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Priming immunization against cholera toxin and *E. coli* heat-labile toxin by a cholera toxin short peptide-β-galactosidase hybrid synthesized in *E. coli*

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A synthetic oligodeoxynucleotide encoding for a small peptide was employed for the expression of this peptide in a form suitable for immunization. The encoded peptide, namely, the region 50–64 of the B subunit of cholera toxin (CTP3), had previously been identified as a relevant epitope of cholera toxin. Thus, multiple immunizations with its conjugate to a protein carrier led to an efficient neutralizing response against native cholera toxin. Immunization with the resulting fusion protein of CTP3 and β-galactosidase, followed by a booster injection of a sub-immunizing amount (1 μg) of cholera toxin, led to a substantial level of neutralizing antibodies against both cholera toxin and the heat-labile toxin of *Escherichia coli*.
Key words: recombinant DNA/synthetic vaccine/*lacZ* fusion/cholera-*coli* vaccine/gene expression.

Introduction

Novel approaches to vaccine production include two main avenues of research: the use of synthetic peptides which correspond to relevant antigenic determinants for induction of anti-viral or anti-bacterial immunity (Arnon *et al.*, 1983), and the application of genetic engineering methodology for insertion of the relevant genes into an appropriate vector (e.g., vaccinia virus) that could be utilized for vaccination (Smith *et al.*, 1983; Panicoli *et al.*, 1983). In the present study we attempted to bridge the synthetic and recombinant DNA approaches by using a product of a synthetic cholera toxin-relevant 'gene' fused to a truncated *lacZ*, to elicit an anti-cholera immune response.

Cholera toxin is an 84 kd protein composed of one A subunit of 27 kd and five B subunits each of 11.6 kd. The A subunit, although synthesized as a single polypeptide chain, is usually proteolytically nicked to form two disulphide-linked polypeptides: A_1 (22-kd) and A_2 (5-kd) (Gill, 1976; Mekalanos *et al.*, 1979). The A_1 polypeptide is an enzyme that promotes the activation of adenylate cyclase in target cells by catalysing the ADP-ribosylation of a GTP-binding regulatory component of the cyclase complex (Gill and Merlin, 1978). The resulting accumulation of cyclic AMP in the intestinal mucosa leads to the severe fluid loss characteristic of cholera. Each B subunit has a high binding affinity for the toxin's cell surface receptor, ganglioside GM_1 (Holmgren *et al.*, 1975). Neutralizing antibodies raised against the holotoxin react mainly with the B subunit (Finkelstein, 1973). Moreover, antibodies to the B subunit are capable of neutralizing the biological activity of the intact toxin, and hence its native pentamer (choleragenoid) is currently being used for vaccination (Glass *et al.*, 1984). However, the protection afforded by it, or even by the intact isolated cholera toxin or toxoid, is incomplete and of relatively short duration, and it has been proposed that synergism between the toxin and other components of *Vibrio cholerae* is necessary for higher efficacy (Svennerholm *et al.*, 1982). It has also been argued that only a live vaccine will be effective in inducing the essential local immune responses in the intestine (Levine *et al.*, 1979).

In recent studies (Mekalanos *et al.*, 1983; Kaper *et al.*, 1984) attempts were made to use the recombinant DNA methodology to prepare a live oral cholera vaccine. A pathogenic strain of *V. cholerae* was attenuated by deletion of DNA sequences encoding the A_1 subunit of the cholera enterotoxin. A restriction endonuclease fragment encoding the A_1, but not the A_2 or B sequences, was deleted *in vitro* from cloned cholera toxin genes. The mutation was then recombined into the chromosome of *V. cholerae* Ogawa 395, a pathogenic strain that confers complete immunity to subsequent infection following an initial clinical infection. The resulting strain, however, still does not offer an acceptable vaccine; although of significant protective capacity, it caused diarrhoeal problems in more than half of the human volunteers (Kaper *et al.*, 1985).

