Vaccines

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A. Introduction

*Corynebacterium diphtheriae*, *Clostridium tetani*, and *Bordetella pertussis* produce potent toxins that are entirely or in part responsible for the severe diseases caused by these microorganisms. The clinical manifestations of diphtheria and tetanus can be thoroughly reproduced by the systemic administration of the toxins. Both diseases can be prevented by administration of toxin-neutralizing antibodies or by immunization with partially purified and detoxified toxins (toxoids). Diphtheria and tetanus vaccines are commonly considered as composed only of the detoxified form of the toxins. However, it should be emphasized that diphtheria and tetanus vaccines usually contain approximately only 75% and 50% of detoxified toxins, respectively. The remaining impurities are antigens produced by the bacteria that may play a role in controlling colonization.

Pertussis is a more complex disease since the pertussis toxin is the main, but not the only, molecule contributing to toxicity induced by the bacterium. Administration of the pertussis toxin by the systemic route reproduces the systemic symptoms of the disease, but not the local toxicity and cough. Administration of toxin-neutralizing antibodies has been shown to reduce the duration and the severity of the disease. Immunization with detoxified toxin either alone or combined with other purified antigens from *B. pertussis* has been shown to prevent the disease.

In order to be safely administered as vaccines toxins need to be detoxified. Detoxification of bacterial toxins was a milestone in vaccine development. Consequently, in this chapter we propose to trace the past and present of the vaccines that have been developed to defeat these diseases and conclude that a bright future lies ahead.

The starting point of such a story is definitely the discovery by Ramon (1924) in France and Glenny and Hopkins (1923) in the United Kingdom of an effective method to detoxify tetanus and diphtheria toxins based on formaldehyde treatment. The detoxification of these two toxins allowed their use as vaccines and the introduction of mass immunization, which led to the almost complete eradication of both diseases from developed countries. Diphtheria (D) and tetanus (T) vaccines, which are used mostly for infant immunization, combined with whole cell pertussis (P) as DPT, are still prepared using the
method described by Ramon. New generation vaccines, such as the acellular pertussis vaccines, may contain either the toxoid detoxified by the old method of Ramon, or nontoxic mutants produced by the genetically manipulated of the micro-organism. The latter molecules have been reported to be better immunogens than the classical chemically detoxified toxoids, and to provide excellent protection from disease.

Programs to provide diphtheria and tetanus vaccinations have been among the safest and most successful campaigns against infectious diseases. Both diseases have almost disappeared from those countries in which appropriate immunization is carried out (PAPPENHEIMER 1984; BIZZINI 1984).

In developing countries where vaccination is not yet fully practiced both diseases are still responsible for a remarkably high number of deaths (RAY et al. 1983; BIZZINI 1984): it is estimated that between 300000 and one million cases of tetanus occur annually, 80% of them being newborn children (neonatal tetanus).

In the early 1990s a massive epidemic of diphtheria occurred in the former Soviet Union, causing more than 50000 cases and several thousand deaths over 4 years. This outbreak of diphtheria showed in a definitive way that diphtheria vaccination of infants and booster of the adults is still a must for controlling the disease.

B. Diphtheria Toxin

Diphtheria toxin is a 58.35 kDa protein molecule that is released into the supernatant by toxigenic C. diphtheriae strains (PAPPENHEIMER 1977; COLLIER 1982). The toxin is synthesized as a single polypeptide chain of 58.35 kDa that, following mild trypsin treatment and reduction of a disulfide bond, can be divided into two functionally different moieties: fragment A and fragment B of 21.15 and 37.2 kDa, respectively (PAPPENHEIMER 1977; RATTI et al. 1983; GREENFIELD et al. 1983).

The three-dimensional structure of the molecule has shown that the toxin contains three different domains: the C (catalytic) domain, corresponding to fragment A; the T (transmembrane or translocation) domain, composed of nine α-helices (TH1–TH9); and the carboxyterminal R (receptor binding) domain (CHOE et al. 1992; BENNETT et al. 1994).

Fragment A is a NAD⁺-binding enzyme that catalyzes the transfer of the ADP-ribosyl group of the NAD molecule, to a posttranslationally modified histidine residue (diphthamide) of elongation factor 2 (EF2), a GTP-binding protein involved in protein synthesis in eukaryotic cells (BROWN and BODLEY 1979; VAN NESS et al. 1980). The EF2-ADP-ribose complex is inactive, and consequently diphtheria toxin causes inhibition of protein synthesis and cell death. It has been shown that in vitro a single molecule of fragment A is enough to kill one eukaryotic cell (YAMAIZUMI et al. 1978). This observation is correlated with the findings of in vivo studies showing that diphtheria toxin
is one of the most potent bacterial toxins. In fact, the minimal lethal dose is below 0.1 μg/kg body weight (PAPPENHEIMER 1984).

Although the entire lethal activity of diphtheria toxin resides in fragment A, fragment B is required for receptor binding and for translocation of fragment A across the cell membrane. The COOH-terminal domain involved in receptor binding (R) and the NH2-terminal hydrophobic domain (T), which is involved in the interaction with the cell membrane, mediate this process. The toxin receptor is the heparin-binding EGF-like growth factor precursor (NAGLICH et al. 1992; BROWN et al. 1993; IWAMOTO et al. 1994; HOOPER and EIDELS 1995), that is present on most mammalian cells but is not found in murine cells.

The diphtheria toxin gene is carried by a family of closely-related bacteriophages (corynebacteriophages), that are able to integrate into the bacterial chromosome and convert nontoxigenic, nonvirulent *C. diphtheriae* strains into toxigenic, highly virulent species (FREEMAN 1951; UCHIDA et al. 1971).

