AN ASSAY METHOD FOR LEUKOCYTE PYROGEN

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There is good evidence that when blood is incubated at 37°C with bacterial endotoxin, a pyrogen is liberated from neutrophil polymorphonuclear leukocytes and can be demonstrated in the supernatant plasma after centrifuging (1, 2). This pyrogen has been called leukocyte (or endogenous) pyrogen, and it is believed that the fever which follows the intravenous injection of endotoxin into experimental animals or man is caused by the release of leukocyte pyrogen (3, 4). The present paper describes a procedure which allows the quantitative assay of leukocyte pyrogen in mixtures of leukocyte pyrogen and endotoxin.

Pyrogens are assayed biologically by injecting them into rabbits and measuring the fever which results. It is easy to show qualitative differences between the febrile responses to leukocyte pyrogen and endotoxin, but all these distinctions become blurred when the endotoxin is dissolved in a medium containing protein (5).

Atkins and Snell (5) claimed that if a rabbit was given a large dose of endotoxin intravenously, its febrile response to small doses of endotoxin in plasma was reduced or abolished for about 24 hr afterwards, while the response to leukocyte pyrogen was little altered. They called this state of reduced response to endotoxin the endotoxin-refractory state, and suggested that fevers seen in normal rabbits could be regarded as due to the combined effect of leukocyte pyrogen plus endotoxin, while fevers in endotoxin-refractory rabbits were due to leukocyte pyrogen only. It was therefore possible to estimate endotoxin and leukocyte pyrogen separately. This method was examined and found to be unsuitable for quantitative purposes. A modified assay procedure based on that of Atkins and Snell was worked out, and was shown to be valid by formal statistical methods.

Methods

Rabbits were of mixed breeds, but mostly New Zealand whites. They were not used for assay purposes until they weighed at least 2.5 kg, and once selected were used exclusively and regularly for assay work. Assays were normally performed every other day; it was found that daily assay caused rapid deterioration in health. The rabbits were put into rows of wooden stalls, restrained by a collar around the neck. Room temperature was thermostatically controlled between 68° and 70°F. Rabbits' temperatures were measured by rectal thermistors

inserted at least 6 cm and connected to a 16 point Kent potentiometric recorder. The temperature of each animal was printed on a moving chart every 8 min, automatically providing a graph of temperature against time. All injections were given intravenously, and were diluted to a convenient volume (usually 2.5 ml) with saline. Two injections of leukocyte pyrogen were given on each assay day; it was found that there was little difference between morning and afternoon responses (6). Care was taken that each pyrogen was injected into rabbits whose experience of assay work was similar; it was found that rabbits new to assay work responded less to leukocyte pyrogen than did experienced animals (6). It was found that the correlation between response and body weight was very poor, so each rabbit was given the same dose of pyrogen regardless of weight (6). When only two pyrogens were being compared, they were assayed in the same group of animals, using a simple cross-over design.

It was not possible to ensure that the thermistors used recorded actual temperature changes. A correction coefficient was therefore determined weekly for each thermistor using a stirred thermostatically controlled water bath and an accurate mercury thermometer. All experimental readings were corrected before use (7).

Blood was obtained from a stock of healthy donor rabbits, usually by puncture of the central ear artery, occasionally by cardiac puncture. All blood was heparinized. White blood cells were counted in a Neubauer chamber using 2% acetic aid as diluent. Differential counts were done by examining 200 consecutive white cells on a Leishman stained film.

Metal apparatus and glassware, including syringes, were rendered sterile and pyrogen-free by baking at 170°C for at least 3 hr in a hot air oven. Some disposable syringes and needles were also used. Solutions of 0.9% saline, heparin, pentobarbitone, and lignocaine were obtained from commercial sources.

Three endotoxins were used: the first was a proteus endotoxin dispersed on mannitol ("E" pyrogen, Organon, Crown House, Mordern, Surrey, England); the second was typhoid vaccine (Burroughs Wellcome & Co., London); and the third was a purified lipopolysaccharide from *E. coli* (Dr. Elizabeth Work).

All experiments were done using aseptic technique; controls were included to exclude contamination with external endotoxin or microorganisms.

Leukocyte pyrogen was made by incubating fresh rabbit blood with either 0.003 μg/ml of proteus endotoxin or 0.001 μg/ml of *E. coli* endotoxin for 24 hr at 37°C. The supernate was removed after centrifuging and stored at 4°C until used. All injections were measured in terms of volume.

Fever height was read directly from the chart and was defined as the difference between the mean temperature in the 30 min preceding injection and the maximum temperature attained in the 90 min following injection. Bornstein et al. (8) showed that dose–response curves for leukocyte pyrogen were similar whether response was expressed as fever height or fever area. This was confirmed and it was shown that from the statistical point of view, height and area were about equally useful for assay purposes (9). Because fever height was so much easier to measure, it was used in preference to area throughout this work.

Results were analyzed by conventional statistical methods, using four decimal places throughout and correcting the final result to two decimal places. In all graphs, mean fevers are plotted together with their 95% confidence limits.

