Electron Microscopic Observations of Human Leucocytes

*II. Appearance in Naturally Occurring Fevers**

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Believing as I do in the continuity of nature, I cannot stop abruptly where our microscopes cease to be of use. Here the vision of the mind authoritatively supplements the vision of the eye.

John Tyndall
Address at Belfast, 1874

When human leucocytes are artificially stimulated *in vivo* or *in vitro* by a bacterial pyrogen, they release, without destroying themselves, a pyrogenic substance that differs chemically and biologically from the original bacterial pyrogen (Snell *et al.*, 1956; Cranston *et al.*, 1956). Although leucocytic pyrogen has not been seen, or at least recognized, by light or phase microscope, a finely granular extracellular material (fig. 1) is consistently visible by electron microscope in artificially stimulated leucocyte preparations that we know to be pyrogenic (Goodale, Fillmore, and Hillman, 1962).

We are reasonably certain that the granular material is a genuine cellular product in response to the stimulation and, although definite proof is lacking, that it represents, at least in part, leucocytic pyrogen. If the artificially induced granular material does indeed represent leucocytic pyrogen, and if leucocytic pyrogen is responsible for naturally occurring human fevers, it would follow that the same granular material should also be visible in leucocyte preparations from febrile patients with a variety of diseases. The purpose of this study is to report the findings by electron microscope in preparations of human leucocytes that have been "naturally" stimulated *in vivo* by pathological processes ranging from infections to terminal carcinomas.

Materials and Methods

All equipment was sterilized and made pyrogen-free by heating at 180° for 2 hours. Glassware was siliconized. A 3% dextran solution (molecular weight 186,000 in 0.9% saline, from Pharmachemical Corporation, Bethlehem, Pa.), was usually used to sediment the erythrocytes. This solution was tested in rabbits to ensure its freedom from pyrogens, as was the autoclaved 0.9% saline that was used for control purposes. The patients (table 1) were drawn from the medical and surgical wards of the Albany Medical Center Hospital, without selection as to age, type of disease, or treatment but with regard only to the height and duration of the fever.

Blood (20 ml) was drawn from an antecubital vein of each febrile patient into a glass syringe with the use of heparin (10,000 U.S.P. units per 10 ml) as the anticoagulant. When the patients had been afebrile for at least 48 hours (except for patient ※17 in table 1, who had been afebrile 20 hours) 20 ml of blood were again drawn and a control leucocyte preparation made by exactly the same method as that used for the febrile leucocyte preparation. Many patients died before becoming afebrile, so that from them control samples could not be obtained. The blood was divided into two equal parts and placed in centrifuge tubes. In most cases a volume of 3% dextran equal to the volume of blood was added to sediment the erythrocytes. (In a few instances erythrocyte sedimentation was accomplished over a longer period without the use of dextran.) After sedimentation for 20 minutes in a 37°C water bath, the leucocyte-rich plasma from each sample was aspi-

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rated and centrifuged at 4°C for 5 minutes at 1,000 rpm (250 × g) in an International Refrigerated Centrifuge, model PR-2. Unless otherwise stated, all subsequent steps were carried out at 4°C.

The supernatant fluids were discarded and one of the two cell buttons thus obtained were processed as follows. The cells were washed twice, with 10 ml of 0.9% saline for each wash and then incubated in 10 ml of saline for 30 minutes at 37°C. They were then fixed and embedded exactly as to be described for the cells in the second button. The cells in the second button were fixed immediately for 1 hour with 2% osmium tetroxide in Dalton's buffer (pH 7.4). The osmium-leucocyte mixture was centrifuged at 1,000 rpm for 5 minutes and the supernatant discarded. After suspension and thorough mixing in 10% ethanol, the cells were centrifuged at 1,000 rpm for 3 minutes. In this manner the cells were dehydrated through a series of graded ethanol solutions, washed three times in absolute ethanol, once in equal parts of absolute ethanol and methacrylate (7 parts butyl methacrylate to 1 part methacrylate), and then washed once in the methacrylate mixture alone. Final embedding was in gelatin capsules containing the prepolymerized methacrylate mixture with 1.5% benzoyl peroxide as a catalyst. Polymerization was completed at 55°C overnight.

Sections for both phase and electron microscopy were cut with a Servall Porter-Blum microtome by using glass knives. Sections were mounted on formvar-coated grids and were examined with either a Siemens Elmiskop I (60 KV), RCA EMU-3F (50 KV), or RCA EMU-3G (50 KV) electron microscope. Most grids were examined unstained but a few were stained with uranyl acetate by floating them on a 1% solution for 15 to 45 minutes at room temperature, then washing (by flotation) in distilled water for 1 to 2 minutes. A few additional grids were stained with lead (Karnovsky, 1961). For each patient in whom the granular material was easily found, a minimum of eight and an average of ten "febrile" and a similar number of control (if obtainable) grids were examined. For each patient in whom the granular material was not easily seen or not seen at all, up to 50 grids were examined with an average of approximately 25. Selection of patients occurred only in that we tried to find adult patients with fevers ranging from mild to marked and from a few hours to sometimes several weeks' duration.

Results

The results are summarized in tables 1 and 2. The finely granular extracellular material present in electron micrographs of leucocyte preparations from naturally febrile patients is morphologically identical to that seen in artificially stimulated leucocyte preparations. The granules sometimes appear singly but usually are in clumps or aggregates varying from about 0.1 to 1.0 μ in diameter. Individual granules are of two sizes: the smaller (approximately 50 Å in diameter) make up the great bulk of the aggregates while the larger (400 to 800 Å in diameter) relatively infrequent granules are usually peripherally located. The only morphological difference between the two types of leucocyte preparations is in the leucocytes themselves. Those cells artificially stimulated appear normal (figs. 1 and 2), while those "naturally" stimulated by the febrile process (figs. 3 to 8), almost all contain cytoplasmic vacuoles, from 0.2 to 2.0 μ in diameter, from 1 or 2 to 20 in number, with many of them containing the finely granular material. The number of vacuoles and the amount of granular material in vacuoles were greatest when the blood was drawn as the fever was rising. About half the vacuoles contain, in addition to the granular material, rounded bodies, 800 to 1200 Å in diameter, with distinct external membranes. These bodies are in the same size range as the larger granules of the extracellular material, but whereas the latter have a uniform appearance throughout, those in the vacuoles usually appear empty. In patients afebrile for 48 hours, normal cell structure was observed (fig. 7).

