

Effect of anti-inflammatory agents on the haemorrhagic response of mouse skin to *Bordetella pertussis* heat-labile toxin

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Summary. *Bordetella pertussis* heat-labile toxin (HLT) was assayed by the haemorrhagic response produced by subcutaneous injection into weaned mice. Young mice, 3–5 weeks old of either sex, were highly responsive but they became resistant to HLT as they matured. Two anti-inflammatory agents, prednisolone and meclofenamate, inhibited the skin reactions in young mice. When given intraperitoneally, prednisolone was most inhibitory if it was injected just before, or at the same time as, HLT challenge. Prednisolone given 3 h after challenge, when the skin reactions had started to develop, did not significantly attenuate the final response. Both drugs were even more effective when mixed with the HLT challenge and injected subcutaneously. These findings are discussed in relation to the possible mechanism of action of HLT and to the reported beneficial effects of corticosteroids in the treatment of whooping cough.

Introduction

A major toxic component of *Bordetella pertussis* is the dermonecrotising heat-labile toxin (HLT). The mechanism of action of HLT is unknown but it is lethal when given intraperitoneally (ip) or intravenously to mice, guinea pigs or rabbits. When injected subcutaneously (sc) or intracutaneously (ic) into various experimental animals it causes haemorrhage and necrosis (Munoz, 1971). A sensitive method for detecting HLT activity is by injecting it sc or ic into suckling mice and scoring the haemorrhagic reaction after 24–48 h (Katsampes *et al.*, 1942; Kurokawa *et al.*, 1969; Cowell *et al.*, 1979; Livey and Wardlaw, 1984). Most studies on HLT have involved the use of crude cell extracts in which the activities attributed to HLT have been based on the difference in activity before and after heating at 56°C. However, the purified preparations of Onoue *et al.* (1963) and Nakase *et al.* (1969) retained their dermonecrotising and lethal activities.

The mechanisms of pathogenesis of human pertussis are still obscure and the role of HLT has not been defined (Pittman, 1979). However, the marked effects seen in laboratory animals after injection of HLT strongly suggest that it has a significant part in the disease process (Munoz, 1971; Olson, 1975; Wardlaw and Parton, 1983). Recently, it was shown that prednisolone, given orally or ip before chal-

lenge, would protect mice against the lethal effects of HLT (Parton, 1985). Glucocorticoids such as prednisolone have been reported to be beneficial in the treatment of children with severe pertussis, by reducing the frequency and severity of the coughing spasms and the duration of the illness (Dayal *et al.*, 1969; Zoumboulakis *et al.*, 1973; Barrie, 1982; Dianese, 1982). These findings raise the possibility that the beneficial effects of corticosteroids are due to their counteracting some action of HLT in the child (Parton, 1985).

In the present investigations, HLT was assayed by the haemorrhagic response after sc injection in weaned mice. The ability of a steroidal and a non-steroidal anti-inflammatory agent to inhibit this reaction is reported.

Materials and methods

Heat-labile toxin

A crude cell extract of *B. pertussis* served as a convenient source of HLT. The extract was prepared from *B. pertussis* strain 18334 grown in modified Hornbrook medium (Wardlaw *et al.*, 1976) for 48 h at 37°C, as described previously (Idigbe *et al.*, 1981). Cultures were harvested by centrifugation at 10 000 *g* for 30 min at 4°C. Packed cells were resuspended in saline (NaCl 0.85% w/v), and, after freezing, were disrupted by three passages through an X-press (Life Sciences Laboratories, Sarum Road, Luton). After thawing, the suspension was centrifuged at 100 000 *g* for 30 min at 4°C. The supernate of the

cell lysate (cell extract) was distributed in small amounts and stored at -20°C . For use, it was thawed and diluted in sterile saline. Protein estimation on the cell extract was done by the method of Herbert *et al.* (1971) with bovine serum albumin as a standard.

Skin reactions

These were assessed in 3–4-week-old male HaM/ICR mice (Charles River UK Ltd, Manston Road, Margate, Kent CT9 4LT) except where stated. After ether anaesthesia, groups of five or ten mice were given injections of 0.05 ml of a suitable dilution of the crude extract sc in the dorsal region. After 24 h the animals were killed with CO_2 and the diameters of the haemorrhagic reactions within the skin were measured. In the case of large irregularly-shaped reactions, the mean of the largest and smallest dimensions was determined.

Anti-inflammatory agents

Prednisolone-21-sodium succinate was obtained from Sigma. Sodium meclofenamate monohydrate was a gift from Parke, Davis and Co. Ltd, Usk Road, Pontypool, Gwent. For injection, both were dissolved in sterile saline.

