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Feasibility of testing recombinant oral attenuated *Salmonella* vaccines in rabbits

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This thesis is submitted in fulfillment of the requirements for the degree of M.Sc.

ABSTRACT

Chancroid is a sexually transmitted genital ulcer disease caused by cutaneous or mucosal infection with the fastidious Gram-negative bacterium *Haemophilus ducreyi*. Chancroid is a facilitating cofactor, or a promoter of sexual transmission of the human immunodeficiency virus (HIV). It is prevalent in "intensive" commercial sex workers, particularly in populations with minimal public health surveillance and treatment. Antimicrobial treatment as intervention targeted to commercial sex workers reduces chancroid prevalence and may decrease the incidence of HIV in epidemic areas. However, therapeutic control requires intensive public health intervention, and is subject to treatment failure associated with HIV immunodeficiency and acquired antibiotic resistance. An effective vaccine against chancroid could take the place of therapeutic control programs, offering long-lasting protection without the risk of widespread drug resistance.

Orally administered recombinant attenuated *Salmonella* strains are used as vaccine vectors to deliver heterologous, pathogen-derived antigens to intestinal mucosal associated lymphoid tissue, and to provide vaccine adjuvancy. Chancroid vaccines are tested in a temperature-dependent rabbit model of experimental *H. ducreyi* infection. This model is successful because titred intradermal inocula and the immune response are quantified, and several measures of a disease effect resembling human chancroid are serially recorded. This allows meaningful comparisons of virulence between immunized rabbits and controls, in order to establish vaccine effects. However, testing of recombinant attenuated *Salmonella* strains as vaccine vectors has never been done in rabbits; it is usually done in mice. Anatomic and physiologic differences may limit this

approach to the demonstration of vaccine feasibility in rabbits. A three-part study was designed to assess the feasibility of testing attenuated *Salmonella* vector vaccines in rabbits. The questions asked were, 1) what is the maximum tolerated oral dose and minimum immunogenic oral dose of attenuated *Salmonella* in rabbits, 2) can a recombinant antigen expressed in the attenuated vector be recognized by the rabbit immune system, and 3) will experimental *H. ducreyi* infection in rabbits after oral *Salmonella* vaccination function as a comparative quantitative virulence assay to permit vaccine evaluation? A dose escalating study was conducted, and a maximum dose for consistent tolerance of 10^9 colony forming units (CFU), and a minimum dose for consistent seroconversion and high-level antibody titre (endpoint titre >1:16000) of 10^8 CFU were identified. Expression of tetanus toxin fragment C (TetC), a surrogate for *H. ducreyi* antigens that will eventually be expressed in this system, was achieved in attenuated strain *S. typhimurium* SL3261, and a strong serum antibody response to the recombinant antigen was shown. Finally, the course of experimental *H. ducreyi* infection in rabbits fed the attenuated *Salmonella* vector, the recombinant strain, or phosphate buffered saline (control) was followed. Culture positive, ulcerative disease was quantifiable in all three groups, suggesting that vector-, and recombinant-fed rabbits could serve as appropriate controls in future assessment of recombinant *Salmonella* vectors carrying *H. ducreyi* antigens. This is the first use of a recombinant attenuated *Salmonella* vaccine vector in rabbits. This work provides the necessary background for testing of *H. ducreyi* vaccine antigens delivered by attenuated *Salmonella* strains, and will speed human vaccine development by permitting feasibility to be demonstrated in the rabbit model of chancroid.

ACKNOWLEDGEMENTS

I would like to thank Dr. Bill Cameron for his sound advice, ideas, great patience and support, Dr. Craig Lee, for practical advice, Dr. Wallace Lauzon, anchor of the lab, and Dr. Isabelle Leduc for her support with the rabbit model. The attention and care showed by all the staff of Animal Care Services was greatly appreciated, and the guidance and expertise of Dr. Susan Kilborn, Kim Yates, Eileen Franklin and Monique Boileau, was critical to the success of this work. The Ontario HIV Treatment Network provided financial support; I thank the OHTN, and wish them continued success.

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ABBREVIATIONS USED

ABTS 2.2`-azino-bis (3-ethyl benthizolone-6-sulfonate)

AEC 3-amino-9-ethyl carbazole

ASC antibody secreting cells

CA chocolate agar

CDC U.S. centers for disease control

CFU colony forming units

CPA cyclophosphamide

DTH delayed-type hypersensitivity

EGTA ethylene glycol-0,0'-bis'(2-amino-ethyl)-N,N,N`N`,-tetraacetic acid

EIA enzyme immunoassay

GALT gut-associated lymphoid tissue

GC gonococcal

GUD genital ulcer disease

HIV human immunodeficiency virus

HSV herpes simplex virus

IFN interferon

IL interleukin

LB Luria-Bertani

LPS lipopolysaccharide

MALT mucosa-associated lymphoid tissue

MH Mueller-Hinton

MHC major histocompatibility complex

PAGE polyacrylamide gel electrophoresis

PBS phosphate buffered saline

PMN polymorphonuclear cell

SDS sodium dodecyl sulfate

SGSC *Salmonella* genetic stock centre

SOD superoxide dismutase

TCR T cell receptor

TetC tetanus toxin fragment C

TGMS Tris, gelatin, magnesium sulfate (buffer)

TNF tumour necrosis factor

INTRODUCTION

Attenuated *Salmonella* strains were initially developed to provide a single dose, orally administered, typhoid fever vaccine. Such was their success in eliciting protective immunity against typhoid, that they were adapted as live oral vaccine vectors expressing recombinant antigens to deliver and provide adjuvancy for heterologous pathogen-derived antigens. This tactic has achieved protective immunity against viral, bacterial and protozoan pathogens. Towards developing a recombinant attenuated *Salmonella* vaccine for control of chancroid, a sexually transmitted genital ulcer disease caused by *Haemophilus ducreyi*, a Gram-negative bacterium, I investigated the feasibility of using attenuated *Salmonella* as a vector to vaccinate rabbits, which provide the only available model of inducible immunity to chancroid.

Chancroid

Chancroid (reviewed in (1,2)) is transmitted by sexual contact with a partner infected with *H. ducreyi*. Bacteria are thought to penetrate the epidermis through minor abrasions (3). An inflammatory papule ensues 7-14 days after infection, which rapidly develops into a pustule. This pustule ruptures to form a necrotic, non-indurated ulcer with a ragged, undermined border, and a soft, tender, friable base (4). Without treatment, the disease persists for 3-8 weeks in men, and as long as 6 months in women (5). Inguinal lymphadenopathy occurs in some cases. The affected lymph node may suppurate, or rupture to form a draining but usually sterile bubo characteristic of

chancroid (6). Phagedenic ulcers, caused by superinfection with anaerobic bacteria, may result in necrotic destruction of much tissue (2,6).

Epidemiology

The prevalence and geographic range of chancroid has steadily decreased throughout the 20th century, perhaps due to socioeconomic changes altering patterns of prostitution, cessation of global military troop movements in the mid-1940s, and the advent of antibiotics, (reviewed in (7)). Endemicity is limited to regions of poverty with limited public health infrastructure. In the 1980s and early 1990s, chancroid remained endemic in eastern sub-Saharan Africa, South East Asia and parts of Latin America (8), with pockets of endemicity in the United States (9). Prostitution is an element in the maintenance of endemicity, and in most outbreaks, due to the "core-group" ecology of its transmission (10). For example, reemergence of chancroid in the United States of America was geographically and temporally associated with the exchange of sex for cocaine in "crack houses" in the 1980s (6). Aggressive therapeutic intervention in some centres has reduced the local prevalence of chancroid (11), but it is still very common in parts of Africa as noted in recent studies in Senegal (12) and Madagascar (13).

Chancroid and the human immunodeficiency virus (HIV)

Chancroid is a facilitating co-factor, or promotor of sexual transmission of HIV (14). Several mechanisms have been proposed to explain this finding. Chancroid and HIV share a sexual mode of transmission, and so occur in the same "core groups" of high-frequency STD transmitters (14). The presence of an ulcerative lesion on the

genitals provides a portal of entry for HIV. Inflammatory recruitment of CD4+ T cells, Langerhans cells and macrophages, all target cells of HIV, to the vicinity of the lesion, may further enhance transmission (15). Chancroid also enhances the infectiousness of a person with HIV; HIV can be recovered from the surface of chancroid lesions (16). Finally, HIV-associated immunodeficiency may result in larger chancroid ulcers that take longer to heal, and may be more resistant to treatment (17). These mechanistic arguments may also apply to other genital ulcer diseases such as syphilis and herpes simplex virus (HSV) infection, but there is a strong geographic association between chancroid and HIV; chancroid is common in all countries where the adult prevalence of HIV is greater than 8% (7,18). Control or elimination of chancroid could effectively reduce the heterosexual transmission of HIV where the diseases coexist.

Control of chancroid

Humans are the only natural host of *H. ducreyi*; there are no animal reservoirs (7). Although asymptomatic carriage of *H. ducreyi* has been reported (19), the existence of an ecologically important carrier state is not recognized. To sustain endemicity, a minimum of 15-20 changes in sexual partnerships per year is required (20). Steen argued that, for these reasons, *H. ducreyi* occupies a precarious niche, and is sustainable only in the context of an intensive commercial sex industry in which workers are exposed to many sexual partners in the absence of adequate public health surveillance and treatment. Intervention by vaccination or therapeutic treatment, specifically targeting commercial sex workers, could control outbreaks, or eliminate chancroid altogether (7).

Therapeutic treatment of chancroid with a single dose of some antibiotics can clear chancroid ulcers in a few days. In some centers, syndromic management of genital ulcers and prophylactic treatment of high-frequency transmitters, such as women who work as prostitutes, effectively reduced the incidence of chancroid. The rate of HIV transmission was also reduced (21). In areas where bacterial STD treatment was not effective in reducing the incidence of HIV, herpes simplex virus was the predominant genital ulcer disease, and chancroid was less common (18,22,23).

Single-dose therapies are preferred for use in chancroid endemic areas to assist compliance and practicality of public health treatment-based control programs. Treatment guidelines have been updated (24) to prevent treatment failures observed with HIV-associated immune deficiency (25,26). However, plasmid–associated antibiotic resistance now includes many classes of antimicrobial drugs (27). Resistant strains are widespread, and variable between regions. Gaining specific knowledge of the prevalence and distribution of different resistance phenotypes is laborious because *H. ducreyi* is difficult to isolate and culture, and facilities for antimicrobial susceptibility testing are uncommon where chancroid is endemic (27).

H. ducreyi acquired plasmids encoding TEM-1-type β-lactamase in 1976 (28,29). Two penicillin-binding proteins have also been identified (30). Resistance to tetracycline is associated with the TetM determinant, which is also plasmid-mediated in *H. ducreyi* (31). Another plasmid encodes resistance to sulfonamides, streptomycin and kanamycin (32). Appearance of trimethroprim resistance in Thailand (33) and Rwanda (34) highlighted the emergence of new resistance phenotypes, and rendered an inexpensive therapy (trimethroprim-sulfametrole) ineffective. Although quinolones are still effective

against *H. ducreyi*, *N. gonorrhoeae* has acquired resistance to these agents (35). Horizontal transfer of this resistance phenotype would further limit the treatment possibilities for chancroid.

Currently, the U.S. Centers for Disease Control (CDC) recommends a single oral dose of azithromycin (1g), a single intramuscular dose of ceftriaxone (250mg), a 3 day course of ciprofloxacin (500mg oral twice daily) or a 7-day course of erythromycin (500mg 4 times daily) for treatment of chancroid (24).

A vaccine against chancroid, targeted to core groups of high-frequency STD transmitters, is a practical public-health objective, and could provide the benefits of reduced dissemination of HIV, without the risk of failure due to expanding antibiotic resistance, HIV-associated immunodeficiency, or lack of treatment compliance. An ideal vaccine in this setting would require no cold-chain for distribution, no sterile syringes, and could be delivered in a single dose. These are features of some oral attenuated *Salmonella* vector vaccines.

Towards a vaccine against chancroid

Until recently, mechanisms of chancroid pathogenesis and the nature of its causative bacterium, *H. ducreyi*, were little known. This was an obstacle to vaccine development. Interest in this formerly little-studied infection has now expanded in recognition of its important relationship with HIV transmission. Virulence determinants have been identified and characterized using *in vitro* cell and organ culture models, in animal models and in a human model of disease. The pathogenesis of and immune response to infection are now better understood.

The pathogenesis of and immune response to *H. ducreyi* infection

Early accounts of the histopathological changes that occur on clinical *H. ducreyi* infection describe a trilaminar architecture, with chains of bacteria among a fibrinous, purulent exudate at the ulcer surface, overlying a granulocytic polymorphonuclear cell infiltrate, with a prominent plasma cell infiltrate forming the third layer, suggesting a prominent role for humoral immunity in the pathogenesis *of H. ducreyi* infection (36). In contrast, Magro et al. and King et al. (15,37,38), examined biopsies from culture-confirmed cases of chancroid. They described a lesion histology suggestive of a delayed type hypersensitivity (DTH) cell-mediated immune reaction, with an interstitial and perivascular inflammatory infiltrate extending from reticular to deep tissues, with equal numbers of CD4+ and CD8+ T lymphocytes and macrophages. Few plasma cells were seen. Magro (15) doubted the verity of the trilaminar description for chancroid, suggesting that lesions examined in the former study were not culture-confirmed chancroid.

In a temperature dependent rabbit model (described below), Desjardins et al. (39) studied the histopathology of lesions at 4, 10, 15, and 21 days after infection. They reported a preliminary infiltrate of polymorphonuclear cells (PMN) and macrophages at day 4 and day 10, typical of an acute inflammatory response, followed by an influx of lymphoid cells later in infection (40); CD5+ T cells, the rabbit equivalent of human CD4+ cells, were predominant in the secondary infiltrate.

In a human model (described below), Palmer et al. (41) noted a DTH-like histopathology at the pustular stage of infection similar to that described by Magro in

clinical ulcers. T cells in this model were CD45RO+, with 60-80% CD4+ cells, and 20-40% CD8+ cells, both of the αβ lineage. Cytokine production was measured by reverse transcription-polymerase chain reaction (RT-PCR) of mRNA in biopsies. A mixed or Th1 cytokine profile was seen with interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α). Gelfanova et al. (42) isolated *H. ducreyi*-specific T cell clones from lesions in experimentally infected human subjects inoculated 7-14 days earlier. These cells were CD3+ and predominantly CD4+, and produced gamma interferon (IFN-γ), or IFN-γ plus IL-10, but not IL-4 or IL-5 on stimulation with *H. ducreyi* cell lysates.

Immunological considerations for vaccine development

In clinical infection, a strong serum antibody response to *H. ducreyi* is elicited, but is not protective. Autoinoculation and serial reinfection are common features of chancroid. Several studies suggest a role for cellular immunity in protection against chancroid. Desjardins et al. achieved partial protection in rabbits on parenteral vaccination with a pilus preparation in Freund's adjuvant (40). Clearance of infection coincided with the appearance of plasma cells in the lesion, so a role for humoral immunity was postulated. To better characterize the nature of this protection, a mechanistic study was conducted (39). It revealed that passive transfer of IgG from hyper-immune rabbits to naïve rabbits did not confer protection. Further, rabbits immunized with whole cell or pilus preparations of *H. ducreyi* developed a DTH response to pilus components of *H. ducreyi* injected intradermally. This response was absent in naïve controls. Finally, clearance of infection also coincided with the appearance of CD5+ cells (the rabbit equivalent of CD4+ T cells) in the lesion. These

observations point to the importance of the cellular arm of the immune system in the immune response to chancroid.

San Mateo et al. (43) studied the relative roles of bacterial and host immune response factors in ulcer formation in the pig model of chancroid. Pigs in which immune cell deficiency had been induced by cyclophosphamide (CPA) injection exhibited lesions that did not ulcerate; the surface epithelium remained intact, though some deeper tissue damage was evident. This suggests that an immunopathological mechanism participates in ulcer formation. However, in CPA treated pigs, clumps of *H. ducreyi* bacteria were observed, with cell densities one order of magnitude greater than in untreated pigs; cellular immune mediators, PMN, lymphocytes and monocytes may control *H. ducreyi*, and may play a role in clearing the infection.

Chancroid is considered a cutaneous infection. Indeed, cutaneous lesions do occur on the genitals (e.g. the shaft of the penis, the labia and inner thigh in women), and, in the human model of experimental infection, on the skin of the upper arm. However, Ballard (23) described mucosal lesions due to chancroid, seen intraurethrally in men, and on the cervix in women, and reported that these mucosal lesions did not spontaneously resolve. Whether these are truly non-resolving lesions, or represent repeated autoinoculation at apposed sites is unclear. In either case, secretory IgA and cellular immune mediators at the mucosal surface may help protect against chancroid lesions at mucosal surfaces.

Candidate antigens for chancroid vaccine development

Several *H. ducreyi* antigens have been tested in the rabbit model of chancroid. Some of these were protective, in that the severity and duration of infection was reduced. None of the antigens tested to date have prevented ulcerative disease entirely. Hansen et al. (44) showed that, in contrast to human clinical infection, rabbits were protected against secondary experimental exposure to *H. ducreyi* after initial experimental infection. When they immunized rabbits with *H. ducreyi* cell envelopes they saw similar protection. Desjardins et al. (40) protected rabbits with a pilus preparation, but found that LOS was not protective. Immunization of rabbits with purified recombinant hemolysin protein did not affect lesion formation, but did result in reduced recovery of viable bacteria from lesions on immunized rabbits (45). Interestingly, this effect did not change when the infectious agent was an isogenic mutant strain of *H. ducreyi* deficient in the hemolysin gene (*hhdA*). Recently, Leduc et al. (publication pending) investigated the hemoglobin receptor (HgbA (46,47)), in native and recombinant form and a surface protein of obscure function (D15 (48)) as vaccinogens. All three tested were partially protective in the rabbit model.

An ideal candidate for a subunit recombinant vaccine is conserved between strains and over time to ensure lasting protection against any clinical strains that might be encountered. Several *H. ducreyi* genes appear to be conserved, including *hhdA* (encoding hemolysin (49)), *hgbA* (encoding the hemoglobin receptor (46)), *tdhA* (encoding a heme receptor (50)), *dsrA* (involved in serum resistance (48)), *ftpA* (encoding the pilin monomer (51)) , and *pal* (a membrane lipoprotein (52))(53).