In 14 cases, half of the leucocytes were washed free of plasma and incubated with saline prior to preparation for the electron microscope in order to find out if they released granular material during incubation. Eight of these preparations contained the granular material either within cytoplasmic vacuoles or outside the cell.

Although the quantity of granular material in the leucocyte preparations cannot be accurately assessed, it is possible to say (1) that all preparations of leucocytes collected while the fever was rising or stable (15 cases) contained the granular material, (2) that leucocytes from patients with rising fevers are frequently vacuolated and that the vacuoles often contain granular material, and (3) that granular material is usually not seen in leucocyte preparations collected while the fever is waning (3 of 7 cases) and is infrequently (2 of 12 cases) seen when no fever is present.

In leucocyte preparations from nine patients, phagocytosis of platelets by neutrophils was seen (fig. 8). The platelets were generally intact and could be easily recognized. Platelet phagocytosis

TABLE 1

Data from 22 Patients with Fevers Due to a Variety of Diseases

Clinical Data								Electron Microscope Observations			
								Presence of granular material			
								Febrile specimen[c]			
No.	Age	Sex	Race	Disease	Temperature[a] (°F) at bleeding	Treatment[b]	Condition when afebrile specimens were taken	Fresh	Saline incubation	Afebrile specimens	Phagocytosis of platelets
1	25	F	W	Hodgkin's disease, labial abscess	104, stable	Aspirin Demerol Penicillin	Improved	I.C. E.C.	Not done	None	No
2	27	F	N	Lobar pneumonia	105.6, stable	Penicillin Codeine	Improved	I.C.	Not done	None	No
3	29	M	W	Von Recklinghausen's disease	100.2 ↑	Aspirin Codeine	Improved	I.C.	Not done	None	No
4	40	F	W	Malignant melanoma	102 ↓	Penicillin Streptomycin	Dead[d]	I.C.	None	Not done	Yes
5	40	F	N	Cholangitis jaundice	102.7 ↑	Neomycin	Dead[d]	I.C. E.C.	Not done	Not done	Yes
6	46	F	W	Fracture of humerus	103 ↓	Aspirin Nembutal Codeine	Improved	None	None	None	No
7	47	F	W	Fracture of femur	101.5 ↓	Penicillin Streptomycin	Improved	None	I.C.	None	Yes
8	46	M	W	Acute pyelonephritis	101.3 ↑	Chloromycetin Cortisone	Improved	I.C.	I.C.	None	Yes
9	49	F	W	Hodgkin's disease	101 ↑	Chloromycetin Aspirin Phenobarbital	Improved	I.C. E.C.	I.C. E.C.	None	Yes
10	50	M	W	Thrombophlebitis with popliteal abscess	101 ↑	Penicillin	Improved	I.C. E.C.	Not done	I.C. E.C.	Yes
11	54	M	W	Thrombotic thrombocytopenic purpura with cerebral hemorrhage	101.5 ↓	Cortisone Chloromycetin Aspirin	Dead[d]	None	None	Not done	No
12	54	F	W	Carcinoma of breast with metastases	103 ↓	Penicillin Aspirin Chloromycetin	Dead[d]	E.C.	None	Not done	No
13	57	M	W	Carcinoma of tongue	104, stable	Streptomycin Penicillin Codeine Leucovorin	Dead[d]	I.C. E.C.	Not done	Not done	No
14	60	M	W	Bilateral leg amputation for gangrene Postoperative pneumonia	102 ↓	None	Dead[d]	None	Not done	Not done	No
15	61	M	W	Subacute bacterial endocarditis	100.5 ↓	Penicillin Aspirin	Improved	None	None	None	No
16	62	F	W	Lymphosarcoma	100.5, stable	Aspirin Thorazine Codeine	Dead[d]	I.C.	I.C.	Not done	No

[a] Direction of arrow indicates whether the temperature was rising or falling at the time the blood was drawn; temperatures are oral unless otherwise indicated and taken within 15 minutes of the blood sample.

[b] Treatment listed is that which the patient was receiving when the blood was drawn and for at least the previous 24 hours.

[c] I.C. = intracellular; E.C. = extracellular.

[d] Patient was febrile until death.

TABLE 1—*Continued*

Data from 22 Patients with Fevers Due to a Variety of Diseases

Clinical Data								Electron Microscope Observations			
								Presence of granular material			
								Febrile specimen[c]			
No.	Age	Sex	Race	Disease	Temperature[a] (°F) at bleeding	Treatment[b]	Condition when afebrile specimens were taken	Fresh	Saline incubation	Afebrile specimens	Phagocytosis of platelets
17	67	F	W	Fever of unknown origin	102 ↑	Cytomel	Improved	I.C.	I.C.	E.C.[e]	Yes
18	68	F	W	Carcinoma of breast with metastases	103.4, stable (rectal)	Prednisone	Dead[d]	None	I.C.	Not done	No
19	70	F	W	Mycosis fungoides	100 ↑	Gantrisin Cytoxan	Improved	I.C.	Not done	None	No
20	71	M	W	Congestive heart failure Pneumonia	100.2 ↑	None	Dead[d]	E.C.	I.C. E.C.	Not done	Yes
21	72	F	N	Acute pyelonephritis	101 ↑	None	Dead[d]	I.C. E.C.	None	Not done	No
22	74	M	W	Urinary tract infection	100 ↑	Achromycin	Improved	I.C.	I.C.	None	Yes

[e] Patient was afebrile less than 24 hours.