RESULTS

The first experiments were designed to test the method of Atkins and Snell (5). Figure 1 shows pooled data from many experiments in which the responses to various doses of proteus endotoxin were measured in normal and in endotoxin-refractory rabbits. Each point represents the mean response of eight animals;

the normal response is plotted along the abscissa and the refractory response along the ordinate. The 95% confidence limits are plotted as a rectangle around each point. The dotted line at 45° denotes complete equality, and it is clear

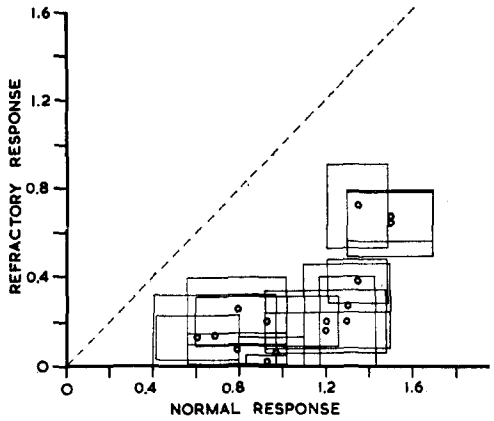


FIG. 1. Responses of normal and endotoxin-refractory rabbits to proteus endotoxin in plasma.

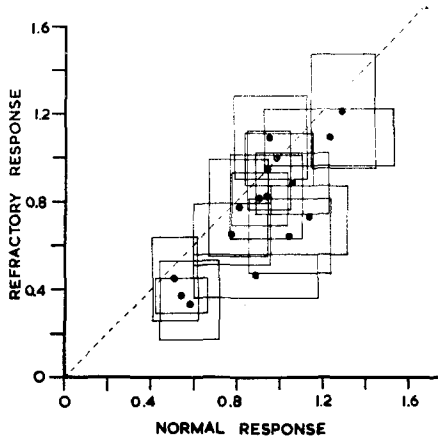


FIG. 2. Responses of normal and endotoxin-refractory rabbits to leukocyte pyrogen.

that there is a significant depression of the response to endotoxin in plasma in refractory animals. However, the response to small doses is not reduced completely to zero, and the response to large doses is not reliably abolished at all. Figure 2 shows a similar diagram in which are plotted the responses to various doses of leukocyte pyrogen in normal and refractory rabbits. There is slight but significant depression of the response to leukocyte pyrogen in endotoxin-refractory rabbits, especially well marked with small doses.

Because of these disappointing results, an attempt was made to improve the assay method. It was known that large quantities of endotoxin could be inactivated by incubation in normal plasma for 24 hr at 37°C (10, 11). This effect was confirmed (Fig. 3) but inactivation was not complete except with the lowest dose (0.01 μg proteus endotoxin). However, if endotoxin was incubated in plasma for 24 hr and then injected into endotoxin-refractory rabbits, no fever resulted even from a dose of 0.1 μg (Fig. 4). This was enough to give 10 normal rabbits a fever of about 1°C.

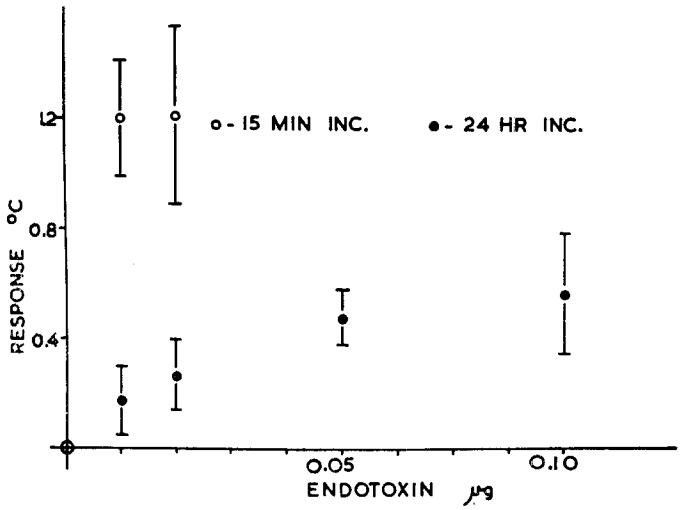


FIG. 3. Partial inactivation of proteus endotoxin by incubation in 1 ml plasma at 38°C for 24 hr. Injection into normal rabbits.

It was then shown that when leukocyte pyrogen was incubated in plasma for 24 hr and injected into endotoxin-refractory rabbits, the response to small doses was moderately reduced, and the response to large doses was little altered (Fig. 5).

It thus seemed that the double procedure of incubation in plasma for 24 hr followed by injection into endotoxin-refractory rabbits could be made the basis of an assay procedure which could measure leukocyte pyrogen in the presence of large quantities of endotoxin. In order to establish this it was necessary to show that dose–response curves for leukocyte pyrogen alone and for mixtures of leukocyte pyrogen and endotoxin coincided. Standard methods of biological assay could be applied provided: (*a*) there was a linear relationship between the febrile response and the dose or log dose of pyrogen given; (*b*) that in the case of a linear dose–response curve the response to zero dose of pyrogen was known; (*c*) that individual responses to the same dose of pyrogen were normally dis-

tributed; (*d*) that the variance of responses to different doses of pyrogen was constant or nearly so.

