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Detection of endotoxin in mice by measurement of endotoxin-induced changes in plasma concentrations of zinc and of the acute-phase protein serum amyloid P-component

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Preparations of six bacterial endotoxins reduced plasma concentrations of zinc and increased concentrations of the acute-phase protein serum amyloid P component (SAP) in mice. The changes were sufficiently sensitive to permit use of either response in tests for endotoxin and hence for pyrogenic contamination.

Pharmaceutical products intended for parenteral administration are tested for pyrogenic contamination, usually with endotoxins (lipopolysaccharides) from Gram-negative bacteria. There are shortcomings in the existing tests for pyrogens. The 'rabbit fever test' is reliable but costly; the Limulus Amoebocyte Lysate (LAL) test for endotoxin gives false negative results with enzyme inhibitors, certain antibiotics and viral vaccines and has proved difficult to standardize (WHO/BS/82.1372).

Boobis & Hartley (1981) reported that dose-dependent hypozincaemia was evoked in mice not only by endotoxins from Gram-negative bacteria but also by a heat-killed suspension of the Gram-positive bacterium *Staphylococcus aureus* which was negative in a LAL test, and by a preparation of streptomycin that caused fever in rabbits but was negative in a LAL test. From results obtained largely with one endotoxin (from *Salmonella abortus equi*), Boobis & Hartley suggested the measurement of hypozincaemia in mice as a sensitive test for detection of pyrogens. Recently we confirmed the sensitivity to endotoxin of plasma zinc concentrations in mice and demonstrated that concentrations of another constituent of mouse plasma, the acute-phase reactant serum amyloid P component (SAP), were likewise very sensitive to endotoxin (Poole et al 1984). We have now measured plasma zinc and SAP responses to six different endotoxins in two strains of mice under carefully controlled conditions and have also investigated the effects on the responses of certain modifications of these conditions to determine the suitability of the responses to serve as the basis of tests for pyrogens.

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MATERIALS AND METHODS

Materials

All needles, syringes, glassware, plastic ware and solutions were sterile and pyrogen-free. The lysate used in the LAL test was purchased from Associates of Cape Cod (Woods Hole, Massachusetts) and had a stated sensitivity of 0·03 endotoxin units ml^{-1} (10 endotoxin units = 1 ng of the USP preparation of *E. coli* 0113 : H10 : K(−)ve, termed EC5). The six endotoxins tested were from *Salmonella abortus equi*, batch M6, kindly donated by Dr C. Galanos, Max-Planck Institute fur Immunbiologie; *Escherichia coli* 055 : B5 (*E. coli* 055 : B5), batch HK 2627, kindly donated by Difco laboratories; *E. coli* 0111(J5)UDP-gel epimerase-deficient (*E. coli* J5); *Serratia marcescens; Shigella dysenteriae* and *Pseudomonas aeruginosa* P2AB-X virulent strain. The endotoxin preparations were of different degrees of purity. The last four endotoxins were materials prepared for a WHO international study (WHO/BS/82.1372) and freeze-dried in ampoules each containing a nominal weight of endotoxin. The preparation of *E. coli* 055 : B5 was from the same batch (HK2627, Difco) as that used in the WHO study and showed similar activity to the earlier preparation in LAL and rabbit pyrogen tests (Table 1). The preparation of *S. abortus equi* was from a different batch to that used in the WHO study, but was prepared in the same laboratory and showed similar activity to the earlier preparation in LAL and rabbit pyrogen tests (Table 1).

Mice

Male mice of the Parkes strain (outbred white mice, purchased from the National Institute for Medical Research, London) and of the C57/BL6 strain

Table 1. Doses of six endotoxin preparations required to depress plasma zinc (by ≥12%), elevate SAP (by about 28% in Parkes mice and by 57% in C57/BL6 mice), evoke a fever of +0·55 °C in rabbits and clot a Limulus Amoebocyte Lysate of stated sensitivity 0·003 ng ml^{-1} *E. coli* 0113 : H10 : K(-)ve endotoxin.