In our laboratory the synthetic approach to vaccination was evaluated (Jacob *et al.*, 1983). As reported previously, six peptides corresponding to various regions of the cholera toxin have been chemically synthesized and used for immunization. The most effective peptide for induction of anti-cholera toxin antibodies was the region 50–64 denoted CTP3. Antibodies against this 15 amino acid residue peptide inhibited efficiently the biological activity of cholera toxin, as manifested in both secretion of fluid into intestinal loops and in adenylate cyclase induction (Jacob *et al.*, 1984a). Furthermore, the sequence of this peptide is completely homologous to the corresponding region in the heat-labile toxin of pathogenic strains of *E. coli*. Consequently, antibodies to the synthetic CTP3 were efficient in cross-reacting with, and neutralizing partially purified *E. coli* LT from several strains (Jacob *et al.*, 1984b). In some cases these antibodies were even more efficient than antibodies to intact cholera toxin.

The working hypothesis here was that the expression of this peptide by bacteria other than *V. cholerae* may provide an appropriate agent for induction of immunity towards cholera and *E. coli* toxins, and that this could be approached by recombinant DNA techniques.

To allow *E. coli* to synthesize CTP3, we constructed plasmids containing a synthetic DNA sequence encoding this peptide fused, in phase, to a truncated *lacZ* gene. The CTP3-β-galactosidase hybrid proteins specified by these plasmids can be more easily detected, assayed and isolated than the free CTP3. Moreover, previous studies showed relatively short peptides fused to β-galactosidase to be immunogenic (Weinstock *et al.*, 1983). One of the plasmids constructed coded for a fused protein initiated three codons upstream to the CTP3 coding sequence. In another plasmid, the CTP3-β-galactosidase coding sequence was introduced within the *E. coli ompF* gene, so as to specify a product initiating with the *ompF* signal peptide that may function to export

the protein into the bacterial outer membrane (Weinstock *et al.*, 1983). The results showed the hybrid β-galactosidases were efficiently recognized by antibodies raised against CTP3, and injection of gel-purified CTP3-β-galactosidase led to an efficient priming immunization against both cholera toxin and the heat-labile toxin of *E. coli*.

Results

Plasmid constructions

The two plasmids constructed for the expression of CTP3 fused to β-galactosidase are shown in Figure 1A.

The vector used for constructing pAM1 is a derivative of pMC1403 (Casadaban *et al.*, 1980) with an *Eco*RI-*Bam*HI fragment containing the λP_R promoter sequence joined to the *lac* operator and a synthetic ribosome-binding sequence, followed by an initiation codon in frame with a truncated *lacZ* gene. The efficiency of the composite regulatory sequence is indicated by the high level of β-galactosidase specified by this vector (Leitner and Caruthers, in preparation). The unique *Bam*HI site overlapping the initiator ATG was used to introduce a 54 bp synthetic DNA duplex coding for CTP3 (Figure 1C).

The design of the DNA duplex includes single-stranded extensions allowing its ligation into a *Bam*HI site. These termini extend the CTP3 coding sequence with codons for Met, Asp and Pro at the 5′ end, and with a Met codon at the 3′ end. The latter Met residue can be useful for the regeneration, if desired, of a CTP3-like peptide by cyanogen bromide cleavage of the hybrid protein. The synthetic DNA sequence is based on the preferred codon usage in *E. coli* (Grosjean and Fiers, 1982) and deviates from the corresponding sequences in the genes coding for the subunit of cholera and *E. coli* enterotoxins. A *Kpn*I site was incorporated into the sequence to facilitate the analysis of recombinants. In addition, care was taken to avoid possible formation of secondary structures that could sequester the Shine-Dalgarno and ATG sequences. Although the synthetic DNA can be inserted into the vector in two orientations, only the correct orientation will yield a fused β-galactosidase. In the opposite orientation, the reading frame is terminated within the insert.

For the construction of pAM2, the synthetic DNA fragment replaced the sequence between the two closely located *Bam*HI sites of pORF1 (Weinstock *et al.*, 1983). Introduced in this way, the sequence of the cloned fragment matches the reading frames of both the upstream *ompF* and the downstream *lacZ* genes.

