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

PART I. MONONUCLEAR AND TEMPERATURE RESPONSES

BY MARY DAWSON and J. P. TODD

From the Royal Technical College, Glasgow

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IN the quantitative assay of pyrogens only the method based on temperature response in the rabbit has so far been examined in detail, and the results obtained by this method suffer from very wide variation. Attempts to find a less variable method of estimation led us to examine the reported[1–17] effects on the white blood-cell picture as a means of controlling the estimation of pyrogen. These workers injected pyrogenic preparations from various micro-organisms, such as *S. typhosa*, *Ps. aeruginosa* and *E. coli*, into different species of animal—the guinea-pig, the rabbit and the dog—and into man. The response generally noted was a leucopenia about an hour after injection, a temperature rise about one and a half to two hours after injection and then a leucocytosis. Some workers[2,12] carried out modified Arneth counts on neutrophil polymorphonuclears and reported a "shift to the left." Some[4,17] reported a fall in the numbers of eosinophils. Various other types of leucocyte have been reported to be affected in the leucopenias and leucocytoses. Finally, reports on the relative sensitivities of temperature rises and white cell counts as responses to pyrogen are conflicting.

In the present work the source, preparation and method of using the pyrogen standard, and the preparation and method of counting of smears are those previously described[18]. It was first established that the error involved in counting cells was less than the normal week-to-week fluctuation in count. To do this the variance in repeated counts of the same smear was compared with the variance in counts of different smears from the same animal at weekly intervals. This was repeated with other animals until it was established that the differences in variance were significant. It was also established that the error involved in counting cells and the normal fluctuation were themselves less than the effect caused by the injection of the doses of pyrogen used. It was also established that the time between injection and maximum white cell change in the differential count was about three hours.

Table I shows the fluctuation at weekly intervals of the small lymphocyte percentages of the rabbit population. The mean percentage of small lymphocytes in rabbits not previously used was not significantly different from that of rabbits used in a previous series of experiments involving the injection of pyrogen.

Repeated puncturing of the ear vein at half-hourly intervals for the removal of blood in itself produced a slight fall in the percentage of small lymphocytes, even without the injection of pyrogen. The change in white cell count was greatest about three hours after injection. Puncture of the

ear vein for the removal of blood was therefore limited to two occasions in each experiment, before injection and three hours after injection.

The fall in the percentage of small lymphocytes after injection was variable. There was, however, a high degree of positive correlation

TABLE I

DISTRIBUTION OF SMALL LYMPHOCYTES IN 25 RABBITS AT WEEKLY INTERVALS. RABBITS MARKED * NOT PREVIOUSLY GIVEN PYROGEN

Rabbit	Small lymphocytes, per cent.					
1	66	81	96	86	78	88
2	81	46	88	83	74	56
3	82	50	78	83	83	58
4	82	62	80	82	75	73
5	98	73	56	73	95	58
6	75	79	74	97	90	89
7	86	86	88	74	91	85
8	54	69	86	89	77	86
9	81	68	67	77	88	83
10	86	82	84	83	67	72
11	87	94	93	88	75	85
12	84	86	62	80	73	75
13	85	79	74	94	85	90
14	78	66	67	55	93	78
15	82	64	87	45	77	82
16	85	83	86	91	90	85
17	86	71	85	72	67	69
18	79	78	92	85	73	78
19	92	80	79	92	82	56
20	76	90	74	77	92	81
21	87	74	86	94	81	87
*22	55	85	75	71	66	63
*23	87	91	76	89	77	83
*24	69	73	87	80	50	54
*25	74	80	96	86	77	78

between the initial percentage of small lymphocytes present in any animal and the subsequent fall in that animal. Thus, it was observed in some rabbits with a high initial percentage, that the fall was actually greater than the total initial percentage of small lymphocytes for other rabbits. For this reason the fall in small lymphocytes was related to the initial percentage by expressing it as a percentage of that figure. This method of expressing the change due to pyrogen gave an answer of smaller variance than that obtained by subtracting the percentage after injection from the percentage of small lymphocytes before injection.

Although no correlation was found between temperature before injection and the extent of the subsequent rise, the accuracy of expressing the rise as a percentage of initial temperature was investigated. Thus the temperature response was expressed in terms analogous to those used for small lymphocyte response, but no increase in accuracy was found.

These small lymphocyte percentage falls and temperature rises measured simultaneously are recorded in Table II. No significant difference was found in the small lymphocyte responses of new and previously used members of the population.

