EFFECT OF PREDNISOLONE ON THE TOXICITY OF *BORDETELLA PERTUSSIS* FOR MICE

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SUMMARY. Prednisolone, given orally or intraperitoneally before challenge, protected mice against the lethal effect of a crude cell extract of *Bordetella pertussis* containing heat-labile toxin (HLT) as the major toxic component. Prednisolone did not diminish the lethal toxicity of heated *B. pertussis* cell suspensions containing pertussis toxin and endotoxin but devoid of HLT. This suggests that the protective effect of the steroid was directed against the HLT. When live bacteria were injected intraperitoneally, prednisolone showed a protective effect against the initial toxæmia. By day 7, however, the protection was no longer evident and the steroid promoted the survival of the organisms within the peritoneal cavity. These findings are discussed in the light of reports of the beneficial effects of corticosteroids in the treatment of whooping cough and in relation to a possible role for HLT in the pathogenesis of the disease.

INTRODUCTION

Bordetella pertussis produces three distinct toxins,—pertussis toxin (or pertussigen), a heat-labile dermonecrotising toxin (HLT) and endotoxin—and a number of other, less well characterised toxic substances (Wardlaw and Parton, 1982 and 1983). Despite much recent investigation and speculation, the role of these toxins in the pathogenesis of whooping cough remains obscure. Pittman (1979) has suggested that the harmful effects of the disease, including the paroxysmal cough, are caused by pertussis toxin which diffuses from the site of infection and acts systemically to cause a variety of pathophysiological changes. These effects on the host persist long after the organisms have been eliminated. However, the primary stimulus to coughing may be local irritation due to local inflammatory disease and locally-acting toxins (Olson, 1975).

Once the paroxysmal stage has been reached, it is generally considered that little can be done to modify the course of the disease (Miller and Fletcher, 1976). Antibiotics may accelerate the elimination of infection, but do not halt the paroxysmal coughing. However, several reports have suggested that corticosteroids should be considered for the treatment of severe pertussis. Zoumboulakis *et al.* (1973) showed that hydrocortisone given intramuscularly over a period of 7–8 days significantly reduced the frequency of coughing, whooping and vomiting and shortened the duration of the

illness, especially in infants under one year old. Both control and steroid-treated groups received erythromycin. The earlier work of Dayal *et al.* (1969) had also suggested that prednisolone with tetracycline was beneficial in the treatment of pertussis. More recently, Barrie (1982) stressed that the paroxysmal cough was suppressed by steroids and cited three cases in 8–13-week-old infants in which oral prednisolone dramatically reduced the severity and number of coughing spasms. Further support for this form of treatment was provided by Dianese (1982).

A major obstacle in pertussis research is the lack of a satisfactory animal model for the paroxysmal coughing syndrome in man. However, each of the three toxins is lethal for mice when injected intraperitoneally and they can be differentiated on the basis of heat stability and, to some extent, on the time taken to cause death (Ishida, 1968). The object of the work reported here was to explore the possible effect of prednisolone on the toxicity of *B. pertussis* cells and cell extracts in mice.

MATERIALS AND METHODS

Strains. *B. pertussis* strain 18334, a vaccine strain, was obtained originally from Connaught Laboratories Ltd, Toronto. Strain 18-323, a challenge strain used for the intracerebral mouse protection test, was obtained from Dr F. Sheffield, National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB.

Cell extract was prepared from *B. pertussis* strain 18334 grown in modified Hornibrook 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 Ltd, 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 Dulbecco A phosphate-buffered saline (Oxoid) (PBS).

Heated cell suspensions were also prepared from *B. pertussis* strain 18334 grown and harvested as described. Cells were resuspended in PBS and heated at 56°C for 30 min. Thiomersal (BDH) was added as a preservative at a final concentration of 0.1 mg/ml and the suspension was freeze-dried in small amounts. It was reconstituted in sterile distilled water immediately before use. The 100°C-suspension was prepared from a batch of the 56°C-heated suspension by steaming for 1 h.