An ideal vaccinogen is also expressed during infection. Elevated serum antibodies to HgbA, TdhA, D15 (an outer membrane protein (48)), Hlp (a lipoprotein (54)), HhdA, CDT (a cytolethal distending toxin (55)), and LOS in patients with chancroid indicates that these factors are expressed *in vivo*. Confocal microscopy of lesion biopsies stained with monoclonal antibodies specific for FtpA, Pal Hlp, MOMP and OmpA2 (the major outer membrane proteins (56)) showed that these too were expressed *in vivo* (57). Further evidence is provided by transcripts, detected by RT-PCR in biopsies from experimentally infected human subjects, including those from *momp*, *ftpA*, *losB* (D-D heptose transferase (58)), *lst* (sialyltransferase (59)), *cdtB* and *hhdA* (60).

A series of experiments in the human model of experimental infection have compared *H. ducreyi* 35000 strains defective in many of these putative virulence factors to the isogenic parent strain. Virulence in the human model affected only in *pal* (61), *hgbA* (62), and *dsrA* (63) mutants, of the 10 tested to date. While these results are not directly indicative of a good vaccinogen, proteins that are so clearly critical in establishing infection are more likely to be conserved, and expressed *in vivo*.

Based on this evidence, suitable candidate genes for experimental *H. ducreyi*/*Salmonella* recombinant vaccines might include *hgbA*, *d15*, *dsrA*, *pal*, *ftpA*, *or tdhA*.

Models of chancroid

For testing of vaccine candidates, an animal model of comparative virulence is a critical tool. Smith (64) described three attributes desirable in an animal model for comparative virulence.

1) Animals, either a natural host or a valid animal model, should be inoculated with graded doses. Quantitation of bacterial inocula is critically important to standardize interpretation of model data within experiments and between laboratories. *H. ducreyi* has a clumping habit of growth (65), possibly due to expression of adhesins on the cell surface (66). Estimates of culture density differ for different media and different measurement techniques (67), and this variation must be considered in experimental comparisons, particularly when experiments may be compared between laboratories.
2) The route of inoculation and inoculum size should mimic those in the natural human disease, and the nature and tempo of disease produced should mimic the natural infection. This provides analogy to the disease of interest.
3) The immune response and the experimental disease effect should be quantifiable in some consistent measures. This permits correlations and comparisons to be made with confidence for functional and mechanistic analysis of factors in pathogenicity and protection, or virulence.

What do these criteria mean in the context of chancroid and its models? I will briefly describe the *in vivo* models for chancroid, and then discuss how two of these accommodate Smith's criteria.

The immunology of mice is well understood, and a mouse model for chancroid would provide insight into the nature of protective immunity. However, attempts to develop a mouse model for chancroid have failed (68,69). Although ulcers were

produced on inoculation of 10^7 CFU, heat killed cells and purified LPS caused similar lesions, showing that ulceration was not due to an infectious process.

Intradermal inoculation of the foreskin of male pig tailed macaques with 10^7 or $2x10^8$ CFU produced lesions similar in appearance to those seen in humans, with culture positivity for up to 20 days and inguinal lymphadenopathy in 4 of 5 animals (70). However, the number of inoculum sites per animal is limited, and the cost of primate husbandry is high. These factors may prohibit samples large enough for statistical relevance.

A swine model, (71) in which the ears were inoculated using an allergy testing device to simulate epidermal abrasions, was also successful. Cost is again a limiting factor.

A human infection model has been very useful in evaluating relative virulence in early infection (41,42,72-75). Volunteers are inoculated using an allergy-testing device on the upper arm, but lesions are not allowed to progress to ulceration. The estimated delivered dose that consistently produces pustules at a tempo similar to that of clinical infection is 30 CFU. This model is limited by its short duration, and for vaccine development in that political and ethical issues preclude the initial testing of prospective vaccines in humans.

The temperature-dependent rabbit model of chancroid was adapted from a model of *H. ducreyi* virulence, used since 1948 (29). By housing rabbits at 15-17°C, Purcell et al. (76) achieved ulcerative disease by intradermal inoculation of 10^5 CFU, compared with 10^8 CFU required for rabbits housed at room temperature. Lesion production is dependent on live organisms, and on *in vivo* replication. This model was adapted by

Desjardins et al. (39,40) to the study of inducible immunity by adding measurement of lesion size to the comparisons between lesions, and culture of a lesion from each inoculum size at each timepoint.

How well do the human and rabbit models live up to Smith's criteria of reliable propagation and quantitation of the pathogen, the ability of either the natural host or animal model to mimic human chancroid with respect to route of infection, inoculum size and pathology, and an easily measured disease effect?

The temperature dependent rabbit model is unique in that a number of titred doses (up to 5) that are quantified by viable count are used, in triplicate sites. This best fulfills Smith's first criterion of graded doses.

The second criterion is that the route of inoculation and the disease produced should mimic human infection. Two techniques are used in these models to apply the bacteria to the tissues: intraepithelial inoculation, in the manner of a tuberculin skin test, or shallow punctures applied using an allergy testing device. Intraepithelial inoculation offers a measurable inoculum (39,40), one of the important criteria described by Smith. The allergy-testing implantation device may better mimic the natural mode of *H. ducreyi* contact, which is presumed to be through superficial abrasions that occur during sexual intercourse. However, the delivered dose can only be estimated (72).

The site of inoculation may also be important. In all models described above, inoculation is at cutaneous sites, and genital inoculation is used only in the primate model (70). Both cutaneous (77) and mucosal (23) lesions are observed clinically. Human genital skin differs in temperature, architecture, and Langerhans cell density from the

skin of the ear of a pig, the back of a rabbit or the arm of a human, and these differences may influence the nature of the disease effect (74).

The inoculum size may also influence the disease observed. The infectious inoculum for natural disease is unknown, but an estimated delivered dose of as few as 30 cells was sufficient for consistent pustule formation in the human model (72). Higher inocula were found to accelerate ulceration. This was undesirable for the purposes of human experimental infection, which is terminated prior to ulceration, but may not be irrelevant to clinical disease. Higher inocula are required for ulceration in non-human models, possibly reflecting the host specificity of *H. ducreyi*. The minimal ulcer producing inoculum for experimental *H. ducreyi* infection in the temperature dependent rabbit model is 10^5 CFU (39,40,48,78). The consistency of disease production is relevant to the efficiency of an experiment, while the threshold inoculum for production of a consistent disease effect must be known in order to allow measurement of effects such as treatment, or vaccination.

Smith's final criterion is that a measurable disease effect is produced. In the temperature-dependent rabbit model, three measures of disease severity are recorded: lesion size, clinical score, and aspiration of a third lesion for culture. Measurements are taken every second day for 20 days. This model is unique in that the multiplicity of inocula, measurements and sampling instances permits robust statistical analyses. New Zealand White rabbits are outbred, and variability in the data collected between individuals from this model is high. Although this may seem a disadvantage of the model, this variability may better reflect that encountered in nature or the human population. Measurements of multiple lesion sites and timepoints for each rabbit, and

comparison of several measures of disease throughout the course of infection, partly compensates for this variability.

The temperature-dependent rabbit model of infection is a powerful tool in the study of inducible immunity for the purposes of chancroid vaccine development. An added criterion for vaccine development is the ability to measure the immune response to the vaccine. Because reagents for qualitative interpretation of the immune response in rabbits are not commercially available, an enzyme immunoassay (EIA) for serum immunoglobulins was developed to detect immune recognition of vaccine antigens (40). Although protective immunity may not be related to the quantity of specific serum antibody, various immune responses do move in concert, and detection of a specific antibody response does signify that an immune response to the vaccine antigen has been elicited. Demonstration of protection from infection and disease is the important practical outcome (and a definition of immunity), and this is measurable with a high degree of confidence in the rabbit model.

A recombinant attenuated *Salmonella* vaccine against chancroid

The impetus for this study was the desire to develop an oral recombinant chancroid vaccine delivered by an attenuated *Salmonella* vector, for use in humans. This vaccination tactic could provide the sterilizing immunity that has eluded us with partially protective parenteral vaccines. If this could be accomplished, a single dose, orally administered vaccine could be distributed without refrigeration to sex workers in endemic areas. I will briefly describe the history and development of attenuated *Salmonella*

vaccines, and their adaptation for use as recombinant vectors, and then discuss how these might provide protection against chancroid.

The study of *Salmonella* enteric fever and a model for typhoid

Salmonella typhi causes typhoid fever, a systemic infection that causes fever, abdominal pain and constipation (79-81), and is host restricted to humans (82). The intracellular survival and proliferation of *S. typhi* in host phagocytic cells is an important feature of the pathology of typhoid fever (83). Non-typhoidal *Salmonella* strains, including *S. typhimurium*, infect a wide range of hosts, and cause localized enteritis (84). However, in certain inbred strains of mice (e.g. BALB/c, C57BL/6), infection with *S. typhimurium* produces an illness resembling enteric (typhoid) fever (81). This is associated with a deficiency in Nramp-1, a polytopic membrane protein expressed in macrophages, and localized to the membrane of the late endosome/early lysosome where it may act as a transporter protein for outward transport of divalent cations (Mg^{++}, Fe^{++}), and inward transport of cytocidal effectors (85-87). Much of the current understanding of *S. typhi* pathogenesis is extrapolated from this experimental model for typhoid fever (82). The interactions between *S. typhimurium* and mouse host defenses are well studied, as they present a popular model for the mechanisms of bacterial pathogenesis (Reviewed in (83,84,88)). But the parallels between the disease and its model may be overstated: Sirard et al. argue that *S. typhi* and *S. typhimurium* differ in the way they interact with the host, and stimulate immune responses (82). For example, virulence in *S. typhimurium*, but not *S. typhi* is regulated by a virulence plasmid (89).

Typhoid fever

Typhoid fever is a food-borne febrile human disease caused by enteric infection with *Salmonella typhi* (reviewed in (79-81)). On ingestion, bacteria surviving passage through the acidic environment of the stomach infect the small intestine, preferentially targeting the gut-associated lymphoid tissue (GALT) at mucosal follicles called Peyer's patches (90,91). Bacterial attachment to the epithelial wall is mediated by fimbriae. *S. typhi* penetrates the gut epithelium, and gains access to the intracellular environment of the submucosal macrophages of the lymphoid follicle by bacterial-mediated endocytosis. *S. typhi* has acquired multiple evasive strategies to protect against the intracellular killing mechanisms of these cells, and proliferates intracellularly, sequestered from extracellular effectors of host immunity. Free bacteria transit from the Peyer's patches via the lymphatics, thoracic duct and bloodstream to the reticuloendothelial tissues, the liver and spleen, where proliferation resumes in phagocytic cells (79). Symptomatic disease (fever, abdominal pain and constipation; (79,80) begins when a systemic invasion is launched from the reticuloendothelial system. Without antibiotic treatment typhoid fever is fatal in 10-15% of cases (80). A "carrier-state" results in 1-6% of untreated cases, in which bacteria are not cleared, but sequestered in the gall bladder, and continue to be shed in feces (79).

Salmonella virulence factors

In order to establish infection, *S. typhi* or *S. typhimurium* must overcome a series of host protective factors. Most virulence associated genes in *S. typhimurium* have a GC content that differs significantly from the remainder of the chromosome, are often

flanked by remnants of bacteriophage or transposon insertion sequences, and by genes that are contiguous in related, non-pathogenic bacteria, and so are considered to have been acquired by horizontal gene transfer.

The acid environment of the stomach is the first barrier encountered when bacteria in food or water are ingested, and, as hypochlorhydria is a host susceptibility factor for *Salmonella* infection, it very likely plays a role in host protection (92). *Salmonella* have developed an adaptive acid-tolerance response to help overcome gastric acidity (93,94).

A layer of mucus and embedded, commensal, non-pathogenic, bacterial flora protects the intestinal epithelium. *Salmonella* penetrate this barrier and attach to the apical surface of enterocytes via fimbriae (95), selectively targeting the lymphoid follicle-associated epithelium (FAE) (96).

To gain access to their cellular hosts, the follicle-associated macrophages, the bacteria must penetrate the gut epithelium: the microfold (M) cells and enterocytes lining the intestinal wall at the Peyer's patches (97,98). Both the epithelial cells and the underlying host macrophages are invaded using a type III secretion system (99). Briefly, bacterial type III secreted proteins enter the host cell, and modify basic host cell functions, including membrane trafficking, signal transduction, and cytokine gene expression. Cytoskeletal rearrangements mediated by the type III proteins result in a deformation of the cell membrane, known as "membrane ruffling", followed by internalization of the bacterium. This *S. typhimurium*-mediated invasion differs from host-cell receptor-mediated phagocytosis (100).

Enteric fever-causing salmonellae (*S. typhi* in humans, *S. typhimurium* in mice) occupy a niche within submucosal and reticuloendothelial macrophages where they are sequestered from the host humoral immune mediators, but must survive microbicidal environment within the phagosome. The battery of genes that protect against intracellular killing are controlled by a two-component regulatory system, consisting of a membrane spanning sensor kinase (PhoQ) that, when triggered, activates a transcriptional regulator PhoP. PhoP activates and represses over 40 genes, the PhoP activated genes (*pag*), and PhoP repressed genes (*prg*). Other protective genes encode a type III secretion system that functions to prevent or delay phagosome/lysosome fusion, and superoxide dismutase (SOD) and catalase which protect against oxidative killing (82).

In innately resistant (wildtype) mice, or in humans, non-typhoidal salmonellae cause self-limited enteritis. Bacterial-mediated endocytosis occurs in M cells and enterocytes as it does in enteric fever. But type III secreted proteins induce the enterocyte or M cell to secrete IL-8, resulting in neutrophil recruitment, and fluid and electrolyte secretion into the gut lumen (84). Clinically, this results in diarrhea and abdominal tenderness.

Live oral vaccines against typhoid fever

One of the two currently licensed typhoid vaccines (Vivotif, Berna, Switzerland), is a live oral preparation of *S. typhi* Ty21a, a chemically attenuated derivative of the pathogenic strain Ty2 (101). The molecular basis for attenuation is unknown. Ty21a is well tolerated (102), but its immunogenicity is moderate. Protective efficacy depends on the formulation (lyophilized vaccine, enteric coated capsule or liquid suspension), and on

the number and timing of repeated doses (103). The liquid suspension achieved between 70 and 80% efficacy in field trials with a 3-dose regimen (104). This protection persists for 5-7 years and the immune response elicited includes serum IgG, mucosal IgA, and strong Th1-biased cell-mediated immune responses (102,105).

Much research has been invested in the development of oral attenuated strains capable of eliciting protective immunity in a single dose by the introduction of defined attenuating mutations.

Hoiseth and Stocker (106) interrupted the metabolic pathway for biosynthesis of aromatic amino acids by deleting *aroA* in *S. typhimurium* LT2, creating strain SL3261. Chorismate, the end product of this pathway, is required for synthesis of 2,3-dihydroxybenzoate and *para*-aminobenzoic acid. While these compounds are available in some growth media, they are limiting in mammalian tissues. Strain SL3261 is avirulent in mice, and protective as a live-oral vaccine. One of the most promising *S. typhi* vaccine strains for use in humans, CVD908, is attenuated by deletion of *aroC* and *aroD* (107). This strain has been shown to be highly immunogenic and safe in human trials, although a clinically silent bacteremia of the vaccine organism was manifest in some subjects (108).

An obligate requirement for adenine is instilled by the deletion of *purA*. This alone does not sufficiently attenuate virulence (109), but a *purA aroA* strain of *S. typhi* was over-attenuated and poorly immunogenic (110). Guanine nucleotide auxotrophy was induced by deletion of *guaBA* genes from *S. typhi* Ty2 (111,112).

Curtiss et al. (113) showed that in *Salmonella*, *cya*, encoding cyclic AMP and *crp*, encoding the cyclic AMP response protein together regulate a diversity of genes and

operons. Deletion of these genes resulted in attenuated virulence, and the attenuated strain was protective as an oral vaccine in mice (113). A corresponding attenuation was tested in *S. typhi* (strain χ^{3927}) (114). Although this strain was insufficiently attenuated, and caused disease in some experimental subjects, introduction of a third mutation in *cdt*, a gene involved in movement of the bacteria between the gut associated lymphoid tissue and the reticuloendothelial organs, rendered the resulting strain, χ^{4073}, avirulent. Its immunogenicity was retained (115). Intracellular survival is dependent on the *phoP/phoQ* two-part regulatory system, governing the expression of several virulence associated genes which enable bacterial survival within the endocytic vacuole of macrophages (116). Deletion of these genes (116), or constitutive expression of their products (117), in *S. typhimurium* creates a strain that is attenuated. Ty800, a *phoP/phoQ*-deleted *S. typhi* strain, shows promise as a human vaccine (118).

HtrA, a serine protease heat shock protein, is required for full virulence in mice. It may protect against intracellular peroxide during infection of enterocytes (119). A *htrA* deletion mutant administered as an oral vaccine protects against wildtype challenge (120,121). This mutation was introduced into *aroC/aroD* mutant CVD908 (CVD908*htrA*; (122)). The resulting strain was strongly immunogenic, and, in contrast with CVD908, no vaccinemia was noted following vaccination.

Recombinant expression of pathogen-derived antigens in attenuated *Salmonella* strains

The unique way these oral typhoid vaccines stimulated the immune system spurred researchers to investigate their adaptation as carriers and "adjuvants" for

recombinant antigens derived from other pathogens. Several recombinant expression systems were devised to balance the need for antigen stability in the vector, with the requirement for high-level expression to ensure immune recognition of the recombinant antigen.

While in general, the quality and strength of the immune response to the vector and the heterologous antigen coincide, introduction of recombinant DNA imposes a metabolic burden on the host cell because cellular machinery is diverted from the maintenance and replication of the host bacterium to support the replication of heterologous DNA, and synthesis of the vaccine antigen (123). This imposes a selective disadvantage on the vaccine vector; if the plasmid is segregated from one bacterium, its progeny are likely to outgrow plasmid-bearing vectors, and this may compromise the immunogenicity of the recombinant protein. The size and copy number of the heterologous DNA and the rate of gene expression determine the magnitude of this burden (124,125).

One strategy for recombinant antigen expression that minimizes the imposed metabolic load is chromosomal insertion (126). The pathogen-derived gene of interest and a suitable promoter are introduced by homologous recombination into the chromosome of the host, using a suicide vector (127). Chromosomal expression is stable, but as the recombinant gene is usually present in a single copy, the level of heterologous antigen expression is low, and this compromises the quality of the immune response to the recombinant protein (128).

