

Review

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Development of acquired immunity to *Salmonella*

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Salmonella enterica serovar Typhi (*S. typhi*) causes human typhoid fever, a serious and widespread disease in developing countries. Other *Salmonella* serovars are associated with food-borne infections. The recent emergence of multi-drug-resistant *Salmonella* strains highlights the need for better preventive measures, including vaccination. The available vaccines against *Salmonella* infection do not confer optimal protection. The design of new *Salmonella* vaccines must be based on the identification of suitable virulence genes and on knowledge of the immunological mechanisms of resistance to the disease. Control and clearance of a vaccine strain rely on the phagocyte oxidative burst, reactive nitrogen intermediates, inflammatory cytokines and CD4⁺ TCR- $\alpha\beta$ ⁺ T cells and are controlled by genes including *NRAMP1* and MHC class II. Vaccine-induced resistance to reinfection requires the presence of Th1-type immunological memory and anti-*Salmonella* antibodies. The interaction between T and B cells is essential for the development of resistance following vaccination. The identification of immunodeficiencies that render individuals more susceptible to salmonellosis must be taken into consideration when designing and testing live attenuated *Salmonella* vaccines. An ideal live *Salmonella* vaccine should therefore be safe, regardless of the immunological status of the vaccinee, but still immunogenic.

Introduction

Salmonella enterica serovar Typhi (*S. typhi*) causes typhoid fever in humans, with an annual global burden of about 16 million cases, leading to 600 000 deaths (Ivanhoff, 1995). Other *S. enterica* serovars (e.g. *S. typhimurium*, *S. enteritidis*) that can be transmitted from domestic animals to humans cause gastroenteritis and represent a serious problem for the food industry (Cooke, 1990; Todd, 1990). Prevention of salmonellosis by implementation of hygiene measures is difficult. Antibiotic treatment of infections can be problematic due to the recent emergence of multi-drug-resistant *Salmonella* strains (Mirza *et al.*, 1996).

Vaccination is an effective tool for the prevention of *Salmonella* infections (Mastroeni *et al.*, 2001). The currently available vaccines against salmonellosis can be broadly divided into three major classes.

(i) Whole-cell killed vaccines

These consist of bacterial cells inactivated with heat or acetone and are administered parenterally. In humans, killed vaccines elicit good antibody responses and confer a moderate degree of protection (Levine *et al.*, 1989). Whole-cell killed vaccines are reactogenic and induce poor cell-mediated immunity (Collins, 1974; Harrison *et al.*, 1997).

(ii) Subunit vaccines

Subunit vaccines, such as the ones based on the Vi polysaccharide of *S. typhi*, are safe, immunogenic and are currently licensed for human use. Vi vaccines confer between 55 and 75% protection against typhoid fever in endemic areas (Acharya *et al.*, 1987; Klugman *et al.*, 1996). The immunogenicity and protective ability of Vi increases when the latter is bound to protein carriers (Kossaczka *et al.*, 1999; Lin *et al.*, 2001; Singh *et al.*, 1999; Szu *et al.*, 1987, 1989). Other subunit vaccines such as the ones based on detoxified LPS, cell extracts, porins, O-polysaccharides and O-conjugates have been tested in experimental models and have proven less efficacious.

(iii) Live attenuated vaccines

The potential superiority of live attenuated vaccines in comparison with inactivated preparations has recently prompted extensive research towards the development of *Salmonella* mutants to be used as vaccines in human and veterinary medicine. To date, the development of live attenuated vaccines against *Salmonella* infections has been based mainly on empirical criteria. The availability of the complete genome sequences for *S. typhi* and *S. typhimurium* and advanced methods to identify virulence genes expressed *in vivo* are useful tools for the generation of attenuated *Salmonella* mutants as potential vaccine candidates (McClelland *et al.*, 2001; Parkhill *et al.*, 2001; Shea *et al.*, 1996; Slauch *et al.*, 1994). However, the rational design and

Abbreviations: CGD, chronic granulomatous disease; DC, dendritic cell.

selection of *Salmonella* strains to be used as potential vaccines must also hinge on knowledge of the pathogenesis and immunobiology of the diseases. In fact, the profile and anatomical location of vaccine-induced immune responses have an enormous influence on whether solid and long-lasting acquired resistance to the pathogen is established in the vaccinated individual.

