Cell-Mediated Immunity and Antibody Responses Elicited by Attenuated *Salmonella enterica* Serovar Typhi Strains Used as Live Oral Vaccines in Humans

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The development of improved typhoid vaccines is a high global public health priority. However, their development has been hampered by a lack of information regarding the specific determinants of protective immunity to *Salmonella enterica* serovar Typhi (S. Typhi) infection in humans. Although antibodies to S. Typhi O, H, and Vi appear to be involved in protection against S. Typhi infection, it is unknown whether such antibodies mediate protection, act in conjunction with other adaptive responses, or serve as a surrogate for the presence of other, more dominant protective immune responses (e.g., cell-mediated immunity [CMI]). CMI responses elicited by immunization of subjects with attenuated S. Typhi oral vaccines include lymphoproliferation; production of type 1 cytokines (e.g., interferon-γ and tumor necrosis factor–α); and classical major histocompatibility complex (MHC) class Ia–restricted and novel, nonclassical MHC class Ib (human leukocyte antigen [HLA]–E)–restricted CD8+ cytotoxic T cell responses. In sum, human immunity to S. Typhi elicited by immunization is unexpectedly broad and complex. However, the immunologic correlates of protection remain largely undefined.

Disappointingly, unlike most other contributors to this special remembrance of Dr. Theodore E. Woodward, I did not have the opportunity to interact with him personally. By the time I joined the faculty at the University of Maryland in 1989, he had already been retired for almost a decade. My knowledge of Dr. Woodward’s impeccable character and commitment to alleviate the scourge of typhoid fever was relayed to me by colleagues who had interacted closely with him, such as Dr. M. Levine. Dr. Woodward’s seminal contributions in the 1960s and early 1970s to understanding the pathogenesis of and host defenses against typhoid fever are well recognized. For more than 16 years, Dr. Woodward’s studies have been a source of inspiration and guidance to me in my research to characterize the immunologic correlates of protection in this disease that affects millions of individuals each year. Thus, it is with great gratitude that I dedicate this review to the memory of Dr. Woodward.

**INTRODUCTION**

*Salmonella enterica* serovar Typhi (S. Typhi), the causative agent of typhoid fever, is an invasive bacterium that rapidly and efficiently passes through the intestinal mucosa of humans, its only natural host, to reach the reticuloendothelial system [1, 2]. It is estimated that ∼21,650,000 episodes of typhoid fever and 216,500 deaths due to the disease occurred in areas of endemicity in 2000 [3]. The risk of acquiring typhoid fever is increased among clinical microbiologists and travelers to regions where the disease is endemic [4]. The emergence of multidrug-resistant strains of S. Typhi [4] has added a sense of urgency to develop more effective typhoid vaccines. An attenuated S. Typhi live oral vaccine would also be a candidate to serve as a carrier of foreign antigens from other pathogens [5]. None of the currently available typhoid vaccines is ideal. Ty21a (the only licensed attenuated live oral vaccine) and purified Vi capsular polysaccharide vaccines are well tolerated...
but only moderately protective [1, 4]. This deficiency has driven efforts to develop improved attenuated S. Typhi vaccine strains. The most promising include CVD 908-htrA, CVD 909, Ty800, and M01ZH09 [1, 4]. Despite recent advances [5–19], the pace of progress in this area of vaccinology has been hampered by a dearth of information about the specific determinants of protective immunity to S. Typhi in humans, particularly cell-mediated immunity (CMI), the focus of this review.

IMMUNE RESPONSES TO S. TYPHI IN HUMANS

Because humans are the only natural host for S. Typhi, and because there is a lack of a suitable small-animal model, the study of immune responses to S. Typhi in humans during the course of infection and vaccination has been imperative but difficult. Immunologic studies in volunteers immunized with purified Vi polysaccharide vaccine and with attenuated live oral vaccine strains have yielded important insights into the protection conferred by these types of vaccines when tested in the field [1, 4]. Immunity to S. Typhi is complex and involves local and systemic antibody and CMI components. Serum antibodies to S. Typhi antigens (e.g., Vi and lipopolysaccharide [LPS] O) likely play an important role in defense against typhoid bacilli when they are extracellular. However, because S. Typhi can persist intracellularly in the human host, thereby avoiding destruction by antibodies and complement, CMI is expected to be essential in eliminating S. Typhi infection. A discussion of current knowledge of immunity to S. Typhi in humans follows.

