

QUANTITATIVE ASPECTS OF THE RELEASE OF LEUKOCYTE  
PYROGEN FROM RABBIT BLOOD INCUBATED  
WITH ENDOTOXIN

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In the preceding paper a method for assaying leukocyte pyrogen was described which could be used in the presence of bacterial endotoxin (1). A simplified method suitable for routine use was also described. In the work reported in the present paper, the simple method was used to demonstrate the quantitative relationships between endotoxin concentration and leukocyte pyrogen yield in whole rabbit blood. These relationships strongly support the view that leukocyte pyrogen is a separate entity from endotoxin.

If endotoxin is added to blood, any which escapes binding to the buffy coat cells is dissolved in plasma. If the mixture is now incubated for 24 hr at 37°C, and injected into endotoxin-refractory animals, any fever which results will not be due to endotoxin unless a very large quantity has been used (1). Assuming the assay method is valid, and that liberation of leukocyte pyrogen is complete at 24 hr, it should be possible to demonstrate three phenomena:

(i) There should be some endotoxin concentration too small to cause significant leukocyte pyrogen release. Higher concentrations should produce some leukocyte pyrogen, but eventually a concentration should be found which causes all leukocytes present to liberate all the pyrogen which they contain, or can make. From this point on there should be no increase in pyrogen yield, no matter how great the concentration of endotoxin.

(ii) If a fixed quantity of endotoxin is incubated with different volumes of blood, then with small volumes an excess of endotoxin will be present, and the amount of pyrogen obtained should be directly proportional to the volume of blood used. With large volumes, insufficient endotoxin will be present to liberate all the leukocyte pyrogen. The graph of pyrogen yield against volume of blood will depart from the straight line. If the experiment is repeated using different fixed quantities of endotoxin then (a) the graphs of pyrogen yield against volume of blood should follow the *same* straight line, (b) the pyrogen yields at the points of equivalence just before the curves depart from the straight line should be directly proportional to the doses of endotoxin used.

(iii) If fairly large doses of different endotoxins are incubated with the same volume of blood, the pyrogen yields should be identical.

*Methods*

These were as described previously (1). The experiments of Sections (i) and (ii) were done using a purified proteus endotoxin ("E" pyrogen, Organon, Crown House, Mordern, Surrey,

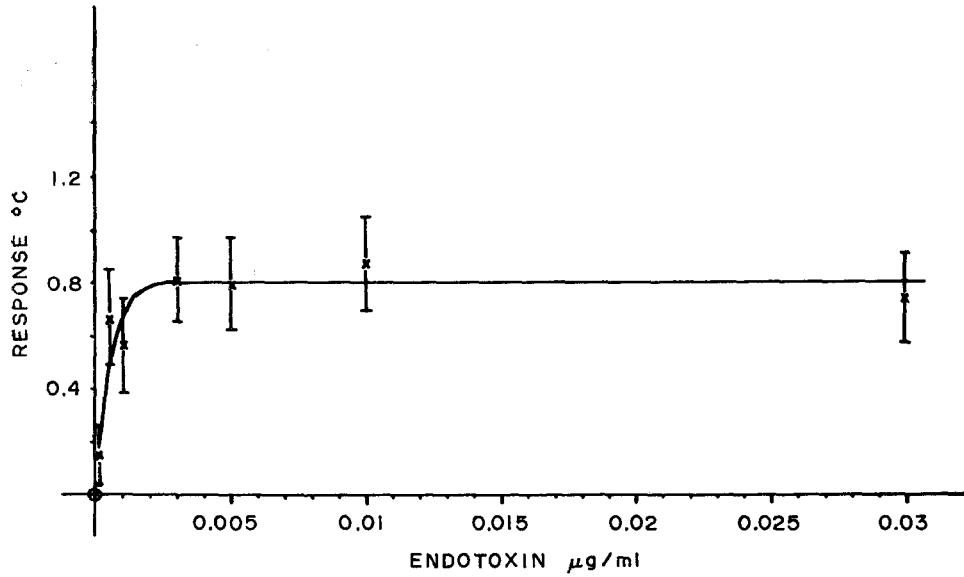


FIG. 1 and 2. Leukocyte pyrogen yield from 1 ml plasma from blood incubated 24 hr at 37°C with various concentrations of endotoxin.

