FAILURE OF RABBIT NEUTROPHILS TO SECRETE ENDOGENOUS PYROGEN WHEN STIMULATED WITH STAPHYLOCOCCI*

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Endogenous pyrogen (EP)[1] was first described by Beeson (1) in saline extracts of rabbit peritoneal exudate cells. These cells were >95% neutrophils, and it was assumed that neutrophils were the source of the pyrogen (1). A series of studies over the last 30 yr has established that EP is the common mediator of almost all febrile states that are not attributable to disorders of the thermoregulatory complex in the nervous system (2). We activated peritoneal exudate cells by using three different stimuli in the presence of radiolabeled amino acids. The EP were subsequently purified to homogeneity (3) and were shown to be radioactive. Furthermore, EP secreted in response to endotoxin, staphylococci, or tuberculin were identical. The purified EP had an ~13,000 mol wt and an isoelectric point of 7.3 (4).

We have recently shown that purified rabbit macrophages secreted four EP. One EP appeared to be identical with the EP previously purified from peritoneal exudate cells that were mostly neutrophils. The other EP were a family of proteins with isoelectric point (pI) values between 5.0 and 5.2. When macrophages were stimulated in the presence of radiolabeled amino acids, the purified pI 5.0–5.2 pyrogens aligned with peaks of radioactivity. This suggested that these EP were also synthesized *de novo* when the macrophages were stimulated. Also, EP from macrophages stimulated with endotoxin co-isoelectric focussed with EP from macrophages stimulated with staphylococci (5).

Because macrophages appeared to make all the known EP, we reinvestigated the question of what EP neutrophils made, if any. We purified neutrophils and monocytes or macrophages from rabbit blood and from peritoneal exudates. We tested the purified neutrophils for pyrogen production and for various other functional activities. The results are described in this paper.

Materials and Methods

Preliminary experiments showed that when peritoneal exudates were induced with glycogen, the cells did not separate well on gradients. We therefore returned to Beeson's original technique (1) of injecting 500 ml of sterile 0.15 M sodium chloride into the peritoneal cavity and removing the cells 16–18 h later. Cell yields were lower, and differential counts showed that ~50% of the

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[1] *Abbreviations used in this paper:* CFU, colony-forming units; EP, endogenous pyrogen(s); FMLP, *N*-formyl Met-Leu-Phe; MEM, minimum essential medium; pI, isoelectric point; PMN, polymorphonuclear cell(s); RBC, erythrocyte(s); ZAS, zymosan-activated serum.

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cells present were mononuclear. Rabbit blood was obtained either from the heart or the central ear artery. It was anticoagulated with 3.8% sodium citrate (10% vol/vol).

All cells were processed in Hanks' minimum essential medium (MEM) (Grand Island Biological Co., Grand Island, N. Y.) that contained no divalent cations and that also contained 10% vol/vol of 3.8% sodium citrate. The pH was adjusted to 7.2 with 1 N HCl before use. This medium was also used in all gradients. When the cells had been purified and washed, they were transferred into the medium appropriate to the test being done. This was RPMI-1640 (Grand Island Biological Co.) that contained 100 U/ml penicillin and 50 μg/ml streptomycin for pyrogen production, and MEM with Earle's salts (Grand Island Biological Co.) for all other functional tests. For tests of superoxide generation, phenol red was omitted.

Peritoneal exudates were centrifuged at 400 *g* for 10 min, and then the supernate was discarded. The cells were washed twice in citrated MEM and suspended at a final concentration of 1×10^7 cells/ml. A small sample of the suspension was deposited onto glass slides with a Shandon cytofuge (Shandon Southern Instruments Inc., Sewickley, Pa.). These slides were stained with Wright's and Giemsa stains, and for alkaline phosphatase activity (6).

Density gradients were constructed using colloidal silica (Percoll, Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.). The material as received from the manufacturer was diluted with one-ninth its vol of 10-fold MEM. This mixture was called 100% Percoll. Lower concentrations were prepared by diluting 100% Percoll with the appropriate volume of citrated MEM. The simplest gradient used was linear from 20 to 100% Percoll. Some step gradients were also used, the most common being equal parts of 20, 50, and 80% Percoll. Washed peritoneal exudate cells were carefully layered over the Percoll in a 250-ml centrifuge bottle and were centrifuged at 400 *g* for 1 h at room temperature. The cells separated into two clearly visible bands that were aspirated separately; the intermediate layer was discarded. The separated cells were centrifuged at 400 *g* for 10 min at 4°C, resuspended in citrated MEM, and again centrifuged. They were then suspended in the appropriate medium. Small specimens were taken for cell counts and smears.