Live challenge suspensions were prepared from *B. pertussis* strain 18-323 grown on Bordet-Gengou agar (Gibco Biocult Diagnostics Ltd, Paisley, Scotland), supplemented with sterile defibrinated horse blood (Gibco) 20% v/v, for 48 h at 37°C. Growth from the plates was resuspended in sterile Casamino acids solution containing Casamino acids (Difco technical) 10g/L, NaCl 5g/L, MgCl₂·6H₂O 0.1g/L and CaCl₂ 0.016 g/L, pH 7.1. The concentration of the cells was standardised against the WHO 5th International reference preparation of opacity (Perkins *et al.*, 1973) to give a notional value of 10¹⁰ cells/ml.

Lethality titrations were done in 3–4-week-old male HaM/ICR mice (Charles River, UK Ltd, Manston Road, Margate, Kent CT9 4LT). Groups of mice of similar weight were given intraperitoneal (ip) injections of graded doses of the *B. pertussis* preparations. Deaths were recorded daily.

Recovery of B. pertussis from the peritoneal cavity. Mice surviving ip challenge with live cells were killed by cervical dislocation. The skin was stripped from the abdomen and 2 ml of sterile Casamino acids solution was injected into the peritoneal cavity along the mid-anterior line. After massage of the abdomen for 30s, 0.5 ml was withdrawn from the pocket of fluid formed at the flank and, of this, 0.1 ml was plated on to Bordet-Gengou agar. Colonies were counted after incubation for 4–5 days at 37°C. The identity of the *B. pertussis* colonies was confirmed by slide agglutination with rabbit antipertussis serum (obtained from C.R. Manclark, Division of Biologics Standards, NIH, Bethesda, MD, USA).

Prednisolone treatment. For oral administration, prednisolone-21-sodium succinate (Sigma)

was dissolved in tap water and offered to the mice *ad libitum* in the usual water bottle. The solution was changed daily. For ip injection, the steroid was dissolved in PBS.

Protein estimations on the cell extract and cell suspensions were done by the method of Herbert *et al.* (1971) with bovine serum albumin as a standard.

Statistical methods. LD50 values and 95% confidence limits were calculated by the probit method (Finney, 1964). For determining the significance of differences between proportion data from the challenge experiments, 2 × 2 contingency tables (Finney *et al.*, 1963) were used.

RESULTS

Effect of prednisolone on the lethal toxicity for mice of B. pertussis cell extract

Graded doses of a crude cell extract of *B. pertussis*, injected ip into 3-week-old mice, were rapidly lethal. Most deaths occurred within one or two days of challenge and usually there were no further deaths after day 3 (table I). By heating the cell extract to 56°C for 30 min, toxicity was greatly reduced; LD50 values of unheated and heated samples were calculated to be 1.34 and > 240 µg of cell-extract protein per mouse respectively. In view of the rapid killing (Ishida, 1968) and the reduction of the toxicity by mild heat treatment (Munoz, 1971), deaths in this assay were assumed to be due primarily to the heat-labile toxin (HLT) of *B. pertussis*.

Mice given prednisolone sodium succinate (150 µg/ml) in their drinking water from day 2 before challenge to day 4 after challenge, were afforded significant protection against the toxin (table I). At day 3, the LD50 value for the cell extract was 3.0 µg protein per mouse. A few more deaths occurred up to day 7, but the protective effect was still significant. No deaths occurred in unchallenged animals receiving prednisolone, but all groups of animals given the steroid failed to gain weight normally.

In the above experiment, the mice received a high dose of prednisolone in their drinking water, equivalent to approximately 0.7 mg of steroid/mouse/day, or 50 mg

TABLE I

Effect of oral prednisolone on the lethal toxicity for mice of B. pertussis cell extract

Treatment	Challenge dose (µg protein/mouse)	Number of survivors on day			LD50 (µg protein/mouse) on day	
		0	3	7	3	7
	6	20	0	0	1.34	1.34
Water only	3	20	2	2	(1.67, 1.09)†	(1.67, 1.09)
(Controls)	1.5	20	6	6		
Prednisolone*	6	20	1	1	3.0	2.72
in drinking water	3	20	10	8	(3.58, 2.52)	(3.28, 2.24)
from day -2 to +4	1.5	20	19	18		

* The average daily intake per mouse at the time of challenge was 0.7 mg prednisolone/mouse/day or 50 mg prednisolone/kg body weight/day.

