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Pyrogen from Mouse Macrophages Causes Fever in Mice (39150)

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Fever is a common physiological response of mammals, and some reptiles, to infectious or inflammatory stimuli (1, 2). Mice, however, usually remain normothermic, or become hypothermic, when exposed to these same agents (3, 4). Fever in rabbits, and presumably in man, is mediated by endogenous pyrogens, small proteins which are released from leucocytes after stimulation by inflammatory agents such as endotoxin or phagocytosis of bacteria. These proteins, after entering the blood, in some way alter the "set-point" of temperature-regulating neurons in the hypothalamus, inducing responses of heat production and conservation, and thus causing fever in the host (5). The observation that under special conditions endotoxin can raise, rather than lower, body temperature in some mice (6–8) led us to postulate that mouse leucocytes, like those of rabbit and man, would produce endogenous pyrogen after stimulation *in vitro*.

Since previous work had indicated that products of mouse macrophages produce unpredictable temperature responses when injected into rabbits, we attempted to detect mouse leucocyte pyrogen by measuring the temperature responses of mice that were maintained at 34–35° (6) and injected with supernatants of cultured mouse macrophages.[1] The results confirmed our hypothesis that mouse macrophages produce endogenous pyrogen and showed that temperature responses in mice can be used to detect small amounts of endogenous pyrogens.

Materials and methods. All materials and reagents used were obtained or rendered pyrogen-free, as described previously (9). Mouse peritoneal macrophages were obtained from normal, 6-week-old female Swiss–Webster mice, using standard sterile techniques. These cells were 50–60% phagocytic macrophages and 40–50% small lymphocytes. Cells were incubated overnight at a concentration of 2.5×10^6 cells/ml in Eagle's Minimum Essential Medium (MEM, Auto-POW, Flow Laboratories, Inc., Rockville, Md.) containing 0.25% (w/v) lactalbumin hydrolysate (10). Heat-killed *Staphylococcus albus*, prepared as described previously (9), were added to some flasks at a multiplicity of 30:1 bacteria to cells to induce phagocytosis. Other flasks contained medium and staphylococci but no cells. After 18 hr of incubation, supernatants were centrifuged, cultured for sterility by inoculation in thioglycollate broth, and tested for pyrogen by injection into mice.

Pronase digestion of culture supernatant was performed as follows: 0.5 mg of pronase (B grade, Calbiochem, San Diego, Calif.) was dissolved in 0.05 ml of saline, preincubated at 37° for 2 hr, and then added to 2.5 ml of pyrogenic supernatant from mouse macrophages. This mixture was then incubated for 6 hr at 37°. Controls prepared and incubated in the same way included pyrogenic supernatant plus 0.05 ml saline and tissue culture medium plus pronase.

Ten-week-old Swiss–Webster mice were placed in individual small cages in an incubator maintained at 34–35°. After 1 hr, they were removed briefly every 10 min for rectal temperature readings, taken by means of a thermistor probe (No. 402, Yellow Springs Instrument Co., Yellow Springs, Ohio) inserted to a distance of 2 cm for about 30 sec and recorded on a Yellow Springs tele-thermometer. Mice were gently restrained by the tail during the pro-

[1] A previous report (Falk, R. J., Lab. Invest. **15**, 1761 (1966)) that products from mouse granulocytes produced changes in the circadian temperature cycle of conditioned mice provided questionable evidence for leucocyte pyrogen, because no temperature response was observed until 5 hr after injection.

cedure, and stroked to minimize activity. After three or four readings, stable baseline temperatures were usually achieved, at levels for individual mice between 37 and 38°. These mice were then injected intravenously with 0.3 ml of a test solution, and their temperatures were monitored every 10 min thereafter for 1 hr or longer. Individual mice received no more than four injections during 4 days and were then sacrificed.

