

Pertussis toxin is required for pertussis vaccine encephalopathy

(post-immunization encephalopathy)

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ABSTRACT A mouse model for encephalopathy induced by pertussis immunization has been described; it has features that closely resemble some of the severe reactions, including seizures and a shock-like state leading to death, occasionally seen after administration of *Bordetella pertussis* (whooping cough) vaccine. Susceptibility to encephalopathy maps to genes of the major histocompatibility complex and correlates as well with the genetic regulation of the level of antibody response to bovine serum albumin. In this study we have investigated which bacterial determinant is responsible for the encephalopathy. Two lines of evidence implicate pertussis toxin as the active bacterial component. Single-site mutants of *B. pertussis* with single affected virulence factors were tested. A mutant that produces a defective pertussis toxin had greatly diminished capacity to induce encephalopathy, whereas a hemolysin- and adenylate-cyclase-deficient avirulent mutant had the same activity in the mouse model as a virulent strain. Purified pertussis toxin plus bovine serum albumin was tested and found to induce the lethal encephalopathy, demonstrating that the toxin was the critical constituent of *B. pertussis* responsible for encephalopathy.

The pertussis vaccine component of diphtheria-pertussis-tetanus (DPT) vaccine is associated with convulsions in one of 1750 doses (1), while severe and permanent neurologic damage has been calculated to occur with one of every 310,000 doses (2). Although the benefits of the current pertussis vaccination program outweigh the risks by a considerable margin (3), development of a safer, efficacious vaccine is an important goal. In the past this effort has been hampered by lack of definitive information about which bacterial antigens were essential for vaccine efficacy and which bacterial products were responsible for the reactogenicity.

A lethal-shock-like syndrome preceded by myoclonic seizures is induced in mice with an appropriate major histocompatibility (*H-2*) gene after immunization with heat-killed *Bordetella pertussis* vaccine and bovine serum albumin (BSA) (4, 5). This model, which resembles post-pertussis immunization encephalopathy, involves daily injections alternating BSA with vaccine for 4 days, then a BSA challenge 5 days later. Death usually results within minutes of the final challenge. Previous studies have shown that the linkage of susceptibility to pertussis vaccine encephalopathy is associated with high antibody responsiveness to BSA. The strain distribution of high immune responsiveness to BSA was identical to that reported by Riley *et al.* (6) and conforms to our results with susceptibility to *B. pertussis* encephalopathy. Thus, *H-2^d* mice were high responders to BSA and highly susceptible to encephalopathy (57/65 died), while *H-2^b* mice

were low responders to BSA and totally resistant to *B. pertussis* encephalopathy induced with whole cell pertussis vaccine (0/44 died) (4, 5). In this study we have attempted to define what component in the pertussis vaccine is responsible for inducing encephalopathy in genetically susceptible mice.

MATERIALS AND METHODS

Measurement of Serum Antibodies. Microtiter plates (Costar, Cambridge, MA) were coated with 1 ml containing 2×10^9 *B. pertussis* organisms for 8 hr or with 50 μ l of BSA (100 μ g/ml) for 1 hr. After unbound antigen had been removed, 50 μ l of diluted serum was added to each well and incubated at room temperature for 1 hr. Bound serum antibodies were identified with β -galactosidase-conjugated sheep anti-mouse (Fab)₂ (Bethesda Research Laboratories). After addition of the conjugate, *p*-nitrophenyl β -D-galactopyranoside was added, and the absorbance at 405 nm was read 2 hr later on a spectrophotometer. All dilutions were tested in triplicate and data are expressed as mean (\pm SD) absorbance units for each serum dilution. Normal mouse serum served as a background control.

Mice. All mice were obtained from the breeding colony of H. O. McDevitt (Stanford Univ.), except SJL/J, which were purchased from Jackson Laboratory (Bar Harbor, ME), and BALB/c, obtained from the Radiobiology Department at Stanford.

Tn5 Insertion Mutants. Tn5 insertion mutants were derived from virulent strain BP338 (7). The organisms were grown on Bordet-Gengou medium as previously described (7) for 2 days at 37°C. The cells were harvested, washed, and suspended to an optical density of 2.0 at 580 nm in phosphate-buffered saline, pH 6.5. This corresponds to 5–10 $\times 10^9$ colony-forming units/ml. To produce a heat-killed vaccine, the bacteria were heated to 56°C for 30 min and 0.02% thimerosal was added. Vaccines were stored refrigerated for up to a week or frozen.

