THE LEUCOCYTE RESPONSE IN THE RABBIT TO PYROGEN FROM *PROTEUS VULGARIS*

PART II. NEUTROPHIL AND TEMPERATURE RESPONSES

BY W. ANDERSON and J. P. TODD

From the School of Pharmacy, Royal Technical College, Glasgow

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THE changes which occur among the neutrophils in the circulating blood of rabbits after the intravenous injection of pyrogen were considered worthy of investigation because there is evidence suggesting a relationship between these changes and the occurrence of fever as a result of a pyrogenic stimulus[1]. Several authors use[2], or have suggested the use[3] of, the alteration in the total number of circulating leucocytes as an index of pyrogenic activity. We have found this index unreliable in rabbits, and therefore decided to trace the changes occurring in the overall lobar configuration presented by the nuclei of the circulating rabbit neutrophils subsequent to the intravenous injection of pyrogen, and investigate its merits and potentialities as an index of activity. Previous reports of the effect of pyrogen on neutrophils are concerned with alterations in total numbers, some including comment upon the obvious addition of young neutrophils with unsegmented nuclei, others describing a "shift to the left," but none reports any attempt to measure this shift.

This report describes an endeavour to measure this "shift to the left" which occurs in rabbits after the intravenous injection of pyrogen, by calculating the percentage fall in the average number of lobes per neutrophil, and to compare this measurement as a possible index of pyrogenic activity with measurement of the average maximal rise of temperature in rabbits.

MATERIALS AND METHODS

The pyrogen preparation used was the dialysed freeze-dried sterile supernatant liquid from a culture of *Proteus vulgaris* grown in a simple glucose-ammonium-salt medium containing nicotinic acid (2×10^{-5}M). Its chemical simplicity and the fact that it can be prepared pyrogen-free commended this medium as an appropriate choice for *Proteus vulgaris*. The results of repeated tests before and after the investigation have satisfied us that this pyrogen preparation maintained its potency unaltered throughout the tests. The medium could not, in itself, be shown to have any detectable effect on temperature or white blood cells in the rabbit, nor indeed to produce any toxic effects after repeated intravenous injection under the conditions of the test.

In the investigation 4 dose-levels were used:—

	Dose	Log dose
A	0·02 ml./kg.	$\bar{2}$·3010
B	0·06324 „	$\bar{2}$·8010
C	0·1125 „	$\bar{1}$·0510
D	0·2 „	$\bar{1}$·3010

It will be seen that log B is equally spaced between log A and log D and that log C is equidistant from log B and log D. D was chosen as the highest dose because it elicited temperature and white blood-cell responses equivalent to about 75 per cent. of the maximal responses which, in our experience, can be elicited by this pyrogenic preparation under these conditions of experiment. The freeze-dried material was dissolved in apyrogenic saline immediately prior to injection. The dilution was such that each dose of original dialysed supernatant (e.g., 0·2 ml./kg., etc.) was contained in 2 ml., so that no matter what the dose, each animal received 2 ml./kg. of solution.

30 previously unused rabbits of both sexes were employed in the investigation and were divided into 6 groups of 5, the members within each group having similar normal values for the average number of lobes per neutrophil. Each animal received one injection per week over 12 weeks and 15 rabbits (3 groups) were used on each occasion; they were denied food during the 36 hours preceding the test. Environmental temperature was maintained at a reasonably constant level throughout.

The technique used in the investigation of temperature response was essentially that developed by Wylie and Todd[4], with certain minor alterations and refinements. This method where each rabbit is accommodated in a specially designed box, provides maximum comfort for the animals and combines with that essential, minimum opportunity for unnecessary and undesirable movement.

Smears of circulating blood were prepared from each rabbit immediately prior to, and about 3½ hours after, injection of pyrogen, and stained with Giemsa's Stain. We have found this stain satisfactory if used in a dilution of 1 in 10 and applied after fixing the rapidly dried smear for 3 minutes in pure methanol. Polynuclear and differential counts were performed on each smear. In performing the polynuclear count we adopted the grouping suggested by Cooke and Ponder[5] in their practical modification of the count introduced by Arneth, and the average number of lobes per neutrophil over 100 neutrophils was calculated for each rabbit (described by these authors as the "weighted mean"). These suggestions of Cooke and Ponder were adopted because they lead to a rapid and sensitive assessment of the overall nuclear picture of the neutrophils.

