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ENGINEERING BACTERIAL TOXINS FOR THE DEVELOPMENT OF NEW VACCINES

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BACTERIAL ADP-RIBOSYLATING TOXINS

ADP-ribosylating bacterial toxins are proteins, produced by pathogenic bacteria, which are usually released into the extracellular medium and cause disease by killing or altering the metabolism of eukaryotic cells. The toxins are usually composed of two functionally distinct domains: a toxic moiety and a vector which have been called domains A and B, respectively. The vector (B) binds the receptors on the surface of eukaryotic cells and delivers the toxic part (A) across the membrane of eukaryotic cells so that it can reach its target proteins[1].

While the properties and the complexity of the vector (B) differ from toxin to toxin and often also within the same family of toxins, all of the A domains have a common mechanism of action: they are enzymes which ADP-ribosylate eukaryotic target proteins which control crucial circuits of eukaryotic cells, such as protein synthesis, transmembrane signaling, oncogenesis, cytoskeleton structure. The target proteins also have a common feature and a common structure: they are GTP-binding proteins. The only ADP-ribosylating toxins characterized both in terms of protein and genetic structure are diphtheria toxin (DT), Pseudomonas exotoxin A (PAETA), pertussis toxin (PT), cholera toxin (CT) and the *E.coli* heat-labile toxin (LT). The main properties of these toxin s are summarized in Table I.

THE ACTIVE SITE OF ADP-RIBOSYLATING TOXINS HAS A COMMON STRUCTURE

The initial comparison of the aminoacid sequences of DT and PAETA did not show sequence homology[2]. However, when by photoaffinity labeling Carroll and Collier showed that Glu148 of diphtheria toxin is functionally equivalent to Glu553 of Pseudo-

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TABLE I

Bacterial toxin	Acceptor protein	Acceptor aminoacid	Effect on eukaryotic cells	Primary	Eukaryotic cell structure receptor
Diphtheria toxin	Elongation factor-2	Diphtamide 715	Inhibition of protein synthesis	Known	14.5 Kd protein?
Pseudomonas ETA	Elongation factor-2	Diphtamide 715	Inhibition of protein synthesis	Known	Not known
Pertussis toxin	Gi, Go, T	Cys 352	Alteration of trans-membrane signal transduction	Known	160 Kd glycoprotein in CHO cells
Cholera toxin	Gs, T (Gi and Go	Arg 201	"	"	Ganglioside GM1>GDb1
E. coli LT1	"	Arg 201	"	"	Ganglioside GM1>GDb1>GM2

monas exotoxin A[3] they realigned the aminoacid sequences using this reference point and found a strong homology between the two toxins[4]. Regions of strong homology were also found between pertussis and cholera toxins[5, 6]. The two groups of toxins (which have a different target) did not show any aminoacid homology.

In the meantime, by chemical modification, photoaffinity labeling and site-directed mutagenesis, a number of aminoacids were identified which are essential for the catalytic activity of the different toxins[2]. Remarkably, these aminoacids were found to be conserved in all enzymes (Fig. 1).

The availability of the three-dimensional structure of PAETA, determined by X-ray crystallography by Allured et al.[7] provided the template for a computer-based molecular modeling of DT, PT and CT. Using the coordinates of ETA we were able to predict the majority of the structure of DT and the structure of the active sites of PT and CT. As shown in Fig. 2, the active site of the four toxins which contain all aminoacids identified in Fig. 1 share a common structure.

SITE-DIRECTED MUTAGENESIS OF THE ACTIVE SITE AND CONSTRUCTION OF NON TOXIC MOLECULES

The identification of the aminoacids which are in the active site of the ADP-ribosylating toxins, provided the rational basis for the mutagenesis of their genes in order to obtain non toxic molecules to be used in vaccines. A number of non toxic molecules have been obtained from diphtheria toxin, pseudomonas exotoxin A and pertussis toxin[8]. In all cases, the substitution of the glutamic acid (residue 6 in Figs. 1 and 2) or its deletion was the mutation most effective in reducing the toxicity of the molecules.