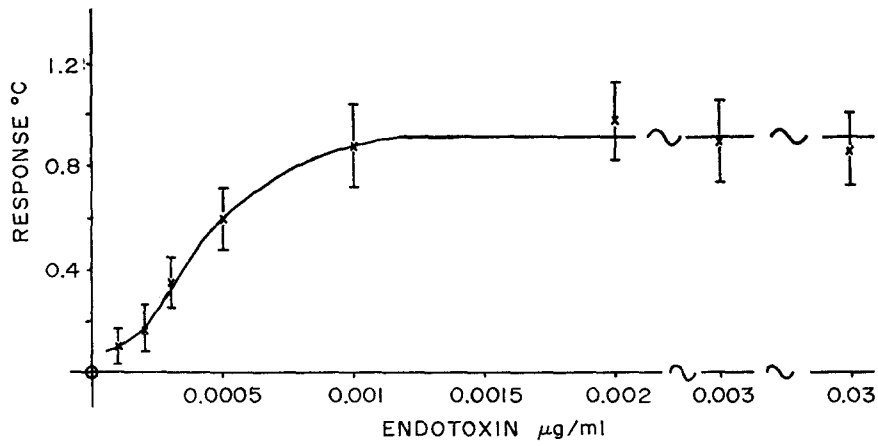


FIG. 2

England) both for experimental incubations and for rendering the assay animals refractory to endotoxin. In Section (iii) the refractory state was produced by 0.05 ml typhoid vaccine (Burroughs Wellcome & Co., London). It was shown in the preceding paper (1) that the leukocyte pyrogen content of a solution is directly proportional to the mean fever which it causes. In all graphs, mean fevers are given, together with 95% confidence limits, and can be regarded as expressing leukocyte pyrogen content in arbitrary units. When necessary, pyrogens were assayed in a small dose to bring the response into the linear portion of the dose-response curve, and the notional response to the original solution obtained by multiplication.

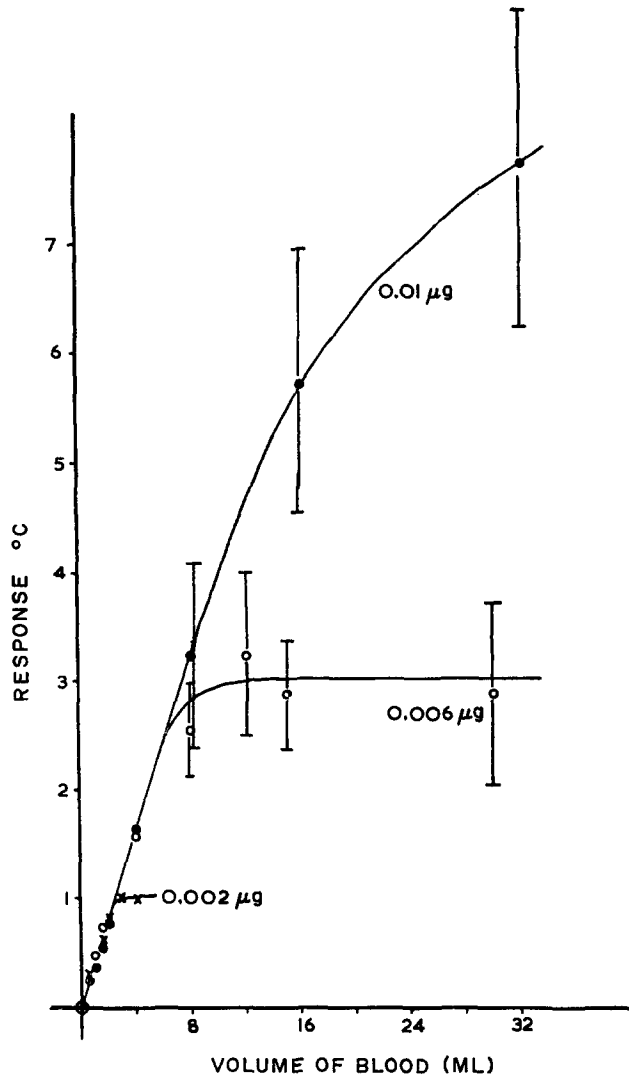


FIG. 3 and 4. Leukocyte pyrogen yields from different volumes of blood incubated with fixed quantities of endotoxin.

