THE BIOASSAY OF HUMAN ENDOGENOUS PYROGEN

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SUMMARY

1. A method is described for the quantitative assay of human endogenous pyrogen in saline and plasma which is valid in the presence of exogenous pyrogens.

2. The value of the assay is demonstrated by a study of the relation between endotoxin concentration and endogenous pyrogen production by human leucocytes in vitro.

Key words: human endogenous pyrogen, bioassay.

Evidence acquired from animal experiments suggests that fever in inflammatory disease is mediated by a circulating endogenous pyrogen. Endogenous pyrogen is released by leucocytes, macrophages in the lung and spleen (Atkins, Bodel & Francis, 1967) and liver Kupffer cells (Dinarello, Bodel & Atkins, 1968) in response to a variety of stimuli. Rabbit endogenous pyrogen appears to be a polypeptide which on intravenous injection produces a monophasic fever with a short latency and duration of effect (Bennett & Beeson, 1953). In rabbits, both the area under the curve of temperature and the maximum rise in temperature during the 90 min after injection are linearly related to the logarithm of the injected dose (Murphy, 1966), and this has been used as a basis for the quantitative assay of rabbit endogenous pyrogen.

Human leucocytes will release endogenous pyrogen in vitro after incubation with bacterial pyrogens (Cranston, Goodale, Snell & Wendt, 1956). Intravenous injection of this endogenous pyrogen into a normal volunteer will produce a monophasic fever of short latency. Bodel & Atkins (1966) have shown that human endogenous pyrogen will produce fever in rabbits when given intravenously and this has subsequently been confirmed by Cooper (1971) who found that rabbits rapidly develop sensitivity fevers to small doses of normal human plasma.

Any assay system for endogenous pyrogen should be unaffected by the presence of endotoxins. Insensitivity to these substances can be produced in two ways. Firstly, 'refractory' rabbits can be used. These are animals that have received a large intravenous (i.v.) dose of bacterial pyrogen 12–24 h before. This renders them relatively insensitive to subsequent
injections of endotoxins (Atkins & Snell, 1963). Secondly, the preparation to be tested can be incubated with normal rabbit plasma for 18–24 h before injection (Murphy, 1966). Normal rabbit plasma can inactivate endotoxin by mechanisms which are incompletely understood.

An assay system for human endogenous pyrogen might be expected to be of value in studying the physical and chemical characteristics, the production and release of human endogenous pyrogen and the pathogenesis of fever in a wide variety of clinical situations. The present work was performed in an attempt to develop such an assay system.

METHODS

The assay was conducted with New Zealand white rabbits of either sex weighing 2.0–3.0 kg. Groups of six rabbits were used for a period not exceeding 5 consecutive days. They were lightly restrained in head stocks in a temperature-controlled laboratory at 18–23°C. During any one day, the laboratory temperature did not fluctuate by more than ±1°C. The animals' rectal temperatures were measured with thermistors (STC G23) inserted 8–10 cm and recorded every 160 s on a sixteen-channel recorder (Kent). The thermistors were calibrated at least once a week, preliminary studies showing that day-to-day changes in sensitivity were less than 5%. Injections were given only when the animals had become accustomed to their surroundings and when their rectal temperatures had been stable for at least 30 min. Animals received two injections per day and at least 3.5 h was allowed to elapse between injections. The maximum possible number of injections that any animal could receive was therefore ten. In many instances, however, the solutions under investigation were assayed in animals rendered refractory to bacterial pyrogens. Refractoriness was induced by the intravenous administration of 1 ml of typhoid vaccine (TAB, Wellcome) on the evening before the assay was performed (Murphy, 1966). It was only possible to use these rabbits for 4 days of assaying, the first day being used to accustom them to the experimental system and to render them refractory.

Human endogenous pyrogen in plasma was prepared by incubating heparinized whole blood in 200 ml centrifuge bottles at 37°C for 18 h with a purified preparation of Proteus endotoxin ('E' pyrogen, Organon Laboratories) at a concentration of 3 ng/ml. The plasma was separated by centrifugation at 2000 g for 20 min and stored at +4°C. Human endogenous pyrogen in saline was prepared in the same way as endogenous pyrogen in plasma up to the start of incubation with Proteus endotoxin. After 2 h incubation (subsequently termed pre-incubation), the plasma was separated and the cells washed three times with NaCl, 150 mmol/l (saline). They were then resuspended in a volume of saline equal to the original plasma volume, and the mixture was incubated for a further 16 h. Subsequent steps were the same as those used to prepare human endogenous pyrogen in plasma. Total leucocyte counts were performed on all blood samples drawn. The leucocyte content of each incubate was calculated, and the dose of endogenous pyrogen expressed as the total number of leucocytes from which the injection volume was derived. All glassware was rendered pyrogen-free by baking at 160°C for 4 h.

