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Salmonella as Live Carrier of Antigens in Vaccine Development

César Gonzalez Bonilla, Ericka Pompa Mera,
Alberto Diaz Quiñonez and Sara Huerta Yepez
División de Laboratorios de Vigilancia e Investigación Epidemiológica
Coordinación de Vigilancia Epidemiológica y Apoyo en Contingencias
Instituto Mexicano del Seguro Social
México

1. Introduction

Attenuated *Salmonella* strains have been extensively used as live carriers of heterologous antigens. In animal models they elicit strong mucosal and systemic immune responses to passenger antigens of a broad variety of pathogens. *Salmonella* has several theoretical advantages over other vaccine vector systems. Among them, especially attractive is the bacterial ability to interact with mucosal and systemic compartments of the immune system and deliver passenger antigens directly to antigen presenting cells (APC) when administered by the oral route. However, despite the promising results in animal models, clinical trails have been disappointing and more research is needed in order to understand the protective immunity mechanisms and solve the main drawbacks of vaccine design. Herein, we summarize the accumulated experience using *Salmonella* as live carrier emphasizing the role of passenger antigen localization into different bacterial compartments. This is an important factor determining the type and quality of the host immune response. The evidence suggests that antigens located on the bacterial surface induce higher antibody responses whereas those located in the cytoplasm elicit better cellular immunity. In order to display recombinant antigens on the bacterial surface we have used outer membrane (OMPs) or autotransporter proteins. Mice immunized with *Salmonella* strains expressing the main B cell epitope from the *Plasmodium falciparum* circumspozoite protein, presented better antibodies response when placed on the bacterial surface as a fusion proteins with OMPs or autotransporters, than the whole recombinant protein located in the bacterial cytoplasm.

2. *Salmonella* as live carrier of antigens

Vaccination is the public health intervention intended to control infectious diseases with the best cost-effective ratio. Among the number of different approaches for vaccine development, live vectors stand as one of the most promising options. They may be defined as attenuated microorganisms which produce heterologous recombinant proteins (or bear plasmids DNA with eukaryote transcription machinery) and elicit immune response to the passenger antigen. Extensive research in the last decade in the fields of molecular biology,

bacterial genetics and immunology has significantly accelerated the use of a number of microorganisms as vaccine vectors. Several pathogenic bacteria can be engineered for attenuation and expression of foreign proteins originally encoded by other microorganisms. Although several attenuated or commensal nonpathogenic bacteria have been employed to express passenger antigens (Kottonet al. 2004; Medinaet al. 2001), enteric Gram negative bacteria, especially *Salmonella*, have been preferred because they are easy to manipulate. However, these vaccine candidates raise safety concerns associated with the release of genetically modified microorganisms into the environment. (Abd Elet al. 2007; Kottonet al. 2004).

An ideal vaccine has to fulfill not only immunogenicity, efficacy and safety requirements, but also has to be inexpensive, stable at room temperature and easy to administer (Polandet al. 2010). Bacterial carriers specifically *Salmonella* strains, attenuated by genetic engineering, have several advantages over other vaccine types because they can be administered by the oral route and are more stable at room temperature than other vectors, such as viruses. Recent research have developed more stable strains, one example is a live attenuated *S.* Typhi Ty21a vaccine treated by foam drying, a modified freeze drying process, formulated with trehalose, methionine, and gelatin, is stable for approximately 12 weeks at 37°C and its derivatives expressing foreign antigens, such as anthrax, are immunogenic (Ohtakeet al. 2011).

Persistent and effective immune responses require initial activation of innate immunity. Bacterial pathogen associated molecular patterns (PAMPS) recognized by pattern recognition receptors (PRRs) in macrophages, dendritic cells (DC) or epithelial cells (ECs) determine the nature and extent of the adaptive response through membrane associated and soluble cytokine signaling. For example, Toll-like receptors (TLRs) TLR4 and TLR2 deficient mice immunized with *S* Typhi porins, which have been found to elicit maturation of $CD11c^+$ conventional DC, showed impaired B-cell response, characterized by reduction of IgG antibody titers to porins, specially of the IgG3 isotype (Cervantes-Barraganet al. 2009). Other authors have investigated the role of TLRs responses to an attenuated *S.* Typhimurium BRD 509 expressing the saliva-binding region (SBR) of *Streptococcus mutans*. Using TLR2, TLR4 and MyD88 deficient mice, they demonstrated that the induction of a serum IgG2a (type 1 response) to the passenger antigen involved TLR2 signaling, whereas the response to *Salmonella* involved signaling through TLR4 (Salamet al. 2010). Thus, antigen specific T and B cells are activated in a coordinated manner to achieve optimal primary and secondary immune responses. The induction of memory is a key characteristic of an effective vaccine (Chenet al. 2010; Medinaet al. 2001). It has been demonstrated that primary effector T cell activation to Salmonella depends on the innate function of B cells (though TLR signaling mediated by MyD88) whereas the induction of T cell memory mediated by antigen specific presentation by the BCR (Barret al. 2010). Moreover, the cell wall of Gram negative bacteria promotes Th1 responses. For instance, an *Escherichia coli* strain expressing an ovalbumin (OVA) allergenic peptide on the bacterial surface, as a fusion protein with the *S.* Typhi OmpC porin, reduced the lung inflammatory response in mice allergic to OVA, with a significant decrease of IL-5 mRNA and induction of IFN-γ mRNA in cells from bronchio alveolar lavages and specific anti-OVA IgE reduction (Yepezet al. 2003).

