OBSERVATIONS ON THE PRODUCTION OF PYROGENIC SUBSTANCES BY RABBIT AND HUMAN LEUCOCYTES

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As fevers may arise from divers causes, the concept of a single common endogenous mediator is rather attractive. The basis of this concept is the finding of Bennett and Beeson (2) of pyrogenic material in sterile extracts of rabbit leucocytes. They did not obtain pyrogens in extracts of other rabbit tissues; this suggests that leucocytes are an integral part of the mechanism of fever (1). The present investigations show that, in the absence of added bacterial substances, rabbit leucocytes do not contain an active pyrogen, though they can form one on incubation; this process can be inhibited. Human leucocytes neither contair nor produce pyrogen.

The properties of leucocytic pyrogens differ from those of the pyrogens obtained from Gram-negative bacteria, as shown in Table I.

Pyrogenic agents with the same properties as leucocytic pyrogens have been found in a number of different situations; in the past, the term endogenous pyrogen has been indiscriminately applied to these, regardless of their origin. Whether or not these pyrogens are the same as leucocytic pyrogen is uncertain, and in the present state of our knowledge, it is impossible to say whether leucocytes play any part in their production *in vivo*. For this reason we wish to distinguish pyrogens by their origin, as well as by their properties, and to use the following nomenclature:

Bacterial pyrogen: Pyrogenic material obtained from killed bacteria.

Leucocytic pyrogen: The pyrogenic material derived from white cells to which no known pyrogens have been added.

Leucocytic-bacterial pyrogen incubate (LBP): The pyrogenic material obtained when leucocytes and bacterial pyrogen are incubated together.

Circulating pyrogen: The pyrogen present in the blood of febrile animals.

Methods

Animals.—

Hybrid rabbits, with weights ranging from 1.6 to 3.6 kg. were used, both as donors of white cells, and to assay the pyrogenic effects of various preparations. To suppress parasitic infections (8), particularly with *Eimeria stiedae*, nitrofurazone (5-nitro-2-furaldehyde semicarba-

zone) 6 to 11 parts in 100,000 by weight was added to the food of all animals, except in experiments specifically mentioned. Animals which were not given nitrofurazone are referred to as untreated animals. Treatment with nitrofurazone did not affect the temperature response to intravenous injection of bacterial or leucocytic pyrogens (see Results).

Assay for Pyrogenicity.—

Preparations were injected intravenously at a dose equivalent to 2×10^7 leucocytes per kg. of recipient and the rectal temperature response measured.

The assay rabbits were kept in a room whose temperature varied between 19 and 23°C., and during any one assay the room temperature varied less than $\pm$ 1°C. These rabbits were restrained either by the conventional stocks or by a light chain around the neck.

Rectal temperatures were measured to within $\pm$ 0.1°C. using copper constantan thermocouples enclosed in polyethylene tubing. These were inserted 5 to 10 cm. into the rectum and taped to the tail. Readings were taken at 15 minute intervals. Injections were given when the temperatures had been stable within 0.3°C. for any hour, and measurements were made until any fever had subsided, or, if no apparent fever developed, for at least 4 hours after injection.

The febrile response was measured as the fever index, the area under the elevated portion

TABLE I

Pyrogen	Stability to heat (90°C.—30 min.)	Relative latency between intravenous injection and onset of fever	Production of fever in tolerant recipients	Production of tolerance on repeated injection
Bacterial	Stable	Long	Depressed	Yes
Leucocytic	Unstable	Short	Unaffected	No

After Wood, W. B., Jr., *Lancet*, 1958, 2, 53.

of the temperature time curve (9); and expressed in degree centigrade-hours (°C. hours). Two observers measured the areas independently and their assessments seldom differed by more than 10 per cent (9). Fever index was used as a parameter, because, in an initial series of experiments on 36 non-tolerant rabbits, given different doses of bacterial pyrogen (pyrexal, the lipopolysaccharide from *Salmonella abortus equi*, Westphal and Lüderitz, 10), it was found that fever index was more closely correlated with the log dose of pyrogen than was the maximum temperature rise. The correlation coefficient relating area to log dose was +0.910; that relating maximum temperature rise to log dose was +0.672. These correlation coefficients differ significantly ($t = 2.82, p < 0.01$).

The apparent fever index was measured in 50 uninjected rabbits, in order that allowance could be made for variations not due to the injection of pyrogens. The mean fever index was 0.25, S.E. $\pm$ 0.07°C. hours.

Sterilization: All equipment was sterilized and rendered pyrogen-free by heating to 150°C. for 2 to 3 hours: 0.9 per cent NaCl solution (henceforth referred to as saline) and distilled water were tested for freedom from pyrogens. Other substances such as the starch used to produce peritoneal exudates were heated as solids to 150°C. for 2 to 3 hours to render them sterile and pyrogen-free. They were then dissolved and the solutions were proven to be free of pyrogen by injection into rabbits.