The chromosome of *C. diphtheriae* contains two primary attachment sites (attB1 and attB2) (RATTI et al. 1983; RAPPUOLI and RATTI 1984). The presence of the sites on the chromosome allows the stable integration of two copies of corynephage DNA into the bacterial chromosome (RAPPUOLI et al. 1983). Because each phage carries one copy of the tox gene, double lysogens produce twice the toxin produced by monolysogens. This property has been very important for obtaining strains hyperproducing CRM 197, a nontoxic form of diphtheria toxin that is used as a carrier for *Haemophilus influenzae* type B (ANDERSON et al. 1987; EGAN et al. 1995; ROTHBROCK et al. 1995) and for meningococcal conjugate vaccines (COSTANTINO et al. 1992). CRM 197 has also been proposed as a new vaccine against diphtheria (RAPPUOLI 1990; PODDA and RAPPUOLI, unpublished data).

### C. Tetanus Toxin

Tetanus toxin is a potent neurotoxin that is synthesized intracellularly by *Clostridium tetani* as a single polypeptide chain of 150.5 kDa. After cell lysis the toxin is released into the medium and cleaved by endogenous proteases generating an NH2-terminal light chain of 52.3 kDa (fragment A) and a COOH-terminal heavy chain of 98.3 kDa (fragment B–C). A disulfide bridge holds together light and heavy chains. The intact, unnicked toxin can be recovered from the washed bacteria by extraction with a neutral, hypertonic solution. Treatment of the toxin with papain yields an NH2-terminal fragment A-B of 99 kDa and a COOH-terminal fragment C of 51.5 kDa (BIZZINI 1978, 1984; FAIRWEATHER et al. 1986).

Tetanus toxin binds to gangliosides and proteins of peripheral nerve terminals through the COOH-terminal of fragment C; it is then internalized and translocated through retrograde transport to the spinal cord neurones, where it produces a spastic paralysis accompanied by convulsions (BIZZINI 1978).
Purified fragments A and B–C are nontoxic, but if they are reassociated, toxicity is restored.

The toxin can be neutralized by monoclonal antibodies binding the A, B, or C fragments, suggesting that in theory any of the three nontoxic fragments could be used as a vaccine (Volk et al. 1984). Fragments A, B, and C have been successfully used to immunize mice against tetanus (Bizzini 1984).

The gene encoding tetanus toxin, contained within a large plasmid of 75 kb, has been cloned and sequenced (Eisel et al. 1986; Fairweather and Lyness 1986; Nieman et al. 1988). The knowledge of the sequence of this gene led to the cloning of genes coding for the related botulinum toxin produced by A–G serotypes. Despite the lack of high homology within the amino acid sequences of these neurotoxins, a conserved “HEXXH” motif was identified in the light chain. This motif is typically present in the active site of metalloproteases. On the basis of this evidence it was demonstrated that the light chain of tetanus toxin is a zinc-metalloprotease able to cleave with remarkable specificity synaptobrevin, an integral membrane protein of the presynaptic vesicle (Schiavo et al. 1992; Montecucco and Schiavo 1994), thus interfering with the release of neurotransmitters.

D. Production, Effectiveness, and Problems of Conventional Diphtheria and Tetanus Vaccines

I. Production and Detoxification of Diphtheria and Tetanus Toxoids

To produce a conventional diphtheria vaccine, the hypertoxinogenic C. diphtheriae strain PW8 (Rappuoli et al. 1983; Park and Williams 1986) is grown in fermentors for 36–48 h until the concentration of the toxin in the supernatant reaches 150–250 Lf/ml (1 flocculation unit, or Lf, corresponds to 2.5 µg diphtheria toxin) (Bizzini 1978). Although several semisynthetic media for the industrial growth of diphtheria have been described so far, many of the manufacturers still grow the PW8 strain in a medium containing enzymatic digests of beef (Lingood 1941). This method of growing bacteria is likely to change in the near future due to the need to remove any substance of bovine origin from the production media of biologicals, following the epidemic of bovine spongiform encephalopathy in the United Kingdom in the 1990s. Once the fermentation is complete, the bacteria are removed by centrifugation or filtration, and formaldehyde is added to the supernatant to a final concentration of 0.75%. The supernatant is then stored for 4–6 weeks at 37°C to allow complete detoxification of diphtheria toxin.

Similarly, to produce the conventional tetanus vaccine the highly toxigenic Harvard strain of C. tetani is grown in a semisynthetic medium (usually modified from Mueller and Miller 1945) in a fermentor for about 1 week until the bacteria lyse and release tetanus toxin into the supernatant. The average yield obtained under these conditions is approximately 60–80
Lf/ml (Bizzini 1984). The culture is then filtered, and the filtrate containing the toxin is detoxified by adding formaldehyde to a final concentration of 0.5%. The pH is adjusted to 7.6 and the supernatant is then stored at 37°C for 4 weeks to allow the complete detoxification of the tetanus toxin.

During the detoxification process formaldehyde reacts with the toxin molecules, peptones, and other proteins present in the medium (Blass 1964; Blass et al. 1967; Bizzini and Raynaud 1974; Sinkovic 1975; Bizzini 1984; Pappenheimer 1984). The formulation proceeds in two steps: the first step involves the reaction, which is very rapid and reversible, mainly with the ε-amino groups of lysine; the second step involves a slower reaction of the unstable product generated in the first step, with another amino acid side chain containing a reactive hydrogen (indole ring of Trp, amide group of Asn and Gln, phenol ring of Tyr, ε-NH₂ group of Lys, imidazole group of His, and guanidine group of Arg) (Fraenkel-Conrat and Olcott 1948; Bizzini and Raynaud 1974; Sinkovic 1975).