Expression of the ctp-lacZ hybrid genes

Expression was monitored by β-galactosidase assays of permeabilized transformed bacteria, and by immunoblot analysis of electrophoretically resolved protein extracts.

β-galactosidase activity assays (Miller, 1972) of *E. coli* MC1061 transformed with pAM1 gave 7500 − 8500 units and the activity of TK1046 transformed with pAM2 was 3500 − 5000 units.

The results of immunoblot analysis of pAM1 and pAM2 transformants (Figure 2) show a high-intensity, specific labelling with the antiserum against the CTP3 peptide, of a protein band corresponding in position to β-galactosidase. (Immunoblotting with this antiserum resulted in a few additional labelled bands, but these were of low intensity, and probably non-specific, since they were observed with normal serum as well.) This CTP3-β-galactosidase fusion product gave a similar strong reaction with antiserum against pure β-galactosidase, and a weak reaction with antiserum against intact cholera toxin.

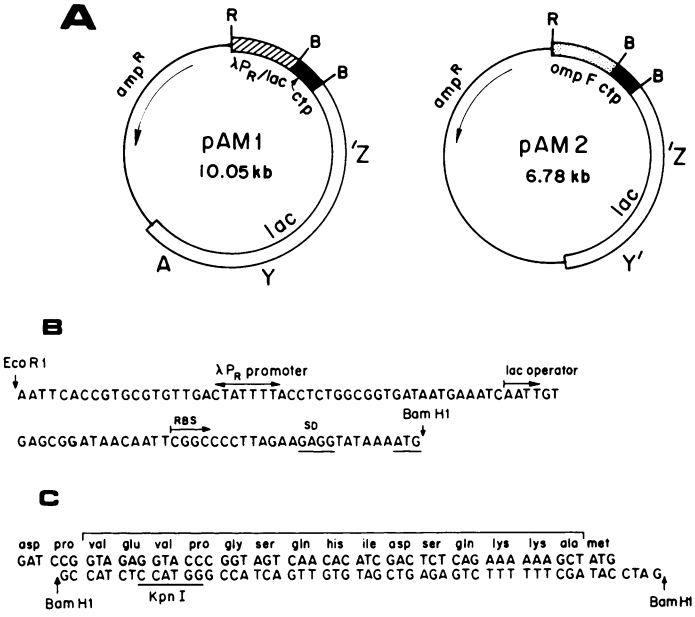

Fig. 1. Structure of vectors for the expression of CTP3-β-galactosidase fusion products. **(A)** General structure of expression vectors. Hatched box, composite λP_R/Lac promoter (in pAM1); dotted box, *ompF* promoter and amino-terminal coding sequence (in pAM2); filled boxes, CTP3 coding sequence (ctp); open boxes, parts of the *lac* operon; line, part of pBR322 sequence. Restriction sites: R, *Eco*RI, B, *Bam*HI. **(B)** Sequence of the regulatory region in PAM1. Arrows designate cleavage sites by the indicated restriction enzymes. RBS, ribosome-binding site; SD, Shine and Dalgarno sequence. **(C)** Sequence of the synthetic, 54 bp DNA duplex coding for CTP3 (ctp). The amino acid sequence corresponding to CTP3 is overlined. Arrows designate cleavage sites by *Bam*HI.