Preparation and Collection of Peritoneal Exudates: Exudates were prepared by injecting a 3 per cent solution of starch, in saline, into the peritoneal cavity of the rabbit (5). This solution was employed because the injection of saline alone produced exudates containing few

leucocytes. The addition of starch to the saline gave exudates with a rich yield of leucocytes; differential cell counts showed that 80 to 90 per cent of the cells were polymorphs.

The rabbit was anaesthetized with 25 mg./kg. nembutal given intravenously. The belly and left side of the thorax were clipped free of hair, and the skin washed with alcohol. A fold of skin and of abdominal muscle was lifted between the thumb and finger, and shaken gently to ensure that no gut was included in it. A 17 standard wire guage transfusion needle was introduced through the fold of peritoneum and 200 ml. sterile pyrogen-free starch solution was introduced into the peritoneal cavity by means of a standard blood transfusion set or a 100 ml. syringe. The former method was more convenient.

12 to 16 hours later the rabbit was anaesthetized with nembutal and exsanguinated by cardiac puncture. In some experiments the animal was dipped in antiseptic solution at this stage. The skin of the abdomen was cleansed with alcohol and a midline incision 4 to 6 inches long was made down to the peritoneum. This membrane was picked up with tissue forceps and incised; the wound edges were held apart and upwards. To prevent the formation of large fibrin clots, 200 ml. saline at 2–4°C. was poured into the peritoneal cavity and mixed with the abdominal contents by gently rocking the carcass. The gut was gently held to one side with a retractor and the fluid aspirated into a Buchner flask or an intravenous infusion bottle. Opening the belly and aspirating its contents were done under a perspex hood to reduce the possibility of contamination.

After exudates had been sucked out, the abdominal cavity was rinsed with sufficient chilled saline to make the total aspirated volume 540 ml. The body was then examined for the presence of coccidial lesions and injuries to the viscera.

Isolation of Leucocytes from Peritoneal Exudates: The exudates were centrifuged at 650 g (1600 R.P.M. M.S.E. centrifuge) for 10 minutes at 2–4°C. The supernatant was discarded, and the cells were pooled and dispersed. The pooled cells were then washed three times with 200 ml. lots of saline.

The washed cells were suspended in approximately 20 ml. of saline. Samples were counted in duplicate and the total leucocyte yield was calculated.

Care was taken at all stages to keep the temperature of the cells below 5°C. except when otherwise stated.

Treatment of the Washed Cells: Cells were incubated in sealed ampoules or in pyrex tubes which were closed with aluminium caps. The tubes or ampoules were immersed in a waterbath at 36–37°C. The time of incubation varied from 1 to 48 hours.

Cells were disintegrated by ultrasonic vibration (M.S.E. ultrasonic disintegrator, 75 watt, 19 kc./sec.). The sterilized stainless steel cylindrical probe (¾-inch diameter) was dipped into 5 ml. of cell suspension, which was cooled in ice. The machine was switched on for three periods of 1 minute each, separated by 15 second intervals. This treatment destroyed over 98 per cent of fresh cells, or cells which had been frozen and thawed, and over 90 per cent of cells which had been heated to 56°C. for 30 minutes. Some cell suspensions were frozen in solid CO_2-ethanol mixture, then thawed gently, and the procedure repeated three times. Other cell suspensions were heated in capped pyrex tubes or ampoules, using a waterbath maintained within ± 0.5°C. of the desired temperature.

Isolation of Leucocytes from Rabbit Blood.—

Blood was collected from anaesthetized rabbits (nembutal 25 mg./kg. intravenously) by cardiac puncture, using heparin, 1000 units/100 ml. as an anticoagulant. The blood was centrifuged at 900 g (2,000 R.P.M. M.S.E. centrifuge) for 10 minutes at 2–4°C. The buffy coat was aspirated, along with red cells and some plasma, and was transferred to hour-glass shaped tubes. It was again centrifuged, so that the white cells, which accumulated in the waist of the tube, could readily be removed. They were then washed in the same way as leucocytes obtained from peritoneal exudates.

Experiments with Human Leucocytes.—

Human leucocytes were obtained from healthy volunteers or from afebrile patients without infective diseases. Blood was taken from the antecubital vein, using standard blood transfusion equipment, with heparin, 1000 units/100 ml. as anticoagulant. The blood was centrifuged in the cold, at 900 g and the buffy coat was aspirated, together with variable amounts of red cells and plasma. In three experiments, the cells were washed three times in saline, and incubated at 37°C. for 2 hours in this medium. In other experiments, the buffy coat was used without washing. Mechanical destruction of cells was attempted by shaking the buffy coat in a closed tube with glass beads, 1 to 2 mm. in diameter, for 6 hours at 4°C.