Expression from a high-copy number plasmid with a strong promoter allows high-level expression of the heterologous antigen for maximal immune recognition, but the

metabolic burden on the cell results in strong selective pressure favouring plasmid loss (125,129). Lower copy-number plasmids have been developed in which stabilizing factors have been introduced to ensure that those that have lost the plasmid do not outgrow plasmid-bearing cells.

In the vaccine vector population, plasmid stability is promoted if loss of the plasmid is detrimental to the bacterial vector strain (125,129). This is the logic supporting balanced lethal mutations: a gene essential for bacterial viability is introduced into the expression plasmid. The host vector is deleted of this gene, and so is reliant on the plasmid to complement this lethal mutation. Balanced lethal mutation of the β-semialdehyde dehydrogenase (*asd*) gene is commonly used in attenuated *Salmonella* (115,130,131).

Similarly, Galen et al. (129) used a naturally occurring postsegregational killing system (132). Synthesis of a lethal pore-forming protein encoded by *hok* is blocked by a small antisense mRNA transcribed from *sok*. *sok* mRNA is highly susceptible to nuclease degradation, and so must be constitutively expressed from the plasmid to prevent *hok* translation. Plasmid loss results in cell death.

Another stabilizing tactic is to delay synthesis of heterologous proteins until the bacterial vector has reached the intracellular environment of the follicle-associated submucosal macrophages, where the induction of immunity is thought to occur (125,129). Inducible promoters are activated by the environmental conditions found within the endocytic vacuole of the submucosal or reticuloendothelial macrophages. These include anaerobiasis, nutrient limitation, or the presence of nitrates or reactive oxygen species, and the promoters adapted for this purpose are derived from genes that

are activated in these conditions (133,134). The best characterized is p*nirB*, the promoter from the nitrate reductase gene of *E. coli* (135) which is activated by high environmental nitrate concentrations, and by anaerobiasis. Chatfield et al. (136) first used p*nirB* to drive expression of *tetC*, encoding the C fragment of tetanus toxin (tetanus toxoid) in attenuated *Salmonella*.

Several other promoters of this ilk have been used since. Dunstan et al. (137) compared p*nirB* with p*pagC* from a PhoP-activated *Salmonella* gene, which is induced by growth in medium lacking $MgCl_2$, and p*kat*, which is induced by H_2O_2 in the medium. They found that the p*pagC* promoter was most efficient in eliciting protective immunity, followed by p*nirB*. Orr et al. tested anerobically-induced promoter p*dmsA* (138), and Roberts et al. (139) used the promoter from the heat shock protein, p*htrA* to direct recombinant gene expression. In comparison with p*nirB*, p*htrA* was more effective. However, p*nirB* remains the most commonly used.

Somatic expression, surface display, or secretion?

A final consideration in directing recombinant expression of the pathogen-derived antigen from the *Salmonella* vector strain is the cellular location of antigen expression. Heterologous antigens can be expressed within the cytoplasm, secreted from the cell, or embedded in the cell surface (125). Several groups have suggested that export of the heterologous antigen from the cytoplasm makes the vector strain more immunogenic. Haddad et al. (140) expressed malarial antigens cytoplasmically, and at the cell surface. Although the level of surface expression was 10- to 100-fold lower than cytoplasmic expression, the immune response to the two strains was similar. This suggests that

immunogenicity of the antigen is enhanced by surface expression, but also suggests that immunogenicity does not absolutely require surface expression. Low-level surface expression may engender a lesser metabolic burden, with equivalent immune recognition; this strategy could also promote plasmid stability. However, surface expression, especially at high levels, may compromise the integrity of the cell membrane (125).

Two groups have succeeded in salvaging poorly immunogenic cytoplasmic antigens by engineering their secretion. Hess et al. (141,142) inserted listeriolysin into a truncated *E. coli* hemolysin A gene. The product of this manipulation was secreted, and the antigen protected against infectious challenge. Russmann et al. (143) achieved secretion of short immunodominant epitopes of the murine lymphocytic choriomeningitis virus via *Salmonella* type III secretion machinery. This resulted in a class I-restricted CTL response that protected against lethal intracerebral challenge. Secretion techniques are limited by the size of protein that can be accommodated, but could be useful for pathogens in which immunodominant peptide epitopes are known.

TetC as a test antigen

Towards the development of a single-dose oral tetanus vaccine for use in the developing world, one of the first recombinant antigens tested in an attenuated *S. typhimurium* vaccine was the C fragment of tetanus toxin (TetC) (136). TetC expression in attenuated *Salmonella* has since been incorporated into several studies of the immune response to vaccination (128,139,144,145). It is also used as part of a fusion protein to rescue less immunogenic heterologous antigens, acting as an adjuvant (146-149).

The immune response to oral attenuated *Salmonella* vaccines

Studies of the mechanisms of immunity to *Salmonella* vaccines are confused by differences in study design. The route of infection (intraperitoneal, intravenous or oral) differs, and this may modify the immune response elicited. Also, results may differ between studies in which naïve hosts are infected with attenuated strains, and those in which vaccinated hosts are challenged with virulent bacteria. In naïve hosts infected intravenously with attenuated strains, the αβ T cell receptor (TCR), the class II major histocompatibility complex (MHC II), and the gamma interferon receptor (IFN-γR) are required for clearance, suggesting a role for IFN-γ producing, MHC II-restricted T cells (150). γδ TCR and β2 microglobulin knockout mice could clear infection, indicating that MHC class I restricted or γδ T cells are not required for clearance (151). Costimulation of T cells via CD28 is required for clearance of this primary "immunizing" infection, but B cells are not. However, on challenge with virulent *Salmonella* strains, immunized mice required specific antibody for protection (152). Depletion of CD4+ T cells, IFN-γ or IL-12 impaired clearance of secondary infection in vaccinated mice (153). TNF-α also seems to be required for clearance (154). Unlike *Listeria*, *Salmonella* lacks hemolysin, and so cannot enter the cytoplasm from the phagosome. For this reason, *Salmonella* antigens were assumed not to be displayed with MHC I (150). However, passive transfer of both CD4+ and CD8+ cells were required for protection from oral challenge (153), and CD8+ T cells are induced in response to recombinant antigens expressed from a *Salmonella* carrier (155). Lo et al. (156) found that β2 microglobulin knockout mice were more susceptible to oral infection, and described *Salmonella*-specific cytotoxic T lymphocytes. It is not clear how *Salmonella* antigens are processed by the MHC I

pathway. Lo et al. (156) found CTLs that were MHC Ib-restricted, complexed with a non-classical MHC molecule, which may not require *Salmonella* to escape the vacuole in which it divides. Others have speculated that the *Salmonella*-containing vacuole may be "leaky", or that *Salmonella* antigens may reach the classical MHC I processing pathway when phagocytic cells clean up host macrophages killed by *Salmonella*-mediated apoptosis (157).

The immune response to oral vaccination may vary with the mutation used to attenuate the vaccine strain. For example, *phoP/phoQ* deleted strains appear to favour antibody responses (158), but *aro* gene deletions, and those in *htrA*, stimulate both cell mediated and humoral responses (148,159). The immune response to *aro*-deleted strains is usually described as Th1-biased, characterised by carrier and antigen specific $CD4^+$ T lymphocyte production of proinflammatory cytokines such as gamma interferon (IFN-γ) and IL-2, with serum antibodies, predominantly of the IgG2a subtype (159). However, some groups have also detected Th2 type cytokines (IL-4, IL-5), with induction of serum IgG1, and IgE, suggesting a more balanced response (148). *Salmonella* vaccines are hailed as mucosal vaccines, and *Salmonella* and guest-antigen specific antibody secreting cells (ASC) are seen, with homing receptors directing their migration to intestinal, genital and oral mucosal surfaces (160-162).

Application of recombinant *Salmonella* to chancroid vaccine development: statement of objectives

As *Salmonella* vaccines elicit a strong, Th1 biased, cell-mediated immune response, they may be especially adept at eliciting protection against *H. ducreyi*, which

induces a DTH-like response on infection. A *Salmonella* vector, engineered to deliver *H. ducreyi* antigens, and to provide adjuvancy for these antigens, may protect against experimental *H. ducreyi* infection in rabbits. In order to apply an oral recombinant attenuated *Salmonella* vaccine to chancroid, a preliminary study of the feasibility of this approach in rabbits was required.

The two most-studied systems are *S. typhimurium* in innately susceptible mice, and *S. typhi* in humans. These cause enteric fever in their respective hosts, but infection of other species results in localized gastroenteritis. Rabbits provide the only suitable model for chancroid vaccine development. Anatomical and physiological differences between mice and rabbits may not allow a "mouse" vaccine strain to achieve the desired vaccine effect safely in rabbits. Even if an anti-*Salmonella* antibody response could be elicited, it is not clear whether *S. typhimurium* could effectively deliver a recombinant antigen if unable to invade macrophages, proliferate intracellularly, and invade systemically. Non-specific effects of the vector strain, or a recombinant derivative carrying an irrelevant antigen, might impair the progression of disease in experimental *H. ducreyi* infection of rabbits, and compromise the rabbit model as a quantitative assay of comparative virulence. Such effects would prevent the use of these strains as controls, and might compromise future studies to assess the feasibility of recombinant *H. ducreyi* vaccines.

To address these concerns, I conducted a study to answer 3 important questions that address the feasibility of using attenuated *Salmonella* vaccines in the rabbit model of chancroid.

1) What is the maximum tolerated oral dose, and minimum immunogenic oral dose of attenuated *Salmonella* in rabbits?
2) Can delivery of a heterologous antigen be effective as an immunogen in this system?
3) Will experimental *H. ducreyi* infection in rabbits after oral *Salmonella* vaccination function as a comparative quantitative virulence assay to permit vaccine evaluation?

MATERIALS AND METHODS

Strains and culture conditions

Salmonella typhimurium SL3261 (his G46 (del) aroA 554), with permission from Dr. B.A.D. Stocker (106) *S. typhimurium* LB5010 (*galE* r-m+)(163), and bacteriophage P22 (HT int-ve) (164) were ordered from the *Salmonella* Genetic Stock Centre (SGSC) at the University of Calgary, Calgary, AB. Plasmid pTETnir15 (amp^r) was kindly supplied in *E. coli* and *S. typhimurium* BRD805 by Dr. D. Pickard (Imperial College, London). *S. typhimurium* SL3261(pTETnir15) was constructed in this study (see below).

S. typhimurium and *E. coli* strains were maintained in frozen stocks at -70°C, and grown at 37°C on Luria Bertani (LB) agar (Difco, Sparks, MD). LB broth cultures were inoculated from a single colony, and grown at 37°C with shaking at 200 rpm. Ampicillin was added at 100 μg/mL where required for selection of plasmid-bearing strains (LB+amp).

For anaerobic induction of tetanus toxin fragment C (TetC) expression, cultures were grown in boiled LB+amp with 1% agar. After inoculation, sterile mineral oil (Sigma) was layered on the surface of the medium, and tubes were incubated overnight, at 37°C, in a candle jar.

Haemophilus ducreyi 35000 was isolated from a Winnipeg chancroid outbreak in 1975 (29). It is now a laboratory standard strain, and is virulent in humans (Cameron, D.W., Unpublished data) (74), and in the rabbit model of chancroid (39,76).

Stocks from our laboratory, and that of Dr C. Elkins (University of North Carolina at Chapel Hill, Chapel Hill NC), maintained at -70°C, were grown on chocolate

agar (CA: GC agar base [Difco] with 1% [w/v] bovine hemoglobin [BBL/Beckton Dickinson, Cockeysville, MD]), supplemented with 5% fetal bovine serum (FBS; Gibco/BRL, Rockville, MD) and 1% IsoVitaleX (BBL/Becton Dickinson, Cockeysville, MD) at 33°C in 5% CO_2. Forty-eight-hour growth was swabbed into broth media. Broth cultures were grown at 33°C with shaking at 175 rpm. GC broth (15g/L proteose peptone [Difco] with 23mM K_2HPO_4, 7.3 mM KH_2PO_4 [both from Fisher Scientific, Fair Lawn, NJ], and 86 mM NaCl [BDH, Toronto, ON]), supplemented with 5% FBS and 1% IsoVitaleX was used for EIA antigen preparation. Mueller-Hinton broth (BBL/Becton Dickinson), supplemented with 50% α-minimum essential medium (Gibco/BRL) and 20% FBS was used to prepare rabbit inocula.

Animal strains and husbandry

All animal experiments were approved by the Animal Care Protocol Review Committee of the University of Ottawa (Protocol MI-89). Forty-two age-matched male New Zealand White rabbits, between 2.2 and 2.5 kg in weight, were purchased from Charles River Co. (St. Constant, QC). The animals were allowed to rest for one week after arriving at the animal care facility. Rabbits were housed individually, in wire cages (Hoeltge, Inc., Cincinnati, OH) in an 11.7 m^2 room, and fed and watered *ad libitum*. In addition, each rabbit was provided with an alfalfa hay cube or a carrot, on alternate days of the week. For environmental enrichment, "bunny blocks" and "jingle balls" were provided (Bio-Serv, Frenchtown, NJ). If rabbits were eating poorly, a treat of canned pineapple or pumpkin, frozen in cubes in an ice cube tray was provided.

Preparation of inocula fed by oral gavage

A 12% (w/v) aqueous solution of $NaHCO_3$ (EM Science/BDH, Toronto, ON) was filter-sterilized, and 5mL aliquots were drawn into 10mL syringes on the day of oral inoculation.

A 50mL culture of *S. typhimurium* SL3261 or *S. typhimurium* SL3261 (pTETnir15) was grown to mid-log phase (OD_{600} between 0.5 and 1.0 as measured on a Varian DMS 200 spectrophotometer, [Varian, Ottawa, ON]), blanked against the growth medium, in polystyrene cuvettes [Sarstedt, St. Leonard, QC]). This culture was chilled overnight (4°C), then used to seed a fresh LB broth culture that was grown to an OD_{600} of approximately 1. The bacteria were pelleted, and resuspended in phosphate buffered saline (PBS; Sigma, Oakville, ON) to the highest required density in a 50mL polypropylene centrifuge tube. Inocula of lower concentration were prepared by serial dilution in PBS.

Bacterial suspensions and PBS (for sham-immunized controls) were held on ice until five minutes before administration. The inoculum was warmed, prior to feeding 5mL to each rabbit. Material remaining in the tube was kept on ice. Tenfold dilutions in PBS were plated on LB or LB+amp agar to measure the viable inoculum.

Animal inoculations by oral gavage

Thirty rabbits were inoculated in groups of six. Food was removed from the cages 12 hours before the operation. Each rabbit was sedated with 25-35 mg/kg ketamine-HCl ("ketalean" Bimeda/MTC, Cambridge, ON), 2mg/kg midazolam ("versed", Sabex, Boucherville, QC), and 0.5 mg/kg atropine sulfate (MTC pharmaceuticals, Cambridge,

ON) by intra-muscular injection. Six was a manageable number for the technically complicated oral gavage operation. Within each experiment, rabbits were anesthetized 10-15 minutes apart. This allowed sufficient time perform each manipulation, even if problems arose. Two operators were required.

Baseline blood samples were drawn (3mL drawn from the ear artery) before a French #12 infant feeding tube (Benlan Inc., Oakville, ON), lubricated with K-Y jelly (Johnson and Johnson, New Brunswick, NJ), was introduced into the stomach. Correct placement of the tube in the stomach was ensured by marking on the tube the distance from the rabbit's mouth to its last rib, then inserting the tube to this mark. Placement was confirmed when stomach contents were seen, drawn by capillary action into the tube.

5mL of 12% $NaHCO_3$ were introduced into the tube, and flushed into the stomach with 10mL air before the tube was removed. Thirty minutes were allowed to pass before the bacteria were administered to ensure that the bicarbonate solution had sufficiently mixed with and neutralized stomach acid. One rabbit died, apparently from asphyxiation, immediately after the administration of this sodium bicarbonate feed. In subsequent experiments, the feeding tube was removed for the duration of the 30-minute interval before each rabbit was fed *S. typhimurium* SL3261 suspended in 5mL PBS (23 rabbits) or 5mL PBS alone. Isoflurane ("Aerrane", Janssen, Toronto, ON), with 100% O_2 (Praxair, Ottawa, ON) was administered as required to maintain sedation: As the *Salmonella* inoculum (or PBS) was fed nearly an hour after the rabbits were initially sedated, on reinsertion of the feeding tube, rabbits awakened and struggled or bit the tube. This caused bleeding from the throat in 3 rabbits. In each case, this bleeding stopped within 5 minutes. Isoflurane with 100% O_2, administered 5 minutes before the second intubation,

maintained sedation and prevented rabbits from struggling on reinsertion of the tube. The feeding tube was reinserted and 5mL of a bacterial suspension or PBS were administered. Again, the tube was flushed with 10mL air and removed.

The volumes administered did not seem to cause discomfort. 10mL of air was used to flush the tube after each fed volume. Air was used, instead of water or PBS, to avoid abdominal discomfort from ingestion of too large a fluid volume.

A French #12 (large) feeding tube was used to avoid buckling of the tube inside the esophagus, and consequent aspiration of gavage material. For future work, I would suggest a smaller tube; French #8 is the size recommended (165).

Wellness monitoring

The rabbits were closely monitored on the day of the gavage, and daily for a minimum of 7 days following. The animal care staff, which is familiar with the normal health parameters of rabbits, recorded wellness data on a chart defining 10 indicators of wellness. Body mass, rectal temperature, hydration, attitude and demeanour, nasal discharge, piloerection, volume of fluid consumed, volume and type of food consumed, and fecal production (volume and consistency) were recorded daily. Treats were provided, and Ringer's saline (Baxter, Toronto, ON) was administered subcutaneously when required.

Enzyme immunoassay (EIA) antigen preparation

S. typhimurium SL3261 was grown in broth to an OD_{600} of 1. Bacteria were harvested by centrifugation, washed four times in PBS (3000 x g, 10 min.), then resuspended in PBS with 1% (w/v) sodium dodecyl sulfate (SDS; ICN, Aurora, OH.). The cell suspension was sonicated on ice 3 times for 30 seconds with 15 seconds between pulses, rocked for 2 hours at room temperature, then centrifuged at 50000 x g at 4°C for 90 minutes. The supernatant (crude soluble antigen) was harvested, and the protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL). The antigen was stored at -20°C.

H. ducreyi crude soluble antigen was used as a heterologous negative control to assess the specificity of the immune response to oral inoculation with *S. typhimurium* SL3261. The control antigen was prepared exactly as described above for the test antigen from *H. ducreyi* cultures grown in supplemented GC broth.

Tetanus toxin fragment C (TetC), purified from a papain digest of tetanus toxin, was purchased from Calbiochem (San Diego, CA).

