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Loss of adjuvanticity in rats for the hyperacute form of allergic encephalomyelitis and for reaginic antibody production in mice of a phenotypic variant of *Bordetella pertussis*

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Summary. The adjuvanticity of a phenotypic (C-mode) variant of *B. pertussis*, known to be deficient in certain immunological and physiopathological properties, was compared to that of the normal (X-mode) strain. The X-mode vaccine was a potent adjuvant for induction of hyperacute experimental allergic encephalomyelitis to guinea-pig spinal cord in Lewis rats whereas C-mode vaccine was inactive. X-mode vaccine was also highly active in the induction of reaginic (both IgE and IgG1) antibodies to ovalbumin in mice while C-mode vaccine caused only a transitory increase in the IgE level. These data support the view that an adjuvant component of *B. pertussis*, which is probably identical with the histamine-sensitizing and leukocytosis promoting factor, is much diminished in C-mode cells while the lipopolysaccharide adjuvant remains unchanged.

INTRODUCTION

The immunological and physiopathological properties of *Bordetella pertussis* cells can be manipulated phenotypically by altering the composition of the growth medium (Lacey, 1960; Pusztai & Joó, 1967). For example, the simple replacement of 5 g/l NaCl by the same weight-concentration of $MgSO_4 \cdot 7H_2O$, in either solid or liquid culture media, leads to cells with greatly reduced virulence, immunogenicity and content of agglutinogen (Lacey, 1960). The production of histamine-sensitizing factor (HSF), mouse-protective antigen (PA) and 28,000 and 30,000 mol. wt polypeptides in the cell-envelope is greatly diminished (Parton & Wardlaw, 1975; Wardlaw, Parton & Hooker, 1976), as well as the production of heat-labile toxin (HLT) (Livey, Parton & Wardlaw, 1978), lymphocytosis-promoting factor (LPF) (E. O. Idigbe, R. Parton and A. C. Wardlaw, unpublished results), and adenylate cyclase (Parton & Durham, 1978). Cells from the NaCl media were designated X-mode (X = xanthic), from their yellowish colour, while those from $MgSO_4$-containing (and other) media were called C-mode (C = cyanic) from their bluish appearance (Lacey, 1960). Lacey introduced the term 'antigenic modulation' for these reversible phenotypic changes in *B. pertussis* cultures, to distinguish them from the phase variation of Leslie & Gardner (1931) which appears to involve permanent loss of properties. Although the mechanism of change may be different, C-mode and Phase IV organisms resemble one another closely in

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lacking many of the activities possessed by their X-mode, Phase I counterparts.

B. pertussis cells exhibit, in addition to the above, a variety of adjuvant effects (reviewed by Morse, 1976). The object of this investigation was to compare the adjuvant activities of X-mode and C-mode vaccines in two systems: the production of the hyperacute form of experimental allergic encephalomyelitis (EAE) by guinea-pig spinal cord in Lewis rats (Levine & Wenk, 1965) and the induction of reaginic antibodies to ovalbumin in mice (Mota & Peixoto, 1966; Clausen, Munoz & Bergman, 1969).

MATERIALS AND METHODS

Pertussis vaccines and extract

B. pertussis strain No. 18334 was grown in X- and C-media as described previously (Wardlaw *et al.*, 1976). X-mode vaccine was made from cells grown in litre amounts of X-medium for 72 h at 35° on an orbital shaker (80 r.p.m.). The cells were harvested by centrifugation, suspended in saline, heated for 30 min at 56° and preserved with thiomersal (0·1 mg/ml final concentration). C-mode vaccine was prepared similarly, except that the culture medium contained 5 g/l $MgSO_4 \cdot 7H_2O$ instead of 5 g/l NaCl. The cell content of both vaccines was standardized before heating by comparison with the International Opacity Standard (Perkins, Sheffield, Outschoorn & Hemsley, 1973). One International Opacity Unit is equivalent to approximately 10^9 organisms/ml. The C-mode vaccine had only about 2% of the HSF activity of the X-mode on an equal opacity basis.