Blood was easily separated into monocytes and polymorphonuclear cells (PMN), but the latter cells were contaminated with all the erythrocytes (RBC). Purified PMN could be obtained only by procedures that led to excessive losses. Because pyrogen production is uninfluenced by the presence of blood or RBC (7), we devised the following procedure: 50 ml of 100% Percoll and 100 ml of 75% Percoll were layered in a 250-ml centrifuge bottle, and 100 ml of rabbit blood, diluted 1:1 with citrated MEM, was layered on top. The bottles were centrifuged at 400 *g* for 1 h at room temperature. The upper band of cells was harvested and was used for pyrogen production after the washing procedure described above. The intermediate layer was discarded. The RBC/PMN layer was washed once in citrated MEM, diluted again in citrated MEM and cell counts and differentials were done. It was then divided into equal halves; one was transferred to RPMI-1640 and used for tests of pyrogen production. The other half was diluted with 6% dextran (average 234,000 mol wt) (Sigma Chemical Co., St. Louis, Mo.) in 0.15 M sodium chloride to make a final dextran concentration of 2%. The supernate was removed as soon as the bulk of the RBC had settled. The cells were centrifuged down at 400 *g* for 10 min, and exposed to 0.03 M sodium chloride for 30 s. Tonicity was restored with 0.27 M sodium chloride, the cells were then centrifuged at 400 *g* for 10 min at 4°C and were suspended in MEM at a concentration of 4×10^7/ml.

Opsonized zymosan particles were prepared by exposing 100 mg of zymosan (Sigma Chemical Co.) to 10 ml of normal rabbit serum for 30 min at 37°C. The particles were centrifuged down and washed once with 0.05 M sodium chloride. The serum was kept frozen at −20°C and is referred to as zymosan-activated serum (ZAS). For experiments on phagocytosis, cells were diluted to 5×10^6/ml in MEM and exposed to 10 opsonized zymosan particles per cell for 15 min in a roller tube system at 37°C. Smears were made, and the number of particles taken up per cell was determined.

Superoxide generation was measured by mixing 5×10^6 cells with 0.1 μmol of cytochrome *c* and 5×10^7 opsonized zymosan particles in a total vol of 1 ml. Controls included tubes in which the cells or the opsonized zymosan particles were omitted, or to which 10 μmol of superoxide dismutase (Sigma Chemical Co.) had been added. The mixtures were incubated for 15 min at 37°C and then centrifuged. The supernates were aspirated, and the optical density

was read at 550 nm. Only that fraction of the OD that was inhibited by superoxide dismutase was used to calculate rates of superoxide production. Results were expressed as nanomoles of superoxide generated per minute per 10^7 cells, and assays were usually done in duplicate.

Chemotaxis was measured in 1% agarose gels (Seakem, Marine Colloids Div., FMC Corp., Rockland, Maine) that contained 0.25% gelatine (BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville, Md.) and MEM. Wells were 2 mm in diameter, and were usually set 5 mm apart. Some experiments with ZAS as the chemotactic agent were done with wells 2.5 mm apart. Three rows of four wells could be cut on a single 3- × 1-in. slide. 4×10^5 cells in 10 μl MEM were added to the central wells, and 10 μl MEM was added to all lower wells. To the upper wells were added varying amounts of chemotactic agents or MEM. The slides were incubated at 37°C for 2 h in 5% CO_2, and were then flooded with methanol and left overnight. The agarose layers were stripped off, and the adherent cells were stained with Wright's stain. The distance moved towards buffer or chemotactic agent was measured with an ocular micrometer. Assays were done at least in quadruplicate; the average distance moved and its standard error are the values used in the tables.

Bacterial killing was measured with *Staphylococcus epidermidis* opsonized with normal rabbit serum. 5×10^6 cells were suspended in 1 ml MEM with 5×10^6 colony-forming units (CFU) of opsonized *S. epidermidis.* Control tubes contained bacteria but no cells. The mixtures were tumbled at 37°C for 2 h; samples were diluted and plated at 0, 1, and 2 h. The number of CFU in cell and control tubes at the various times was determined, and killing was expressed as the percentage of reduction in CFU in the cell tubes as compared with the corresponding controls.