## RESULTS

(i) Figure 1 shows the results of an experiment designed to test the first deduction. A series of bottles containing 32 ml of fresh heparinized rabbit blood and various concentrations of proteus endotoxin were incubated for 24 hr at 37°C. At the end of the incubation the cells were centrifuged down and 1 ml doses of the supernatant plasma injected into endotoxin-refractory rabbits. The graph shows quite clearly that the pyrogen yield reached a maximum of

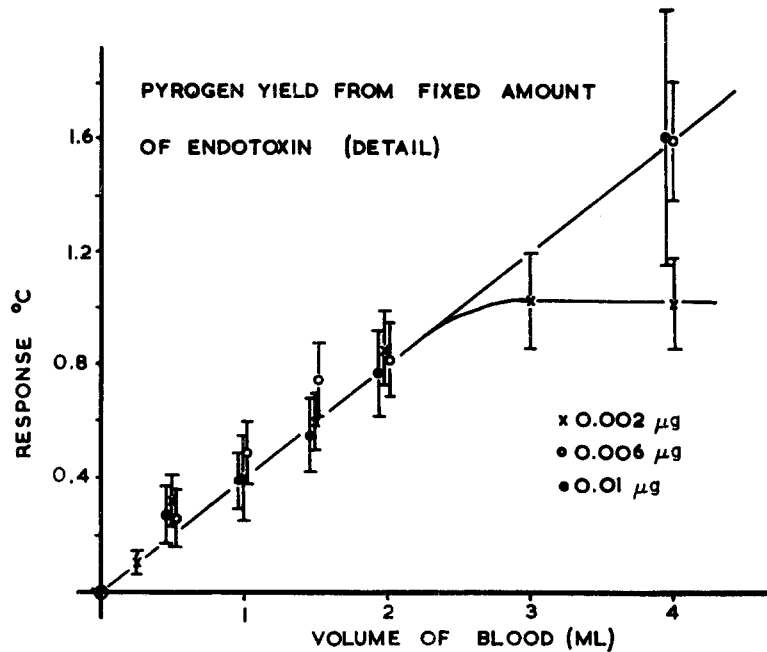


FIG. 4

about 0.8°C when the endotoxin concentration was about 0.002 µg/ml. There was no increase in pyrogen yield even with a fifteenfold increase in endotoxin concentration. This was confirmed by analysis of variance.

To delineate more precisely the area of change in the curve, the experiment was repeated using slightly different concentrations of endotoxin. The results are shown in Fig. 2 and completely confirm the first experiment, the concentration of endotoxin just sufficient to cause a maximal yield of leukocyte pyrogen being 0.001 µg/ml.

(ii) Figures 3 and 4 show the results of three separate experiments designed to test the second deduction. The three fixed quantities of endotoxin chosen were 0.002, 0.006, and 0.010 µg. The endotoxin was added to different volumes

of whole blood and incubated for 24 hr. Each experiment was done using pooled blood from six rabbits, and final white cell counts were  $10,000 \pm 1,000$  in all experiments. Individual variation was thus minimized. Doses of plasma between 0.25 and 1 ml were injected directly. Larger and smaller amounts were assayed by proportions (see Methods). This explains how fevers of  $3^\circ$  and  $5^\circ\text{C}$  came to be measured, and why the confidence limits of these large fevers were

