2–3 days; for mouse tumor cell lines, only one-half the volume of supernate was replaced with fresh medium at each time interval.

Mouse tumor cell lines (J-774, PU5-1.8, WEHI-3, P388 D1, RAW-8, and R-8) were obtained from Dr. Peter Ralph, Walker Laboratory, Sloane-Kettering Institute for Cancer Research, Rye, N. Y. The characteristics and origins of these cells have been described previously (12–16). Briefly, J 774, PU5-1.8, and P388 D1 cells resemble macrophages or histiocytes; WEHI is a myelomonocytic leukemia cell line; RAW-8 and R-8 cells were derived from mouse lymphomas and resemble lymphocytes. Cells were maintained in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N.Y.) with 10% heat-inactivated, filtered fetal calf serum, 50 U penicillin/ml and 50 μg streptomycin/ml. For cell transfer, $\cong$ 2 ml of supernate containing $\cong 10^4$ cells were transferred weekly to new incubation flasks with 30 ml fresh medium. Before transfer of J-774, PU5-1.8, or WEHI-3 cells, which grow as loosely adherent cell layers, cells were detached from the flask surface by vigorous pipetting.

For experiments with the histiocytic and myelomonocytic cell lines, flasks of a single line with nearly confluent, adherent cell layers were selected, usually 3–4 days after seeding. All supernatant medium was discarded, and flasks were rinsed four times with RPMI (without serum) warmed to 37°C. 10 ml RPMI with 2.5 mg/ml lactalbumin hydrolysate (ICN Nutritional Biochemicals, Div. International Chemical & Nuclear Corp., Cleveland, Ohio), pyrogen-free (PF) medium, were then added. In some experiments, 10% endotoxin free, heat-inactivated fetal bovine serum (Flow Laboratories, Inc., Rockville, Md.; lot 4055 1190) was used in place of lactalbumin. In other experiments, heat-killed staphylococci, prepared as described previously (11), were added to one of a pair of flasks at an estimated ratio of 10:1 bacteria to cells. The flasks were incubated for 18 or 42 h. For experiments with R-8 and RAW-8 cells which grow in suspension, 3–4 days after seeding the flask contents were removed, centrifuged at 100 *g* for 10 min, and the supernatant medium was discarded. The cells were resuspended in RPMI without serum, centrifuged at 100 *g* for 10 min, and this washing procedure was repeated three times. Cells were finally suspended in 10 ml PF medium and equal aliquots were incubated in new flasks for 18 or 42 h.

Human tumor cell lines (Caki-1, Caki-2, and SK-HEP-1) were obtained from Dr. Jörgen Fogh, also of Walker Laboratory. The characteristics and origins of these lines have been previously reported (17). Caki-1 and Caki-2 cells, derived from clear cell renal adenocarcinomas, were grown in McCoy's 5 A medium (modified) (Grand Island Biological Co.), containing 15% filtered fetal calf serum, 50 U penicillin/ml, and 50 μg streptomycin/ml. SK-HEP-1 cells, derived from an adenocarcinoma of the liver, were similarly maintained except that Eagle's minimal essential medium (MEM; Auto-POW, Flow Laboratories, Inc.) plus 2 mM L-glutamine was substituted for McCoy's medium. For transfer of cells, flasks with confluent layers were either treated with Viokase (11) (Grand Island Biological Co.) for Caki-1 and SK-HEP-1 lines, or cells were detached by scraping with a rubber policeman, for Caki-2 cells. One half of the cells recovered from each flask were transferred to new flasks containing 20 ml of the appropriate medium.

For experiments with human tumor cell lines, all supernate was removed from flasks with confluent cell layers, and the cells were rinsed three times with medium containing no serum. Flasks were then reincubated for 18 to 42 h with 5 ml of medium containing 15% pooled normal human serum. 5 ml medium with 15% human serum was also incubated as a control.

3T3-SV 40 mouse fibroblasts (Swiss-Webster origin) were obtained from B. Dorman, Department of Biology, and Dr. Paul Lebowitz, Department of Medicine, Yale University. Cells were grown in MEM (see above) with 2 mM L-glutamine, 10% fetal calf serum, 50 U penicillin, and 50 μg streptomycin/ml. Once a week, cells were transferred using Viokase as described above. For

experiments, the same techniques were used for rinsing and incubating cell layers as those described for human tumor cell lines, except that 10% rather than 15% serum was used.

At the end of all incubations, supernates were removed, centrifuged at 800 or 2000 *g* for 20 min, and assayed for pyrogen content. Cells were also removed and counted, using a hemocytometer or Coulter counter (model Z_F; Coulter Electronics Inc., Hialeah, Fla.), and viability was measured in most experiments by exclusion of 1% eosin dye. When present, nonadherent cells were collected by initial centrifugation of the supernates at 100 *g*; adherent cells were obtained by scraping or by treatment with Viokase. For cells incubated with *Streptomyces albus*, percent phagocytosis was estimated from Wright's-stained coverslip smears. This varied from 59–77% in all experiments.

Heat and Pronase Treatment of Pyrogens. 50 mg of pronase (B-grade; Calbiochem, San Diego, Calif.) was dissolved in 5 ml saline, and preincubated for 2 h at 37°C. 0.05 ml was then added to 2.5 ml of a pyrogenic supernate and the mixture was incubated for 4 h at 37°C. Another 2.5 ml of the pyrogenic supernate was incubated for 3 h at 37°C, and then for 1 h at 56°C. A third aliquot was incubated for 4 h at 37°C with no additions. As a control, 2.5 ml tissue culture medium containing no pyrogen, with 0.05 ml pronase added, was also incubated at 37°C for 4 h.