Cells were tested for the ability to secrete EP by stimulating them with 50 CFU opsonized *S. epidermidis* per leukocyte. Leukocytes were suspended at a concentration of $1–2 \times 10^6$/ml in RPMI-1640, 100-ml portions were placed in 850-cm^2 roller tissue culture bottles (Corning Glassworks, Science Products Div., Corning, N. Y.), and the staphylococci were added. The flasks were gassed with 5% CO_2 in air, and rotated at 37°C for 24 h. The culture fluids were removed, centrifuged at 27,000 *g* for 20 min at 4°C, filtered through an 0.45-μm membrane filter, and stored at 4°C.

Pyrogenic activity was determined by direct intravenous injection of supernates of stimulated leukocytes. Supernates from large numbers of PMN occupied an inconveniently large volume, and sometimes caused low, irregular, febrile responses (Results). Therefore, these supernates were concentrated with ammonium sulfate precipitation. Ammonium sulfate, which had been baked at 170°C for 3 h to render it sterile and endotoxin free, was added to the supernate in the amount of 561g/liter (80% saturation at room temperature). The mixture was allowed to stand for 18 h at 4°C. The precipitate was centrifuged down at 27,000 *g* for 60 min at 4°C, dissolved in a small quantity of sterile water, and dialysed against 0.15 M sodium chloride with a dialysis membrane whose average pore size corresponded to a 3,000 mol wt (Spectrapor 3, Spectrum Medical Industries Inc., Los Angeles, Calif.). The concentrated dialyzed supernates were filtered through a 0.45-μm membrane filter and were tested for pyrogenicity by intravenous injection into rabbits.

If an injection caused a measurable mean febrile response (>0.2°C), we calculated the number of cells that would have secreted 1°C unit of pyrogen (8). If there was no measureable febrile response (<0.1°C in all cases), we assumed that we would have detected any mean febrile response of 0.3°C or greater, and used the 0.3°C figure to calculate a minimal number of cells per pyrogen unit. For PMN, we quote two values, one obtained by direct injection of culture fluids, the other (usually larger) obtained by injection of ammonium sulfate-precipitated material.

As far as possible, we used cells from the same rabbits both for tests of pyrogen production and for tests of typical neutrophil activities such as phagocytosis. To make clear which experiments were done with which batch of cells, we have used corresponding experiment numbers in all tables. Any experiment with an E number was done with peritoneal exudate cells, any experiment with a B number with blood cells.

Results

The efficiency of cell separation attained is shown in Table I. Neutrophils were invariably obtained free of macrophages (<0.5% in each of nine experiments as judged

TABLE I

Separations Achieved with Percoll Gradients for Rabbit Peritoneal Exudate and Blood Cells

Experiment	Band	Number of cells $\times 10^8$	PMN	M*	L
E1	T	2.2	61	30	9
	B	2.4	100	—	—
E2	T	4.9	0	87	13
	B	4.2	100	—	—
E3	T	15	10	85	6
	B	5.3	100	—	—
E4	T	4.2	3	94	3
	B	7.9	100	—	—
B1	T	18.4	8	21	71
	B	16.0	80	0	20
B2	T	8.2	1	32	68
	B	18.0	77	0	23
B3	T	—	—	—	—
	B	2.4	91	0	9
B4	T	16	13	40	45
	B	46	93	0	7
B5	T	12	12	13	75
	B	8	59	0	41

* M, macrophages; L, lymphocytes; T, top band of cells on the gradient; B, bottom band.

by Wright's, Giemsa, and alkaline phosphatase staining). In experiments with blood cells, a few lymphocytes contaminated the neutrophil preparations. Macrophages were usually contaminated with small numbers of neutrophils, but there was no noticeable difference in pyrogen yield whether or not a few neutrophils were present. Macrophages were invariably heavily contaminated with lymphocytes; these were generally ignored (9). A few experiments were done in which macrophages were allowed to adhere to the culture bottles for 24 h, and the nonadherent cells were discarded. The results were no different whether or not lymphocytes were removed.

The neutrophils isolated on these gradients were ~85% viable, as judged by their ability to exclude Buffalo Black. In all experiments, there was avid phagocytosis of opsonized zymosan particles. The number of particles per cell after 15 min of incubation was 1.3 averaged over all the experiments. There was no obvious difference in this regard between blood and peritoneal exudate neutrophils.