The final result of the formaldehyde treatment can be the formation of an internal link within the protein molecule, a cross-link between different protein molecules, which then form dimers or oligomers, or a cross-link between the protein molecule and peptides or amino acids present in the medium (Rappuoli 1990). When the formaldehyde is added to the crude culture supernatant containing the toxin molecules, this results in the coating of the toxoid by peptides deriving from the beef meat used to prepare the medium. These peptides are unnecessary antigenic determinants of bovine origin which might be responsible for some of the side effects associated with diphtheria and tetanus vaccination, especially in the adult population, which has been repeatedly exposed to bovine antigens.

To overcome this problem and to produce better vaccines, the toxins may be purified before the addition of formaldehyde. This process, which is now used by very few vaccine manufacturers, is likely to become of general use in the near future.

II. Production and Detoxification of Purified Diphtheria and Tetanus Toxoids

The conventional method of preparation used for diphtheria and tetanus vaccines entails a large amount of impurities, some of which are covalently linked to the toxoids and therefore impossible to remove. An easy way to avoid this problem is to treat with formaldehyde the purified proteins instead of the crude culture supernatant. In fact, when purified proteins are formaldehyde-treated, only intra- and intermolecular bridging can occur.

Several methods have been described for the purification of diphtheria and tetanus toxins, and most of them are based on a diafiltration of the culture supernatant, precipitation by ammonium sulfate, and if necessary a further purification step on a gel filtration or on an ion-exchange chromatography (Bizzini 1984; Pappenheimer 1984). Using these methods, diphtheria and
tetanus toxins can be obtained at a purity ranging from 85% to 95%. The partially or highly purified toxin preparations can then be detoxified by the conventional formaldehyde treatment.

The purity of the diphtheria toxoid prepared by detoxification of purified toxin is much higher than that of the conventional diphtheria vaccines. Purity ranges from 85% to 95%, whereas the purity in the conventional vaccines does not exceed 60%.

Early studies on the formulation of purified diphtheria toxin showed that the toxoids can revert to toxicity if diluted, or if the excess of formaldehyde is removed (LINGOOD et al. 1963; STAINER 1968; AKAMA et al. 1971; PAPPENHEIMER 1984). At a later stage it was shown that if 0.025 M lysine is used in the reaction, the toxoids obtained were stable and immunogenic. An alternative way to detoxify toxins has been developed by Relyveld and is based on the use of glutaraldehyde (STAINER 1968; RELYVELD 1969; PAPPENHEIMER 1984). Diphtheria and tetanus toxins can be completely and irreversibly inactivated by treatment for a short time with 0.025 M or 0.0025 M glutaraldehyde, respectively. The presence of lysine is still necessary for the irreversible detoxification of diphtheria toxin, but not for tetanus toxin. The vaccines obtained by glutaraldehyde detoxification of purified tetanus and diphtheria toxins (called POLAN for polymerized antigen) (RELYVELD et al. 1983; RELYVELD and BEN EFRAIM 1983; BIZZINI 1984) have been successfully used for the immunization of large groups of infants and children in France without noticeable adverse reactions.

E. Future Prospects

I. Engineered Live-Attenuated Strains

Infection with pathogens can efficiently stimulate both humoral and cellular immunity. In recent years it has been possible to construct fully defined attenuated micro-organisms and to test them as potential live vaccines. An example of this approach is the use of genetically defined attenuated Salmonella strains as oral vaccines. Such strains undergo a limited subclinical infection in mice and provide solid immunity to salmonellosis (CHATFIELD et al. 1992a). These strains have been also used as carriers for heterologous antigens and the fragment C of tetanus toxin has been an excellent model antigen. In fact, FAIRWEATHER et al. (1990) reported that fragment C could be expressed within a Salmonella typhimurium aro A strain under the control of the tac promoter, and that this strain could be used to immunize mice both orally and intravenously. After two oral immunizations with the recombinant Salmonella strain, mice developed high anti-tetanus antibody titers and were 100% protected by challenge with tetanus toxin. This approach was further refined by the use of the nirB promoter, which is repressed under aerobic conditions and is activated under anaerobic conditions. With this promoter, a single oral dose of S. typhimurium aroA aroD expressing fragment C was sufficient to completely
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protect mice from tetanus toxin challenge (Chatfield et al. 1992b). Fragment C has been also expressed in attenuated strains of S. typhi (Chatfield et al. 1992a), the human pathogen responsible for typhoid fever, increasing the possibility of obtaining an oral typhoid-tetanus vaccine a step closer to reality.

II. Recombinant Molecules

Ever since the initial cloning and sequencing of the genes encoding diphtheria and tetanus toxins, parts of the two molecules have been expressed in Escherichia coli using a wide variety of vectors (Leong et al. 1983; Greenfield et al. 1984; Eisel et al. 1986; Fairweather et al. 1986, 1987; Cabiaux et al. 1988; Nieman et al. 1988). For diphtheria these molecules have been tested for enzymatic activity of fragment A (Leong et al. 1983), functionality of fragment B (Cabiaux et al. 1988), the possibility of using them as specific tumor-killing agents (Murphy et al. 1986; Bacha et al. 1988; Murphy and Vanderspek 1995; Negro and Skaper 1995), and their ability to induce protective antitoxic antibodies. So far, the attempts to induce neutralizing antibodies against diphtheria using recombinant molecules have met a poor success.

In marked contrast, for tetanus the possibility of using recombinant molecules as vaccines has been successful. Fragment C of the tetanus toxin has been produced in a soluble form using E. coli and Pichia pastoris expression systems (Fairweather et al. 1987; Makoff et al. 1989; Clare et al. 1991). Recombinant fragment C produced in this way retains all the biological properties of native fragment C, including the internal disulfide bridge structure and the ganglioside binding activity. Moreover, it is as effective as native fragment C derived from tetanus toxin in inducing protective antibodies in mice. No tetanus molecules analogous to the diphtheria toxin CRMs have been produced from C. tetani, mostly because of the absence of efficient genetic mechanisms to manipulate the bacterium. However, recombinant CRMs have been generated by site-directed mutagenesis of the glutamic acid in position 234 located within the HEXXH motif in the light chain. The Glu 234→Ala substitution renders the light chain nontoxic and, in combination with native heavy chain, a completely nontoxic molecule able to induce protective immunity in mice can be generated (Li et al. 1994). The use of recombinant molecules would solve some of the problems associated with the production of the conventional vaccine as, for instance, the containment necessary to grow C. tetani, the long fermentation periods (1 week), the low yield of purified toxin, the low purity of the toxoid, and the handling of large volumes of highly toxic materials.