Osmotic damage to white cells was caused by the addition of 9 volumes of distilled water to the buffy coat at room temperature. In other experiments cell damage was caused by heating to 56°C. for 30 minutes. In all these experiments the white cells were counted before and after the procedure, though the latter count was difficult and certainly inaccurate, mainly because of leucocyte agglutination. Each preparation was reinjected into the donor, whose temperature response was measured at 1 minute intervals with oral and rectal thermocouples, until 1 hour had elapsed after injection. Thereafter the rectal temperature was measured at hourly intervals, with a clinical thermometer.

RESULTS

The Effects of Nitrofurazone Treatment of the Assay Rabbits, on Their Febrile Response to Bacterial Pyrogens

To decide whether nitrofurazone treatment affected the assay of pyrogens, five treated and five untreated rabbits were each injected intravenously with 0.1 μg./kg. of bacterial pyrogen, and their febrile responses compared. The average fever index of untreated animals was 5.46, s.e. $\pm$ 0.38°C. hours while that of treated animals was 6.04, s.e. $\pm$ 0.78°C. hours. It was therefore concluded that treatment with nitrofurazone did not vitiate the assay of pyrogens.

Yield of Leucocytes from Peritoneal Exudates.—

Cell counts were performed on washed white cells obtained from peritoneal exudates. Owing to losses in washing, these counts underestimate the number of cells originally present in the exudates. As counts were usually performed on pooled exudates, the yield of cells from individual exudates is unknown.

The average yield of cells per exudate was 8.97 $\times$ 10^8 in 35 untreated animals. In 24 animals treated with nitrofurazone the average yield was 7.22 $\times$ 10^8. There was considerable variation in cell count from experiment to experiment, and it appears unlikely that nitrofurazone treatment affected the yield of white cells. In the exudates from treated and untreated animals the cells were principally polymorphs, which accounted for 80 to 90 per cent of the total.

Pyrogenic Properties of Washed Cells from Peritoneal Exudates.—

(a) Cells from Rabbits not Treated with Nitrofurazone

Washed cells, kept in saline at 4°C. after harvesting, caused little or no fever when injected intravenously into rabbits, in a dose of 2 $\times$ 10^7 cells/kg.

The mean fever index of 12 injections was 0.49, s.e. ± 0.17°C. hours, a response which did not differ significantly from that of uninjected rabbits ($t = 0.18$, $p > 0.10$).

If the cells were incubated in saline at 37°C. for 1 hour the resultant fever index was 1.25, s.e. ± 0.10°C. hours (42 injections). This response differs significantly from that following the injection of unincubated cells ($t = 3.64$, $p < 0.001$) and the response of uninjected rabbits ($t = 8.13$, $p < 0.001$). Similar results were obtained when the cells were incubated in physiological salt solution (120 mM NaCl, 12.5 mM—Na_2HPO_4, 3.12 mM—NaH_2PO_4, 1.2 mM—$MgSO_4$, 4.8 mM—KCl, 2.5 mM—$CaCl_2$, glucose 1 mg./ml. medium pH 7.4) both in the presence or absence of dextran 6 per cent w/v, and under both aerobic and anaerobic conditions. Thus incubation of leucocytes is necessary for pyrogen to appear.

Accordingly, an experiment was carried out to determine the yield of pyrogen after incubation for periods of 1½ to 48 hours.

Groups of four test rabbits were injected intravenously with 2×10^7 washed cells/kg., which had been incubated for different times, and, as shown in Fig. 1, the fever resulting from their injection differed little, after incubation from 1½ to 48 hours. Though there was a slightly greater fever index after the injection of cells incubated for 6 to 18 hours, this difference was not significant. Thus incubation for periods of longer than 1½ hours did not increase the yield of pyrogen, nor was there any indication that prolonged incubation resulted in its loss or degradation.

Over the range 0.8 to 6.4×10^7 cells per kg., the febrile response was related to the number of cells, as shown in Fig. 2. It is not possible to be certain whether this relationship is linear or logarithmic over this range, and both relations are shown in the figure. The pyrogen present in these preparations was in all cases inactivated by heating to 90°C. for 30 minutes.

Because incubation was necessary to demonstrate the presence of a pyrogenic agent in these cells, it seemed important to determine whether incubation merely facilitated release of a pyrogen from within the cell, or whether it was necessary for the formation of a pyrogen; for this reason the effects of cell damage were investigated.

