

***In Vitro* Expression of the *ctxB* Toxin Gene Towards the Development of a DNA Vaccine Against Cholera**

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Abstract

The complete eradication of cholera is an unachievable goal because it is now firmly established that there are environmental reservoirs for *Vibrio cholerae*. Although there are effective treatments for this disease, they are expensive and impractical in times of epidemic. All these points lead to the fact that the development of a safe, cheap, and efficient vaccine is probably the best solution to the problem. A new generation of vaccine, termed DNA vaccine, would probably be a better alternative to the traditional vaccines. In this study, the focus is on the *ctxB*, the gene encoding the B subunit cholera toxin as a potential candidate for the DNA vaccine against cholera. The *ctxB* gene is required for the binding of the Cholera Toxin (CT) to the eukaryotic cell and facilitates the entry of the active toxin (CTXA) into the host cell which causes the profuse diarrheal symptom. The *ctxB* gene was cloned in pVax 1 (Invitrogen), and proven to be in the correct orientation. Subsequently, expression of the B subunit toxin *in vitro* was successfully carried out using 10 and 20 ml of Effectene™ (Qiagen) reagent with 0.4 mg pVax/*ctxB*, 90 hours post transfection in COS-7 cells. The results showed that the pentamer size of the *ctxB* (58 kDa) was expressed instead of its single monomer of 11.6 kDa. This means that the mechanism of the eukaryotic expression system *in vitro* was able to produce this end-product by successfully processing the binding of five single peptides of the *ctxB*. Further investigations involving this potential DNA vaccine against cholera is currently underway, including the production of antibody in animal models.

Introduction

Cholera is an acute infection of the intestine caused by the bacterium *Vibrio cholerae*. It is characterized by numerous, voluminous watery stools (rice water stools), often accompanied by vomiting (bicarbonate loss). Although there are measures to control the spread of cholera, we are still plagued by this dreadful disease. A half million cases have been reported to the World Health

Organization (WHO) between 1998 to 2000 with 20,000 deaths. The reported overall case-fatality rate (CFR) for 1999 has remained stable at 3.6%, in which the Asian continent showed a 61% increase compared to 1998. To make matters worse, the number of deaths notified to WHO due to this disease has also doubled (WHO, 2000). For Malaysia, the most recent major outbreak of cholera was in May 2001 with 94 confirmed cases and one death (The Star, 2001). The high number of cholera cases reported signifies cholera to be one of the significant gastrointestinal diseases in Malaysia.

The mechanism by which *Vibrio cholerae* causes cholera is well established. Its virulence factors are cholera enterotoxins (*ctxAB*), zonula occludens toxin (*zot*) (Fasano *et al.*, 1991), accessory cholera enterotoxin (*ace*) (Trucksis *et al.*, 1993), heat stable and heat labile enterotoxins (LT), toxin coregulated pilus (*tcp*), core encoded pilin (*cep*), hemolysin/cytotoxin and Shiga-like toxin (O'Brien *et al.*, 1984). The cholera *ctxAB*, *zot*, *ace*, *cep* and another gene *orfU*, are found to be located on a 4.5 kb segment termed the CTX element in which the order of the genes is *cep-orfU-ace-zot-ctxAB* (Ghosh *et al.*, 1996). This cholera enterotoxin includes a polymeric protein with a molecular weight (MW) of 85,000 composed of two major regions, A and B. These two regions produce the most important toxins that are responsible for the acute diarrhoea symptoms, *ctxA* and *ctxB*. The A and the B regions of the cholera enterotoxin are arranged in a single transcriptional unit with the A cistron preceding the B cistron (Ghosh *et al.*, 1996). The A region (27,000 MW), responsible for the biologic activity of the enterotoxin, is linked by noncovalent interactions with the B region (58,000 MW), which is composed of five identical noncovalently associated peptide chains of 11,600 MW. The A subunit, although synthesized as a single polypeptide chain, is usually proteolytically nicked to form two disulfide-linked polypeptides, A1 (22,000 MW) and A2 (5,000 MW) (Mekalanos, *et al.*, 1983). The A1 enzyme promotes the activation of adenylate cyclase by inactivating a GTPase in the cyclase complex which leads to the excessive production of cyclic adenosine 51-monophosphate (cAMP). Subsequent cAMP-mediated cascade of events lead to the hypersecretion of chloride and bicarbonate followed by water, resulting in the characteristic isotonic voluminous cholera stool. The B region has high affinity for a G_{M1} gangloside receptor on the surface of toxin sensitive cells. It alone has no enterotoxic activity, but facilitates the entry of the A subunit into the eukaryotic cell membrane (Spangler, 1992; Ganguly and Kaur, 1996).