Comparison of the coefficient of variation in the temperature and small lymphocyte responses, Table III, shows that at the high dose level the white cell method is the more accurate. At the other two dose levels the methods are of equal accuracy.

TABLE II

CHANGES IN SMALL LYMPHOCYTES PER CENT. (L) AND TEMPERATURE ° C. (T) IN 19 RABBITS GIVEN 3 DOSES OF PYROGEN

Rabbit No.	High dose				Middle dose				Low dose			
1 L	1·13	1·24	1·18	1·23	0·89	0·31	0·90	0·76	0·70	1·43	0·80	0·54
T	51	81	82	68	49	33	71	24	34	65	56	14
2 L	1·26	1·45	2·28	0·85	0·86	0·32	1·05	0·26	0·43	0·81	0·97	0·30
T	81	83	46	68	74	20	27	15	11	72	64	32
3 L	1·30	1·53	1·63	1·21	1·09	0·88	1·44	0·97	0·81	1·06	1·41	0·93
T	87	86	90	63	67	−1	60	50	39	45	49	35
5 L	0·96	0·95	1·13	0·99	1·29	0·44	0·73	0·86	0·55	0·56	0·74	−0·21
T	95	84	77	93	88	54	57	62	90	72	87	14
6 L	1·40	1·76	1·40	1·13	1·47	0·93	1·47	1·20	1·23	1·36	1·55	0·12
T	51	71	74	53	32	30	44	70	37	51	78	34
7 L	1·32	1·34	1·49	1·17	1·21	1·29	1·24	0·73	1·16	0·71	1·41	0·19
T	81	85	86	84	83	78	63	45	50	43	85	44
8 L	1·27	1·24	1·27	1·52	1·13	1·16	1·15	0·61	0·73	0·59	0·34	1·16
T	85	85	82	73	79	68	64	59	80	45	15	55
9 L	0·81	1·36	1·21	1·16	1·07	0·87	1·31	0·74	0·75	0·12	0·05	1·06
T	85	74	76	40	54	71	49	41	27	−16	−14	47
10 L	1·03	0·94	1·68	1·76	1·35	1·39	1·01	1·13	0·97	0·75	0·17	1·14
T	75	76	75	87	61	74	45	1	78	22	27	42
11 L	1·91	2·14	1·60	1·51	1·75	2·16	1·67	2·21	1·68	1·80	1·26	2·44
T	83	89	76	76	78	71	74	28	15	30	13	46
12 L	0·27	0·78	0·26	0·11	0·67	1·65	1·38	0·21	0·70	1·31	1·73	1·00
T	17	85	63	69	67	49	48	26	25	20	14	25
14 L	1·24	0·33	1·45	1·31	1·38	1·37	0·99	1·26	1·21	0·09	0·67	0·86
T	91	82	90	85	53	67	77	65	21	35	63	68
15 L	2·10	1·87	1·48	1·44	1·88	1·11	1·96	1·36	1·20	0·41	1·21	1·48
T	78	72	78	62	67	58	50	60	44	44	75	33
16 L	0·47	0·13	0·28	1·64	1·11	1·09	0·64	1·32	0·50	0·38	1·50	2·08
T	92	93	74	90	75	77	78	87	69	65	82	69
17 L	1·08	0·40	0·81	1·24	1·42	1·37	1·19	1·17	1·06	1·46	0·95	0·79
T	83	71	82	72	72	62	89	77	58	33	86	25
21 L	0·58	0·93	0·44	0·64	1·06	0·97	1·80	1·00	0·50	0·60	0·70	0·03
T	83	64	70	72	71	55	91	80	84	51	64	33
22 L	1·13	0·95	1·41	1·51	1·48	1·82	1·82	1·36	1·47	1·53	1·69	1·01
T	90	98	85	85	78	75	60	76	34	39	77	31
23 L	1·53	1·21	1·72	1·50	1·20	1·61	1·50	0·86	1·02	0·75	0·87	0·96
T	91	28	87	61	68	43	72	62	38	21	36	22
24 L	1·24	0·69	0·88	1·15	1·06	1·25	1·44	1·41	1·25	1·01	1·08	0·90
T	78	60	65	71	62	42	85	65	70	12	31	25

TABLE III

COMPARISON OF COEFFICIENT OF VARIATION IN TEMPERATURE AND SMALL LYMPHOCYTE RESPONSES

	High dose	Middle dose	Low dose
Mean temperature response	1·18	1·18	0·93
Standard deviation	0·46	0·41	0·50
Coefficient of variation per cent.	39	35	54
Mean small lymphocyte percentage fall	76	59	44
Standard deviation	15	20	24
Coefficient of variation per cent.	20	34	55