In all experiments, neutrophils purified on gradients and stimulated with opsonized zymosan, made superoxide (Table II). The basal rate of superoxide production varied. Peritoneal exudate neutrophils usually produced some superoxide spontaneously, whereas blood neutrophils did not. Purified neutrophils made as much or more superoxide as did the unpurified peritoneal cells, even when the mixtures were adjusted to contain equal numbers of neutrophils.

The results of the chemotactic assays are shown in Table III. Peritoneal exudate neutrophils responded well to *N*-formyl Met-Leu-Phe (FMLP), but not at all to ZAS or to antigen-antibody complexes. Blood neutrophils were also found to respond well to FMLP; but initially their response to ZAS was variable, although some responses were clearly positive. It turned out that this variable response of blood neutrophils to

TABLE II
Superoxide Production by Rabbit Neutrophils Purified from Blood or Peritoneal Exudates

Experiment	Superoxide production by resting cells	Superoxide production by stimulated cells
	nM/10⁷ cells/min	*nM/10⁷ cells/min*
E3	0.65	1.0
E4	0.5	1.2
E6	ND	1.13
E7	0.7	1.6
B1	ND	1.18
B2	<0.1	0.89
B4	ND	1.3
B5	0.15	0.38
B6	<0.1	0.66

ND, not done because insufficient cells were available.

TABLE III
Distances Moved Toward Various Chemotactic Substances By Neutrophils Purified from Peritoneal Exudates and from Blood

Experiment	Stimulus			
	Buffer	2.5×10^{-8} M FMLP	1×10^{-8} M FMLP	ZAS
E3	2.8 ± 0.1	10.5 ± 0.2	9.0 ± 0.4	2.8 ± 0.1
E4	5.2 ± 0.2	7.9 ± 0.2	7.1 ± 0.5	ND
E6	2.6 ± 0.1	5.3 ± 0.5	5.8 ± 0.2	2.8 ± 0.4
E7	2.7 ± 0.1	8.1 ± 0.2	7.2 ± 0.2	2.4 ± 0.1*
B1	3.5 ± 0.1	8.5 ± 0.3	5.5 ± 0.5	3.4 ± 0.3
B2	2.5 ± 0.2	8.4 ± 0.5	9.0 ± 0.5	2.9 ± 0.4
	2.0 ± 0.1	ND	ND	4.6 ± 0.5‡
B4	5.6 ± 0.2	10.4 ± 0.3	7.9 ± 0.2	10.4 ± 0.3*

All FMLP (and unmarked ZAS column) experiments were done with wells cut 5 mm apart and with an incubation period of 2 h. ND, not done.
* Wells 2.5 mm apart, incubated 2 h.
‡ Wells 5 mm apart, incubated 3 h.

the C5a chemoattractants was because we had cut the wells 5 mm apart and were incubating for only 2 h. If we incubated longer, or used wells only 2.5 mm apart, we got good chemotactic responses to ZAS with blood neutrophils. The chemotactic responses of purified neutrophils were, on the whole, better than those of unpurified cells, even when the suspensions were adjusted to contain equal numbers of neutrophils.

Neutrophils from blood or from peritoneal exudates killed *S. epidermidis* efficiently in all experiments (Table IV).

In all of these experiments, we were never able to demonstrate convincing evidence for pyrogen production by purified neutrophils, whether they came from blood or peritoneal exudates (Table V). Supernates from 4×10^7 neutrophils or less were invariably negative. When we injected supernates of large numbers of cells (2.4×10^8) we sometimes saw low, irregular fevers (Fig. 1). These did not appear to be

TABLE IV

Killing of S. epidermidis by Neutrophils Purified from Blood and from Peritoneal Exudates

Experiment	Percentage of killing	
	1 h	2 h
E7	88	95
B1	85	95
B2	88	ND
B3	88	92

ND, not done.

TABLE V

Pyrogen Liberation by Macrophages and Monocytes Isolated on Gradients, and Failure to Demonstrate Pyrogen Production by Neutrophils Isolated on the Same Gradients

Experiment	Cell type		
	PMN		Monocyte-macrophage
	DI	AS	
E1	$>7 \times 10^8$	$>1 \times 10^9$	2.8×10^6
E2	$>2 \times 10^7$	$>1.1 \times 10^8$	2.6×10^6
E3	$>3 \times 10^7$	$>1.2 \times 10^8$	2.7×10^6
E4	$>5 \times 10^7$	$>2 \times 10^8$	3.9×10^6
B1	$>1 \times 10^8$		1.9×10^6
B2	$>1.7 \times 10^8$		2.5×10^6
B4	$>1 \times 10^9$		3.2×10^6
B5	$>5 \times 10^7$	$>4 \times 10^8$	1.0×10^6