Pertussis Toxin. Pertussis toxin was concentrated on hydroxylapatite and then purified by chromatography on a fetuin affinity system (5, 8).

Immunizations. For induction of encephalopathy mice were given killed *B. pertussis* organisms suspended in 0.2 ml of phosphate-buffered saline on days 0 and 2 by injection into the tail vein. On days -1, +1, and +6 each mouse received 1 mg of BSA in 0.2 ml of phosphate-buffered saline intraperitoneally (i.p.). Mice were observed for the next 2 hr for seizures, shock, and death. For induction of BSA sensitivity, mice were immunized ip with 100 μ g of BSA in 0.2 ml of phosphate-buffered saline emulsified in complete Freund's adjuvant. Mice were reimmunized i.p. 21 days later with 100 μ g of BSA in 0.1 ml of phosphate-buffered saline.

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Abbreviation: BSA, bovine serum albumin.

RESULTS AND DISCUSSION

Mice with $H-2^{d,s,k}$ haplotypes, either high or intermediate in susceptibility to encephalopathy, were good responders to BSA, while C57BL/10 (B10) mice, resistant to encephalopathy, were poor responders to BSA (Fig. 1). The correlation between genetically controlled responsiveness to BSA and susceptibility to *B. pertussis* encephalopathy is striking. In Table 1 the antibody response to *B. pertussis* in two susceptible strains, BALB/c and DBA/2 (both $H-2^d$), is compared to that in two resistant strains, B10 and BALB/b (both $H-2^b$). Responses to *B. pertussis* were comparably strong for all strains, including those highly susceptible to encephalopathy and those that were resistant. Thus, genetic susceptibility to vaccine encephalopathy correlated with immune responsiveness to BSA and not to *B. pertussis*.

We next analyzed which components of *B. pertussis* might contribute to development of encephalopathy in this model. Transposon Tn5 was used to derive stable pertussis toxin mutants. Two of these mutant strains of *B. pertussis* were compared for their encephalopathic potential. BP338 is a virulent-phase *B. pertussis* strain, while BP357, derived from BP338, produces a defective pertussis toxin that lacks one of the toxin subunits (7). Strain BP348 is a hemolysin⁻ adenylate cyclase⁻ mutant but produces normal amounts of pertussis toxin (7). The location of the Tn5 insertion mutations has been determined by using a ³²P-labeled DNA probe specific for Tn5 to hybridize to chromosomal DNA restriction fragments separated by electrophoresis (7). On these Southern hybridizations the *Eco*RI restriction fragment containing the site of the Tn5 insertion is distinct in the pertussis toxin mutant BP357 and differs from the insertion site in BP348 (7).