Assays for Intracellular Pyrogen. Flasks of J-774 cells were chosen 3 or 4 days after cell transfer, and cells were incubated in PF medium for 18 or 42 h, as described above. Supernatant medium was removed for subsequent pyrogen assay, and cell layers were rinsed three times with warmed RPMI medium. 5 ml RPMI was then added to the flask, and the cells were detached with a rubber policeman. In some experiments, this cell suspension was transferred to a duplicate flask and additional cells were collected. Cell numbers in the suspensions, both total and percent viable, were determined as described above. The mixture was disrupted by five cycles of freeze-thawing in dry ice–acetone, centrifuged at 2,000 *g* for 20 min, and the supernate was then assayed for pyrogen content.

Sephadex Filtration of Pyrogenic Supernates. PF medium was added to flasks of J-774 cells as described previously, and after a 48-h incubation, it was removed and stored at −20°C. A pool of 100 ml medium was assayed for pyrogen content and then concentrated to 2.5 ml by evaporation through dialysis tubing in front of a fan. This material was filtered through a 48 × 2.5-cm Sephadex G-75 column, prepared as described previously (18) and equilibrated with phosphate-buffered saline, pH 7.4, containing 2.5 mg/ml lactalbumin hydrolysate. Sodium azide was omitted. 5-ml effluent fractions were collected, and 0.3-ml vol were injected for pyrogen assay. Three experiments with J-774 pyrogen were carried out, using two different columns. Human monocyte pyrogen was also prepared as described previously (18), and 1.5 ml containing four rabbit doses was filtered separately on one of the columns. Where pyrogen assays of individual tubes were carried out at several dilutions, a "derived" (ΔT) was calculated by taking the average values for peak ΔT and multiplying by the dilution. Markers to determine exclusion and filtration volumes for different molecular weights included blue dextran, ovalbumin (Worthington Biochemical Corp., Freehold, N. J.), and 2 × crystallized trypsin and ribonuclease A (both from ICN Nutritional Biochemicals). Protein markers were applied singly to each column after completion of the pyrogen studies using buffer containing no lactalbumin, and their filtration volumes were determined by measurement of optical density at 290 λ.

Results

Histiocytic and Myelomonocytic Tumor Cell Lines. Supernate from tissue culture flasks was obtained 24 or 48 h after incubation of the cells in PF culture media, and was tested in mice for pyrogenicity. The results of a representative experiment with J-774 cells are shown in Fig. 1. Although the supernate removed after 24 h of incubation was only equivocally pyrogenic, supernate from the 48-h incubation clearly produced a febrile response in mice. The shape of the fever curve, which peaked at 20 min after injection and rapidly returned to baseline or below, is identical to that produced by injection of mouse macrophage pyrogen, obtained after stimulation of normal peritoneal macrophages by phagocytosis (10, 11). Injection of media alone (control) caused no elevation of temperature. When results of a series of experiments were exam-

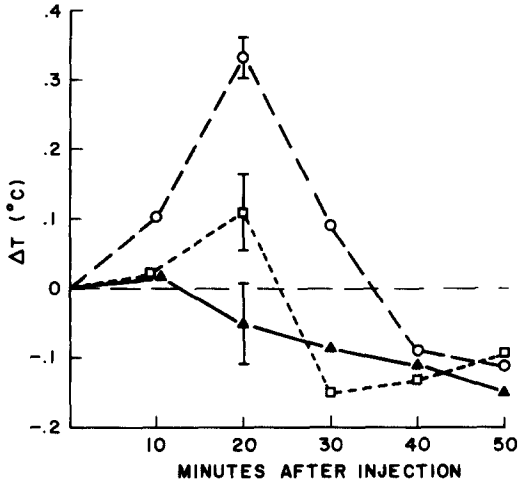

FIG. 1. Spontaneous pyrogen release by histiocytic tumor cells (J-774) during in vitro incubation. Average temperature change (ΔT) from baseline in groups of 11–12 mice after injection of culture supernates from 24-h (□) and 48-h (○) incubations of cells, or control media (▲), is shown. Each supernatant sample was derived from 1–5 × 10^5 viable cells. SEM at 20 min are shown by brackets.

ined, pyrogen was clearly detectable in medium from 24-h incubations, but larger amounts were always present after 48-h incubations. In addition, the same results were obtained when PF media contained fetal bovine serum in place of lactalbumin. These results suggest that J-774 cells spontaneously release pyrogen into the medium during in vitro culture.

Similar experiments were carried out with three other mouse cell lines: PU5-1.8 and P 388 D_1, which have the characteristics of histiocytic tumors, and WEHI-3, previously characterized as a myelomonocytic leukemia. In each instance, we obtained findings which were essentially identical to those described above for J-774 cells. A summary of our results is shown in Fig. 2. Pyrogen was present in supernates of 48-h incubations of all four cell lines. We initially tested the culture supernates in a wide range of doses, injecting media derived from 5 × 10^4–10^6 viable cells (complete data not shown). Since subsequent studies showed that maximal temperature elevations in the mice occurred regularly when media representing about 1–5 × 10^5 cells per dose was injected, this dilution of supernate was then tested most extensively for each cell line.