EIA

Serum was collected weekly, before and for four weeks following oral gavage, and stored at -20°C. *S. typhimurium* SL3261 crude soluble antigen was diluted in 0.1M carbonate buffer (3.75 mM Na_2CO_3, and 8.72 mM $NaHCO_3$ [both from BDH]; pH 9.6) to a concentration of 50ng/μL. *H. ducreyi* 35000 crude soluble antigen was diluted to 20 ng/μL, and TetC to 10 ng/μL in the same buffer. The test volume was 150 μL for *S. typhimurium* SL3261 and *H. ducreyi* 35000 EIAs, and 50 μL for the TetC EIA. One

volume of the appropriate antigen was added to each well of a ProBind 96 well flat-bottomed polystyrene assay plate (Falcon, Franklin Lakes, NJ), and allowed to bind overnight at 4°C. Plates were washed 3 times in wash buffer (0.1% Tween 80 [Sigma] in PBS), and one volume of 2% (w/v) bovine serum albumin (BSA, Sigma) in PBS (blocking agent) was added to each well. Plates were incubated for 1 hour at 37°C, and then washed 3 times. One volume of sample buffer (1% Newborn Calf Serum [NBCS; Gibco], 0.1% Tween 80 in PBS) was added to each but the first row of wells. For *S. typhimurium* and *H. ducreyi* EIAs, 2 volumes of a 1:250 dilution of each serum sample in sample buffer was loaded in triplicate columns in the first row. The serum was diluted two-fold, serially, from 1:250 to 1:32000 down the plate. On each plate, one column of blank wells received only sample buffer at this step (negative control). For *S. typhimurium* SL3261 EIA, one column of wells received sera drawn at week 4 from the rabbit that had received the highest dose of *S. typhimurium* SL3261 ($1.8x10^{11}$ CFU) as a positive control. For *H. ducreyi* EIA, the positive control was serum from a rabbit parenterally immunized with 100 μg of the *H. ducreyi* subunit antigen HgbA in Freund's adjuvant, and boosted four weeks later with 100 μg HgbA in Freund's incomplete antigen (Leduc et. al., submitted for publication). For the TetC EIA, two volumes of serum, diluted 1:50, were added to the first row in triplicate columns, and similarly diluted two-fold down the plate from 1:50 to 1:6400. Again, one column of wells received sample buffer only, and in one column, a positive control anti-TetC polyclonal antibody (see Western blot below) was diluted from 1:250 to 1:4000. Sera were allowed to bind for 30 minutes at 37°C. Plates were washed 3 times before one volume of a secondary antibody (Goat anti-rabbit immunoglobulins, conjugated to horseradish peroxidase; BioSource,

Camarillo, CA), diluted to 1:2000 in sample buffer was added to each well. The plates were incubated at 37°C for 30 minutes, then washed 3 times. One volume of the colour substrate, ABTS (0.02% [w/v] 0.36mM 2,2'-azino-bis [3-ethyl benthizoline-6-sulfonate] [Roche, Laval, QC] in 0.1M citric acid/0.02M sodium phosphate [BDH; pH 4.25] with 0.03% hydrogen peroxide [Sigma]), was added to each well, and the plates were incubated at room temperature for 25 minutes. The optical density of each well was read at 405nm in a microplate reader (Model 3550, BioRad Laboratories, Richmond CA.). Endpoint titres were calculated from curves made by plotting OD_{405} against dilution. The dilution immediately before that at which the response was extinguished was identified as the endpoint dilution.

Introduction of plasmid pTETnir15 into *S. typhimurium* SL3261

Plasmid pTETnir15 contains the gene for tetanus toxin fragment C (TetC), with expression controlled by the *nirB* promoter from the nitrate reductase gene of *E. coli*. It also includes an ampicillin resistance gene for *in vitro* selection. For experimental consistency, the plasmid was introduced into *S. typhimurium* SL3261, the attenuated strain for which the optimal dose range had been determined. The plasmid was isolated from its *E. coli* host using a midi-prep kit (Invitrogen, Burlington, ON). DNA concentration and purity were determined using a Genequant spectrophotometer (Amersham-Pharmacia, Baie d'Urfe, QC). Plasmid manipulations were as described in Anderson et al. (166).

S. typhimurium LB5010, used as an intermediate host, is a semi-rough mutant with a partial LPS deficiency (*galE*), and is restriction incompetent, but modification

competent (r-, m+)(163). Cells were washed twice in ice-cold water, and once in cold water with 10% (v/v) glycerol. Cells were suspended in an equal volume of the final wash solution, and 60μL aliquots were stored at -70°C.

200ng of plasmid were added to one aliquot of washed cells, just prior to electroporation in a Cell-porator (Gibco) with 4 kΩ resistance, and 330 μF capacitance. Transformed cells were immediately transferred to 1mL LB broth with 20mM glucose, and shaken for 2 hours at 37°C before plating on LB +amp.

Transformed *S. typhimurium* LB5010 were grown overnight in broth. Phage P22 stock was diluted tenfold in TGMS buffer (0.01mM Tris-HCl, pH 7.4, with 0.25% $MgSO_4$, 0.59% NaCl, and 0.1% gelatin). 100μL of overnight culture were added to10μL of each phage dilution between 10^{-1} and 10^{-6} and incubated at 37°C for 45 minutes. 3mL of melted top agar were added and the mixture was layered onto an LB+amp plate and incubated 4-6 hours at 37°C or until visible plaques appeared. The highest phage dilution to have visible plaques was harvested into TGMS buffer. If no plaques were visible, the plate with the lowest phage dilution was harvested. 50μL chloroform was added, and the mixture was centrifuged at 15000xg for 15 minutes at 4°C. The clear supernatant (phage lysate) was stored in a glass tube, with 50μL chloroform, at 4°C.

100 μL of *S. typhimurium* SL3261 overnight culture was added to 10μL neat phage lysate, and to each of the ten-fold dilutions of the lysate between 10^{0} to 10^{-4}. The mixture was incubated at 37°C for 25 minutes. 1 mL of LB broth with 5mM EGTA (ethylene glycol-0,0'-bis'[2-amino-ethyl]-N,N,N',N',-tetraacetic acid) was added and the incubation continued for 1h. 100μL of this culture was spread on LB+amp agar with

5mM EGTA (LB+amp+EGTA), and incubated overnight at 37°C. Colonies were passaged twice on LB+amp+5mM EGTA before further analysis.

Lipopolysaccharide (LPS) silver stain

To determine whether the the transduced clone had a "smooth" phenotype (with wildtype LPS), an SDS-PAGE gel was silver stained using the method of Tsai and Frash (167). Briefly, S. *typhimurium* SL3261, putatively containing plasmid pTETnir15, was suspended in Laemmli SDS-PAGE loading buffer (168) and boiled. 0.01% (w/v) Proteinase K was added, and the sample was incubated at 60°C for 1 hour before loading onto an acrylamide gel. SDS-PAGE for LPS silver stains, Coomassie blue stains, and Western blots was run on a BioRad mini protean II gel apparatus, as described in the product literature (169), using Laemmli buffers (168,170). The gel was fixed overnight in 40% [v/v] methanol with 5% [v/v] acetic acid. The LPS was oxidized for 5 minutes in 0.7% [w/v] periodic acid, with 40% [v/v] ethanol, and 5% [v/v] acetic acid, and then the gel was washed three times for 15 minutes in water. The silver stain was prepared by adding 2 mL concentrated ammonia to 28 mL 0.1M NaOH, then 5 mL 20% (w/v) $AgNO_3$ while stirring. Finally, the volume was brought to 150 mL with ddH_2O. The washed gel was agitated vigorously in this solution for 10 minutes, and then washed again three times for 10 minutes. The stain was developed in 50mg citric acid and 0.5mL 37% (v/v) formaldehyde in 1L ddH_2O until the LPS bands showed strongly against the clear background of the gel. The gel was dried onto filter paper for preservation.

Analysis of TetC expression

Anaerobic growth or a high nitrate environment activates the *nirB* promoter, which controls expression of TetC in pTETnir15. The negative control *S. typhimurium* SL3261 (not transduced), *S. typhimurium* SL3261 (pTETnir15), and the *E. coli* (pTETnir15) and *S. typhimurium* BRD805 hosts in which the plasmid had been supplied were compared. Plasmid-containing strains were grown both aerobically and anaerobically. Proteins in whole cell lysates were separated by SDS-PAGE, and visualized with Coomassie Brilliant Blue/Bismarck Brown (171). A duplicate gel was electroblotted at 100 volts, for 1 hour onto a nitrocellulose membrane (E-bond, Amersham-Pharmacia) using a BioRad mini-protean II assembly. Blotted membranes were stained with Ponceau-S (Sigma) to confirm that the proteins had been transferred to the blot, and to mark the position of molecular weight markers on the membrane. The membrane was blocked in 2% skim milk in PBS at 37°C. A commercially prepared polyclonal antibody raised in rabbits against tetanus toxin peptides 1300-1314 conjugated to KLH, and affinity purified (α-TetC; Biogenesis, Kingston, NH), was suspended at 1:16000 in the blocking buffer. The antibody was allowed to bind for one hour at room temperature. The membrane was rinsed in 0.2% Tween 20 in PBS before blotting for one hour at room temperature with a secondary antibody (Goat anti-rabbit immunoglobulin-HRP, as used in EIAs above) at 1:2000 in 1% skim milk/PBS. The membrane was rinsed in PBS, then TetC was detected colorimetrically with AEC (0.12% (w/v) 3-amino-9-ethyl carbazole (AEC) with 30% (v/v) N,N-dimethylformamide in 0.05M sodium acetate, pH 5.0).

Growth curves

H. ducreyi 35000 isolates from the University of Ottawa, or from the University of North Carolina at Chapel Hill were grown for 12 hours in 60 mL MH or GC broth. Samples were drawn at 3, 6, 8, 9, 10, 11 and 12 hours. At each sampling, the optical density at 600 nm OD_{600} was measured, and serial dilutions were plated to determine the viable count. Mean OD_{600} and plate counts (CFU/ml) were plotted against time (n=5). Peak viable growth (yield), persistence of logarithmic growth, and the maximum growth rate were compared between laboratory isolates (Ottawa and North Carolina), and between growth media (GC and MH), using Student's t-test. A Gram stain from each time point showed growth characteristics.

Preparation of *H. ducreyi* inocula

H. ducreyi cultures were harvested at the late log phase of growth (12 hours) in supplemented Mueller-Hinton broth, pelleted by centrifugation, then resuspended in Mueller-Hinton broth at 10^7 CFU/mL. Ten-fold serial dilutions yielded cell suspensions at 10^6, 10^5, and 10^4 CFU/mL. 100μL of each suspension was diluted in Mueller-Hinton broth and the dilutions were plated on CA to estimate the delivered inoculum size from colony counts. Each suspension was drawn into a 1mL tuberculin syringe used to inoculate the rabbits.

Rabbit Inoculations

The temperature dependent rabbit model of *H. ducreyi* infection has been described in detail (39,76). Rabbits were challenged with *H. ducreyi* as described by

Desjardins et. al. (40) The temperature in the rabbit room was reduced to 14±1°C one week before infection (Thermo Air Plus air conditioning unit). This temperature was maintained for the remainder of the experiment. Rabbits' backs were shaved 2 days before inoculation with *H. ducreyi* and every day thereafter. Four triplicate inocula (10^6, 10^5, 10^4, or 10^3 CFU) were injected, in 100μL volumes, intradermally in a 3x4 grid drawn on each rabbit's back. An operator blinded to the gavage treatment observed lesions every other day for 20 days. Two lesions at each inoculum were measured using electronic calipers, and assigned a clinical score (1= redness, 2=induration, 3=suppuration, 4= ulceration). The third lesion was cultured by injection of 100μL PBS into the lesion followed by aspiration of PBS, blood and inflammatory exudate from the lesion. This aspirate was plated on CA, and colonies were counted 2 days after plating. Culture positivity was determined by the presence of one or more colonies characteristic of *H. ducreyi* (small, gray colonies that can be pushed intact across the plate with the point of a wire probe).

The temperature-dependent model of *H. ducreyi* infection: technical considerations

New Zealand White rabbits are not truly inbred, and there is significant variability between animals. In large samples, the effect of this variability is overcome, but in the small samples necessitated by the complexity of intragastric feeding, they can confound experimental results. The most sensitive confounding parameter of this assay is the temperature-dependent nature of the model. *H. ducreyi* grows best between 33 and 35°C. Temperatures of 37°C, even for short periods, are lethal. The rabbits are housed at 14°C, and their backs are shaved daily to maintain a skin temperature permissive for the growth

of *H. ducreyi*. However, some rabbits have a thick-shafted fur, growing quickly from densely spaced follicles, which is not easily shaved clean. Others have sparse downy fur that is easily removed. Lesions on densely furred rabbits are less severe and of shorter duration than in downy-furred rabbits (data not shown). I speculate that the skin of the densely furred rabbits is insulated, and so maintains a surface temperature less permissive for the pathogen. Regular shaving with a sharp blade is required to minimize this effect. The timing of initial hair removal may also be important. The metabolic state of the *H. ducreyi* inoculum may be critically important for the success of this model; the timing of culture harvest should be informed by growth curves generated under the same culture conditions in which the experimental inoculum is grown.

RESULTS

Determination of an optimal dose range for *S. typhimurium* SL3261 administered to rabbits

Rabbit doses

S. typhimurium SL3261 was administered to rabbits in experimental groups of six. There were few precedents for the use of oral attenuated *S. typhimurium* in rabbits. One, Boedeker et. al. (172), used an oral gavage dosage of 10^{10} CFU for *S. typhimurium* (H68) carrying the same attenuating mutation as SL3261 (*aroA*). As no illness was reported in this paper, and immunological data were inconclusive, I used 10^{10} CFU as the median of a dose range for the first two groups of six rabbits. Because a dose higher than 10^{11} CFU was impracticably "thick", two-fold dilutions from a suspension of $2x10^{10}$ CFU/mL (to yield a dose of 10^{11} CFU in the 5mL feed volume) was the first dose range tested (in experimental groups 1 and 2).

Rabbits fed these doses became severely ill, as described below. In the next two experimental groups, five-fold dilutions from $2x10^{8}$ CFU/mL (to yield a dose of 10^{9} CFU in the 5mL inoculum) were used. The 10^{9} CFU maximum in these experiments was lower than the lowest dose that caused illness in the first two rabbit groups, and the dose range was wider. No rabbits showed signs of serious illness in these experiments, though some discomfort, probably due to residual anesthetic effects was noted the day after gastric intubation. A fifth experimental group was fed either ~$1.2x10^{7}$ CFU, or PBS. These rabbits were later challenged with *H. ducreyi* as part of a later experiment, but

observations on their wellness and immune response to oral inoculation were included in this dose-determination.

Grouping of rabbits for dose-range comparisons

As the cell density of the inoculum could only be estimated by optical density measurements prior to administration, it was important to confirm the viable cell density by plate culture titration. The doses given, as calculated by viable counts, are represented in Figure 1. It was decided to compare 6 logarithmic dose ranges. Group 1 included doses $>10^{10}$ CFU (n=7); group 2, $>10^{9}$ to 10^{10} CFU (n=4); group 3, $>10^{8}$ to 10^{9} CFU (n=3); group 4, $>10^{7}$ to 10^{8} CFU (n=5); group 5, $>10^{6}$ to 10^{7} CFU (n=4); group 6 PBS controls (n=9).

Wellness observations

For all rabbits, a wellness chart was maintained for at least seven days after the day of gavage. Parameters observed were body weight, body temperature, hydration, attitude and demeanor, nasal discharge, piloerection, feces production (volume produced and consistency), and food and water consumption. All rabbits were well hydrated, but Ringer's saline was administered if a rabbit appeared uncomfortable. There were no signs of nasal discharge or piloerection. Rabbits remained alert and active, even during other signs of illness, except where noted. Wellness data for each rabbit (organized by dose group) are summarized in Table 1, Figure 2, and Figure 3. Illness was marked only in the first two dose groups.

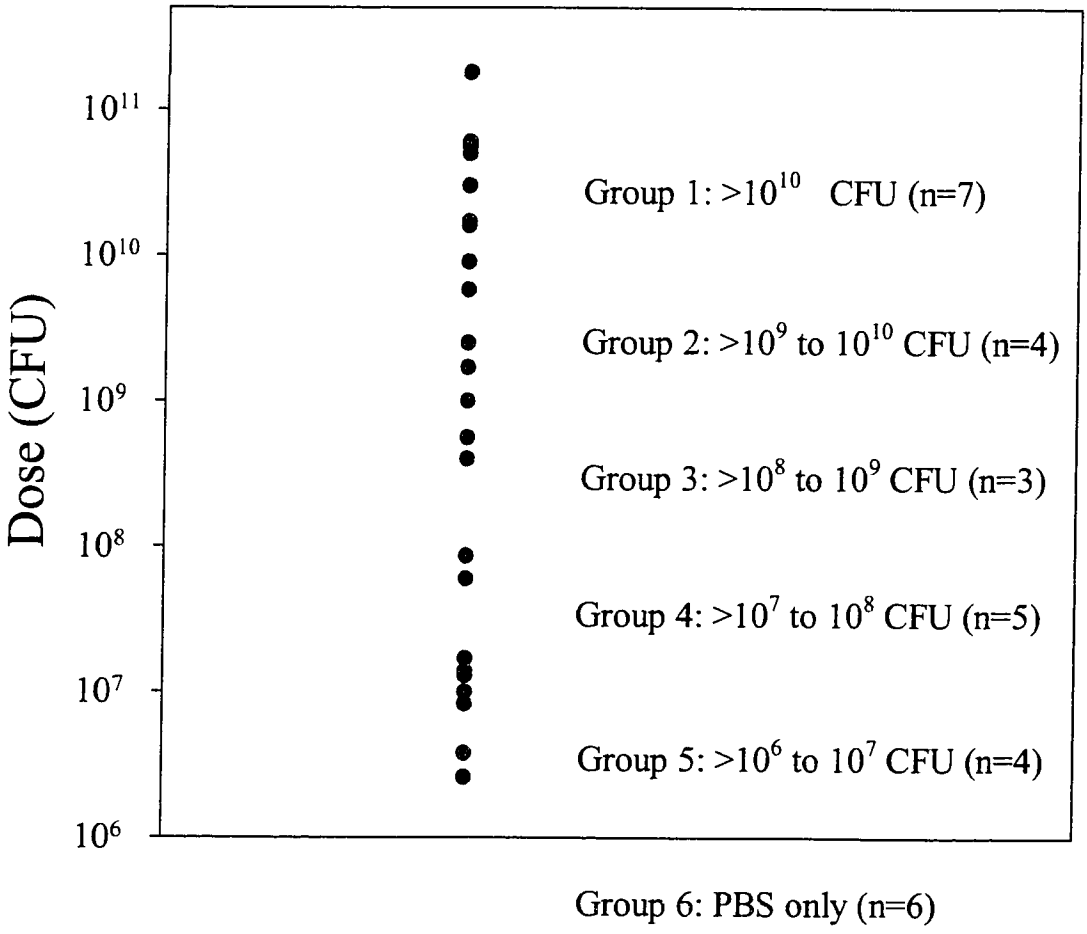

Dose (CFU)
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
Group 1: $>10^{10}$ CFU (n=7)
Group 2: $>10^{9}$ to 10^{10} CFU (n=4)
Group 3: $>10^{8}$ to 10^{9} CFU (n=3)
Group 4: $>10^{7}$ to 10^{8} CFU (n=5)
Group 5: $>10^{6}$ to 10^{7} CFU (n=4)
Group 6: PBS only (n=6)

Fig. 1. Doses received by individual rabbits. An aliquot of gavage material was diluted in PBS, and plated on LB agar to estimate the concentration of viable organisms in the cell suspension by colony forming unit density on culture. For analytic purposes, each rabbit (●) was assigned one of six groups based on these viable count-confirmed titres as shown.

