THE ASSAY OF BACTERIAL PYROGENS

BY M. DAWSON and J. P. TODD

From The School of Pharmacy of Glasgow Royal Technical College

Received July 4, 1952

THE methods based on temperature response at present in use in the testing of solutions for pyrogen are useful as limit tests for fever-producing effect, but for estimation of effect involving comparison of preparations which do not differ markedly they are of little value for two reasons—the lack of a stable reference standard and the variation in the temperature responses of the rabbit. This work describes the preparation of a standard and its use in investigating variations in rabbit responses.

THE PREPARATION OF A STANDARD

As a source of standard we chose first *Escherichia coli* because it had been shown to produce pyrogen copiously,[1] to grow well in simple media of known chemical composition and to be relatively non-pathogenic. The pyrogenic supernatant liquid from cultures of this organism was, however, found to be unstable to even mild degrees of heat, whether the liquid was heated at the *p*H value of the growth, 4·7 to 4·9, or whether it was adjusted to *p*H 7 before attempting to concentrate by heating. The results of heating are shown in Table I.

TABLE I

LOSS OF PYROGENIC EFFECT FROM *Escherichia coli* PROVISIONAL STANDARD ON HEATING UNDER REDUCED PRESSURE

Dilution required that response might fall within the quantitative range	Time of heating (minutes)	Temperature °C.	Average rise in temperature in groups of 5 rabbits	
			Solution before heating	Solution after heating
0·2 per cent.	120	55	0·49	0·37
0·2 ,, ,,	45	50	1·13	0·47
0·2 ,, ,,	20	40	1·19	0·47
0·1 ,, ,, *	20	40	0·94	0·58

* Solution adjusted to *p*H7 before heating.

The pyrogenic effect also decreased on storage (Table II).

Attempts were made to store this pyrogen in the dry state by adsorbing it on asbestos pads and storing these in a desiccator. Complete adsorption of pyrogen on to a 3·6-cm. asbestos pad took place from 100 ml. of solution of pyrogen at *p*H 4·7 to 4·9, which was the normal *p*H value of a 4-days' growth of *E. coli*. Complete elution took place at *p*H 9 to 12. The dried pad retained the activity but the eluate soon decomposed. This method of storing pyrogen was soon abandoned as it was not convenient to elute the pyrogen and free the solution from asbestos fibres before each experiment. Table III compares the residual activity after storing the pyrogen on the pad and in the eluted form.

TABLE II

LOSS OF PYROGENIC EFFECT FROM *Escherichia coli* PROVISIONAL STANDARD ON STORAGE

Dilution	Period of storage days	Average rise in temperature in groups of 5 rabbits	
		Solution before storage °C.	Solution after storage °C.
1·0 per cent.	6	0·65	0·16
0·2 „ „	7	*1·56	0·83
0·2 „ „	12	*1·56	0·35
0·2 „ „	9	0·67	0·14
1·0 „ „	40	0·85	0·38

* Same solution.

TABLE III

COMPARISON OF THE LOSS OF PYROGEN IN THE ADSORBED AND ELUTED STATES ON STORAGE FOR 5 DAYS

Average rise in temperature in groups of 5 rabbits due to		
Pyrogen adsorbed, immediately eluted and immediately injected	Pyrogen stored on a pad for 5 days, eluted and immediately injected	Pyrogen adsorbed, immediately eluted and eluate stored for 5 days
0·77	0·80	0·31
0·95	0·75	0·49

No attempt was made to freeze-dry this preparation on account of its lack of stability.

E. coli was now discarded as a source of pyrogen and a standard prepared from *Proteus vulgaris*. The organism was grown in simple medium and separated from the liquid by continuous, high-speed centrifuge. The liquid was filtered through sterile, unglazed porcelain candles into sterile freeze-drying tubes and spin-freeze-dried. After the secondary drying under vacuum and with phosphorus pentoxide the ampoules were sealed by fusion of the glass and tested for faulty sealing by a high-frequency, glow-discharge tester. No loss of pyrogen occurred in the freeze-drying process and the material suffered no obvious storage loss during the 20 months it was used as the standard for the temperature response experiments. In carrying out these experiments the supply of this standard was exhausted, the amount prepared being limited by the capacity of the freeze-drying unit.