Lymphocytic Tumor Cell Lines. Spontaneous pyrogen release during in vitro culture was demonstrated in similar experiments using cells from one of two mouse cell lines previously characterized as closely resembling lymphocytic lymphoma cells (Fig. 3). Unlike the results with the macrophage lines discussed above, pyrogen in the PF culture medium of RAW-8 cells was more easily detectable after 24 h than after 48 h incubation. However, maximal fevers were produced as before by dosages of supernate corresponding to 1–5 × 10^5 cells/dose. We were unable to demonstrate any pyrogen release at either 24 or 48 h into PF media by the other lymphocytic tumor line, R-8, although doses of supernate corresponding to 10^5–10^6 viable cells were examined.

Effect of Phagocytic Stimulation on Pyrogen Release by Histiocytic Tumor

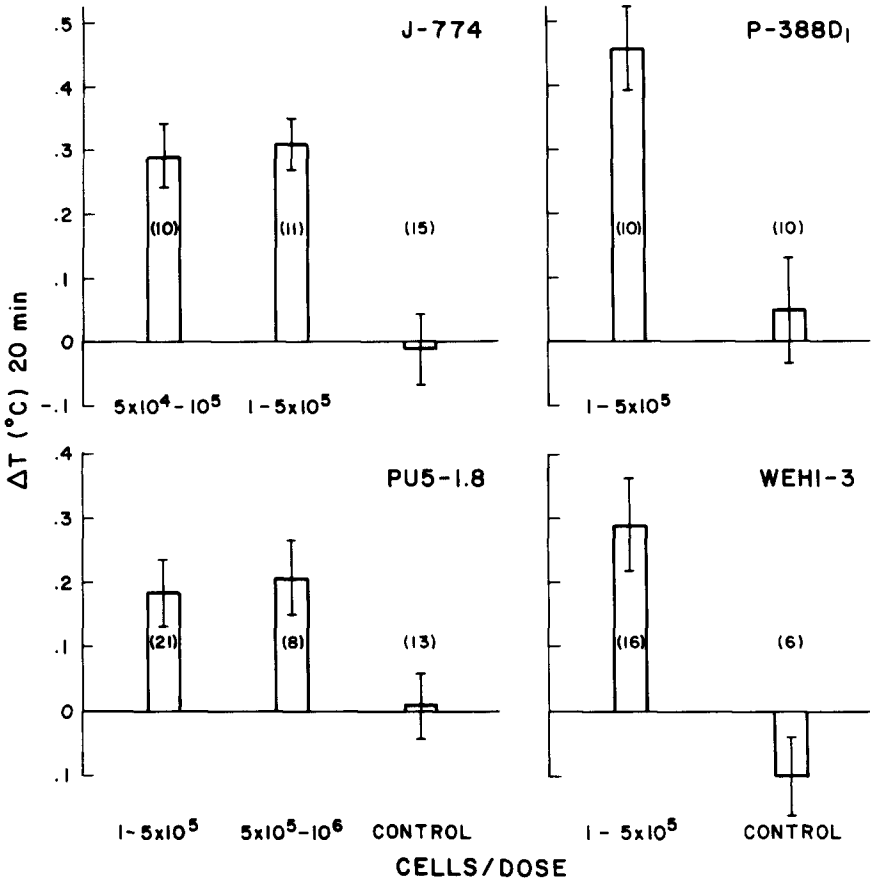

FIG. 2. Spontaneous pyrogen release by cells from three histiocytic tumor cell lines (J 774, P-388 D_1, PU5-1.8) and one myelomonocytic leukemia line (WEHI-3). In this and subsequent figures, average maximum temperature elevation (ΔT) ± SEM, 20 min after injection of culture supernates into groups of mice, is shown by the height of the bars, and numbers of mice in parentheses. Cells/dose, shown below the bars, indicates the number of viable cells recovered after incubation from which that dose of supernate was derived. In this experiment, cells were incubated in PF media for 48 h, and the supernate was diluted as needed and injected for assay. Control injections were of media incubated without cells.

Cells. We studied the effect of phagocytosis of staphylococci on pyrogen release by two histocytic cell lines, J-774 and PU5-1.8. In both cases, we noted increased fevers in the mice after injection of supernates from cells stimulated by ingestion of bacteria compared to the same numbers of cells releasing pyrogen spontaneously in culture (Fig. 4). Although the mouse assay for pyrogen is not quantitative over a large dose range, it is roughly quantitative over a two- to three-fold range between minimal and maximal temperature elevation, when increasing amounts of pyrogen are injected (10). These results, therefore, suggest that there were small, probably one- to twofold, increases in the amounts of pyrogen released by these cells after particle ingestion. We did not study either of the lymphocyte-like lines after incubation with staphylococci since we presumed they were nonphagocytic; R 8 cells do not ingest bacillus Calmette-Guérin (BCG) (19).

