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-α/-β 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 (Kossaczk 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
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; Ylid 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 NRAMP 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 gp91phox−/− 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 antibacterial 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 β1 chain (IL-12R β1) 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).

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The ability to respond to LPS appears to be important for host resistance to Salmonella. Lps^+/− 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-αβ+ T cells. In fact, H2-Ab1- /− /− mice (lacking mature CD4+ TCR-αβ+ T cells) and TCR-β−/− mice (lacking TCR-αβ+ 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., 1991). TCR-γδ+ T cells appear during infection and probably play a role in resistance to salmonellosis in Nramp1−/− 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-2d, H-2v and H-2w), intermediate (H-2a, H-2b, H-2k), or low (H-2l) (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 HLA-DQA1*0308 were associated with susceptibility to typhoid fever, whereas HLADRB1*04, HLADQB1*0401/2 variants were associated with resistance to the vaccine strain (Cao et al., 1993). Class Ib CD8+ T cells. In fact, H2I−/− mice (lacking mature CD8+ T cells) and TCR-αβ+ 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., 1991). 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).

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 & Thornes, 1996; Harrison et al., 1997; Kantele et al., 1986, 1991; Kuusi et al., 1979; McSorley et al., 2000; Szttein 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; Szttein et al., 1994). Class Ib CD8+ T cells capable of lysing Salmonella-infected target cells also appear after vaccination (Lo et al., 1999, 2000; Paatti et al., 2002; Pope et al., 1994; Salerno-Goncalves et al., 2002; Szttein 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; Robertson et al., 1982a; Segall & Lindberg, 1993; Stabel et al., 1993; Szttein 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 reinfesting 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 & Espinasse-Maes, 1992; Schafer & Eisenstein, 1992; Tite et al., 1991). Long-term protection against Salmonella requires the antigen-specific recall of immune cells, 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 intravenously 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 nude (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 (Sztein 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 (Sztein et al., 1995). This is further supported by the fact that CD4+ T cells obtained from B-cell-deficient Ig-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 Ig-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 vaccines

The mouse typhoid model and clinical observations in immunocompromised patients have unravelled a number of important immunodeficiencies 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, administration 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 (Chukravortty 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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