Freeze-drying of eluate from pads in an attempt to prepare a purer standard was not a success, as shown in Table IV. Neutralisation of the eluate before freeze-drying did not prevent loss of pyrogen.

A new standard was prepared from *P. vulgaris*. The culture was centrifuged and the supernatant liquid filtered as before. In order to obtain a purer product the filtrate was dialysed through cellophane to free it from inorganic salts. It was then re-sterilised by filtration and freeze-dried. No pyrogen was lost during drying and no obvious storage loss occurred while this standard was in use for the leucocyte response experiments.

TABLE IV

LOSS OF PYROGEN DURING FREEZE-DRYING OF ELUATE

pH value of solution before drying	Average rise in temperature in groups of 5 rabbits	
	Eluate before drying	Eluate dried and reconstituted
9·9	0·86	0·34
10·4	*1·33	0·69
6·7	*1·33	0·75

* Same solution.

RABBIT TEMPERATURE RESPONSE TO PYROGEN STANDARD

Animals. 25 rabbits, adult, either sex, weighing about 2·5 kg.

Method. The animals were placed in boxes adjustable for size and held lightly and comfortably in a normal sitting position and the temperatures were read by thermocouple junctions balanced against a junction in a water bath of known temperature, as described by us elsewhere.[1]

When the rabbit basic temperature was reached it was noted and the pyrogenic solution then injected, *via* the marginal ear vein, at 37° C. and diluted to a volume of 2 ml./kg. of body weight. Temperatures were read half an hour after injection and then at 10-minute intervals until they had risen to a peak and had begun to show a definite fall. The rabbits were kept awake throughout the experiment. Each of 25 rabbits was injected 4 times with each of 3 dose levels of pyrogen standard, the doses being 0·2 ml./kg., 0·06324 ml./kg. and 0·02 ml./kg., the middle dose being chosen so that its logarithm was equidistant from that of the other two.

TABLE V

TEMPERATURE INCREASES DUE TO INJECTIONS OF PYROGEN STANDARD

Dose	0·02 ml./kg.	0·06324 ml./kg.	0·2 ml./kg.
Mean of 100 responses	0·90	1·14	1·21
Standard deviation	0·36	0·34	0·39
Mean of 25 mean responses ..	0·90	1·14	1·20
Standard deviation	0·24	0·26	0·30

Results. The temperature increases due to these injections are shown in Table V along with their standard deviations. In every case this is a large fraction of the response and it is questionable whether a test showing a deviation of this magnitude can be regarded as of value except for limit tests, as used in the B.P. It is not sufficiently accurate for systematic work involving comparisons of solutions of approximately the same concentration.

Investigation of Results. Increased response or decreased variance would lessen the error. Seibert[2] showed that the response could not be increased beyond a maximum by further increase in dose and Wylie and Todd[1] found that maximum under the present conditions of experiment to be 1·3° C. For this reason causes of variance were sought in

order to lessen the error by their elimination. The possibilities considered were (*a*) variance within rabbits and variance between rabbits, (*b*) variance due to breed, sex, weight and colour.

(*a*) Analysis of variance of temperature response within and between rabbits showed the latter to be the greater. Comparison of an unknown sample of pyrogen with a standard would therefore be more accurate if carried out on the same rabbits.

(*b*) Dutch and Blue Fox rabbits were the predominating breeds in the population and no difference between the responses of the 2 groups was brought to light by t-tests which showed 30 to 40 per cent. probability of the 2 samples coming from the same population. Similar results were obtained from a comparison of the responses of bucks and does (40 to 50 per cent.) and those of dark-eared rabbits and light-eared rabbits which it was thought might radiate differently (70 to 80 per cent.). Pearson's correlation coefficient was also calculated to see if the following pairs of measurements were related, basic temperature and rise in temperature, weight and basic temperature, and weight and rise in temperature. The results were inconclusive.