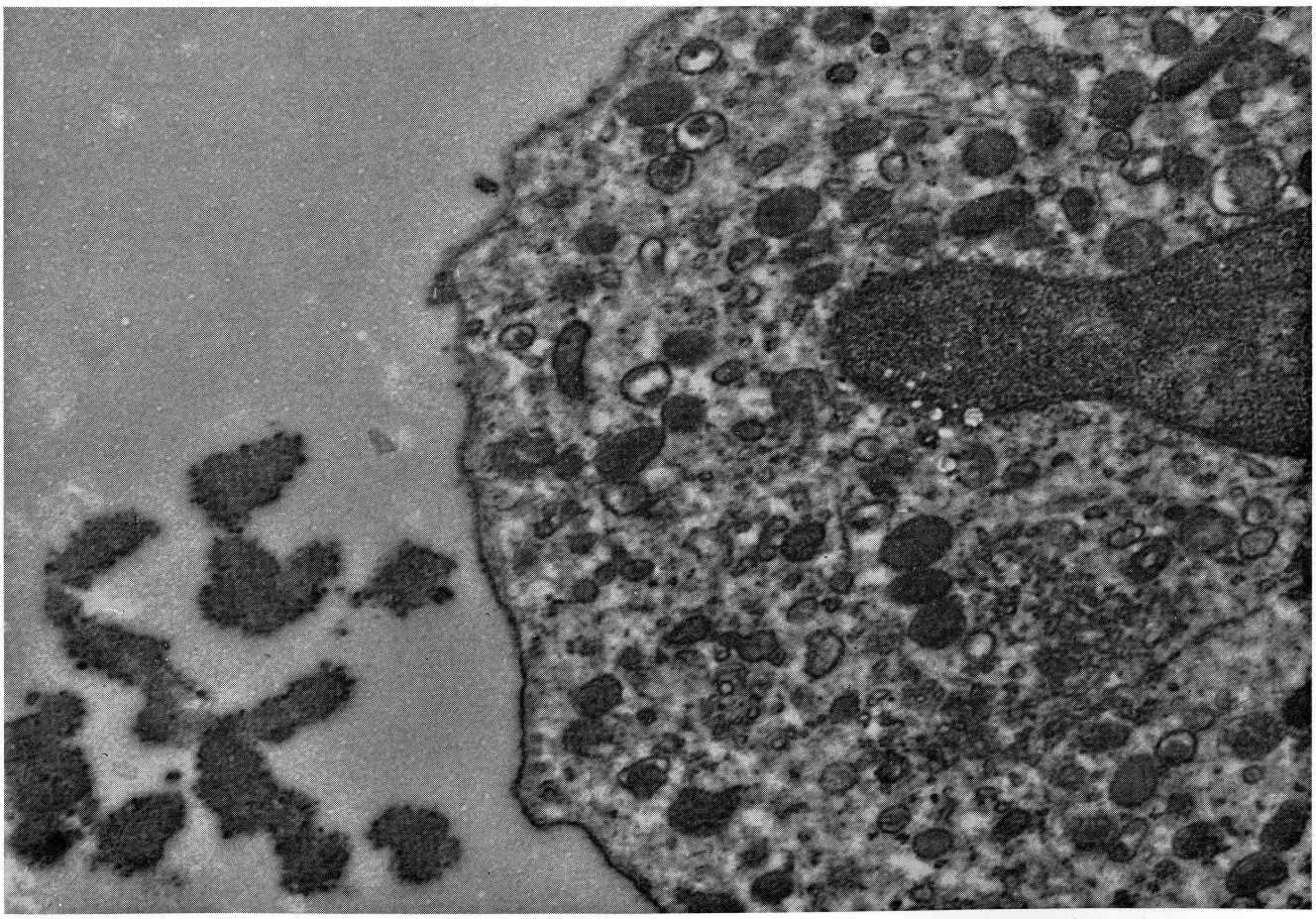


Fig. 1—Portion of neutrophil, artificially stimulated *in vitro* by incubation for 1 hour with bacterial endotoxin (Lipexal). To the left of the cell are aggregates of granular material which may represent, in part at least, leucocytic pyrogen. It is morphologically indistinguishable from the granular material seen in leucocyte preparations "naturally" stimulated *in vivo* by a variety of disease processes (figs. 3 to 7). Unstained, × 27,840.

TABLE 2

Summary of Electron Microscopic Findings in Leucocyte Preparations from 22 Febrile Patients.

Blood samples for controls could be obtained from only 12 patients because the remaining 10 patients died before becoming afebrile.

Temperature of Patient	Presence of Granular Material	
	Yes	No
Rising or at peak.....	15*	0
Falling.....	3*	4
Afebrile controls.....	2	10

* In one of these cases granular material was not seen in the fresh specimen but was present within leucocytes in the saline-incubated specimen.