Among the many non toxic derivatives obtained, the one which has been studied in more detail is PT-9K/129G, a non toxic mutant of pertussis toxin which is being actively tested in clinical trials as a new vaccine against whooping cough. This mutant contains

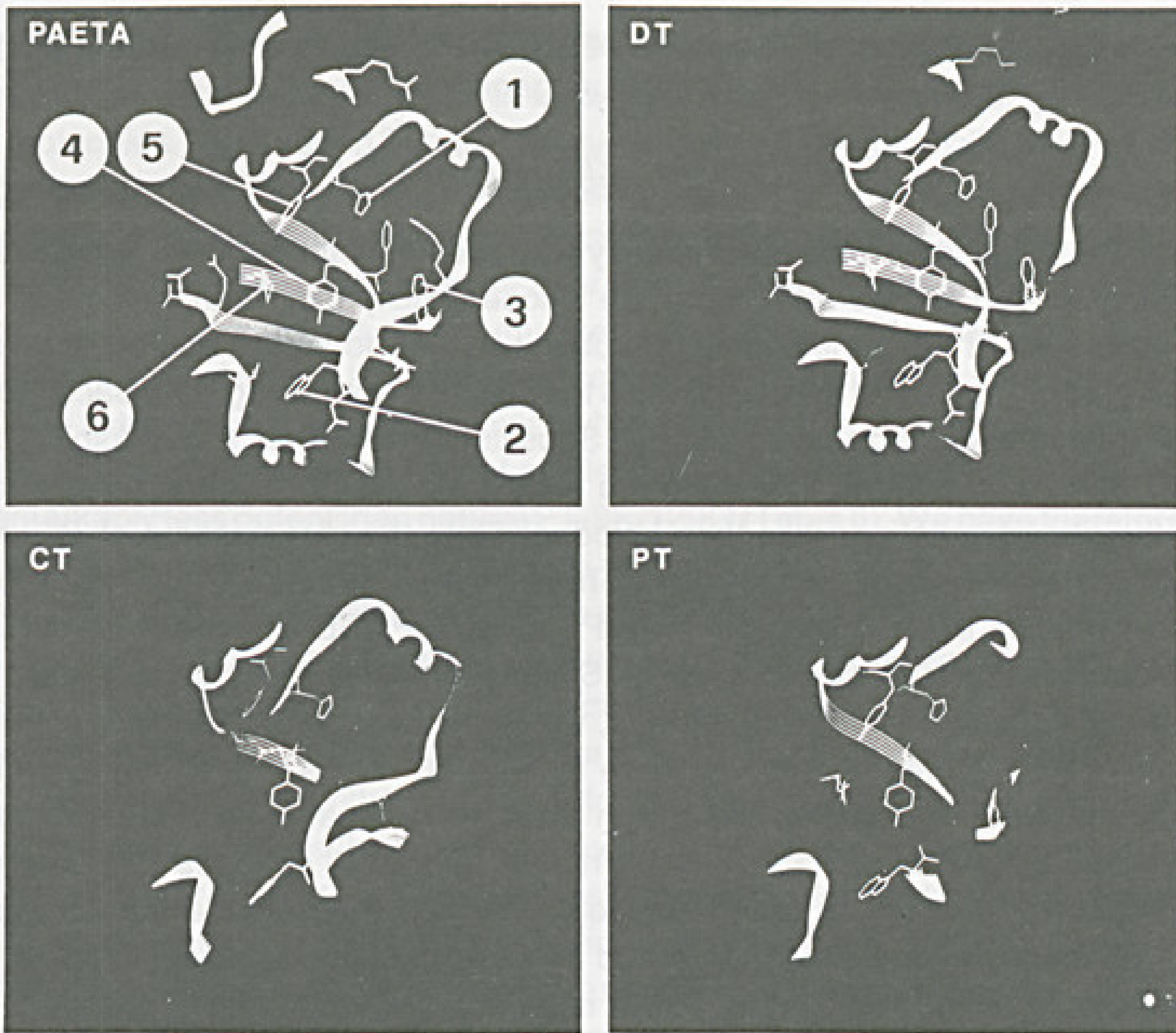

Figure 1 - Homologies between the three regions forming the active site of Pseudomonas exotoxin A (PAETA) and corresponding regions of diphtheria toxin (DT) pertussis toxin (PT), cholera toxin (CT) and E. coli LT toxin (LT).