Each table entry is the number of cells required to make 1 U of pyrogen (1 U of pyrogen causes a mean febrile response of 1°C when injected intravenously into a rabbit). DI, estimate obtained by direct injection of cell supernates; AS, estimate obtained after ammonium sulfate precipitation. In experiment B4, the PMN fraction was put up at 1.2×10^7 cells/ml. This enabled us to test the supernate of 2.4×10^8 cells by direct injection.

attributable to EP, and were not seen in material precipitated with ammonium sulfate. Furthermore, even when accepted at face value, they indicated that $>1 \times 10^9$ PMN would be needed to secrete 1 U of pyrogen.

It was possible that ammonium sulfate would not precipitate minute amounts of EP from large volumes of dilute solution, or that some enzyme in neutrophil supernates would render the EP inactive when they were coprecipitated. We therefore undertook a special experiment (Table V, B5). The neutrophil fraction contained 8×10^8 PMN, and was put up in 400 ml RPMI. 10 ml of this supernate, corresponding to 2×10^7 PMN, was tested by direct injection in four rabbits and was negative. The remaining 360 ml was divided into two equal halves. 0.1 ml of a purified pI 7.3 EP preparation, which contained five 1°C units of pyrogen was added to one half. The other half served as a control. Both preparations were precipitated with ammonium sulfate, and the precipitates were dissolved in water, dialyzed against saline, and filtered as usual. Each preparation was then injected intravenously into five rabbits.

The preparation to which pyrogen had not been added was completely negative,

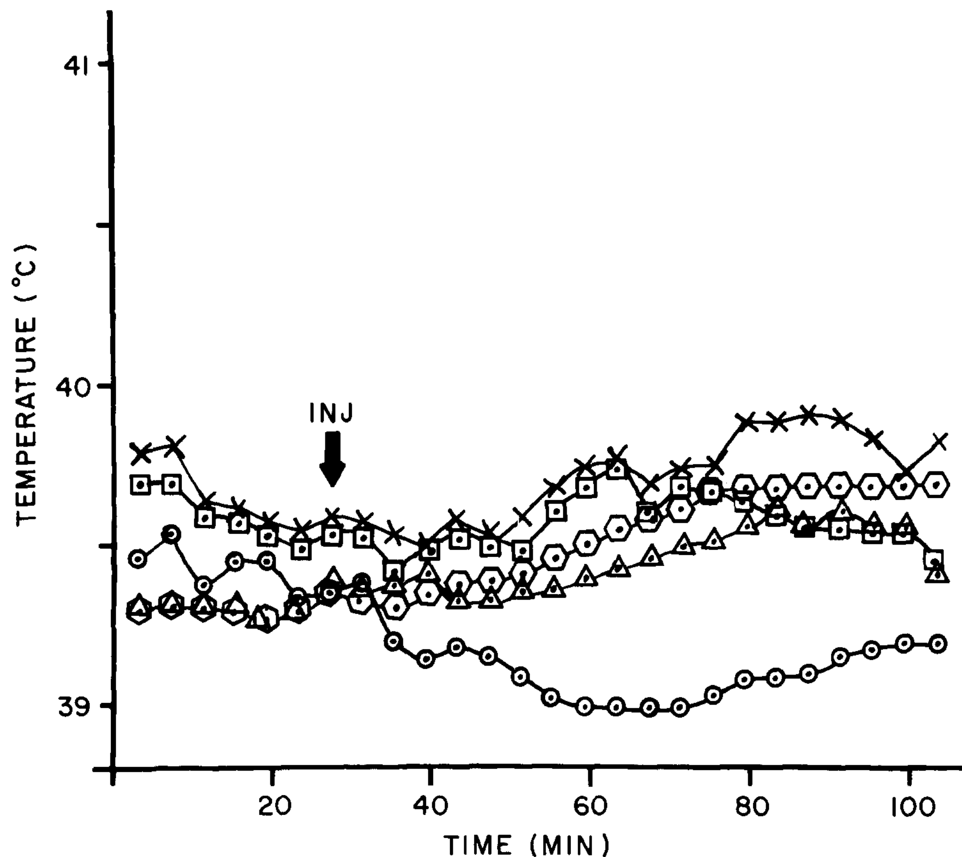

Fig. 1. Temperature responses of five rabbits injected intravenously with the supernate of 2.4×10^8 PMN. Inj, injection given. From experiment B4.

although each rabbit had received the proteins secreted by 6.4×10^7 PMN (Fig. 2). The five rabbits injected with the preparation to which five 1°C units of pyrogen had been added developed a mean febrile response of 1.11°C (Fig. 3).