Serum Antibodies

The exact role of serum antibodies in protection against typhoid fever remains unclear. The fact that purified Vi polysaccharide administered as a parenteral vaccine is efficacious against typhoid fever demonstrates that serum Vi antibodies can mediate protection. In contrast, the live oral vaccine strain Ty21a, which lacks Vi antigen, also confers a moderate level of long-lived protection [1, 4, 20]. With Ty21a, the rate of seroconversion of serum IgG O antibody, although not believed to be the operative immune mechanism elicited by attenuated strains, correlated with the level of protection conferred by some Ty21a formulations and immunization schedules [1, 4]. However, relapses of acute typhoid occur despite the presence of elevated titers of antibodies against O, H, and other S. Typhi antigens [21–24]. Moreover, the seroconversion rate of IgG O antibodies did not predict the poor efficacy of other formulations [25]. The appearance of antibodies specific to outer membrane proteins of S. Typhi (e.g., OmpC) and heat shock proteins (e.g., GroEL) in patients with typhoid fever is a clear indication that the immune response to S. Typhi is not restricted to LPS, H, and Vi components. Collectively, these observations suggest that, although serum antibodies to S. Typhi antigens, including O, H, and Vi, may play a role in protection against S. Typhi, they might not represent the dominant protective immune response that eventually eliminates the bacteria from the host. Because some IgG subclasses are better suited to antibacterial activities (e.g., neutralization and opsonization) than others, the proportion of the IgG subclasses (IgG1–IgG4) may be important in determining the functionality of the response. Similarly, antibody avidity, which measures the strength of the attachment of antibodies to their antigen and is highest after B cells have been adequately primed, is an important measurement of the strength of the anamnestic response. Regrettably, no information is available on IgG subclasses or the avidity of antibodies elicited in typhoid vaccine recipients or patients with typhoid fever. Such information might better define the role of serum antibodies in protection from disease.

Systemic CMI

Both CD4+ helper T cells and CD8+ cytotoxic T cells might play key roles in defense against S. Typhi.

Helper T cell responses. Clinical trials, including studies of volunteers experimentally challenged with wild-type S. Typhi, performed in the 1960s and 1970s by Theodore Woodward and colleagues at the University of Maryland, provided seminal information about infection and immunity and suggested a key role for CMI [1, 2, 21, 26]. However, because, at that time, techniques to measure antibacterial CMI were rudimentary, limited CMI data were obtained. Those studies showed that typhoid fever conferred only 30% protection against typhoid fever on experimental rechallenge, and correlations were not observed between antibody responses to O and Vi and protection [22, 24]. Detailed measurements of CMI to S. Typhi have subsequently been performed during the past 15 years in subjects orally immunized with attenuated S. Typhi live vaccine strains at the Center for Vaccine Development.

We showed that immunization with attenuated S. Typhi strains, including Ty21a, CVD 906, CVD 908, CVD 908-htrA, and CVD 909, resulted in the appearance in peripheral blood of sensitized lymphocytes that exhibit significantly increased lymphoproliferative responses and Th1-type cytokine production patterns (i.e., IFN-γ in the absence of IL-4 and IL-5) in response to S. Typhi antigens [6, 8, 14–16, 18, 19, 27, 28], [R. Wahid, R. Salerno-Gonçalves, and M.B.S., unpublished data]. These results were confirmed by others [10, 29, 30]. We have demonstrated that PBMCs from Ty21a and CVD 909 vaccine recipients also secreted IL-1β, TNF-α, and IL-10 after in vitro incubation with S. Typhi flagella [14, 19]. Moreover, we identified CD3+CD4+CD8-CD56- T helper cells as the predominant IFN-γ-secreting effector cell population responding to soluble S. Typhi antigens in CVD 908-htrA [6] and CVD 909 vaccine recipients (figure 1A and 1B) (R. Wahid, R. Salerno-Gonçalves, and M.B.S., unpublished data). Lundin et al. [10] and Kilhamn et al. [29] reported that both CD4+ and CD8+ T cells from
Ty21a vaccine recipients secrete IFN-γ in response to particulate S. Typhi antigens.

The observed Th1-type responses are likely to be important in resistance to S. Typhi by a variety of mechanisms, including augmentation of macrophage bactericidal activity and enhancement of antigen presentation. The importance of Th1-type responses in resistance or susceptibility to S. Typhi is supported by associations between susceptibility to S. Typhi infection in HIV-positive patients and IL-12 receptor–deficient subjects and by the demonstration of human leukocyte antigen (HLA) haplotypes and TNF-α polymorphisms that either are protective or predispose individuals to typhoid fever.