(*a*) The relation between the dose of pyrogen given and the response observed varied according to circumstances. In normal rabbits (i.e., not refractory or tolerant to endotoxin), there was a linear relationship between the log dose

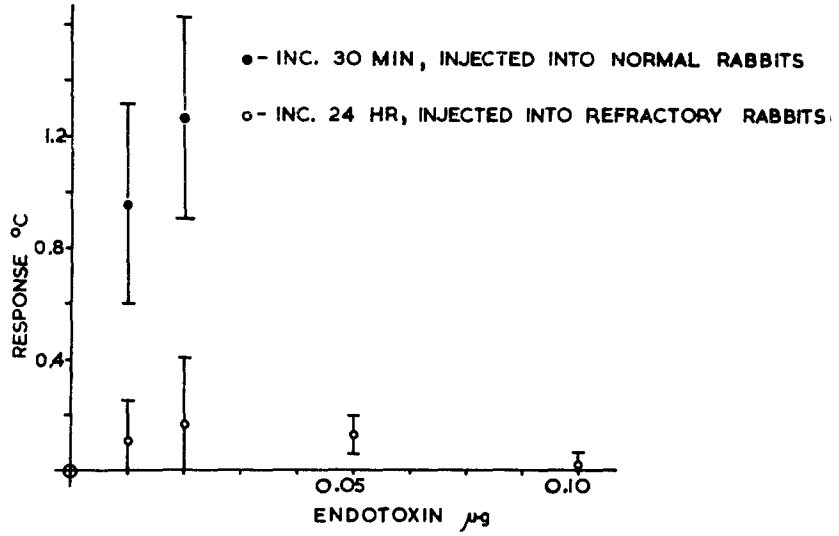


FIG. 4. Complete inactivation of proteus endotoxin by incubation in 1 ml plasma at 38°C for 24 hr and injection into endotoxin-refractory rabbits.

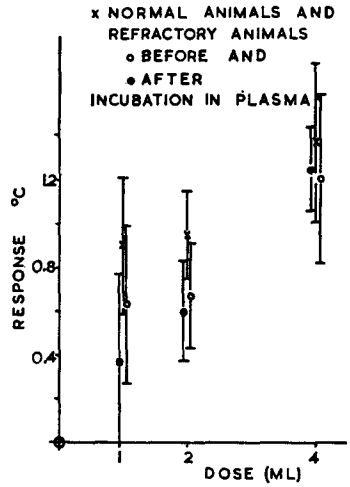


FIG. 5. Responses to leukocyte pyrogen little affected by incubation in plasma for 24 hr and injection into endotoxin-refractory rabbits.

and the mean fever height. Figure 6 shows log dose–response lines for two different groups of 16 rabbits. The lines have been shown to be valid by analysis of variance.

However, when leukocyte pyrogen was incubated in plasma for 24 hr, and then injected into endotoxin-refractory rabbits, the response to small doses was depressed, while the response to large doses was little altered. The dose–response curve, sigmoid in normal rabbits, became flattened, and for responses between 0° and 1°C could be regarded as linear. Figure 7 shows a dose–response line for

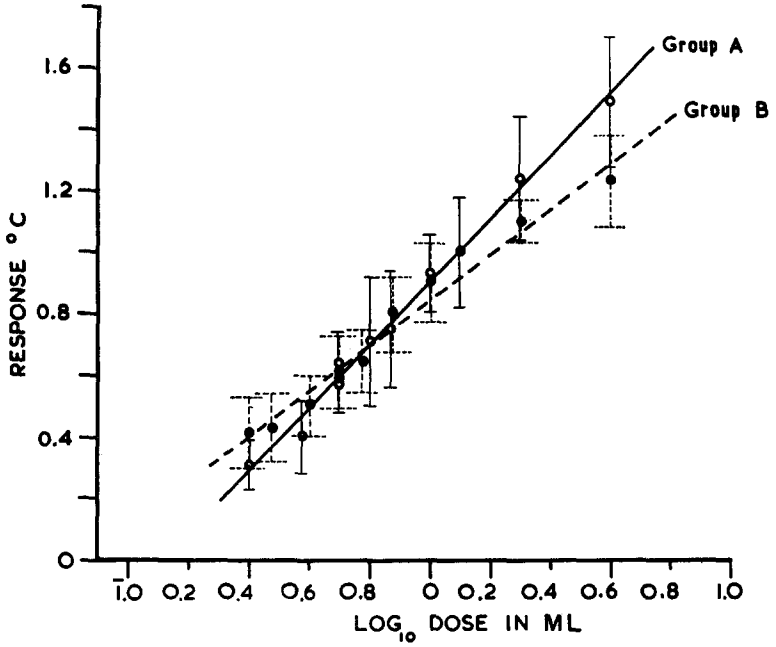


FIG. 6. Log dose–response lines for leukocyte pyrogen in two groups (A and B) of normal rabbits.

leukocyte pyrogen assayed in this way. Linearity was confirmed by analysis of variance. Thus, under the proposed assay conditions, there was a linear relationship between response and dose.

(*b*) A group of 32 rabbits was given 2 ml of sterile pyrogen-free saline intravenously and the mean deviations from base line during the 90 min following injection were measured, taking account of size, duration, and sign of deviations. The values on four occasions (mean ± standard deviation) were: +0.0225 ± 0.12°C, +0.015 ± 0.09°C, +0.011 ± 0.10°C, −0.002 ± 0.09°C.

These deviations are so near zero as to be of no practical importance, and the response to zero dose may be taken to be zero. This is confirmed by the fact that the dose–response lines appear to pass close to the origin. Experimental

responses therefore need not be corrected for base line shifts during assay, as these cancel out on average.