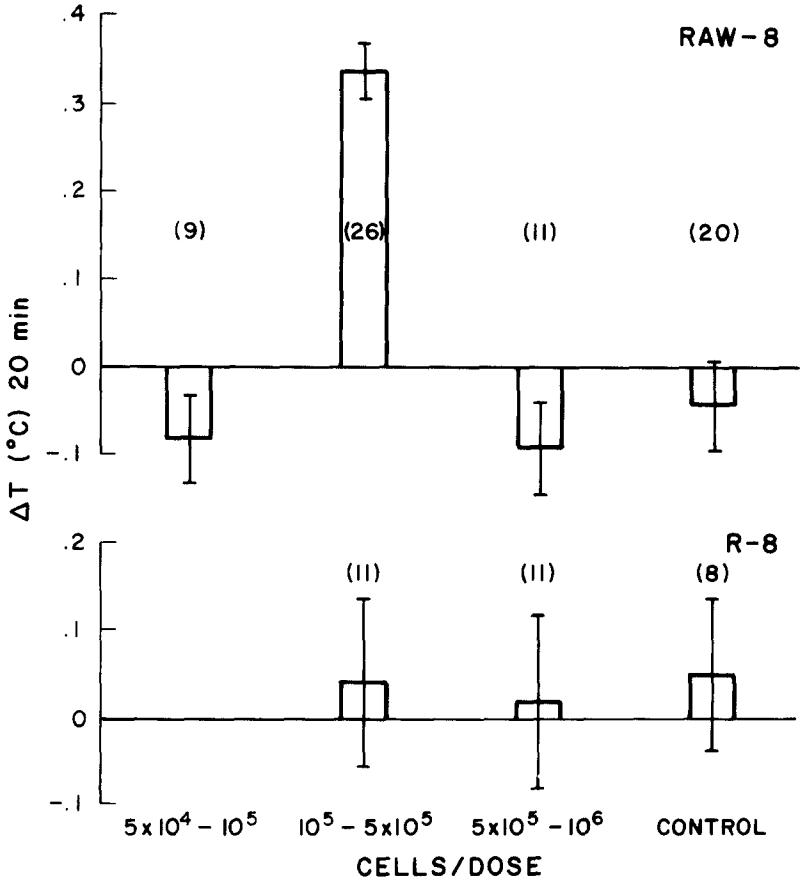

FIG. 3. Spontaneous pyrogen release by one of two lines of mouse lymphocytic-type tumor cells (RAW-8, R-8) during in vitro culture. Cells were incubated in PF media for 24 h before supernate was assayed. See Fig. 2 for other details.

Characteristics of Tumor Cell Pyrogens. As is illustrated in Fig. 1, the type of febrile response induced by the pyrogens from all five of these cell lines was identical to that produced in mice by normal leukocyte EP (10, 20). The pyrogens in flask supernates of the mouse tumor cells appeared to be more stable than those present in crude supernates of stimulated mouse macrophages, since they lost little activity during storage at −20°C for 1 wk, or −70°C for over 3 wk, whereas mouse macrophage pyrogen loses activity within days at 4°C and within 1 wk at −70°C (unpublished observations). However, the tumor cell pyrogens were inactivated by heating to 56°C for 1 h, and by incubation with pronase (Fig. 5). These characteristics suggest that they are proteins, like other endogenous pyrogens of leukocyte origin (7).

We obtained an estimate of the molecular weight of the pyrogen released by J-774 cells by filtration of a concentrate of culture supernate through a Sephadex G-75 column (Fig. 6). Two peaks of pyrogenic activity were present, one at a mol wt of ≅ 30,000, and the other at a mol wt ≥60,000, close to the exclusion volume of the column. Because the pyrogen assay in mice is not easy to

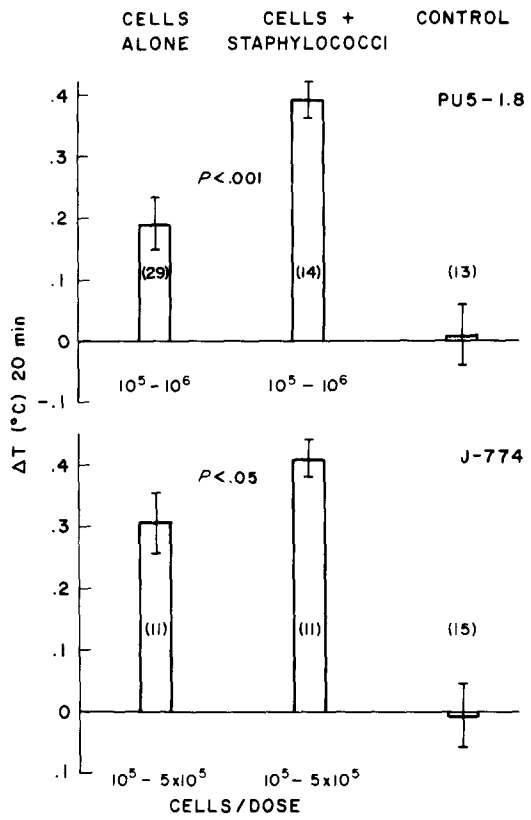

FIG. 4. Comparison of spontaneous pyrogen release by cells from two histiocytic tumor cell lines (PU5-1.8 and J-774) with that induced by phagocytosis of heat-killed staphylococci. Cells were incubated in PF media for 48 h after addition of bacteria or an equal volume of saline. The number of cells containing bacteria was estimated to be between 59 and 77% in all experiments. *P* values were calculated using Student's *t* test for small samples. See Fig. 2 for other details.

quantitate, it is not certain how much of the total pyrogenic activity was associated with each peak. However, the amounts appeared to be similar. By contrast, when supernate from human monocytes was applied to the same column, the two expected peaks of activity were noted, at mol wt of ≅ 14,000 and 40,000, and the quantity of the 14,000 mol wt pyrogenic species was clearly greater, as reported previously (18).