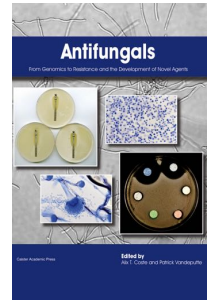
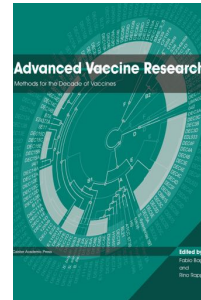
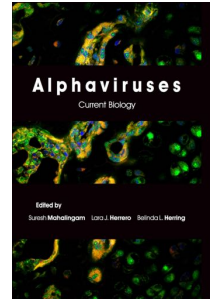
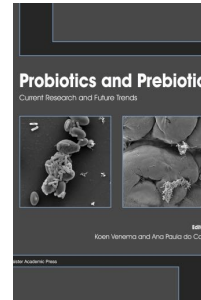
Despite the effective treatments against cholera, the disease still afflicts people in the poor and developing countries. The best alternative is to provide a cholera vaccine that will immunize the public before an outbreak occurs. Several cholera vaccines have been created (Ghosh *et al.*, 1996; Favre *et al.*, 1996; Taylor *et al.*, 1997;

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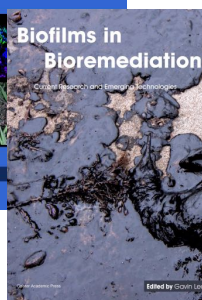
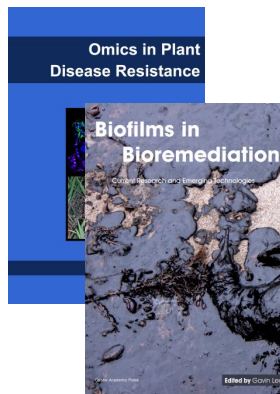
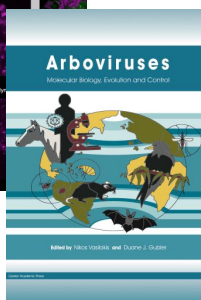
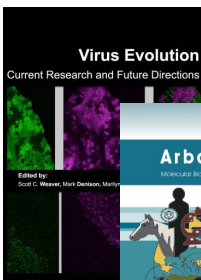
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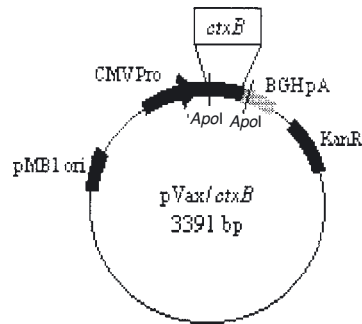


FIG. 1. Diagram of the pVax1 vector with the cholera *ctxB* gene insert of 391 bp. pMB1 ori: Origin of replication; CMV Pro: CMV immediate-early promoter, *ctxB*: cholera toxin B gene insert, BGH pA: bovine growth hormone gene (for polyadenylation signals), Kan R: Kanamycin resistance gene. Digestion with *Apo I* produces 410 bp and 2981 bp bands.