(*c*) Figure 8 shows the distribution of 118 responses to 1 ml of leukocyte pyrogen in endotoxin-refractory rabbits. The normal distribution was shown to

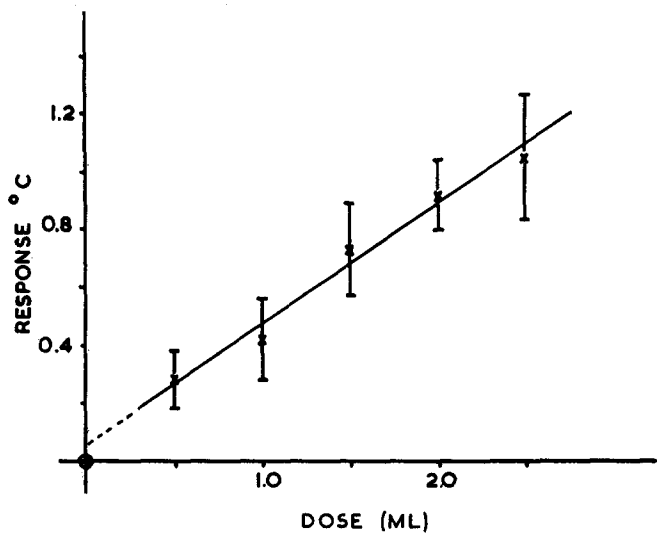


FIG. 7. Dose–response line for leukocyte pyrogen in endotoxin-refractory rabbits.

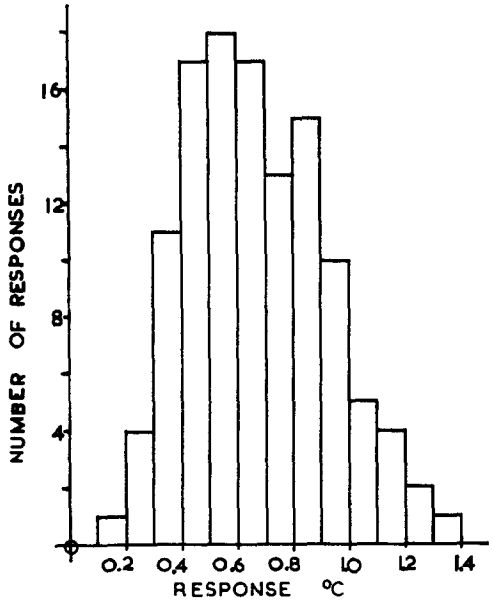


FIG. 8. Distribution of 118 responses to leukocyte pyrogen in endotoxin-refractory rabbits.

fit adequately by the chi square test. (Total $\chi^2 = 10.51$ with eight degrees of freedom.) Logarithmic transformation of the responses was tried and gave a distribution which was definitely skewed.

(*d*) The requirement of constant variance at all dose levels was only approximately true. It is quite clear from experience in assay work that large fevers

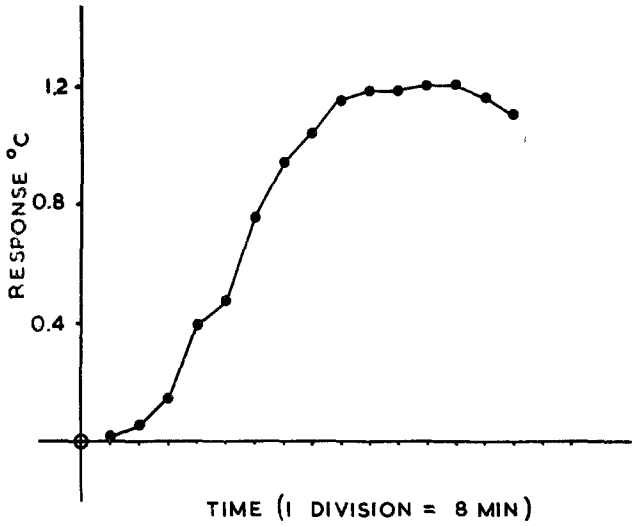


FIG. 9. Response of normal rabbits to 10^{-3} μg *E. coli* endotoxin. Mean fever curve (8 rabbits).

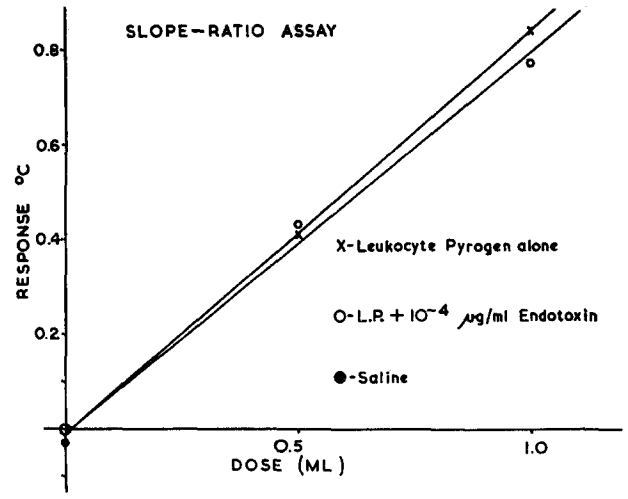


FIG. 10. Comparison of leukocyte pyrogen with leukocyte pyrogen + 10^{-4} μg/ml *E. coli* endotoxin.

have greater variances than small ones. The line of Fig. 7 was formally tested by Bartlett's test, which showed no significant change of variance from one dose level to another. However, this is merely evidence that the change of variance was not gross, and not that there was no change at all. In practice, uncertainty resulting from change of variance from one dose level to another affects mainly the fiducial limits, and not the potency ratio itself.