Mechanism of Production of Tumor Cell Pyrogen. We have done some preliminary studies to examine the mode of production of pyrogens by mouse tumor cells. When flasks containing J-774 or RAW-8 cells in PF media were placed at 4°C, supernates remained PF, in contrast to supernates of parallel flasks incubated as usual at 37°C. In addition, more pyrogen appeared in flasks of histocytic and myelomonocytic tumor cells as incubation continued for 48–72 h. Although we usually chose flasks containing moderate to large numbers of cells at 3, 4, or 5 days after seeding, pyrogen production appeared to progress during continued incubation regardless of the initial age of the culture. When rinsed cell layers were disrupted by freeze-thawing, and the resulting super-

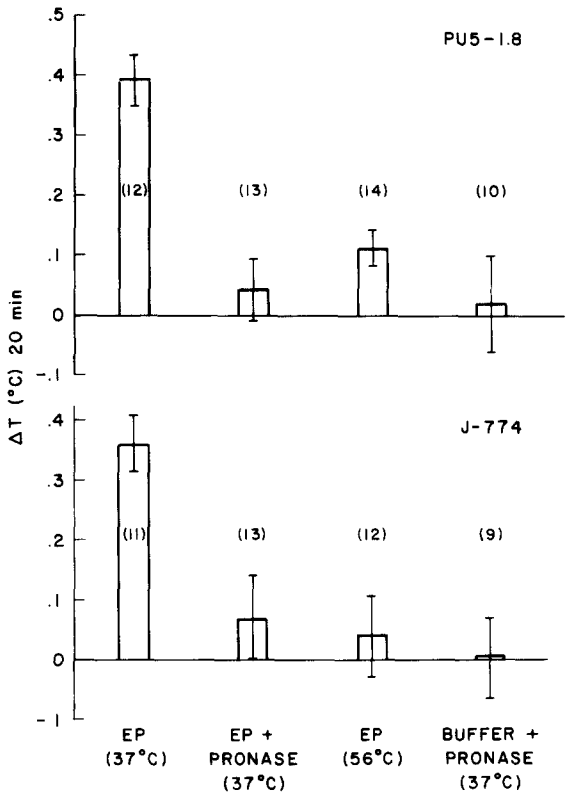

FIG. 5. Inactivation of pyrogenic supernates (EP) from two mouse histiocytic tumor cell lines (PU5-1.8 and J-774) by incubation with pronase or by heating for 1 h at 56°C. See text for details.

nates were assayed for progen content, at no time were significant amounts of intracellular pyrogen detected (Table I), even though pyrogen was present in the culture supernates of the same cells (positive assay results are underlined). The character of the PF medium in which cells were cultured to collect pyrogen for assay did not appear to affect pyrogen production, since we obtained identical results using medium containing either lactalbumin hydrolysate or a PF bovine serum. These findings, therefore, suggest that active production and secretion of pyrogen by these cells is occurring spontaneously and continuously during in vitro culture.

Comparison of Pyrogen Production with Other Characteristics of Mouse Tumor Cell Lines. As shown in Table II, pyrogen was produced by all tumor cell lines which possessed properties of macrophages, including the capacity for phagocytosis of bacterial particles or zymosan, presence of IgG receptors on the cell surface, and secretion of lysozyme. Although RAW-8 cells resemble lymphocytes morphologically, they secrete lysozyme in culture, and therefore presumably contain cells with macrophage characteristics (16). The cell line R-8 has not to date been reported to have any macrophage characteristics, and is presumably composed of lymphocyte-derived cells; cells of this line did not produce pyrogen.

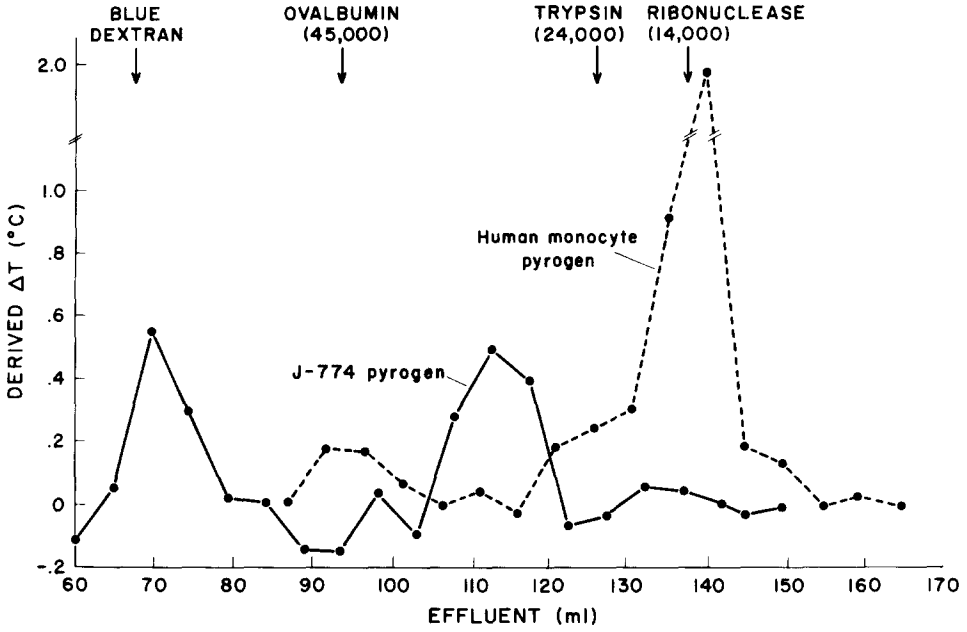

FIG. 6. Sephadex G-75 filtration of pyrogenic supernates from J-774 cells and stimulated human blood monocytes. 5-ml fractions were collected and 0.3-ml samples (undiluted or diluted 1:2-1:8) were injected into mice for pyrogen assay. Derived change in temperature (ΔT) was calculated as described in Materials and Methods. Each point represents the average temperature responses of 4-9 mice. Peak elution volumes of marker proteins are indicated by arrows.