The present article focuses on those aspects of the development of immunity to *Salmonella* that are relevant to vaccine design.

In vivo pathogenesis of *Salmonella* infections

After oral infection, *Salmonella* invades M cells and epithelial cells and passes through the Peyer's patches, mesenteric lymph nodes, lymphatic vessels and the blood stream (Carter & Collins, 1974). An alternative mechanism of invasion has been described where *Salmonella* is engulfed by dendritic cells (DCs) at the mucosal surface (Rescigno *et al.*, 2001) and is then transported from the gastrointestinal tract to the bloodstream by CD18⁺ phagocytes (Vazquez-Torres *et al.*, 1999). After interacting with complement factors, the bacteria reach an intracellular location within macrophages, polymorphonuclear cells (GR1⁺), DCs (CD11c⁺ MHC-II⁺) and occasionally B220⁺ B cells (Biozzi *et al.*, 1960; Dunlap *et al.*, 1991; Liang-Takasaki *et al.*, 1983; Saxen *et al.*, 1987; Warren *et al.*, 2002; Yrlid *et al.*, 2001). Later in the infection, the bacteria are localized in discrete infection foci consisting of inflammatory phagocytes that are surrounded by normal tissue (Richter-Dahlfors *et al.*, 1997). The numbers of infected phagocytes and foci in a *Salmonella* infection increase in parallel with the number of viable bacteria present in the tissues. Bacterial growth results in the distribution of bacteria to uninfected cells and in the formation of new pathological lesions that always contain small numbers of bacteria (our unpublished results). Therefore, effective vaccine-induced immunity needs to control bacterial growth at each focus of infection and must hinder the redistribution of *Salmonella* to new foci.

Knowledge of the anatomical sites where protective immunity must operate is also relevant to vaccine design. For example, using live attenuated *Salmonella* vaccines in mice, it is easier to induce protection against oral challenge than intravenous challenge (Hormaeche *et al.*, 1991). This suggests that local immunity in the gut or in the associated lymphoid tissue must play an important role in acquired protection against the disease.

Mechanisms that control growth and clearance of the vaccine in tissues

The importance of understanding how the growth of a *Salmonella* vaccine strain is controlled by the host is twofold. Firstly, it provides an indication of which mechanisms of innate and acquired immunity are triggered during primary infection with the vaccine and, secondly, it allows the

identification of potential immunodeficiencies that would make administration of a particular live attenuated vaccine dangerous.

Early innate immunity

In the mouse typhoid model, *Salmonella* growth in the tissues is controlled by the *NRAMP1* gene during the first few days of the infection. *NRAMP* homologues have been identified in several animal species. This gene encodes a divalent metal (Fe²⁺, Zn²⁺, Mn²⁺) pump phosphoglycoprotein (90–100 kDa) that is rapidly recruited to the bacteria-containing phagosome (Vidal *et al.*, 1993). Recent studies in humans suggested no association between polymorphic alleles within or near *NRAMP* and susceptibility to typhoid fever (Dunstan *et al.*, 2001b) however, not excluding a possible correlation between *NRAMP1* and clinical parameters such as severity of disease or incubation time.

The control of *Salmonella* growth in the early phases of a primary infection requires reactive oxygen intermediates generated via the phagocyte NADPH oxidase (*phox*), as shown by the extreme susceptibility to salmonellosis displayed by gp91*phox*^{-/-} mice (Mastroeni *et al.*, 2000b). *Salmonella* can evade killing by inhibiting the localization of the NADPH oxidase to the phagosome. This is achieved by using genes within the pathogenicity island 2 (SPI-2) (Vazquez-Torres *et al.*, 2000). Reactive oxygen intermediates also appear to be important for resistance to *Salmonella* in humans. In fact, chronic granulomatous disease (CGD) patients are deficient in the NADPH oxidase and are susceptible to recurrent microbial infections, including salmonellosis (Curnutte *et al.*, 1989; Mouy, 1989).