It was now possible to confirm the validity of the assay method by formal statistical methods. When there is a linear relationship between dose and

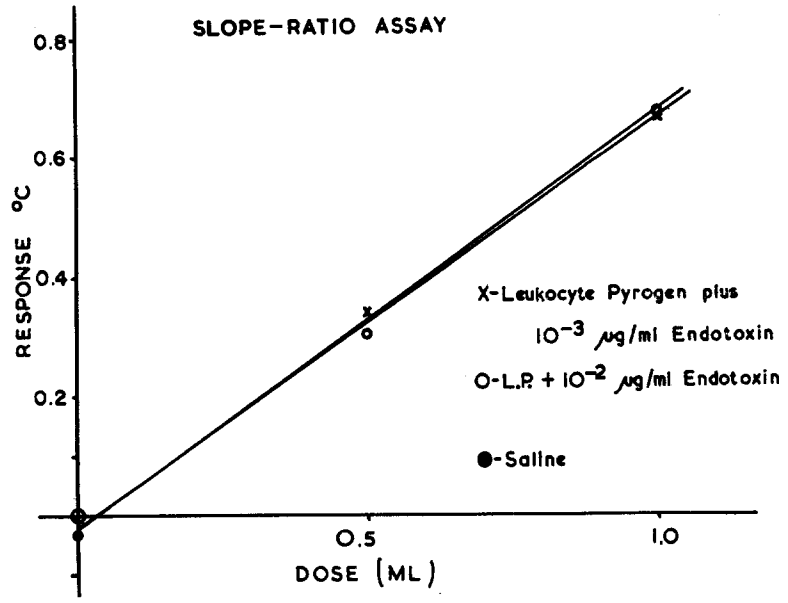


FIG. 11. Comparison of leukocyte pyrogen + 10^{-3} µg/ml endotoxin with leukocyte pyrogen + 10^{-2} µg/ml *E. coli* endotoxin.

response, the standard procedure is the five point slope ratio assay, using two doses of each preparation and one zero dose (saline) (12). Two regression lines are calculated subject to the condition that they shall intersect at zero dose. The ratio of potency of the two solutions is estimated by the ratio of the slopes of the regression lines, and the variance of this ratio can be estimated by Fieller's theorem (13). Two tests of validity must be made. The slope of the regression line for the standard preparation must differ significantly from zero, and this is tested by calculating a quantity called g, which should be <0.1. If $g > 0.1$, modified formulas for the potency ratio and its variance must be used, and if $g > 1.0$, the whole assay is invalid. It must also be shown that the two regression lines are valid, and this can be done by analysis of variance.

Experiments were conducted using a stock of leukocyte pyrogen which had

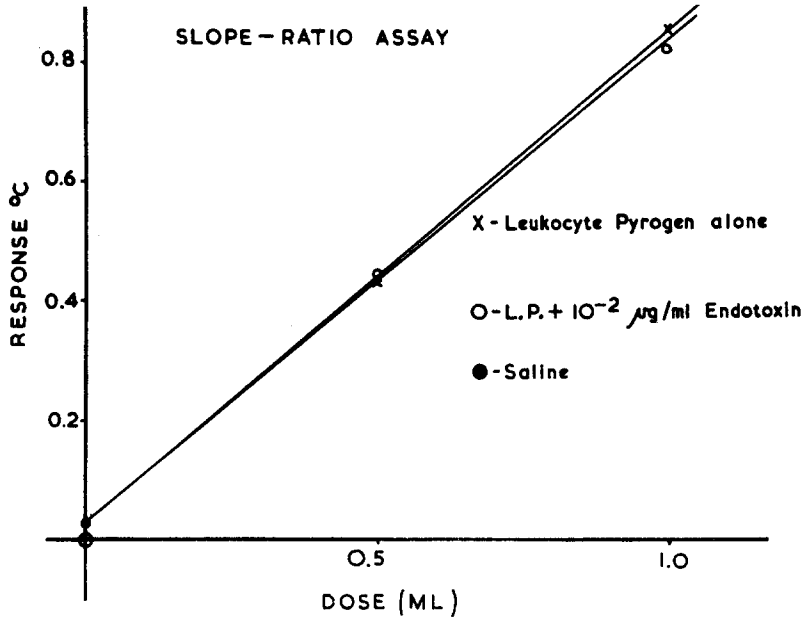


FIG. 12. Comparison of leukocyte pyrogen alone with leukocyte pyrogen + 10^{-2} µg/ml *E. coli* endotoxin.

been made by incubating fresh whole rabbit blood with 10^{-3} µg/ml *E. coli* endotoxin at 37°C for 24 hr. The blood was then centrifuged and the supernatant plasma contained leukocyte pyrogen. Aliquots of this were incubated for another 24 hr at 38°C with and without the addition of more endotoxin. The resulting solutions were then compared by five point slope-ratio assay in rabbits who had been made endotoxin-refractory by the injection of 0.05 ml typhoid vaccine. Each pair of solutions was given to the same 16 rabbits and the order of material given and dose injected was randomized. 10^{-3} µg *E. coli* endotoxin given to normal rabbits caused a fever of more than 1°C (Fig. 9). The results of the slope-ratio assays are shown in Figs. 10–13 and show clearly that leukocyte pyrogen alone assays almost identically with mixtures of leukocyte pyrogen and *E. coli* endotoxin in concentrations up to 10^{-1} µg/ml. The exact figures and fiducial limits are given below; for each assay $g < 0.1$ and analysis of variance confirmed validity.