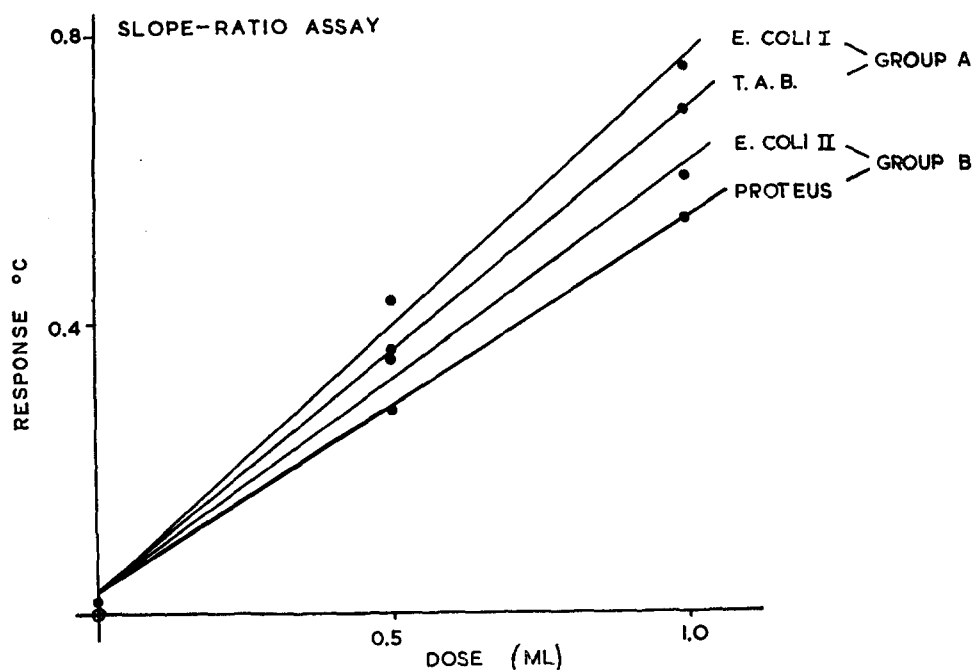


FIG. 5. Leukocyte pyrogen yields from plasma of the same blood incubated 24 hr with four different endotoxins. Groups A and B are two different groups of 16 rabbits each. T.A.B., typhoid vaccine.

so wide. The results were according to expectation. All three curves followed the same straight line at first, and this was confirmed by analysis of variance. The points where the three curves left the straight line were approximately  $0.9^\circ$ ,  $2.3^\circ$ , and  $4.5^\circ\text{C}$ , which were in tolerably good proportion to 0.002, 0.006, and  $0.010 \mu\text{g}$ .

(iii) An experiment to test the third deduction is shown in Fig. 5. Four endotoxins were used: typhoid vaccine; proteus endotoxin; and two *E. coli* endotoxins, one only moderately purified and the other very pure. For each a dose was selected adequate to produce complete liberation of pyrogen from blood. Arterial blood from four rabbits was pooled, and an aliquot incubated

with each endotoxin for 24 hr. The pyrogenicity of the supernatant plasmas was then compared in endotoxin-refractory rabbits by slope-ratio assay.

In each group of animals there is no significant difference between the two plasmas. The ratio of the largest to the smallest slope is 1.40, which is significant.

However, from experiments in which the two groups were given the same pyrogen, it was known that the response of Group A averaged 1.25 times that

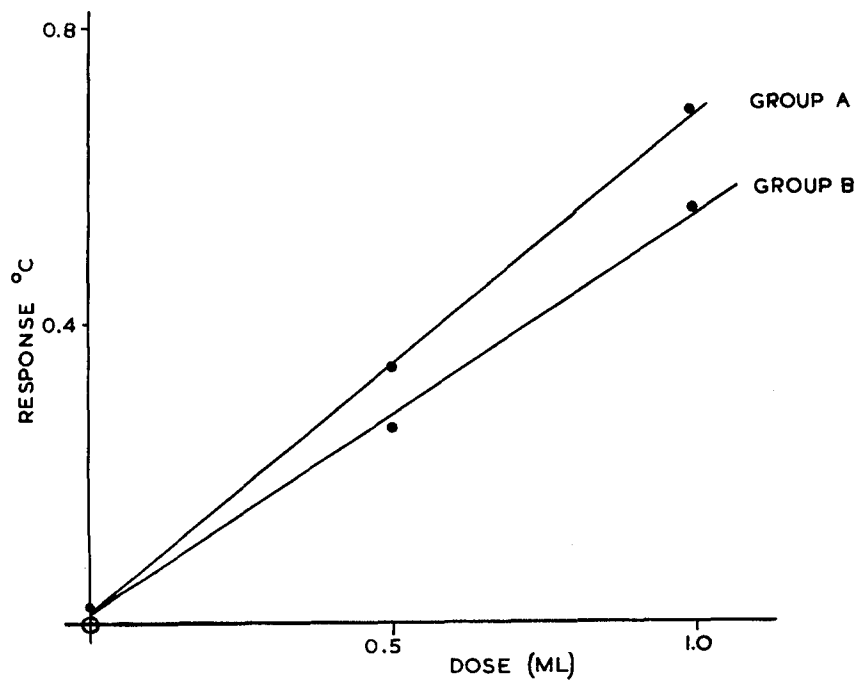


FIG. 6. Responses of Groups A and B to the same leukocyte pyrogen preparation.

of Group B (Fig. 6). When the responses and slopes of Group B were multiplied by 1.25, no significant difference remained (Fig. 7).