Priming immunization

Immunization of rabbits with whole cells of *E. coli* TK1046 transformed with pAM2 ($1-5 \times 10^9$ cells/rabbit/injection), did not lead to an immune response against CTP3, but did result in antibodies against β-galactosidase (results not shown). In the case of the *E. coli* MC1061 transformed with pAM1, the fused CTP3-β-galactosidase protein was purified from the bacterial extract and used to immunize rabbits. The results (Table I) show that although immunization with this protein as such did not lead to a significant titre of anti-cholera toxin antibodies (Group D), it did result in an efficient priming immune response. Thus, when following the immunization procedure, or even after a single administration of the fused CTP3 protein, the rabbits were boosted with a minute quantity (1 μg) of intact cholera toxin, a substantial antibody level was obtained (Groups A and B). These antibodies cross-reacted to a high extent with the heat-labile toxin (LT) of *E. coli* and were capable of efficiently neutralizing the biological activities of cholera toxin. The extent of toxin neutralization by these antisera reached values of 50 − 90%, as compared with 100% inhibition by hyperimmune anti-CT serum, which served as a positive control and was included in each assay (Group H). A similar effect was obtained when boosting was performed with LT (Group C). The antibody titre obtained in this case was similar to those observed in Groups A and B, but in each case the reaction with the homologous toxin was slightly higher than the cross-reaction. The antibodies of Group C were very efficient in neutralization of cholera toxin, as manifested in the inhibition of both fluid secretion into intestinal ligated loops, and adenylate cyclase induction.

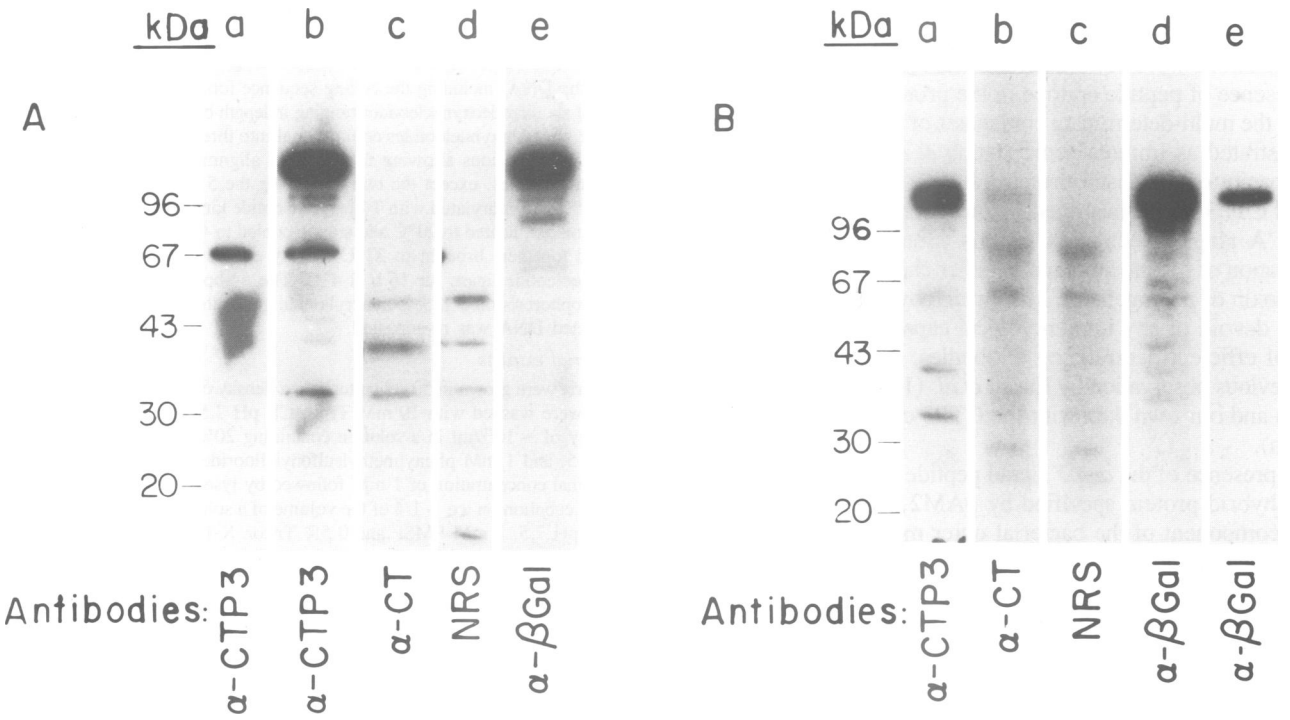

Fig. 2. Immunoblot analysis of bacteria containing plasmids pAM1(A) and plasmid pAM2(B), with various antisera. **(A) Lane a**, parent bacterial strain MC1061 with no inserted plasmid, **lanes b – e**, bacterial strain MC1061 containing pAM1, were developed with the indicated polyclonal serum. **(B) Lanes a – d**, bacterial strain TK1046 containing pAM2, developed with the indicated polyclonal serum; **lane e**, purified β-galactosidse, developed with polyclonal anti β-galactosidase serum.

