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Immunogenicity of specific *Bordetella pertussis* surface antigens in diphtheria–tetanus–pertussis (DTP) vaccines

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SUMMARY

The predominant causative organism of whooping cough in Australia is of a serotype which has normally been associated overseas with unvaccinated communities. Australian DTP vaccines pass the statutory mouse test for *Bordetella pertussis* potency but this test is now believed to be relatively insensitive to certain factors, especially the major type-specific agglutinogens, which are presumably also important in the human host–parasite relationship. Because endemic *B. bronchiseptica* infections make some laboratory animals unsatisfactory for testing *B. pertussis* agglutinin responses, we have developed a test in which young farm sheep were immunized with vaccines. Type-specific agglutinins in their sera were assayed after absorption of non-specific agglutinins by suspensions of selected bordetella strains. Three well-reputed European DTP vaccines and two recent batches of Australian DTP vaccine were tested and compared thus. All evoked significant agglutinin responses to the main agglutinogens.

INTRODUCTION

Three major *Bordetella pertussis* serotypes (namely types 1,2; 1,2,3; 1,3) are responsible for most cases of human whooping cough and, in the past, marked changes in the serotype balance of *B. pertussis* field strains have been associated with deficiencies in the serotype makeup of the pertussis vaccines then in use (Preston, 1966; Preston & Carter, 1986). The serotype makeup of *B. pertussis* field strains in Australia was, for many years, similar to that in comparable communities overseas (Blaskett, Gulasekharam & Fulton, 1971; Preston & Carter, 1986) but, over the past 10 years, there has been a steady drift towards the predominance of type-1,2 infecting strains (Blaskett, 1979). This was puzzling because, if this swing were vaccine induced, it should have resulted from deficiencies in the vaccines used in the early 1970s but, apart from a slight *increase* in antigen 2 in 1969 (Blaskett *et al.* 1971; fig. 1), we could find no evidence of significant changes having been made in our *B. pertussis* vaccine production or testing procedures during the 8 years prior to, or during the early stages of, this latest antigenic swing. Furthermore, routine serotyping of our vaccine-production strains of *B. pertussis* and of the freshly prepared vaccine harvests, using standard slide-agglutination tests (Preston, 1970*a*), indicated that the vaccines have had a proper balance of the major agglutinogens 1, 2 and 3 at the time of manufacture.

Demonstration of continued agglutinability of DTP vaccines during normal storage is not a statutory requirement and has, indeed, proven difficult in our experience (unpublished), particularly with vaccines near the ends of their stated shelf lives. This has been due to auto-agglutination, incomplete agglutination and 'prozone' formation in conventional slide or 'tube' agglutination tests conducted by the procedures of Preston (1970*a*), Preston & TePunga (1959) or Manclark & Meade (1980). The vaccines had, however, all passed the statutory mouse intracerebral challenge test for immunogenic potency (British Pharmacopoeia, 1980).

Unfortunately, although this test was apparently validated in the MRC pertussis vaccine trials of the 1950s (Whooping Cough Immunization Committee *et al.* 1956), it now appears that it is particularly sensitive to one of the several toxins produced by *B. pertussis* (Robinson & Irons, 1983) and not so sensitive to those surface antigens, such as the filamentous haemagglutinin (FHA) and the major agglutinogens, which we now believe are important as 'adhesins' in experimental and, presumably, natural *B. pertussis* infections (Preston & Stanbridge, 1976; Ashworth *et al.* 1982; Robinson, Irons & Ashworth, 1985).

It is also possible that too much reliance had been placed on direct agglutination by typing sera for controlling serotype makeup of vaccines. In some situations, such as with some *Salmonella typhi* strains, the higher the content of Vi antigen (which, like the major *B pertussis* agglutinogens, is also a K or capsular antigen), the *lower* the titre of agglutination by anti-Vi sera (Kauffmann, 1954). Whether a comparable phenomenon occurs with *B. pertussis* is not known but it is known that surface antigens can be masked *in vitro* in some *B. pertussis* strains (Preston & TePunga, 1959; Holt, 1968) while, in some other strains, antigens detectable by direct agglutination may fail to evoke significant immune responses *in vivo* (Bronne-Shanbury, 1976). Moreover, surface antigens have been shown to elute readily from *B. pertussis* and may block agglutination of whole cells (Maitland & Guerault, 1958; deBock & van Dam, 1960). We therefore considered it important to demonstrate satisfactory agglutinin-inducing capacity by the *B. pertussis* component of CSL triple antigens, in addition to routine testing of fresh *B. pertussis* vaccine harvests for type-specific agglutinability.