Table 1. Wellness parameters for rabbits in each of 6 dose groups

Group No.	1	2	3	4	5	6
Dose range	$>10^{10}$ CFU	$>10^{9}$ to 10^{10} CFU	$>10^{8}$ to 10^{9} CFU	$>10^{7}$ to 10^{8} CFU	$>10^{6}$ to 10^{7} CFU	PBS
No. in group	7	4	3	5	4	6
Rectal temperature (mean number of days ≥40°C)	1.3	0.2	0.3	0.2	0.5	0.2
Mean weight change (kg)	-0.15	+0.16	+0.10	+0.16	+0.05	+0.07
Feces (mean abnormal days)	4.4	1.8	1.0	1.0	1.0	1.0
Appetite (mean days without chow)	6.7	4.0	1.0	1.0	1.0	1.0
Water (mean days drinking ≤1/4 bottle)	5.7	5.0	1.0	1.2	1.5	1.2

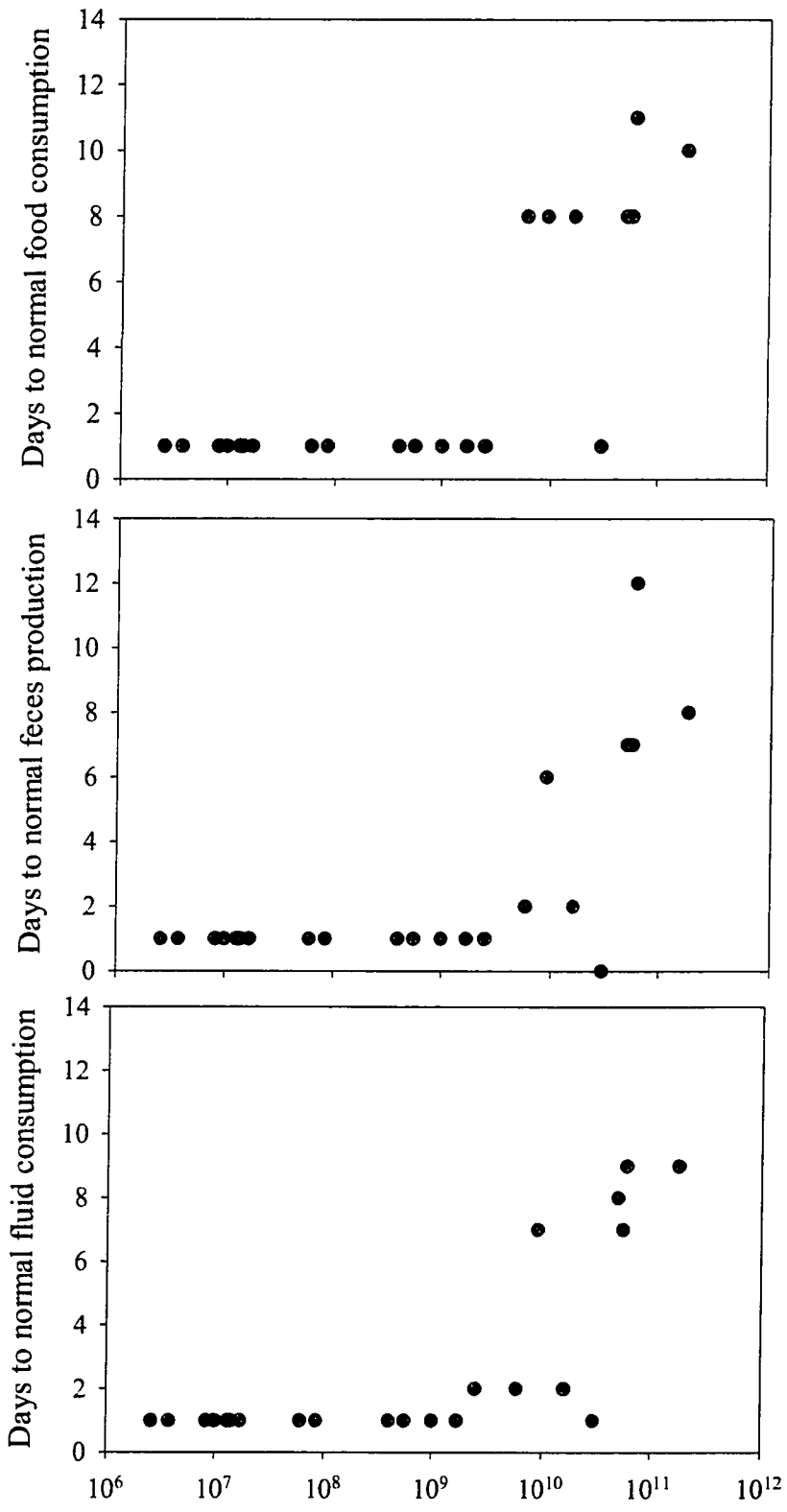

Days to normal food consumption
Days to normal feces production
Days to normal fluid consumption
0
2
4
6
8
10
12
14
10^6
10^7
10^8
10^9
10^{10}
10^{11}
10^{12}
Dose (CFU)

Fig. 2. The dose-related endpoint for clinical tolerance is 10^9 CFU for rabbits fed *S. typhimurium* SL3261 by oral gavage. Of 7 rabbits in dose group 1 ($>10^{10}$ CFU), one died. 5 of the 6 surviving rabbits, and 2 of 4 rabbits in dose group 2 ($>10^9$ to 10^{10} CFU) were ill for 7 to 12 days following inoculation. All other rabbits were well from the second day after oral gavage until the end of the experiment. Illness was characterized by anorexia (A.), tarry malformed feces or diarrhea produced in small quantity (B.), and poor fluid consumption (C.).

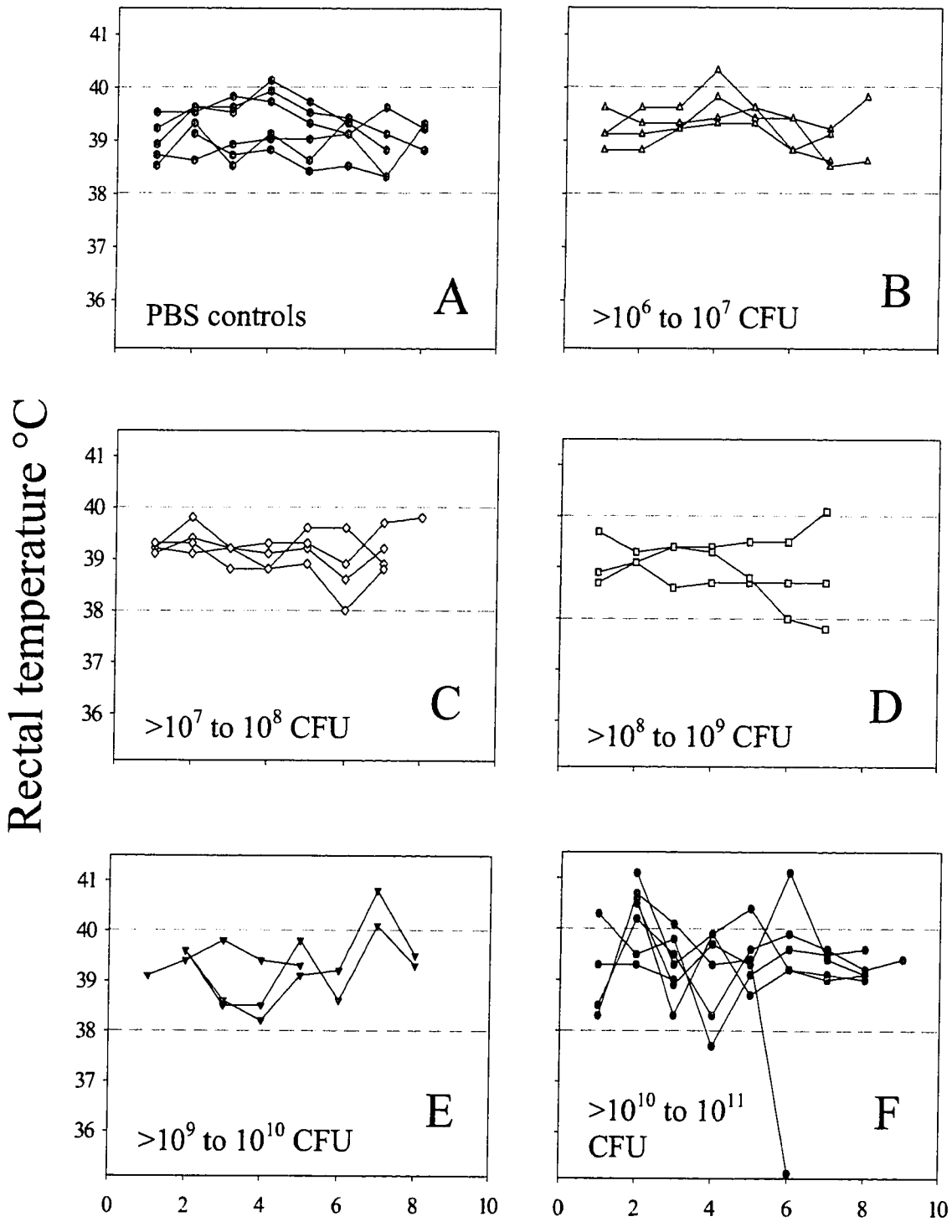

Rectal temperature °C
41
40
39
38
37
36
PBS controls
A
>10^6 to 10^7 CFU
B
>10^7 to 10^8 CFU
C
>10^8 to 10^9 CFU
D
>10^9 to 10^10 CFU
E
>10^10 to 10^11 CFU
F
0
2
4
6
8
10
Days following oral gavage

Fig. 3. Hectic fever in rabbits fed *S. typhimurium* SL3261 at high doses. Rectal temperature was recorded daily for 7-9 days after oral gavage. Each rabbit is represented by a plot, and plots are grouped by dose. A. Controls (group 6), fed PBS only; B. group 5, fed $>10^6$ to 10^7 CFU; C. group 4, fed $>10^7$ to 10^8 CFU; D. group 3, fed $>10^8$ to 10^9 CFU; E. group 2, fed $>10^9$ to 10^{10} CFU; and F. group 1, fed $>10^{10}$ CFU. The upper (— —) and lower (— —) "normal" limits of body temperature are shown for reference.

Groups 3-6, fed PBS or doses >10^6 CFU to 10^9 CFU.

No evidence of illness was seen.

Group 2, doses >10^9 CFU to 10^{10} CFU.

Two rabbits (fed 2.5x10^9 and 1.7x10^9 CFU) showed no sign of illness, and two (fed 5.8x10^9 and 9.0x10^9 CFU) were ill, with the symptoms described below.

Group 1, doses >10^{10} CFU.

One rabbit (fed 3x10^{10} CFU) showed no evidence of illness. All others showed evidence of gastrointestinal upset, with signs peaking between 5 and 7 days. Specifically, spiking fevers were noted, with anorexia, poor water consumption, and paucity of feces, with malformed pellets or in extreme cases a tarry black diarrhea. One rabbit (fed 1.7x10^{10} CFU) died 6 days after immunization. Necropsy showed evidence of septic shock and disseminated intravascular coagulation.

Serum EIA to detect specific antibody against *S. typhimurium* SL3261 crude soluble antigen

In order to determine whether oral doses were immunogenic, sera were assayed for antibody to *S. typhimurium* SL3261 crude soluble antigen. Blood samples were drawn before, and weekly for four weeks following, oral immunization with *S. typhimurium* SL3261. A serum EIA against a crude soluble antigen preparation of *S. typhimurium* SL3261 was used to determine the endpoint titre for serum from each rabbit at each timepoint. These data were grouped according to the dose groups described

above, and the endpoint titres are shown in Figure 4A. Sera from PBS-fed rabbits did not react with the antigen. Groups 4 and 5 ($>10^6$ to 10^8 CFU) responded weakly to inoculation. In dose group 4, and to a lesser extent group 5, some rabbits showed a high titre antibody response to the vaccine strain, and others showed little or no response. This distribution is evident in Figure 4B and C where the data are described using vertical point plots and box plots. A positive response considered to be one in which all rabbits in the group had SL3261-specific antibody of high titre. Note that dose groups 1, 2 and 3 ($>10^8$ CFU) have a similarly strong specific antibody response to the crude soluble antigen preparation.

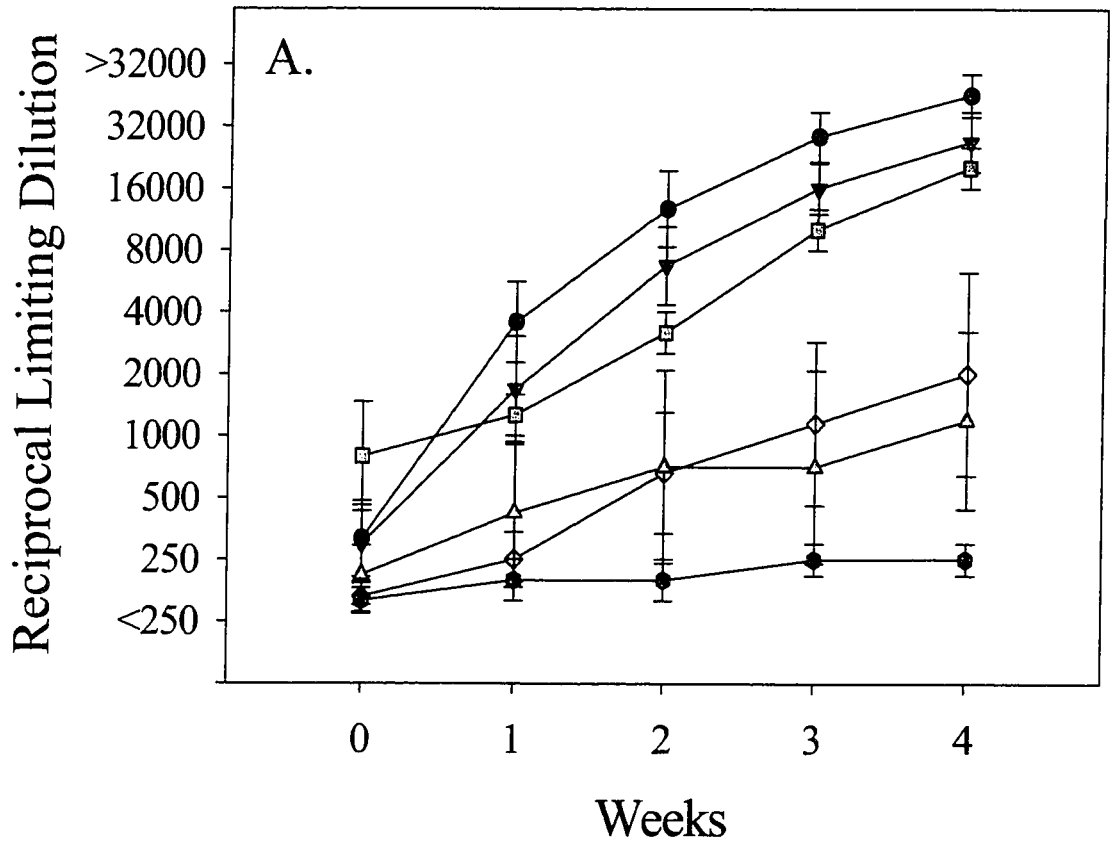
Optimal dose range for rabbits fed *S. typhimurium* SL3261

Rabbits in dose groups 3-6 tolerated the vaccine strain well. Rabbits in dose groups 1-3 showed a consistent, strong immune response to the soluble portion of the vaccine antigen. Given these results, a dose range of $>10^8$ to 10^9 CFU (dose group 3) was chosen as the target dose in the following experiments.

