Expression of the Vibrio cholerae *Toxin B Subunit in the Live Recombinant Vector* Salmonella typhi *Ty21a*

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ABSTRACT

The strategies to develop effective bacterial vectors have mainly relied on the genetic manipulation of selected microorganisms to ensure the correct presentation to the immune system of convenient amounts of foreign antigens. A new approach consisting in the oral delivery of bacterial vectors carrying adequate quantities of foreign antigens previously expressed *in vitro,* has been developed. *In vitro* expression of the cholera toxin binding subunit (CTB) by exogenous induction was studied in the recombinant vector *Salmonella typhi* Ty21a, in order to create an experimental tool to further evaluate such an approach. Parameters related to the effectiveness of immunization such as plasmid stability, bacterial viability, and timing of induction, were evaluated as elsewhere, while CTB expression and intracellular location were assayed by GM1-ELISA in samples harvested hourly from cultures induced at 2, 3, 4, and 5 h after inoculation, and were grown for six more hours. CTB expression kinetics was significantly dependent on the timing of induction. The maximal average activity—800 ng/mL of culture—was reached between 2 and 3 h after induction and a lower value of 750 ng/mL remained steady. Seventy percent of the total activity was periplasmic. No differences were detected between induced and control cultures, but in antigen expression. These results are of practical value for the experimental testing of the suggested approach.

Keywords: CTB, IPTG induction, protein expression, *Salmonella typhi* Ty21a, vaccine vectors

Biotecnología Aplicada 2000;17:7-10

RESUMEN

Expresión de la subunidad B de la toxina de *Vibrio cholerae* en el vector vivo recombinante *Salmonella typhi* Ty21a. Las estrategias para el desarrollo de vectores bacterianos efectivos, se han basado fundamentalmente en la manipulación genética de microorganismos seleccionados para garantizar una correcta presentación, al sistema inmune. En este estudio se desarrolló una nueva estrategia basada en la liberación oral de vectores bacterianos que portan cantidades adecuadas de antígenos foráneos previamente expresados *in vitro*. Se estudió la expresión *in vitro* por inducción exógena de la subunidad de unión de la toxina del cólera (CTB) en el vector recombinante *Salmonella typhi* Ty21a, con el propósito de crear una herramienta experimental para evaluar este procedimiento. Se evaluaron parámetros relacionados con la efectividad de la inmunización, como la estabilidad del plásmido, la viabilidad bacteriana y el tiempo de inducción. La expresión de la CTB y su localización intracelular, se analizaron mediante un GM1-ELISA a partir de muestras tomadas cada 1 h de cultivos inducidos a las 2, 3, 4 y 5 h después de la inoculación, y crecidos durante otras 6 h. La cinética de expresión de CTB dependió en gran medida del estadio de inducción. La actividad máxima promedio (800 ng/mL de cultivo) se alcanzó entre 2 y 3 h despúes de la inducción, se mantuvo estable en 750 ng/mL y 70% de la actividad se localizó en el periplasma. Entre los cultivos inducidos y los controles, sólo se observaron diferencias en la expresión del antígeno. Estos resultados son de valor práctico para evaluar experimentalmente el procedimiento sugerido.

Palabras claves: CTB, expresión de proteínas, inducción con IPTG, *Salmonella typhi* Ty21a, vectores vacunales

*I*ntroduction

Salmonella-based live vectors stand as a promising strategy for several groups involved in the Children's Vaccine Initiative Program [1–3].

Salmonella typhi Ty21a, a bacterial strain attenuated by non-specific chemical mutagenesis, has become a widely used human oral typhoid vaccine since the 1970's [4–6]. It has made this and other salmonella strains attractive for the development of live vaccine vectors for mucosal immunization [7–10].

Several genetic approaches such as the use of single or multiple chromosome-integrating constructions, and the development of auxotrophic mutant strains [11–14], have been designed to overcome the problems of insufficient *in vivo* antigen expression because of plasmid unstability.

Recently, a cardinal different approach not based on genetic manipulations but on a new understanding of the requirements for inducing antigen-specific mucosal antibodies, has been advanced by Cárdenas *et al.* [15, 16]. Using a recombinant salmonella strain, a direct correlation between the level of humoral response induced and the initial amount of antigen presented to the gut-associated lymphoid tissue (GALT), was observed after oral immunization of mice. This correlation was not affected by the plasmid unstability *in vivo*.

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It was concluded that the critical event for induction of an adequate antigen-specific humoral immune response was the priming effect caused by the amount of foreign antigen initially presented to the mucosal inductive sites, and not the persistence of its presentation as it has commonly been accepted. This may open new opportunities for the use of *S. typhi* Ty21a strain and simpler genetic constructions for the development of live vaccine vectors.