† Figures in brackets are the 95% confidence limits.

steroid/kg body wt/day over a 7-day period. However, these daily intakes are calculated from the consumption of drinking water up to the time of challenge, and, after challenge, sick animals drank less than less severely affected animals. In groups of mice given graded doses of prednisolone from day 2 before challenge to day 4 after challenge, doses of >3.75 mg/kg body wt/day still gave significant protection (fig. 1).

Prednisolone also protected mice against HLT challenge when given by the ip route. Fig. 2 shows the effect of two doses (1.4 mg/mouse) of prednisolone given ip either 24h and 3h before challenge or 3h and 24h after challenge compared with control animals given diluent only. In this experiment, the challenge dose was slightly less toxic than in previous experiments and late deaths occurred in all three groups. Prednisolone was highly protective at day 3 and day 7 if given before challenge, but less effective if given after challenge. In the latter groups, protection was highly significant at day 3 but not significant at day 7, with 9 out of 20 animals surviving compared with 5 out of 20 in the control group.

Effect of oral prednisolone on the lethal toxicity for mice of heated cell suspensions

B. pertussis cell suspensions were heated either to 56°C for 30 min or to 100°C for 1h. The former treatment destroys HLT but leaves active pertussis toxin and endotoxin, whereas the latter treatment allows only endotoxin to persist, because pertussis toxin is destroyed by heating to 80°C for 30 min (Wardlaw and Parton, 1982). When these suspensions were used to challenge prednisolone-treated and normal mice,

FIG. 1.—Effect of graded doses of oral prednisolone on the lethal toxicity for mice of *B. pertussis* cell extract. All animals were challenged with $3\ \mu\text{g}$ of cell-extract protein. Shaded area represents survivors on day 3, unshaded area represents survivors on day 7; * = highly significant ($p \leq 1\%$), † = significant ($p \leq 5\%$) difference from corresponding group of animals receiving no steroid.

FIG. 2—Effect of prednisolone given ip before or after challenge with *B. pertussis* cell extract. Prednisolone (1.4 mg/mouse) was given 24h and 3h before challenge or 3h and 24h after challenge with 3 μ g of cell-extract protein. Shaded area represents survivors on day 3, unshaded area represents survivors on day 7; control mice received 0.5 ml of PBS 24h and 3h before challenge; * = highly significant ($p \leq 1\%$) difference from corresponding group of control animals.

no protective effect was seen in the steroid-treated groups (table II). In all groups, most of the deaths had occurred by day 3, which is characteristic of deaths due to endotoxin rather than to pertussigen (Ishida, 1968); only a few deaths occurred between day 3 and day 7. The boiled cell suspension was less toxic than the suspension that had been heated to 56°C, but the suspensions were prepared and tested separately. Challenge with the cell suspension that had been heated to 56°C produced no significant difference in toxicity in the control animals and the group treated with steroid. With the boiled suspension, however, the mice treated with prednisolone were almost twice as sensitive to the challenge. No deaths were seen in unchallenged animals given water or steroid for the duration of the experiment.

Effect of oral prednisolone on the lethal toxicity for mice of live B. pertussis given by the ip route

When mice were challenged ip with live cells of *B. pertussis* strain 18323, those treated with prednisolone were afforded significant protection at day 3 (table III). By day 7, however, many more deaths had occurred in the groups treated with steroid and the protective effect was no longer evident.