Statistical analysis

The mean diameters of skin reactions in test and control groups were compared by Student's *t* test. Diameters of reactions were shown to be normally distributed by Rankit analysis (Bliss, 1967).

Results

Effect of age of mice on skin reactions to HLT

Although suckling mice (<3 weeks old) have been used previously for detecting HLT activity, the possibility of using random groups of weaned (>3 -week-old) mice was investigated. The age of the animals had a marked effect on the haemorrhagic response to the cell extract (fig. 1). Groups of 10 male or female mice of different age ranges were given sc injections of cell extract containing $1.2\text{ }\mu\text{g}$ of protein and, the diameters of the haemorrhagic reactions within the skin were measured after 24 h. Mice 3–5 weeks old showed the largest reaction, c. 17–20 mm in diameter. Older mice were less responsive; little if any reaction was seen in animals older than 7 weeks. There was no significant difference in the responsiveness of male and female mice. When cell extracts were injected ic into the shaved backs of mice, reactions in young and old mice were of similar diameter and severity to those seen after sc injection (data not shown). In all subsequent experiments, HLT samples were injected sc into male mice, 3–4 weeks old.

The heat lability of the toxic activity was demonstrated by heating the cell extract to 56°C for 20 min. No haemorrhagic effect was seen with such heated samples, even with a dose 400-fold higher than that used above.

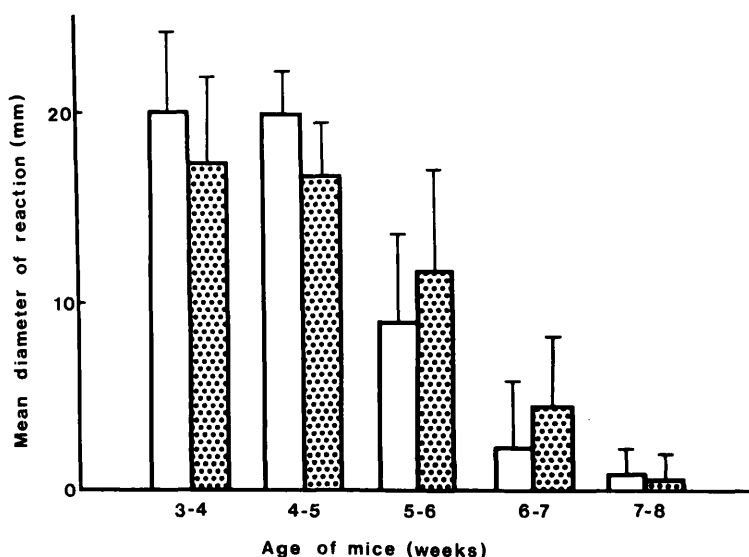


Fig. 1. Effects of age and sex of mice on the haemorrhagic reaction to *B. pertussis* cell-extract (containing $1.2\text{ }\mu\text{g}$ of protein) injected sc. The unshaded and shaded areas are the results for groups of 10 female and male mice respectively. The bars represent 1 SD.

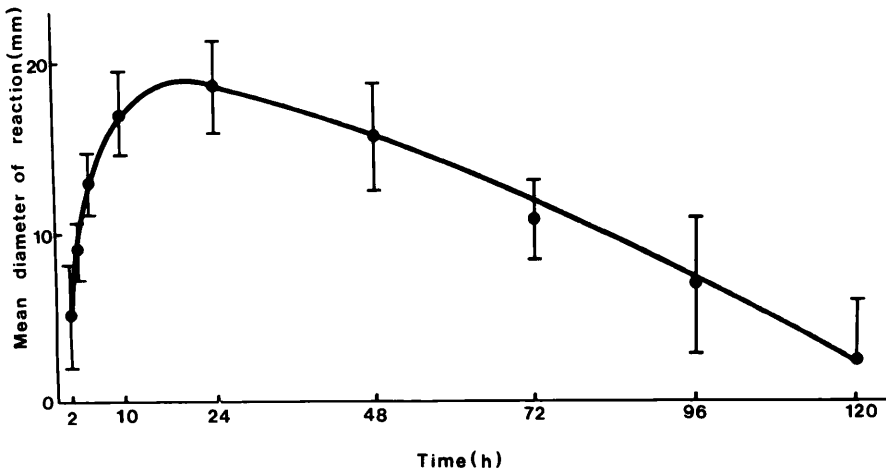


Fig. 2. Time course of the haemorrhagic response to *B. pertussis* cell-extract injected sc. Groups of five mice were used and each mouse received cell-extract containing $1.2 \mu\text{g}$ of protein. The bars represent ± 1 SD.