(*a*) Leukocyte pyrogen + 10^{-4} µg/ml endotoxin compared with leukocyte pyrogen alone:

$$R = 0.95 \pm 0.19$$

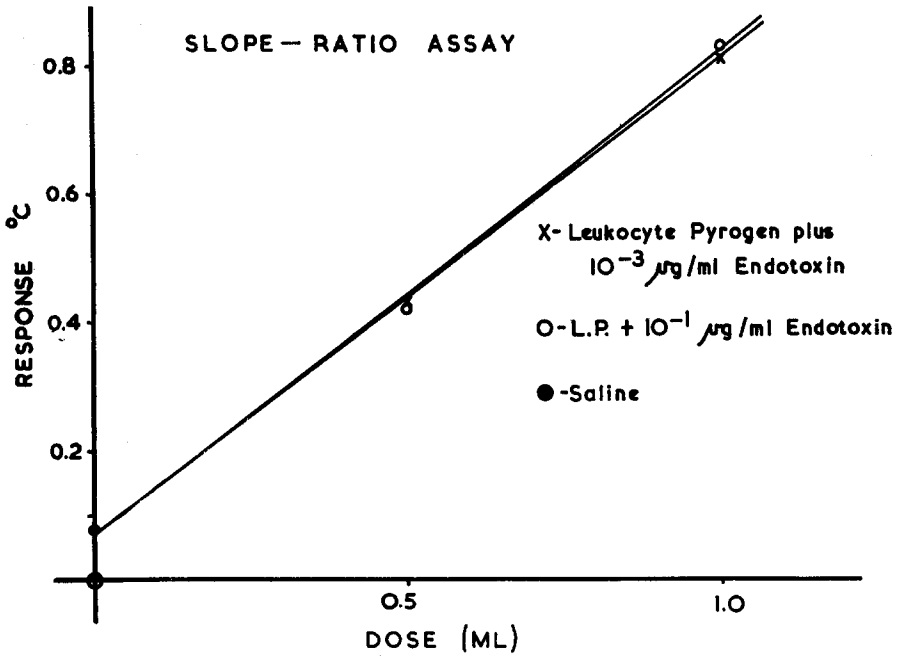


FIG. 13. Comparison of leukocyte pyrogen + 10^{-3} µg/ml endotoxin with leukocyte pyrogen + 10^{-1} µg/ml *E. coli* endotoxin.

(*b*) Leukocyte pyrogen + 10^{-2} µg/ml endotoxin compared with leukocyte pyrogen + 10^{-3} µg/ml endotoxin:

$$R = 0.98 \pm 0.20$$

(*c*) Leukocyte pyrogen + 10^{-2} µg/ml endotoxin compared with leukocyte pyrogen alone:

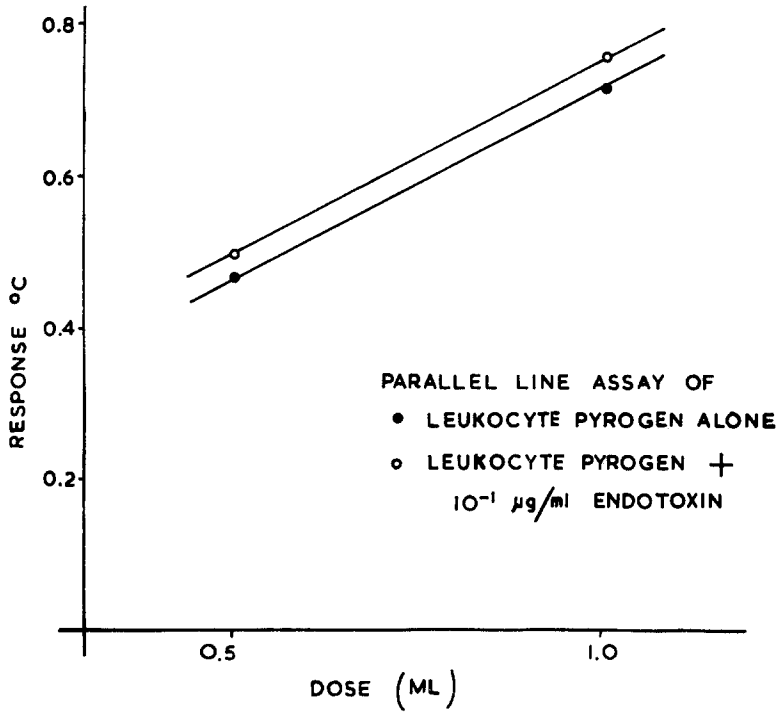
$$R = 0.98 \pm 0.20$$

(*d*) Leukocyte pyrogen + 10^{-1} µg/ml endotoxin compared with leukocyte pyrogen + 10^{-3} µg/ml endotoxin:

$$R = 1.02 \pm 0.23$$

These results were very satisfactory, but it was thought necessary to confirm that the validity of the assay method extended to concentrations of endotoxin as high as 10^{-1} µg/ml. Four further experiments were therefore undertaken in which leukocyte pyrogen alone was compared with leukocyte pyrogen + 10^{-1} µg/ml endotoxin. Unfortunately, the results when obtained showed serious departures from the usual linear relationship between dose and response. It is

thought that the reason for this was that, due to pressure of work, the assay animals were being used daily; all previous work had been done allowing a day of rest between assays. Several rabbits became obviously unwell, and most were not eating or drinking normally. Daily assay was clearly too much for them, and it was demonstrated that after a week's rest, the dose–response curve had resumed its usual linear shape (14).