Methods of measuring the response other than by simple rise in temperature were now considered. These were the use of only the maximum temperature attained as opposed to the use of the difference between this and the temperature at injection, and a measure taking into account not only the height of the rise but also the time taken to reach it. Emmens[3] says that the measure of the response after test is as useful as a comparison of the before-test and after-test states where the first is variable and the two are correlated. Applying this to pyrogen, the results were noted for the maximum temperature attained after each injection. These results (Table VI) show that the maximum temperature gives no real information and that the basic temperature must be taken into account.

300 graphs were drawn plotting rise against time until the maximum temperature was attained. From this point a perpendicular was dropped to the time axis and the area enclosed measured by planimeter. The average area and its standard deviation for each of the 3 dose levels was calculated and found to be as variable as height of rise alone and therefore of little use in a quantitative assay. The magnitude of the standard deviation in the results of all the above experiments led us to believe that temperature response in the rabbit is not an accurate method to use for the quantitative assay of pyrogen.

TABLE VI

MAXIMUM TEMPERATURES ATTAINED AFTER INJECTION OF PYROGEN STANDARD

Dose ml./kg.	Mean of 100 maxima °C.
0·02	39·44
0·06324	39·72
0·2	39·47

PRELIMINARY REPORT ON RABBIT LEUCOCYTE RESPONSE TO INJECTED PYROGEN

Pyrogen has several pharmacological properties, the main properties being an effect on the white blood cell picture,[4,5,6,7,8,9,10,11] inhibition of thermal panting in dogs,[12] ulcer inhibiting action,[13] an effect on peripheral

circulation,[14] reduction of gastric acid secretion[15] and reactions of tissues to the administration of pyrogen.[16] Of these it was decided that changes in the white cell picture as the basis of a method of assay warranted investigation. No quantitative examination seems to have been carried out on the changes in the relative numbers of the different types of white cells due to pyrogen.

Animals. 25 rabbits, adult, either sex, weighing about 2·5 kg. Some had been members of the population used in the first part of this work. Others were new, replacing those whose ear veins had become occluded due to repeated injection.

Standard pyrogen. The standard pyrogen used for these experiments was the dialysed standard previously described.

Methods. Some preliminary work on differential white cell counts was done. This established that (*a*) the error in repeated readings of the same smear was less than the difference between smears from the same rabbit on successive days and that (*b*) this in turn was less than the difference between smears before and after injection of pyrogen. The white cell count did not, of course, fluctuate as rapidly as temperature, and the departure from normal was greatest about 3 hours after injection.

In the main investigation the population of 24 was given 4 injections each, at weekly intervals, of 0·2 ml./kg. of standard. The temperature responses were measured as before and, at the same time, differential white cell counts were made from drops of blood from the marginal ear veins, the cells being stained with Leishman's stain and examined at a magnification of 600. Smears were made before injection and 3 hours after injection. In the differential counts the cells counted were classed as large lymphocytes, small lymphocytes, monocytes, eosinophils, basophils and neutrophils.

The usual number of cells counted in differential white counts is 300. Error may be introduced by the tendency of small lymphocytes to stay at the beginning of the smear or to be drawn along the centre and for granulocytes to be drawn to the end of the smear or to lie along the edges. To avoid this error strips across each end and the middle of the smear were counted and, if by then a total of 300 had not been attained, 2 intermediate strips between the centre and each end were added. This gave various totals of more than 300 for each smear. To make the results more readily comparable, all the individual cell counts were expressed as percentage of the total number counted, thus giving figures for the percentage of large lymphocytes, etc.

Results. Normally small lymphocytes predominate. After injection a fall in the percentage of small lymphocytes and a rise in the percentage of neutrophils occurred. In the other less numerous types there were no significant differences. We considered from the general appearance of the smears that there was probably an absolute as well as the measured relative increase in the neutrophils but the present work is restricted to differential counts and their use as an index of pyrogenic effect. Total counts were not carried out.

The changes in percentage of small lymphocytes were first considered.