Several routes of immunization have been assessed with *Salmonella* live carriers. The oral route elicits protective immunity. For instance, mice immunized orally with *S.* Typhiumurium or *S.* Typhi expressing the fullength *B. anthracis* protective antigen (PA)

were protected against a lethal challenge with aerosolized *B. anthracis* spores (Stokeset al. 2007). However, the intranasal route is more immunogenic. Indeed, mucosal immunity is most effectively induced when antigens are delivery directly in mucosa, i.e. by oral, intranasal, intrarectal, or intravaginal routes. (Galenet al. 1997). The eye conjunctiva has also demonstrated to be a feasible administration route. Attenuated *Salmonella* vaccine strains administered by eyedrops induced LPS-specific antibodies and protection to the oral challenge with virulent *Salmonella* in mice. Eyedrop vaccinations do not deliver antigens into the CNS as noted with the intranasal route.(Seoet al. 2010)

A needleless delivery system has been developed recently, consisting in a micro wave controlled explosion which disrupts the skin barrier. This system was used to immunize mice with a *S.* Typhimurium vaccine strain pmrG-HM-D (DV-STM-07) with the idea to place the bacteria in the epidermis where resident Langerhans cells may uptake them more efficiently and present the bacterial antigens to lymphocytes (Jagadeeshet al. 2011)

There are a number of attenuated *S.* Typhi strains with defined mutations constructed by genetic engineering. Some have been tested in humans demonstrating immunogenicity and acceptable safety profile, such as *S.* Typhi Ty800, which is mutated in *phoP/phoQ.(Hohmannet al. 1996) or* M01ZH09, a *S.* Typhi (Ty2 aroC-ssaV-) ZH9 (Tranet al. 2010). *Other* attenuated *Salmonella* strains have disrupted the *aroC* and *aroD genes*. The interruption of the biosynthetic pathway of aromatic metabolites results in a bacterial nutritional dependence on *p*-aminobenzoic acid and 2,3-dihydroxybenzoate, substrates not available to bacteria in mammalian tissues (Hoisethet al. 1981). As result, the *aro*-deleted bacteria are not able to proliferate within mammalian cells. However, the organisms survive intracellularly long enough to stimulate immune responses. Inactivation of either *aroC* or *aroD* independently results in attenuation, but deletions in both genes reduce the possibility of virulence restoration by recombination. Two vaccine strains harboring deletion mutations in *aroC* and *aroD* have been evaluated as candidate live oral vaccines in adult volunteers (Bumannet al. 2010; Gonzalezet al. 1994; Tacketet al. 2000; Tacketet al. 2007).

Attenuated *S.* Typhi vaccines have been engineered to express and deliver passenger antigens (proteins and DNA encoded) of a number of pathogens, as the measles virus hemagglutinin, the *Bacillus anthracis* protective antigen (PA), the *Plasmodium falciparum* circumsporozoite surface protein (tCSP), the nucleocapsid (N) protein of severe acute respiratory syndrome-associated coronavirus (SARS-CoV), or the HPV16 L1 protein (L1S).(Chinchillaet al. 2007; Frailleryet al. 2007; Galenet al. 2004; Luoet al. 2007; Pasettiet al. 2003)

3. Influence of passenger antigen location on the immune response

There is increasing evidence that expression level and antigen location determine vaccines efficacy. It is important to achieve passenger antigen expression in the desired bacterial compartment under constitutive or inducible conditions, in order to regulate antigen production. (Bumann 2001; Galenet al. 2001; Kurlandet al. 1996; Pathangeyet al. 2009). Insufficient expression interferes with the immune response to passenger antigens and there is a general notion that high antigen production by live vectors may result in better immune response. Therefore, the production of antigens from high copy number plasmids is apparently the best designing approach. However, excessive expression drives to increased metabolic load, plasmid loss and toxicity (Galenet al. 2001; Pathangeyet al. 2009) Antibody

responses to antigens delivered by S. Typhi live vectors are inversely related to the metabolic burden imposed by antigen production, and may be improved when antigens are expressed from low-copy-number plasmids and exported out of the cytoplasm (Galenet al. 2010). Three solutions are proposed to solve this problem: 1) chromosomal integration of heterologous genes, 2) On/off recombinant protein production by using *in vivo*-inducible promoters, and 3) Plasmid stabilization systems. Although both strategies are intended to limit heterologous gene expression, the second strategy has the additional advantage that protein is preferentially produced at the appropriate host environment, such as acidic vacuoles in macrophages. In addition, when plasmid stability is maintained in the absence of antibiotics, there is flexibility for the introduction of a variety of passenger genes without the need to use chromosomal integration systems.

Thus, S Typhi ZH9 (Ty2 Delta aroC Delta ssaV) producing the B subunit of *Escherichia coli* heat-labile toxin or hepatitis B virus core antigen from the bacterial chromosome using the *in vivo* inducible ssaG promoter, stimulated potent antigen-specific serum IgG antibodies to the heterologous antigens (Stratfordet al. 2005).