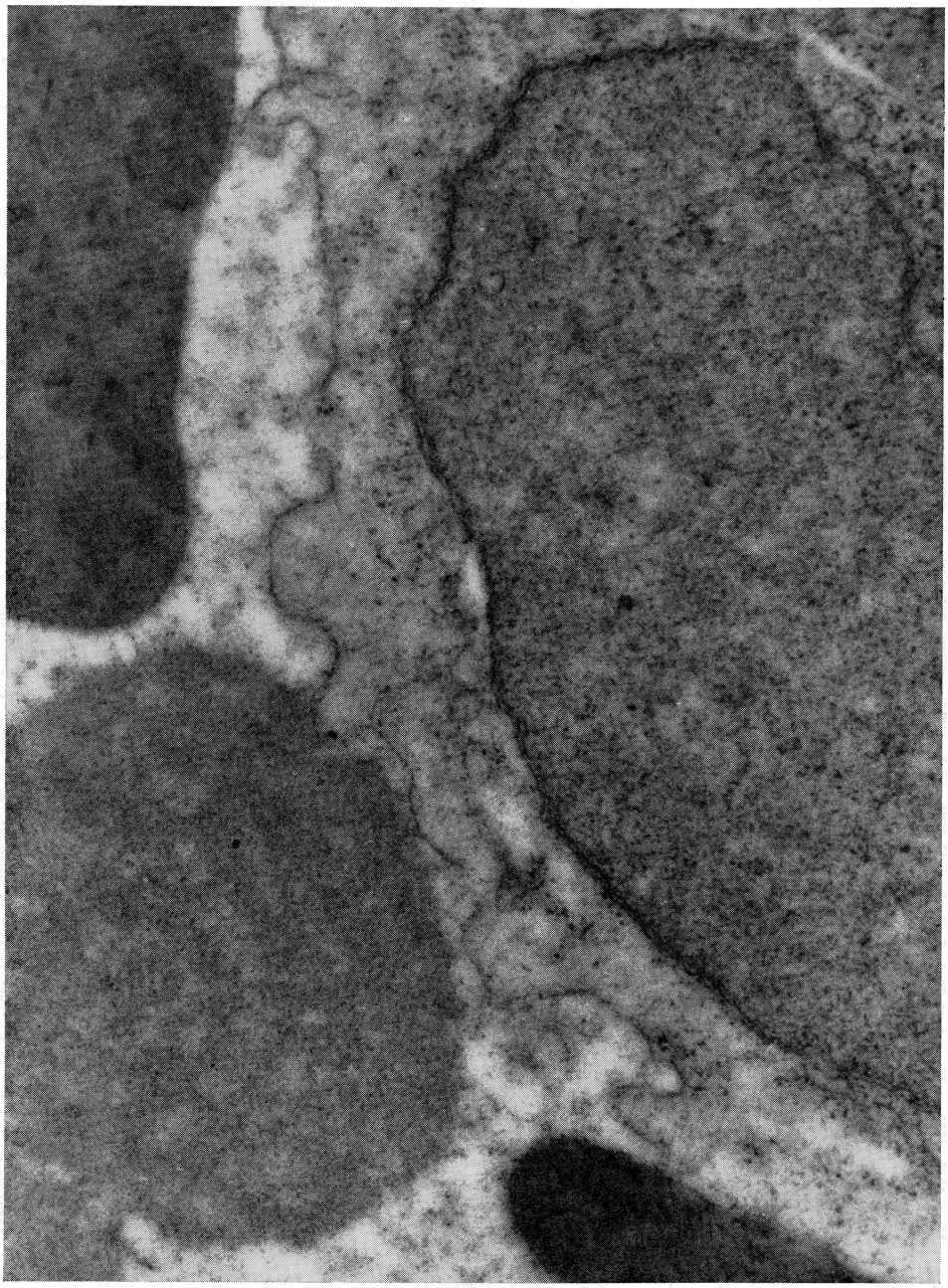
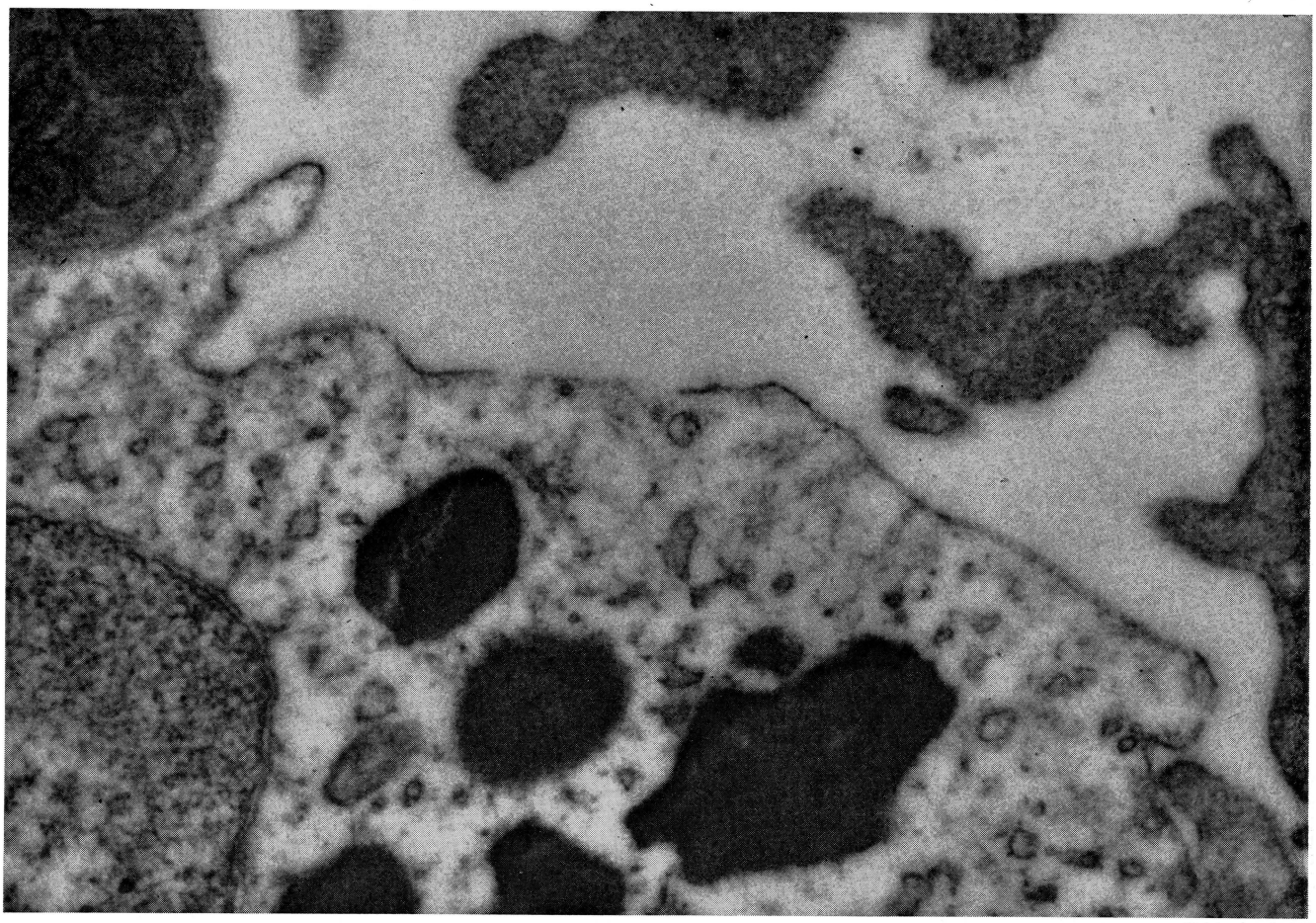