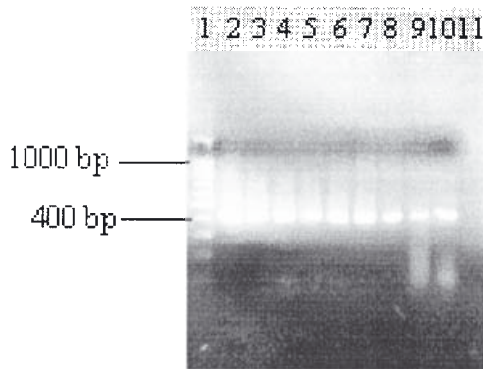


FIG.2. Analysis of PCR products using primers SL1 and SL2. Lane 1 contains a 100 bp molecular weight marker (Fermentas), lanes 2 to 10 represent PCR products from different samples of *Vibrio cholerae*, and lane 11 shows the negative control.

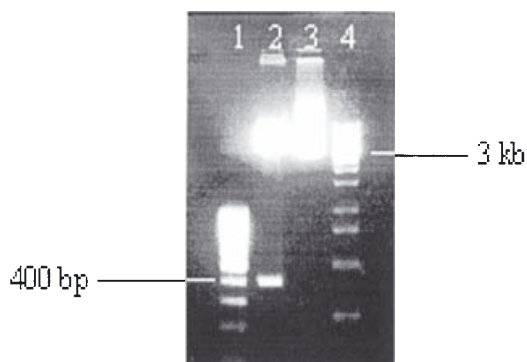


FIG 3. Restriction digest analysis of pVax/*ctxB* and pVax/con using *Apo I*. Lane 1 and lane 4 contain 100 bp and 1 kb molecular weight marker respectively. Lane 2 shows the digestion product of pVax/*ctxB* and lane 3 represents the digestion of pVax/con.

Trach *et al.*, 1997), yet they are not effective and potent enough (WHO, 2000). Fortunately, due to the unmet needs of the old and new epidemics of infectious diseases, as well as the advent of molecular biology, a new era of vaccinology has been stimulated. This novel approach employs plasmid DNA, engineered to express one or more genes of the pathogen in mammalian cells. The proteins expressed lead to a stronger and persistent cell-mediated and humoral immune response compared to the conventional and the recombinant vaccines. This unique approach to immunization, termed DNA vaccination, may overcome deficits of the traditional antigen-based methods and provide safe and effective vaccines.

The purpose of this study is to produce a functional DNA plasmid vaccine carrying a *ctxB* gene which would be expressed in mammalian cell culture, as a potential DNA vaccine candidate against cholera. The B subunit of cholera subunit was chosen as the antigen to induce immunity against cholera due to several reasons: (i) Infected individuals mount an immune response to the cholera toxin primarily recognize the B subunit (Heyningen, 1974) which means that it has a possible role in immunity to natural infections. (ii) The B subunit alone has no enterotoxic activity, but crucial in facilitating entry of the A subunit into the eukaryotic cell membrane; the isolated A subunit is nonreactive with intact cells (Spangler, 1992). Thus, it poses no harm to the host. (iii) The nucleotide sequence of the B subunit has 80 percent homology with that of *Escherichia coli* subunit B heat-labile enterotoxin (LTB) (Spangler, 1992; Lockman and Kaper, 1983). Therefore, it might also induce immunity against the LTB toxin in cells with coupled infections. (iv) The most important reason is that random mutations in the *ctxB* gene are not common (Olsvik, 1993). For these reasons, CTXB is deemed suitable as an antigen to induce immunity against cholera.

Results

Confirmation of Correct PCR Product

Nine randomly chosen *Vibrio cholerae* samples were subjected to PCR and all of the samples produced the expected *ctxB* amplicon of 391 bp (Figure 2). The PCR product was cloned into the pCR®2.1 TOPO plasmid and sent for automated sequencing. The DNA sequence analysis confirmed that the amplicon was the *ctxB*, with the AUG sequence upstream of the real initiation codon of the *ctxB* gene being eliminated. The analysis also revealed that the strain belongs to the Classical biotype of *Vibrio cholera*, as shown in our previous study (Hon *et al.*, 2000). These results show that the new primers have successfully amplified the gene of interest.