These vectors would play two circumstantial roles. Firstly, they may be producers of enough quantities of antigens expressed intracellularly under controlled *in vitro* conditions. Secondly, they may work as a kind of Trojan horse to carry and target *in vitro* expressed antigens to inductive sites at mucosal surfaces *in vivo*.

These criteria and previous expression results of the cholera toxin binding subunit (CTB) in several bacterial systems [17–20], prompted the selection of *S. typhi* Ty21a as a bacterial strain, the pUC-derived pJF1 plasmid with the *tac* promoter and carrying the $lacI^q$ repressor gene as genetic construction, and CTB gene preceded by the *E. coli* heat-labile toxin (LTB) binding subunit gene secretion signal, as foreign antigen.

The present study has two major objectives: firstly, to study the expression of CTB using isopropyl-β-D-thiogalactopyranoside (IPTG) at different times of growth, and to determine the parameters related to its use as a live vector vaccine. Secondly, to propose its use as an experimental tool valuable for further immunological studies.

Materials and Methods

Bacterial strain and plasmid

The human typhoid live vaccine strain *S. typhi* Ty21a from the Enterprise for the Manufacturing of Biologicals "Carlos J. Finlay", Havana, Cuba, was phenotypically and genotypically tested for the metabolic transformation of different sugars present in the testing media [21] and for its mutation in the *galE* gene [22], respectively. This strain genetically transformed with the pJF1 plasmid—a pUC-derived vector carrying the genes encoding the LTB secretory signal peptide and CTB antigen, both under the control of the IPTG-inducible *tac* promoter—was kindly provided for this work [17, 18, 20, 23].

Media, growth kinetic conditions and induction by IPTG

Luria-Bertani (LB) medium was prepared and used as described by Bridson [21]. It was supplemented with 100 µg/mL of ampicillin (Boehringer Mannheim GmbH, Germany), unless otherwise were stated. An overnight culture was used as inoculum and the initial optical density (OD) was adjusted to 0.1 at 550 nm. Separated cultures of 25 mL of medium in 250-mL erlenmeyers were grown under standard conditions —250 rpm, 37 ºC in a Shaker Incubator (G76D New Brunswick Scientific, USA)—, were induced with IPTG (Sigma, USA) at 2, 3, 5, and 5 h of culture to a final concentration of 100 µM. Cultures were incubated for six more hours. Samples were harvested hourly to determine OD at 550 nm, viability, plasmid stability, total cell protein concentration and CTB activity, as described [23]. As negative control, an identical culture was kept under the same conditions described, but no IPTG was added.

CTB activity

The assay was performed as previously reported [15, 24]. Briefly, the pellet from 1 mL of culture was suspended in 300 µL of phosphate-buffered saline with 0.05% Tween 20 (PBS-T), and lysed by sonication in a ultrasonic cell disrupter (MSE Soniprep 150, UK) in a burst of 30 s on ice. This mixture was diluted 1:2 in PBS-T buffer and 100 µL were added to the previously GM1-coated wells. Purified GM1 ganglioside was kindly supplied by the Department of Enteropathogenic Bacteria, Finlay Institute [25], and a 2-µg/mL GM1 coating concentration was used elsewhere. After incubation at 37 ºC for 1 h, the plates were washed three times with PBS-T and bound CTB was detected by addition of anti-CTB conformational specific monoclonal antibodies (MAb) diluted 1:2000 in PBS-T and incubation for 1 h at 37 ºC. Thereafter, the plates were washed as described before. The anti-CTB MAbs were supplied by the Department of Monoclonal Antibodies at the National Center for Scientific Research and at Finlay Institute in Havana, Cuba. An anti-mouse IgG (Fc-specific) peroxidase conjugate (Sigma, USA) was added to a final dilution of 1:20,000 and incubated at 37 ºC for 1 h. After washing, 100 µL of substrate buffer containing 0.04% *o*-phenylenediamine dihydrochloride (OPD) (Sigma, USA) were added to each well. The plates were incubated in the dark at 37 ºC for 30 min and the reaction was stopped by adding 25 µL of 2.5 N HCl. ODs were determined at 492 nm in an ELISA reader (Anthos Labtec Instruments, Austria). CTB activity was quantified by using purified CTB (Sigma, USA) as standard, and the values were expressed as nanograms of CTB either per milliliter of culture or per milligram of total cell protein, based on ELISA results.

Total cell protein determination

Determinations were carried out by the method proposed by Lowry *et al.* [26], with a previous perchloric acid treatment. A sample of 1 mL of culture was centrifuged for 10 min at 5500 x*g* at room temperature, and the pellet was suspended in 5 mL of 0.25 M perchloric acid (Sigma, USA) and kept on ice for 30 min with frequent agitation. This volume was centrifuged at 3500 x*g* for 10 min at 4 ºC. The resultant pellet was vigorously suspended in 5 mL of 0.5 M perchloric acid and incubated for 15 min at 70 ºC in a water bath. After centrifugation, the supernatant was discarded and the pellet was vigorously suspended in 1 mL of 1 M NaOH (Merck, Germany). The mixture was incubated at 80 ºC in a water bath until the sample was totally clarified, and then stored at 4 ºC until use.