B. pertussis saline extract was made as previously described (Munoz & Hestekin, 1963) from Phase 1 *B. pertussis* cells grown in medium low in Mg. It was stored as a lyophilized powder at room temperature.

Guinea-pig cord emulsion

This was prepared from young (about 2 months old) Hartley strain guinea-pigs by the method of Levine, Wenk, Devlin, Pieroni & Levine (1966). Briefly, a suspension of spinal cord was made in physiological saline (100 mg/ml), heated at 60° for 75 min and homogenized with a Teflon grinder. It was forced once through a 19 gauge needle and kept frozen until needed.

Induction of hyperacute EAE

Female Lewis rats were injected intraperitoneally with 25 mg guinea-pig spinal cord emulsion mixed with various doses of *B. pertussis* vaccines or cell–extract in a total volume of 4 ml. The animals were observed daily for symptoms of hyperacute EAE.

Method of scoring EAE

EAE was scored on the basis of rapidity of onset and maximum severity of paralysis. Severity was rated from 0 to 4 as follows: 0, no symptoms; 1, rough hair coat, slight ataxia and slight paralysis of tail and hind legs; 2, definite paralysis of tail and hind legs, but retaining ability to move about on four legs; 3, complete paralysis of tail and both hind legs but capable of moving about by means of front legs; 4, totally paralysed, lying on side and showing a slight bloody exudate from nose and eyes.

Rapidity of onset was scored on the basis of the day when the rat first showed paralysis. Paralysis at 5 days post-inoculation was scored as 5 for rapidity; at 6 days the score was 4, at 7 days the score was 3 and so on. The rapidity scores and maximum severity scores were added together to give the overall EAE score for each rat.

Production of reaginic antibodies

Mice were of a closed colony bred from the Ham I/CR strain (Charles River U.K. Ltd, Manston Road, Margate, Kent). Groups of ten 6 week old females were injected intraperitoneally with doses of *B. pertussis* vaccine and 200 μg ovalbumin (Sigma Chemical Co.) in saline in a total volume of 0·5 ml. Animals were bled after 7 or 21 days by cardiac puncture and individual sera collected and stored at −20°.

Measurement of reaginic antibodies

Homocytotropic antibodies were measured by passive cutaneous anaphylaxis (PCA), using a hairless strain of mouse (hrhr) as recipients. Antisera were diluted 1/5 or 1/10 in Dulbecco A phosphate buffered saline (PBS) and 0·05 ml amounts injected intradermally into the dorsal surface. Each mouse was injected at four sites and each antiserum was injected into two recipients. After 2 or 48 h, animals were challenged intravenously with 1 mg ovalbumin in 0·2 ml of PBS containing 0·5% w/v Evans blue dye. Reactions were clearly visible on the mouse skin surface as well defined blue areas. The animals were killed 30 min after challenge and the diameter of blueing measured. A positive response was taken as an area of blueing at least 5 mm in diameter in one or both recipients. With a 48 h sensitization period, the reaction is specific for IgE antibodies; IgG1

antibodies were detected by heating the diluted antisera at 56° for 4 h and using a 2 h sensitization period (Lehrer, 1977). The heat treatment completely abolished the 48 h PCA reactions.

RESULTS

Since the object of the investigation was to make a quantitative comparison of the relative adjuvanticity of X- and C-mode pertussis vaccines, the dose of vaccine in the injection mixtures given to the different groups of animals was varied while the dose of neuroantigen or ovalbumin was kept constant.

Induction of hyperacute EAE

Groups of five rats were given 25 mg spinal cord alone, and mixed with X-mode or C-mode pertussis vaccine in doses of $0{\cdot}5 \times 10^9$, 5×10^9 and 50×10^9 cells. A further group of animals received spinal cord with 200 μg of a saline extract made from X-mode *B. pertussis* cells. The results with forty rats observed over 19 days are summarized in Table 1. Spinal cord alone produced only minimal and transitory symptoms in a single animal and yielded an overall EAE score of 3. X-mode vaccine showed a pronounced dose-related adjuvant effect, with the lowest dose giving a score of 7 and the two higher doses, scores of 25. At the highest dose all animals died.