Cloning of *ctxB* Gene into pVax

The *ctxb* segment from the pCR®2.1 TOPO was then subcloned into the *Hind* III site of linearized pVax. Upon digestion analysis with *Apo* I, the purified pVax/*ctxB* plasmid showed a distinct *ctxB* band at 410 bp and the linearized plasmid at 2.9 kb while the pVax/con did not show the existence of an insert. The production of the 410 bp band proved that the insert was in the correct orientation (Figures 1 and 3).

A highly purified pVax/*ctxB* and pVax/con with A₂₆₀/A₂₈₀ ranging from 1.7 to 1.9 extracted using the Endofree™ Plasmid Kit (Qiagen) were used for the transfection experiments. Pyrogenic endotoxins were minimized from the plasmid preparation for the transfection of sensitive eukaryotic cells such as the COS-7. Even though high levels of RNase A were used in the first step of the plasmid purification procedure, the eluted plasmid was completely free from RNase. Before proceeding with the transfection experiments, the endotoxin-free plasmid was checked for the insert and was sequenced again to confirm that no mutation has occurred in the *ctxB* gene that might disrupt the expression of the protein. Results from sequence analysis proved that the cloned *ctxB* gene contains no mutation, with no extra AUG sequence upstream of the initiation codon, and is in the correct orientation for transcription (Figure 4).

Expression of the *ctxB*

Using the Effectene™ reagent, the cells were observed to be healthy with no signs of toxicity or contamination seen during the incubation period and before extraction. *ctxB* gene expression was not seen after 48 hours of incubation from all the lanes (figure not shown). However, after ≈ 90 hours of transfection, faint bands at 58 kDa that were parallel to the *ctxB* band from the *Vibrio cholerae* positive control were seen in lanes 7 and 8 (Figure 5). The results showed that the *ctxB* gene has been expressed from the pVax 1 plasmid using 0.4 μg of DNA with 10 and 20 μl of the Effectene reagent after 90 hours of incubation.

The reagent and transfection conditions yielding the positive result were repeated to validate and confirm the expression of the *ctxB* gene from the pVax 1 vector. For this purpose, the vector without the insert (pVax/con) and the extract of another diarrheal causing bacteria, *Escherichia coli*, were used. In this experiment, COS-7 cells were transfected with 0.4 μg of pVax/*ctxB* and pVax/con with 10 and 20 μl of the Effectene™ reagent. Extracts were prepared at 90 hours post transfection and analyzed for the expression of the *ctxB* gene product by Western blot. Figure 6 is a Western blot analysis which shows a specific 58 kDa gene product that was expressed at low levels in the cells transfected with pVax/*ctxB* but not in cells transfected with pVax/con and in untreated cells (no-reagent-no-DNA control). The specificity of the *ctxB* gene control from the *Vibrio cholerae* isolate is shown when no band at 58 kDa appeared in the *Escherichia coli* extract lane. The results of figures 5 and 6 show that the *ctxB* gene has been successfully expressed by the pVax/*ctxB*.

Discussion

Primers CTX7 and CTX9B adopted from Olsvik Ø., *et al* (17), used in our previous study were not suitable to produce the PCR product for expression as these primers include the AUG sequence located 4 bases upstream of the initiation codon of the *ctxB* which may interfere with the translation process. Thus, primers SL1 and SL2 were designed to overcome this problem. Restriction endonuclease sites and a Kozak consensus sequence were also added to primers SL1 and SL2 to facilitate cloning

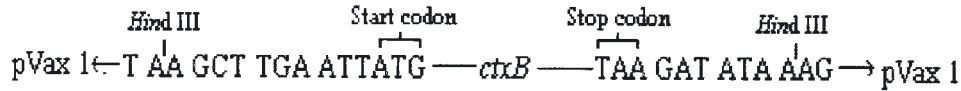


FIG 4. Fragment of *ctxB* in pVax 1 vector.