It is seen from Figure 1 that the white cell method was quantitative over the dose range used whereas the temperature method failed to distinguish between the middle and high doses. Each point on the graph is the mean of the responses to 76 injections. When the responses were calculated in terms of total mononuclears, that is, large lymphocytes, small lymphocytes and monocytes, the points obtained coincided with those for small lymphocytes. It was established that the three white cell

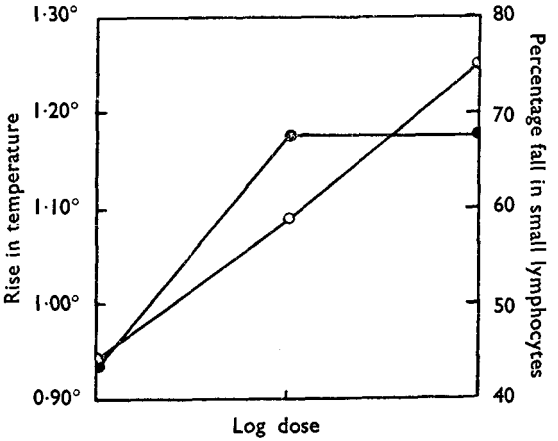

FIG. 1. Comparison of the rise in temperature and the percentage fall in small-lymphocytes as responses in the same experiments.
— ● — ● — rise in temperature.
— ○ — ○ — percentage fall in small-lymphocytes.

responses were significantly different and that a straight line fitted the data.

Comparison of the figures within any one column of Table II shows that rabbits of high sensitivity and of low sensitivity are encountered in both responses. It was established by calculation of Pearson's correlation coefficient that, in any one rabbit, the magnitude of the response by one method was not necessarily related to the magnitude of the response by the other method.

Analysis of total variance in the small lymphocyte responses into between rabbit and within rabbit components showed that between rabbit variance existed significantly.

SUMMARY

1. Rabbit leucocyte response to a freeze-dried pyrogen from *Proteus vulgaris* was investigated and differential counts were carried out.

2. The relative percentage of small lymphocytes fell, the maximum fall occurring about three hours after injection. The fall was expressed as a percentage of the initial level.

3. At the high dose level injected, the small lymphocyte percentage fall had a coefficient of variation of 20 per cent. and the temperature rise

a coefficient of 39 per cent. At the two lower dose levels the responses were of equal variability.

4. The small lymphocyte response remained quantitative over the three doses studied but the temperature response failed to distinguish between the middle and high doses chosen.

We wish to thank Mr. J. C. Eaton of the Mathematics Department for advice on the statistical analysis of the results, Mr. W. H. Martin of the Electrical Engineering Department for advice on the welding of thermocouples, Dr. J. Wallace of the Glasgow and West of Scotland Blood Transfusion Service for the use of a freeze-drying unit, the Trustees of the McCallum Bequest for the provision of a refrigerator, and one of us (M. D.) wishes to thank the Trustees of the Wellcome Pharmaceutical Research Fellowship.

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Discussion

DR. J. R. HODGES (London) said that he felt that the effects on the leucocytes described by Miss Dawson were not specific effects of pyrogen. The subjection of an animal to any form of stress causes the secretion of adrenocorticotrophic hormone by the anterior pituitary gland. The adrenocorticotrophic hormone stimulates the production of adrenocortical steroids which cause a fall in both the lymphocyte and eosinophil counts. This was probably the effect observed by Miss Dawson. It would not occur in hypophysectomised animals.

MISS DAWSON replied that the experiments were conducted on normal rabbits. The object was to find a method which would be quantitative when a larger dose was injected than could be measured by temperature. They were aware of the described effect on the lymphocytes. None of the rabbits was hypophysectomised.

THE CHAIRMAN added that it was known that one of the effects of pyrogen was the stimulation of the hyphophysis with the physiological effect mentioned. It was also a relevant criticism that, due to excitement or

other causes, and due to the complex mechanisms, there could be a lymphocyte fall. At the same time, it was extremely unlikely that this would occur in all of a group of rabbits which had been properly treated, with due regard to the excitability of the rabbits. The rabbit had not a very stable temperature-regulating system; one had only to knock it over the ear to elevate its temperature. But when they worked with a group of rabbits they ruled out the possibility of such occurrences, as far as possible. Unless such an occurrence as Mr. Smith had mentioned—the flooding of the laboratory—took place, it was unlikely that all the rabbits would be affected. With the quiet atmosphere in the room, this excitement did not occur, in any event. On studying the paper it would be seen that the experimental conditions were very reasonable.

