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 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-αβ T cells and are controlled by genes including Nramp1 and MHC class II. Vaccine-induced resistance to reinfecion 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.

(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 (Kossacka 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
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 (Biuzzi et al., 1960; Dunlap et al., 1991; Liang-Takasaki et al., 1983; Saxen et al., 1987; Warren et al., 2002; Yilid 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 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 (Biuzzi et al., 1960; Dunlap et al., 1991; Liang-Takasaki et al., 1983; Saxen et al., 1987; Warren et al., 2002; Yilid 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.

**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 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 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).
The ability to respond to LPS appears to be important for host resistance to *Salmonella*. Lps<sup>−</sup> 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<sup>+</sup> TCR-αβ<sup>−</sup> T cells. In fact, H2<sup>L</sup>-A<sup>β</sup>−<sup>−</sup> mice (lacking mature CD4<sup>+</sup> TCR-αβ<sup>+</sup> T cells) and TCR-β<sup>−</sup> mice (lacking TCR-αβ<sup>+</sup> 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-γδ<sup>+</sup> T cells appear during infection and probably play a role in resistance to salmonellosis in NRAMP<sup>+</sup> 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<sup>A</sup>, H-2<sup>D</sup> and H-2<sup>N</sup>), intermediate (H-2<sup>B</sup>, H-2<sup>F</sup>, H-2<sup>M</sup>, H-2<sup>D</sup> and H-2<sup>C</sup>-p) or low (H-2<sup>F</sup>-p) (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 HLA-DRB1*0301/6/8, HLA-DQB1*0201-3 and HLA-DQA1<sup>A</sup>/C0<sup>Æ</sup>/C226<sup>Æ</sup> probably play a role in resistance to salmonellosis in *Salmonella* animals and humans exposed to live attenuated 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; Svensson et al., 1979; Watson et al., 1992).

**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, lipopolysaccharide proteins, 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 II CD8<sup>+</sup> 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; 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., 1988; Oggunniyi et al., 1994; Robertsonson et al., 1982a; b; Segall & Lindberg, 1993; Stabel et al., 1993; Szttein et al., 1994; Taylor et al., 1998; Udhayakumar & Muthukaruppan, 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; Svensson 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 (Burchier & 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 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 (Szein 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 (Szein 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 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.

References


Development of acquired immunity to Salmonella


Development of acquired immunity to Salmonella