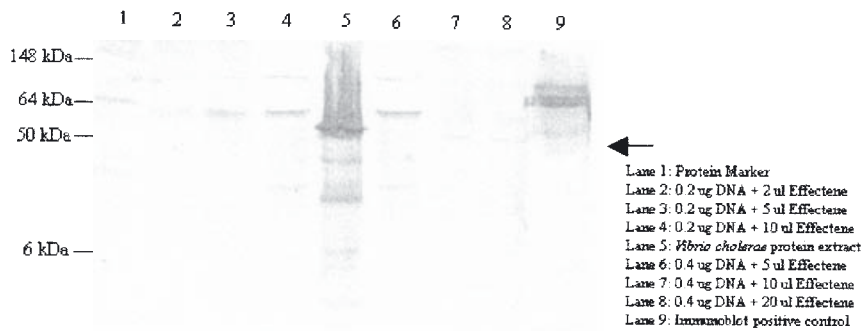


FIG 5. Western blot analysis of COS-7 cells transfected with pVax/*ctxB* using the Effectene (Qiagen) method after 90 hours post transfection. Lane 1 contains a prestained broad range protein molecular weight marker. Lanes 2 to 4 and 6 to 8 represent the protein extracts of transfected COS-7 cells with variable amount of DNA and Effectene reagent. Lane 5 shows a protein extract of *Vibrio cholerae* isolate and lane 9 contains a IgG monoclonal antibody positive control provided by the detection kit. Arrow indicates the 58 kDa band of *ctxB*. Transfection experiments represented in lanes 7 and 8 show positive result for the expression of the *ctxB* gene.

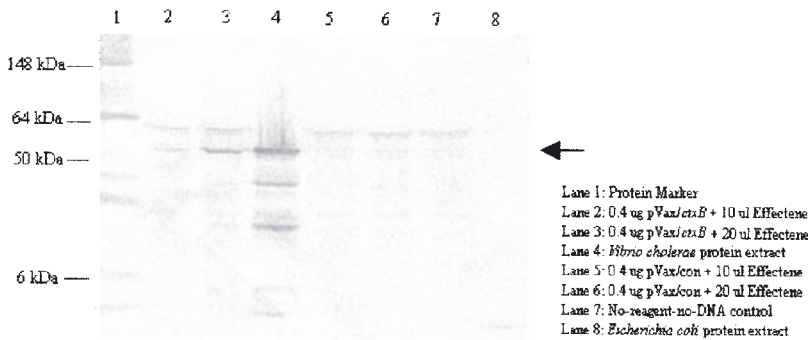


FIG 6. Confirmation of pVax/*ctxB* expression Lane 1 contains a prestained broad range protein molecular weight marker. Lanes 2 and 3 represent the extracts of transfected COS-7 cells using 0.4 μ g pVax/*ctxB* with 10 μ l and 20 μ l Effectene, respectively. Lane 4 shows a protein extract of *Vibrio cholerae* isolate. Lane 5 and 6 show the extract of transfected COS-7 cells using 0.4 μ g pVax/con with 10 μ l and 20 μ l Effectene, respectively. Lane 7 represents the untreated cells and lane 8 is a protein extract of *Escherichia coli*. A band at 58 kDa appears in lanes 2 and 3 which are parallel to the *ctxB* band on the *Vibrio cholerae* protein extract shows that the *ctxB* has been expressed by the pVax/*ctxB*. No band appears at 58 kDa in the *Escherichia coli* lane showing that the monoclonal anti-*ctxB* IgG is specific for the B subunit of *Vibrio cholerae*. Arrow indicates the 58 kDa band of *ctxB*.

and to increase the efficiency of expression. The best annealing temperature for both primers was found to be at 52°C. Upon sequencing, primers SL1 and SL2 were confirmed to produce the *ctxB* gene. These primers produced 391 bp of PCR product, 69 bp less than the one produced by CTX7 and CTXB9. Out of this 391 bp product, 374 bp constitute the open reading frame (ORF) of the *ctxB* gene whereas the extra 17 bp are the *Hind* III restriction enzyme sites sequences.