Before commencing the investigation the rabbits were conditioned to the procedure to be adopted in the tests and normal values were worked out from smears taken during this conditioning period under the experimental conditions prevailing during the subsequent tests, i.e., with thermocouples inserted. We found that this approximately constant degree of stress to which they were subjected did not deflect the count or upset the rectal temperature of the animals beyond normal limits.

Normal values. Average number of lobes per neutophil:—Mean value for 30 rabbits, 2·30; Coefficient of variation = 10 per cent.

It was found that some animals gave consistently lower values than others, e.g., the group of 5 Beveren rabbits gave a mean normal value of 2·13, whereas the 5 Dutch gave a mean normal value of 2·59. The means for the other 4 groups lay between the values for the 2 groups cited. It

was also found that within the range of these normal values, percentage changes in the average number of lobes per neutrophil, observed for each breed after the intravenous injection of any one dose-level of pyrogen, did not differ significantly.

The error observed in counting one smear several times was shown to be less than that involved in counting smears prepared from the blood of one rabbit at different times (weekly intervals). Both of these errors in turn were less than the difference between counts performed on smears prepared before and after injection of the smallest dose-level of pyrogen used in the investigation.

Results

Total white cell counts were not performed at each test because of the time-consuming technique, especially when 15 animals were being used at once, and when the information provided by them was not required in the calculation of change occurring in the average number of lobes per neutrophil. Several were made, however, and the results obtained are recorded in Table I. The post-injection counts were made between 3 and 4 hours after injection of pyrogen.

TABLE I

INCREASES IN TOTAL NUMBERS OF LEUCOCYTES IN RABBITS, 3 TO 4 HOURS AFTER INJECTION OF VARIOUS DOSE-LEVELS OF PYROGEN. TEMPERATURE RESPONSES ARE ALSO GIVEN

Dose, ml./kg.	Increase in white blood cells/cu. mm.	Rise in rectal temperature, °C.
0·2	14,400	1·22
	14,800	1·57
	6,600	1·30
0·1125	7,200	1·17
	6,400	1·49
	13,200	1·10
0·06324	3,000	0·77
	8,000	1·18
	13,000	0·81
0·02	1,400	0·34
	—	0·95
	800	0·45

Preliminary investigation showed that maximal deflection of the average number of lobes per neutrophil could be expected to occur between 3 and 4 hours after injection of pyrogen. Then, after a period of instability, the count commenced an upward return to normal and attained a steady pre-injection level about 4 to 6 days later (see Fig. 1).

Our end-point was therefore the value given by a smear taken about 3½ hours after injection. We have measured the response as the percentage fall in the average number of lobes per neutrophil, i.e., the difference between pre- and post-injection readings expressed as a percentage of the pre-injection reading. The deflection of the count can be shown to be due to the addition of 1- and 2-lobed neutrophils—mostly 1-lobed—to the circulating blood giving as a result, a lower value for the average number of lobes per neutrophil.

It was observed that, when a double-peaked temperature response occurred, it was advisable to wait until after the second peak had passed before removing the sample of blood, otherwise the smear contained very few white blood cells (about 200 or less white blood cells in one smear—which made counting extremely tedious and difficult—as opposed to 60 to 80 per strip of smear in one taken after the second peak). When the biphasic temperature response occurred, the second peak appeared about 3 hours