MR. A. L. BACHARACH (Greenford) suggested that if Dr. Hodges's criticism, as he understood it, were valid it might strike at the roots of biological assay. Miss Dawson had described an attempt to establish a method of assay; its validity depended on the planning of the investigation and a satisfactory outcome to the statistical analysis of results. For example, it was no criticism of the growth response method for measuring vitamin A to establish that, had there been treatment with the growth-promoting hormones of the anterior pituitary, there would also have been an increase in weight, in spite of the vitamin A deficiency because the internal evidence of the assay showed a quantitative relation between the amount of vitamin used and the measured response. It was that, and nothing more, that Miss Dawson claimed to have established. He argued that to criticise a method of assay based upon a response to a particular stimulus, chemical or biological, simply because other stimuli could reproduce the same response in other circumstances or the same circumstances, was not justifiable if most biological assays were to be acceptable at all. Mr. Whittet had suggested that pyrogen-free water must be sterilised after "distillation" and that pyrogen-free solutions must be sterilised immediately after preparation. If water was distilled in a properly baffled still and it was received in a container itself sterile—if, in fact, it really was distilled and not in part splashed—he found it difficult to understand why it should be necessary to re-sterilise it, since pyrogens are not volatile in steam.

MR. WHITTET replied that it was partly an additional precaution. If the water were collected aseptically and the container closed immediately there would be little chance of the preparation being contaminated, but often the preparation of water for injection was only a preliminary to the manufacture of various other preparations. It was essential that sterilisation should take place the same day in those circumstances. He asked Miss Dawson if the leucocyte method had been compared with the rise in temperature method in a series of pyrogens from various organisms. It would be interesting to see whether there was correlation in large surveys. Many people were of the opinion that the substances causing hyperthermia and those causing the leucocyte effect were not the same.

THE CHAIRMAN said this had been done only for one pyrogen because of the enormous labour involved. They were fairly confident that if it applied to one it applied to others.

DR. J. I. M. JONES (Park Royal) endorsed what was said about the validity of criteria used in biological assays; if they established a satisfactory correlation between two sets of observations it would be justifiable to use one to prognosticate the second. That was what they did in biological assay. On the other hand, he supported Dr. Hodge's criticism from another angle. In the case mentioned, only at two levels was correlation established. It was always possible to get a straight line between two points. He did not think they were justified in concluding from the evidence that the leucocyte response and the temperature response could be used, the one to prognosticate the other over the complete range of doses. At high doses and low doses there might not be correlation, but he also suggested, after eliminating high and low doses, that certainly more than two points were needed to establish the correlation.

Miss DAWSON said she used three doses, not two.

DR. JONES interjected that only two correlated.

MISS DAWSON explained that limitation of material had restricted her to three doses. She hoped to correct this in future.

THE CHAIRMAN said the leucocyte test might become important in the therapeutic use of pyrogen should it be desirable to suppress the pyrogenic response. It might then be necessary to follow the blood picture as the only means of controlling an adequate dosage. Within limits, they had covered this range. There was justification in what Dr. Jones had said, but the work had supplied a basis for encouraging the use of the method, although it would be wrong to pretend that the correlation had been established further.

DR. W. L. M. PERRY (Mill Hill) said that the fact that none of these extracts of bacteria were of known purity must be taken into account. Dr. Hodges had pointed out that the impurities present might themselves produce stress. That was a valid criticism of the method as long as the substances were known to be impure.

DR. HODGES said that, while he agreed with much that Mr. Bacharach had said about the requirements of a biological assay, he considered that the most important requirement was that it should possess a reasonable degree of specificity. The method described by Miss Dawson might be a very good one for assaying pure pyrogen preparations, but pharmacists were more interested in the detection of pyrogens in drugs. The injection of the drug itself would obscure the whole effect by stimulating the very sensitive mechanism by which adrenocorticotrophic hormone is released.

DR. JONES said that if adrenocorticotrophic hormone was injected, then for the period specified in the B.P. tests there was a fall and not a rise in temperature. The rise did not take place until after 3 hours.

DR. WORRALL asked if pyrogen-containing water had been administered by mouth to very young animals. This might have a bearing on infantile

diarrhœa, a problem troubling many bacteriologists. Pyrogen might be the cause.