The pVax/*ctxB* construct contains two *Hind* III restriction enzyme cutting sites flanking the *ctxB* gene, one 10 bp upstream of its initiation codon and the other one 6 bp downstream of its stop codon. There is another stop codon in the pVax at the *Xba* I restriction site (88 bp downstream of the *ctxB* stop codon), which is supposed to avoid a "read through" during the translation process. However, this extra stop signal is not in frame with the *ctxB* gene. It was discovered that there were two unique *Apo* I restriction sites at 10 bp downstream of the *ctxB* start codon and 48 bp downstream of its stop codon (located on the plasmid). Restriction cuts by this enzyme to the plasmid with the *ctxB* inserted in the correct orientation would yield 2 bands; 410 bp and 2981 bp, as shown in figure 1. However, plasmids with *ctxB* inserted incorrectly would yield 64 bp and 3327 bp bands. A plasmid without an insert would produce a band at 3391 bp (linearized plasmid). Thus, this enzyme was used to identify clones that have the insert and the orientation of the insert simultaneously.

The *ctxB* gene was successfully expressed in COS-7 cells using the Effectene™ (Qiagen) transfection method 90 hours post transfection. Usually, only 48 hours of incubation was sufficient for a gene to be expressed (Sambrook, 1989). This delay might be due to the low transfection efficiency and a very low level of expression after 48 hours post transfection in which the expressed protein could not be detected by the immunoblot immunoperoxidase detection method (Western MAX™ Kit, Amresco). It is likely that after 90 hours post transfection, enough of the expressed protein has accumulated in the cells and the amount was sufficient for the detection. Other methods of detection, such as chemiluminescent or radioactive which are more sensitive could probably be used to detect the expression earlier even at low levels.

It was found that 0.4 µg of pVax/*ctxB* with 10 and 20 µl of the Effectene™ reagent showed positive results for expression. Upon repeating the experiment to confirm the earlier results, it was observed that 0.4 µg pVax/*ctxB* with 20 µl of the Effectene™ reagent gave the most optimal conditions with a higher intensity of the *ctxB* band on the immunoblot compared to the 0.4 µg pVax/*ctxB* with 10 µl of the transfection reagent seen. Even though the protein expressed could be detected, it was still expressed at a lower level compared to the *ctxB* from the crude protein extract of *Vibrio cholerae*. Nevertheless, one should not correlate or predict the efficiency of pVax/*ctxB* expression *in vitro* to the *in vivo* study that will follow later, as the expression systems and the environments are not similar. There is less evidence demonstrating that the level of expression *in vitro* correlates directly with immunogenicity *in vivo*. Constructs that are more potent *in vivo* have

generally been shown to express better *in vitro*; however, the converse may not be true, and systematic studies of this issue have not been reported (Caulfield *et al.*, 1998). In addition, only small amounts of the *ctxB* protein or any immunogen of DNA vaccine could elicit immune responses. This trend is due to the adjuvant effect of the DNA vaccine that could amplify the response (Kowalczyk and Ertl, 1998; Shroff *et al.*, 1999).

In this study, a crude protein extract of *Vibrio cholerae* was used as a positive control to determine the correct size of the *ctxB* compared to the expressed *ctxB* from the pVax/*ctxB*. The expression results showed that the most intense band of the *Vibrio cholerae* protein extract was at 58 kDa, which is the size of the B subunit pentamer protein. However, no bands were seen in the lane with *Escherichia coli* crude protein extract which means that the primary monoclonal *ctxB* IgG (anti-mouse) has specifically bound to the *ctxB* protein on the PVDF membrane. All other bands which appeared were considered as background staining which was due to the poor signal-to-noise ratio. Overdevelopment of the substrate in the detection step could also be one factor of the high background.