In contrast, similar graded doses of C-mode vaccine exhibited much lower adjuvant activities with a maximum score of only 7 and no deaths. Saline extract at 200 μg was a more potent adjuvant than X-mode vaccine. It will be noted that in all groups of animals, except where deaths occurred, paralysis scores reached their peak around day 9–10, while by day 19 post-injection the animals had recovered.

Similar results to the above were obtained in another experiment involving forty rats with the same batches of X-mode and C-mode vaccines made from *B. pertussis* strain 18334. In this case, however, the

Table 1. Comparative adjuvant activities of X-mode and C-mode *B. pertussis* vaccines and saline extract for induction of hyperacute EAE

Adjuvant	Total paralysis score* for five rats on day									Total EAE score for five rats‡
	5	6	7	8	9	10	12	14	19	
None (cord alone)	0	0	0	1	1	1	0	0	0	3 (−)
X-mode vaccine ($0{\cdot}5 \times 10^9$)	0	0	0	0	0	2	6	5	0	7 (NS)
X-mode vaccine (5×10^9)	0	0	1	6	14	15	9	2	0	25 (S)
X-mode vaccine (50×10^9)	0†	0	0	0	4	11	6+2D	2D	—§	25 (S)
C-mode vaccine ($0{\cdot}5 \times 10^9$)	0	0	0	0	1	2	2	0	0	4 (NS)
C-mode vaccine (5×10^9)	0	0	0	1	3	4	2	1	0	7 (NS)
C-mode vaccine (50×10^9)	0	0	0	0	1	1	1	0	0	2 (NS)
B. pertussis saline extract (200 μg)	0	2	10	13	20	18	6+D	2+D	0	36 (S)

* D, dead

† One animal was found dead 3 days after injection, presumably due to vaccine toxicity. The remaining four animals died of hyperacute EAE before day 19.

‡ Significance (in parentheses) of the difference between the total EAE score of the group compared with cord-alone controls as determined by the Mann–Whitney *U*-test (Campbell, 1974): S, significant at the 5% probability level; NS, not significant.

§ All dead.

dose of spinal cord was eight times higher and by itself produced hyperacute EAE with a maximum paralysis score of 15 in five rats on day 11. Addition of C-mode vaccine did not increase this, nor were there any deaths. In contrast, eight out of fifteen rats receiving spinal cord plus three levels of X-mode vaccine had died by day 14.

Production of reaginic antibody to ovalbumin

Groups of ten mice were injected intraperitoneally with 200 μg ovalbumin alone or with ovalbumin mixed with graded doses of either X-mode or C-mode pertussis vaccines. Table 2 summarizes the results with 180 mouse sera obtained in two experiments 21 days after immunization and subjected to tests with sensitization times of 2 h and 48 h at a dilution of 1/10. In the 48 h PCA test, sera raised against ovalbumin alone showed only three responders out of twenty, indicating that a single dose of 200 μg ovalbumin was a poor inducer of IgE antibodies. Ovalbumin mixed with C-mode pertussis vaccine was no better even at an adjuvant dose of $2{\cdot}5 \times 10^9$ cells. At the highest level of X-mode vaccine ($2{\cdot}5 \times 10^9$) 85% of the mice produced detectable IgE antibodies to ovalbumin.

In 2 h PCA tests with the same antisera, but heated to 56° for 4 h to destroy IgE, similar results were obtained. X-mode vaccine was a potent adjuvant for antibodies presumed to be IgG1, whereas C-mode vaccine had no significant activity.