**Classical (major histocompatibility complex [MHC] class Ia–restricted) cytotoxic T cell responses.** The ability of CD8+ cytotoxic T cells to eliminate cells infected with intracellular organisms is a fundamental immunologic effector mechanism in protection against intracellular infectious agents. Because S. Typhi is an intracellular pathogen, cytotoxic T cell responses likely play a crucial role in limiting progression of typhoid infection, by destroying host cells harboring bacilli. We developed a cytotoxic T cell assay and showed, in subjects immunized with CVD 908 or CVD 908 expressing the circumsporozoite protein of *Plasmodium falciparum*, the presence of classical MHC class Ia–restricted CD8+ cytotoxic T cell effectors able to lyse S. Typhi–infected targets [17]. We have extended these observations by demonstrating the ability of attenuated S. Typhi strains Ty21a, CVD 908-htrA, and CVD 909 to elicit cytotoxic T cell responses mediated by CD3+CD8+CD56+ T cell receptor α/β–positive cells in vaccine recipients [6, 11, 19]. This cytotoxic T cell cytotoxicity is granule dependent (i.e., mediated by perforin/granzyme) rather than due to Fas/Fas ligand interactions [9].

A second key effector mechanism of CD8+ T cells is cytokine production, IFN-γ production in particular. We developed an enzyme-linked immunospot assay to measure quantitatively the ability of CD8+ T cells to secrete IFN-γ after in vitro exposure to S. Typhi–infected autologous targets (phytohemagglutinin-expanded PBMCs) and observed that immunization with attenuated S. Typhi strains Ty21a, CVD 908-htrA, and CVD 909 elicited up to 550 IFN-γ–producing spot-forming cells per 10^6 PBMCs [6, 19]. Similar frequencies were observed by flow cytometry. Production of IFN-γ, as determined by enzyme-linked immunospot assay and measurement of cytotoxic T cell activity, correlated closely in individuals [11]. Although the predominant IFN-γ–producing effector population was consistently found to be CD3+CD8+CD4– T cells, some vaccine recipients also exhibited significant increases in CD3+CD4+CD8– IFN-γ–producing T cells (figure 1C–1F).

Even though we observed significant correlations between (1) IFN-γ production in response to soluble antigens and (2) cytotoxic T cell activity and IFN-γ production in response to S. Typhi–infected targets, no correlations were observed between CMI and serum antibody responses [6, 8, 18]. These observations emphasize the critical need to identify immunologic surrogates of protection of attenuated typhoid vaccine candidates as they move into field trials of efficacy. Antibody and CMI responses in vaccinated volunteers must be correlated with a lowered incidence of typhoid fever in comparison with that among control subjects.

**Nonclassical (HLA-E–restricted) cytotoxic T cell responses: a novel effector mechanism elicited in subjects immunized with the Ty21a typhoid vaccine.** We have recently described a novel mechanism that might be important in resistance to S. Typhi infection in humans—that is, the presence in subjects immunized with Ty21a of a novel subset of nonclassical human HLA-E–restricted anti–S. Typhi–specific CD8+ T cells able to kill S. Typhi–infected targets regardless of whether they share classical MHC class Ia molecules [9]. Extensive studies, including the use of the S. Typhi–infected EBV-LCL line 721.221.AEH (which does not express HLA-A, -B, -C, or -G) as targets, indicated that HLA-E molecules are associated with...
anti–S. Typhi–specific cytotoxic T cell killing and IFN-γ production [9]. Moreover, we identified CD3⁺CD8⁺CD4⁺CD56⁻ T cells as the effectors and demonstrated that this phenomenon is proteasome dependent and granule dependent. Increases in the net frequency of IFN-γ–producing spot-forming cells were observed in the presence of targets coated with peptides that contain S. Typhi GroEL HLA-E–binding motifs. Thus, not only natural killer cells but also a subset of CD8⁺ T cells can recognize bacteria-derived peptides in the context of HLA-E molecules, and these effectors may contribute to host defense against intracellular bacteria. Because nonclassical MHC class Ib molecules (i.e., HLA-E) are less polymorphic than classical MHC class Ia molecules, they likely present a more conserved set of peptides present in microorganisms. HLA-E–restricted cytotoxic T cells may bridge innate and adaptive immune responses and represent a mechanism complementary to classical MHC class Ia–restricted responses in protecting subjects from typhoid [9].