Differential growth of *H. ducreyi* 35000 from two laboratories in two broth media

Growth curves

As input inoculum is a determining variable in output observations of the rabbit model of *H. ducreyi* infection, it is important that its measurement is reproducible within


A.
Reciprocal Limiting Dilution
>32000
32000
16000
8000
4000
2000
1000
500
250
<250
0
1
2
3
4
Weeks

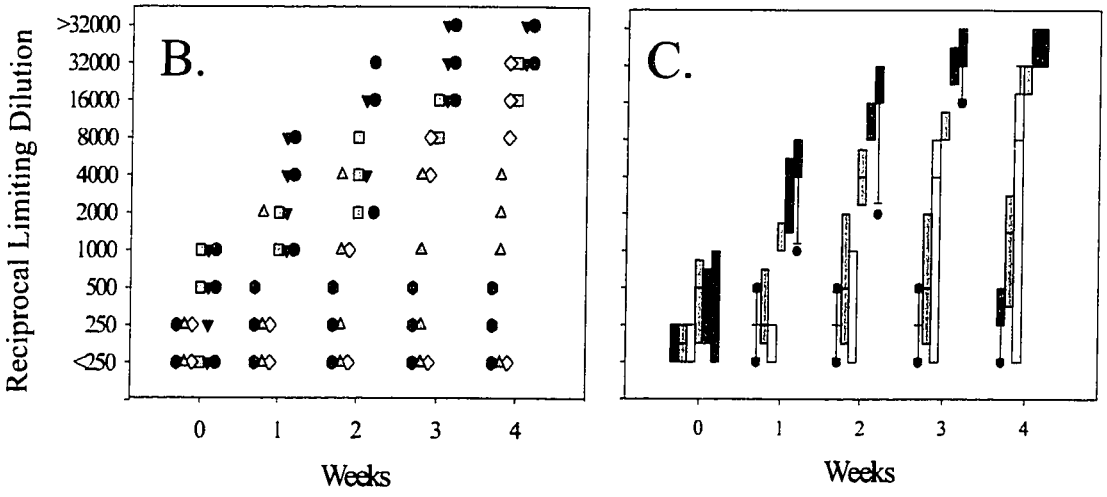

B.
Reciprocal Limiting Dilution
>32000
32000
16000
8000
4000
2000
1000
500
250
<250
0
1
2
3
4
Weeks
C.
0
1
2
3
4
Weeks

Fig. 4. The minimal 100% immunogenic dose is 10^8 CFU for rabbits fed *S. typhimurium* SL3261 by oral gavage. The serum antibody response by endpoint titration to SL3261 crude soluble antigen was measured by EIA. (A.) Mean ± SEM for [—●—] Group 6 (controls) fed PBS only; [—▲—] group 5, fed $>10^6$ to 10^7 CFU; [—◇—] group 4, fed $>10^7$ to 10^8 CFU; [—□—] group 3, fed $> 10^8$ to 10^9 CFU; [—▼—] group 2, fed $>10^9$ to 10^{10} CFU; [—●—] group 1, fed $>10^{10}$ CFU. At the threshold of immunogenicity (group 4), rabbits responded to vaccination either strongly, or not (did or did not seroconvert). Vertical point plots (B.) individual rabbits represented by points, with symbols identifying dose group. Box plots (C.) show median and interquartile range. Whiskers represent the 10^{th} and 90^{th} percentile, and outliers are represented by symbols.

produced and between laboratories. To further investigate this, 12-hour growth curves were produced for *H. ducreyi* 35000. Growth characteristics of stocks used in our laboratory were compared in two media, Mueller-Hinton broth and GC broth, and by two different estimates of culture density with stocks donated by a collaborator (Dr. C. Elkins). Differential growth characteristics were noted when culture density was measured by plate titration to identify viable cells (Figure 5), but not when cultures were measured by optical density (Figure 6). Some of these differences were statistically significant (Student's t-test; Table 2).

Gram stains

To understand why different measures of cell density in broth culture might result in such different findings, Gram stains taken at each sampling time during culture were compared. Figure 7 shows a representative comparison between Gram-stained slide smears prepared from mid-log broth culture for *H. ducreyi* 35000 grown in Mueller-Hinton broth (A) and in GC broth (B). Cells in short chains of two or three, interspersed with large clumps of cells, characterize growth in Mueller-Hinton broth compared with long paired chains of cells ("railway tracks") grown in GC broth. The North Carolina and Ottawa strains did not appear to differ in this respect.

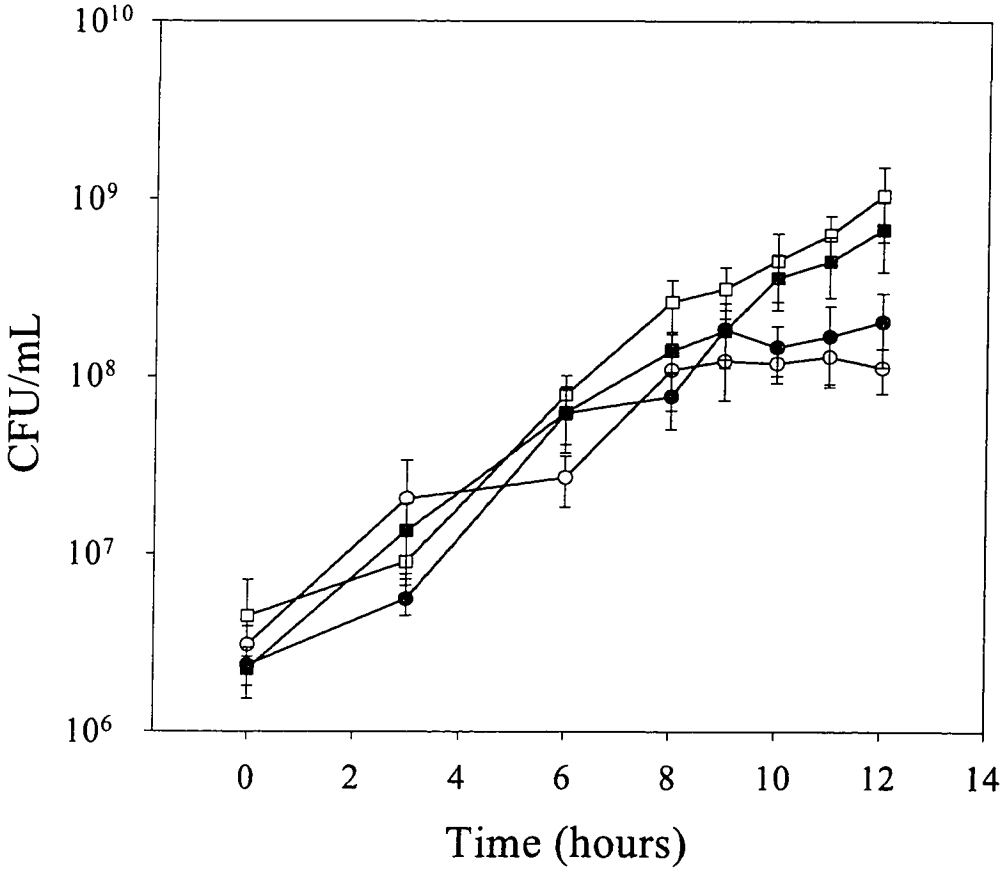

10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
CFU/mL
0
2
4
6
8
10
12
14
Time (hours)

Fig. 5. A comparison of growth characteristics for *H. ducreyi* isolates from two laboratories, grown in two broth media as measured by plate titration. [—□—] North Carolina (NC) isolate grown in GC broth; [—○—] NC isolate grown in MH broth; [—■—] Ottawa isolate grown in GC broth; [—●—] Ottawa isolate grown in MH broth.

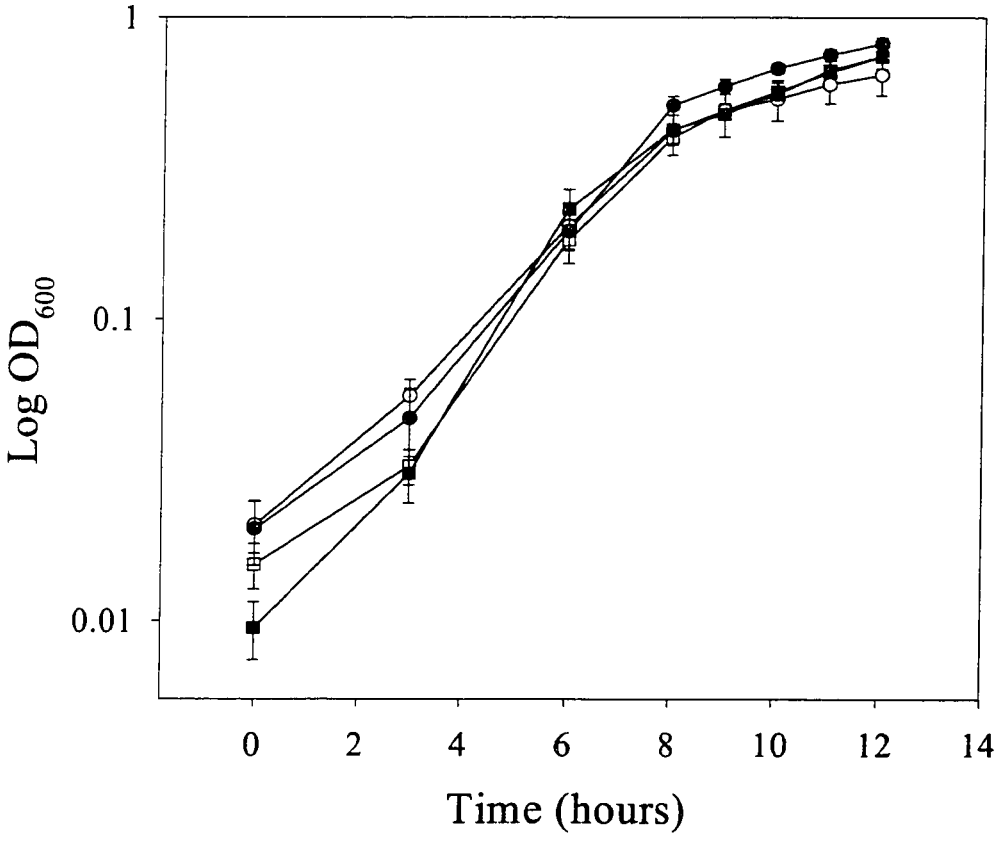

1
0.1
0.01
Log OD600
0
2
4
6
8
10
12
14
Time (hours)

Fig. 6. A comparison of growth characteristics for *H. ducreyi* isolates from two laboratories, grown in two broth media as measured by optical density at 600 nm (OD_{600}). [—□—] North Carolina (NC) isolate grown in GC broth; [—○—] NC isolate grown in MH broth; [—■—] Ottawa isolate grown in GC broth; [—●—] Ottawa isolate grown in MH broth.

Table 2. Comparison of three growth parameters for *H. ducreyi* 35000 from two laboratories in two broth media as measured by plate titration. Values are mean ± standard deviation. Matching symbols indicated values that are significantly different ($p<0.05$, Student's t-test).

Condition	Persistence of Logarithmic Growth (hours)	Peak Viable Growth (Yield in CFU/ml)	Growth Rate at midlog phase ($hours^{-1}$)
35000 Ottawa grown in GC broth	10.8 ± 0.80 *	$9.2 \pm 3.4 \times 10^8$ ∞	0.223 ± 0.023 †
35000 Ottawa grown in MH broth	8.0 ± 0.95 *	$2.4 \pm 1.3 \times 10^8$ ∞	0.241 ± 0.014 ‡
35000 NC grown in GC broth	11.0 ± 1.0 •	$12.8 \pm 5.3 \times 10^8$ ¶	0.128 ± 0.022 †Θ
35000 NC grown in MH broth	8.8 ± 0.95 •	$1.5 \pm 0.3 \times 10^8$ ¶	0.306 ± 0.008 ‡Θ


A.

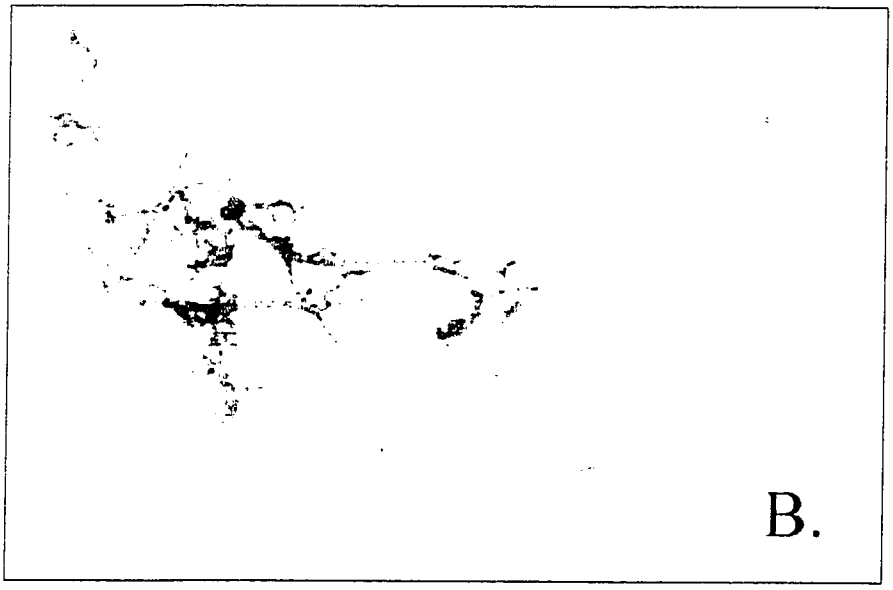

B.

Fig. 7. Representative Gram stains from mid-log phase cultures of *H. ducreyi* 35000. Cells grown in MH broth appear in short chains (arrows) and large clusters at 8 hours incubation (A). Cells grown in GC broth differe in appearance as long streptobacillary patterns (B).

Construction and characterization of the recombinant strain *S. typhimurium* SL3261(pTETnir15)

Plasmid pTETnir15 carries the gene for the C fragment of tetanus toxin (TetC) under the control of the *E. coli nirB* promoter. TetC was used in these experiments as a surrogate for the *H. ducreyi* antigens that will eventually be tested in this system to show that a recombinant antigen is recognized, and an immune response mounted to it, and in anticipation of its use as a heterologous negative control antigen in future experimental vaccinations. To ensure that rough mutations are not selected by electroporation, the plasmid was isolated from its *E. coli* host, and introduced into an intermediate strain (LB5010) by electroporation. The plasmid was then transferred into *S. typhimurium* SL3261 by transduction using phage P22 (see methods). The integrity of the recombinant strain SL3261(pTETnir15) was assessed by LPS silver stain and TetC expression analysis.

Characterisation of LPS

Electroporation selects for LPS-deficient cells. To ensure that the recombinant product of the plasmid transfer has wildtype LPS, electroporation into the intermediate strain LB010 was followed by phage transduction into *S. typhimurium* SL3261. Phage P22 uses as its cellular receptor the intact LPS molecule. Transduction of the vaccine strain SL3261 with a phage lysate of the intermediate, LB5010, ensures that cells with a "smooth" phenotype are selected. Full-length LPS is required for viability in vivo (166), so the LPS of transduced strain *S. typhimurium* SL3261(pTETnir15) was assessed by SDS-PAGE and silver staining (Figure 8). It appeared to be intact, as evidenced by the high

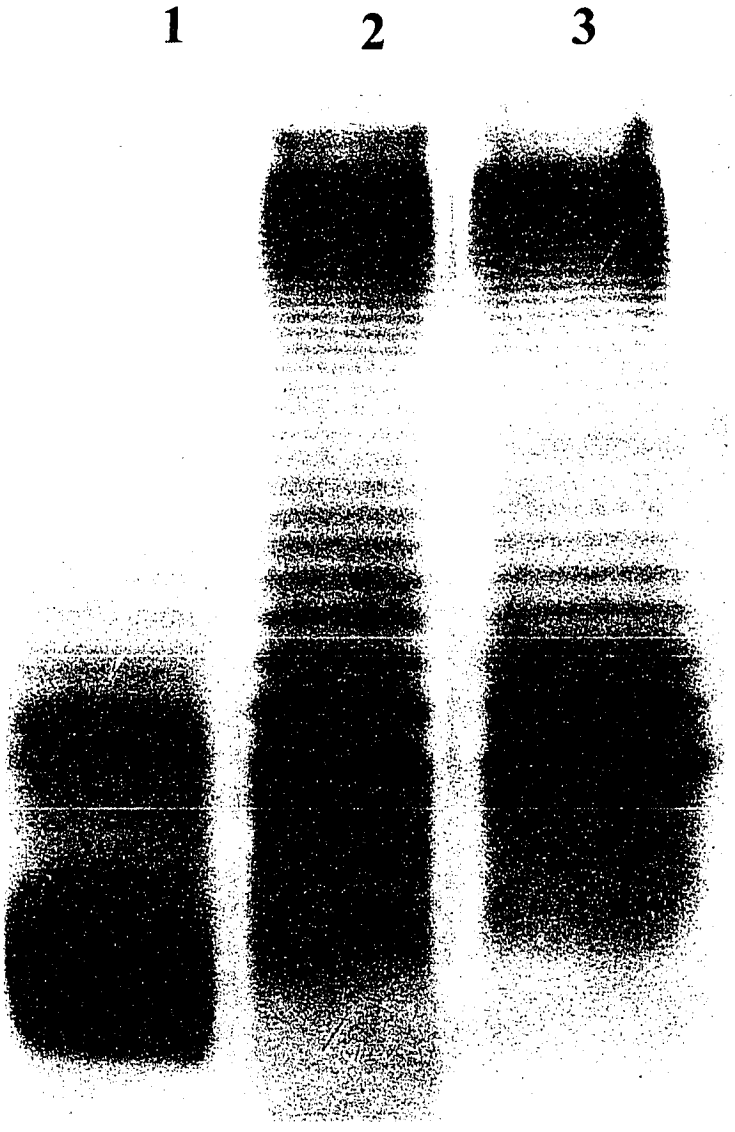

1
2
3

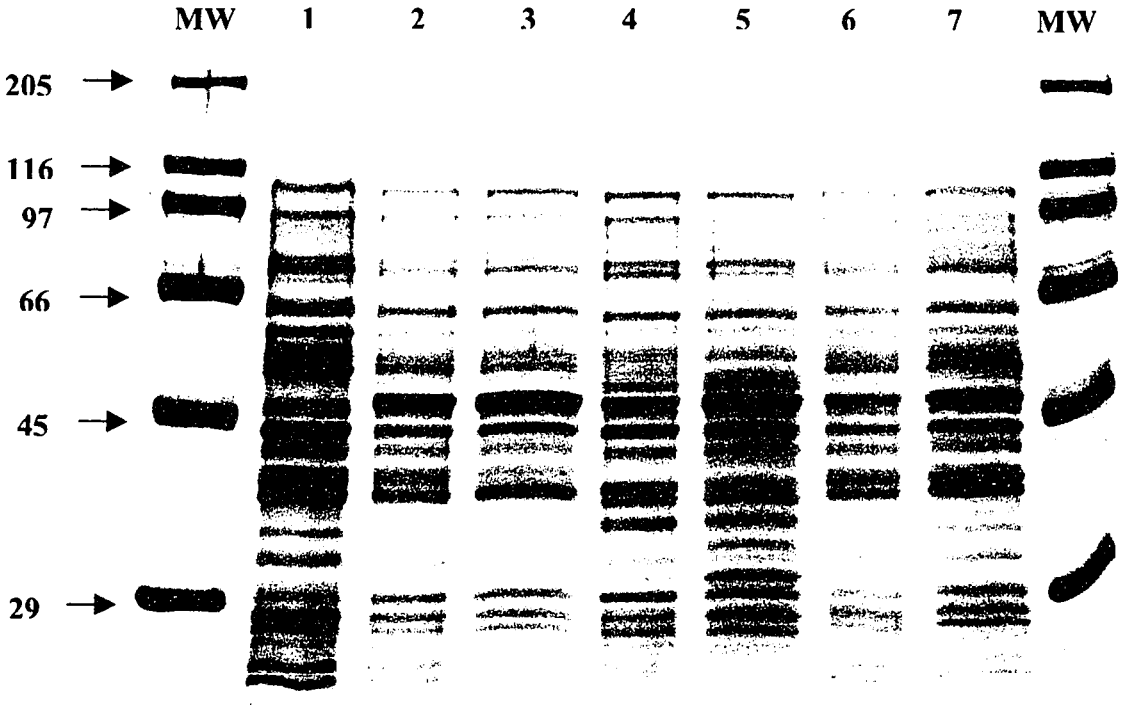
Fig. 8. Recombinant strain *S. typhimurium* SL3261(pTETnir15) has "smooth" LPS. Proteinase K-treated whole cell lysates were fractionated by SDS-PAGE. LPS was visualized by silver staining. Lane 1, LPS-mutant *S. typhimurium* LB5010; Lane 2, recombinant strain SL3261(pTETnir15); Lane 3, parent strain SL3261.

molecular weight ladder pattern seen on the stained gel, similar to that of the parent strain. The intermediate, LB5010, because of its *galE* mutation, has prominent low molecular weight bands, with only a faint laddering at higher molecular weights.