Escherichia coli, which has high degree of homology of its B subunit heat labile enterotoxin (*etxB*) with the *ctxB* (Spangler, 1992), was used to validate the expression of the *ctxB* gene from the *Vibrio cholerae*. It has been estimated that there is 82% homology at the amino acid level between the *etxB* and *ctxB* (Williams *et al.*, 1999). The absence of any band in the *Escherichia coli* protein extract lane, and the appearance of the 58 kDa band of *ctxB* in the *Vibrio cholerae* protein extract lane in the Western blot analysis showed that the primary monoclonal *ctxB* IgG was only specific for the *Vibrio cholerae* B subunit enterotoxin.

As have been already mentioned, the *ctxB* gene comprises of five identical B subunits, with a size of 11.6 kDa each. The expressed protein of the *ctxB* gene might appear as 11.6 kDa, or the total of the five subunits which is 58 kDa. This study showed that only the 58 kDa size of the protein was successfully expressed. The presence of the 58 kDa CTXB shows that the expression system mechanism has bound the single peptide of the *ctxB* and this might give implications to the immunogenicity of the protein *in vivo*. Another important consideration upon expressing of any exogenous protein from mammalian cells is the glycosylation factor. Secreted proteins, membrane proteins, and proteins targeted to vesicles or certain intracellular organelles are likely to be glycosylated (Varki and Freeze, 1994). When expressing a glycosylated protein in a heterologous expression system, it may be desirable to determine whether the protein is glycosylated properly. An improper glycosylated protein might appear at a different size than the expected one. In this study, the glycosylation of the protein was not being examined. However, the extent or the pattern of the protein glycosylation, if it ever occurred, would not appear to cause any problems as the expected size of CTXB, 58 kDa, was obtained.

This study has shown that the expression of CTXB protein from pVax 1 carrying the *ctxB* gene was obtained by using 0.4 µg of the DNA with 10 and 20 µl of Effectene

reagent in COS-7 cells. This expression study also shows that the pVax/*ctxB* construct developed was able to function *in vitro* even with a low amount of protein expressed. The functionality of the pVax/*ctxB* *in vivo* is currently under study. It is hoped that the B subunit of this cholera toxin when expressed in animals will establish adequate levels of antibody and a primed population of cells, which would rapidly expand in numbers upon second contact with the "real" pathogen. The detection of immune responses against the antigen will prove that the pVax/*ctxB* is able to induce immunogenicity in animal models.

Experimental Procedures

Bacteria and Plasmid

Twenty *Vibrio cholerae* clinical isolates (one each of *Inaba* and *Bengal*, and 18 of *Ogawa* from the 1995, 1996 and 1998 outbreak strains) were obtained from the Medical Microbiology Department, Universiti Malaya and the Bacteriology Division, Institute for Medical Research, Malaysia. Some of the bacteria were maintained in prepared thiosulphate-citrate-bile salts sucrose (TCBS) selective-differential agar (Bacto®, Difco Laboratories) while others were stored on a slant agar covered with mineral oil at room temperature. Genomic DNA of the bacteria was extracted using Qiagen Tissue Extraction Kit Method, according to the manufacturer's protocol.

Plasmids pCR®2.1 TOPO and pVax 1 were purchased from Invitrogen. The pCR®2.1 TOPO is a cloning vector while pVax 1 is a eukaryotic expression vector derived from pcDNA 3.1 (Invitrogen) which is specifically designed for use in the development of DNA vaccines. This vector contains the cytomegalovirus (CMV) immediate early promoter and 3' polyadenylation signal from the bovine growth hormone. TOP 10 F' competent cells which were used in the subsequent cloning reaction were supplied together with the plasmids.

Synthetic Oligodeoxynucleotide Primers

Oligodeoxynucleotides used as primers for amplification were custom made by Operon Technologies, Inc., USA. The sequence of the primers are SL1 (5'-CCC AAG CTT GAA TTA TGA TTA AAT TAA AAT TTG-3') and SL2 (5'-CCC AAG CTT TAT ATC TTA ATT TGC CAT ACT AA-3'). These primers were developed to exclude the AUG sequence located upstream of the *ctxB* initiation codon, as reported in our previous study (Hon *et al.*, 2000). A *Hind* III site was incorporated in these primers for purposes of subcloning and analysis, and a Kozak consensus sequence was added preceding the initiation codon for efficient protein expression in mammalian cells. Successful amplification using these primers would produce a 391 bp product of the *ctxB* gene.