Results. In the first experiments, mice were injected with supernatants of cultured mouse macrophages, and their temperature responses were monitored. The average results of three such experiments are shown on the left in Fig. 1. Whereas injection of supernatant from nonstimulated macrophages, and from medium incubated without cells, caused no significant change in temperature of mice, injection of supernatant from stimulated macrophages produced a prompt, monophasic fever, which reached a peak at approximately 20 min and rapidly subsided. This short latent period and rapid defervescence after iv injection is characteristic of responses in rabbits to endogenous pyrogens produced by rabbit or human cells (5). Although occasional small temperature elevations were observed following injection of such supernatants in mice kept at 25°, only mice kept at temperatures above 30° consistently gave febrile responses.

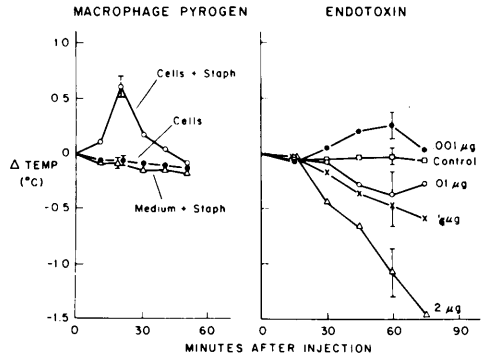

FIG. 1. Temperature responses in mice to intravenous injection of incubation medium from mouse macrophages (left) or various doses of endotoxin (right). Each point represents the average change in temperature from baseline of 7–15 mice. SEM are shown at the time of maximum fever. Macrophages were incubated alone (CELLS) or with heat-killed staphylococci (CELLS + STAPH); medium with staphylococci (MEDIUM + STAPH) served as control. Supernatant from 7–8 × 10^5 peritoneal cells, of which about 4 × 10^5 were phagocytic macrophages, equaled one dose. *E. coli* endotoxin (Difco) was diluted in saline; 0.3 ml, containing 2.0 to 0.001 μg endotoxin, or saline alone, was injected intravenously into each mouse.

To compare these responses to those which would be produced by injection of endotoxin, dilutions of *E. coli* lipopolysaccharide (Difco) were tested in the same manner, except that temperatures were taken every 15 min, since in initial experiments changes in temperature were noted to occur more slowly. The average responses in groups of mice to these injections are shown on the right in Fig. 1. Marked hypothermia occurred in almost all mice receiving 2 μg endotoxin and in some mice in each group receiving smaller doses. Small elevations of temperature, which reached a maximum between 30 and 60 min, occurred in half the mice receiving the smallest dose. These results, like those reported by others (6–8), clearly distinguish the temperature responses to injection of endotoxin from those described above to products of stimulated mouse macrophages.

The temperature responses of mice to varying doses of leucocyte products were next examined (see Fig. 2, left). Mouse macrophages to which staphylococci had been added were incubated at concentrations up to 5 × 10^6 cells/ml, and supernatants were collected as usual. Serial twofold dilutions were then made in tissue culture media, and 0.3-ml samples were injected into mice. In each experiment, the dilution producing maximum average temperature elevation in a group of mice was assigned the value of 4X, and compared with more and less concentrated solutions of the same material, where available. As shown in Fig. 2, highly concentrated solutions containing pyrogen caused no average temperature rise in mice; low fevers occurred in some animals and moderate hypothermic responses occurred in others. Dilutions of the pyrogenic material, however, as shown by responses to 4X, 2X, and 1X doses, produced characteristic responses of decreasing magnitude.