III. Mucosal Vaccination

It is clear that the development of mucosal (oral or intranasal) tetanus and diphtheria vaccines would be a significant advantage in global immunization...
against both diseases. Nevertheless, it is known that nonliving antigens are poor mucosal immunogens, unable to induce local and systemic immune response when delivered at mucosal sites. The coadministration of nonliving antigens with cholera toxin (CT) or heat-labile toxin (LT), induce in the host the ability to mount both a mucosal and systemic immune response. Conversely, the toxicity of both LT and CT has precluded their use as adjuvants in humans. Using computer modeling to predict the mutations that would generate nontoxic derivatives of LT (PIZZA et al. 1994), LT mutants able to retain the adjuvant properties of the wild-type molecule have been produced. These mutants have been tested in combination with purified recombinant fragment C of tetanus toxin as a mucosal vaccine in intranasally immunized mice. In this experiment fragment C induced high levels of mucosal and systemic antibodies that were able to protect all mice from challenge with tetanus toxin (DOUCE et al. 1995, 1997). This result shows that mucosal vaccines against tetanus have become more feasible by the use of newly developed mucosal adjuvants.

To improve vaccination in developing countries, where there is the logistic difficulty of delivering the two or three doses necessary to give protection, the development of single dose vaccines against diphtheria and tetanus has been proposed. In order to achieve this goal efforts are being focused on developing controlled-release formulations which can deliver the necessary vaccine in a single dose. Most of the work on controlled-release vaccines has concentrated on the use of poly(lactide-coglycolide) (PLG) polymers (COHEN et al. 1991). Single-dose vaccines may become a reality in the future since many of the technical problems of the stability of micro-encapsulated antigens are being solved (SCHWENDEMAN et al. 1995).

IV. CRM 197

Following nitrosoguanidine mutagenesis of corynephage βtox+, several phages have been isolated that encode for nontoxic proteins, which are immunologically related to diphtheria toxin (UCHIDA et al. 1971, 1973a–c; LAIRD and GROMAN 1976). These proteins are generally called cross-reacting materials (CRMs).

CRM 197 contains a single glycine to glutamic acid substitution in position 52 that makes the fragment A unable to bind NAD+ and therefore enzymatically inactive (PAPPENHEIMER et al. 1972; GIANNINI et al. 1984). Being enzymatically inactive and therefore nontoxic but otherwise identical to diphtheria toxin, CRM 197 has been proposed as a natural candidate to develop a new vaccine against diphtheria. However, structural differences between CRM 197 and diphtheria toxin make this mutant more susceptible to proteases and less immunogenic than diphtheria toxoid (PAPPENHEIMER et al. 1972). After stabilization with formalin, CRM 197 becomes more immunogenic and able to induce protective antibody titers against diphtheria (PORRO et al. 1980); however, its potency per microgram of protein is still slightly lower than that of
diphtheria toxoid. A formalin-stabilized form of CRM 197 has been successfully used in a clinical trial combined with tetanus toxoid, as a booster in young adults (Podda and Rappuoli, unpublished data).

F. Diphtheria and Tetanus Toxoids as Carriers for Polysaccharide Vaccines

Invasive bacterial diseases caused by pneumococci, meningococci, and *H. influenzae* type b can be prevented by serum antibodies against the capsular polysaccharide of these organisms (MacLeod et al. 1945; Robbins et al. 1984; Robbins and Robbins 1986). These polysaccharides are high molecular mass polymers of repeating oligosaccharide units that induce only a primary antibody response in adults but are not immunogenic at all in infants younger than 2 years of age (MacLeod et al. 1945; Robbins et al. 1984). The reason probably lies in the nature of the major histocompatibility complex, which binds peptides but not sugars and polysaccharide molecules. Consequently, the immunogenicity of the polysaccharide is mostly independent of helper T cells. This problem has been overcome by conjugation of carrier proteins containing T-cell epitopes to the polysaccharides. Conjugates between CRM 197, tetanus, or diphtheria toxoids, and capsular polysaccharides have been developed. They are immunogenic in infants and induce a memory response (MacLeod et al. 1945; Robbins et al. 1984).

Today they are among the most successful vaccines, recently introduced for immunization of all infants.

G. Pertussis

I. The Disease

Pertussis, or whooping cough, is a disease caused by *B. pertussis*, a gram-negative bacterium which adheres to the cilia of the upper respiratory tract of humans, colonizes this tissue and releases a number of virulence factors responsible for the local and systemic damages associated to the disease (Weiss and Hewlett 1986; Rappuoli 1994). The disease is characterized by long-lasting paroxysmal cough, accompanied by whoops, vomiting, cyanosis, and apnea. The most common complications are pneumonia, seizures, encephalopathy, and death. Antibiotic treatment is very effective in clearing the bacteria but has little consequence on the disease, mostly because the disease is usually diagnosed when the bacteria have already released the toxins. *B. pertussis* infects virtually all children who are not immune; however, the disease can occur at any age. Mortality is frequent under 1 year of age and occurs mostly in developing countries, where incidence can be as high as 1/100. In developed countries the frequency is between 1/1000 and 1/10000; of the
infants in Western countries who contract the disease in the first year of life 50% are hospitalized and 1% die. After the first year of life only 4% of children with the disease are hospitalized. It has been estimated worldwide that whooping cough causes approximately 300000 deaths each year (Müller et al. 1986; Sutter and Cochi 1992).