There is unfortunately no direct assay procedure for antibodies to individual *B. pertussis* agglutinogens. All smooth *B. pertussis* strains contain a mixture of surface agglutinogens, some found in all *B. pertussis*, some peculiar to particular strains of it and some shared with other *Bordetella* species or even, to some extent, with other genera. The assay method generally adopted has been to immunize animals in a standard fashion with the test vaccines and to subject the resulting immune sera to a somewhat cumbersome series of cross-absorptions, in order to remove unwanted antibodies and leave just those desired (Eldering, Hornbeck & Baker, 1957; Preston, 1970*b*; Bronne-Shanbury, 1976). These would then be assayed by direct bacterial agglutination, using appropriate *B. pertussis* serotypes as test 'antigens'.

Laboratory rodents have usually been used for raising antisera for these purposes but mice have been reported to give unacceptably high non-specific losses of antibody in the absorption steps (Agarwal & Preston, 1976) whilst many rabbits and guinea-pigs, apart from being expensive to keep under proper conditions, possess significant pre-immunization titres to *Bordetella bronchiseptica*,

an ubiquitous animal pathogen (Goodnow, 1980) which shares minor agglutinogens with *B. pertussis* (Eldering, Hornbeck & Baker, 1957). Injection of DTP vaccine into such animals would presumably induce anamnestic responses to these shared bordetella antigens, distorting the immune response to the major immunogens. We turned to farm sheep both for preparing typing sera and for assaying specific agglutinin-producing capacity of vaccines because they seemed not to have the disadvantages cited for laboratory rodents and to have the additional advantages of sturdiness and ability to provide large blood samples.

Since there were no recognized reference preparations for use in the agglutinin-production test, we compared Australian DTP vaccines with DTP from three well-reputed overseas manufacturers.

MATERIALS AND METHODS

Receipt, culturing, serotyping and preservation of *B. pertussis* field isolates were as previously described (Blaskett, Gulasekharam & Fulton, 1971) except for provision of cultures by several additional laboratories in Victoria and Queensland and the use of lyophilized typing sera from Professor Zakharova, WHO Pertussis Laboratory, Gamaleya Institute, Moscow, USSR. The latter sera were reconstituted as recommended on the ampoule labels. Agglutinogen 7 was determined in *B. pertussis* isolates by means of hyperimmune rabbit anti-*B. parapertussis* 5952 serum, prepared by Mr L. Fulton, CSL.

Agglutinin titres of sheep sera were determined by a micro-agglutination tray test, slightly modified from that of Manclark & Meade (1980) by using formalinized test 'antigens' (cf. Preston & TePunga, 1959), by scoring agglutination grades more finely around the endpoint (generally to 0·25 grade intervals, based largely on size and appearance of any pellets), and by interpolation of the endpoint where there was no well showing exactly the desired '+' (= 1·0) reaction (cf. Holt, 1968). The '+' reaction was assigned to any well with a continuous sheet of agglutinated cells plus a central pellet roughly half the diameter of that in the corresponding negative ('saline control') well. High titre and low titre control sera were included with each assay, as a check on the performances of the bacterial suspensions used as 'test antigens'.

Agglutinability of stored DTP vaccines was quantitated by titration against WHO typing sera or anti-5952 (parapertussis) serum, using a modification of the sheep serum agglutinin-assay procedure, by which the overnight standing period at 37 °C was extended by 2 days at 4 °C, to permit 'false positives' to settle into negative buttons whilst the stronger 'true positives' remained essentially unchanged. Test antigens for the agglutination tests were prepared from *B. pertussis* strains Gl.353 (type 1), 360E (type 1,2), H36 (type 1,3) and BR2 (type 1,2,3), *B. parapertussis* strain NCTC 5952 and *B. bronchiseptica* strain BR179.