#### DISCUSSION

These experimental results confirm the three predictions made at the outset. The concentration of proteus endotoxin which causes maximal liberation of leukocyte pyrogen from blood is thirty times less than that which has been demonstrated to be completely inactivated by the assay procedure. With typhoid vaccine and *E. coli* endotoxin, the shape of the curves has been found to be identical, and the smallest concentration causing complete leukocyte

pyrogen liberation is a thousand times less than that inactivated by the assay procedure. Leukocyte pyrogen therefore cannot be endotoxin as added at the beginning of incubation.

It is more difficult to exclude the possibility that leukocyte pyrogen represents endotoxin modified in some way by leukocytes. However it is not easy to imagine what transformation endotoxin could undergo which would result in the precise

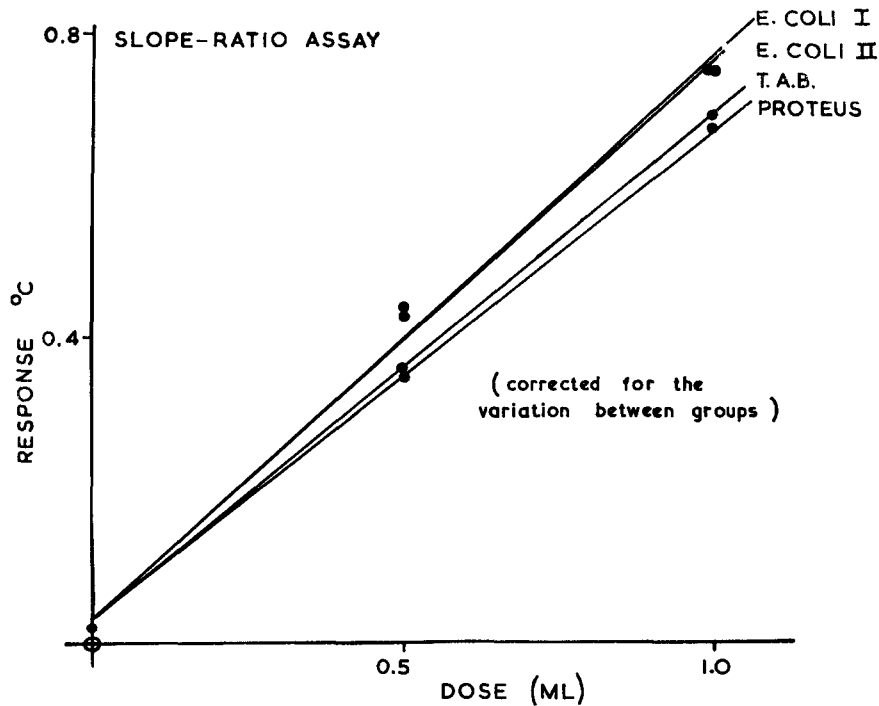


FIG. 7. Data of Fig. 5 corrected for variation between the two groups of assay rabbits.

quantitative relationships shown above. The yield of leukocyte pyrogen is completely independent of endotoxin concentration over a thousandfold range. The yield of pyrogen is directly proportional to the volume of blood used (and therefore the number of leukocytes present), and different quantities of the same endotoxin yield the same amount of pyrogen when incubated with blood. Lastly, endotoxins from three different organisms, one a crude cell-body vaccine, the others purified in varying degree, yield the same amount of pyrogen when incubated with blood. These results are more easily explained on the assumption that endotoxin causes blood cells to liberate a pyrogen which is a separate entity.

SUMMARY

This paper describes the relations between the endotoxin concentration and leukocyte pyrogen yield when endotoxin is incubated with whole rabbit blood for 24 hr. The results provide strong evidence for the view that leukocyte pyrogen is not modified endotoxin, and also show that the simplified assay method described in the previous paper works reasonably well.

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