Fig. 2—To the left and below the visible portion of the lymphocyte, and between parts of two erythrocytes, is an aggregate of granular material about 1.0 μ in diameter. In this case 10 ml of whole blood from one of the authors was incubated for 1 hour *in vitro* with 0.5 μ of bacterial endotoxin (Lipexal) and then returned intravenously to the donor. Twenty-five minutes later chills and rising fever began, at which time blood for this preparation was drawn. The granular material here is made up of fine particles averaging approximately 50 Å diameter. Stained with uranyl acetate, × 48,000.



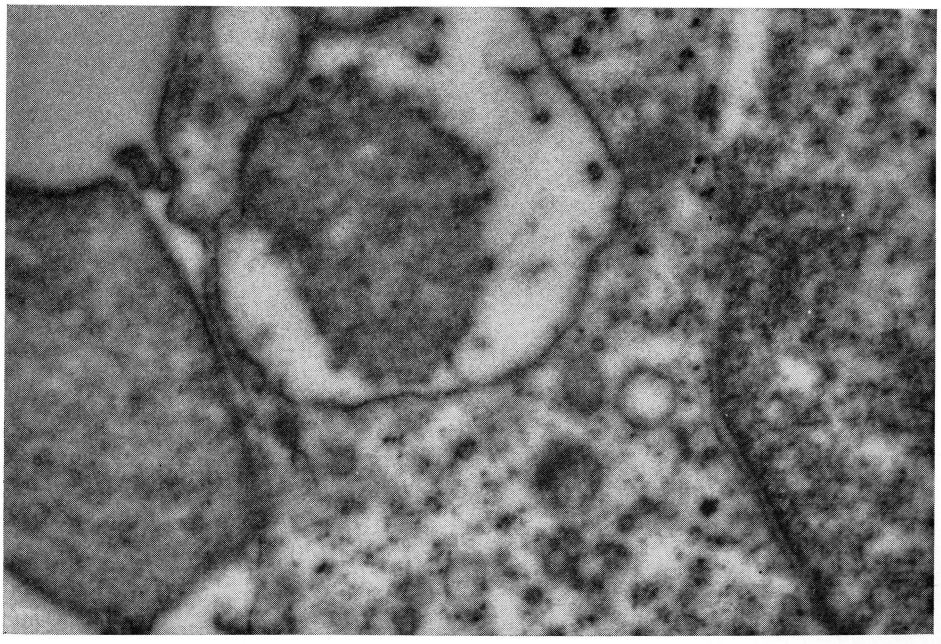