Cultures of the *B. pertussis* and *B. parapertussis* strains were kindly provided by Dr N. Preston, Manchester University, UK; strain BR179 was isolated at CSL from a sick guinea-pig; strain BR2 was a CSL vaccine production strain. Cultures used in the sheep experiments were grown on Oxoid charcoal agar with 10% defibrinated sheep blood. DTP vaccine samples were obtained through normal commercial channels and were stored at 4 °C.

For the agglutinin production studies, young Romney Marsh wethers from a

flock showing no serological evidence of *B. bronchiseptica* infection were assigned at random to groups of five. Each sheep was given two 0·5 ml doses of DTP intramuscularly at an interval of 4 weeks and, 2 weeks later, 100 ml blood samples were taken from the jugular vein. Sera were stored at −20 °C and were subsequently agglutinin-assayed individually or as pools of five sera. These pools were absorbed successively three times with *B. bronchiseptica* (absorption Stage I) to remove antibody to antigen 7, then assayed against BR179 (to demonstrate removal of anti-7) and against Gl.353 (to determine anti-1 agglutinins). Following this, they were absorbed three times with Gl.353 (absorption Stage II), to remove anti-1, leaving only anti-2 and anti-3. Residual agglutinins were titrated against *B. pertussis* Gl.353, 360E and H36 suspensions. The quantities of absorbing suspensions needed for each serum absorption step were calculated from the agglutinin titres, according to Holt (1968). On average, a total of 800×10^9 *B. bronchiseptica* and 6000×10^9 *B. pertussis* Gl.353 were used per ml of serum.

Bacteria for the absorptions were grown with shaking at 36 °C in liquid medium (Cohen & Wheeler, 1946), the cultures killed with neutral formalin (Cruickshank *et al.* 1975), at 0·4% final formaldehyde concentration, supernantants removed by centrifuging and sedimented cells resuspended to $250–1000 \times 19^9$ cells/ml (determined by opacity in a colorimeter) in isotonic saline containing 0·2% neutral formaldehyde.

RESULTS

Serotype drift in B. pertussis *field isolates*

Table 1 extends our previously published results from serotyping of Australian *B. pertussis* field isolates (Blaskett, Gulasekharam & Fulton, 1971; Blaskett, 1979), and includes 310 more isolates examined during 1978–85. Of these, 224 were typed in our laboratory, 44 (isolated 1981–2) by Dr N. Preston, Manchester for Dr M. Gapes, Royal Alexandra Hospital for Children, Camperdown, NSW (Gapes, 1984) and 42 (isolated 1984–5) by Dr Gapes herself (personal communication, 1985). Over the last decade, serotype 1,2 has achieved and maintained a clear predominance amongst the field isolates. The once dominant 1,3 serotype is now of minor though significant occurrence and serotype 1,2,3 has been all but eliminated. The three laboratories' results have agreed closely (1977–80 *vs.* 1981–5). The relative proportions of type 1,2 are now very similar for each state although we did observe (unpublished) differences in the years when resurgence of type 1,2 commenced in each state.

Modified agglutination tests on stored DTP vaccines

Results of titrations conducted with whole and centrifuged DTP preparations are shown in Table 2.

Type-specific antigens 1, 2 and 3 and the genus antigen 7 were detected in two stored CSL vaccines studied but agglutination was less intense (shown by lower antibody titres) with these than it was with the formalinized suspension of freshly grown *B. pertussis* BR2 (type 1,2,3,7), which was a vaccine production strain included in the test for comparison purposes. Generally, removal of supernatants from the stored vaccines resulted in their agglutination to slightly higher antibody titres. Addition of the supernatants to freshly grown *B. pertussis* BR2 cells, lowered the titres in all cases.

Table 1. B. pertussis *serotype drift with time*

Years	No. of isolates	% of each serotype		
		1,2	1,2,3	1,3
1950–59	41	73	20	7
1960–71	258	3	24	73
1971–77	212	43	22	39
1977–80	316	84	3	13
1981–85*	86*	87*	1*	12*
Total	913			

* The 1981–85 data, portion only of which has been published (Gapes, 1984), was kindly provided by Dr M. Gapes, Royal Alexandra Hospital for Children, Camperdown, NSW. Half these strains were typed at Manchester University and half at Royal Alexandra Hospital, Sydney.

