

Reducing the endotoxic activity of pertussis vaccine

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SUMMARY

Unadsorbed, regular production pertussis vaccine was treated with polymyxin B sulphate at concentrations of 25, 50 and 100 $\mu\text{g/ml}$. The toxic activity of treated and untreated vaccines was compared using both the limulus amoebocyte lysate test and the mouse-weight-gain test. Protective efficacy was also assessed by the mouse protection test. No discernible effect on either toxicity or efficacy of the pertussis vaccine was observed. When the vaccine was treated with 5000 $\mu\text{g/ml}$ of polymyxin, endotoxic activity assessed by the limulus lysate test appeared to be abolished.

INTRODUCTION

Endotoxin, as an inherent component of Gram negative bacterial vaccines (WHO Secretariat, 1953; Peltola, 1978) or as a contaminant of non-bacterial vaccines (Knight & Lucken, 1977; Kuronen *et al.* 1977; Geier, 1978) is an unwanted source of adverse reactions. Recent methods of reducing the amount of endotoxin in vaccines have met with limited success (Nagel, 1970; Reichelderfer *et al.* 1975; Spasojevic, 1977; Nagel & de Graaf, 1978; Nakase, 1978). The present study examines the new approach of using a specific endotoxin-inactivator to achieve this purpose. Pertussis vaccine was chosen as the prototype endotoxic vaccine and attempts were made to reduce its endotoxin content using the antibiotic polymyxin, an agent with an extensive reputation as a powerful endotoxin disruptor and inactivator (Lopes & Inniss, 1969; Bannatyne *et al.* 1977; Cooperstock, 1974; Corrigan & Bell, 1971; From, Good & Fong, 1972; Bannatyne, Harnett & Cheung, 1977; Bannatyne & Cheung, 1979).

MATERIALS AND METHODS

Unadsorbed, regular production pertussis vaccine (Connaught Laboratories, Toronto) was allowed to react for 3 h at 4 °C with polymyxin B sulphate (Aerosporin) at concentrations of 25, 50 and 100 $\mu\text{g/ml}$. The toxic activity of treated and untreated vaccines was compared using both the limulus amoebocyte lysate (LAL) test and the mouse weight-gain test. The protective efficacy of the paired vaccines was determined by the mouse protection test. Pertussis vaccine treated with 500 μg of polymyxin/ml was tested for endotoxin activity by the LAL

test. Female Swiss white mice (ICD-CDI strain) weighing 15.0 ± 2.0 g were used in all the mouse experiments.

Limulus Amoebocyte Lysate Test

Twofold dilutions from 1:2 to 1:2048 of polymyxin-treated and untreated pertussis vaccine were prepared in pyrogen-free water using pyrogen-free test tubes. Aliquots (0.2 ml) of these vaccine dilutions were incubated with 0.2 ml of limulus amoebocyte lysate (Microbiological Associates) at 37 °C for 1 h. Appropriate positive and negative controls including the National Reference Endotoxin, EC-2, and polymyxin alone were tested in parallel. Tubes were examined hourly for 6 h and at 18 h for the presence of firm clot.

Mouse Weight-Gain Test

Preliminary experiments in the ICD-CDI mouse strain indicated that the LD₅₀ for polymyxin was 18 mg/kg. The WHO-approved procedure for the mouse weight-gain test was followed (WHO Secretariat, 1953). Mice were tested in groups of 100. They were weighed at the beginning of each experiment. Each mouse was injected intraperitoneally with one half volume (0.5 ml) of a single human dose of polymyxin-treated (25, 50 and 100 µg/ml) or untreated vaccine. The total weight of the group was determined at 3 and 7 days after inoculation. The criteria for lack of toxicity of the injected products were (1) no weight loss in the group at 3 days. (2) not less than a 3.0 gram average weight-gain per mouse at 7 days. (3) not less than 95% survival. The significance of the observed differences between each of the treated and untreated vaccine groups in the weights at 3 and 7 days was evaluated by the Null hypothesis.

Mouse Protection Test

The WHO-approved modification of Kendrick's original intraperitoneal-immunization/intracerebral challenge test was used (WHO Secretariat, 1953). Lyophilised vials of a single lot of *Bordetella pertussis* strain 18-353 were prepared and used throughout the experiment. Preliminary experiments showed that the LD₅₀ of *B. pertussis* 18-353 for the mouse strain was very close to 1.0×10^8 cells and the ImD₅₀ for the vaccine was just less than a 1:4 dilution. Mice were tested in batches of 100 and randomized with regard to their distribution into groups, shelf position, order of immunization and order of challenge. They were immunized with one ImD₅₀ dose in 0.5 ml of treated or untreated vaccine. After 14 days they were challenged intracerebrally with one 200 LD₅₀ dose of the *B. pertussis* challenge suspension (2.0×10^8 cells). Mice were observed for 14 days and the mortality/protection rates recorded. The significance of the difference in protection rates between the treated and untreated vaccines was determined by the Chi-square calculation.

RESULTS

In the mouse weight-gain test both untreated and polymyxin-treated vaccines fulfilled the three criteria for freedom from toxicity (Table 1). No statistical evidence of diminished toxicity on behalf of any of the polymyxin-treated vaccines in the 3 and 7 day weight criteria of toxicity was observed (Table 2).