The Effect of Freezing and Thawing, and Homogenizing of Rabbit Leucocytes on the Production of Leucocytic Pyrogen

A pool of white cells was divided into five equal parts. Two parts were incubated at 37°C. for 1 hour; one was cooled, the other was frozen and thawed repeatedly and then re-incubated for a further hour. Two other parts were repeatedly frozen and thawed, and then one of them was incubated at 37° for 1 hour. The fifth part was left as a control. Finally all five parts were ultrasonically disintegrated and the preparations stored at −20°C. For assay each

of the five samples was divided into six aliquots, one of which was heated to 85–90°C. for 30 minutes. The results are shown in Fig. 3. None of the heated material gave rise to fever. The two preparations which produced fevers were those which had undergone incubation at 37°C. before freezing and thawing.
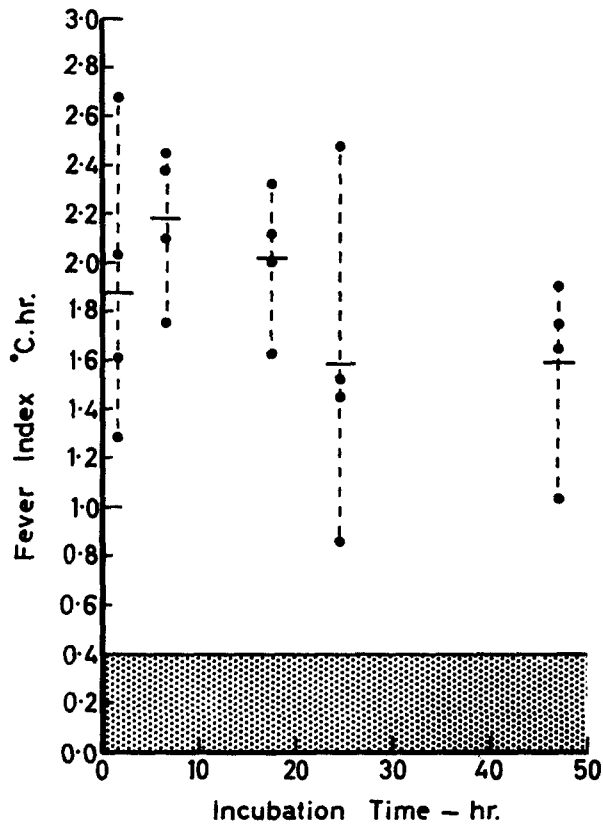

Fig. 1. The fever responses produced by intravenous injection into rabbits of suspensions of 2 × 10⁷ rabbit leucocytes which had been incubated in 0.9 per cent NaCl solution for various times. Each individual fever index (●) is shown, and the horizontal lines denote the mean index for each incubation time. The stippled area denotes the maximum fever index expected from a group of uninjected animals.

The other preparations gave responses which did not differ significantly from those expected in uninjected rabbits. The implications of the results are that disintegration of white cells releases negligible amounts of pyrogen, and that freezing and thawing white cells seriously impairs their ability to release pyrogen on subsequent incubation. Furthermore neither freezing and thawing, nor ultrasonic disintegration seriously degraded leucocytic pyrogen which had already been released from the cells.

The Effect of Heating to 56°C. on Rabbit Leucocytic Pyrogen and Its Production.—

A pool of leucocytes was divided into four samples. One was incubated for 1 hour at 37°C., then heated to 56°C. for 30 minutes and further incubated for 1 hour at 37°C. The second sample was incubated at 37°C. for 1 hour, after which it was immediately stored at 4°C. A third fraction was heated to 56°C. for 30 minutes and then
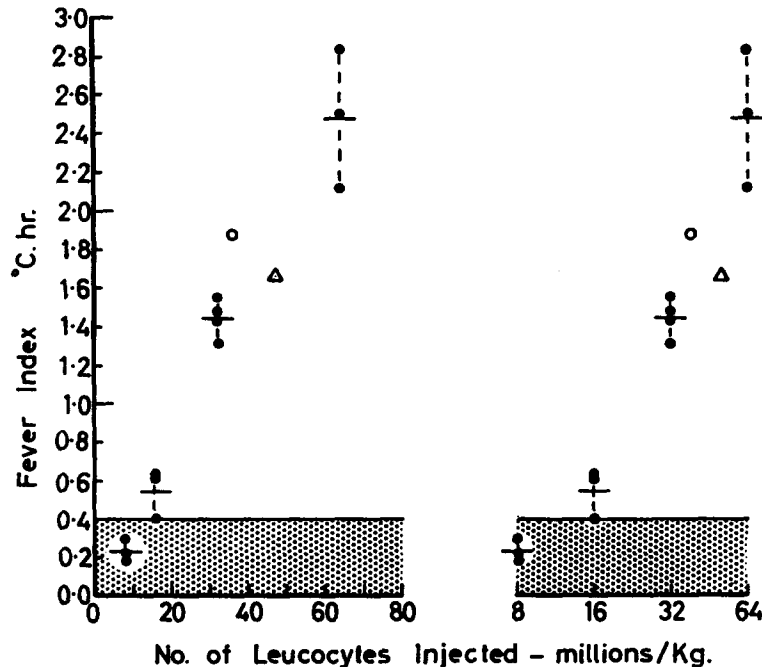