FIGS. 14–17. Comparisons of leukocyte pyrogen alone with leukocyte pyrogen + 10^{-1} µg/ml *E. coli* endotoxin using parallel line assay.

Whatever the cause of the change of shape of the dose–response curve, the results could not be used as slope-ratio assays. Accordingly, the zero values were discarded, the results plotted against log dose, and the potency ratio estimated by parallel line assay. Two lines through the points are calculated subject to the condition that they are parallel. The potency ratio is estimated as the antilogarithm of the horizontal distance between the lines. The validity of the assay can be checked by analysis of variance (15). The results are shown in Figs. 14–17 and the precise values given below. When a regression of response on log dose is used, g is too large to neglect in the calculations so the values were calculated from the exact form of Fieller's equation.

(*a*) $R = 1.045$ $0.81 < R < 1.36$

(*b*) $R = 1.14$ $0.91 < R < 1.48$

(*c*) $R = 1.13$ $0.85 < R < 1.60$

(*d*) $R = 1.10$ $1.01 < R < 1.39$

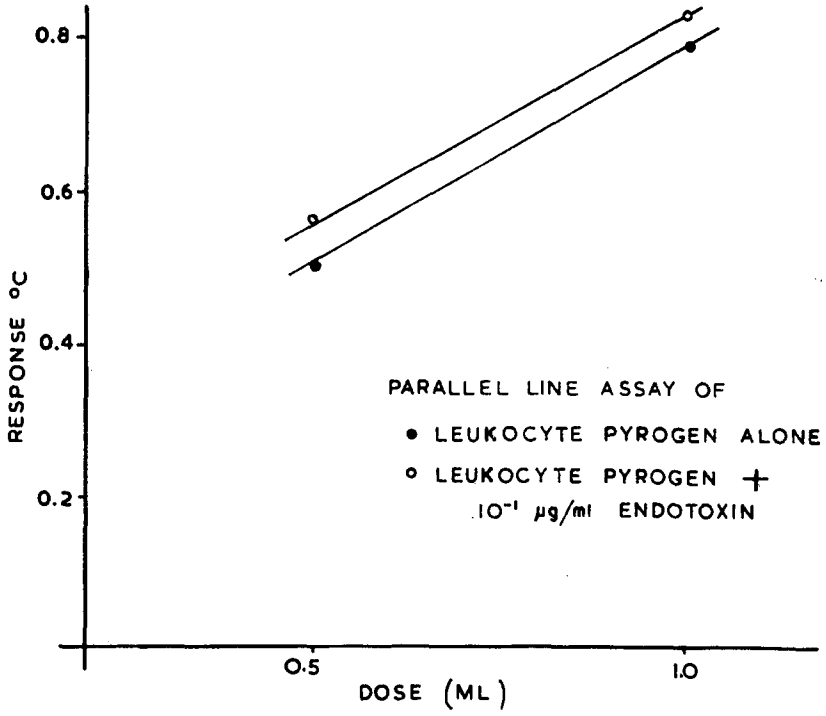


FIG. 15

Using all five experiments in which leukocyte pyrogen was compared with leukocyte pyrogen + 10^{-1} µg/ml endotoxin, the mean potency ratio is $R = 1.087$, and the true value almost certainly lies between 1 and 1.20.

DISCUSSION

The method developed above enables one to assay leukocyte pyrogen accurately in the presence of huge quantities of endotoxin. 10^{-1} µg of endotoxin is sufficient to give 100 rabbits a fever exceeding 1°C, but causes a mean error of only 9% in the assay of leukocyte pyrogen. Smaller quantities are quite undetectable. Therefore, if experimental materials are incubated in plasma for 24 hr and injected into endotoxin-refractory rabbits, the resulting fevers may

be attributed with confidence to leukocyte pyrogen, provided the concentrations of endotoxin used have not exceeded the known limits of validity of the assay method. In passing, it should be noted that the validity of the method is only certain under the precise conditions described above: when the refractory state is induced by proteus endotoxin and tested with proteus endotoxin, or when the refractory state is induced by typhoid vaccine and tested by *E. coli* endotoxin. It cannot be assumed to be a general method of inactivating *all* endotoxins without confirmation in the particular case.