The differences between the percentages of small lymphocytes before and after injection were extremely variable from one rabbit to another. It was considered that this was because the percentage before injection was itself a variable. To overcome this difficulty the differences were expressed as percentages of the small lymphocyte percentage before injection. These results are referred to as the percentage falls in small lymphocyte count. The mean percentage fall in small lymphocytes in the 96 responses was 75 per cent. with a standard deviation of 15. When the average for each rabbit was calculated from its 4 results and the 24 averages considered, the mean fall was still 75 per cent. with a smaller standard deviation, in this case 9.

Similar calculations were done for "total mononuclears," i.e., large and small lymphocytes and monocytes. The mean of the 96 percentage falls was 75 per cent. with a standard deviation of 14. The mean of the 24 was 75 and the standard deviation 9. The temperature results obtained at the same time as the white blood cell counts were comparable with those in Table V, the population mean being 1·18° C. and the standard deviation 0·37° C. considering mean rises, 0·45° C. considering individual rises. It was established that there was no correlation between temperature rise and white blood cell change, i.e., a rabbit sensitive to pyrogen by one response was not necessarily sensitive by the other.

Discussion

In this preliminary investigation these figures seem to indicate that small lymphocyte count is a more accurate method of assay of pyrogen than temperature measurement. The standard deviation of the temperature responses is a larger fraction of the response than in the case of white cell responses giving an assay with wider limits of error. Work is in progress on the effect on the differential count of different dose levels of pyrogen and of pyrogen from different organisms.

Summary

1. The preparation of a provisonal standard pyrogen has been described.

2. Temperature rises and white blood cell changes in the rabbit in response to this pyrogen standard have been investigated.

3. A smaller variance was found in white blood cell changes than in temperature rises.

The authors gratefully acknowledge the help of J. C. Eaton, M.A., of the Mathematics Department of Glasgow Royal Technical College for advice on the statistical analysis of the results, of W. H. Martin, B.Sc., D.R.T.C., of the Electrical Engineering Department for advice on the welding of thermocouples, of J. Wallace, B.Sc., M.D., of the Glasgow and West of Scotland Blood Transfusion Service for use of thefreeze-drying unit, of the Trustees of the McCallum Bequest for the provision of a refrigerator, and one of us (M.D.) thanks the Trustees of the Wellcome Pharmaceutical Research Fellowship, during the tenure of which this work was carried out.

REFERENCES

1. Wylie and Todd, *Quart. J. Pharm. Pharmacol.*, 1948, **21**, 240.
2. Seibert, *Amer. J. Physiol.*, 1925, **71**, 621.
3. Emmens, *Principles of Biological Assay*, Chapman and Hall, London, 1948, p. 191.
4. Co Tui, Hope, Schrift and Powers, *J. Lab. clin. Med.*, 1944, **29**, 58.
5. Young and Rice, *ibid.*, 1944, **29**, 735.
6. Chapman, *Quart. J. Pharm. Pharmacol.*, 1942, **15**, 361.
7. Bose and Ahuja, *Indian J. med. Res.*, 1944, **32**, 9.
8. Bandelin, *J. Amer. pharm. Ass., Sci. Ed.*, 1945, **34**, 48.
9. Dorche and Castaing, *Ann. pharm. franç.*, 1950, **8**, 353.
10. Co Tui and Schrift, *J. Lab. clin. Med.*, 1942, **27**, 569.
11. Lonsen and Liebert, *Illinois med. J.*, 1949, **96**, 186.
12. Rall and Wells, *J. Pharmacol.*, 1950, **98** (1), 27.
13. MacGinty, Wilson and Rodney, *Proc. Soc. exp. Biol. N.Y.*, 1949, **70**, 169.
14. Pinkston, *Amer. J. Physiol.*, 1934, **110**, 448.
15. Blickenstaff and Grossman, *Amer. J. Physiol.*, 1950, **160**, 567.
16. Windle, Chambers, Ricker, Ginger and Koenig, *Amer. J. med. Sci.*, 1950, **219**, 422.

DISCUSSION

The paper was read by MISS M. DAWSON.