S. Typhimurium aroA (STM-1) expressing *Mycoplasma hyopneumoniae* antigens form plasmid or chromosomal systems were administered to mice. Whereas no significant immune response was detected with the plasmid based expression, systemic IgM and IgG responses were detected with the chromosomal integration system which used strong promoters (Maticet al. 2009).

A plasmid maintenance system has been tested in *S.* Typhi CVD 908-htrA consisting in the deletion of genes encoding catalytic enzymes and addition of random segregation function of multicopy plasmid (Galenet al. 2010). Other option to achieve plasmid stability relies on the development of plasmid trans- complementation of lethal deletions in the live vector. Thus, plasmids encoding the single-stranded binding protein (SSB), an protein involved in DNA replication were used to transform *S.* Typhi CVD 908-htrA and CVD 908, and used to deliver anthrax toxin from *Bacillus anthracis* as a foreign antigen in mice (Galenet al. 2010).

A dual system to achieve increased antigen expression was developed by chromosomal integration of the T7 RNA polymerase gene (T7pol) in *S.* Typhi CVD908. The T7pol gene was amplified from *Escherichia coli* BL21(DE3) and inserted by homologous recombination in the bacterial chromosome under the control of the inducible nirB promoter. The resulting strain, S. typhi CVD908-T7pol, was able to trans-complement two plasmids bearing the luc or the lacZ reporter genes controlled by the T7 promoter under anaerobic culture conditions (Santiago-Machucaet al. 2002)

Other factor influencing protein expression efficiency include differences of codon usage between the native passenger gene and that host chromosome. Although so far exclusively applied to *Escherichia coli*, codon harmonization may provide a general strategy for improving the expression of soluble, functional proteins during heterologous host expression (Angovet al. 2011)

It has been suggested that passenger antigens delivered by attenuated *Salmonella* strains induce better systemic and mucosal immune responses when displayed on the bacterial surface (Chenet al. 2000; Leeet al. 2000; Ruiz-Perezet al. 2002). A variety of surface display systems have been described (Samuelsonet al. 2002). The most widely used have been fimbria and outer membrane proteins (OMPs), including porins and autotransporters. (Kjaergaardet al. 2002; Klemmet al. 2000; Krameret al. 2003; Rizoset al. 2003)

Passenger fusion proteins (peptides-flagellin) have demonstrated to enhance the immunogenicity of vaccine peptides (Newtonet al. 1989; Newtonet al. 1991a; Newtonet al. 1991b; Stocker 1990; Stockeret al. 1994). In these models the heterologous peptide is fused in-frame to the central hypervariable domain of *Salmonella* FliCd flagellin, which is derived from *S.* Müenchen and expressed by an attenuated *S.* Dublin strain. The chimeric flagellins are exported to the bacterial surface where the subunits assemble into the flagellar shaft without a significant impact on bacterial motility and host tissue colonization (Newtonet al. 1989; Stockeret al. 1994). Nonetheless, previous results showed that the genetic fusion may not enhance antigen-specific antibody responses in mice immunjized by the oral route with recombinant *S.* Dublin (De Almeidaet al. 1999; Sbrogio-Almeidaet al. 2001). Interestingly, the genetic background of both the mice and the *Salmonella* strains affected the immunogenicity of flagellins (Sbrogio-Almeidaet al. 2004). Indeed, recent evidence indicates that *Salmonella* flagellin administered by the oral route may trigger immunological tolerance in healthy mice, although the precise mechanism underlying this response remains unknown (Sanderset al. 2006)

However, in some cases exported, secreted proteins may induce stronger antibody response. (Galenet al. 2001).

Table 1 shows some examples of the influence that heterologous protein location in *Salmonella* has on the type of immune response, which are described as follows.

Antigen displayed	Location in bacterial carrier	Immune response	Reference
TGEV C and A epitopes	Fimbria and outer membrane (MisL)	Humoral	(Chenet al. 2007)
NS3 Dengue Virus	Outer membrane (MisL)	CTL	(Luria-Perezet al. 2007)
Ea1A y EaSC2 *Eimeria stiedae*	Cytoplasm	CTL	(Vermeulen 1998)
LTB *E. coli*	Periplasm	Humoral and cellular	(Takahashiet al. 1996)
SERP and HRPII *P. falciparum* antigens	Outer membrane (OmpA)	Humoral	(Schorret al. 1991)
p60 *L. monocytogenes*	Cytoplasm	CTL	(Gentschevet al. 1995)

Table 1. Influence of heterologous protein location in *Salmonella* carrier, on the immune response.

Attenuated *S.* Typhimurium CS4552 (crp cya asd pgtE) was constructed expressing transmissible gastroenteritis virus (TGEV) C and A epitopes fused to the passenger domain of the MisL autotransporter or to the 987P FasA fimbriae subunit under the control of in vivo-induced promoters. The antibody response between both expression systems was compared. Mice vaccinated with the recombinant bacteria displaying the antigens in fimbriae presented the highest level of anti-TGEV antibodies with the epitopes expressed in fimbriae. This result suggests that polymeric display could induce better immune responses towards specific epitopes (Chenet al. 2007).