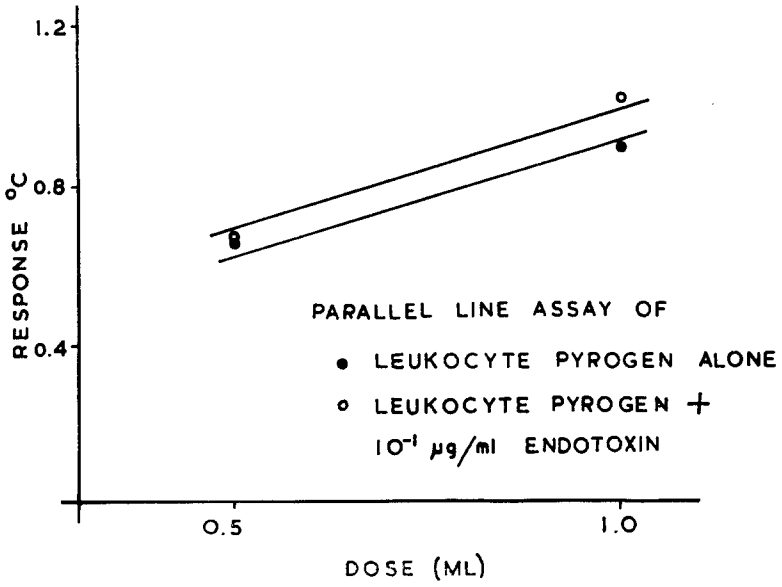


FIG. 16

Previous methods have not been shown to be valid by formal statistical methods. That of Fessler et al. (16) depends on the assumption that endotoxin in plasma is not heat-labile, which has been shown to be false (5). The endotoxin refractory state as described by Atkins and Snell (3) certainly exists, but the difference in responses to endotoxin and leukocyte pyrogen is not absolute, and when one simply has two mean responses, each of which has a large standard error, it is not possible to infer the proportions of endotoxin and leukocyte pyrogen in an unknown mixture. One can use the method to say whether or not a given pyrogen is mostly endotoxin, but it is not suitable for quantitative purposes. The method of Atkins and Wood (3) has not been examined; however, even in their published figures it is apparent that endotoxin fevers are not entirely abolished, and no indication was given of the dose range over which

the method is valid. It is also rather cumbersome to use in practice because of the requirement for serum from endotoxin-tolerant rabbits.

Slope-ratio assays may be done using any number of doses of the two solutions, the simplest arrangement being a three point assay, in which the response to one fairly large dose of each solution is measured, and also the response to zero dose. In practice, the response to zero dose may be taken to be zero, for the mean base line deviations shown above are very small compared to the usual fevers measured. The potency ratio of two solutions of leukocyte pyrogen is then given by the ratio of the mean fever heights which they cause.

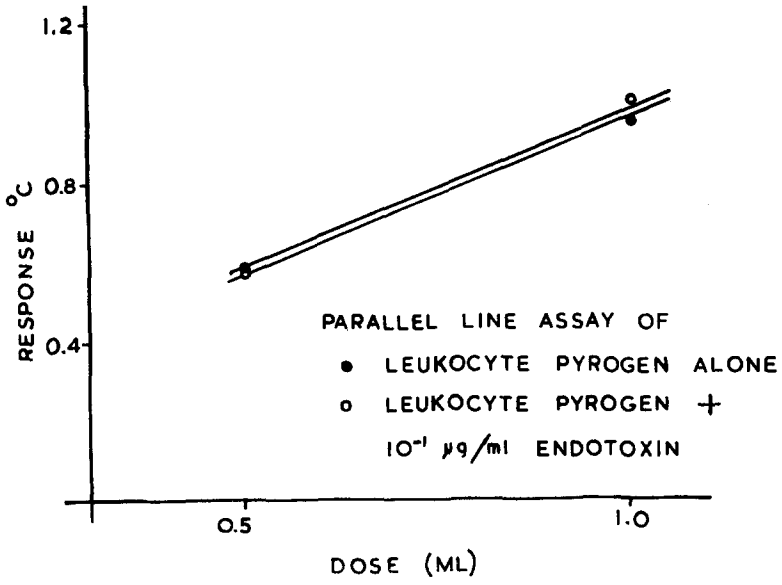


FIG. 17

However, the assumption of a zero response vitiates the whole logical basis of the assay, for no regression of response on dose has been demonstrated in the particular assay under consideration. Furthermore, there is no way of checking from the internal evidence of the assay the validity of the other assumptions: normal distribution; constant variance; and linearity of dose–response curve. Thus the simple comparison of one dose of each pyrogen cannot be regarded as giving any exact estimate of the amount of pyrogen which each contains, for the estimate is not susceptible to mathematical analysis. Provided it is clearly recognized that the data are at best semiquantitative, and no great reliance is placed on the exact numbers obtained, the method can be used with results which seem in practice to be quite good. Furthermore, a good deal of pyrogen work consists of showing that one pyrogen is or is not significantly different

from another. For this purpose, the t test is absolutely valid; indeed, even if the numerical values are regarded as no more than indications of rank order, analysis by nonparametric methods will show the differences equally well. From the practical point of view, the simple two-point comparison takes two-fifths of the time required for a full scale slope-ratio assay.

It should be noted that the simple comparison of one dose of each solution of leukocyte pyrogen provides an approximate value for the potency ratio only under the circumstances of the present assay. When leukocyte pyrogen is obtained from some source known to be free from endotoxin, and is injected into normal rabbits, the dose–response curve is not linear, but logarithmic, and other arrangements will have to be made.

SUMMARY

A method for assaying leukocyte pyrogen is described which is shown to remain valid despite the presence of very large amounts of bacterial endotoxin. It uses a combination of two procedures to inactivate endotoxin: incubation in normal rabbit plasma for 24 hr at 37°C, and injection into rabbits rendered refractory to endotoxin. The validity of the assay was confirmed by formal statistical methods. A simplified assay method is also described which lacks full statistical validity, but is more suitable for routine use.

The work described in this paper was done while the author held a Medical Research Council Fellowship in Clinical Research. The results are presented only in the form of diagrams; the full results with statistical calculations may be found in the author's D. Phil. thesis, which is in the Bodleian Library, Oxford.

I have been helped in the course of this work by many people, and would expecially like to thank Dr. E. S. Snell for introducing me to pyrogen work, and Mr. C. Hanson, Mr. P. Pollitt, and Mr. G. Barnard for technical assistance.

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