Table 2. *Agglutinabilities of stored vaccines and effects of supernatant (S/N) removal*

Suspension	Treatment	Specific agglutinin titres*			
		anti-1	anti-2	anti-3	anti-7
DTP TA181	None	155	90	145	9
DTP TA181	S/N removed	380	120	250	27
Pertussis vaccine Pool 399	None	140	100	140	15
Pertussis vaccine Pool 399	S/N removed	250	135	150	15
Freshly grown *B. pertussis* BR2	None	440	1200	750	170
Freshly grown *B. pertussis* BR2	TA181 S/N added	250	150	620	85
Freshly grown *B. pertussis* BR2	Pool 399 S/N added	250	210	475	75

* The anti-1, anti-2 and anti-3 sera, made up as recommended by the suppliers, represented 1/25, 1/25 and 1/50 dilutions, respectively, of cross-absorbed, hyperimmune donkey sera (Zakharova, personal communication, 1978). The titres presented above have been calculated on an 'original whole serum' basis.

Comparison of DTP vaccines by means of agglutinin-production studies in sheep

Two weeks after the second vaccine doses, the pre-absorption ('Stage O') micro-agglutination titres averaged 2000 (range low hundreds to middle thousands, in individual sheep) against suspensions of three major *B. pertussis* serotypes but they averaged only 130 (range 50–700 in individual sheep) against *B. bronchiseptica* (refer Table 3). The latter titres are considered due to antibody to the bordetella genus-antigen 7.

Treatment of the sera with suspensions of *B. bronchiseptica* BR179 ('absorption stage I') removed anti-7 agglutinins and reduced the average agglutinin titres for *B. pertussis* Gl.353 (which possesses surface antigens 1 and 7) from 1600 to 1000; these post-absorption agglutinin titres were attributed to antibodies to the species agglutinogen 1. The next absorption steps ('Stage II'), in which 'Stage I' sera

Table 3. B. pertussis *agglutinins* in sera of sheep‡ immunized with DTP vaccines: effects of agglutinin absorptions by* B. bronchiseptica *and* B. pertussis *serotype 1,7*

Vaccine details															
Code...	A			B			C			D			E		
Manufacturer...	Commonwealth Serum Labs, Melbourne, Australia						Wellcome Foundation, London, UK			Institute Merieux, Lyons, France			Swiss Serum & Vaccine Institute, Berne		
Lot no....	190.5			191.5			A58049			Y0086			24.348		
Expiry...	11/85			1/86			4/86			2/87			3/86		
Agglutinin-absorption stages†	0	I	II	0	I	II	0	I	II	0	I	II	0	I	II
Test antigens															
BR179 (7, etc.)	**120***	<10	<10	**110**	<10	<10	**180**	<10	<10	**130**	<10	<10	**100**	<10	<10
Gl.353 (1,7)	2500	**1200***	<20	1200	**920**	<20	1400	**950**	<20	1500	**1200**	<20	1500	**810**	<20
360E (1,2,7)	2000	n.t.	**970***	910	n.t.	**520**	2200	n.t.	**440**	1800	n.t.	**1000**	1300	n.t.	**240**
H36 (1,3,7)	3700	n.t.	**760***	1800	n.t.	**350**	4100	n.t.	**1400**	3600	n.t.	**450**	2600	n.t.	**160**

* Titres cited are means of from 2–5 assays, covering two series of agglutinin absorptions. The anti-7, anti-1, anti-2 and anti-3 titres are shown in bold type.

† Agglutinin-absorption stages: 0, unabsorbed sera from the immunized sheep; I, stage 0 sera absorbed with *B. bronchiseptica* BR179 (to remove anti-7); II, stage I sera absorbed additionally with *B. pertussis* Gl.353 (to remove anti-1).

‡ Sera from these sheep, prior to immunization, gave titres <10 against BR179 and Gl.353 (360E and H36 n.t.).

n.t., not tested.

were treated with suspensions of *B. pertussis* strain Gl.353, removed anti-1 and left only anti-2 and anti-3 of the major *B. pertussis* agglutinins (Table 3). Treatments I and II combined to reduce the average agglutinin titres against *B. pertussis* strains 360E (antigens 1, 2 and 7) and H36 (antigens 1, 3 and 7) from 1600 and 3000 respectively, to about 600 in each case.