Since endogenous pyrogens produced by rabbit and human leucocytes are inacti-

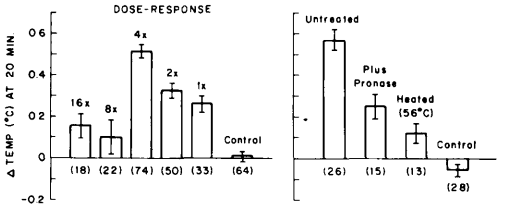

FIG. 2. Temperature responses in mice to intravenous injection of supernatant from mouse macrophages incubated with heat-killed staphylococci, or medium plus staphylococci (control). Average change in temperature from baseline at 20 minutes after injection ± SEM is shown by the height of the bars, and numbers of mice, in parentheses below the bar. In the dose-response experiment (left), 0.3 ml volumes of serial, twofold dilutions of supernatant were injected into groups of mice. The dilution giving maximum average temperature elevation at 20 min was assigned the value of 4X, and compared with more or less concentrated samples. The 4X dose generally contained supernatant from 2–4 × 10^5 phagocytic macrophages. On the right, temperature responses are shown to injection of supernatant, incubated alone (UNTREATED), incubated with pronase (0.2 mg/ml), or heated at 56° for 1 hr.

vated by heating and by incubation with pronase or trypsin (5), supernatant from stimulated mouse macrophages was tested for pyrogenic activity after heating at 56° for 1 hr and after pronase digestion. The results, shown on the right in Fig. 2, indicate that the activity of the supernatant is diminished by treatment with pronase, as well as by heating at 56° for 1 hr ($P = <.001$ for each treatment). These findings suggest that mouse macrophage pyrogen has an essential protein moiety which is heat-labile.

Our results indicate that although fever in mice is rare, mouse macrophages resemble rabbit and human mononuclear phagocytes in their capacity to release an endogenous pyrogen after phagocytosis *in vitro*. Presumably, therefore, mice ordinarily fail to develop fever not because pyrogen production is absent, but because of unusual thermoregulatory responses. Mice have a high thermal conductance, and in the laboratory reach conditions of thermal neutrality only at temperatures above 30° (11). A peripheral vasodilatation, which results from systemic endotoxin (12), may also occur with other pyrogenic stimuli. In cooler environments, this induced peripheral vasodilatation may counteract the central regulatory impulses for heat conservation, resulting in normothermia or hypothermia. Overriding peripheral vasodilatory responses, either to large amounts of endogenous pyrogen or to other materials released by stimulated macrophages in high concentrations, may explain the failure of mice to develop fever when injected with large doses of pyrogenic supernatants.

Calculations using the data shown in Figs. 1 and 2 indicate that products of 1–2 × 10^5 stimulated mouse macrophages produce fever in a mouse. By contrast, products of 0.5–1 × 10^6 stimulated rabbit or human monocytes are usually required to induce fever in a rabbit. To determine whether mice could be used to assay human pyrogen, we measured temperature responses in mice after injection of supernatants from human blood monocytes incubated *in vitro*, either alone or with heat-killed staphylococci. Parallel assays of the same materials were carried out in rabbits. The results showed that mice reacted to supernatants containing human leucocyte pyrogen with febrile responses which were indistinguishable from those shown in Fig. 1 for mouse leucocyte pyrogen. Supernatants derived from 4–6 × 10^3 stimulated monocytes produced average peak temperature elevations 20 min after injection of 0.54° in 24 mice. Control supernatants were nonpyrogenic. When compared with responses in rabbits, these figures indicate that the assay of human pyrogen in mice is 100- to 200-fold more sensitive.

Our experiments show that mice resemble other mammals in the capacity of their leucocytes to make pyrogen, and in their ability to develop fever after injection of this protein, under controlled conditions. The model may be useful for investigating the pathogenesis of immune fevers, using cells from genetically defined strains of mice, and in detecting small amounts of human pyrogen in clinical fevers.

Summary. Mouse peritoneal macrophages, after phagocytosis, release an endogenous (leucocyte) pyrogen. Intravenous injection of stimulated cell culture supernatant produces a prompt, monophasic fever in mice maintained in a 35° environment.

The pyrogen is distinct from endotoxin, and resembles cell pyrogens of other species in heat-lability and pronase sensitivity. Human leucocyte pyrogen produces identical responses in mice. Measurement of fever in mice appears to provide a sensitive biological assay for endogenous pyrogens.

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