TABLE II
Effect of oral prednisolone on the lethal toxicity for mice of heated B. pertussis cell suspensions

Treatment	Challenge	Challenge dose (μg protein/ mouse)	Number of survivors on day			LD50 (μg protein/mouse) on day	
			0	3	7	3	7
Water only (Controls)	Cell suspension heated to 56°C for 30 min	675	10	1	0		
		337	10	7	7	346	265
		169	10	9	8	(454, 254)†	(316, 223)
Prednisolone* in drinking water from day -2 to +7		675	10	1	1		
		337	10	8	8	389	368
		169	10	9	8	(524, 292)	(514, 263)
Water only (Controls)	Cell suspension heated to 100°C for 1h	2700	10	5	5	2297	2225
		1350	10	7	6	(3024, 1744)	(2969, 1668)
		675	10	10	10		
Prednisolone* in drinking water from day -2 to +7		2700	10	2	2		
		1350	10	3	3	1323	1323
		675	10	9	9	(1846, 947)	(1846, 947)

* Dosage as in table I.
† Figures in brackets are the 95% confidence limits.

TABLE III

Effect of oral prednisolone on the lethal toxicity for mice of live B. pertussis given by the ip route

Treatment	Challenge dose (number of organisms, 10^9 /mouse)	Number of survivors on day			LD50 (number of organisms, 10^9) on day	
		0	3	7	3	7
Water only (Controls)	5	10	0	0	2.04	1.95
	2.5	10	6	4	(2.45, 1.7)†	(2.4, 1.58)
	1.25	10	9	9		
Prednisolone* in drinking water from day -2 to +7	5	10	3	1	5.13	2.29
	2.5	10	10	5	(6.72, 3.9)	(3.21, 1.63)
	1.25	10	10	8		

* Dosage as in table I.

† Figures in brackets are the 95% confidence limits.

In a further experiment, peritoneal washings were obtained from surviving animals at day 7 to determine if steroid treatment allowed survival or multiplication of *B. pertussis* in the mouse peritoneal cavity. The results are shown in table IV. With this challenge, over 50% of the animals in each group survived to day 7. Again, more prednisolone-treated mice than controls were alive on day 3, but by day 7 the number of survivors in each group was similar. When counts were made on Bordet-Gengou medium from samples of peritoneal wash fluid, *B. pertussis* could be recovered from most of the animals. With the higher dose of prednisolone, a large number of organisms was isolated from each of the survivors. Few, if any, colonies were obtained from control mice and the lower dose of prednisolone gave an intermediate result.

TABLE IV

Effect of oral prednisolone on the lethal toxicity for mice and survival of live B. pertussis† injected ip

Treatment	Number of survivors on day			Median number of cfu recovered per 0.1 ml of peritoneal wash fluid
	0	3	7	
Water only (Control)	15	11	10	3
Prednisolone* 5 mg/kg body wt/day	15	13	11	55
Prednisolone* 50 mg/kg body wt/day	15	15	9	> 200

* Given in drinking water from day -2 to +7. Dosage calculated from the water consumption up to the time of challenge.

† Challenge dose was *c.* 2.5×10^9 cfu.

DISCUSSION

Glucocorticoids are valuable in the treatment of many diseases in which immunological or inflammatory activity contributes to the pathology, although they can also have undesirable effects such as lowering resistance to infection (Kass and Finland, 1958; Baxter and Rousseau, 1979). Their reported beneficial effects in the treatment of whooping cough (Zoumboulakis *et al.*, 1973; Barrie, 1982) prompted the present investigation into the effect of prednisolone on the various toxic activities of *B. pertussis* for mice.

The paroxysmal coughing stage of pertussis starts at approximately the same time as the pathogen is being eliminated from the host (Pittman, 1970). Thus, there is strong evidence that a long-acting toxin contributes to the harmful effects of the disease. Pittman (1979) suggested that pertussis toxin is the bacterial component responsible. However, it seems likely that more than one component of the organism is necessary for the pertussis syndrome since other *Bordetella* species, which lack pertussis toxin, can occasionally give rise to a paroxysmal cough, although usually in a mild form. Moreover, the cough may be re-activated by succeeding and unrelated infections (Olson, 1975). Thus, the primary stimulus to coughing may be local irritation but the special features of the paroxysmal stage may only occur when pertussis toxin is also present.