II. History of Acellular Vaccines

Vaccination is the only way to control pertussis. Mass vaccination using killed bacteria (cellular vaccine) was introduced in the 1950s and reduced by 99% the incidence of diseases in infants.

In spite of its efficacy, the vaccine composed of whole, inactivated B. pertussis cells is not widely used, mostly because of the fear of the side effects which have been associated with it. Most of these are mild reactions such as redness, swelling, and fever and occur in 30%–70% of vaccinees. However, the vaccine has also been associated with more severe reactions, such as neurological damages and death, which have been reported with a frequency of 1/100000 and 1/300000, respectively (Miller et al. 1981). Although never proven to be caused by the vaccine, these reactions have caused a drop in vaccine uptake and stressed the need for a new vaccine, devoid of side effects and possibly composed of well-defined and purified antigens.

Many molecules produced by B. pertussis and involved in the virulence of the bacterium, have been identified as candidates for inclusion in a vaccine against whooping cough. These include molecules involved in the adhesion of the bacteria to the eukaryotic cells and to the cilia of the upper respiratory tract, and molecules which cause local and systemic damage of the host. Using a combination of pertussis toxin, filamentous hemagglutinin, and an outer membrane protein that has been subsequently characterized, named pertactin or 69K, partially purified from culture supernatants of B. pertussis and detoxified with formaldehyde, Y. and H. Sato developed a new vaccine, which has been used in Japan since 1981 in children over 2 years of age. Following the Japanese example, many laboratories developed acellular vaccines containing pertussis toxin, filamentous hemagglutinin, and other purified B. pertussis antigens, such as the fimbriae or agglutinogens, and 69 K (Moxon and Rappuoli 1990; Rappuoli 1996).

In order to be included in vaccines pertussis toxin needs to be detoxified. Y. Sato used formalin treatment to make the toxin inactive, but this procedure, although effective for diphtheria and tetanus, was not entirely satisfactory for pertussis toxin, which in some cases showed some reversion to toxicity (Storsæter et al. 1990). To obtain completely detoxified molecules, a number of other chemical methods were used: these included stronger formaldehyde treatments, hydrogen peroxide (Sekura et al. 1988; Krantz et al. 1990), tetratinromethane (Winberry et al. 1988), and glutaraldehyde. In our laboratory we have opted to use genetic tools instead of chemical reagents to detoxify pertussis toxin.
III. Pertussis Toxin

Pertussis toxin is a complex bacterial protein toxin of 105 kDa composed of five noncovalently linked subunits termed S1–S5 (TAMURA et al. 1982). It is organized into two functional domains called A and B. The A domain, which is composed of the S1 subunit, is an enzyme of 26.22 kDa with ADP-ribosyltransferase activity. It binds NAD and transfers the ADP-ribose group to a cysteine residue present in a...XCGLX motif, located at the carboxyterminal region of the α-subunit of many GTP-binding proteins, such as Gt and G0, that are involved in signal transduction in eukaryotic cells, altering their response to extracellular stimuli. The B domain is a nontoxic oligomer formed by four distinct subunits, termed S2–S5, of 21.92 kDa, 21.86 kDa, 12.06 kDa and 11.77 kDa, respectively, which are present in a 1:1:2:1 ratio (TAMURA et al. 1982). The B oligomer binds the receptor on the surface of eukaryotic cells and facilitates the translocation of the enzymatically active subunit across the cell membrane so that it can reach the target G proteins.

The crystal structure of the protein has been solved (STEIN et al. 1994). The genes coding for the five subunits of pertussis toxin are clustered in a 3.2-kb fragment of the chromosomal DNA, with the typical organization of a bacterial operon. The PT operon was sequenced by two different research groups in 1986 and was described to contain the genes in the following order: S1, S2, S4, S5, S3 (LOCHT and KEITH 1986; NICOSIA et al. 1986). Each gene contains a sequence coding for a signal peptide. The five subunits are cotranslationally secreted into the bacterial periplasm where the protein is assembled into the holotoxin. Secretion of the toxin occurs only after assembly of the holotoxin, or of the B domain, and requires the product of the nine-genes ptl operon, which is located downstream from the PT operon and is regulated by the same promoter (COVACCI and RAPPUOLI 1993; WEISS et al. 1993).

IV. Genetic Detoxification

Following the cloning and sequencing of the 3.5-kb DNA fragment containing the five PT genes (LOCHT and KEITH 1986; NICOSIA et al. 1986), the attempt to express the operon in E. coli failed. The individual subunits of the pertussis toxin were successfully expressed in E. coli and Bacillus subtilis; however, these recombinant molecules were unable to induce protective immunity because protection was found to be mediated by conformational epitopes which are present in the entire, assembled pertussis toxin but absent in the individual recombinant subunits (BARTOLONI et al. 1988). The failure to induce a good protective immunity with recombinant subunits expressed in E. coli and B. subtilis suggested that the ideal vaccine should be a PT molecule whose toxicity had been eliminated by the genetic manipulation of the gene coding for the S1 subunit. Therefore, to obtain a molecule suitable for vaccine use we
decided to modify the PT genes in the chromosome of *B. pertussis* in order to obtain a molecule fully assembled but devoid of toxicity.

For this purpose, we and other investigators generated a number of recombinant Sl molecules containing amino acid substitutions and tested their enzymatic activity (PIZZA et al. 1988; BARBIERI and CORTINA 1988; CIEPLAK et al. 1988; LOCHT et al. 1989).