Fig. 2. Fever responses in rabbits to intravenous injection of suspensions of various numbers of rabbit leucocytes, each suspension having been incubated in 0.9 per cent NaCl solution for 1 hour. Each individual index is shown (●) and the mean for a given concentration of cells is shown (—). The left hand picture shows fever index plotted against the dose of cells, and the right hand plot is of fever index against log number of cells. The stippled area denotes the maximum fever index expected from a group of uninjected rabbits. O = mean results taken from King and Wood, 1958, (6); △ = mean results taken from Collins and Wood, 1959, (3).

incubated for 1 hour at 37°C., and the fourth was heated to 56°C. for 30 minutes. Then all four were centrifuged at 650 g for 15 minutes at 2°C. The supernatants were aspirated and stored at 4°C. Each preparation was injected into four rabbits in a dose equivalent to 4 × 10^7 cells/kg. Fig. 4 shows the treatment undergone by each preparation and the resultant fevers obtained. The preparations which had been incubated at 37°C. before heating to 56°C. gave rise to substantial fevers. Comparison of preparations 1 and 2 suggest that heating to 56°C. may cause a little degradation of the fever principle previously released during incubation. When cells were heated to 56°C. before incubation no pyrogen was subsequently formed. Heating the cells to 56°C. did not release any significant amount of pyrogen.
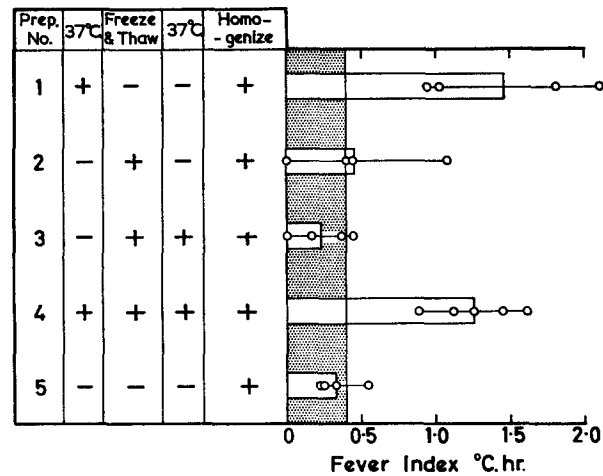
FIG. 3. Fever responses to intravenous injection into rabbits of a standard dose of rabbit leucocytes which had undergone various treatments (see text). Horizontal rectangles denote the mean fever index. Individual observations shown thus (o—o). The stippled areas denote the maximum fever index expected from a group of uninjected rabbits.
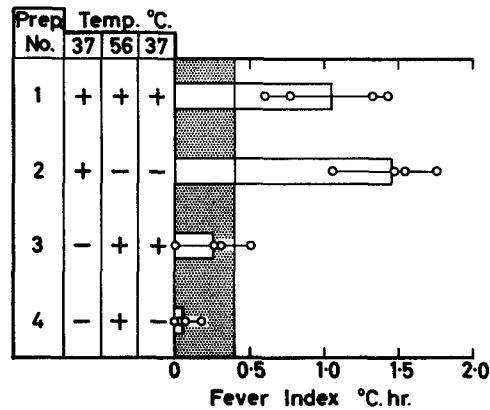

FIG. 4. Fever responses to intravenous injection into rabbits of a standard dose of rabbit leucocytes which had undergone various treatments (see text). Conventions as in Fig. 3.

This experiment shows that heating leucocytes to 56°C. for 30 minutes prevents the formation of leucocytic pyrogen but does not destroy leucocytic pyrogen once it has been formed.

The Effects of Ageing on the Ability of Leucocytes to Form Pyrogen.—

Leucocytes from untreated rabbits were kept at 4°C. for 7 to 14 days before incubation at 37°C. for 1 hour. They were then injected in a dose of 2 × 10⁷