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                   1
ETA    436  F V G Y H G T F L E
DT      17  F S S Y H G T K P G
PARS   857  R L L W H G S R T T
PT      31  N V L D H L T G R S
CT      40  N L Y D H A R G T Q
LT      40  N L Y D H A R G T Q
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                        2     3 4                  5
ETA    463  D A I W R G F Y I ...(7).. Y G Y
DT      47  D D D W K G F Y S ...(9)...... Y
PT      23  F T A W G N .....
CT *   124  I Y G W Y R .....
CT *   171  H R A W R E .....
PARS   893        ... G I Y F A ....(9)..... Y
PT      85        ... I G . Y I Y ..(6)... F Y
CT      81        ... S T Y Y I Y ..(8)..... F
LT      81        ... S T Y Y I Y ..(8)..... F
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                       6
ETA    549  G G R L E T I L G W
DT     144  S S S V E Y I N N W
PARS   983  L L Y N E Y I V Y D
PT     125  T Y Q S E Y L A H R
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ETA    555  I L G W P L A
DT     150  I N N W E Q A
PT     104  Y F E Y V D T
CT *   122  S Q I Y G W Y
CT *   124  I Y G W Y R V
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Figure 2 - Structure of the active sites of PAETA, DT, CT and PT, obtained using the coordinates of PAETA. The numbers identify the aminoacids shown in Fig. 1.

two aminoacid substitutions (Arg9 —> Lys9 and Glu129 —> Gly) which make this molecule absolutely non toxic but fully immunogenic [9-10].

GENETIC DETOXIFICATION OF PT

The failure to induce protective immunity with recombinant subunits suggested that the ideal vaccine should be a PT molecule whose toxicity has been eliminated by genetic manipulation of the gene coding for subunit S1. To do so, we and other investigators generated a number of recombinant S1 molecules containing aminoacid substitutions and tested their enzymatic activity. Substitution of either Arg9, Asp11, Arg13, Trp26, or Glu129 was found to reduce the enzymatic activity to undetectable levels[8]. Each of the above mutations was then introduced into the chromosome of *B. pertussis* whose wild type genes had been deleted. These new *B. pertussis* strains were found to produce molecules indistinguishable from PT in SDS-PAGE which had a toxicity that ranged 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 PT double mutants that were at least 10^6 times less toxic than wild type PT (Table II)[9]. Such molecules, being non toxic, were ideal candidates for new vaccines provided they had maintained the correct B- and T-cell epitopes and were able to induce protective immunity in animal models. The non toxic double mutant shown in Table II was found to have the same B- and T-cell epitopes as wild type toxin, to induce toxin-neutralizing antibodies, and to protect mice from the intracerebral challenge[9, 10].

GENETICALLY DETOXIFIED MOLECULES ARE SAFER AND MORE IMMUNOGENIC THAN CHEMICALLY DETOXIFIED TOXINS

Formaldehyde and glutaraldehyde are usually used to detoxify toxins for vaccine purposes. To find whether PT-9K/129G is more immunogenic than chemically inactivated per-