TABLE I

*Differences in Intra- and Extracellular Pyrogen from J-774 Cells Incubated for Either 18 or 42 h**

	Cell incubation	Cells × 10⁴/dose‡			Controls§
		5–10	11–49	49–100	
	h				
Supernate of disrupted cells	18	0.02 ± .07 (6)‖	−0.04 ± .05 (8)	−0.01 ± .06 (7)	
	42	−0.01 ± .05 (7)	0.00 ± .05 (15)	−0.10 ± .03 (11)	
Culture supernate	18	ND¶	0.18 ± .04 (10)	ND	−0.11 ± .03 (32)
	42	ND	0.29 ± .03 (23)	ND	

* Average change in temperature from baseline 20 min after injection, ± SEM.
‡ Cells from which supernate was derived for injection.
§ Injection of medium alone.
‖ Number of injections.
¶ ND, not determined.

Studies of Pyrogen Release by Human Carcinoma Tumor Cell Lines and Viral-Transformed Mouse Fibroblasts. Since patients with tumors other than leukemias and lymphomas occasionally suffer from prolonged, unexplained fevers, we also studied the capacity for pyrogen production by cells from three tumor cell lines derived from human carcinomas, two renal cell carcinomas (CAKI-1 and CAKI-2), and one hepatoma (SK-HEP-1). In addition, we tested

TABLE II
Mouse Tumor Cell Lines

Cell line	Origin	Morphologic and growth characteristics	Functional properties	Lysozyme secretion	LAF secretion (LPS added)	Pyrogen secretion	
						Spontaneous	After phagocytosis
J774	Balb/c derived from reticulum cell sarcoma, ascites form (13, 19)	Well-differentiated histiocytic lymphoma; loosely adherent (21)	Phagocytic: BCG, zymosan, latex, ab-coated erythrocytes, staph; ab-dependent target cell lysis (19, 21)	++ (16, 19)	++ (24)	++	+++
PU5-1.8	Balb/c derived from lymphoma PU 5-1, ascites form (16)	Histiocytic, loosely adherent	Phagocytic: BCG, zymosan, latex, staph; produces CSA after LPS (25)	++ (16, 19)	+ (24)	+	++
P388D1	DBA/2 derived from methylcholanthrene-induced lymphoid tumor, ascites form (12)	Histiocytic, loosely adherent	Phagocytic: BCG, latex, zymosan, staph; surface C_3 and F_c IgG receptors; "Nonspecific" esterase +; lyses ab-coated erythrocytes (12, 19)	++ (16)	++ (24)	++	ND
WEHI-3	Balb/c cloned from a myelomonocytic leukemia (14)	Monocytic, loosely adherent, differentiates into granulocytic and monocytic colonies (22)	Phagocytic: BCG, zymosan, latex; produces CSA (19, 22)	+ (19)	+ (24)	++	ND
RAW8	Balb/c derived from Abelson leukemia virus-induced lymphoma (15, 23)	Lymphocytic; nonadherent		+ (16)	ND*	+	ND
R8	Balb/c × C 57 BL/6 derived from Abelson virus-induced lymphosarcoma, "B" lymphoma (15)	Lymphocytic; nonadherent	Not phagocytic for BCG (19)	− (16)	ND	−	ND

* ND, not determined.

the hypothesis that viral transformation might confer the potential for pyrogen production on a mouse fibroblast cell line previously shown not to produce pyrogen spontaneously (11). We therefore tested incubation media obtained after 18- and 42-h incubations from the three human carcinoma cell lines, and after 18-h incubations from 3T3-SV 40 transformed mouse fibroblasts. We injected dosages of supernate derived from 0.5–49 × 10^4 cells for CAKI-1 and -2 lines; 1–>50 × 10^4 cells for the SK-HEP-1 cells, and 5–>100 × 10^4 cells for the mouse fibroblasts. 6–20 mice were used for each assay. In no instance were we able to demonstrate spontaneous pyrogen release by any of these cell lines. Although the carcinoma cell lines were of human origin, we have shown elsewhere (20) that pyrogen released by human leukocytes is easily detectable in mice.

Discussion

Unexplained fevers occur commonly in diseases such as acute leukemias, Hodgkin's disease, and other lymphomas (1–3), most of which are characterized by proliferation of phagocytic cells of bone marrow origin, including granulocytes, monocytes, and macrophages or histiocytes. Normal cells of these types

produce pyrogen only after stimulation with an exogenous agent, such as endotoxin or phagocytosis, and production of pyrogen ceases after 1–2 days (7). In the studies reported here, we have demonstrated that three mouse tumor cell lines with characteristics of macrophage or histiocytic tumors, and one identified as a myelomonocytic leukemia, release pyrogen during in vitro culture. Moreover, this release appears to occur spontaneously and continuously during cell growth and multiplication. This conclusion is supported by our data showing that increasing amounts of pyrogen relative to cell numbers appear in the culture media with time, that similar amounts of pyrogen are produced by cells chosen either 3, 4, or 5 days after seeding, and that pyrogen accumulates in culture media which is identical to standard growth media except for the absence of endotoxin. Thus, these tumor cells appear to have retained the capability to produce pyrogen that is characteristic of their nonmalignant cells of origin, but to have acquired an altered mechanism for production of pyrogen, since they apparently require no initiating stimulus, and production does not cease after 24–48 h.