cells per kg. Three separate preparations of leucocytes were used, and in each the cells formed leucocytic pyrogen when fresh. Twelve injections of fresh cells were given; the mean febrile response was 1.24, s.e. ± 0.17°C. hours. Eleven preparations from the same cells were injected after ageing; these injections were followed by a significantly smaller febrile response of 0.59, s.e. ± 0.12°C. hours ($t = 3.27, p < 0.01$). This response was not significantly greater than that of rabbits which had not been injected.
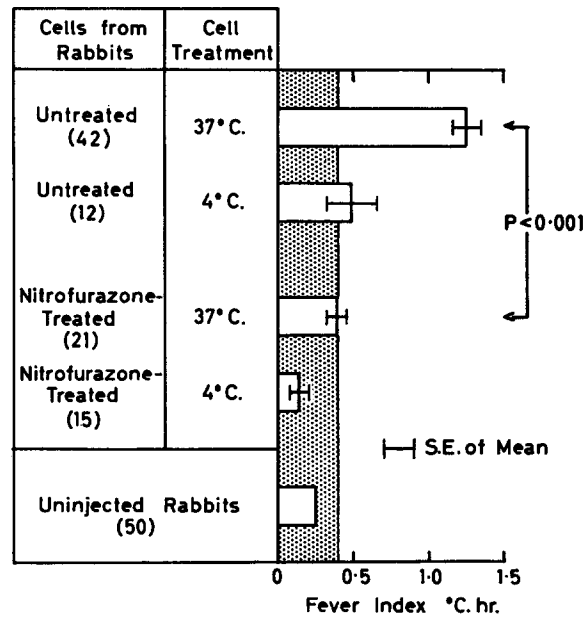

FIG. 5. The effects of treatment with nitrofurazone upon the pyrogenicity of rabbit leucocytes. Figures in parentheses indicate the number of assay rabbits injected. Conventions as in Fig. 3.

(b) *The Effects of Treatment with Nitrofurazone Upon the Formation of Leucocytic Pyrogen*

Washed white cells were obtained from peritoneal exudates of rabbits maintained on continuous treatment with nitrofurazone. Direct injection of such cells, in a dose of 2×10^7 cells/kg., without prior incubation, was followed in 15 recipients by a mean fever index of 0.14, s.e. ± 0.06°C. hours (Fig. 5). The mean fever index, after the injection of cells incubated at 37°C. for 1 hour into 21 recipients, was 0.39, s.e. ± 0.07°C. hours. Though the difference between these indices is significant ($t = 2.72, p < 0.01$) neither of them differs significantly from that observed in uninjected rabbits. Thus, there may be a very small yield of pyrogen when cells from treated animals are incubated, but

this is so small as to be of doubtful importance, and is significantly less than that following the injection of incubated cells from untreated animals.

It is thus apparent that treatment of the donor animals with nitrofurazone almost entirely abolishes the pyrogenicity of all preparations. A number of investigations were carried out in an attempt to decide the reason for this.

The first possibility is that the exudates were contaminated by microorganisms sensitive to nitrofurazone and that the pyrogenic agent liberated by cells from untreated animals was due to contamination. Samples of 8 peritoneal exudates, taken from untreated rabbits at the time of harvesting, and cultured in Robertson's cooked meat medium and on blood agar plates aerobically and anaerobically showed no growth. 115 samples of leucocyte preparations from untreated animals were cultured the same way. Though no samples showed growth after culture for 48 hours, 18 (15.5 per cent) of them showed very slight growth thereafter. The incidence of fevers after injection of sterile preparations did not differ significantly from that after the injection of preparations which gave positive cultures ($X^2 = 0.095, 0.9 > P > 0.5$). Whether or not fever developed was related to the treatment of the white cells and not to the results of the culture. It appears unlikely that the bacterial contamination encountered bore any relation to pyrogenicity.

The possibility was also investigated that the pyrogenicity was due to contamination by $E. stiedae$, a parasite commonly infecting rabbits. The liver was removed from one rabbit which had not been treated with nitrofurazone and which was found to have liver lesions. Using aseptic precautions, one of the lesions was evacuated, and the sporocysts were suspended in saline. The cysts were counted, and a specimen was cultured in broth. This culture was sterile.

One sample of sporocysts, suspended in saline, was incubated alone, and injected intravenously into assay rabbits in doses of 1450 sporocysts/kg. These did not produce fever. The same number of sporocysts was disintegrated ultrasonically and injected intravenously into 4 rabbits. This preparation did not cause fever. In a further experiment 25,000 cysts were added to 200 ml. starch solution, and this mixture was used to prepare a peritoneal exudate in a rabbit receiving nitrofurazone. The cells were obtained from this exudate in the usual way. These cells did not cause fever after incubation and injection into assay rabbits. It therefore appears unlikely that sporocysts of $E. stiedae$, as found in liver, are responsible for the pyrogenic properties of leucocytes obtained from the peritoneum of untreated rabbits.

Leucocytes from untreated rabbits were incubated $in vitro$ with nitrofurazone, in a concentration of 0.01 per cent (w/v). When injected, these cells gave a significantly smaller fever than control cells incubated without nitrofurazone. Cells incubated with nitrofurazone gave a fever index which was not significantly different from that observed in uninjected rabbits.