Time course of skin reactions

Fig. 2 shows the time course of the response to the cell extract injected sc. Numerous petechiae were visible at the site of injection within 2 h of challenge. Thereafter, there was a rapid increase in the diameter and severity of the reaction, with the most pronounced reaction, a dark haemorrhagic zone with some oedema, at *c.* 24 h. By day 5 after challenge the reactions had largely disappeared.

Inhibition of skin reactions by prednisolone or meclofenamate

A high dose of prednisolone sodium succinate (3 mg/mouse) given ip reduced significantly the haemorrhagic response to HLT; the degree of reduction depended on the time of administration of the drug (fig. 3). The greatest inhibition occurred when the prednisolone was given 1 h before, or at the same time as, the HLT challenge. When given

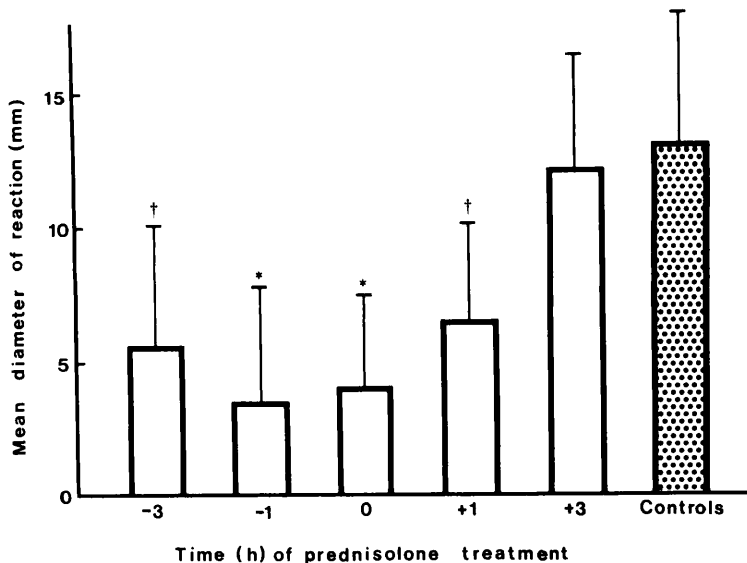


Fig. 3. Effect of prednisolone sodium succinate on the haemorrhagic reactions due to HLT. The drug (3 mg/mouse) was given ip before or after or at the time of challenge. Groups of 10 mice were given cell-extract containing $0.6 \mu\text{g}$ of protein/mouse by sc injection at time 0. By Student's *t* test for difference between test and control groups: * = highly significant ($p \leq 0.01$); † = significant ($p \leq 0.05$). The bars represent ± 1 SD.

1 h after challenge, there was still a significant reduction in the eventual response; but when given 3 h afterwards, no significant protection was seen.

A non-steroidal anti-inflammatory agent, sodium meclofenamate, also inhibited the haemorrhagic response to HLT. The effects of prednisolone and meclofenamate were compared by injecting graded doses of the drugs ip into groups of mice 1 h before challenge with HLT. Table I shows the diameters of the skin reactions in these mice compared with controls. In both cases, large amounts of the drug (≥ 1 mg/mouse or *c.* 70 mg/kg of body weight) were required to give a significant reduction in the diameters of the skin reactions. Prednisolone was also effective at a similar dose level when administered orally, by incorporating the drug in the drinking water for 2 days before challenge. A dose of prednisolone sodium succinate 1.5 mg/mouse/day gave a highly significant reduction in skin response ($P < 0.01$) whereas with 0.75 mg/mouse/day, there was no significant reduction ($P > 0.05$).

Prednisolone and meclofenamate were more effective when mixed with HLT before injection sc (table II). Presumably, much higher local concentrations of the drugs were achieved. A 250- μ g dose of prednisolone sodium succinate gave a significant reduction in the diameter of the reaction, and higher doses inhibited the reaction completely. Sodium meclofenamate was even more active. The reactions were completely inhibited by 25 μ g of the drug; and at 6.25 μ g, the lowest dose tested, there was a highly significant reduction in skin reaction.

Table I. Effect of ip prednisolone or meclofenamate on the haemorrhagic reactions to *B. pertussis* cell extract injected sc into groups of five mice*

Treatment	Drug dose (mg/mouse)	Mean diameter (mm) of skin reaction \pm SD (and significance†)
Prednisolone	3	7.8 \pm 5.4 (S)
sodium succinate	1.5	10.0 \pm 4.1 (NS)
	0.75	13.6 \pm 3.6 (NS)
Sodium	1	9.8 \pm 3.2 (S)
meclofenamate	0.5	12.6 \pm 3.9 (NS)
	0.25	13.2 \pm 4.5 (NS)
Saline	(control group)	15.0 \pm 3.1

* Each mouse received cell-extract containing 0.6 μ g of protein 1 h after injection of drug or saline.