We had no difficulty demonstrating pyrogen production by monocytes and macrophages isolated on Percoll gradients. Furthermore, the number of macrophages required to make 1°C unit of pyrogen was reasonably constant (Table V), averaging $\sim 2.5 \times 10^6$ cells. Because it was possible that some kind of cooperative interaction between neutrophils and macrophages and/or lymphocytes was required before neutrophils could make pyrogen, we tried the effect of adding back macrophages and lymphocytes to gradient-purified neutrophils. Pyrogen formation was observed, but the amount was exactly what would have been expected on the basis of the number of macrophages added (Table VI).

Discussion

The neutrophils isolated during these experiments appeared to be normal by a variety of functional tests. The most important of these tests was probably that of superoxide generation. Because superoxide is unstable, a positive result in this test

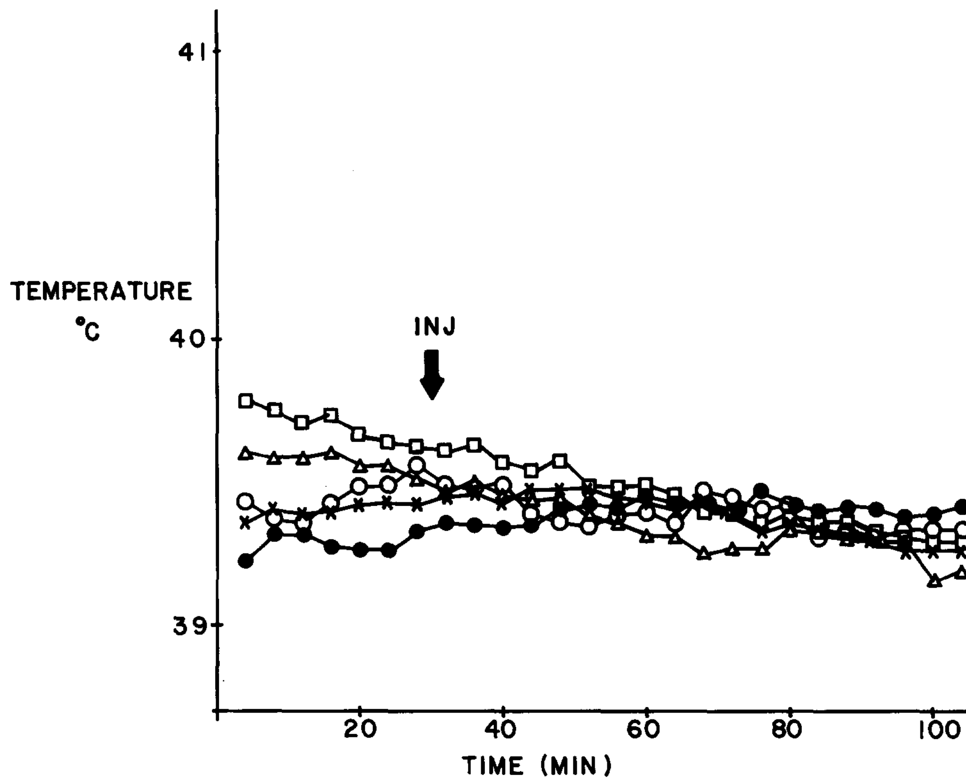

FIG. 2. Temperature responses of five rabbits injected intravenously with the supernate of 6.4 × 10^7 PMN, concentrated by ammonium sulfate precipitation. Inj, injection given. From experiment B5.

meant that the cell was alive and functional at the time the test was done. Bacterial killing, for example, could conceivably be mediated by substances synthesized and stored in granules by a cell dead at the time of test. However, it is difficult to see how dead cells could phagocytose, demonstrate random motility, or respond to chemotactic agents.

In the case of neutrophils from peritoneal exudates, the cells used to make pyrogen were treated in exactly the same way as those used for functional tests. In the case of blood neutrophils, this was not possible because the complete purification of neutrophils led to heavy losses, and the number left was too small to test for pyrogen secretion in an adequate manner. Therefore, we took advantage of the fact that RBC are irrelevant to pyrogen production (7), and used the RBC/PMN layer directly for tests of pyrogen secretion. We reasoned that neutrophils in the RBC/PMN mixture were unlikely to do worse in functional tests than neutrophils subjected to extensive further processing.