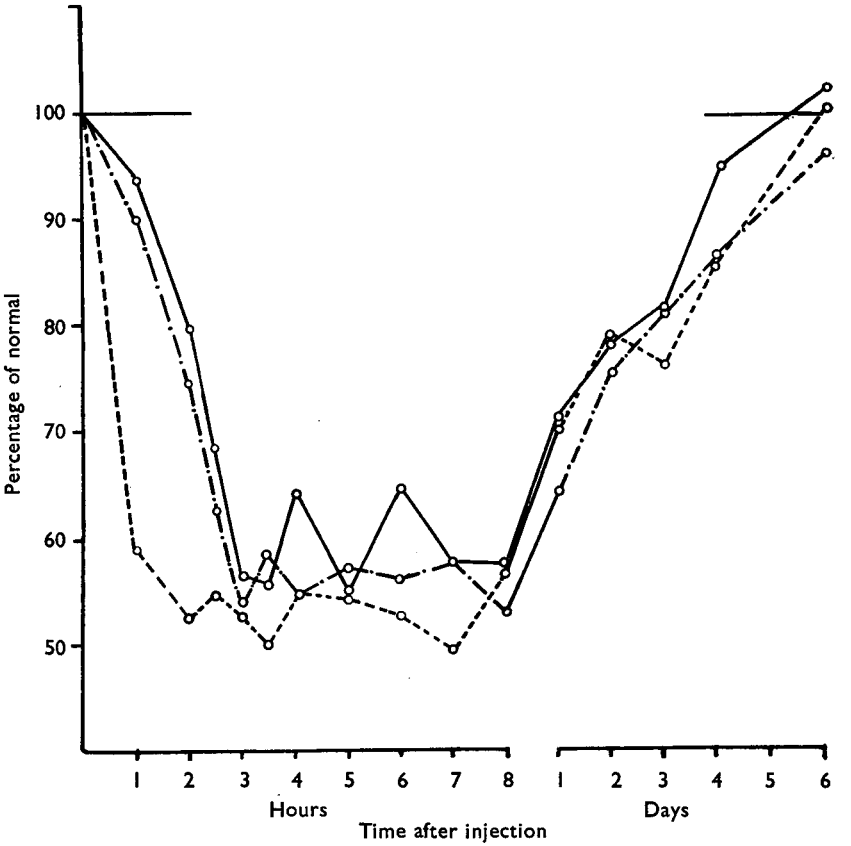

FIG. 1. Graph showing depression in average number of lobes per neutrophil after injection of pyrogen (0·2 ml./kg.) and the course of return to normality.

O——O Rabbit number 1
O—·—O ,, ,, 4
O - - O ,, ,, 5

after injection. After the second peak had passed, an abundance of young neutrophils made their appearance.

COMPARISONS OF INDICES OF PYROGENIC ACTIVITY INVESTIGATED

(See Tables II, III and IV and Figures 2, 3 and 4)

The results for each dose level are means of 90 readings (i.e., 360 experiments) and in each case the means can be shown to differ significantly from each other. Significant correlation was found to exist between each of the 3 different indices and the logarithms of the doses and also between temperature response and both white blood-cell responses. Analyses of variances showed that, in the case of all 3 indices, between-rabbit variance exists significantly. The comparison of different pyrogenic preparations would therefore be more accurate if the same rabbits were used, provided

they are not used often enough or within close enough intervals to establish a significant degree of tolerance.

Inspection of the regression lines suggests that, to a first approximation, straight lines fit the 3 sets of data. Analyses of variances to check linearity give the following values for F ($n_1 = 2$, $n_2 = 356$):—small lymphocyte percentage fall:—2·53; percentage fall in average number of lobes per neutrophil:—3·46; average maximum rise in temperature:—6·2. It is seen that 2·53 is just below the 5 per cent. probability level, 3·46 is just above it and 6·2 between the 1 per cent. and 0·1 per cent. levels.

TABLE II

TEMPERATURE RESPONSE IN THE RABBIT TO VARIOUS DOSE-LEVELS OF PYROGEN

Dose, ml./kg.	Average maximal rise in rectal temperature	
	Mean °C.	Coefficient of variation per cent.
0·2	1·38	21
0·1125	1·25	21
0·06324	0·95	30
0·02	0·61	36

TABLE III

LEUCOCYTE RESPONSE IN THE RABBIT TO VARIOUS DOSE-LEVELS OF PYROGEN PERCENTAGE FALL IN AVERAGE NUMBER OF LOBES PER NEUTROPHIL

Dose, ml./kg.	Percentage fall in average number of lobes per neutrophil	
	Mean per cent.	Coefficient of variation, per cent.
0·2	35·6	22
0·1125	29·8	28
0·06324	25·2	33
0·02	16·8	48

TABLE IV

RESPONSE IN THE RABBIT TO VARIOUS DOSE-LEVELS OF PYROGEN SMALL LYMPHOCYTE PERCENTAGE FALL (6)

Dose, ml./kg.	Small lymphocyte percentage fall	
	Mean	Coefficient of variation, per cent.
0·2	56·5	27
0·1125	49·8	39
0·06324	44·8	41
0·02	27·0	62

The ratio of the standard deviation of the scatter about the regression line to the difference between the means for maximum and minimum doses was calculated for each index of activity. This confirmed the impression given by the coefficients of variation that the order of accuracy for the 3 indices is: rise in temperature, percentage fall in the average number of lobes per neutrophil, and small lymphocyte percentage fall.