The CHAIRMAN observed that it was interesting to see that the standard pyrogen was sufficiently stable to permit it to be freeze dried, and there should be no difficulty in reproducing it.

DR. J. C. DARE (Kippax) drew attention to the change in the situation during the past twelve months in relation to the determination of bacterial pyrogens. Pyrogen preparations were being used in the United States, and it was now becoming urgent that an adequate quantitative method of calculating the potency of pyrogen preparations should be developed as distinct from a test for their absence. The authors suggested that temperature measuring methods were not of much use for preparations which did not differ markedly. It depended on what was meant by the word "markedly." There were at least three teams of workers in this country who had been studying the question of a standard, and all had arrived at the conclusion that a dried preparation of *Proteus vulgaris* was the most promising. The question of whether temperature measurements were going to be adequate or whether some other method, as had been suggested, was to be used, was rather an open one. He felt that much more information was needed about differences of blood counts before accepting that method as being superior to the temperature test. With the temperature measuring equipment which would shortly become available it would be possible, for all practical purposes, to make an error-free determination of temperature, but that could not be said about cell counts. In a recent paper it was shown that the standard deviation of the differential count was of the order of $\pm$ 7 per cent. if the cells in which one was interested constituted 50 per cent. of the white cells. If the proportion of cells in which one was interested was only 10 per cent. of the white cell count, then a standard deviation of about $\pm$ 21 per cent. was obtained. The lymphocyte count of the rabbit was usually between 50 and 60 per cent. of the total white cell count. If that were reduced by 75 per cent. it was getting down to about 12·5 to 15 per cent.

of the total. In other words, there would be a standard deviation and error in the counting in the initial stages of $\pm$ 7 per cent. which would be correspondingly larger after the depression. The full potentialities of the temperature-measuring methods had not yet been investigated. By appropriate design of experiments to allow for individual animal variations the standard deviation could be improved. Limits of error would be somewhere between 50 and 200 per cent. of the true value. Those were wide limits, and he asked the authors what they meant by "markedly," because for human beings it was necessary to increase the dose threefold in order to obtain a significant change in response.

It had previously been shown that the lymphocyte count falls after a stress stimulus, becoming minimal 3 hours after the stimulus. Had the authors any evidence to show that the reduction in the proportion of lymphocytes which they observed 3 hours after an injection was, in fact, due to pyrogen and not to the stimulus given by the process of injecting the solution?

DR. K. BULLOCK (Manchester) said that the complex filtrate used raised the temperature and altered the white cell count. There was no correlation between those effects, yet the authors assumed that the pyrogen effect could be assayed on white cells. Why did they assume that one substance caused both effects?

MR. K. L. SMITH (Nottingham) said that the main use of the pyrogen test was qualitative. The authors had detected the activity of their standard pyrogen by means of a quantitative test. He had attempted to establish a response curve, and his slope was about as good as that of the authors, namely, a tenfold change in dilution gave a 0·3° C. change in temperature. The authors' standard deviation was greater than his. The changes of dose should have been carried out earlier because it was the relation of the standard deviation to the slope which gave the accuracy of the assay.

MR. T. D. WHITTET (London) said he was convinced that there was more than one pyrogen. He wondered whether the absence of correlation between the two factors was general and whether the pyrogen from one organism was consistently more effective in giving one response than the other.

MISS M. DAWSON, in reply, said that the standard deviation of the temperature response could be reduced considerably by appropriate grouping of the rabbits once an idea of the individual response was obtained. This was, however, an inconvenient method to use, depending as it did on the continued presence of the same rabbits in the population. With regard to the phrase "markedly different", to find how close in pyrogen content two samples may be and yet be distinguishable depends on the number of rabbits used in the test. The number of rabbits required in the sample to show a given difference in response with a given probability might be ascertained, as is well known, by applying the t-test in reverse. The lack of correlation between the temperature response and the white cell count in any one rabbit might be a reflection on the observed instability of the rabbit's temperature-regulating mechanism. The question of comparing pyrogens from different organisms had not been investigated in the present work.