Table I. Priming effect achieved by immunization with CTP3-β gal fusion protein.

Group	Priming antigen	Boosting antigen	Number of responders[a]	Antibody titer[b]		CT-induced cAMP reduction (%)	Inhibition of CT-induced fluid secretion (%)
				Versus CT	*Versus* LT		
A	CTP3-β gal (multiple inoculations)	CT (1 μg)	1/2	120 – 180	70 – 90	61 – 81	70 – 80
B	CTP3-β gal (single inoculation)	CT (1 μg)	2/2	120 – 180	90 – 150	65 – 93	50 – 93
C	CTP3-β gal (single inoculation)	LT (1 μg)	2/3	90 – 110	100 – 150	58 – 73	50 – 80
D	CTP3-β gal (multiple inoculations)	–	0/3	< 30	< 15	0	0
E	β gal (100 μg) (two inoculations)	CT (1 μg)	0/2	< 10	< 10	0	0
F	–	CT (1 μg)	0/2	< 10	0	0	0
G	–	LT (1 μg)	0/2	0	0	0	0
H	CT (100 μg)	CT (100 μg)	positive control	650 – 950	500 – 810	100[c]	100[c]

[a]Responders are defined as those rabbits whose sera lead to toxin neutralization (> 20%).
[b]Antibody titre is defined as the serum dilution yielding 50% binding. Numbers represent range in different bleedings of different rabbits in each group.
[c]Reduction in CT activity by positive control hyperimmune anti-CT, taken as 100% inhibition. All other inhibition values are relative to this value.

The priming effect thus obtained is specific to the CTP3 peptide sequence in the fused protein, since a similar pre-immunization with pure β-galactosidase (Group E), or a single inoculation of either CT (Group F) or LT (Group G), at a low dose without priming, gave no anti-toxin antibody response whatsoever.

Discussion

The data reported above demonstrate that a synthetic oligodesoxynucleotide encoding for a small peptide can be employed for the expression of this peptide in a form suitable for immunization. The encoded peptide, CTP3, constitutes a region that has been previously identified as a relevant epitope of cholera toxin (Jacob *et al.*, 1983).

In previous applications of the recombinant DNA approach to vaccine development, the genetic material inserted into the expressing vector has usually been of extensive length, encoding for at least an entire antigenic protein and sometimes including an entire viral genome (Smith *et al.*, 1983; Bachrach, 1982). An exception to this is a recent study in which a relatively short fragment of a clone was isolated and inserted into an *E. coli* expression plasmid, for potential use as a malaria vaccine (Young *et al.*, 1985). The starting point in the present study was the identification of a short epitope on the antigenic protein that could be encoded by a relatively short synthetic oligodeoxynucleotide. The advantage of this strategy lies in the ability to induce only a relevant immune response of a predetermined specificity, without resorting to the intact antigenic protein that might be toxic; furthermore, it could lead to better cross-reaction with related proteins (Jacob *et al.*, 1984b; Shapira *et al.*, 1984).

The soluble CTP3-β-galactosidase hybrid protein extracted

from the bacteria transformed with pAM1 did not lead to detectable anti-peptide antibody production even when two booster immunizations were administered. The reason for that might be the presence of peptide epitope in the protein only once, in contrast to the multi-determinant conjugates of CTP3 which had been demonstrated as immunogenic (Jacob *et al.*, 1983). However, the capacity of the fused protein to elicit an immune response toward CTP3 is demonstrated by the observed specific priming effect. A single inoculation of this protein, followed by administration of minute amounts of either cholera toxin or the heat-labile toxin of pathogenic *E. coli* (which by themselves are completely devoid of any immunological capacity) results in a high level of efficient neutralizing antibodies. This effect is similar to a previous observation by Emini *et al.* (1983) in the polio virus system and our own data with the CTP3 conjugates (unpublished data).