TOLERANCE

It was observed after the investigation had terminated that 8 of the population of 30 rabbits were beginning to show diminished responses to the highest dose level of pyrogen. These responses differed significantly from those elicited by the same dose at the end of the test. Rabbits which had been rested for 3 months gave the expected response. Advantage was taken of this condition of incipient tolerance in the 8 rabbits and their sera were tested for antibodies using the white-ring precipitation test. The presence of antibodies to our pyrogen could not be demonstrated in any of the 8 sera. We conclude therefore that for this level of tolerance the presence of antibodies to the pyrogen is not essential.

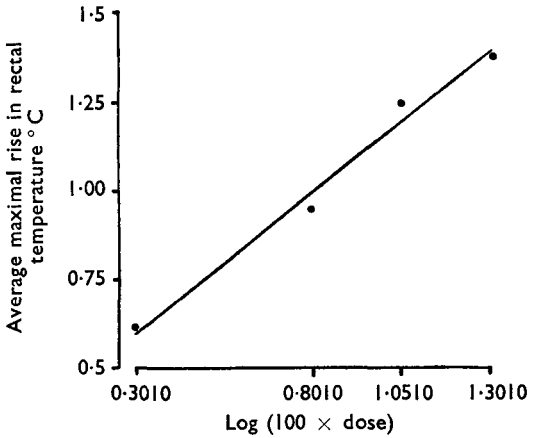

FIG. 2. Log dose response curve for pyrogen. Temperature response. Each experimentally determined point represents the mean of 90 observations.

$$Y = 0{\cdot}36 + 0{\cdot}79x \qquad \sigma_r = 0{\cdot}27$$

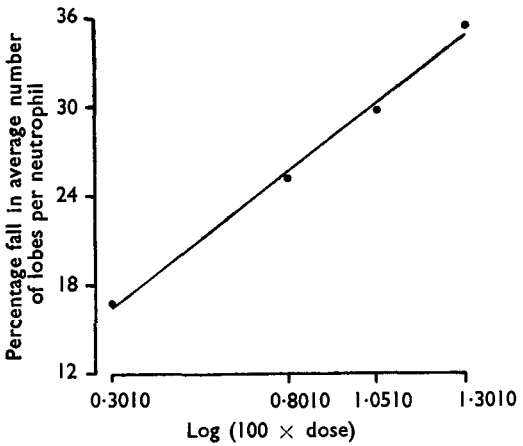

FIG. 3. Log dose response curve for pyrogen. Response measured as percentage fall in average number of lobes per neutrophil. Each experimentally determined point represents the means of 90 observations.

$$Y = 12{\cdot}83 + 18{\cdot}33x \qquad \sigma_r = 8{\cdot}24$$

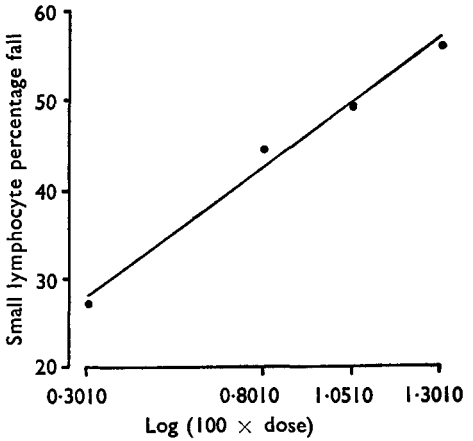

FIG. 4. Log dose response curve for pyrogen. Response measured as small lymphocyte percentage fall. Each experimentally determined point represents the mean of 90 observations.

$$Y = 19{\cdot}46 + 29{\cdot}03x \qquad \sigma_r = 17{\cdot}6$$

DISCUSSION

In the light of recent views[7,8] which have cast doubt upon the production of temperature-rise as a direct effect of pyrogen, it seems reasonable to attempt a measurement of response using an index based on an effect which, in view of these doubts, is not less likely to be attributable, directly or indirectly, to pyrogen. It appears from our results that in estimation of

pyrogenic effect, measurement of the average maximum temperature rise in rabbits affords accuracy not exceeded by any other method of estimation hitherto investigated, but, on the other hand, our results also suggest that an alternative method possessing an almost equivalent degree of accuracy exists in measuring the left-handed deflection of the polynuclear count, by computing the percentage fall in the average number of lobes per neutrophil which occurs in rabbits about 3½ hours after injection of pyrogen.