RESULTS

Assay of human endogenous pyrogen in saline

Fig. 1 shows the pooled results of a preliminary experiment in which nine non-refractory rabbits received human endogenous pyrogen in saline. An intravenous injection of a volume of
Assay of human endogenous pyrogen

Fig. 1. Mean response of a group of rabbits to an intravenous injection of human endogenous pyrogen in saline. Ordinate, change in temperature (in °C). Vertical lines represent ±1 SEM, n = 9.

Table 1. Assay of human endogenous pyrogen in saline

<table>
<thead>
<tr>
<th>Effect metamer</th>
<th>Correlation coefficient</th>
<th>Slope</th>
<th>Intercept</th>
<th>λ</th>
<th>L</th>
</tr>
</thead>
<tbody>
<tr>
<td>90 min fever height</td>
<td>+0.67</td>
<td>+0.61</td>
<td>-3.86</td>
<td>0.48</td>
<td>2.09</td>
</tr>
<tr>
<td>(P &lt; 0.001)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90 min 'fever area'</td>
<td>+0.71</td>
<td>+41.05</td>
<td>-267.3</td>
<td>0.42</td>
<td>2.42</td>
</tr>
<tr>
<td>(P &lt; 0.001)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Analysis of the relationship between log dose of human endogenous pyrogen and two measures of febrile response. For definition of λ and L see the text.

supernatant derived from 9.2 × 10^7 leucocytes was given and the changes in temperature plotted at 10 min intervals. After a latency of about 10 min the mean temperature rose to a maximum at 40–60 min and declined thereafter. After injection (3 h) the mean rectal temperature was not significantly different from that at the time of injection (t = 1.11, P > 0.10).

Fig. 2 shows the log dose–effect relationship for a five-point assay of human endogenous pyrogen in saline. In this experiment, only one dose was administered per day and the dose was expressed as the number of leucocytes from which the volume of injectate was derived. The
effect is expressed both as the maximum rise in temperature (in °C) during the 90 min period after the injection (the 90 min fever height) and as the integral (°C min⁻¹) of the temperature rise during the same period (the 90 min 'fever area'). It can be seen from Fig. 2 (see Table 1) that there is a significant relationship between the logarithm of the dose of human endogenous pyrogen and both the 90 min fever height and the 90 min 'fever area'. The indices of precision λ and L (1/λ) (Gaddum, 1953) are slightly better by using the 90 min 'fever area' as the effect metamer.

In contrast, Fig. 3 shows the response to control intravenous injections prepared in the same way as human endogenous pyrogen in saline, except that no endotoxin was added before pre-incubation. There was no significant correlation between the logarithm of the dose and the 90 min fever height (r = -0.026; P > 0.10).
Assay of human endogenous pyrogen in plasma

The results of two three-point assays of human endogenous pyrogen in plasma by using non-refractory animals are shown in Table 2 (assays 1 and 2). The dose of endogenous pyrogen was again expressed as the common logarithm of the number of leucocytes from which the injectate was derived. The two assays were conducted on different groups of rabbits. There was a highly significant correlation between the 90 min fever height and the dose of endogenous pyrogen for both assays \((P<0.001)\), but the values for \(\lambda\) and \(L\) suggest that there was some decrease in precision associated with the assay of human endogenous pyrogen in plasma as compared with human endogenous pyrogen in saline (cf. Table 1).

### Table 2. Assay of human endogenous pyrogen in plasma

<table>
<thead>
<tr>
<th>Assay</th>
<th>Refractory (R) or non-refractory (NR) animals</th>
<th>Correlation coefficient</th>
<th>Slope</th>
<th>Intercept</th>
<th>(\lambda)</th>
<th>(L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NR</td>
<td>+0.91</td>
<td>+0.74</td>
<td>-4.79</td>
<td>0.51</td>
<td>1.95</td>
</tr>
<tr>
<td>2</td>
<td>NR</td>
<td>+0.85</td>
<td>+0.83</td>
<td>-5.53</td>
<td>0.58</td>
<td>1.72</td>
</tr>
<tr>
<td>3</td>
<td>R</td>
<td>+0.90</td>
<td>+0.80</td>
<td>-5.36</td>
<td>0.62</td>
<td>1.61</td>
</tr>
<tr>
<td>4</td>
<td>R</td>
<td>+0.72</td>
<td>+0.82</td>
<td>-5.58</td>
<td>0.61</td>
<td>1.64</td>
</tr>
<tr>
<td>5</td>
<td>R</td>
<td>+0.55</td>
<td>+0.70</td>
<td>-4.29</td>
<td>0.51</td>
<td>1.95</td>
</tr>
</tbody>
</table>