Endotoxin	Zinc depression Parkes	C57/BL6 (ng/mouse)	SAP elevation Parkes	C57/BL6 (ng/mouse)	Rabbit fever (ng kg^{-1})	(+) LAL endpoint (ng ml^{-1})
E. coli 0111(J5)UDP-GED	0·5	0·5	<0·5	0·5–1	0·06[a]	0·0016
E. coli 055:B5	5–10	1–5	5–10	1–5	0·6[b]	0·003
S. abortus equi	<0·5	5	5	5	<0·5[c]	0·003
Ser. marcescens	10	0·5	<0·5	<0·5	0·6[a]	0·05
Sh. dysenteriae	10	5	10	5–10	1·3[a]	0·4
P. aeruginosa	1	5–10	5–10	5–10	32[a]	0·2

[a] Data contained in the interim report of a WHO study of Limulus Amoebocyte Lysate (LAL) and rabbit tests for pyrogens (WHO Working Document WHO/BS/82.1372).

[b] Data also contained in the interim report (WHO/BS/82.1372) pertaining to an ampouled preparation of *E. coli* 055 : B5 (batch HK2727, Difco laboratories) and confirmed at NIBSC for the preparation of *E coli* 055 : B5 (HK2627) used in the present study.

[c] Unpublished data kindly supplied by Dr P. Weidner (GMN, West Germany)

N.B. The six endotoxin preparations used were of different degrees of purity and had not been calibrated in terms of a defined unit of endotoxin. Therefore the activities shown above pertain only to these preparations of the endotoxins.

(inbred black mice purchased from Olac Ltd, Bicester, Oxon), 30–40 g, were housed 50 mice per cage at 23 ± 2 °C with water and food (Diet R & M no. 1, BP Nutrition) freely available, and subjected to natural light and dark.

Procedure

Twenty-four hours before endotoxin was injected, 40 mice were taken at random from one cage and housed two per cage at 23 ± 1 °C, with free access to water and food (Diet R & M No. 1, BP Nutrition). Endotoxins were injected intraperitoneally in a volume of 0·5 ml pyrogen-free sterile saline (150 mM NaCl); control injections were of 0·5 ml of the saline only. The doses of endotoxin were 0·5, 1 and 5 ng/mouse or 5, 10 and 50 ng/mouse. Each dose was given to 10 naive mice and each set of doses was given in at least one experiment in each mouse strain. At intervals after (4 h, 24 h) and in some experiments 1 h before injection of endotoxin, mice were bled from the orbital sinus (100 µl) into glass capillaries (Gelmen-Hawksley Ltd) coated with heparin and the blood centrifuged at 1720*g* for 15 min. Plasma concentrations of zinc and of SAP were measured as described previously (Pepys 1979; Poole et al 1984).

In other experiments, Parkes mice were injected with *E. coli* 055 : B5 (1 and 10 ng/mouse), *S. abortus equi* (1 and 2 ng/mouse) or *Sh. dysenteriae* (10 and 20 ng/mouse). In some of these experiments, mice were stressed in one of four ways before and after injection. The stresses were (i) low ambient temperature: 12 ± 1 °C for 24 h before and 4 h after injection; (ii) food deprivation for 24 h before and 4 h after injection; (iii) water deprivation for 24 h before and 4 h after injection, and (iv) noise-stress (BBC Radio 1) for 4 h before injection and 4 h after injection. The sound pressure level was 80–85 dB with peaks up to 100 dB, quantified by a sound level indicator (type 1048E, Dawes Instruments Ltd, London) with 'C'-weighting: 50 Hz–5 kHz flat, −10 dB at 15 kHz. In two sequences of four experiments, one in Parkes mice and the other in C57/BL6 mice, performed at weekly intervals, the same mice were used with random assignment to dose (1 or 10 ng *E. coli* 055 : B5 endotoxin or saline control) on each occasion.

A LAL test was carried out on the six endotoxin preparations using a procedure similar to that described previously (Prior & Spagna 1979). A rabbit pyrogen test was carried out on the preparation of *E. coli* 055 : B5 as described in the European Pharmacopoeia (Volume II, 1971).

Statistical analysis

Analysis of variance of logarithms of responses was used to examine both intra- and inter-experimental differences following preliminary analysis of responses for outliers and heterogeneity of variances (Gaines Das & Rice 1985).