In other experiments where a total of 220 sera were obtained at 7 days rather than 21 days after immunization, a different pattern emerged (Table 3). These sera were tested at a dilution of 1/5. No IgE responders were detected after immunization with ovalbumin alone, whereas a significant proportion of animals responded to mixtures of ovalbumin and the various doses of either X- or C-mode pertussis vaccine. Although an adjuvant dose–response effect was apparent, the highest doses of adjuvant, whether X or C, did not give the greatest response. Maximum adjuvant activity was provided by $2{\cdot}5 \times 10^8$ cells of either vaccine. With this dose of X-mode vaccine, 26/30 mice

Table 2. PCA tests with sera taken from mice 21 days after injection of ovalbumin and graded doses of X-mode or C-mode *B. pertussis* vaccines

Sensitization period in PCA test (h)	*B. pertussis* vaccine as adjuvant	No. of mice responding/No. immunized with ovalbumin (200 μg) and pertussis vaccine at (No. of cells)				
		0	$2{\cdot}5 \times 10^6$	$2{\cdot}5 \times 10^7$	$2{\cdot}5 \times 10^8$	$2{\cdot}5 \times 10^9$
48	X-mode		7/20 (35%)	8/20 (40%)	14/20 (70%)	17/20 (85%)
(for IgE)	C-mode		3/20 (15%)	1/20 (5%)	3/20 (15%)	2/20 (10%)
	None	3/20 (15%)				
2	X-mode		3/20 (15%)	6/20 (30%)	11/20 (55%)	8/10 (80%)
(for IgG1)	C-mode		1/20 (5%)	1/20 (5%)	1/20 (5%)	1/20 (5%)
	None	1/20 (5%)				

Table 3. Forty-eight hour PCA test with sera taken from mice 7 days after injection of ovalbumin and graded doses of X-mode or C-mode *B. pertussis* vaccine

B. pertussis vaccine as adjuvant	No. of mice responding/No. immunized with ovalbumin (200 μg) and pertussis vaccine at (No. of cells)				
	0	$2{\cdot}5 \times 10^6$	$2{\cdot}5 \times 10^7$	$2{\cdot}5 \times 10^8$	$2{\cdot}5 \times 10^9$
X-mode		5/30 (17%)	14/30 (47%)	26/30 (87%)	19/30 (63%)
C-mode		3/20 (15%)	6/20 (30%)	10/20 (50%)	8/20 (40%)
None	0/20				

(87%) became responders whereas only 10/20 (50%) of C-mode recipients responded.

When the same 7 day sera, after heating to 56° for 4 h, were subjected to the 2 h PCA test, no reactions were observed in any of the 220 sera, indicating that the reactions given by unheated sera had been due to IgE alone and that the IgG1 response to the injection mixtures had not yet developed.

DISCUSSION

There is good evidence that *B. pertussis* vaccines made from Phase I (X-mode) cultures contain two quite distinct adjuvant substances, one heat-stable and the other heat-labile (Pieroni & Levine, 1966; Murgo & Athanassiades, 1975). The former, which is stable to boiling for 1 h and an effective adjuvant for diphtheria toxoid in guinea-pigs, is a lipopolysaccharide (Farthing, 1961). The heat-labile adjuvant, which is destroyed by heating for 30 min at 80°, operates in experimental systems where boiled pertussis vaccine is inactive. Among these systems are tetanus toxoid protective immunity in CFW mice (Pieroni & Levine, 1966), hyperacute EAE with guinea-pig spinal cord in Lewis rats (Levine *et al.*, 1966), and induction of IgE, active anaphylaxis and mouse protective activity (Munoz & Bergman, 1977). There are numerous other systems where pertussis vaccine has been used as an adjuvant but where the effect of heating at 80° was not reported. It seems likely that in some systems both of the adjuvants affect the immune response in different ways and to different extents. For example, with the haemagglutinin response to sheep erythrocytes in mice, heating the pertussis vaccine used as adjuvant for 40 min at 100° reduced significantly but did not abolish its adjuvant activity (Murgo & Athanassiades, 1975).