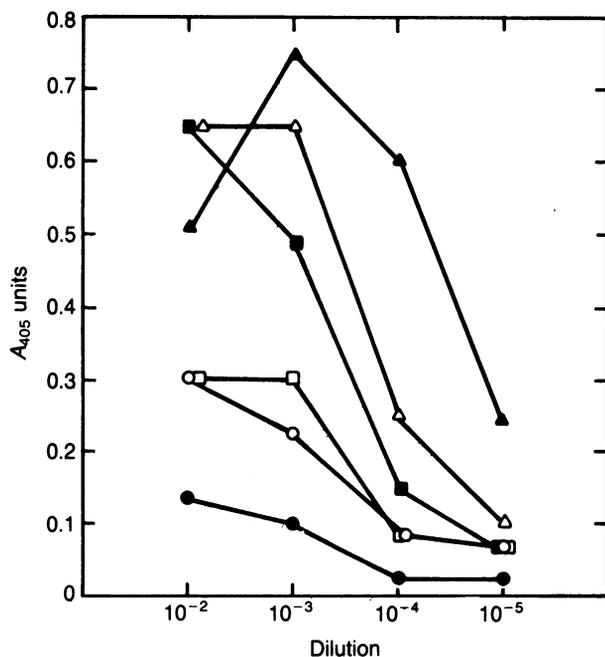


FIG. 1. Anti-BSA antibody level at various serum dilutions in several inbred mouse strains. ●, B10 ($H-2^b$); ○, B10.D2 ($H-2^d$); □, B10.S ($H-2^s$); ■, B10.BR ($H-2^k$); △, BALB/c ($H-2^d$); and ▲, SJL/J ($H-2^s$). Five mice per strain were immunized i.p. with 100 μ g of BSA in 0.2 ml of phosphate-buffered saline emulsified with complete Freund's adjuvant. Mice were boosted on day 21 with 100 μ g of BSA in phosphate-buffered saline and bled for antisera on day 29. The antibody response to BSA was determined by an ELISA (5). Absorbance at 405 nm was determined with a spectrophotometer. Results are expressed as mean absorbance. Normal mouse serum served as a background control. At 10⁻² and 10⁻³ dilutions the response of B10 differs from that of all other strains ($P < 0.01$), except B10.S ($P < 0.06$) (Student's *t* test).

Table 1. Anti-*B. pertussis* antibody responses in mice with different genotypes

Strain	Anti- <i>B. pertussis</i> antibody, A_{405} units		
	1:200	1:400	1:800
BALB/c ($H-2^d$)	1.2 \pm 0.05	0.90 \pm 0.18	0.60 \pm 0.18
DBA/2 ($H-2^d$)	1.2 \pm 0.17	1.04 \pm 0.28	0.70 \pm 0.16
BALB/b ($H-2^b$)	1.1 \pm 0.11	0.68 \pm 0.13	0.41 \pm 0.04
B10 ($H-2^b$)	0.98 \pm 0.21	0.78 \pm 0.22	0.61 \pm 0.22

Results are mean \pm SD, five mice per group. Mice were immunized with heat-killed *B. pertussis* (3×10^{10}) intravenously on days 0 and 2. On days -1 and +1 mice were given 1 mg of BSA i.p. Mice were bled for anti-*B. pertussis* determination on day 6. Serum titers for anti-*B. pertussis* antibody were measured by an ELISA (5).

Fig. 2 illustrates the dose of BP348, BP357, or BP338 required to induce encephalopathy and death in this model. Fifty percent of mice were killed with vaccine made with virulent-phase BP338 when the dose was between 7.5×10^7 and 1×10^8 heat-killed organisms, while for BP348 50% were killed at a dose between 10^8 and 2×10^8 organisms. BP357, the toxin-deficient strain, killed 50% of the mice at a dose 5×10^9 organisms. The pertussis toxin mutant was significantly less potent than wild type (0/10 vs. 8/9 dead, $P < 0.001$ at 5×10^8 organisms, 0/11 vs. 7/11 dead, $P < 0.002$ at 10^8 organisms).

Since BP357 produced far less encephalopathogenicity than BP338, we asked whether pertussis toxin could substitute for BP338 in inducing encephalopathy, using the standard protocol. Table 2 reveals that pertussis toxin can be substituted for *B. pertussis* organisms in the induction of encephalopathy. BSA is still necessary for expression of encephalopathy and death in this model, when pertussis toxin is substituted for *B. pertussis*. Encephalopathy occurred in 14/15 BALB/c mice given pertussis toxin and BSA and in 0/10 given toxin without BSA. Interestingly, pertussis toxin plus BSA caused encephalopathy in 10/15 B10 ($H-2^b$) mice and 4/10 BALB/b ($H-2^b$) mice given the same regimen. Zero of nine B10 died when given pertussis toxin without BSA.

The peculiar relationship between BSA and *B. pertussis* in producing encephalopathy is enigmatic. Why might anti-BSA antibodies be critical in encephalopathy development? We have been unable to produce encephalopathy with anything other than *B. pertussis* and BSA immunization. BSA has been obtained from numerous sources (4, 5). We have substituted *Escherichia coli* endotoxin (0/10 died), myelin basic protein (0/10 died), lysozyme (0/5 died), and the IgM phosphocholine-binding myeloma MOPC 104E (0/10 died) for BSA in the standard protocol in susceptible $H-2^d$ or $H-2^{s/d}$ mice, with no resultant encephalopathy. Likewise, we have substituted the synthetic antigen (T,G)AL [poly(LLys) backbone, poly(DLAla) side chains terminated with poly(LTyr, L-Glu)] which elicited a strong antibody response in $H-2^b$ mice, and could not induce encephalopathy with the standard protocol (0/10 died). In contrast, mice of the $H-2^b$ strains become encephalopathic and die when given both *B. pertussis* vaccine and high-titered anti-BSA antisera (5). We have also considered whether *B. pertussis* that is grown in medium containing bovine protein hydrolysate might cross-react with BSA, a suggestion of E. A. Kabat (personal communication). After immunization with *B. pertussis* alone, antibody response to BSA was no different from normal mouse serum (data not shown). This indicated that immunization with *B. pertussis* alone did not lead to an antibody response to BSA.