RESULTS

Geometric mean plasma zinc concentrations of control mice were significantly different between experiments in either mouse strain ($P < 0·01$), and slightly lower in C57/BL6 mice than in Parkes mice ($P < 0·05$, Fig. 1). Variances of log zinc concentra-

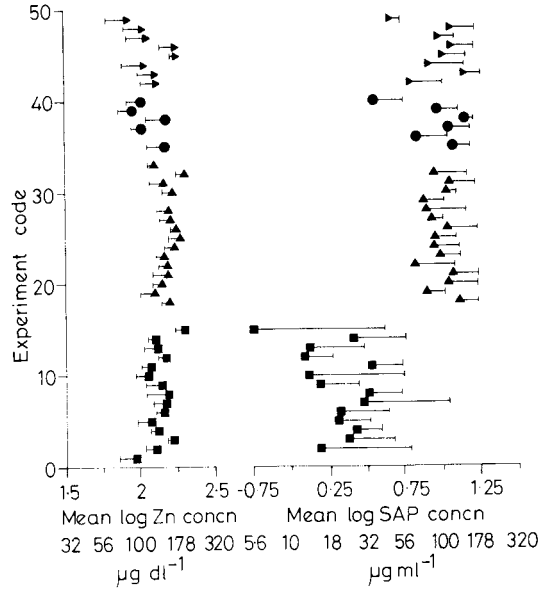

FIG. 1. Distribution of mean log zinc and mean log SAP concentrations of control groups each of ten mice injected with saline in different experiments. Key: ■ C57/BL6; ▲ Parkes; ● Parkes, pre-bled; ▶ Parkes, stressed. Horizontal bars represent standard deviations.

tions in mice injected with saline or endotoxin were similar within and between experiments except that in both strains log zinc concentrations of mice injected with *P. aeruginosa* endotoxin were more variable ($P < 0{\cdot}01$). The pooled variance ($\times 10^5$) for log zinc concentrations, excluding experiments with *P. aeruginosa*, was 491 (range 202–782).

Means of log SAP concentrations for control mice differed between experiments ($P < 0{\cdot}01$) for either strain (Fig. 1) and means in C57/BL6 mice were lower than those in Parkes mice ($P < 0{\cdot}01$). Log SAP concentrations were much more variable ($P < 0{\cdot}001$) than log zinc concentrations, especially in C57/BL6 mice, and were heterogeneous.

For each response in each mouse strain, the minimum doses of the six endotoxins that evoked 'significant' zinc and SAP responses are given in Table 1. These are the doses for which the differences between means of log responses for mice treated with this or a larger dose of endotoxin and for mice in the relevant control group were significant ($P < 0{\cdot}05$).

In experiments in which mice were bled before injection, including experiments in which mice were stressed, plasma zinc concentrations measured after injection of endotoxin or saline were consistently lower ($P < 0{\cdot}01$, Fig. 1) and more variable than those measured in experiments in which mice were not pre-bled. In contrast neither means nor variances of log SAP concentrations in these experiments were consistently different from those obtained previously. In most of these experiments, zinc and SAP concentrations in mice injected with endotoxin differed significantly ($P < 0{\cdot}05$) from concentrations in mice injected with saline in the same experiment (Table 2).

Table 2. Results of experiments with four different stresses (described in detail in Methods). Plasma zinc and SAP concentrations expressed as geometric mean (10^3 × variances of log responses) in ten Parkes mice injected with *E. coli* 055:B5 endotoxin. Zinc concentrations were measured 1 h before and 4 h after injection of endotoxin. SAP concentrations were measured 24 h after injection of endotoxin.

Stress	Injection	Zinc before injection (μg dl⁻¹)	Zinc after injection (μg dl⁻¹)	SAP after injection (μg ml⁻¹)
None	Saline	178 (3)	149[b] (15)	141[a] (3)
	E. coli 055:B5 (1 ng)	192 (5)	120 (9)	118 (34)
	E. coli 055:B5 (10 ng)	184 (4)	119 (3)	149 (18)
Cold (+12 °C)	Saline	99 (5)	84[c] (19)	46[b] (5)
	E. coli 055:B5 (1 ng)	102 (2)	82 (25)	58 (27)
	E. coli 055:B5 (10 ng)	100 (6)	70 (10)	88 (15)
−Food	Saline	177 (2)	174[b] (9)	114[b] (23)
	E. coli 055:B5 (1 ng)	202 (8)	142 (11)	158 (4)
	E. coli 055:B5 (10 ng)	226 (6)	149 (3)	149 (24)
−Water	Saline	154 (5)	112[b] (16)	94[b] (12)
	E. coli 055:B5 (1 ng)	149 (11)	86 (6)	143 (33)
	E. coli 055:B5 (10 ng)	150 (3)	97 (20)	120 (16)
+Noise	Saline	135 (11)	105[b] (14)	113[d] (26)
	E. coli 055:B5 (1 ng)	124 (8)	90 (9)	122 (24)
	E. coli 055:B5 (10 ng)	128 (9)	82[c] (8)	150 (16)