In preliminary studies, we have determined that production and secretion of the tumor cell pyrogens is an active, energy-requiring process. Cells kept at 4°C failed to release pyrogen. At no time were we able to detect significant levels of intracellular pyrogen in the one cell line examined, even though pyrogen was present in increasing amounts in the extracellular medium. Small but significant increases in pyrogen production occurred after incubation of cells from two histiocytic tumor lines with staphylococci, during which time most cells ingested bacteria. These results are similar to those obtained in studies of pyrogen synthesis and release by normal granulocytes and monocytes (7), indicating that the tumor cells retain some of the properties associated with pyrogen production of their cells of origin. On the other hand, as noted above, they appear to have lost at least a part of the normal control mechanisms for initiation and cessation of transcription of the pyrogen molecule, presumably a gene-mediated function (26, 27).

The pyrogens released by these tumor cell lines appear similar to the endogenous pyrogens that are produced when normal phagocytic cells of bone marrow origin are stimulated by phagocytosis or other inflammatory agents. Pyrogen produced by the macrophage tumor lines J-774 and PU5-1.8 were inactivated by heating at 56°C for 1 h and by a 4-h incubation with pronase. All pyrogens produced febrile responses characteristic of EP in mice, as illustrated in Fig. 1. These properties are the same as those of endogenous pyrogens derived from normal human (20), rabbit (P. Bodel, unpublished observations) and mouse (10) granulocytes, monocytes, or macrophages. The estimated molecular weights of pyrogens produced by J-774 cells were, however, larger than those reported for all other endogenous pyrogens previously studied, including those produced by granulocytes, monocytes, and macrophages of human or rabbit origin (18, 28–30). We have not yet determined the molecular weight of mouse macrophage pyrogen because of the lability of the molecule; possibly, pyrogens from mouse leukocytes will also prove to be of unusual size. Alternatively, pyrogenic molecules produced by tumor cells may have different physico-chemical characteristics from pyrogens produced by their nonmalignant cells of origin.

When tumor lines of carcinomas or viral-transformed fibroblasts were studied, we failed to detect any pyrogen in culture supernates. Thus, although many carcinomas are associated with unexplained fever in occasional patients, our studies do not provide support for the hypothesis that such tumor cells can produce pyrogen. These data extend our previous observations that HeLa cells and fibroblasts did not produce pyrogen, even after ingestion of particles (11). Possibly, fever in patients that is associated with the growth of carcinomas is a result of host cell interactions with the tumor, producing stimulation of host granulocytes, monocytes, or macrophages to produce pyrogen, rather than the result of the release of pyrogen by the tumor cells themselves.

The same cell lines that released pyrogen in our studies have been shown by others to secrete lysozyme, and in all but one instance (RAW-8), to have other macrophage characteristics, detailed in Table II. Two of the cell lines produce colony-stimulating activity (CSA), either spontaneously (19), or after addition of agents such as lipopolysaccharide (LPS) or zymosan (25). Lymphocyte-activating factor (LAF) is produced by all four macrophage tumor cell lines, after stimulation by LPS (24). It is noteworthy that tumor cells of presumed B-lymphocyte origin (R-8) did not produce pyrogen in our experiments. Normal lymphocytes have never been found to produce pyrogen, even after antigenic or blastogenic stimulation (7).

Our results raise the possibility that tumor-associated fever in patients with certain malignancies, such as some acute leukemias and lymphomas, may be due to the production of endogenous pyrogen by the malignant cells of granulocytic or monocytic origin. By extrapolation from studies of the cell lines WEHI-3 and RAW-8, it seems likely that the pyrogen molecules can be produced by immature cells at a stage of maturation preceding differentiation into granulocytes or monocytes. The hypothesis that malignant granulocytes and macrophages release pyrogen spontaneously during growth would be consistent with the observation that tumor-associated fever occurs in patients with these diseases during relapse, or at times of disease activity, but not during remissions (1–3). Preliminary studies have also shown that the human histiocytic tumor cell line, U-937 (16), also releases pyrogen during in vitro culture (P. Bodel and P. Ralph, unpublished observations). Such cells may provide an easily available source of human EP for laboratory investigations.

Summary

Tumor-associated fever occurs commonly in acute leukemias and lymphomas. We investigated the capacity for in vitro production of pyrogen by three mouse histiocytic lymphoma cell lines (J-774, PU5-1.8, p 388 D_1), one myelomonocytic line (WEHI-3), and two lymphoma-derived lines, RAW-8 and R-8. Pyrogen was released spontaneously into the culture medium during growth by all cell lines with macrophage or myeloid characteristics including lysozyme production; R-8 cells, of presumed B-lymphocyte origin, did not produce pyrogen. When injected into mice, the pyrogens gave fever curves typical of endogenous pyrogen, were inactivated by heating to 56°C and by pronase digestion, and appeared to be secreted continuously by viable cells. Two pyrogenic molecular species produced by J-774 cells were identified by Sephadex filtration, one of mol wt $\cong$ 30,000,

and the other ≥60,000. By contrast, three carcinoma cell lines of human origin and SV-40 3T3 mouse fibroblasts did not produce pyrogen in vitro. These results suggest that some malignant cells derived from phagocytic cells of bone marrow origin retain their capacity for pyrogen production, and may spontaneously secrete pyrogen during growth.

The author thanks Ms. Heather Miller for carrying out the reported work on human cell lines and fibroblasts, and Doctors Elisha Atkins and Robert M. Donaldson for their helpful criticism during preparation of the manuscript.

Received for publication 9 January 1978.