The active material is admittedly impure, as are most other preparations in use at the present time, and the extent of impurity is unknown, but we offer the fact that there is correlation of the 3 effects between each other over 4 dose-levels, as evidence that these effects derive from the injection of pyrogen. Furthermore, in preliminary investigations using a more highly purified pyrogen from *Proteus vulgaris* (and one from *E. coli*) we have observed that all 3 responses are equally evident after injection.

Summary

1. The effect of 4 dose-levels of pyrogen from *Proteus vulgaris* on the polynuclear count in rabbits has been investigated quantitatively and the effect measured as the percentage fall in the average number of lobes per neutrophil.
2. The simultaneous effect of pyrogen on temperature and on the percentage of small lymphocytes was also recorded.
3. Correlation was found between the 3 effects.
4. The merits of each of these 3 indices of pyrogenic activity have been assessed comparatively.
5. The presence of antibodies to pyrogen could not be demonstrated in rabbits which showed a small degree of tolerance.

It gives us pleasure to express our indebtedness to Miss Anne C. Inglis, B.Sc., M.P.S., for her patient assistance in performing many of the counts and to Mr. J. C. Eaton, M.A., for advice on the statistical analysis of the results.

One of us (W. A.) thanks the Council of the Pharmaceutical Society for the provision of an Educational Grant during the tenure of which this work is being carried out.

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DISCUSSION

The paper was presented by MR. W. ANDERSON.

DR. J. G. DARE (Leeds) said that this was the third paper by Professor Todd and his colleagues in which they had discussed the small lymphocyte percentage fall as a measure of pyrogenic activity. In the first paper they

had found no correlation between temperature and percentage lymphocyte fall; in the second paper they had found partial correlation, and in the present paper complete correlation. No reference was made in the paper to the different results obtained previously. Although the paper dealt mainly with a different index, in view of the discrepancy in the results with the other index the authors should offer a rational explanation as to why the present findings for this correlation should be accepted. In temperature response one often found double peaks; and in looking for the maximum response one must take the higher figure. The frequency of the occurrence of this double peak with *Proteus vulgaris* was related to the dose. Did the authors find a higher incidence of double peaks as the dose increased or was the incidence randomly scattered throughout the doses?

DR. G. E. FOSTER (Dartford) raised the question of the detection of pyrogens in antipyretic drugs, for which the method given in the paper might be useful. It would be interesting if some of Professor Todd's pyrogen could be mixed with such a drug to see whether the pyrogen could be detected by its effect on the white cells.

MR. T. D. WHITTET (London) said that at the Symposium held the previous December it was stated that calcium gluconate caused a temperature fall. This had been confirmed. He had found that chlorpromazine would completely abolish the pyrogenic response. Was the blood picture affected by chlorpromazine? Referring to Menkin's pyrexin, he said that, in America, strongly pyrogenic tissue exudate had been produced, free from bacterial pyrogen. Did the same effects occur with such substances as pyrexin and the leucocyte extract of Bennett?

MR. A. BRAGG (Liverpool) asked the age of the culture used for the preparation of the freeze-dried material. What were the time and temperature factors involved in the freeze-drying treatment?

MR. ANDERSON, in reply, said the object of the paper was to investigate the effect on neutrophils and had the authors wished to hide their previous conclusions they would not have referred to the small lymphocytes at all. A careful inspection of the various relationships between the three indices would show that what had changed was the temperature response. An improvement in the temperature response had resulted in correlation being established between temperature and small lymphocyte percentage fall. Replying about the double peaks, he said that in calculating the results he had used the maximum temperature attained, as shown in the tables. He agreed that the highest dose level of pyrogen elicited the greatest number of double peaks. The smallest dose gave an incidence of 8 per cent. of double peaks, the middle two doses gave 35 per cent. and the highest dose gave 28 per cent. This last result was surprising, and they concluded that the slightly smaller incidence at the highest dose level was probably due to the fact that the first peak was so high that there was then a flattening-out effect. They had visualised the admixture of their pyrogen with several types of drugs, notably those which themselves produced a white cell effect and those which produced a temperature effect. It had been reported that chlorpromazine produced an effect on white cells. In

regard to the age of the culture, he said that the organism was grown for three days at 37° C. in the medium stated.

PROFESSOR J. P. TODD, in reply, commenting on Dr. Dare's references to their previous work, said that Mr. Anderson had employed the term correlation with a different meaning from that used by Miss Dawson in one of the earlier papers.