(c) Pyrogenicity of Leucocytes Obtained from Rabbit Blood

In general, it was observed that leucocytes obtained from the peripheral blood of rabbits behaved in a similar fashion to those obtained from peritoneal exudates. Washed leucocytes, obtained from peripheral blood, were injected

intravenously in a dose of 2×10^7 leucocytes kg. Unincubated cells from rabbits treated with nitrofurazone gave a mean fever index of 0.17°C. hours (8 injections); cells from untreated rabbits gave a mean fever index of 0.25°C. hours (4 injections). After incubation, cells from untreated rabbits caused fever in four animals, the mean fever index being 0.99°C. hours, whereas cells from nitrofurazone-treated rabbits were ineffective; eleven injections were given, and a mean fever index of 0.26°C. hours was observed. The difference between the effects of incubated cells from treated and untreated rabbits was significant ($t = 3.38$, $p < 0.01$).

Nitrofurazone diminished the pyrogenic properties of leucocytes obtained from blood, as well as those obtained from peritoneal exudates.

(d) Human White Cells

In all, 19 experiments were carried out using human white cells. In four experiments the cells of the buffy coat were damaged by shaking (see Methods). The initial total leucocyte counts varied between 1.9 and 5.0×10^8. Injection of one preparation was followed, 1 hour later, by a very slight temperature rise, 0.4°C. The fever index was 0.85°C. hours. In the other three experiments no fever developed.

In nine experiments, cells were subjected to osmotic damage. The initial white cell counts ranged from 2.0 to 16.5×10^8, with a mean of 5.15×10^8. With one exception, these preparations were not pyrogenic. One subject had a temperature rise of 0.3°C., 2 hours after injection; the measured fever area was 0.78°C. hours. Three buffy coat preparations heated to 56°C. for 30 minutes before injection, were not pyrogenic. In these preparations, the initial total leucocyte counts varied between 3.03 and 12.75×10^8 cells.

Three experiments were carried out with washed and incubated white cells. In these experiments the total leucocyte counts before incubation were 1.95, 6.75, and 9.95×10^8. After incubation, the preparations were centrifuged at 2000 R.P.M. for 5 minutes at 2°C., and the supernatant aspirated. These supernatants were then injected into the recipients. In none of these experiments did any fever develop.

DISCUSSION

Ample evidence exists that a pyrogen can be obtained from rabbit leucocytes (2, 3, 7).

The part played by such a pyrogen in the fevers of infection remains uncertain. Using several methods, we have been unable to detect any such pyrogen in preparations of human leucocytes. It is possible that this failure may have been due to inadequate dosage, though doses in excess of 1.5×10^7 cells/kg. have been used in some experiments. As man is, in our experience, considerably more sensitive to bacterial pyrogen than rabbits, it would appear

that this is unlikely to be the explanation. Certainly in man, leucocytes in doses of approximately 2 to 4 × 10⁶/kg. can, on incubation with bacterial pyrogens, produce LBP. Because of this difference between man and the rabbit, the conditions under which leucocyte pyrogen is obtained from rabbit cells were investigated.

It is clear that this pyrogen does not exist preformed within the leucocytes, but is produced during incubation: it cannot be detected in washed leucocytes which have been kept in the cold, even though these be fragmented before injection. Disintegration did not affect pyrogen which had already been formed. The process of formation of rabbit leucocyte pyrogen can be inhibited by freezing and thawing, or by heating to 56°C., though it appears to be insensitive to the electrolyte and glucose composition of the incubating medium and to the presence or absence of oxygen. Though Collins and Wood, (3), found that serum or plasma inhibited the release of leucocytic pyrogen, we have found that dextran is ineffective; the inhibitory effect of serum may be due to its beneficial effects on viability of cells, or to the presence of a specific inhibitor.

Nitrofurazone exerts an inhibitory effect, whether administered to the donor animal, or added *in vitro*. It does not modify the febrile response of animals given bacterial pyrogens. Nitrofurazone has a wide antibiotic spectrum, and its inhibition of pyrogen formation by leucocytes might be attributable to this. This would imply that the formation of leucocytic pyrogen is due to the presence of microorganisms.

That contaminating microorganisms are absent can never be positively proven, but the following evidence makes such contamination unlikely.

1. Unincubated cells do not contain significant amounts of pyrogen, and the time course of pyrogen formation with incubation is unlike that which might be expected with the multiplication of microorganisms.

2. Similar yields of pyrogen have been obtained in different laboratories; our results are compared with those of Wood and his colleagues (3, 7), Fig. 2.

3. Microscopy and cultures failed to provide significant evidence for microbiological contamination.

4. Sporocysts of *E. stiedae*, the most likely culprit, were not pyrogenic when tested in several ways.

The formation of leucocytic pyrogen is not due to the use of starch, nor to the peritoneal inflammation, because a similar yield of pyrogen was obtained from peritoneal and from circulating leucocytes. The inability of aged leucocytes to form leucocytic pyrogen suggests that viable cells are necessary for its production. From the evidence of heating cells to 56°C. or freezing and thawing them, it seems likely that an enzymatic process is involved, though this has not been demonstrated.