**Longevity and clonality of anti–S. Typhi CMI responses.** Other information needed to foster vaccine development involves study of the longevity and clonality of anti–S. Typhi CMI responses elicited by immunization. We have begun to address these issues by evaluating the presence and T cell receptor Vβ gene usage of specific CD8⁺ T cells from healthy adults 5–40 months after oral immunization with Ty21a [7]. Functionally, Vβ has been associated with the antigen-recognition process; specific recognition of peptide results in clonal expansion of subsets of Vβ antigen-specific T cells. Thus, studies of the Vβ repertoire of anti–S. Typhi–specific T cell subsets after immunization with Ty21a and other attenuated S. Typhi strains could provide important insights into the clonality and characteristics of T cell responses to S. Typhi and how they change over time. Although responses were oligoclonal, CD8⁺ T cells from several Vβ families participated. Moreover, CD3⁺CD8⁺ clones derived from these subjects exhibited a T effector/memory phenotype (i.e., CCR7⁻CD27⁻CD45RO⁻CD62L⁻) and coexpressed gut homing molecules (e.g., high levels of integrin α4/β7, intermediate levels of CCR9, and low levels of CD103). These observations support the notion that effector T cells encompassing a broad T cell receptor Vβ repertoire and able to express gut homing molecules are involved in the host’s response to S. Typhi after vaccination with Ty21a [7]. Notably, these cells were circulating >3 years after immunization. Future studies of the induction, expansion, and persistence of anti–S. Typhi–specific memory T cell populations will assess the potential for long-term protection elicited by immunization with attenuated S. Typhi. That CD8⁺ effector T cells elicited in subjects immunized with attenuated strains of S. Typhi include cells restricted by classical, as well as nonclassical, HLA molecules and have at least 2 distinct effector functions (i.e., cytotoxic T cell activity and IFN-γ production) suggest that these responses play a crucial role in limiting the progression of typhoid fever by destroying host cells harboring bacteria.

**Immune Responses in the Gut Microenvironment**

Effector immune responses in the gut microenvironment are likely to be important in protecting the host against S. Typhi. Enumeration of gut-derived circulating IgA antibody–secreting cells (ASCs) detected among PBMCs 7–10 days after ingestion of live oral typhoid vaccines gives an estimate of the degree of priming of the local intestinal immune system. These cells are believed to home to the lamina propria of the intestinal mucosa and other mucosal sites where they synthesize and release IgA antibody. The magnitude of the IgA ASC responses against O antigen after immunization with different formulations and immunization schedules of Ty21a correlated with efficacy in field trials [1, 2, 4]. However, increases in IgA ASC responses did not correlate with serum anti–S. Typhi LPS O responses [31]. Newer, more immunogenic, attenuated S. Typhi strains (e.g., CVD 908, CVD 908–htrA, CVD 909, Ty800, and M01ZH09) also stimulate strong IgA ASC responses after administration of just a single oral dose [4, 8, 16, 28]. Anti–S. Typhi–specific secretory IgA has been detected in the intestinal lavage fluid and stool specimens of subjects exposed to S. Typhi [1, 2, 16]. In contrast, except for the preliminary studies on homing molecules expressed on circulating anti–S. Typhi–specific T cells outlined above, no information is available on the CMI to S. Typhi antigens or ASCs in the gut mucosa of patients with typhoid fever or subjects immunized with attenuated S. Typhi. This remains a much-needed area of investigation. It is critical to identify which systemic responses, if any, correlate with gut mucosal responses and protection. This information will be of key importance in guiding us in the selection of the best systemic immune responses to evaluate in future vaccine clinical studies in typhoid-endemic areas.

**CONCLUDING REMARKS**

Despite progress, our knowledge of which immune responses, particularly CMI responses, lead to protection from S. Typhi infection or disease remains incomplete. Critical areas that need to be addressed include the characteristics, magnitude, and longevity of the memory T and B cell populations induced by immunization; the avidity and subclasses of anti–S. Typhi antibodies; the specific antigens and epitopes that play key roles in protection; the molecules or combination of molecules that determine the gut homing potential of circulating anti–S. Typhi–specific B and T cells; the S. Typhi–specific responses in the gut microenvironment; and the role of HLA-E–restricted responses in anti–S. Typhi immunity. This information is critical for establishing the immune responses likely to correlate with protection from infection with S. Typhi and for the ac-
elerated design of more effective attenuated typhoid fever vaccines.

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