The presence of the *ompF* signal peptide at the amino terminus of the hybrid protein specified by pAM2, could make the protein a component of the bacterial outer membrane (Weinstock, 1984). Nevertheless, immunization with the intact transformed bacteria did not lead to anti-peptide immune response. This could be explained either by an insufficient quantity of the protein or by inefficient transport of the hybrid protein into the bacterial outer membrane as previously observed with other fusion products (Weinstock, 1984).

In the case of cholera and other diseases of the gastrointestinal tract, local immunity was demonstrated to be paramount (Newby, 1984). The advantage of the approach employed here is that the bacteria expressing the relevant peptide may be used as oral vaccine. This can be achieved either by the use of an organism with the peptide exposed on its surface, or when the fusion protein remains cytoplasmatic but would be released after disintegration of the bacteria by digestive enzymes. The use of a non-pathogenic *E. coli* could be advantageous to the use of a genetically manipulated *Vibrio* (Kaper *et al.*, 1984).

Also of relevance is the high cross-reactivity of the elicited antibodies with the heat-labile toxin of *E. coli* indicating that the suggested approach might be suitable for providing a wide-range vaccine against toxigenic *E. coli*. Moreover, the cross reaction of anti-CTP3 with the porcine LT (unpublished data), could be of high significance since it may lead eventually to the production of a veterinary vaccine as well.

In conclusion, we have shown that oligonucleotides encoding relatively small peptides can be used for vaccine development providing that such peptides are endowed with relevant immunological properties. The availability of synthetic peptide antigens for the elucidation of the antigenic properties of proteins and their application as synthetic vaccines (Arnon *et al.*, 1983) can thus be combined with genetic engineering for providing better vaccines in the future.

Materials and methods

Cholera toxin was purchased from Schwartz/Mann. Purified human LT holotoxin was a generous gift from F.A. Klipstein, Rochester, NY.

Bacterial strains and growth conditions

E. coli strains MC1061 [*hsdR* Δ(*lac*) × 74]; MH1000 [*ompB101* Δ(*lac*) × 74] and TK1046 (*ompR*^{cs}1Δ(*argF-lac*) were grown in LB. For selection of transformants, media contained 200 μg/ml penicillin G, and Lac$^+$ colonies were detected on X-gal plates. The first two strains were grown at 37°C; and TK1046 was grown at room temperature. To induce expression from the *ompF* promoter in TK1046, the transformants were incubated at 37°C for 1 h.

Plasmid constructions and transformation

Recombinant DNA procedures were essentially as described (Maniatis *et al.*, 1982). In transformation of TK1046, incubation at 42°C was omitted and replaced by a 5-min incubation at room temperature.

Preparation of the synthetic DNA duplex

A 54-bp DNA, including the coding sequence for CTP3, was prepared by ligation of six oligodeoxynucleotides ranging in length between 14 and 22 nucleotides. These oligodeoxynucleotides could anneal into three duplexes, each with single-stranded extensions allowing their correct alignment. 50 pmol of each of the oligonucleotides, except the two containing the 5′-termini of the end product, were 5′-phosphorylated with T4 polynucleotide kinase and complementary pairs were mixed, heated to 90°C and slowly cooled to 4°C. The annealed pairs were mixed together, brought to 37°C, slowly cooled to 4°C and ligated with T4 polynucleotide ligase for 16 h at 4°C. The 54-bp product was isolated by gel electrophoresis on a 12% polyacrylamide gel in the presence of 7 M urea. The extracted DNA was re-annealed.