The second example is a *S.* Typhimurium SL3261 producing a fusion protein designed to destabilize the phagosome membrane and allow a dengue epitope to reach the cytosol. The fusion protein was displayed on the bacterial surface though MisL and the passenger alpha domain contained a fusogenic sequence, a NS3 protein CTL epitope from the dengue virus type 2 and a recognition site for the protease OmpT. The passenger antigen was released to the milieu, processed through the MHC class I-dependent pathway and simulated cytotoxic T lymphocytes (CTLs).(Luria-Perezet al. 2007)

Eimeria stiedae antigens Ea1A and EaSC2, a parasite refractile body transhydrogenase and a lactate dehydrogenase, respectively were expressed in *S.* Typhimurium, and used to immunize chickens. The challenge with the parasite demonstrated oocyst output reduction related with $CD4^+$ and $CD8^+$ T cells activation (Vermeulen 1998)

The fourth example in **Table 1** is a comparison between the immune response elicited in mice immunized with native *Escherichia coli* enterotoxin (LT) or with *S.* enteritidis expressing the heat-labile toxin B subunit (LT-B) in the bacterial periplasm. Both antigens elicited mucosal IgA antibodies directed to different LT-B epitopes, and serum IgG antibodies to the same immunodominant LT-B epitopes. The same single T-cell epitope was recognized by immune lymphocytes purified from mice immunized with either antigen (Takahashiet al. 1996)

Immunogenic epitopes of the *Plasmodium falciparum* blood stage antigens SERP and HRPII were expressed on the surface of the attenuated *S.* Typhimurium SR-11 strain as fusion proteins with OmpA from *Escherichia coli*. Mice immunized orally with the bacterial recombinants produced anti-SERP and anti-HRPII IgG and IgM antibodies. (Schorret al. 1991)

Finally, an attenuated *S.* Dublin aroA strain which secretes an active listeriolysin from *Listeria monocytogenes* is partially released from the phagosome into the cytoplasm after uptake by J774 macrophage cells. This is an attractive approach to evoke CTLs responses to passenger antigens through the MHC class I-dependent antigen processing pathway (Gentschevet al. 1995)

4. Display of antigens on the bacterial surface

Following, some of our experience with cytosolic versus surface display of antigens will be described with more detail. The same antigen and live vector were used with the only difference in the bacterial compartment where the antigen was expressed.

It has been demonstrated that *Salmonella typhi* OMPs, porins, and particularly OmpC induce protective immune response in a murine model of infection (Gonzalezet al. 1993; Gonzalezet al. 1995; Isibasiet al. 1988; Isibasiet al. 1992; Isibasiet al. 1994). Porins are OMPs which conform diffusion channels for low molecular weight molecules into the bacterial cell. The tertiary structure is a barrel conformed by 16 anti parallel β sheets with external and internal loops. These external loops have permissive regions were heterologous peptides can be inserted (Vegaet al. 2003; Yepezet al. 2003; Zenteno-Cuevaset al. 2007)

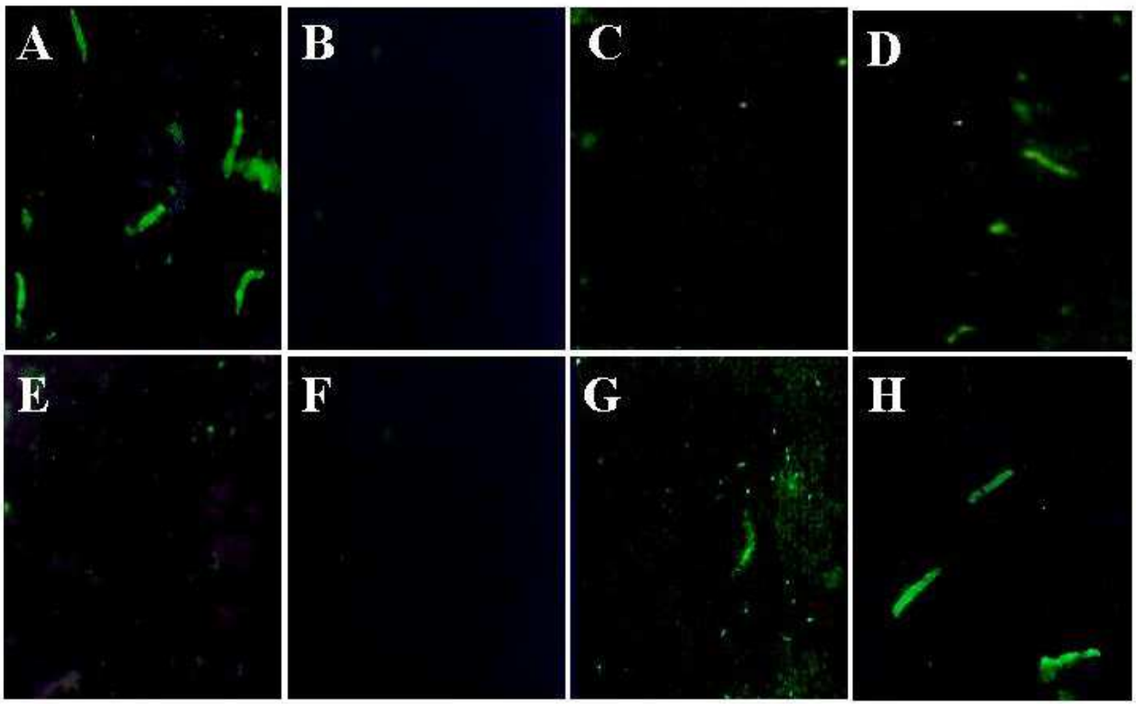
Considering that the major B cell epitope from the *Plasmodium falciparum* circumpsporozoite protein (CSP), the Asp-Ala-Asp-Pro (NANP) repeating sequence, has been inserted in permissive sites of *Pseudomonas aeruginosa* OMP OprF (Wonget al. 1995) we decided to introduce the NANP encoding sequence in the of *S.* Typhi OmpC porin. The NANP3 *P.*