The mutations which render the Sl subunit nontoxic were then introduced into the chromosome of *B. pertussis* using homologous recombination (PIZZA et al. 1989). These new *B. pertussis* strains were found to produce molecules indistinguishable from PT in sodium dodecyl sulfate–polyacrylamide gel electrophoresis, but that had a toxicity ranging from 0.1% to 10% of wild-type PT. Since even 0.1% of the toxicity is by far too high for a molecule to be used in a vaccine, we combined some of the above mutations and obtained several PT double mutants. One of these, the PT 9K/129G mutant, containing mutations of arginine in position 9 to lysine and glutamic acid in position 129 to Gly, resulted to have lost all toxic properties of PT, and maintained all the antigenic properties.

Once the safety and antigenic properties of the PT 9K/129G mutant were demonstrated, the molecule was produced in large scale and formulated in an anti-diphtheria, tetanus, and pertussis (DTaP) vaccine. The anti-pertussis component was either made by the PT 9K/129G alone (10 μg), or by the PT 9K/129G in combination with filamentous hemagglutinin (FHA) and 69 K (5, 2.5, and 2.5 μg each, respectively). The vaccines were tested for safety and immunogenicity in phase I and phase II studies (PODDA et al. 1990, 1991, 1992, 1993, 1994) with optimal results before being used for larger comparative international studies.

### V. Acellular Vaccines Proposed

During the period 1980–1989 many acellular vaccines were developed by vaccine manufacturers. All of them contained either detoxified pertussis toxin alone or combined with FHA, with pertactin or 69 K, and with the agglutinogens. Some of the acellular vaccines developed are reported in Table 1. As shown, the pertussis toxin included in the vaccines has been detoxified with a variety of chemical methods, including formaldehyde, glutaraldehyde, tetranitromethane and hydrogen peroxide. The Chiron vaccines were the only ones containing genetically detoxified pertussis toxin.

### VI. Clinical Trials

Following the Japanese experience, an efficacy study was organized in 1986 in Sweden to test two vaccines: one containing formalin-treated PT, and the other containing formalin-treated PT and FHA. Although infants were immunized only with two doses, both vaccines were able to protect infants from disease (54% and 69%, respectively) (Ad Hoc Group 1988). At the end of this
Table 1. List of acellular vaccines and their composition

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Vaccine composition</th>
<th>Antigen quantity (µg)</th>
<th>PT detoxification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chiron (C-1)</td>
<td>PT</td>
<td>10</td>
<td>Genetic</td>
</tr>
<tr>
<td>North American Vaccine (NAV)</td>
<td>PT</td>
<td>40</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>Swiss Serum &amp; Vaccine Institute (SSVI-1)</td>
<td>PT</td>
<td>50</td>
<td>Tetranitromethane</td>
</tr>
<tr>
<td>Connaught (US)/Biken (CB-2)</td>
<td>PT, FHA</td>
<td>23.4, 23.4</td>
<td>Formaldehyde</td>
</tr>
<tr>
<td>Michigan Dept Public Health (Mich-2)</td>
<td>PT, FHA</td>
<td>25.0, 25.0</td>
<td>Formaldehyde</td>
</tr>
<tr>
<td>Pasteur Merieux (PM-2)</td>
<td>PT, FHA</td>
<td>25.0, 25.0</td>
<td>Glutaraldehyde</td>
</tr>
<tr>
<td>SmithKline Beecham Biologicals (SKB-2)</td>
<td>PT, FHA</td>
<td>25.0, 25.0</td>
<td>Formaldehyde + glutaraldehyde</td>
</tr>
<tr>
<td>Chiron (C-3)</td>
<td>PT, FHA, 69K</td>
<td>5.0, 2.5, 2.5</td>
<td>Genetic</td>
</tr>
<tr>
<td>Lederle-Praxis Biologicals (LPB-3)</td>
<td>PT, FHA, 69K</td>
<td>10.0, 20.0, 5.0</td>
<td>Formaldehyde</td>
</tr>
<tr>
<td>SmithKline Beecham Biologicals (SKB-3)</td>
<td>PT, FHA</td>
<td>25.0, 25.0, 8.0</td>
<td>Formaldehyde + glutaraldehyde</td>
</tr>
<tr>
<td>Connaught Laboratories (Canada) (CLL-3)</td>
<td>PT, FHA, FIM2, FIM3</td>
<td>10.0, 5.0, -5.0*</td>
<td>Glutaraldehyde</td>
</tr>
<tr>
<td>Porton International (Por-3)</td>
<td>PT, FHA, FIM2, FIM3</td>
<td>10.0, 10.0, −10.0*</td>
<td>Formaldehyde</td>
</tr>
<tr>
<td>Lederle Praxis/Takeda (LPT-4)</td>
<td>PT, FHA, 69K, FIM2</td>
<td>3.5, 35.0, 2.0, 0.8</td>
<td>Formaldehyde</td>
</tr>
<tr>
<td>Connaught Labs. (Canada) (CLL-4)</td>
<td>PT, FHA, 69K, FIM2, FIM3</td>
<td>20.0, 20.0, 3.0, 5.0*</td>
<td>Glutaraldehyde</td>
</tr>
</tbody>
</table>

*FIM2 + FIM3.

†The composition of the vaccine used in the Sweden I trial was 10.0, 5.0, 3.0, 5.0.
trial acellular vaccines were not licensed because the observed efficacy was not considered to be high enough, and the absence in the study of an arm immunized with the whole cell vaccine did not allow to compare the efficacy of acellular vaccines with that of the vaccine already in use. In addition, during the study the formalin detoxified pertussis toxin showed some reversion to toxicity and this suggested that further development of acellular vaccines was still necessary (STORSÆTER et al. 1990).

In 1990 the National Institute of Allergy and Infectious Diseases, performed a large scale phase II trial in the United States to compare the safety and the immunogenicity of most of the acellular vaccines available at that time in order to select the vaccines to be subsequently used in new efficacy studies. Thirteen acellular vaccines (Table 1) were tested and compared with two whole cell vaccines. In each arm 120 infants were immunized with three doses of each vaccine. Eleven of the acellular vaccines contained chemically detoxified PT, and two vaccines contained genetically detoxified PT. The results showed that all acellular vaccines were much safer (DECKER et al. 1995) and more immunogenic than the whole cell vaccines (EDWARDS et al. 1995).