Analysis of the relationship between log dose of human endogenous pyrogen in plasma in refractory and non-refractory rabbits. Six animals were used in each assay. For definition of \(\lambda\) and \(L\) see the text.
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Assay of human endogenous pyrogen in refractory rabbits

The value of a quantitative assay for human endogenous pyrogen would be greatly enhanced if it could be performed in the presence of contaminating bacterial pyrogens. The dose–effect relationship between human endogenous pyrogen in plasma and the 90 min fever height was therefore studied in refractory rabbits since such animals are very considerably less sensitive to bacterial pyrogens (Murphy, 1966).

Fig. 4. Dose–effect curve for human endogenous pyrogen in non-refractory (●) and refractory (○) rabbits. Vertical lines represent ± 1 SEM. Details of dose as for Fig. 2.

Fig. 4 (assays 2 and 3 in Table 2) shows the log dose–effect relationship between log dose of endogenous pyrogen and fever height for a three-point assay in a group of rabbits before and after they had been rendered refractory. There were no significant differences in regression intercepts ($t = 0.28, P > 0.10$), or slopes ($t = 0.47, P > 0.10$) between the two assays. Assays 4 and 5 in Table 2 were conducted on different groups of six rabbits; both were three-point assays with human endogenous pyrogen in plasma derived from different volunteers. The dose of endogenous pyrogen was expressed as the common logarithm of the number of white blood cells from which the injectate was derived.

To determine whether the temperature response of rabbits varied during a 4-day assay period, a group of six refractory animals received human endogenous pyrogen in plasma derived from one volunteer at a dose equivalent to $10^{7.50}$ white blood cells twice daily for 4 days. A similar group of refractory rabbits received the same volume of normal human plasma twice daily for 4 days. The results are shown in Fig. 5. For human endogenous pyrogen in plasma (Fig. 5a) there was no significant difference between the morning responses on different days ($F = 0.42, P > 0.05$) or between the afternoon responses ($F = 0.60, P > 0.05$). It was clear, however, that the temperature responses of rabbits to this dose of human endogenous pyrogen
Assay of human endogenous pyrogen

Fig. 5. Temperature response to a fixed dose of human endogenous pyrogen (a) or normal human plasma (b) in a group of non-refractory rabbits. Vertical lines represent ±1 SEM. Open areas, morning responses; hatched areas, afternoon responses. Injections were given twice daily for 4 consecutive days.

were significantly lower in the afternoon than the morning (on paired results, mean = −0.22°C, SEM ±0.04; t = 5.64, P<0.001). There was no evidence to suggest that refractory rabbits developed sensitivity fevers in response to twice daily intravenous injections of normal human plasma (see Fig. 5b).

As additional confirmation that the pyrogenic material in plasma and saline used to construct these dose–effect curves was endogenous rather than exogenous pyrogen, the effect of heat and trypsin treatment on the pyrogenticity of human endogenous pyrogen in saline was studied; these procedures greatly decrease the pyrogenticity of endogenous but not exogenous pyrogen (Bode & Atkins, 1966). A comparison was made between the febrile responses (90 min fever height) of six refractory rabbits to human endogenous pyrogen in saline, human endogenous pyrogen in saline incubated for 30 min at 70°C, human endogenous pyrogen in saline incubated with the same volume of normal rabbit plasma and a 5 ml saline control. Similarly febrile responses were measured in a group of six refractory rabbits given human endogenous pyrogen in saline, human endogenous pyrogen in saline incubated firstly with bovine pancreatic trypsin (Sigma, 0.13 mmol/l) for 24 h, and then with an equal volume of normal rabbit plasma (to diminish the effects of any possible endotoxin contamination of the trypsin). A control injection of 5 ml of saline was also given to this group of rabbits. The results are shown in Table 3. There were no significant differences in the febrile responses between
TABLE 3. Effect of trypsin treatment, heat and normal rabbit plasma on the pyrogenicity of human endogenous pyrogen in saline