Adaptive responses

The phase of early innate immunity is followed by activation of a complex host response that suppresses the growth of bacteria in tissues. This response does not require T or B cells and coincides with the infiltration of macrophages in infected tissues and with the formation of macrophage-rich granulomas. The concerted action of several cytokines, including tumour necrosis factor (TNF)- α , interferon (IFN)- γ , interleukin (IL)-12, IL-15 and IL-18, is essential for the adaptive phase of the immune response. TNF- α is involved in the formation and persistence of granulomas as well as in the regulation of NADPH oxidase-mediated killing of *Salmonella* by macrophages (Vazquez-Torres *et al.*, 2001). IFN- γ is produced presumably by natural killer (NK) cells in response to IL-12 and IL-18 and mediates the upregulation of nitric oxide synthase (iNOS)-dependent macrophage anti-bacterial mechanisms (Mastroeni *et al.*, 1998).

IFN- γ and IL-12 are crucial for host resistance to *Salmonella* infection in humans. IFN- γ -receptor ligand-binding chain (IFN- γ R1) deficiency or signalling chain (IFN- γ R2) deficiency, IL-12-receptor β 1 chain (IL-12R β 1) deficiency as well as deficiencies in the IL-12 p40 subunit predispose humans to salmonellosis (Altare *et al.*, 1998; de Jong *et al.*, 1998; Picard *et al.*, 2002).

The ability to respond to LPS appears to be important for host resistance to *Salmonella*. *Lps^{d/d}* mice are hyporesponsive to LPS due to mutations in the gene that encodes Toll-like receptor 4 (*tlr4*). These mice cannot control *Salmonella* growth, because of defects in macrophage functions (Hormaeche, 1990; O'Brien *et al.*, 1982). Although LPS responsiveness is essential for host resistance in primary infections, vaccine-induced immunity normally develops in mice lacking functional TLR4 (Eisenstein *et al.*, 1984a).

Clearance of bacteria

Clearance of a vaccine strain from tissues requires the CD28-dependent activation of CD4⁺ TCR- $\alpha\beta$ ⁺ T cells. In fact, H2I-A β ^{-/-} mice (lacking mature CD4⁺ TCR- $\alpha\beta$ ⁺ T cells) and TCR- β ^{-/-} mice (lacking TCR- $\alpha\beta$ ⁺ T cells) do not eliminate the bacteria from tissues but show a progressive, usually fatal, increase in bacterial loads in the late stages of infection (Hess *et al.*, 1996). TCR- $\gamma\delta$ ⁺ T cells appear during infection and probably play a role in resistance to salmonellosis in *NRAMP1*^{susceptible} mice (Hess *et al.*, 1996; Mixter *et al.*, 1994; Weintraub *et al.*, 1997).

Clearance of infection is under the control of MHC genes. In B10 congenic mice, the clearance rate of *Salmonella* can be high (H-2^j, H-2^q and H-2^u), intermediate (H-2^d, H-2^f, H-2^k, H-2^p, H-2^r, H-2^s and H-2^v) or low (H-2^b) (Hormaeche *et al.*, 1985; Nauciel *et al.*, 1988). A correlation between MHC class II and III genes and the incidence of typhoid fever has also been described in humans. In fact, the polymorphic allele variants HLA-DRB1*0301/6/8, HLA-DQB1*0201-3 and TNF-A*2 (-308) were associated with susceptibility to typhoid fever, whereas HLADRB1*04, HLADQB1*0401/2 and TNF-A1 (-308) were associated with resistance to the disease (Dunstan *et al.*, 2001a).

Immunological correlates of protective immunity

Following immunization with protective live attenuated *Salmonella* vaccines, long-lasting immunological memory develops in animals and in humans. Serum and mucosal responses elicited by live vaccines are directed towards a broad spectrum of antigens, including LPS, Vi, porins, outer-membrane proteins, lipoproteins, heat-shock proteins, flagella and fimbriae (Brown & Hormaeche, 1989; Cooper & Thorns, 1996; Harrison *et al.*, 1997; Kantele *et al.*, 1986, 1991; Kuusi *et al.*, 1979; McSorley *et al.*, 2000; Szein *et al.*, 1994). In animals and humans exposed to live *Salmonella*, cellular responses are of the Th1 type, as indicated by delayed-type hypersensitivity (DTH) and by the predominant production of IL-2 and IFN- γ upon *in vitro* restimulation of immune T cells (Harrison *et al.*, 1997; Szein *et al.*, 1994). Class Ib CD8⁺ T cells capable of lysing *Salmonella*-infected target cells also appear after vaccination (Lo *et al.*, 1999, 2000; Pasetti *et al.*, 2002; Pope *et al.*, 1994; Salerno-Goncalves *et al.*, 2002; Szein *et al.*, 1995). The T-cell responses are directed towards several antigens, including protein antigens, porins, flagellar epitopes and pilin and, surprisingly, also to LPS and to the Vi surface polysaccharide (Cao *et al.*, 1992; Collins, 1974;