† By Student's *t* test for difference between test and control groups: HS=highly significant ($p \leq 0.01$); S=significant ($p \leq 0.05$); NS=not significant ($p > 0.05$).

Table II. Effect of prednisolone or meclofenamate on the haemorrhagic reactions to HLT, when the drug was mixed with cell extract and injected sc into groups of five mice*

Treatment	Drug dose (μ g/mouse)	Mean diameter (mm) of skin reactions \pm SD (and significance†)
Prednisolone	500	no reactions
	250	12.4 \pm 2.1 (S)
sodium succinate	125	14.8 \pm 2.6 (NS)
	62.5	16.8 \pm 2.2 (NS)
Sodium	25	no reactions
meclofenamate	12.5	5.8 \pm 1.8 (HS)
	6.25	8.0 \pm 2.0 (HS)
Saline	(control group)	16.6 \pm 2.2

* Each injection mixture contained 0.6 μ g of cell-extract protein.

† See footnote to table I.

Discussion

The mechanism of action of HLT is unknown and there is no in-vitro assay for the toxin. The haemorrhagic response to *B. pertussis* cell extracts injected sc into 3–4-week-old mice, as described here, provided a convenient way of detecting HLT activity. Previous workers have used suckling mice (Katsampes *et al.*, 1942; Kurokawa *et al.*, 1969; Cowell *et al.*, 1979; Livey and Wardlaw, 1984) but it is not clear from the literature that mice become less responsive with age. Violle (1950) reported that mice were unresponsive to HLT but the age of the animals was not specified. The reason for this loss of sensitivity to HLT as the mice mature is not known, but preliminary experiments have shown that the same phenomenon occurs also in Wistar (albino) and DA (agouti) rats of different ages (unpublished observations). It is interesting to note the parallel between the HLT responsiveness of these animals and the known age-related severity of pertussis in children (Miller and Fletcher, 1976). With another toxic product of *B. pertussis* (variously called pertussigen, pertussis toxin, histamine-sensitising factor, lymphocytosis-promoting factor, etc.) generally regarded as having a central role in the pathogenesis of whooping cough, mice become more responsive to its histamine-sensitising properties as they mature (Munoz and Bergman, 1968).

The anti-inflammatory agents prednisolone and meclofenamate inhibited the haemorrhagic effect produced by HLT in mouse skin. In a previous report (Parton, 1985), prednisolone was shown to

protect mice against the lethal effect of HLT. In both cases, prednisolone was most effective if given just before, or at the same time as, the HLT challenge. When drug treatment was delayed until 3 h after challenge, no significant protection was given. In the case of the skin response, the reactions would have started to appear by this time. This suggests that some chain of events had been started by HLT and could not be reversed by these drugs.

Both prednisolone and meclofenamate prevent the generation of arachidonic acid metabolites which are potential mediators of inflammation (Nickander *et al.*, 1979). Corticosteroids are thought to inhibit the first step in this reaction, the release of arachidonic acid from membrane phospholipids in target cells. Non-steroidal anti-inflammatory agents such as meclofenamate are thought to act primarily to inhibit prostaglandin synthetase, but, like corticosteroids, they may have other actions (Hart and Huskisson, 1984). Thus, it is possible that one or more of the arachidonic acid metabolites plays an important role in the lethal and skin-haemorrhagic effects of HLT in the mouse. These studies do not, however, shed any light on the primary sites of HLT activity because many non-specific stimuli can promote the arachidonic acid cascade.

Nakase and Endoh (in press) described the effects of purified HLT on surgically-exposed peripheral

blood vessels of guinea-pigs and suckling mice. Within 15 min, HLT had induced a sustained vasoconstriction which eventually led to haemorrhage and oedema. It is interesting to note that certain prostaglandin, thromboxane and leukotriene derivatives of arachidonic acid are potent vasoconstrictors (Nickander *et al.*, 1979; Samuelsen, 1983). It may be possible to identify the important mediators by examining the effects of other, more specific inhibitors or mimetics of these agents in the mouse model described here.

In a review on the treatment of whooping cough, Broomhall and Herxheimer (1984) suggested that carefully designed clinical trials should be set up in which several forms of treatment, including corticosteroids, could be assessed. If HLT has an important role in the pathogenesis of pertussis, as seems possible (Munoz, 1971; Olson, 1975; Wardlaw and Parton, 1983), the present work suggests that non-steroidal anti-inflammatory agents also may be worthy of investigation. Further studies on the effects of a range of anti-inflammatory agents on the various pathophysiological activities of *B. pertussis* toxins in mice could help in the selection of the most likely candidates.

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