falciparum CSP was inserted in the predicted external loop 5. A site directed mutagenesis was achieved by an overlapping PCR in two amplification rounds. In the first amplification two products were generated separately from plasmid pST13 (bears the complete *S.* Typhi OmpC porin and was kindly donatd by Dr. Felipe Cabello Felipe C. Cabello, New York Medical College) a 5` *omp*C moiety bearing the NANP3 sequence in the 3`end, and a 5´moiety with the NANP3 sequence in the 5´end. In the second amplification both moieties were used to generate a fusion product which was digested and religated to pST13, resulting plasmid pST13-NANP. Functionality of the hybrid *omp*C-NANP gene was assessed by Northern blot using RNA obtained from *Escherichia coli* UH312 transformed with pST13 or pST13-NANP. The autoradiography revealed a more intense band in *Escherichia coli*-pST13 as compared with the same strain transformed with pST13-NANP. Thus, suggesting that the *omp*C-NANP hybrid gene is transcribed less efficiently than the *omp*C native gene.

Protein extracts were obtained from *Escherichia coli* UH302 and *Salmonella typhi* CVD908 transformed with pST13 or pST13-NANP, and the OmpC-NANP fusion protein expression was estimated by SDS-PAGE. When the porinless *Escherichia coli* UH302 strain was transformed with pST13, produced large amounts of the 36 kDa protein. Nevertheless, when transformed with pST13-NANP a faint 36 kDa band was observed. The lower protein production found in the strains transformed with pST13-NANP is consistent with the Northern blot analysis. No differences in OmpC expression was observed between the *Salmonella typhi* CVD908 strains transformed with pST13 or pST13-NANP

Groups of five BALB/c mice were immunized with *Escherichia coli* UH302 transformed with pST13 or with pST13-NANP and *S.* Typhi CVD908, CVD908-pST13-NANP, CVD908ΩCSP (bears the whole CSP integrated in the bacterial chromosome)(Gonzalezet al. 1994), CVD908ΩCSP-pST13-NANP. Seven days after the last immunization, antibodies against *Plasmodium falciparum* sporozoites were assessed by IFA. Neither preimmune sera nor sera from mice immunized with *E. coli* UH302-pST13 or *Salmonella typhi* CVD908 recognized sporozoites, and some structures suggesting sporozoites were observed with sera from mice immunized with *E. coli* UH302-pST13-NANP (**Figure 1**). Interestingly, *S. typhi* CVD908ΩCSP (cytosolic CSP expression) was unable to induce antibodies against the parasite and similarly to *E. coli* UH302-pSt13-NANP, sera from mice immunized with *S. typhi* CVD908-pST13-NANP (epitope surface expression) showed some structures resembling parasites. Nevertheless, only mice immunized with *Salmonella typhi* CVD908ΩCSP-pST13-NANP (both surface and cytosolic expression) clearly depicted sporozoites comparable to the positive controls reveled with 2A10 antibody (**Figure 1**). These data were consistent with the measurement of antibodies by ELISA. Serum of BALB/c mice immunized with S. Typhi CVD908, CVD908ΩCSP, CVD908-pST13-NANP, and CVD908ΩCSP-pST13-NANP were collected, and antibodies to (B)4MAPs (a branched peptide containing the NANP) sequence were determined. *S.* Typhi CVD908ΩCSP, which produce a cytosolic CSP from a chromosomal integrated gene, did not elicit measurable antibodies under the experimental conditions for this experiment (1:25 to 1:250). *S.* Typhi CVD908-pST13-NANP, which displayed the NANP epitope on the bacterial surface, elicited mild antibody response (titer 1:25), whereas *S.* Typhi CVD908ΩCSP-pST13-NANP, which produced the epitope on the bacterial surface and the whole CSP in the cytosol, raised the highest antibody response (titer 1:200) after both o.g or i.p immunization (**Figure 2**). Taken together these data suggest, as stated earlier, that the epitope expressed on the bacterial surface may exhibit antigenic

identity with the native CSP in *P. falciparum* sporozoites and that both surface and cytosolic expression elicited better antibody response.