A comparison of the immunogenicity of the pertussis toxin used in the vaccines in the study is reported in Table 2. This trial demonstrated unequivocally that the genetically detoxified pertussis toxin induced anti-PT levels that in enzyme-linked immunosorbent assay (ELISA) and toxin neutralization were 5- to 20-fold higher than those induced by chemically detoxified forms of PT present in the other vaccines.

Table 2. Immunogenicity of genetically (Chiron) versus chemically (all others) inactivated PT in phase II and phase III trials (from EDWARDS et al. 1995; GRECO et al. 1996; GUSTAFSSON et al. 1996). See Table 1 for vaccine identification

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Immunogenicity PT (phase II NIH)</th>
<th>Immunogenicity (phase III efficacy studies)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute value Units/µg protein</td>
<td>Absolute value Units/µg protein</td>
</tr>
<tr>
<td></td>
<td>ELISA CHO</td>
<td>ELISA CHO</td>
</tr>
<tr>
<td>C-1</td>
<td>180 1035</td>
<td>18.0 103.5</td>
</tr>
<tr>
<td>C-3</td>
<td>99 487</td>
<td>19.8 97.4</td>
</tr>
<tr>
<td>CB-2</td>
<td>127 841</td>
<td>5.4 36.0</td>
</tr>
<tr>
<td>SKB-2</td>
<td>104 530</td>
<td>4.1 21.2</td>
</tr>
<tr>
<td>SKB-3</td>
<td>54 205</td>
<td>2.1 8.2</td>
</tr>
<tr>
<td>SSVI-1</td>
<td>99 259</td>
<td>1.9 5.1</td>
</tr>
<tr>
<td>PM-2</td>
<td>68 432</td>
<td>2.7 17.2</td>
</tr>
<tr>
<td>Mich-2</td>
<td>66 327</td>
<td>2.6 13.0</td>
</tr>
<tr>
<td>LPB-3</td>
<td>39 163</td>
<td>3.9 16.3</td>
</tr>
<tr>
<td>CLL-3</td>
<td>38 158</td>
<td>3.8 15.8</td>
</tr>
<tr>
<td>CLL-4</td>
<td>36 142</td>
<td>3.6 14.2</td>
</tr>
<tr>
<td>Por-3</td>
<td>29 118</td>
<td>2.9 11.8</td>
</tr>
<tr>
<td>LPT-4</td>
<td>14 116</td>
<td>4.0 33.1</td>
</tr>
</tbody>
</table>

ND = not done
After the comparative phase II study described above four vaccines were selected to be tested in efficacy trials. Two of them, the Connaught five-component vaccine and the SmithKline Beecham Biologicals two-component vaccine, were tested in an efficacy trial performed in Sweden (Sweden I in Table 3), while the Chiron vaccine was tested in Italy, in parallel with the three-component vaccine produced by SmithKline Beecham Biologicals (Italy in Table 3). This was a unique opportunity to test two vaccines containing exactly the same components (PT, FHA, and 69K) but differing in the method used to detoxify PT and in the amount of antigens present (25, 25, and 8 μg in the SmithKline Beecham Biologicals vaccine, and 5, 2.5, and 2.5 μg in the Chiron vaccine, respectively). The results of the Italian and Swedish trials clearly showed that all acellular vaccines have a greatly superior safety to that of the whole cell vaccines (e.g., fever occurred in 40% of infants vaccinated with whole cell vaccines and in 5%–8% of infants vaccinated with acellular vaccines). All acellular vaccines showed efficacy; however, the most efficacious vaccines were the three-component vaccine of Chiron, containing the genetically inactivated PT (84.2%), the SmithKline Beecham Biologicals vaccine containing the formalin detoxified PT (83.9%), and the five-component of Connaught Laboratories, also containing formalin-inactivated PT (85.2%). The SmithKline Beecham Biologicals vaccine containing the PT and FHA alone showed a surprisingly low efficacy (58.9%). The whole cell vaccine performed very poorly (only 36.1%–48.3% protection) (GRECO et al. 1996; GUSTAFSSON et al. 1996). As previously observed in phase II studies, the genetically detoxified PT was the most immunogenic in these studies, inducing a superior ELISA and toxin neutralizing antibody titers (Table 2; GRECO et al. 1996). This superior immunogenicity may be responsible for other important features observed in the study: the vaccine containing genetically detoxified PT was able to protect starting from the first vaccination dose, and showed a longer lasting protective immunity (SALMASO et al. 1998). In addition, it showed a lower reactogenicity.