MISS DAWSON said she did not know of any comprehensive work where the pyrogen was administered orally to young animals.

MR. G. MILNE (Glasgow) said Mr. Whittet had confessed that he held no brief for charcoal as an adsorbing agent in the preparation of pyrogen-free water. He was not sure whether the speaker had meant pyrogen-free solutions of medicaments. In the preparation of anticoagulants containing sodium acid citrate and glucose for blood transfusion in large quantities much had to be left to unskilled labour, under supervision. They could not afford to omit the charcoal process when they did not know very much about the pyrogen content of the solid medicaments used. Pyrogens were classed chemically as polysaccharides, but in blood transfusion work that term had a specific meaning. Blood group specific substances in red blood cells were all polysaccharides, and those substances were known to be antigenic. In the large-scale use of these pyrogenic substances for artificial fever production were there any signs of antigenicity? Dextran, which was also a polysaccharide, had recently been reported to be antigenic.

MR. WHITTET said that citrate was likely generally to be contaminated, but in view of the fact that really good quality dextrose is rarely pyrogenic —and the same applied to sodium chloride—he was not satisfied that they should use the charcoal method for these routine preparations for administration in saline solution. It was essential for citrate, however. The British Pharmacopœia should describe pyrogen-free drugs which, if stored properly, could be used with the certain knowledge that they would not be pyrogenic.

In reply to Dr. Worrall he said that, at University College Hospital, Hartmann's solution was given to children with gastro-enteritis. This solution was readily contaminated with *E. coli*, which was a pyrogen-producing organism. They now issued a sterile solution of that type for oral use, the whole of which had to be used immediately the container was opened, and the incidence of some minor reactions had fallen considerably.

THE CHAIRMAN said Mr. Milne had raised the vexed question of the antigenic properties of pyrogen. There were differences of opinion on this subject. Dr. Dare, who had worked with one type of standard of his own manufacture, prepared from the whole cell, considered that it showed some antigenic properties. He emphasised again the different natures, already described, of the various pyrogen preparations. Their own preparation did not produce any antigenic response to pyrogen. Contaminating substances might do so.

MR. WESLEY (Sidcup), speaking from the point of view of a large-scale manufacturer of calcium gluconate, asked whether it was considered reasonable for a customer to demand a guarantee of freedom from pyrogen on supplies of the order of a ton.

MR. WHITTET said that in his earlier suggestions he had been thinking of hospital pharmacies. It would obviously be difficult to give the

guarantee for large quantities. As a rule, the manufacturers could take care of their own side of the work. They would probably have methods of eliminating pyrogen.

MR. P. J. FOWLER (Bristol) said that as a hospital pharmacist he was interested in avoiding pyrogens. Had Mr. Smith experience of samples of dextrose-salines which had been pyrogenic on the production line, and what was the source of the pyrogen? If it were the drugs, how were they stored?

MR. SMITH replied that no special treatment was used to render glucose pyrogen-free, but the sodium chloride used was heated almost to redness for a short time. It was more economical to throw away a whole batch of pyrogenic glucose-saline than to try to trace the source of the pyrogen.

MR. FOWLER thought the value of the information lay in avoiding pyrogen in other batches.

MR. SMITH added that if a batch of glucose-saline was pyrogenic a check would be made to determine if the same batch of glucose were involved. If it were, it would be discarded.

EVENING SESSION

Chairman: DR. H. O. J. COLLIER

The following 2 papers were read:

RABBIT RESPONSES TO HUMAN THRESHOLD DOSES OF A BACTERIAL PYROGEN

BY J. G. DARE and G. A. MOGEY

From the Department of Pharmacology, University of Leeds

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THERE are very few quantitative data on the effect of bacterial pyrogens in man. Even less information is available on the relationship between effective doses in man and rabbit. Co Tui and Schrift[1] stated that the rabbit is one-third as sensitive to pyrogen as man; they suggested, in consequence, that to test intravenous solutions for human use 50 to 100 ml./kg. must be given to rabbits. But the number of observations on which their result is based is so small, and the variation of these responses can be so large, that the significance of this result is doubtful. Lees and Levvy[2], on the other hand, stated that a dose of 20 ml. per rabbit was sufficient to detect whether enough pyrogen was present to cause a response in man.

In view of the scarcity and the contradictory nature of the evidence available, and because of the differences in magnitude of the rabbit response in varying experimental conditions, or from repeated administration of pyrogen, we decided to determine the minimal effective dose