Thus, there is still no obvious explanation for why BSA may be critical for *B. pertussis* encephalopathy in mice. BSA may also be important in human pertussis vaccine encephalopathy. As we discussed previously (4, 5), almost all babies exposed to cows' milk have serum antibodies (IgG, IgA, and

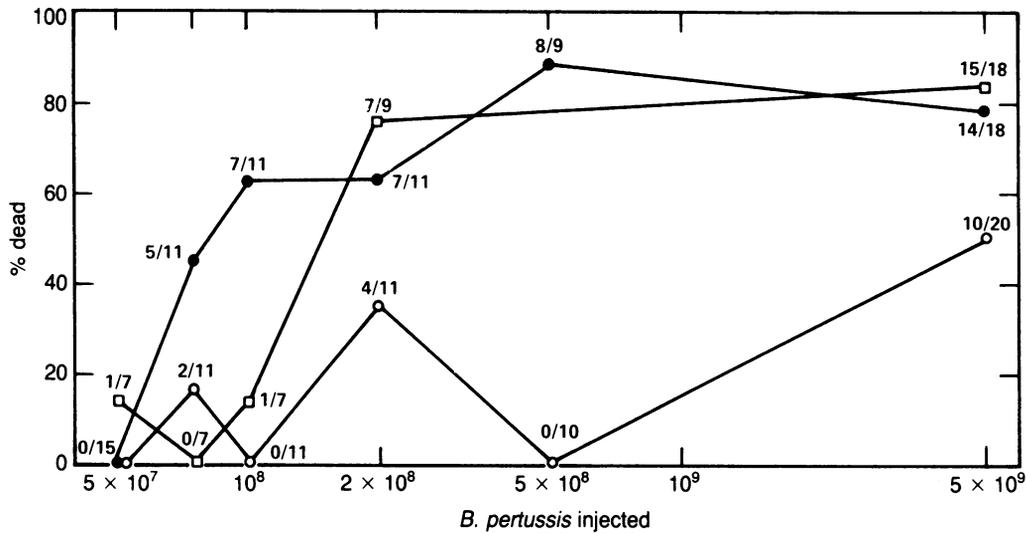


FIG. 2. Comparison of encephalopathic potential of Tn5 mutants of *B. pertussis*. BALB/c mice were given various intravenous doses of *B. pertussis* from different strains on days 0 and 2. On days -1, +1, and +6, 1 mg of BSA was given i.p. Death within 2 hr of injection was recorded on day 6 (4). ●, BP338 (Tox⁺, Adc⁺, Hly⁺); □, BP348 (Tox⁺, Adc⁻, Hly⁻); ○, BP357 (Tox⁻, Adc⁺, Hly⁺). Phenotypes: Tox, toxin; Adc, adenylate cyclase; Hly, hemolysin.

IgM) to BSA (9, 10). Even breast-fed babies have these serum antibodies, which are probably secondary to sensitization to BSA in the mother's milk (10, 11).

We have shown that a particular component of *B. pertussis*, pertussis toxin, when combined with BSA sensitization can induce encephalopathy. This regimen with purified toxin not only is lethal in those strains susceptible to encephalopathy induced with whole cell vaccine but also can kill mice in *H-2^b* strains that are remarkably resistant to encephalopathy induced with whole cell vaccines (0/44 *H-2^b* mice killed with whole cell vaccine). It is rather remarkable that pertussis toxin plus BSA can be lethal in certain strains in which whole cell vaccines have no effect. It is also striking that the toxin is not lethal unless BSA is administered in the regimen. *B. pertussis* mutants induced with transposon Tn5 that have a defective pertussis toxin have much less encephalopathic potential than other wild-type *B. pertussis* organisms. Thus, we hypothesize that pertussis toxin contained in *B. pertussis* vaccine plus antibody to BSA triggers the encephalopathy. Part of the genetic susceptibility to encephalopathy resides in the capacity of the vaccine recipient to mount an anti-BSA response, while another component of susceptibility resides in the amount of pertussis toxin in the vaccine.