<table>
<thead>
<tr>
<th>Material</th>
<th>log Dose* (equivalent no. of WBC)</th>
<th>Febrile responses (90 min fever height; °C ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous pyrogen in saline</td>
<td>8.0</td>
<td>1.29 ± 0.19</td>
</tr>
<tr>
<td>Endogenous pyrogen incubated at 70°C for 30 min</td>
<td>8.0</td>
<td>0.21 ± 0.09</td>
</tr>
<tr>
<td>Endogenous pyrogen in saline incubated with normal rabbit plasma</td>
<td>8.0</td>
<td>1.12 ± 0.22</td>
</tr>
<tr>
<td>Saline</td>
<td>0</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>Endogenous pyrogen in saline</td>
<td>7.63</td>
<td>0.67 ± 0.07</td>
</tr>
<tr>
<td>Endogenous pyrogen in saline incubated with trypsin (0.13 mmol/l)</td>
<td>7.63</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>Saline</td>
<td>0</td>
<td>0.08 ± 0.04</td>
</tr>
</tbody>
</table>

* In 5 ml of saline.

endogenous pyrogen in saline with and without normal rabbit plasma ($t = 0.65$, $P > 0.10$). Pyrogenicity of endogenous pyrogen in saline was, however, significantly decreased both by trypsin treatment and by incubation at 70°C for 30 min ($t = 7.87$, $P < 0.001$; $t = 5.14$, $P < 0.001$ respectively); after either treatment, the pyrogenicity of endogenous pyrogen in saline was not significantly different from saline controls ($t = 0.14$, $P > 0.10$ for trypsin treatment; $t = 1.08$, $P > 0.10$ for incubation at 70°C).

Relationship between endotoxin concentration and endogenous pyrogen released by human leucocytes

In previous experiments, the febrile responses to endogenous pyrogen derived from different quantities of leucocytes were examined. It is also possible to derive different amounts of endogenous pyrogen from a fixed number of leucocytes by varying the degree of stimulation of these cells. This was achieved by incubating different quantities of endotoxin with a fixed number of leucocytes. Venous blood (560 ml) was withdrawn from a volunteer into a heparinized, pyrogen-free container. The blood was divided into eight 70 ml portions each of which was incubated with different concentrations of Proteus endotoxin (10, 3, 1, 0.8, 0.5, 0.3, 0.2 and 0.1 ng/ml whole blood). The blood was incubated at 37°C for 18 h, the plasma separated by centrifugation at 2000 g for 20 min and stored at +4°C. The plasma was assayed by using a group of six refractory rabbits on a cross-over basis and at a dose equivalent to $10^{7-49}$ leucocytes (equal to 4 ml of plasma). The result is shown in Fig. 6. There was a highly significant correlation between the common logarithm of the concentration of endotoxin and the 90 min fever height ($r = 0.89$, $P < 0.001$).
DISCUSSION

The investigation described in the present paper demonstrated that there is a significant relationship between the logarithm of the dose of human white blood cells (in both plasma and saline) and the 90 min fever height in both refractory and non-refractory rabbits. In addition, it has been shown that different amounts of endogenous pyrogen derived from a fixed number of leucocytes can be assayed. The observations that the pyrogenicity of the samples investigated were unaltered by incubation in normal rabbit plasma and abolished by trypsin treatment or heating to 70°C for 30 min show that the febrile responses elicited were due to endogenous as opposed to exogenous pyrogen (Bodel & Atkins, 1966).

It is customary to assess the precision of an assay by the ratio of the root mean square of the residual variance and the slope of the regression line (λ), or the reciprocal of this (L) (Gaddum, 1953). Gaddum recommended that λ should be less than 0.15 and it is clear from the results in Tables 1 and 2 that λ is much higher than this; for assaying human endogenous pyrogen in plasma mean was 0.57 (SEM ±0.05). The principal explanation for this is the inter-individual differences in temperature responses to intravenous human endogenous pyrogen. The assay method would therefore not detect much less than twofold changes in endogenous pyrogen concentration. The threshold of the assay system is relatively high and the method will not detect circulating endogenous pyrogen in plasma from febrile patients (Cranston, Rawlins, Luff & Duff, 1971).

The results of the present study underline the importance of several factors if the method is to be used successfully. Firstly, because of the differences in the slopes of the log dose–effect regression lines amongst different groups of assay rabbits (see Table 2) it is not possible to make quantitative comparisons of 90 min fever heights between groups. Secondly, the importance of assaying material on a cross-over basis is apparent from the results shown in Fig. 5. Thirdly, whilst it has been shown that normal rabbits become rapidly sensitized to human plasma (Cooper, 1971), this does not appear to occur when refractory rabbits are used (see Fig. 5).
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REFERENCES