Post-absorption titres representing antibody to the three major *B. pertussis* agglutinogens are shown in bold type. All vaccines have evoked significant levels of agglutinins to antigens 1, 2 and 3, as well as to the minor antigen 7.

DISCUSSION

Our serotyping results have shown that, since 1950, *B. pertussis* field strains in Australia have undergone two significant antigenic swings. The first, in 1959–60, paralleled the worldwide swing to predominance of type 1,3 field strains (Blaskett, Gulasekharam & Fulton, 1971); the second, a swing back to type 1,2 predominance, commenced in 1972 and appeared to be peculiar to Australia (Blaskett, 1989). Type 1,2 *B. pertussis* is still the predominant cause of whooping cough in Sydney, Australia (Gapes, 1984) and is reportedly now common in New Zealand though type 1,3 remains the most frequent isolate there (Taylor, 1984). Overseas, serotype 1,2 has seemed to predominate only in unvaccinated or inadequately vaccinated communities (Preston, 1976). In Finland, resurgence of serotype 1,2 was associated with the use of vaccines defective in eliciting agglutinins to antigen 2 (Kuronen & Huovila, 1979) and, possibly because many parents rejected vaccination of their children, this serotype recently regained predominance in UK (Preston & Carter, 1986). However, the serotype drift commenced in Australia before the UK vaccine reactogenicity controversy caused the Australian DTP immunization schedule to be reduced from four doses to three (Communicable Diseases Committee, 1978) and before it affected vaccine acceptance by parents. A fairly high immunization rate seems to have been maintained, nonetheless; of 200 children attending a Melbourne public hospital recently, at least 75% were fully immunized (Blaskett, Marley & Roberton, 1986).

On the basis of slide agglutination tests on stored DTP vaccines, Gapes (1984) suggested that the Australian vaccines must have been antigenically defective. We now regard such tests as unsatisfactory for stored DTP or pertussis vaccines. Indeed, we have demonstrated the presence of soluble blocking antigen in the supernatants of two stored Australian DTP vaccines and, by procedures other than slide agglutination, we have also demonstrated that the *B. pertussis* cells in these stored vaccines were in fact agglutinable by *B. pertussis* typing sera. We have moreover demonstrated that two recent batches of Australian and three of European DTP vaccines, when injected into sheep, all evoked the production of significant levels of antibody to the three major *B. pertussis* type-specific antigens 1, 2 and 3 and to the genus antigen 7. In each case, the Australian vaccines evoked agglutinin titres within the ranges produced by the European vaccines.

On the basis of the comparative agglutinin-production tests in sheep, one would expect the Australian vaccines to be as effective as their European counterparts in preventing whooping cough in children. Whether the Australian DTP vaccines

produced from essentially the same *B. pertussis* strains as those tested in this study, but several years *prior* to the swing back to type 1,2 *B. pertussis* predominance, would have performed as well in agglutinin-production tests is a question that cannot be answered but we do know that the majority of *B. pertussis* isolates we have typed, from that or other periods, have come from unimmunized or only partially immunized children (Blaskett & Fulton, 1976; unpublished results), while documented and anecdotal data in a recent Melbourne study (Blaskett, Marley & Roberton, 1986) suggested that fully immunized children were at least 80% protected against whooping cough.

Why *B. pertussis*-type 1,2 now predominates in Australia is still unclear. Overseas, it predominates only in countries in which either vaccination acceptance (Preston & Carter, 1986), or the vaccine itself (Kuronen & Huovila, 1979), has been less than satisfactory. Unrecognized changes in vaccine composition may have occurred in Australia over the years, resulting in changed immunogenic potencies but, regardless, DTP batches have been subject to the statutory mouse intracerebral challenge test for many years. It is, of course, possible that even though the two recent batches of CSL vaccines showed good immunogenicity for antigens 1, 2 and 3, this has not always been the case. Any further serotype changes observed following continued use of highly agglutinogenic vaccines will therefore be of considerable significance regarding the possible causation of the current type-1,2 predominance in Australia.

The sheep system employed in this study has demonstrated its usefulness for comparing the *B. pertussis* immunogenicities of commercial DTP vaccines and could prove a useful adjunct in the development of new and improved pertussis vaccines.

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