The only functional test with which we had trouble in getting positive results was the chemotactic response to ZAS. The chemoattractant in this case was presumably C5a, and this has an ~15,000 mol wt. FMLP has a 438 mol wt, and, therefore, one would expect FMLP to diffuse much faster than C5a. Part of our troubles was, thus, a result of cutting the wells too far apart. However, even when that was corrected,

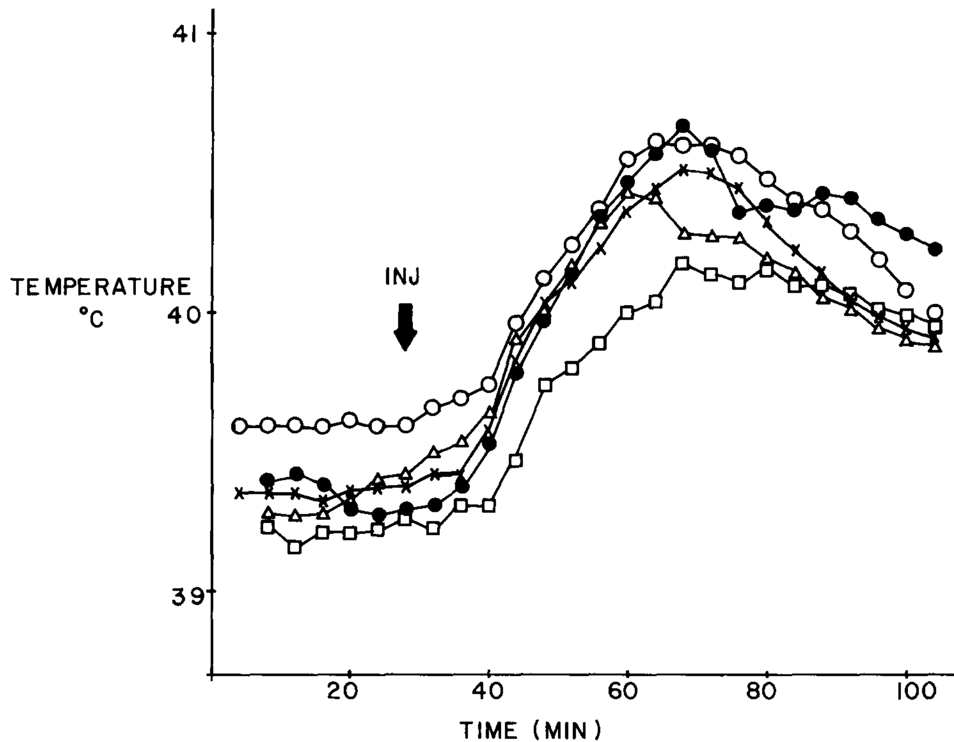

FIG. 3. Temperature responses of five rabbits injected intravenously with the supernate of 6.4×10^7 PMN plus 1 U of pyrogen, concentrated by ammonium sulfate precipitation. Inj, injection given. From experiment B5.

TABLE VI
Failure of Monocytes to Stimulate Pyrogen Production by Neutrophils

Cell mixture	Number of cells per 1°C unit of pyrogen
Original blood (unfractionated)	3.4×10^6 monocytes
Monocyte fraction	3.3×10^6 monocytes
Neutrophil fraction	$>2.1 \times 10^8$ PMN
Neutrophils + monocytes reconstituted in original proportions	3.4×10^6 monocytes

Each table entry is the number of cells required to make 1°C unit of pyrogen.

peritoneal exudate PMN did not respond to ZAS, whereas blood PMN did. It seemed likely that the exudate PMN were unresponsive to C5a because they had accumulated in the peritoneum in response to it (10), and had become deactivated to complement but not to bacterial stimuli (11). The blood PMN, not having been exposed previously to C5a, responded normally.