A subsequent study was then organized in Sweden (Sweden II, Table 3) to compare the relative efficacy of the two-component acellular vaccine from SmithKline Beecham Biologicals, the recombinant three-component from Chiron, the five-component from Connaught, and a whole-cell vaccine produced by Wellcome. The study was not placebo-controlled, and therefore no absolute efficacy could be calculated. Given the large population samples involved (nearly 90,000 children), the study had the statistical power to discriminate even very small differences in efficacy. The results confirmed that the two-component SmithKline Beecham Biologicals vaccine was poorly efficacious, and therefore the arm containing this vaccine was discontinued during the trial. The remaining vaccines (Chiron’s recombinant three-component, Connaught’s five-component, and Wellcome’s whole-cell) were all very efficacious in protecting from disease. The small but statistically significant differences observed indicated that among the very efficacious vaccines the efficacy was superior in the arm vaccinated with the whole-cell, five- and three-component vaccines, respectively.
<table>
<thead>
<tr>
<th>Trial</th>
<th>Design</th>
<th>Schedule</th>
<th>Vaccines used</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH studies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweden I</td>
<td>Prospective, cohort, randomized, double-blind, placebo-controlled</td>
<td>3 doses: 2, 4, 6 months</td>
<td>DT (SBL), DTa2P (SKB-2), DTa5P (CLL-4), DTwP (CLI)</td>
<td>58.9%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DTa3P (C-3)</td>
<td>0.31*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DTa2P (SKB-2)</td>
<td>1.0*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DTwP (Well)</td>
<td>0.13*</td>
</tr>
<tr>
<td>Sweden II</td>
<td>Prospective, cohort, randomized, double-blind</td>
<td>3 doses: 3, 5, 12 months; 2–4, 6 months</td>
<td>DTa3P (C-3), DTa5P (CLL-4), DTa2P (SKB-2)</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DTwP (Well)</td>
<td>0.13</td>
</tr>
<tr>
<td>Italy</td>
<td>Prospective, cohort, randomized, double-blind, placebo-controlled</td>
<td>3 doses: 2, 4, 6 months</td>
<td>DT (C), DTa3P (C-3), DTa3P (SKB-3), DTwP (CLI)</td>
<td>84.2%</td>
</tr>
<tr>
<td>Other studies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gothenburg, Sweden</td>
<td>Prospective, double-blind, placebo-controlled</td>
<td>3 doses: 3, 5, 12 months</td>
<td>DT (SS), DTa1P (NAV)</td>
<td>71%</td>
</tr>
<tr>
<td>Erlangen, Germany</td>
<td>Prospective, cohort, randomized, double-blind, household contact study</td>
<td>4 doses: 2, 4, 6 months with booster at 18 months</td>
<td>DT, DTa4P (LPT-4), DTwP (L)</td>
<td>81.5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>90.8%</td>
</tr>
<tr>
<td>Senegal</td>
<td>Prospective, cohort, randomized, double-blind</td>
<td>3 doses: 2, 4, 6 months</td>
<td>DTa2P (PM-2), DTwP (PM)</td>
<td>86%</td>
</tr>
<tr>
<td>Mainz, Germany</td>
<td>Prospective, blinded, household contact study</td>
<td>4 doses: 3, 4, 5 months with booster before 25 months</td>
<td>DT (BW), DTa3P (SKB-3), DTwP (BW)</td>
<td>88.7%</td>
</tr>
<tr>
<td>Munich, Germany</td>
<td>Case-control</td>
<td>9, 16, 24 months with booster at 15–25 months</td>
<td>DT, DTa2P (CB-2), DTwP (BW)</td>
<td>82%</td>
</tr>
</tbody>
</table>

^a Relative risk after third dose using the 3, 5, and 12 month schedule.

Abbreviations: SBL, Swedish Bactercology Laboratories; CLI, Connaught Laboratories Inc.; Well, Wellcome; SS, Statens Seruminstitut; L, Lederle; BW, Behringwerke.
The overall conclusion from these trials confirm that while three-component vaccines are excellent, the addition of other antigens may increase slightly the protective effect, and that the best protection can still be achieved with the whole-cell vaccine. However, the small differences found between the efficacious vaccines tested in the Sweden II trial are only a statistical curiosity, and they have no relevance for the selection of an efficacious vaccine to be used. Other criteria such as safety, duration of protection, etc., should be also considered. The design, schedule of immunizations, and results of the efficacy trials performed in Italy and Sweden are summarized in Table 3.

VII. Other Clinical Studies

In addition to the above trials which were sponsored and performed under the supervision of the National Institutes of Health (United States), using consistent criteria of disease definition and protocol, several other clinical studies have been performed using different standards, different study designs, and often different schedules of immunization. The additional studies are also reported in Table 3. These trials provide some information about the vaccines used, but the values obtained cannot be compared to those obtained in the Italian and Swedish trials reported in Table 3. For example, one obvious discrepancy between the two trials is the different efficacy between the two-component vaccine of SmithKline Beecham Biologicals in Sweden (58.9%), and the two-component vaccine of Pasteur Merieux in Senegal (86%, Table 3). Given the difference in trial design, it is not clear whether the manufacturing of the Pasteur Merieux vaccine produces a two-component vaccine superior to that manufactured by SmithKline Beecham Biologicals, or whether the absolute efficacy reported for the trial in Senegal has been overestimated.

The development of an acellular vaccine containing genetically detoxified PT represents a new milestone in vaccine development and proves several new concepts that should be taken into account for future vaccine development and for selection of vaccine usage: (a) The native conformation of the genetically detoxified pertussis toxin results in a superior immunogenicity. In addition to the higher antibody titers achieved with a very low dose of PT, in the phase III trial in Italy, the vaccine containing the genetically detoxified PT was able to confer protective immunity starting after the first vaccine dose, allowing the protection of infants in the first few months of life, when the disease is most dangerous. (b) The native conformation of the genetically pertussis toxin induced a better immunological priming against the natural molecule that resulted in a longer lasting protective immunity. (c) The superior immunogenicity of genetically detoxified PT allowed to use a lower antigen dose in the vaccine, which resulted in a lower frequency of common side effects such as fever, redness and swelling. (d) Only genetic detoxification can guarantee the absolute absence of active pertussis toxin, a molecule present in whole-cell vaccines. The absence, even in minimal amounts, of active pertussis toxin is crucial in novel vaccines, because this toxin has been shown to cause
anaphylaxis and permanent modification of the nerve-mediated permeability of the intestine (MUNOZ et al. 1987; KOSECKA et al. 1994). Chemical detoxification is less reliable, and active pertussis toxin has been reported in several acellular vaccine preparations (MILLER et al. 1995), while on another occasion, reversion to toxicity has been reported (STORSAETER et al. 1990).

References


Toxin-Based Vaccines (Diphtheria, Tetanus, Pertussis)


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