Characterisation of TetC expression

To ensure that the transduced strain expressed the TetC, whole cell lysates from overnight cultures, fractionated by SDS-PAGE, were stained with Coomassie Brilliant Blue/Bismarck Brown or electroblotted onto nitrocellulose membranes for Western blotting with a commercial, affinity purified, polyclonal anti-TetC antibody (Figure 9). The parent strain SL3261 was compared with the recombinant SL3261(pTETnir15), and the strains *E. coli* (pTETnir15) and *S. typhimurium* BRD805, in which the plasmid was provided. In anticipation of superior protein expression in anaerobically grown cultures of plasmid-containing strains, aerobic and anaerobic cultures were compared. On Coomassie-stained gels, at 50 kDa, a band, closely migrating with another at approximately 45 kDa, was observed (Figure 9A). Although partially obscured by the comigrating band, the 50 kDa band appeared in plasmid-containing strains, but was not detected in the parent strain. Because the quantity of cell lysate loaded into the wells was not standardized, no assessment of the quality of anaerobic induction could be made. A Western blot, using a commercial polyclonal anti-TetC antibody, showed that the 50 kDa band was indeed TetC, and that it was produced only in plasmid-carrying strains (Figure 9B).

A.



B.

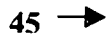


Fig. 9. The recombinant strain *S. typhimurium* SL3261(pTETnir15) can express TetC. Whole cells grown aerobically or anaerobically were lysed and fractionated by SDS-PAGE. Proteins were stained with Coomassie Brilliant Blue/Bismarck Brown (A). A duplicate gel was electroblotted onto a nitrocellulose membrane. The membrane was probed with an affinity-purified polyclonal antibody against TetC (B). Lane 1, SL3261; lane 2, SL3261 (pTETnir15) grown aerobically; lane 3, SL3261 (pTETnir15) grown anaerobically; lane 4, *E. coli* (pTETnir15) grown aerobically; lane 5, *E. coli* (pTETnir15) grown anaerobically; lane 6, *S. typhimurium* BRD805 (aerobic); lane 7, BRD805 (anaerobic).

A comparison of controls 1: Does the temperature-dependent rabbit model of experimental *H. ducreyi* infection function as a quantitative assay of comparative virulence in rabbits "immunized" with the *S. typhimurium* attenuate, or the recombinant strain?

For the purposes of the experimental rabbit model of *H. ducreyi* infection, and for evaluation of "control" data against future comparative assays of *H. ducreyi* virulence, I carried out 2 trial experimental runs to ensure that feeding rabbits the vaccine vector would, in itself, not alter virulence in the rabbit model of *H. ducreyi* infection.

Influence of the live *Salmonella* vector on the natural history of *H. ducreyi* infection

In a preliminary experiment, I challenged 3 rabbits that had been fed *S. typhimurium* SL3261 and 3 PBS-fed controls with *H. ducreyi* (experimental group 5 from the dose-determination experiment). A *Salmonella* dose range of 10^8 to 10^9 CFU was targeted (based on the results of the first four experimental dosage groups). However, this dosage was not achieved, possibly due to a dilution error. Viable counts revealed the mean delivered dose to be 1.2×10^7 CFU. However, the *Salmonella*-fed rabbits did mount a moderate serum antibody response to homologous *S. typhimurium* SL3261 crude soluble antigen as measured by EIA (Figure 10). The titre continued to increase gradually from 4-8 weeks (not shown). I did not detect serum antibodies against the control, *H. ducreyi* strain 35000 crude soluble antigen in *Salmonella*- or PBS-fed rabbits (Figure 11), suggesting that the anti-SL3261 response was specific. The course of experimental challenge infection in the two groups is described in Figure 12. Small numbers of

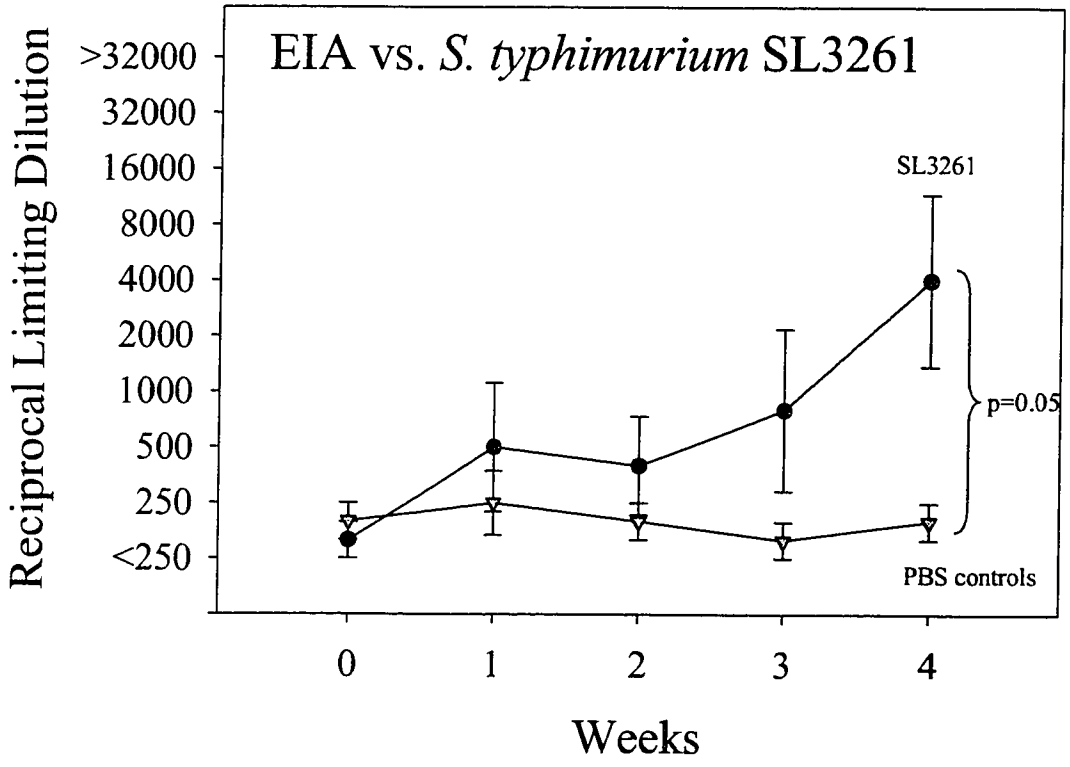

EIA vs. S. typhimurium SL3261
Reciprocal Limiting Dilution
>32000
32000
16000
8000
4000
2000
1000
500
250
<250
SL3261
p=0.05
PBS controls
0
1
2
3
4
Weeks

Fig. 10. Three rabbits fed $1.2x10^7$ CFU *S. typhimurium* SL3261 developed a moderate serum antibody response against SL3261 crude soluble antigen. No increase in titre was seen in serum from 3 rabbits fed PBS only. Mean endpoint titre by serum EIA ± SEM, n=3 in each group. [—●—] rabbits fed *S. typhimurium* SL3261; [—▽—] rabbits fed PBS.

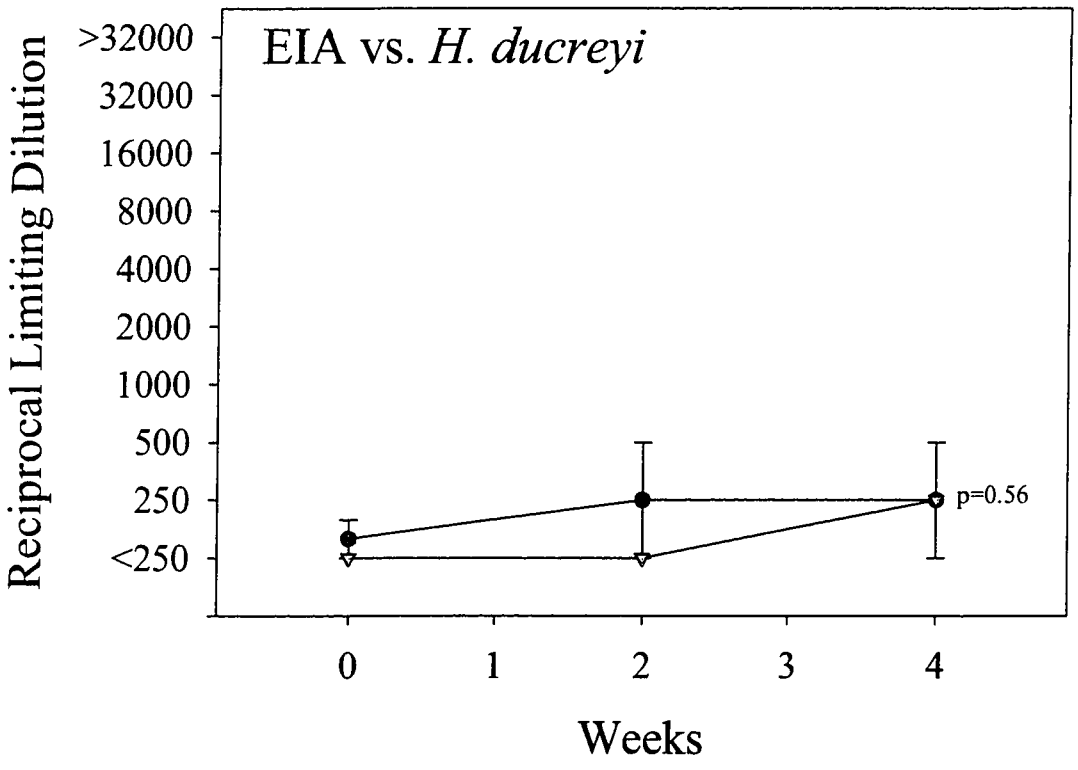

EIA vs. H. ducreyi
Reciprocal Limiting Dilution
>32000
32000
16000
8000
4000
2000
1000
500
250
<250
p=0.56
0
1
2
3
4
Weeks

Fig. 11. Rabbits dosed with *S. typhimurium* SL3261 or PBS by oral gavage had no cross-reacting antibody response against *H. ducreyi* crude soluble antigen before challenge with *H. ducreyi*. Mean endpoint titre by serum EIA against *H. ducreyi* crude soluble antigen ± SEM, n=3 in each group. [—●—] rabbits fed *S. typhimurium* SL3261; [—▽—] rabbits fed PBS.

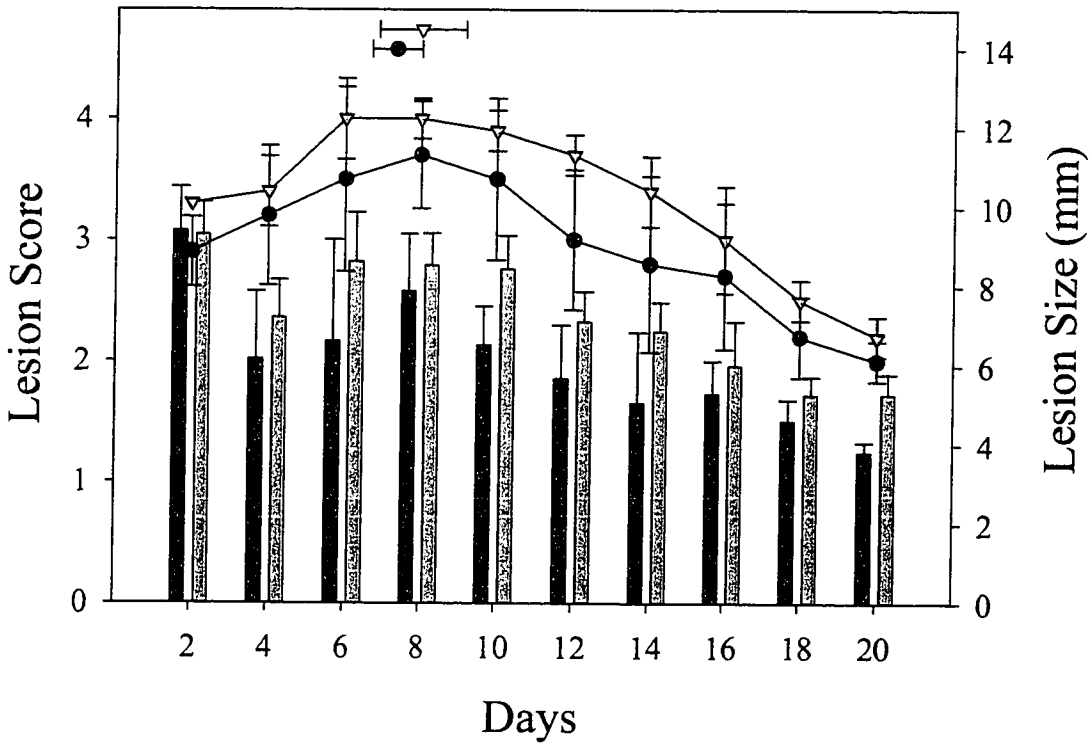

Lesion Score
Lesion Size (mm)
Days
0
1
2
3
4
0
2
4
6
8
10
12
14
2
4
6
8
10
12
14
16
18
20

Fig. 12. *S. typhimurium* SL3261 administered by oral gavage does not impair *H. ducreyi* infection in the temperature-dependent rabbit model of chancroid. Three rabbits fed 1.2×10^7 CFU *S. typhimurium* SL3261, and 3 fed PBS were challenged intradermally with *H. ducreyi* 35000 (data from sites inoculated at 10^6 CFU *H. ducreyi*). As this run of experimental infection produced sizably less disease effect than previously observed in our laboratory, interpretation is limited. Mean ± SEM for [▬] lesion size for *S. typhimurium* SL3261-fed rabbits; [▭] lesion size for PBS-fed rabbits; [—●—] lesion score for *S. typhimurium* SL3261-fed rabbits; [—▽—] lesion score for PBS-fed rabbits; [●] days culture positive for *S. typhimurium* SL3261-fed rabbits; [▽] days culture positive for PBS-fed rabbits.

rabbits in each group preclude comparative statistical analysis. In other studies, the minumum ulcer producing inoculum for *H. ducreyi* was between 10^4 and 10^5 CFU (40,48). In this experimental run, ulceration was consistently achieved only at sites inoculated at 10^6 CFU in both SL3261-fed and PBS-fed rabbits. This limits analysis. The experiment was repeated (below), with more convincing results.

Immunogenicity of the heterologous antigen TetC after oral vaccination with live attenuated *S. typhimurium* SL3261(pTETnir15) in rabbits

In order to show that a recombinant antigen, expressed in attenuated *Salmonella* strain SL3261, could be delivered to the rabbit immune system on oral inoculation, the recombinant strain SL3261(pTETnir15) was administered to rabbits, and the TetC-specific antibody response to inoculation was assessed.

Vaccination and immune response

Four rabbits were fed SL3261(pTETnir15), 4 were fed SL3261, and 4 controls were fed PBS by oral gavage. The delivered dose was estimated at 2.0×10^9 CFU by viable count for both parent and recombinant strains. Although this is just above the tolerance endpoint identified above, no rabbits were subsequently ill. The serum antibody response against *S. typhimurium* SL3261 crude soluble antigen, unrelated control *H. ducreyi* 35000 crude soluble antigen, and TetC purified from a papain digest of tetanus toxin (Calbiochem) was titrated by EIA endpoint dilution. Rabbits fed the parent or recombinant *Salmonella* strain developed a strong serum antibody response against *S.*

typhimurium SL3261 crude soluble antigen (Figure 13), which was maintained for 8 weeks (not shown). PBS-fed rabbits had no antibody response out to week 4. In rabbits fed SL3261, there was a biologically weak but significant (Student's t-test p=0.03) increase in antibody titre against *H. ducreyi* antigen at week 4, compared with PBS-fed controls (Figure 14). This may be due to cross-reactive antigens, and is likely not biologically significant. Finally, rabbits fed the TetC-producing recombinant strain *S. typhimurium* SL3261(pTETnir15) developed an obvious and consistent serum antibody response against tetanus toxin fragment C (Figure 15). This response was absent in rabbits fed the parent strain.

A comparison of controls 2: influence of the recombinant vaccine *S. typhimurium* SL3261(pTETnir15) and the parent *S. typhimurium* SL3261 on the natural history of *H. ducreyi* infection in the rabbit model

In the second trial run of the experimental model of *H. ducreyi* infection, the three groups of 4 rabbits described above were challenged intradermally with *H. ducreyi* 35000. The severity and duration of infection in PBS-fed rabbits and in rabbits fed SL3261 or SL3261(pTETnir15) is described in Figure 16. Although the sample size in this experiment is insufficient for meaningful comparisons or determination of differences, these trials show that the disease of interest (ulcer formation and culture positivity) can be produced in SL3261- or SL3261(pTETnir15)-"immunized" rabbits. These would be appropriate controls for those immunized with an *H. ducreyi* antigen-bearing recombinant *Salmonella*.