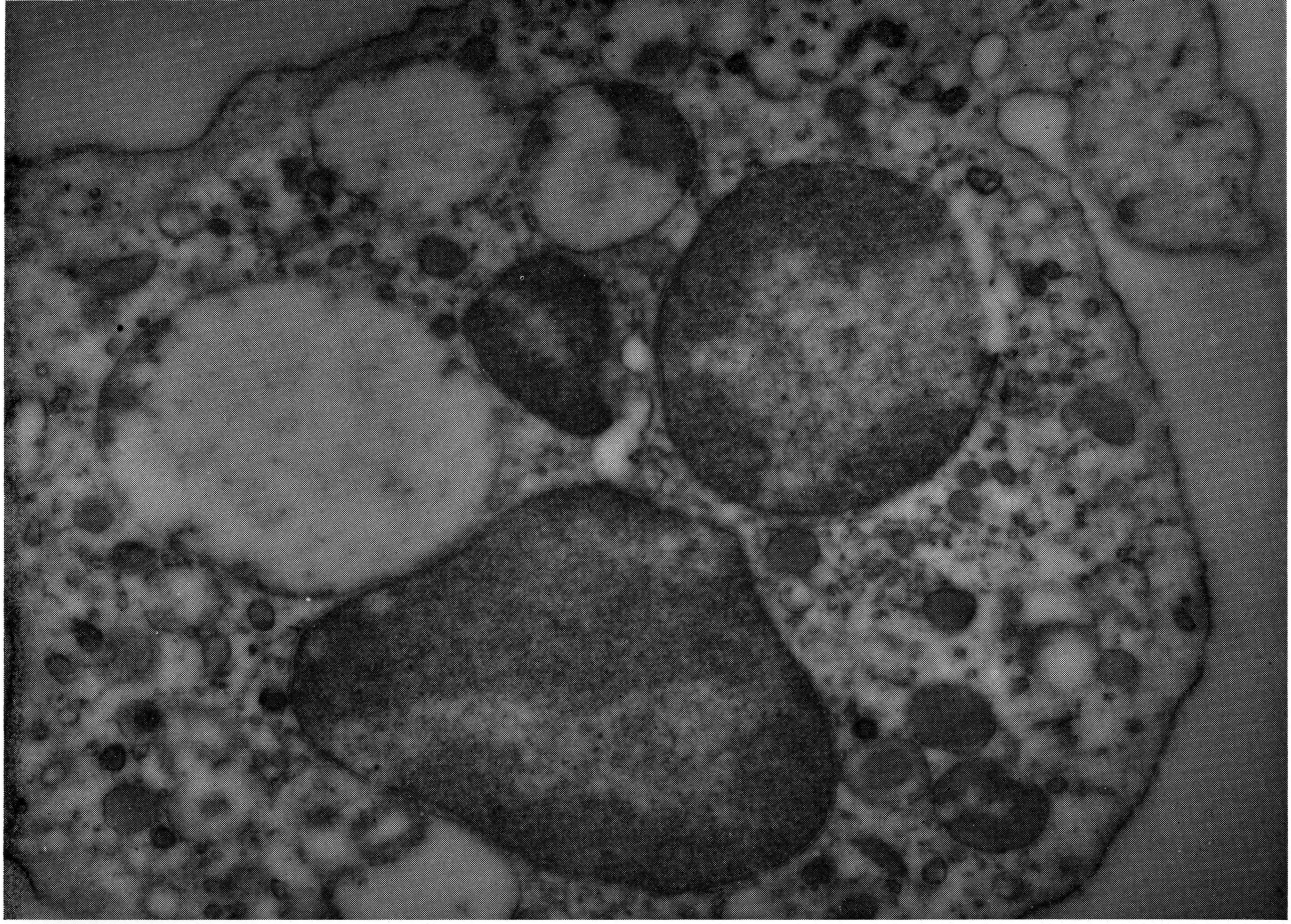
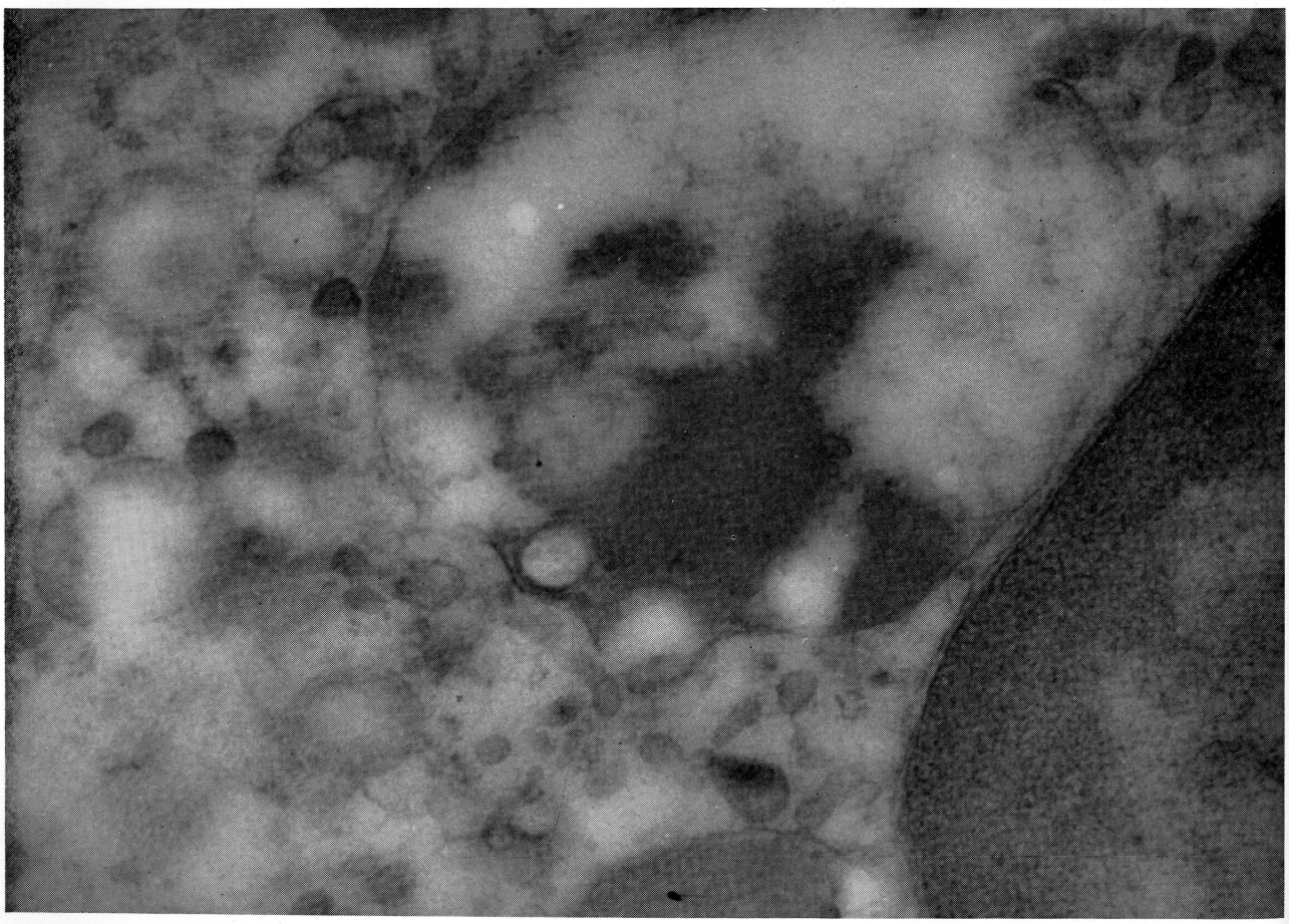


Fig. 3 (Above)—Eosinophil from patient 9, table 1. Blood was drawn when temperature was 101°F and rising. Irregularly shaped aggregates of granular material appear to the right above the cell. Individual particles comprising these aggregates are relatively coarse, averaging approximately 400 Å. Stained with uranyl acetate, × 60,800.

Fig. 4 (Left)—Portion of monocyte from patient 1, table 1, showing the granular material within a cytoplasmic vacuole. The sample was drawn as the temperature was stable at 104°F. Unstained, × 56,000.