Failure to demonstrate EP production by direct injection of supernates from known numbers of stimulated cells seems unequivocal. All such experiments were negative, except B4, where we injected the supernate from 2.4×10^8 PMN into each rabbit. We think that the low-grade, prolonged febrile responses shown in Fig. 2 were probably

not caused by EP. The onset was delayed, the duration of the fevers was prolonged, and not all rabbits injected developed fever. We think it more likely that these minimal responses were caused by the injection of 20 ml of crude supernate that contained bacterial and cellular debris. The largest mean febrile response observed was 0.39°C (five rabbits); another experiment showed 0.24°C (five rabbits). These values were inflated by the convention of recording only positive fevers (negative values count as zero). Even if these responses were accepted at face value, $>1 \times 10^9$ PMN would be required to produce 1°C unit of EP. This is from 300 to 500 times the number of macrophages that can secrete 1 U of pyrogen, and we cannot exclude 0.2–0.3% contamination of our neutrophil preparations by macrophages.

Failure to demonstrate EP in ammonium sulfate precipitates was significant only if ammonium sulfate did in fact precipitate EP. We have clear evidence that 60% ammonium sulfate precipitated both species of EP from solutions that contained 1–10 U/ml, and that recovery was essentially quantitative (D. F. Hanson. Unpublished observations.). However, in the experiments of this paper, we were trying to precipitate 1–5 U of EP from several hundred milliliters of culture fluid, and we had no evidence that ammonium sulfate precipitated EP from solutions so diluted. Support for the idea that ammonium sulfate would have precipitated EP had they been present was obtained from the experiment in which we added 5 U of EP to a RBC/PMN supernate, and were able to recover 5.5 U after ammonium sulfate precipitation. No quantitative significance can be attached to these figures, because one cannot get quantitative results from small amounts of pyrogen. However, the yield was clearly high.

Taken together, the results from direct injection of culture fluids and of ammonium sulfate-precipitated material suggested that if PMN made any EP at all, they did so at least 100 times less efficiently than did macrophages. Once one questions Beeson's (1) original assumption that the pyrogen he found came from the neutrophils that constituted 95% of the cell population that he extracted, no hard evidence that neutrophils make pyrogen can be found. W. Barry Wood's 20 yr of work was done with unpurified peritoneal exudate cells, as was ours, until very recently. Many workers have noted that neutrophils make much less pyrogen per cell than do macrophages (12, 13). Reference to those papers shows that the neutrophil populations discussed contained some macrophages. We are well aware that neutrophils may yet be found to produce pyrogen under some circumstances. We used only one of the many stimuli that cause the secretion of EP, and it is conceivable that some other stimulus may be found to work, or that neutrophils purified in some different way will prove to be active with staphylococci. Nevertheless, the stimulus and the conditions of incubation were those that we and other pyrogen workers have used for years in the routine production of EP.

On the other hand, there is no doubt that purified macrophages make pyrogen (5, 12, 13). Furthermore, they have been shown to make all the known endogenous pyrogens (5). And in these experiments, we had no difficulty demonstrating pyrogen production by macrophages purified on colloidal silica gradients. Macrophages respond to most of the chemotactic stimuli that attract neutrophils, and small numbers of macrophages are likely to be found with neutrophils in all inflammatory exudates. It seems entirely possible that all pyrogen production by neutrophil-rich exudates is attributable to the few macrophages that they contain.

Summary

Cells obtained from acute peritoneal exudates in rabbits were separated into neutrophil and mononuclear populations by centrifugation on colloidal silica gradients. When these populations were separately incubated in tissue culture medium in the presence of opsonized *Staphylococcus epidermidis*, endogenous pyrogen was secreted only by the adherent cells of the mononuclear population. Pyrogen production by neutrophils could not have amounted to as much as 1% of the pyrogen produced by macrophages. When mononuclear cells were added back to purified neutrophils. no pyrogen was produced that could not be accounted for by the number of macrophages added.

Rabbit blood cells were similarly fractionated on colloidal silica gradients. Again, endogenous pyrogen was made only by the adherent mononuclear population.

The neutrophils isolated on these gradients appeared to be morphologically normal and were 85% viable as judged by dye exclusion. They showed normal random motility. Both blood and exudate neutrophils responded chemotactically to *N*-formyl Met-Leu-Phe, and blood neutrophils responded chemotactically to zymosan-activated serum. Both kinds of neutrophils phagocytosed zymosan particles and both killed opsonized *S. epidermidis* in a roller tube system. Both blood and exudate neutrophils showed normal superoxide production when stimulated with opsonized zymosan particles.

This evidence suggests that macrophages are the only source of endogenous pyrogens, and that pyrogens secreted by cell populations that are rich in neutrophils are to be attributed to the monocytes or macrophages that they contain.

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