References

1. Raab, S. P., P. D. Hoeprich, M. M. Wintrobe, and G. E. Cartwright. 1960. The clinical significance of fever in acute leukemia. *Blood.* **16**:1609.
2. Lobell, M., D. R. Boggs, and M. M. Wintrobe. 1966. The clinical significance of fever in Hodgkin's disease. *Arch. Intern. Med.* **117**:335.
3. Boggs, D. R., and E. Frei, III. 1960. Clinical studies of fever and infection in cancer. *Cancer (Phila.).* **13**:1240.
4. Berger, L., and M. W. Sinkoff. 1957. Systemic manifestations of hypernephroma. A review of 273 cases. *Am. J. Med.* **22**:791.
5. Berman, C. 1958. Primary carcinoma of the liver. *In* Advances in Cancer Research. J. P. Greenstein and A. Haddow, editors. Academic Press, Inc., New York. **5**:67.
6. Fenster, L. F., and G. Klatskin. 1961. Manifestations of metastatic tumors of the liver. *Am. J. Med.* **31**:238.
7. Atkins, E., and P. Bodel. 1973. Fever. *In* The Inflammatory Process. B. W. Zweifach, L. Grant, and R. T. McCluskey, editors. Academic Press, Inc., New York. **3**:467.
8. Bodel, P. 1974. Pyrogen release *in vitro* by lymphoid tissues from patients with Hodgkin's disease. *Yale J. Biol. Med.* **47**:101.
9. Bodel, P. 1974. Studies on the mechanism of endogenous pyrogen production. monocytes. *J. Exp. Med.* **140**:954.
10. Bodel, P., and H. Miller. 1976. Pyrogen from mouse macrophages causes fever in mice. *Proc. Soc. Exp. Biol. Med.* **151**:93.
11. Bodel, P., and H. Miller. 1977. Differences in pyrogen production by mononuclear phagocytes and by fibroblasts or HeLa cells. *J. Exp. Med.* **145**:607.
12. Koren, H. S., B. S. Handwerger, and J. R. Wunderlich. 1975. Identification of macrophage-like characteristics in a cultured murine tumor line. *J. Immunol.* **114**:894.
13. Ralph, P., and I. Nakoinz. 1975. Phagocytosis and cytolysis by a macrophage tumor and its cloned cell line. *Nature (Lond.).* **257**:393.
14. Warner, N. L., M. A. S. Moore, and D. Metcalf. 1969. A transplantable myelomonocytic leukemia in Balb/c mice: cytology, karyotype, and muramidase content. *J. Natl. Cancer Inst.* **43**:963.
15. Ralph, P., I. Nakoinz, and W. C. Raschke. 1974. Lymphosarcoma cell growth is selectively inhibited by B-lymphocyte mitogens: LPS, dextran sulfate and PPD. *Biochem. Biophys. Res. Commun.* **61**:1268.
16. Ralph, P., M. A. S. Moore, and K. Nilsson. 1976. Lysozyme synthesis by established human and murine histiocytic lymphoma cell lines. *J. Exp. Med.* **143**:1528.
17. Fogh, J., and G. Trempe. 1975. New human tumor cell lines. *In* Human Tumor Cells In Vitro. ed. Jorgen Fogh, editor. Plenum Publishing Corporation, New York. 115.
18. Haeseler, F., P. Bodel, and E. Atkins. 1978. Characteristics of pyrogen production by

isolated rabbit Kupffer cells *in vitro*. *J. Reticuloendothel. Soc.* In press.
19. Ralph, P., and I. Nakoinz. 1977. Direct toxic effects of immunopotentiators on monocytic, myelomonocytic, and histiocytic or macrophage tumor cells in culture. *Cancer Res.* **37**:546.
20. Bodel, P., and H. Miller. 1978. A new sensitive method for detecting human endogenous (leucocyte) pyrogen. *Inflammation*. In press.
21. Ralph, P., J. Prichard, and M. Cohn. 1975. Reticulum cell sarcoma: an effector cell in antibody-dependent cell-mediated immunity. *J. Immunol.* **114**:898.
22. Metcalf, D., M. A. S. Moore, and N. L. Warner. 1969. Colony formation in vitro by myelomonocytic leukemia cells. *J. Natl. Cancer Inst.* **43**:983.
23. Ralph, P., and I. Nakoinz. 1974. Lipopolysaccharides inhibit lymphosarcomas of bone marrow origin. *Nature (Lond.)* **249**:49.
24. Lachman, L. B., M. P. Hacker, G. T. Blyden, and R. E. Handschumacher. 1977. Preparation of lymphocyte-activating factor from continuous murine macrophage cell lines. *Cell. Immunol.* In press.
25. Ralph, P., H. E. Broxmeyer, and I. Nakoinz. 1977. Immunostimulators induce granulocyte/macrophage colony-stimulating activity and block proliferation in a monocyte tumor cell line. *J. Exp. Med.* **146**:611.
26. Bodel, P. 1970. Studies on the mechanism of endogenous pyrogen production. I. Investigation of new protein synthesis in stimulated human blood leucocytes. *Yale J. Biol. Med.* **43**:145.
27. Nordlund, J. J., R. K. Root, and S. M. Wolff. 1970. Studies on the origin of human leukocytic pyrogen. *J. Exp. Med.* **131**:727.
28. Bodel, P. T., A. Wechsler, and E. Atkins. 1969. Comparison of endogenous pyrogens from human and rabbit leucocytes utilizing Sephadex filtration. *Yale J. Biol. Med.* **41**:376.
29. Kozak, M. S., H. H. Hahn, W. J. Lennarz, and W. B. Wood, Jr. Studies on the pathogenesis of fever. XVI. Purification and further chemical characterization of granulocytic pyrogen. *J. Exp. Med.* **127**:341.
30. Dinarello, C. A., N. P. Goldin, and S. M. Wolff. 1974. Demonstration and characterization of two distinct human leukocytic pyrogens. *J. Exp. Med.* **139**:1369.