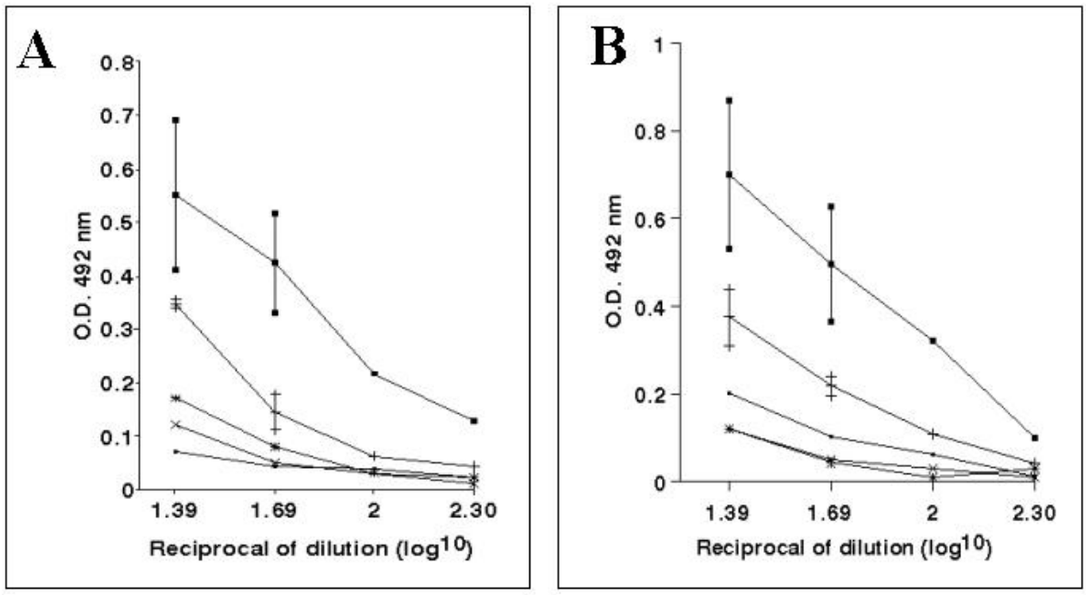


A) Positive control, sporozoites revealed with the 2A10 monoclonal antibody (which recognizes the (NANP)n repeat of Plasmodium falciparum circumsporozoite surface protein); **B)** Negative control, preimmune sera; **C)** *Escherichia coli* UH302,-pST13; **D)** *Escherichia coli* UH302-pST13-NANP; **E)** *Salmonella typhi* CVD908; **F)** *Salmonella typhi* CVD908ΩCSP; **G)** *Salmonella typhi* CVD908-pST13-NANP; **H)** *Salmonella typhi* CVD908ΩCSP-pST13-NANP.

Fig. 1. Antibodies against *Plasmodium falciparum* sporozoites elicited by the immunization of BABL/c mice with bacterial strains expressing the NANP epitope on the bacterial surface were assessed by immunofluorescence assay. The antibody response was compared between strains with cytosolic expression of CSP, surface expression of $(NANP)_3$, or both surface and cytosolic expression.

Western blot was performed revealing OmpC with a rabbit hyperimmune anti-OmpC serum or a cocktail of monoclonal antibodies against OmpC. The rabbit anti-OmpC serum showed two clear bands in the *Salmonella typhi* strains due to cross reactivity with other porin (probably OmpF, which is 35KDa). *Escherichia coli* UH302-pST13 and pST13-NANP revealed a single band. The cocktail of monoclonal antibodies against OmpC revealed a single band and demonstrated that native OmpC and the OmpC-NANP fusion protein display similar molecular weight. It is important to notice that the western blot from these protein extracts using monoclonal antibody 2A10, which recognizes the *Plasmodium falciparum* CSP, failed to recognize the $(NANP)_3$ epitope in the fusion OmpC-NANP protein. Nevertheless, the flow cytometry performed with *Escherichia coli* UH302 transformed with pST13 or pST13-NANP using the same 2A10 monoclonal antibody revealed that *Escherichia coli* UH302-pST13-NANP displays the chimeric OmpC with the *Plasmodium falciparum*

NANP epitope on the bacterial surface. These data could be explained by the low expression levels of the ompC-NANP fusion protein, but may be related to conformational changes in the SDS-PAGE.



A). Antibodies against $(B)_4$MAPs in mice immunized orogastrically with *Salmonella typhi* strains as described elsewhere (Gonzalezet al. 1998) assessed by ELISA; **B)**. Mice immunized intraperitoneally with (▪) CVD908; (+) CVD908-pST13-NANP; (∗) CVD908ΩCSP; (■) CVD908ΩCSP-pST13-NANP; (×) Preimmune sera.

Fig. 2. Comparison between the antibody response against $(B)_4$MAPs, a tetramer branched synthetic peptide containing $(NANP)_3$ in each of the four branches (kindly donated by Dr. Elizabeth Nardin, Department of Medical and Molecular Parasitology. New York University School of Medicine, New York, NY), elicited in mice immunized with *Salmonella typhi* CVD908 expressing the CSP in the cytosol, the $(NANP)_3$ epitope on the bacterial surface or from both bacterial compartments.