Table 1. Mouse weight-gain test for toxicity

Polymyxin μg/ml	Weight in grams						Deaths	
	Day 0		Day 3		Day 7		Untreated vaccine	Treated vaccine
	Untreated vaccine	Treated vaccine	Untreated vaccine	Treated vaccine	Untreated vaccine	Treated vaccine		
25	1590.8 (100)*	1525.5 (100)	1672.4 (100)	1625.3 (93)	1996.2 (100)	1830.6 (93)	0	0
50	1725.6 (100)	1640.0 (100)	1852.2 (100)	1845.8 (100)	2128.2 (100)	2105.1 (100)	0	0
100	1485.4 (99)	1523.2 (101)	1577.9 (99)	1585.3 (101)	1773.8 (98)	1840.0 (100)	1	1

* Numbers in parentheses.

Table 2. Null hypothesis statistics for the treated and untreated vaccines in the mouse weight-gain tests for toxicity

Polymyxin μg/ml	Standard error of the difference in proportions		Observed difference in the proportions	
	3 days	7 days	3 days	7 days
	25	0.13	0.14	0.02
50	0.10	0.15	0.05	0.05
100	0.08	0.15	0.02	0.01

Table 3. Mouse protection test

Polymyxin μg/ml	Per cent mortality	
	Untreated vaccine	Treated vaccine
25	21.4	17.0
50	27.8	32.0
100	30.0	38.0

The mouse protection tests demonstrated no significant alteration in protective effect between the untreated and polymyxin-treated (25, 50 and 100 μg/ml) vaccines (Table 3). Chi-square values with one degree of freedom were 0.33, 0.24 and 1.02 respectively i.e. non-significant at the 1% level.

In the LAL test the untreated vaccine gave a firm clot up to a dilution of > 1:2048. The treated vaccines (25, 50 and 100 μg/ml) showed no reduction in endotoxic activity, giving a firm clot to the same dilution. Vaccine treated with a low therapeutic dose of polymyxin such as might be administered to a 2-3 month old infant (5000 μg/ml vaccine = approx. 1.0 mg/kg/dose) showed complete inhibition of LAL clot formation.

DISCUSSION

Concern for the endotoxin content of vaccines has generated the development of screening tests for pyrogens and endotoxin in biologicals, (Wong *et al.* 1973; Cooper & Pearson, 1977; Geier, Stanbro & Merrill, 1978; Knight & Lucken, 1977; Rastogi, Hochstein & Seligmann Jr., 1977) and has stimulated a search for methods of reducing the amount of endotoxin in these products (Nagel, 1970; Reichelderfer *et al.* 1975; Spasojevic, 1977; Nagel & de Graaf, 1978; Nakase, 1978). These methods have been only partially successful and vaccine reactions due to endotoxin remain common. This is particularly true of pertussis vaccine (Ishida, 1968; Kurokawa *et al.* 1968, 1969; Pieroni & Levine, 1976) which ranks second only to cholera vaccine in terms of endotoxin content. In the present study a novel approach to the problem was explored and de-endotoxification was attempted through the use of the powerful endotoxin disruptor and inactivator polymyxin (Lopes & Inniss, 1969; Bannatyne *et al.* 1977; Cooperstock, 1974; Corrigan & Bell, 1971; From *et al.* 1972; Bannatyne, Harnett & Cheung, 1977; Bannatyne & Cheung, 1979).

Experiments to examine the effect of polymyxin on the toxic and protective aspects of pertussis vaccine were limited by the nature of the standard test systems for these properties – the mouse assay, and by the smallness of the polymyxin dose tolerated by mice in the vaccine. At the minute doses permissible (25, 50 and 100 $\mu\text{g}/\text{ml}$ of vaccine) no discernible effect on either toxicity or efficacy of the pertussis vaccine was observed.

In the more sensitive LAL test for endotoxin where the effect of more realistic detoxifying concentrations could be tested, pertussis vaccine treated with 5000 μg of polymyxin/ml was non-reactive. In view of the knowledge of polymyxins action on endotoxin (Lopes & Inniss, 1969; Koike, Iida & Matsuo, 1969) it is suggested that this finding represents biological inactivation of pertussis endotoxin and the creation of a pertussis vaccine of diminished toxicity. There remain, however, reservations about such a polymyxin-containing vaccine. Most importantly is whether the reduction in endotoxin content is accompanied by a reduction in the amount of protective antigen and associated with a drop in protective efficacy. The answer to that question awaits the introduction of an acceptable alternative to the mouse protection test of vaccine efficacy. Secondly, although a pertussis vaccine containing 5000 μg of polymyxin/ml does not pose a toxic threat to recipients (this concentration is equivalent to a conservative, safe single dose of 1 mg/kg to a 5.0 kg infant) the removal of polymyxin from the product would render the vaccine more attractive. Additional studies to explore possible methods of achieving this are needed. The present study presents preliminary evidence that a reduction in the endotoxin content of pertussis vaccine can be accomplished with polymyxin and offers a new approach to decreasing the toxicity of Gram negative vaccines.

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