Bacterial extracts

Bacteria were grown in 50 ml cultures to a density of ~5 × 10^8 cells/ml. Pelleted cells were washed with 10 mM Tris-HCl, pH 7.5, and resuspended to a final density of ~10^{10}/ml in a solution containing 20% sucrose, 30 mM Tris-HCl, pH 7.5, and 1 mM phenylmethylsulfonyl fluoride (PMSF). EDTA was added to a final concentration of 1 mM followed by lysozyme to 100 μg/ml. After 30 min incubation on ice ~ 1/4 of the volume of a solution containing 50 mM Tris-HCl, pH 7.5, 1 mM PMSF and 0.5% Triton X-100 was added and incubation on ice was continued for 15 min. After addition of $MgCl_2$ to a final concentration of 25 mM, DNase I and RNase A, to a final concentration of 20 μg/ml each were added and the mixture was shaken overnight at 4°C and cleared by a short centrifugation.

Immunoblotting

Bacterial protein extracts were subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel and blotted onto nitrocellulose sheets, probed with the indicated antibodies and subsequently with ^{125}I-labeled *Staphylococcus aureus* protein A.

Purification of CTP3-β-galactosidase-fused protein

Cell extracts of bacteria from a 1 l culture were electrophoresed on preparative 7.5% SDS-polyacrylamide gels. Each gel included a single lane with pure β-galactosidase as a marker. The band corresponding to the fused protein was carefully cut out of the gel and placed in a dialysis bag containing 15 ml of the buffer Tris-glycine, (pH 8.6, 0.1% SDS). The protein was electroeluted by applying 50 mA for 3–4 h. The eluted material was extensively dialysed against 10% methanol and freeze-dried and the protein content was determined.

Immunization procedures

Anti-CTP3 antisera were prepared by immunization of rabbits with a conjugate of CTP3 attached to tetanus toxoid (CTP3-TT). Anti-CT and anti-β-galactosidase antibodies were prepared by immunization of rabbits by multi-site intradermal injection of 100 μg of the respective antigen in Freund's complete adjuvant, twice with a 4-week interval.

Rabbits were immunized either once or several times (see Table I) by multi-site intradermal injections of 1.5–2.5 mg of purified fusion protein diluted 1:1 with Freund's complete adjuvant. Four weeks later (when immunized once) or 2 weeks following the final innoculation (when a multiple immunization schedule was used) a single challenge with 1 μg of CT or LT, diluted 1:1 with Freund's incomplete adjuvant, was injected intradermally. Ten, 30 and 60 days later rabbits were bled and sera were collected.

β-galactosidase assay

The assay was performed on permeabilized whole cells according to the procedure described by Miller (1972).

Cyclic AMP assay

Two methods to determine cAMP induction were used. (i) Kidney cells of White Rock chickens were prepared as described (Jacob *et al.*, 1984a). Adenylate cyclase was activated by incubation of 2.5 × 10^5 cells with 50 ng CT. At the end of the 3-h incubation period, the cells and medium were extracted with 10% perchloric acid. cAMP was assayed by radioimmunoassay according to Harper and Brooker (1975) as previously described (Jacob *et al.*, 1984a). (ii) Mouse thymocytes were isolated from 4–6-week old SJL mice according to Zick *et al.* (1979). Adenylate cyclase was activated by incubation of 1.25 × 10^7 cells with 100 ng CT for 2 h. At the end of incubation period the cells were ruptured by addition of 0.1 ml of 0.1 N HCl to the pellet and boiled for 3 minutes (95°C). The boiled samples were transferred to 4°C and neutralized with 30 μl of 0.25 M Tris, 20 mM EDTA, pH 12.5. The cAMP content in each sample was determined using the assay kit from Amersham (TRK 432).

Ligated ileal loop assay

The assay was performed in 8–12-week-old Sprague-Dawley rats according to Fujita and Finkelstein (1972) with slight modifications as described (Jacob *et al.*, 1983).

Inhibition of cholera toxin biological activity

A fixed amount of CT was pre-incubated with 1:10 dilution of the various antisera, and the residual toxin activity was determined by both cAMP and ligated ileal loop assays. A positive control of hyperimmune anti-CT was included in each assay and considered as causing 100% inhibition.

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