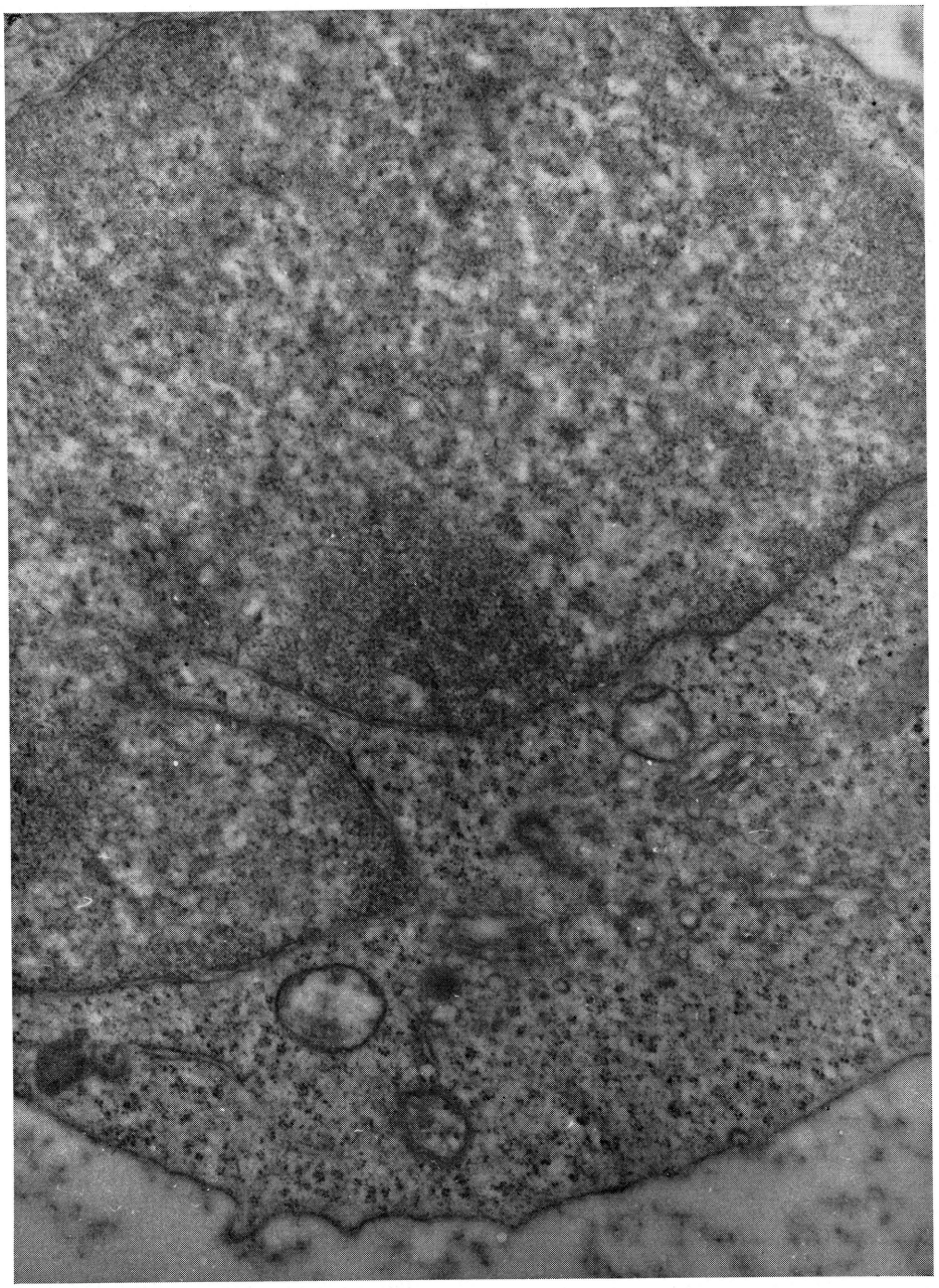
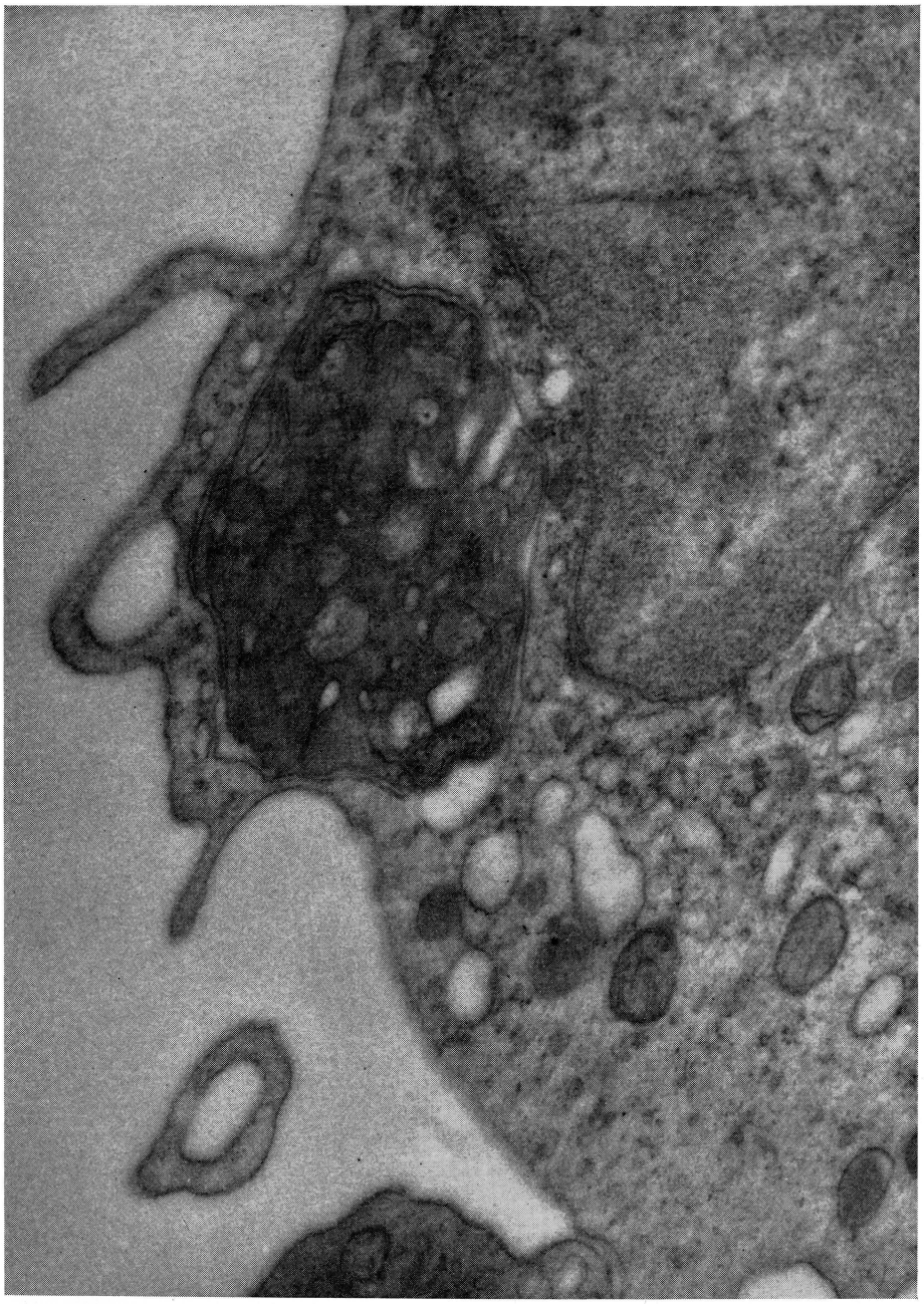