Table 2. Pertussis toxin can substitute for *B. pertussis* in encephalopathy model

Injection	No. dead/no. tested	
	With BSA	Without BSA
Pertussis toxin		
1 μg/ml	3/5	ND
100 ng/ml*	14/15	0/10
10 ng/ml	0/5	0/5
1 ng/ml	0/5	0/5
Phosphate-buffered saline	0/5	0/5
<i>B. pertussis</i> only, 3×10^{10}	3/5	0/5

Mice (BALB/c) were given graded doses of 1 ml of pertussis toxin or *B. pertussis* intravenously on days 0 and 2. On days -1, +1, and +6 some mice were given 1 mg of BSA i.p. The number of dead mice was recorded within 2 hr of the day +6 injection. ND, not done.

*At a pertussis toxin dose of 100 ng/ml on days 0 and 2, with administration of 1 mg of BSA on days -1, +1, +6, 10/15 B10 mice and 4/10 BALB/b mice were dead within 2 hr of day +6 injection.

The mechanism whereby pertussis toxin and anti-BSA antibody combine to produce the damage is still uncertain. Why pertussis toxin plus BSA is encephalopathic even in *H-2^b* mice is also unknown at present. Since *H-2^b* mice make low-level anti-BSA antibody responses, we hypothesize that even they became encephalopathic with sufficient amounts of active pertussis toxin.

Pertussis toxin (8), also referred to as pertussinogen, lymphocytosis-promoting factor, islet cell-activating protein, and histamine-sensitizing factor, is an oligomeric protein toxin with a wide range of physiologic effects, including increased sensitivity to anaphylaxis (12), hyperinsulinemia (13), and increased vascular permeability (14). In addition to encephalopathic signs such as seizures and coma, systemic manifestations prior to death include cyanosis and tachypnea. Postmortem examination of susceptible mice after immunization with *B. pertussis* and BSA reveals widespread vascular congestion and some parenchymal hemorrhage in brain (4). Whether the neurologic signs result from systemic changes such as shock, hypoglycemia, and ischemia (4) is unresolved at present. The molecular basis for the encephalopathic activity of pertussis toxin is not yet known. By ADP-ribosylating the nucleotide-binding protein N_i, pertussis toxin prevents coupling of inhibitory synaptic receptors to adenylate cyclase (15). Pertussis toxin can convert adenosine-induced inhibition of the release of synaptic glutamate in central nervous system nerve terminals to a stimulation of glutamate release (16). Thus, pertussis toxin might induce encephalopathy through modulation of synaptic activity.

The identities of the antigens that are required to confer immunity to *B. pertussis* infection, as well as the roles that specific antigens play in bacterial virulence, can be established only by further examination. Antibody to pertussis toxin was effective for protection against *B. pertussis* infection in mice after either intracerebral or aerosol challenge with live *B. pertussis* (17). Moreover, monoclonal antibody to a subunit of pertussis toxin protected mice against either intracerebral or aerosol challenge with *B. pertussis* (18). In contrast, antibody to filamentous hemagglutinin did not protect against intracerebral challenge with *B. pertussis*. Thus, antibody to certain pertussis toxin epitopes may be sufficient to provide immunity to infection. It is not known at present whether antibody to mutant BP357, which lacks one of the pertussis toxin subunits, can provide effective immu-

nity to *B. pertussis* infection. It is known, however, that the BP357 mutant was severely impaired in its capacity to induce lethal pertussis infection in mice (19). Thus, pertussis toxin has a key role as a virulence factor in *B. pertussis* infection, and antibody to one of its subunits is sufficient to confer immunity. Precise information on the components of the pertussis toxin molecule responsible for antigenicity and protection, for bacterial virulence, and for encephalogenicity will be available with the cloning and sequencing of the pertussis toxin gene.

If the *B. pertussis* mutants deficient in pertussis toxin can immunize against natural *B. pertussis* infection, then this could lead to a *B. pertussis* vaccine safer than the one currently in use. Moreover, the model for *B. pertussis* immunization encephalopathy may serve to screen for the development of safer, genetically engineered *B. pertussis* vaccines. Vaccines containing sufficient pertussis toxin might have encephalopathic potential for genetically susceptible vaccine recipients.

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