We do not know whether incubation of leucocytes causes an unmasking of a "pre-pyrogen" or the synthesis of a new material. These considerations

apply equally, whether leucocytic pyrogen has its origin entirely in the uncontaminated cell, or whether it is directly or indirectly a product of contamination. If contaminants are not the source of leucocytic pyrogen the action of nitrofurazone requires explanation. The two findings that nitrofurazone inhibits the release of leucocytic pyrogen *in vitro* and that it does not affect the *in vivo* response to injected bacterial pyrogen can be interpreted in at least two ways: either release of leucocytic pyrogen is not an essential part of the fever response of the rabbit to injected bacterial pyrogen or the *in vitro* system studied is not representative of conditions *in vivo*. Certainly the conditions of leucocytes *in vitro* are abnormal and nitrofurazone, or serum, may simply alter those abnormal conditions *in vitro* which lead to release of pyrogen. If the action of nitrofurazone on the *in vitro* release of leucocytic pyrogen is dismissed as merely correcting an experimental artefact, then the results of similar *in vitro* studies with leucocytes may only with difficulty be made the basis of any hypothesis of the mechanism of fever *in vivo*. There is however evidence (4) that nitrofurazone does not interfere with the *in vitro* interaction of leucocytes and bacterial pyrogen, L.B.P. being produced in the same way as when nitrofurazone is absent. This favours the first of the two interpretations, namely that leucocytic pyrogen may not be essential to the causation of fever in animals, including man.

SUMMARY

1. The mechanism of release of a pyrogen from leucocytes has been studied in cells obtained from sterile rabbit peritoneal exudates and from rabbit blood. Attempts were made to induce human leucocytes—from blood—to release a pyrogen.

2. Rabbit leucocytes, kept below 4°C., were not pyrogenic and did not release any pyrogen when disintegrated. Incubating such cells, in various media, at 37°C. led to the formation of a pyrogen which was heat-labile. The maximum yield was attained after $1\frac{1}{2}$ hours' incubation.

3. The formation of rabbit leucocytic pyrogen was prevented by freezing and thawing the leucocytes, by heating them to 56°C. for half an hour before incubation, and by ageing them in the cold.

4. Nitrofurazone (5-nitro-2-furaldehyde semicarbazone) prevents the formation of leucocytic pyrogen when given by mouth to the cell-donor animals, or when added to leucocytes *in vitro*.

5. Leucocytes from rabbit blood formed leucocytic pyrogen, on incubation in saline, and this formation was also inhibited by nitrofurazone.

6. No leucocytic pyrogen was released from human leucocytes subjected to mechanical, osmotic, or thermal damage, and it was not formed when the cells were incubated in saline.

7. The source of rabbit leucocytic pyrogen, the action of nitrofurazone on leucocytes, and the supposed role of leucocytic pyrogen in fever are discussed.

BIBLIOGRAPHY

1. Atkins, E., Pathogenesis of fever, *Physiol. Rev.*, 1960, **40,** 580.
2. Bennett, I. L., Jr., and Beeson, P. B., Studies on the pathogenesis of fever. I. The effect of injection of extracts and suspensions of uninfected rabbit tissues upon the body temperature of normal rabbits, *J. Exp. Med.*, 1953, **98,** 477.
3. Collins, R. D., and Wood, W. B., Jr., Studies on the pathogenesis of fever. VI. The interaction of leucocytes and endotoxin *in vitro*, *J. Exp. Med.*, 1959, **110,** 1005.
4. Cooper, K. E., Cranston, W. I., and Fessler, J. H., Interactions of a bacterial pyrogen with rabbit leucocytes and plasma, *J. Physiol.*, 1960, **154,** 22P.
5. Harris, H., 1958, personal communication.
6. King, M. K., and Wood, W. B., Jr., Studies on the pathogenesis of fever. III. The leucocytic origin of endogenous pyrogen in acute inflammatory exudates. *J. Exp. Med.*, 1958, **107,** 279.
7. King, M. K., and Wood, W. B., Jr., Studies on the pathogenesis of fever. V. The relation of circulating endogenous pyrogen to the fever of acute bacterial infections, *J. Exp. Med.*, 1958, **107,** 305.
8. MacDonald, I., Rearing of rabbits free from coccidial infection of the liver (*Eimeria Stiedae*), *J. Animal Technicians Assn.*, 1957, **8,** No. 3.
9. Wendt, F., Snell, E. S., Goodale, F., Jr., and Cranston, W. I., Measurement of the human febrile response to a bacterial pyrogen, *Clin. Sc.*, 1956, **15,** 485.
10. Westphal, O., and Lüderitz, O., Chemische Erforschung von Lipopolysacchariden gramnegativer Bakterien, *Angew. Chem.*, 1954, **66,** 407.