The major toxin of *B. pertussis*, at least for mice, is the heat-labile toxin (HLT). This can be seen from tables II and III which show that the lowest dose of cells heated to 56°C killed only a small proportion of normal mice, whereas the highest dose of live cells, which contained fewer cells, killed all the normal mice. HLT causes inflammation and necrosis when injected into the skin of laboratory animals (Munoz, 1971) and could, therefore, be the primary stimulus to coughing. A role for endotoxin, however, should not be disregarded. It should be noted that the other *Bordetella* species also contain endotoxin and HLT (Munoz, 1971) and that *B. bronchiseptica* causes kennel cough in dogs (Thompson *et al.*, 1976).

In the present investigation, prednisolone, given orally or ip before challenge, was found to protect mice against the lethal effect of HLT. Although this does not prove that the beneficial effects of glucocorticoids on whooping cough are due to counteraction of the activities of HLT, the idea does merit consideration. If the local inflammatory effects of HLT on the respiratory tract were inhibited, then the primary stimulus to coughing might be removed. It is of interest to note that prednisolone prevents the dermonecrotising, as well as the lethal, effects of HLT (unpublished observation).

Oral prednisolone did not appear to have any inhibitory effects on the lethal toxicity of cell suspensions of *B. pertussis* heated to 56°C, a temperature at which activities of both pertussigen and endotoxin are retained. With large doses of boiled cell suspension, where toxicity is presumed to be due to the endotoxin content, the mice treated with prednisolone were more sensitive to the challenge. These findings contrast with those of other workers who have shown that prior administration of glucocorticoids protects mice against challenge with pertussis vaccine (Kind, 1953) or with endotoxin from a variety of gram-negative species (Kass and Finland, 1958). Kind (1953) reported that cortisone protected mice from toxic death following ip injection of a phenol-preserved pertussis vaccine that was presumably devoid of HLT. The details

of these experiments are not clear, but it appears that 1 mg of cortisone was given intramuscularly 18h before challenge and deaths were recorded at 24h.

The reasons for these discrepancies are not known, but may be related to the use of different strains of mice which could differ in their sensitivity to endotoxin (Sultzer, 1972), the period of observation after challenge and differences in glucocorticoid administration. At the doses of steroid used in the present work, mice were already showing inhibition of the normal rate of weight gain at the time of challenge, resulting, no doubt, from a variety of profound changes in their metabolism (Baxter and Rousseau, 1979).

Although corticosteroids may, in some cases, protect mice against the lethal effects of lipopolysaccharide endotoxins, they do not appear to protect against death due to a number of bacterial protein toxins, including those from *Corynebacterium diphtheriae* and *Clostridium perfringens* (Kass and Finland, 1958) which are dermonecrotising. The effect of prednisolone on *B. pertussis* HLT may therefore be unusual and may reflect a different, as yet undefined, mode of action of the toxin.

High doses of live *B. pertussis* injected ip into mice killed the animals rapidly, usually in 1–3 days. Since there is no evidence of bacterial multiplication in the peritoneum, death is thought to be due to the toxin content of the inoculum (Pittman, 1970). In the present study, oral prednisolone gave significant protection to mice up to 3 days after challenge, but thereafter, the protection disappeared. Possibly, the steroid allowed the survival or multiplication of the organisms within the peritoneal cavity by interfering with the normal clearance mechanisms (Fauci, 1979) which, in turn, would increase the toxic burden on the host. Iida *et al.* (1963) have shown that corticosteroids markedly potentiate infection of mice given virulent, or even avirulent, *B. pertussis* intranasally.

In whooping cough, the infection is being eliminated naturally at around the time that treatment usually starts. Where steroids have been used (Dayal *et al.*, 1969; Zoumboulakis *et al.*, 1973; Barrie, 1982; Dianese, 1982) the children were also given antibiotics, thus making it unlikely that the infection would be prolonged or re-activated. In some infections, however, large doses of corticosteroids may so depress host resistance that the efficacy of antibiotics is limited (Kass and Finland, 1958).

Further investigation of prednisolone and related compounds on the various toxic and pathophysiological activities of *B. pertussis* in laboratory animals may yield further insights into the mechanisms of pathogenesis of whooping cough. For this work, purified toxins would be highly desirable.

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