Finally, we will describe some experience with autotransporters for autodisplay of antigens. Autotransporters belong to a family of OMPs, which lack the requirement of specific accessory molecules for secretion through the outer membrane. These proteins bear all necessary signals encoded within the polypeptide itself. They contain a C-terminal domain, (β-domain or translocator domain) which allows the N-terminal α passenger domain to cross from the inner membrane to the periplasmic space. The α-passenger domain is flanked by an N-terminal signal sequence responsible for initial export into the bacterial perplasmic space by a *sec* dependent mechanism. Once in the periplasmic space the C-terminal translocator β-domain forms a barrel and inserts in the outer membrane, and the N-terminal passenger α passenger domain travels through the central pore to the external milieu where exerts its biological function. Once on the surface, the final fate of the N-terminal passenger α passenger domain is determined by the presence of autoproteolytic mechanisms or surface proteases, which cleavage and release the α passenger domain to the external environment. (Finket al. 2001). More than 40 proteins with autotransporting properties have

been characterized (Desvauxet al. 2004; Hendersonet al. 2001). Due the relative simplicity of their transporting mechanism, the β-domain from several autotransporters has been employed translocate and display recombinant passenger proteins on the surface of enterobacteria. We already reported the use of MisL (another member of the AIDA-subfamily) to express foreign immunogenic epitopes on the surface of gramnegative bacteria (Luria-Perezet al. 2007; Ruiz-Olveraet al. 2003; Ruiz-Perezet al. 2002).

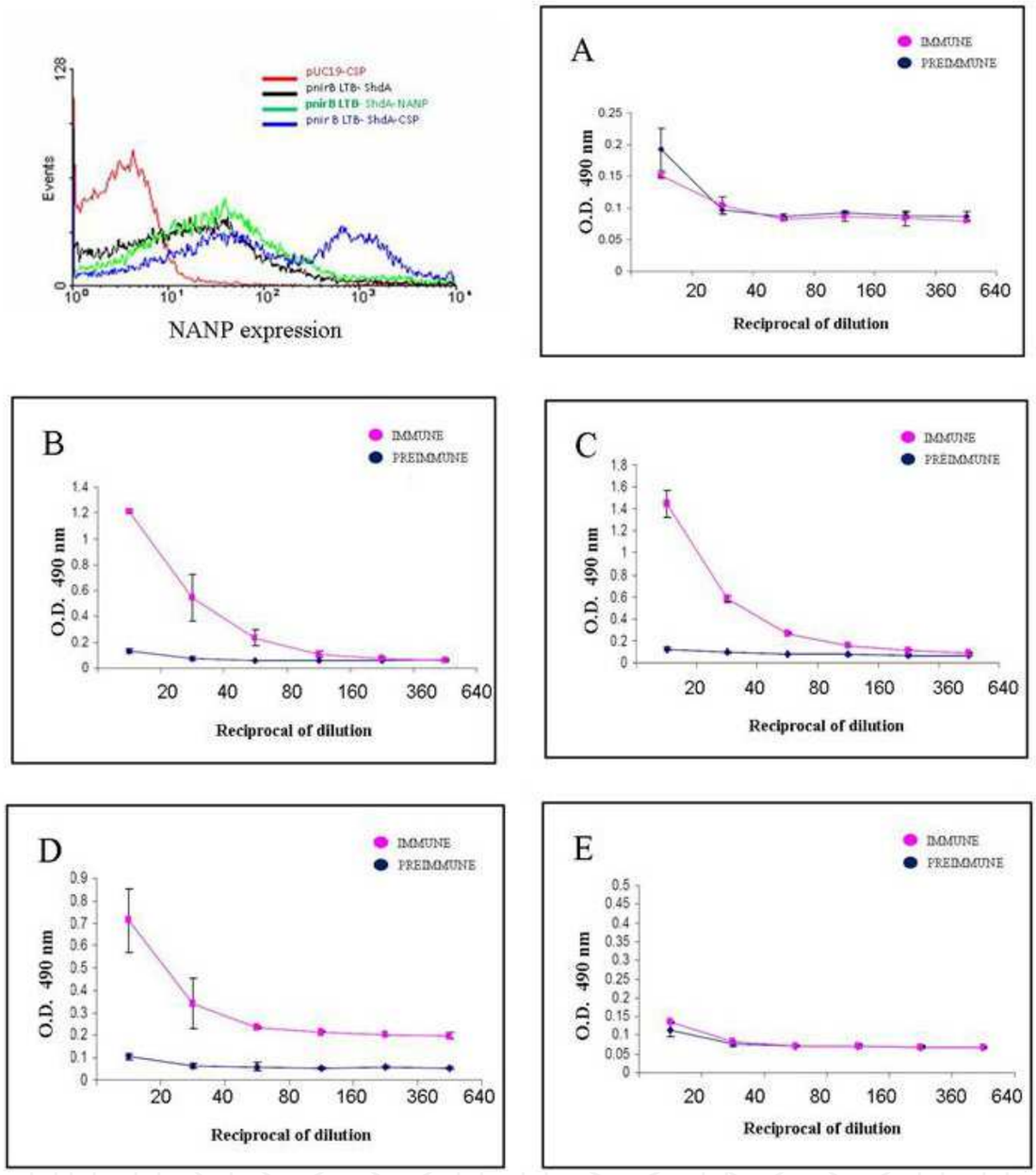
ShdA is other large autotransporter, (Desvauxet al. 2004) identified in *S. enterica* subespecies (Kingsleyet al. 2000), with similar structure to AIDA-I, TibA, and MisL, therefore it has been included also in the AIDA-subfamily. The α-domain is an adhesin (Kingsleyet al. 2000) that mediates bacterial colonization in the host cecum, the main reservoir for *S.* Typhimurium during infection in mice (Kingsleyet al. 2002). In fact, the inactivation of *shdA* produces bacterial number and bacterial permanence in the intestinal mucosa (shedding reduction) (Kingsleyet al. 2000; Kingsleyet al. 2002). The extracellular matrix protein fibronectin is a receptor for the ShdA passenger domain. This was demonstrated by a ShdA–GST (glutathione *S*-transferase) fusion protein which bound fibronectin *in vitro* in a dose dependent manner and was partially inhibited by anti-fibronectin antibodies, suggesting that other receptors may also play a role in ShdA-mediated adherence to the intestinal mucosa (Kingsleyet al. 2004).