[a] This was the largest value obtained in this study for saline-injected Parkes mice.
[b] Geometric mean response significantly < that of one or other or both of the endotoxin-treated groups. $P < 0{\cdot}05$.
[c] Single outlier (plasma zinc > the largest control value) omitted; using all data, g.m. (10^3 variance) = 88 (15).
[d] As 'b' except $P < 0{\cdot}10$.
[e] Variance of zinc concentrations in this experiment was the largest obtained in this study.

For experiments in which mice were pre-bled but not otherwise stressed, the ratio of post- to pre-injection zinc concentration was examined as the 'response'. This response, when compared with post-injection zinc concentration alone, gave no improvement in discrimination between endotoxin treated and control mice within experiments and was not more consistent between experiments.

DISCUSSION

The values and variabilities of SAP concentrations measured in the present study, especially in C57/BL6 mice, were greater than those obtained previously (Pepys et al 1979). In the present study SAP concentrations were measured in mice that had been bled once or twice during the previous 25 h (to

permit plasma zinc measurements). Pre-bleeding had pronounced effects on subsequent plasma zinc concentrations and it is possible that pre-bleeding affected SAP concentrations. Alternatively, it is possible that the mice were suffering from minor infections that elevated SAP concentrations, although the mice appeared to be in good health, displayed normal behaviour and ate and drank normally.

Boobis & Hartley (1981) reported that tolerance to the hypozincaemic effect of endotoxin did not develop in animals injected four or five times at 3-day intervals with low doses (<5 ng *S. abortus equi* endotoxin), but could be induced by high doses (0·5–10 μg endotoxin from *S. abortus equi* or *E. coli*) given over a period of 2–4 days. In experiments in the present study in which mice were used repeatedly at weekly intervals, there was no consistent trend of zinc or SAP responses. Without more information, naive mice are to be preferred for measurements of plasma zinc and SAP concentrations. Woodward et al (1984) showed that in CBA/J mice there was a linear decrease in serum zinc (and presumably plasma zinc) concentrations with advancing age; this would suggest that young mice are to be preferred.

Although zinc and probably SAP concentrations in mice injected with saline were affected by the various stresses applied in the present study, significant responses relative to the control group of both variables to *E. coli* 055 : B5 endotoxin were obtained in most experiments in which animals were stressed. Consequently we consider plasma zinc and SAP responses to endotoxin to be relatively stable considering the severity of the stresses used; whereas the rabbit pyrogen test is affected by even mild stresses such as small changes in ambient temperature, diet and noise.

The pyrogens that contaminate pharmaceutical products are almost invariably endotoxins. We have shown that changes in plasma zinc or SAP concentrations in mice are sufficiently sensitive responses to bacterial endotoxin to permit use of either in a test for endotoxic contamination; previous work indicates that, unlike the LAL test, at least the zinc response is sensitive to a wide range of pyrogenic contaminants (Boobis & Hartley 1981). In this study we tested for contamination by comparing the geometric means of the concentrations of either plasma zinc or SAP measured in two groups each of ten mice, one group injected with test material and the other with pyrogen-free sterile saline. A significant difference ($P < 0·05$) between the means of the log concentrations of the variable measured (zinc or SAP concentration) signalled contamination. This corresponded to a 12% decrease in the geometric mean plasma zinc concentration of the test group relative to that of the group injected with saline and agreed with the results of Boobis & Hartley (1981). We observed significant differences between the groups for SAP concentrations when the geometric mean of the test group was elevated by some 25–50% over that of the control group. These tests were about as sensitive as the rabbit pyrogen test and the data obtained with the preparations of six different endotoxins indicate that orders of relative 'activity' defined in terms of doses required to evoke zinc or SAP responses would be similar to those obtained for rabbit fever and LAL tests.

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