TABLE II

In vivo and *in vitro* properties of PT-9K/129G compared with purified native PT

Property		PT	PT-9K/129G
CHO cell clustered growth	(ng/ml)	0.005	> 5,000
ADP ribosylation	(ug)	0.001	>20
Mitogenicity	(ug/ml)	0.1-0.3	0.1-0.3
Hemagglutination	(ug/well)	0.1	0.1
Affinity constant (anti-S1)	[Ka (L/mol)]	2.4×10^8	6.1×10^8
Affinity constant (anti- PT)	[Ka (L/mol)]	2.0×10^{10}	9.8×10^9
Histamine-sensitization	(ug/mouse)	0.1-0.5	> 50
Leukocytosis stimulation	(ug/mouse)	0.02	> 50
Anaphylaxis potentiation	(ug/mouse)	0.04	> 7.5
Enhance isulin secretion	(ug/mouse)	<1	> 25
In vivo acute toxicity	(ug/Kg)	N.D.	> 1,500

N.D. = not determined

tussis toxin molecules, we treated PT-9K/129G with 0.07% and 0.42% formaldehyde for 48 hours at 37°C and then compared the *in vitro* properties and the immunogenicity or the resulting molecules. As shown in Table III, formaldehyde treatment abolished the hemagglutinating property of PT-9K/129G, decreased its affinity for anti-PT gama globulins and masked the epitope recognized by the protective monoclonal antibody 1B7. The immunogenicity of PT-9K/129G did not decrease after formaldehyde treatment. The ELISA titer of the sera obtained from guinea pigs immunized with natural or formalin-treated molecules were almost identical (Table III). In marked contrast, the ability of the sera to neutralize PT in the CHO assay were dramatically lower when chemically treated molecules were used for immunization. In the intracerebral challenge assay, the formalin-treated molecules were remarkably less potent than natural PT-9K/129G (Table II). We have therefore shown that chemical treatment of toxins for vaccine production induces profound changes in the antigenic properties of the molecules. These changes do not alter the total amount of antibodies induced but change dramatically the quality of the antibodies obtained. As a result, large quantities of chemically treated molecules are required to induce a protective response. Under these conditions, the immune system produces mainly antibodies against non protective epitopes (or with lower affinity for the protective epitopes) and the cellular immunity is overstimulated. Both conditions may favor the appearance of untoward reactions of the Arthus type or delayed typed hypersensitivity. In conclusion, the properties of PT-9K/129G show that molecular genetics has provided new and more efficient tools to inactivate toxins for vaccine use. These molecules have the same conformation as the native proteins and are much better than chemically treated toxins in inducing protective immunity.

TABLE III

Effect of formaldehyde treatment on the properties of the genetically inactivated pertussis toxin mutant PT-9K/129G.

Formaldehyde (%)	Hemagglutination (µg/well)	Affinity Constant: Polyclonal gamma globulins	Affinity Constant: Monoclonal 1B7	Immunogenicity (guinea pigs immunized with 3 µg of antigen): ELISA titer	Immunogenicity: CHO titer	Vaccine Potency (Mice survival after immunization with 5 µg of antigen): Intracerebral challenge
0.0	0.5	$1.15\ 10^9$	$5.5\ 10^7$	3.5	1/2560	13/16
0.07	4.0	$5.26\ 10^8$ $6.75\ 10^7$	-	3.1	1/160	8/16
0.42	>10.0		-	3.3	1/80	1/16

CLINICAL STUDIES

After extensive studies in animal models which have shown that PT-9K/129G is non toxic, immunogenic and is able to protect mice from the infection with virulent *B. pertussis*, we have tested PT-9K/129G in human adult volunteers[11]. The results of this study showed that the vaccine was safe and induced a great increase in antibody titers against PT both in ELISA and CHO neutralization assays. The titers of PT-specific antibodies were higher than those reported in similar studies using higher doses of chemi-

cally detoxified PT. In particular, the ratio between toxin neutralizing titers and total anti PT ELISA titers was the highest so far reported, suggesting that also in man, immunization with a molecule not chemically modified induces antibodies with higher affinity for the native PT. After the successful phase I study in adult volunteers, the vaccine is now being tested in 3- and 15-month old children. So far the results confirm the excellent properties of the new pertussis vaccine.

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