Several autotransporters (Maureret al. 1999) require a link region between the α and β domains for autodisplay. This minimal translocation unit (TU) is necessary to allow folding of the passenger α -domain (Oliveret al. 2003). The role of TU in ShdA still remains to be show. Since autotransporters are able to display heeterologous peptide substituting the α -domain they have been used for the construction of bacterial whole-cell absorbents, study receptor-ligand interactions surface display of random peptide libraries and vaccine development (Lattemannet al. 2000).

We describe here an example of the latter application exposing the NANP immunodominat epitope from *Plamodium falciparum* CSP on the surface of *Salmonella* using an autotransporter. We generated a series of NANP-ShdA fusion proteins containing the β-domain and different truncated α-domains forms under the control of *nirB* promoter (Chatfieldet al. 1992), using the technical approach described elsewhere (Ruiz-Perezet al. 2002).

The flow cytometry **in Figure 3** presents the summary of several assays performed to identify the minimal α-domain amino acid strand necessary for translocation through the ShdA β-domain. *S.* Typhimurium SL3261 was transformed with plasmids bearing different truncated α-domain forms fused to three repeats of NANP [(NANP)3] or the complete CSP. NANP expression on to the surface of the bacteria was determined with a monoclonal antibody. We identified that the minimum translocation unit necessary to translocate the epitope is conformed 16 residues in the α-domain. Interestingly only around 45% of the bacterial strains expressed the antigen on their surface.

BALB/c mice were immunized with different S. Typhimurium SL3261 expressing the full length CSP or the (NANP)3 epitope on the surface and compared with a strain producing the antigen in the bacterial cytosol **(Figure 3 A-E)**. As expected, the strain expressing only ShdA did not elicit antibodies. The strains expressing the NANP or the CSP elicited good antibody response **(Figure 3 B-C)**, whereas the strain producing the CSP in the cytosol was unable to elicit antibodies **(Figure 3 E).** An additional control, autotransporter MisL expressing the NANP epitope, was able as well to elicit antibodies **(Figure 3 D)**.



Serum antibody response elicited by immunization with *Salmonella enterica* serovar Typhimurium SL3261 transformed with differents plasmids. BALB/c mice were immunized o.g. as described elsewhere (Gonzalezet al. 1998) (A) pnirB LTB- ShdA (negative control) (B) pnirB-LTB NANP ShdA; (C) pnirB-LTB CSP ShdA.; (D) pnirB-LTB NANP MisL; (E) pUC19 CSP. Groups of 5 BALB/c mice were immunized orally with two doses of $1x10^{10}$ C.F.U. (15-day interval) of the *Salmonella SL3261* strain transformed with different plasmids. IgG levels were determined one week after last immunization by ELISA as previously described (González et al., 1998). Each graphic represents the serum IgG from one mouse.

Fig. 3. Flow cytometry analysis of strains of *Salmonella enterica* serovar Typhimurium SL3261 transformed with differents plasmids. Plasmid pUC19 CSP corresponding to cytosolic form of antigen, whereas pnir B LTB ShdA-CSP and pnir B LTB ShdA-NANP corresponding to antigen display on the bacterial surface.

5. Conclusion

In summary, there is increasing evidence that antigen location in live bacterial carrier vaccines, in this case attenuated *Salmonella* strains is an important factor determining the type of immune response elicited to the passenger antigen.

6. References

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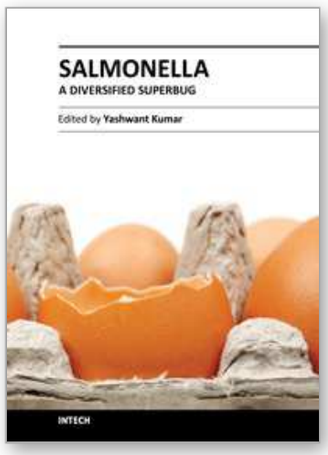

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Salmonella is an extremely diversified genus, infecting a range of hosts, and comprised of two species: enterica and bongori. This group is made up of 2579 serovars, making it versatile and fascinating for researchers drawing their attention towards different properties of this microorganism. Salmonella related diseases are a major problem in developed and developing countries resulting in economic losses, as well as problems of zoonoses and food borne illness. Moreover, the emergence of an ever increasing problem of antimicrobial resistance in salmonella makes it prudent to unveil different mechanisms involved. This book is the outcome of a collaboration between various researchers from all over the world. The recent advancements in the field of salmonella research are compiled and presented.

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