The present studies suggest that *B. pertussis* grown in a medium containing a high level of magnesium does not contain significant amounts of the heat-labile adjuvant. The C-mode vaccine was greatly inferior to X-mode as an adjuvant to accompanying antigens. Exact quantitative comparisons of the relative adjuvant activity of the C-mode and X-mode vaccines are difficult because of the inherent variability of the animal responses and the non-linearity and non-parallelism of the dose–response curves. Nevertheless, for induction of hyperacute EAE in rats, C-mode vaccine gave no significant adjuvant effect over spinal cord alone and had a relative potency of between one-tenth and one-hundredth of X-mode vaccine. The pattern was more complex with reaginic antibodies to ovalbumin in mice and depended on the time of bleeding after the immunizing injection. With 21 day sera, where reaginic antibodies to ovalbumin appeared to be a mixture of IgE and IgG1, C-mode vaccine seemed to have had no adjuvant effect at all compared with antigen alone. In contrast, there was a high percentage of reaginic antibody responders among mice given ovalbumin plus X-mode vaccine. With 7 day sera, however, where the response was purely IgE, both X and C-mode vaccines acted as adjuvants although the latter was less potent. Preliminary experiments (I. Livey and A. C. Wardlaw, unpublished results) suggested that there was little if any loss of lipopolysaccharide from pertussis cells during antigenic modulation. Hence it seems likely that the adjuvant action of C-mode vaccine for reaginic antibodies in 7 day sera may be due primarily to the lipopolysaccharide component. LPS from other bacteria are known to function as IgE adjuvants (Danneman & Michael, 1976) although they are not as effective as the heat labile adjuvant of *B. pertussis* (Clausen *et al.*, 1969). It is not clear, however, why with the C-mode vaccine there should be a higher proportion of IgE responders at 7 days than at 21 days. It is possible that there is a prompt switching off or blocking of IgE antibody production by other immunoglobulins. The complex interplay of doses and timing of antigen and adjuvant which are required in order to maximize the reaginic antibody response has been emphasized (Lehrer, Vaughan & Tan, 1976), and a population of suppressor cells may be affected by *B. pertussis* (Tada, Okumura, Ochiai & Iwasa, 1972). It is possible that C-mode vaccine has only a temporary effect on these cells.

These observations add yet another activity, adjuvanticity, to the list of properties which pertussis cells lose when cultured in a medium high in content of magnesium. Levine & Pieroni (1966) proposed that *B. pertussis* contains a single, unique proteinaceous component responsible for a variety of effects in animals (particularly mice and rats): sensitization to histamine and other vasoactive substances, induction of lymphocytosis, protection against intracerebral challenge with live pertussis and adjuvant activity. Munoz (1976) suggested 'pertussigen' as an appropriate name for the component responsible for their activities. Most of the recent purification studies have tended to support the idea that a single pertussis component is endowed with a variety of biological effects (Sato, Arai & Suzuki, 1974; Lehrer, Tan & Vaughan, 1974;

Lehrer, Vaughan & Tan, 1975; Morse & Morse, 1976; Munoz & Bergman, 1977; Munoz, Bergman, Cole & Ayers, 1978; Yajima, Hosoda, Kanbayashi, Nakamura, Nogimori, Mizushima, Nakase & Ui, 1978a; Yajima, Hosoda, Kanbayashi, Nakamura, Takahashi, & Ui, 1978b). On the other hand, none of the reports appears to have received independent confirmation, and none has dealt with an acceptably homogenous material to allow full corroboration of this view. It would clearly be desirable to have a collaborative investigation of the various purified materials for the several biological activities ascribed to pertussis to see whether all the above activities are localized in a single component. The availability of such material would also be of value in adjuvant studies since it would permit the investigation and utilization of the unique heat-labile adjuvant component of pertussis vaccine uncomplicated by the presence of lipopolysaccharide.

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