Polymerase Chain Reaction (PCR)

For amplification, 14 ng of template DNA, 1 X buffer (100 mM Tris-HCl, 500 mM KCl, 1% Triton X), 1.5 mM MgCl₂, 1.0 mM dNTP mix (10 mM each dNTP), 20 pmol of each primer and sterile deionised water for a final volume of 25 µl (after the addition of polymerase) were used. The reaction mixtures were heated to 95°C for 2 minutes to

denature the DNA and 1.0 U of *Taq* polymerase was subsequently added. All of the PCR components were purchased from Promega. The samples were subjected to 28 cycles of denaturation (95°C for 1 minute), annealing (52°C for 1 minute), and elongation (72°C for 1 minute) using a thermocycler (Mastercycler Gradient, Eppendorf). An additional elongation step at 72°C for 10 minutes was also included. The PCR products were analysed using agarose electrophoresis and quantified using a UV-VIS spectrophotometer (SmartSpec™ 3000, Bio-Rad).

Construction of Plasmid for *ctxB* Gene Expression

The fresh PCR product of 391 bp which gave a discrete band on an agarose gel and had high purity was used for cloning into the pCR® 2.1 TOPO plasmid. After confirmation of the cloned *ctxB* gene by sequencing, the plasmid was digested with *Hind* III enzyme and the fragment was extracted from agarose gel using QIAEX® II Gel Extraction System (Qiagen, Germany). The purified fragment was cloned in the *Hind* III site in the polylinker of plasmid pVax 1. The correct orientation of the insert was checked by restriction digest using *Apo* I and by DNA sequencing. The pVax 1 vector containing the *ctxB* gene was designated as pVax/*ctxB* (Figure 1). All vector constructions and analysis were carried out using standard techniques (19). Plasmid DNA was transformed in the *Escherichia coli* TOP 10 F' cells and cultured in Luria-Bertani (LB) medium in the presence of kanamycin and was purified using a Qiagen Endotoxin Free Plasmid Purification kit (Qiagen, Germany). The DNA concentration was determined by measuring the optical density at 260 nm, and the integrity of the plasmid as well as the absence of contaminating TOP 10 F' DNA or RNA were checked by agarose gel electrophoresis. DNA was stored at -20°C in deionised pure water.

Cell Transfection and Protein Expression

Expression of *ctxB* specific gene product by pVax/*ctxB* was evaluated by a Western Blotting technique in a transient expression system using COS-7 cells. The day before the transfection experiment, 6-well plates were seeded with 4.0 X 10⁵ cells per well in 2 ml of DMEM medium. The cells were incubated at 37°C in a 5% CO₂ incubator until they became 40-80% confluent on the day of transfection. Cells were transfected using the Effectene™ reagent (Qiagen, Germany) according to the manufacturer's optimization protocol, which used 0.2-0.4 µg DNA and 2-20 µl of the Effectene™ reagent in the presence of 10% fetal calf serum. A time scale study (48 versus 90 hours post transfection incubation) was carried out to determine the effect of time on the expression. *Vibrio cholerae* protein extract and plasmid pVax/con, a pVax 1 plasmid without insert, were used as the positive and negative controls, respectively.

The proteins were extracted and loaded in an unstained 12% SDS-Polyacrylamide gel. Electrophoresis was carried out using a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) for 1 hour at 100V. Western™ MAX (Amresco) kit was used to detect the protein expressed on the immunoblot. Basically, this procedure detects the mouse monoclonal anti *ctxB* primary antibody complexed

with the antigen immobilized on the PVDF membrane using a DAB substrate (3, 3'-diaminobenzidine) which gives an alcohol insoluble brown precipitate at the reaction site.

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