Fig. 5 (Left, above)—Neutrophil from patient 8, table 1, showing several large, almost empty vacuoles. Two contain small amounts of granular material and a number of minute, apparently empty vesicles. As this sample was taken the patient was having chills and his temperature was 101.3°F and rising. Unstained, × 18,000.

Fig. 6 (Left, below)—From the same patient as figure 5. Adjacent to the nucleus is a vacuole containing both granular material and a few tiny vesicles. Stained with lead, × 50,000.

Fig. 7 (above)—From the same patient as figures 5 and 6. Blood drawn when he had been afebrile for 48 hours. This monocyte shows typical decrease in size and number of vacuoles in early post febrile period. Unstained, × 32,400.



was apparently not related to the waxing or waning of a patient's fever. However, in every instance in which phagocytosis of platelets was observed, granular material was also seen. No phagocytosis of erythrocytes was seen.

Discussion

If the granular material does, in fact, represent leucocytic pyrogen, why is it not seen in all febrile patients and why is it occasionally seen when patients are afebrile? In the present study it was observed in all 15 patients whose temperature was rising or stable at an elevated level when the blood sample was drawn. However, when blood was drawn as the patient's fever was falling toward normal, the granular material was seen in only three of seven cases, suggesting that its production may cease or be curtailed as the fever-producing stimulus weakens. Actually, in light of previous evidence that leucocytic pyrogen, whether artificially (Goodale and Gander 1962a and 1962b) or naturally induced (Snell, 1961, 1962), is effective in the circulating blood in extremely low concentration, one might anticipate difficulty seeing it in occasional cases.

As part of a previous study (Goodale *et al.*, 1962) we examined leucocyte preparations from 30 healthy afebrile volunteers and saw the granular material in two of them. In these two

Fig. 8—Portion of monocyte from patient 17, table 1. Blood drawn when temperature was 102°F and rising. The phagocytized platelet shows few, if any, signs of disintegration. Although no granular material is visible in this illustration, it was seen in all leucocyte preparations in which phagocytosis of platelets occurred. Unstained, × 60,000.

cases we felt that the granular material might have been due to contamination of the leucocyte preparation by bacterial endotoxin prior to incubation. In the present study there were two patients whose leucocyte preparations contained the granular material, not only when they were febrile but also when they were afebrile. One of the patients (※17, table 1) had been afebrile less than 24 hours and became febrile again the day after her control sample was drawn. The other patient (※10), although his temperature was normal, had a large, open, and infected wound on one leg. Perhaps a threshold exists beneath which there is insufficient circulating pyrogen to elicit a measurable febrile response, but still enough to be sometimes visible by electron microscopy.

The only difference we have noted between leucocyte preparations which have been artificially stimulated *in vitro* to release their pyrogen, and those "naturally" stimulated, is that in the latter the granular material often appears in the cell cytoplasm within vacuoles. Whether it is being formed inside the cell or whether it has been produced at the cell surface and then phagocytized, we do not know. Conditions are undoubtedly more favorable for phagocytosis *in vivo* than *in vitro*.

We are not able at present to assess accurately the amount of granular material present in a leucocyte preparation. Therefore, we can make no statements as to whether height or duration of fever, type of disease, or method of treatment had any detectable effect on the amount or location of granular material. Cortisone has been reported (Atkins *et al*, 1955) to have antipyretic properties. Three of the patients (※8, 11, and 18, table 1) in the present study were receiving either cortisone or prednisone. In patients ※8 and 18, whose temperatures were rising or at peak, leucocytic pyrogen was observed. In patient ※11, whose temperature was falling, leucocytic pyrogen was not seen. From these few observations we can say only that if the granular material is or contains leucocytic pyrogen, then cortisone does not apparently exert its antipyretic effect by preventing the formation or release of the pyrogen. However, the quantification of the granular material must await more precise biological or chemical methods than are now available.

When phagocytosis of platelets by leucocytes occurred, it always occurred in the presence of granular material. Sometimes platelets in advanced stages of disintegration within cytoplasmic vacuoles resembled the granular material. We have observed platelet phagocytosis in only one artificially stimulated leucocyte preparation. Its meaning in the present study is uncertain.

Summary

A granular material was visible by electron microscopy in leucocyte preparations from 17 of 22 febrile patients with a variety of diseases. It was seen in all 15 patients whose temperature was rising when the blood sample was drawn, in 3 of 7 patients whose temperature was falling, and in 2 of 12 afebrile patients.

The granular material is morphologically identical to that seen in leucocyte preparations artificially stimulated *in vitro* by bacterial endotoxins and known to contain leucocytic pyrogen.

The present study would tend to strengthen the association between the granular material and leucocytic pyrogen, but the exact relationship has yet to be proved.

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