Cookson & Bevan, 1997; Galdiero *et al.*, 1998; Hormaeche *et al.*, 1981; Killar & Eisenstein, 1984, 1986; Matsui & Arai, 1989; McSorley *et al.*, 2000; Mukkur *et al.*, 1987; Murphy *et al.*, 1989; Ogunniyi *et al.*, 1994; Robertsson *et al.*, 1982a, b; Segall & Lindberg, 1993; Stabel *et al.*, 1993; Szein *et al.*, 1994; Taylor *et al.*, 1998; Udhayakumar & Muthukkaruppan, 1987; Vordermeier & Kotlarski, 1990a, b; Vordermeier *et al.*, 1990).

Injection with killed *Salmonella* vaccines or purified bacterial components (e.g. porins) gives rise to an IL-4-dominated Th2-type response with low levels of DTH and high levels of specific antibodies of the IgG1 isotype (Galdiero *et al.*, 1998; Thatte *et al.*, 1993).

It is still unclear which antigens are responsible for protection against *Salmonella*. Low levels of resistance against salmonellosis can be induced by administration of flagella, porins or polysaccharide fractions. However, these formulations have been tested mainly as non-living vaccines, making it difficult to ascertain whether poor protection is due to the antigen itself or to the suboptimal vaccine formulation. In the context of live attenuated vaccines, it appears that both immunodominant LPS O-polysaccharide determinants and unidentified non-serotype-specific determinants (presumably proteins) are required to achieve high levels of protection against fully virulent salmonellae (Hormaeche *et al.*, 1991, 1996; Kuusi *et al.*, 1979; Matsui & Arai, 1989; McSorley *et al.*, 2000; Segall & Lindberg, 1993; Svenson *et al.*, 1979; Watson *et al.*, 1992).

Effectors that mediate protection against reinfection in the immunized individual

Resistance to reinfection develops early after the administration of a live *Salmonella* vaccine. The earliest form of vaccine-induced resistance is that seen in chickens immunized orally with live salmonellae, and is probably due to competitive exclusion between the vaccine strain and the reinfecting bacteria in the gut (Berchieri & Barrow, 1990; Cooper *et al.*, 1994). At later stages of infection, resistance to rechallenge is due to the non-specific activation of macrophage functions with the involvement of TNF- α , IL-12, IFN- γ and NK cells (Maskell *et al.*, 1987; Nauciel & Espinas-Maes, 1992; Schafer & Eisenstein, 1992; Tite *et al.*, 1991). Long-term protection against *Salmonella* requires the antigen-specific recall of immunity, with the involvement of both antibodies and T cells in addition to the aforementioned cytokines. In experimental models of *Salmonella* infection, antibodies or immune T cells alone can protect against secondary challenge only in those host-pathogen combinations that involve the use of moderately virulent bacteria or innately resistant hosts (Eisenstein *et al.*, 1984b; Xu *et al.*, 1993). This can explain the moderate efficacy of those human typhoid vaccines based on the Vi polysaccharide antigen of *S. typhi* (Acharya *et al.*, 1987; Klugman *et al.*, 1996) or on acetone-killed whole cells. These vaccines can induce antibody responses, but are believed to be unable to trigger Th1-type immunity (Harrison *et al.*, 1997; Thatte *et al.*, 1993).

Initiation and development of protective vaccine-induced immunity

Dendritic cells (DCs)

The initiation of an immune response usually involves DCs, which are capable of priming naive T cells. *Salmonella* can infect DCs *in vitro* and *in vivo* and can induce activation of and cytokine production by these cells (Hopkins & Kraehenbuhl, 1997; Marriott *et al.*, 1999; Svensson *et al.*, 2000). DCs that phagocytose *Salmonella in vitro* can prime bacterium-specific CD4⁺ and CD8⁺ T cells following administration into naive mice, suggesting a possible role for these cells in the initiation of an immune response to *Salmonella* (Yrlid *et al.*, 2001).