Intracellular distribution of CTB activity

Different cultures were induced with 100 µM IPTG at 2, 3, 4, and 5 h of growth and 1-mL samples were harvested after induction for 3 h. The samples were treated as reported [15, 27]. Briefly, the resultant pellet was suspended in 300 µL TEAN buffer (0.2 M

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Tris pH 8.0, 1 M sucrose, 10 mM EDTA). After 10 min of incubation at room temperature, lysozyme (Boehringer Mannheim GmbH, Germany) dissolved in distilled water was added to a final concentration of 200 µg/µL.

This mixture was further diluted 1:2 in TEAN buffer, incubated for 30 min at 37 ºC and centrifuged at 5000 x*g* for 15 min.

The supernatant containing the periplasmic fraction was collected and stored. The pellet was suspended twice in 300 µL of TEAN buffer and sonicated as described above, but the burst time was reduced to 5 s. The supernatant with the cytoplasmic fraction was collected after centrifugation at 5000 x*g* for 15 min, and stored until use.

The pellet containing the cell wall fraction was suspended as aforementioned, with the exception that TEAN buffer contained 6 M urea, and was left for 1 h at 4 ºC after gentle suspension. After centrifugation at 5000 x*g* for 15 min, the supernatant containing the extracted proteins was collected. This last procedure does not affect CTB activity as previously observed in our laboratory (data not shown). All samples were immediately dialysed overnight against PBS at 4 ºC, aliquoted and stored at -20 ºC until assayed for CTB activity.

Statistical analysis

The standard error, the mean values and the coefficient of variation were calculated for all data. The coefficient of variation never exceeded the 5% value. The mean values of the OD measurements and CTB activity of induced cultures were compared with those of the control, and among themselves by Student's *t* test. Values with $p < 0.05$ were considered statistically significant.

Results

Growth kinetics and plasmid stability

Growth kinetics and viability of *S. typhi* in IPTG-induced cultures were similar to those of the control, and no statistically significant differences in OD measurements were detected during the 11 h of experiment (Figure 1). The mean doubling time was about 37–40 min, the maximal viability of 6×10^{12} cfu/mL was reached after 4 h of growth and remained almost constant until the end of the experiment. The plasmid stability measured in all cultures showed a similar mean value, about 15–20% of the cells became ampicillin-sensitive throughout the experiment.

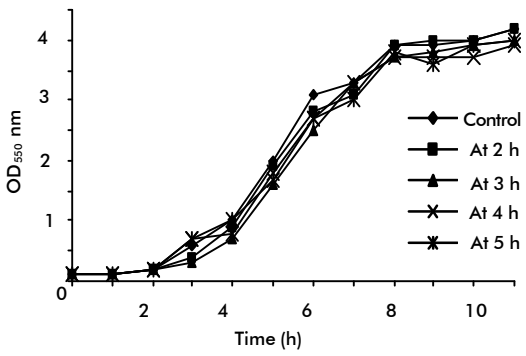

Figure 1. Growth curves of cultures induced with 100 µM IPTG at 2, 3, 4, and 5 h after inoculation. Observe that all the cultures, including the control, showed a similar behavior. The addition of IPTG at such a concentration had no influence on the growth kinetics of recombinant *Salmonella typhi* Ty21a.

Induction of CTB activity

CTB activity was low in non-induced control cultures. Therefore, the differences in CTB activity between induced and control cultures were statistically significant during the experiment. CTB activity levels were different ($p < 0.05$) between cultures induced at different times of growth. A marked tendency for reduction of the statistical significance of these differences with the increase of culture age was consistently observed, and they became no significant in samples taken after 2 h of IPTG addition in cultures induced at 4 and 5 h of growth (Figure 2). A consistent decrease of the maximal CTB activity level was detected with the increase of time at which cultures were induced, the highest activity (975 ng/mL) being detected after 2 h of IPTG addition in cultures induced at 2 h of growth (Figure 2). The maximal mean value of 800 ng/mL was reached 2 h after induction, and 1 h later a value around 750 ng/mL remained steady in all induced cultures until the end of the experiment. The specific activity remained constant after 2–3 h of induction, showing a value of 500 ng mg of total cell protein. In a similar experience, with the addition of 50 µM IPTG to the cultures, all the parameters showed the same behavior, except that CTB activity was approximately 25% lower (600 ng/mL, 390 ng/mg of total cell protein, data not shown).