T cell–B cell interactions in the development of immunity to *Salmonella*

The cross-talk between T and B cells is of fundamental importance for the establishment of solid acquired immunity to salmonellosis.

T cells modulate humoral responses during immunization with live attenuated *Salmonella* vaccines. In fact, athymic *nu/nu* (T-cell-deficient) and CD28^{-/-} mice (with impaired T-cell activation and reduced T cell–B cell co-operation) produce low levels of IgM and IgG3, but little or no IgG1, IgG2a or IgG2b antibodies against *Salmonella* LPS or protein antigens (Mittrucker *et al.*, 1999; Sinha *et al.*, 1997).

B cells can be infected by *Salmonella in vitro* and *in vivo* (Sztejn *et al.*, 1995; Yrlid *et al.*, 2001), raising the possibility that, besides antibody production, B cells may have additional functions in the initiation and modulation of immune responses to *Salmonella*. Epstein–Barr virus-transformed human B-cell lines present *Salmonella* antigens to human T cells, suggesting that B cells may be required for the activation of *Salmonella*-specific T cells (Sztejn *et al.*, 1995). This is further supported by the fact that CD4⁺ T cells obtained from B-cell-deficient *Igh-6*^{-/-} mice immunized with live attenuated *Salmonella* show reduced ability to release the Th1-type cytokines IL-2 and IFN- γ (Mastroeni *et al.*, 2000a). Furthermore, these immunized *Igh-6*^{-/-} mice fail to control the growth of virulent salmonellae in secondary infections (Mastroeni *et al.*, 2000a; McSorley & Jenkins, 2000; Mittrucker *et al.*, 2000).

Consideration of potential hazards determined by immunodeficiencies in vaccinees

The mouse typhoid model and clinical observations in immunocompromised patients have unravelled a number of important immunological determinants of host resistance to *Salmonella* in primary infections. This has contributed to the identification of immunodeficiencies that increase susceptibility to *Salmonella* infection and may represent hazards in the use of live attenuated *Salmonella* vaccines. CGD, defects in IFN- γ or IL-12 secretion or cytokine receptors, HIV infection (and other T-cell defects), diabetes, adminis-

tration of corticosteroids or other immunosuppressive drugs and malignancies predispose animals and humans to *Salmonella* infection (Bodey, 1974; Hohmann, 2001; Ottenhoff *et al.*, 1998). These immunodeficiencies may be latent in the individual at the time of vaccination.

Some widely tested live attenuated *Salmonella* vaccines such as aromatic-dependent (*aroA*) mutants, *htrA* mutants and *aroA htrA* double mutants are still able to cause severe systemic infections in T-cell-deficient, IL-12-deficient or IFN- γ -deficient mice (Hess *et al.*, 1996; Mastroeni *et al.*, 1998; Sinha *et al.*, 1997). SPI-2 mutants of *Salmonella* that are attenuated in severely immunocompromised IFN- γ ^{-/-} mice regain virulence in NADPH-oxidase-deficient or iNOS-deficient animals (Chakravorty *et al.*, 2002; Vazquez-Torres *et al.*, 2000). Some of these vaccines may prove extremely dangerous in CGD patients, in individuals with congenital IFN- γ or IL-12 defects and in individuals with T-cell deficiencies (e.g. HIV-infected patients) (Altare *et al.*, 1998; de Jong *et al.*, 1998; Gotuzzo *et al.*, 1991; Mouy, 1989; Ottenhoff *et al.*, 1998; Picard *et al.*, 2002). It is important to note that some of the immunodeficiencies listed above (e.g. T-cell deficiencies, IFN- γ and IL-12 deficiencies) in humans have been associated predominantly with infections caused by non-typhoidal *Salmonella* strains. The reasons for this are still unclear. One possibility is that primary immunodeficiency is diagnosed more efficiently in developed countries, where *S. typhi* infections are rare due to good sanitation.

Conclusions

The search for an ideal live *Salmonella* vaccine continues. This vaccine should harbour multiple defined mutations in known virulence genes that would render it safe regardless of the immunological status of the vaccinee, but that would not result in excessive attenuation and loss of immunogenicity. This has been difficult to achieve so far. Therefore, in addition to live vaccines, it would be wise to design and/or improve subunit vaccines against salmonellosis that would be usable in situations where live strains may not be suitable.

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