Intracellular distribution of CTB activity

The main CTB activity was found in the periplasmic fraction (Table) irrespective of the stage of growth at which cultures were induced. The same distribution was observed when 50 µM IPTG were added, but CTB activity values were lower (data not shown).

Discussion

No toxic or negative effects related to the expression of CTB activity induced by addition of 100 µM IPTG, were observed over any of the parameters tested. CTB specific activity reached values of 700 ng per milli-

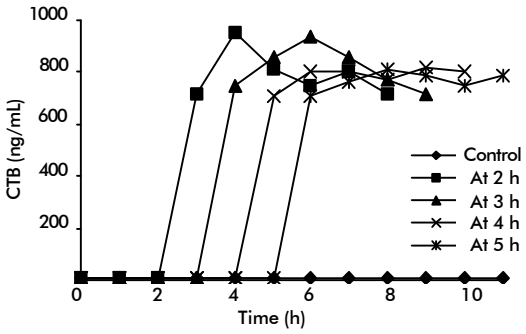

Figure 2. Kinetics of CTB activity expression as measured by GM1-ELISA in samples harvested hourly from cultures of recombinant *Samonella typhi* ty21a induced with 100 µM IPTG at four different times. Note that the maximal CTB activity is reached 2 to 3 h after IPTG addition, and that there are a consistent decrease of maximal CTB activity and differences in CTB activity according to the age of the culture. CTB activity in the control culture remained at low levels during the experiment.

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Table. Distribution percentage of intracellular location of CTB activity in samples harvested 3 h after addition of 100 μM IPTG (final concentration) to recombinant *Salmonella typhi* Ty21a cultures at four different times of growth: 2, 3, 4 and 5 h after inoculation.

Time (h)	Periplasmic	Cytoplasmic	Membranes
2	70	25	5
3	72	23	5
4	71	25	4
5	70	24	6

gram of total cell protein, which are tenfold higher than those reported in a similar system expressing LTB instead of CTB [15].

In a similar experience using 50 μM IPTG for induction, a direct correlation with CTB expression, was observed without detectable influence over the parameters studied (data not shown). The CTB activity in non-induced cultures remained consistently low during the experiment.

These results corroborated the conserved functional efficiency of *lacI*^{q} gene repressor product in this bacterial system, and suggest an increase in the quantity of CTB by a previous IPTG induction *in vitro* before using this strain for oral immunization.

Interestingly, Acosta *et al.* [20] reported the induction of CTB-specific antibodies in mice inoculated with this strain without any previous IPTG induction. This result agrees with the common claiming to replace CTB with less immunogenic antigens, to test the actual capability of these vectors to enhance the immunogenicity of foreign antigens by themselves.

Despite the differences in the genetic systems, the bacterial strains, or the experimental procedures used, the immunogenic and structural similarities between LTB and CTB [28], and the results already reported [15, 20], give a reasonable basis to expect higher antibody responses when previously IPTG-induced bacteria be used for mucosal immunization.

Taken together, these results argue in favour of using this system as an experimental tool for the detailed evaluation of the actual influence that the viability and persistence of salmonella, and the features of heterologous antigen expression have on the immunogenicity of both recombinant antigen and vector.

Received in January, 1999. Accepted for publication in August, 1999.

By changing the concentration of the inducer, the timing of induction, and by deleting the signal peptide sequence from the genetic construction, parameters such as CTB amount and its intracellular location could be changed. Bacteria with different combinations of the aforementioned parameters could be administered either as live, killed, or mixed preparations for assessment of their actual influence upon the *in vivo* immunogenicity of both the bacterial strain and the heterologous antigen.

In this work, the major quantity of recombinant CTB was detected in the periplasmic fraction. This result agrees with the reported conserved functionality of LTB secretion signal peptide in similar Gram-negative bacterial systems [15].

The relationship between cellular location of the recombinant antigen and the characteristics of the elicited T cell in the immune responses, are subject of intense study by different groups [15, 29, 30]. It would be interesting to test the influence of the intracellular location of recombinant antigens on the characteristics of the cellular immune responses.

The extent to which the viability of a salmonella vaccine as a condition for anti-typhoid immunity is also a requirement for its use as an effective carrier of recombinant antigens, is a question that has been recently addressed. In suggestive studies [17, 18], Cárdenas *et al.* found that viability requirement, despite being undoubtedly valid for typhoid vaccine, does not apply completely to the use of these strains as foreign antigen carriers.

Here, we reported the main parameters concerned with the characterization of recombinant antigen expression *in vitro*, which is an important step in the development of a recombinant vaccine vector.

Further immunological studies may be carried out using this vector, such as the influence of antigen location in elicited cellular immune responses, and the actual role of viability, plasmid stability, persistence, and bacteremia on the *in vivo* immunogenicity of carried foreign antigens, which aim not only to evaluate the immune responses induced *in vivo*, but also to address questions of major immunological concern .

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