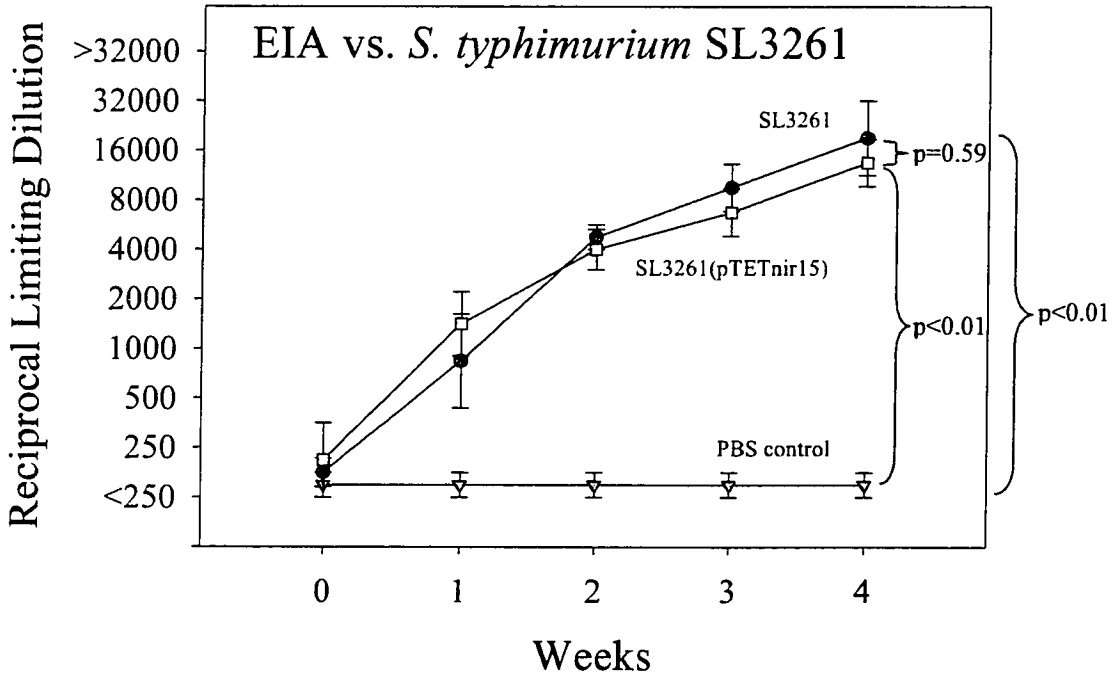

EIA vs. S. typhimurium SL3261
Reciprocal Limiting Dilution
>32000
32000
16000
8000
4000
2000
1000
500
250
<250
SL3261
SL3261(pTETnir15)
PBS control
p=0.59
p<0.01
p<0.01
0
1
2
3
4
Weeks

Fig. 13. Rabbits fed $2.0x10^9$ CFU recombinant SL3261(pTETnir15) or parent strain SL3261, but not rabbits fed PBS, mount a strong serum antibody response against *S. typhimurium* SL3261 crude soluble antigen as measured by EIA. Mean serum endpoint titre ± SEM, n=4 in each group for [—●—] SL3261-fed rabbits; [—▽—] PBS-fed rabbits; [—□—] SL3261 (pTETnir15)-fed rabbits.

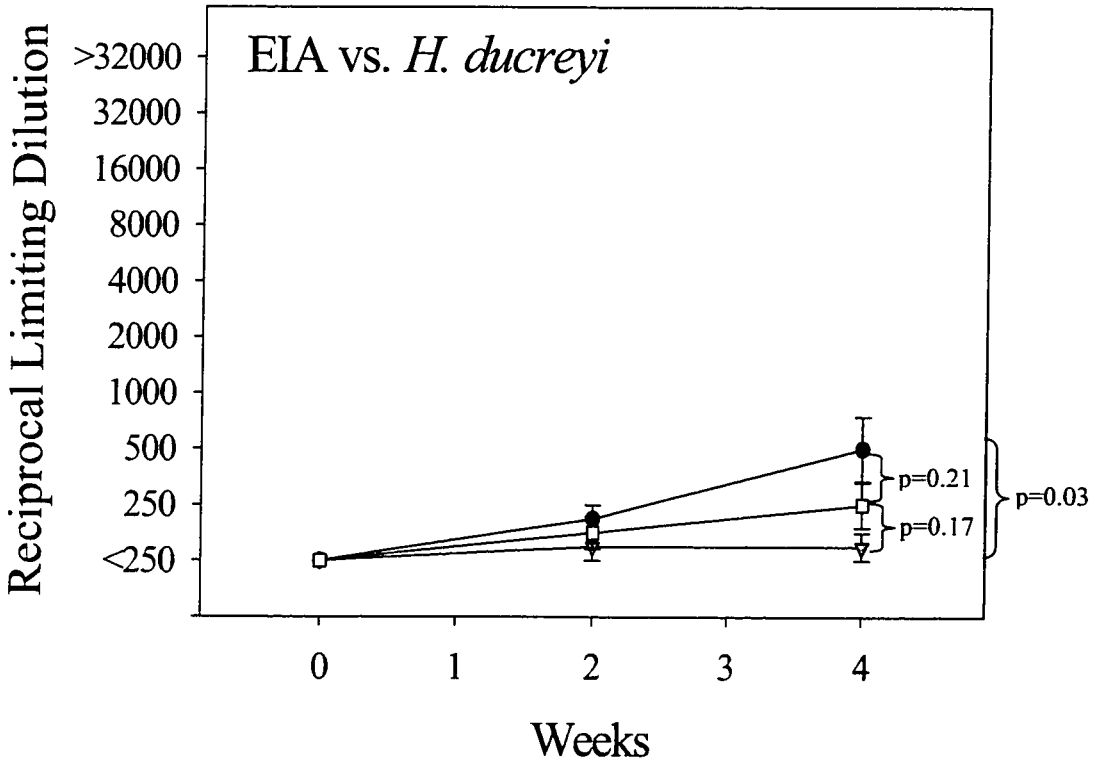
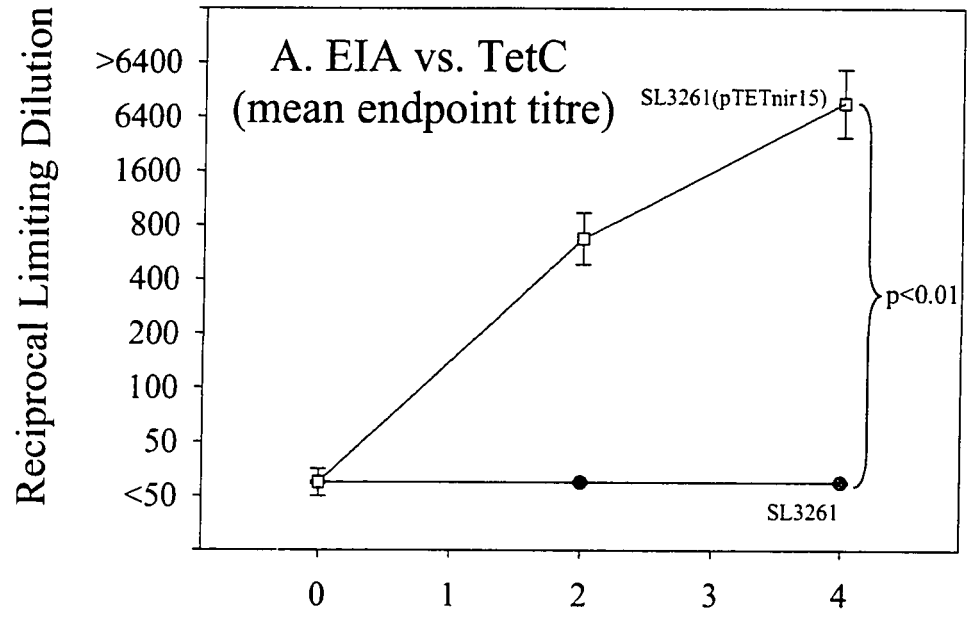

EIA vs. H. ducreyi
Reciprocal Limiting Dilution
>32000
32000
16000
8000
4000
2000
1000
500
250
<250
0
1
2
3
4
Weeks
p=0.21
p=0.17
p=0.03

Fig. 14. Low titre serum antibody response to *H. ducreyi* 35000 crude soluble antigen 4 weeks following *S. typhimurium* SL3261 and SL3261(pTETnir15) inoculation. Mean endpoint titre by serum EIA ± SEM, n=4 in each group for [—●—] SL3261-fed rabbits; [—▽—] PBS-fed controls; [—□—] SL3261(pTETnir15).


A. EIA vs. TetC
(mean endpoint titre)
SL3261(pTETnir15)
SL3261
p<0.01
Reciprocal Limiting Dilution
>6400
6400
1600
800
400
200
100
50
<50
0
1
2
3
4

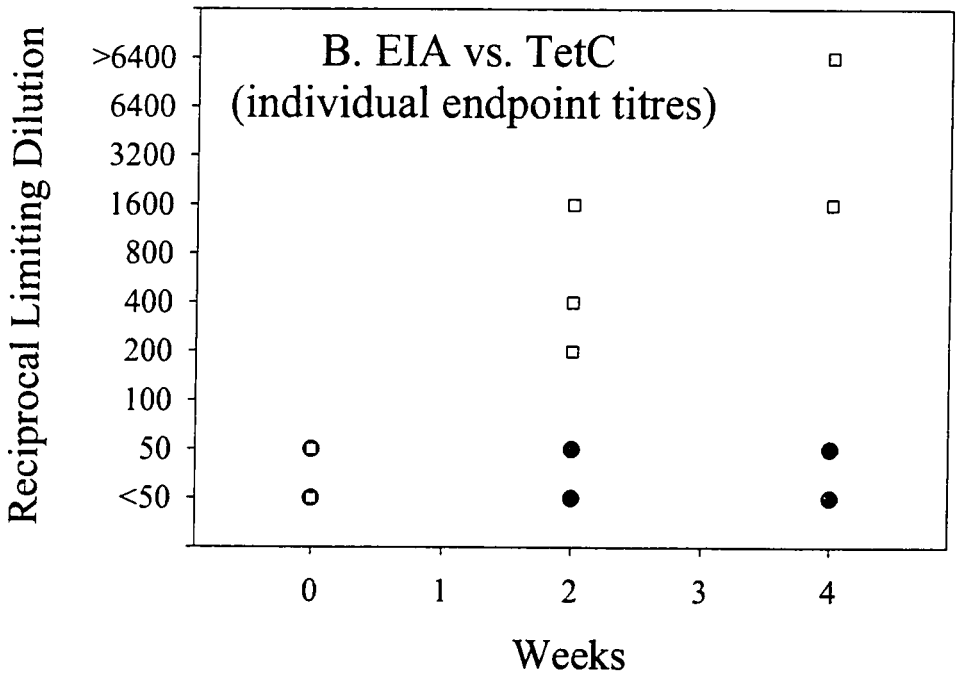

B. EIA vs. TetC
(individual endpoint titres)
Reciprocal Limiting Dilution
>6400
6400
3200
1600
800
400
200
100
50
<50
0
1
2
3
4
Weeks

Fig. 15. Rabbits fed *S. typhimurium* SL3261(pTETnir15) mounted a serum antibody response against TetC, but rabbits fed the parent strain, SL3261, did not. Mean serum endpoint titre by EIA against purified TetC ± SEM (A.) and values for individual rabbits (B.) are shown (n=4 for each group).
[—●—] SL3261-fed rabbits; [—□—] SL3261(pTETnir15)-fed rabbits.

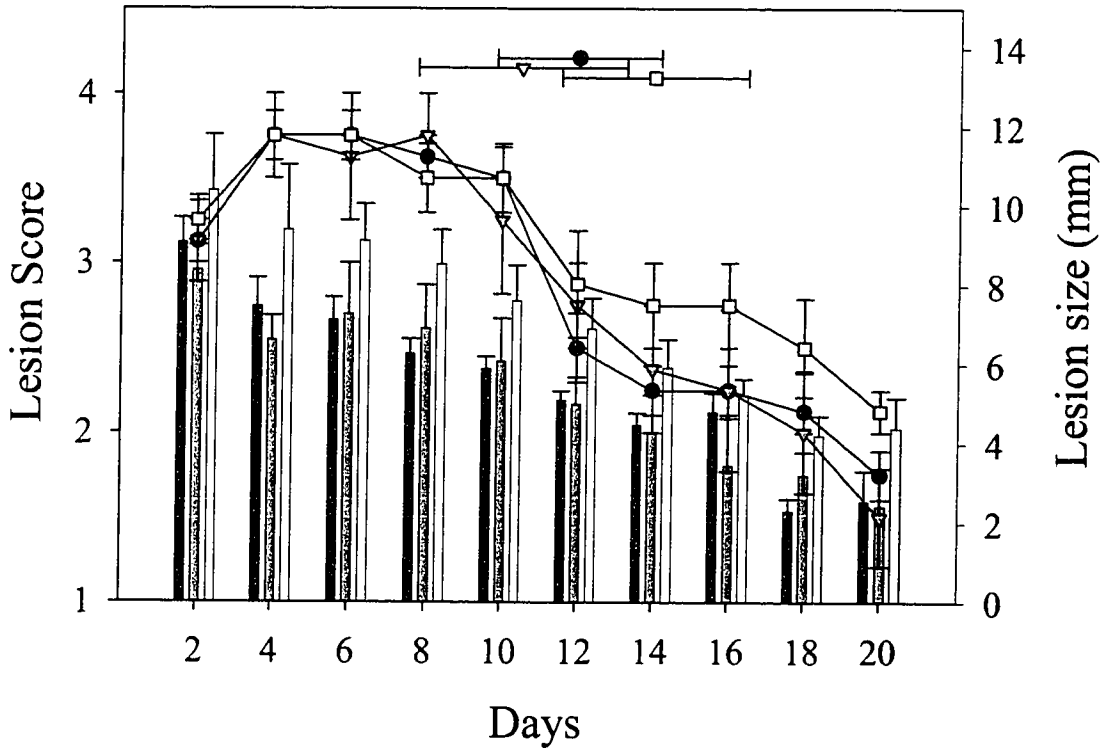

Lesion Score
Lesion size (mm)
Days
1
2
3
4
0
2
4
6
8
10
12
14
2
4
6
8
10
12
14
16
18
20

Fig. 16. In rabbits fed 2.0×10^9 CFU *S. typhimurium* SL3261 or *S. typhimurium* SL3261(pTETnir15), the natural history of *H. ducreyi* infection is not impaired. Shown are data for sites inoculated at 10^5 CFU, the minimum inoculum consistently producing ulcerative disease in this experimental run. Mean values ± SEM are shown (n=4 in each group). [▬] lesion size for SL3261-fed rabbits; [▭] lesion size for PBS-fed rabbits; [▭] lesion size SL3261 (pTETnir15)-fed rabbits; [—●—] lesion score for SL3261-fed rabbits; [—▽—] lesion score for PBS-fed rabbits; [—□—] lesion score for SL3261(pTETnir15)-fed rabbits; [●] days culture positive for SL3261-fed rabbits; [▽] days culture positive for PBS-fed rabbits; [□] days culture positive for rabbits fed SL3261 (pTETnir15).

DISCUSSION

A vaccine against chancroid, targeted to epidemiologic core groups of high-frequency STD transmitters such as networks of prostitutes and their clients could effectively control, or even eliminate chancroid. Vaccine development for chancroid uses the temperature dependent rabbit model of experimental *H. ducreyi* infection. This has several attributes that make it a suitable model for human chancroid, in studies of comparative virulence and inducible immunity that require reproducible quantitation of virulence. Both the route of inoculation and the lesions produced mimic human infection and disease. The input inocula, the serum immune response, cellular immunity (as detected by a delayed type hypersensitivity response) and several measures of disease severity and duration can be quantified in multiple instances, allowing for statistical power despite relatively small samples sizes and considerable variation.

Qualitative aspects of the immune response to vaccination could be more easily elucidated in a mouse model of infection, but attempts to develop a mouse model for chancroid failed (68,69). Recombinant attenuated *S. typhimurium* vector strains are most commonly tested in genetically susceptible mice, because, in these mice, *S. typhimurium* causes an enteric fever disease analagous to typhoid. The invasive nature of this infection may contribute to the immune response elicited to *Salmonella* vectors and the recombinant antigens they deliver. Rabbits have different anatomical and physiological characteristics than mice, and attenuated S. *typhimurium* vectors used may not behave predictably in rabbits due to host-specific virulence requirements. To prepare for studies aiming to adapt the oral recombinant attenuated vaccine vector paradigm for use as a

chancroid vaccine to be tested in rabbits, it was important to answer the following questions:

1) What is the maximum tolerated oral dose, and minimum immunogenic oral dose of attenuated *Salmonella* in rabbits?
2) Can a recombinant antigen expressed in the attenuated vector be recognized by the rabbit immune system?
3) After oral *Salmonella* vaccination, does the temperature dependent rabbit model of experimental *H. ducreyi* infection still function as a quantitative assay of comparative virulence to permit vaccine evaluation?

Determination of an optimal dose range for *S. typhimurium* SL3261 administered to rabbits

Why *S. typhimurium* SL3261?

I began by establishing a well-tolerated, immunogenic dose of the live oral vaccine *S. typhimurium* SL3261 in rabbits. I chose to use this strain for several reasons. SL3261 was one of the first attenuated *Salmonella* strains attenuated by a specific deletion (105), and has been widely used in studies similar to this one, so it is very well characterized. It is attenuated by deletion of *aroA*, a component in the aromatic amino acid biosynthetic pathway. Synthesis of chorismate is impaired, preventing synthesis of α-DHB and *p*ABA, two nutritional requisites of bacteria that are not available endogenously in mammalian tissues. Strains attenuated by deletion of virulence-associated genes or regulons, which combat host defences, may be virulent in

immunosupressed hosts (173), but nutritional auxotrophs may be self limiting in the absence of host protective factors. However, it has been shown that IFN-γ is required for clearance of aroA mutant strains in mice (150). This is an important consideration in a vaccine against chancroid, which will target individuals who are likely to be infected with HIV.

Dose escalating study: tolerance of *S. typhimurium* SL3261 in rabbits

10^8 to 10^9 CFU was the maximal 100% tolerated dose range as analyzed by dose group, but in later experiments, I found that a dose of $2x10^9$ CFU (achieved by targeting a dose of $5x10^8$ CFU) was well tolerated in 4 rabbits. Boedecker et al. (172) inoculated rabbits by oral gavage with 10^{10} CFU of an *aroA* deletion mutant (H68), suspended in a sodium bicarbonate solution. In contrast to the present study, no illness was noted at this dose. Different parameters were measured (weight loss and diarrhea vs. anorexia, abnormal feces production, volume of fluid consumed). Weight loss and diarrhea were noted only in the most severely ill rabbits in our study. As the attenuating deletion was the same in both studies, the vaccine strain may not account for the difference. However, as the genetic background of strain H68 is not described, this is unclear.

In humans, *S. typhi* is particularly successful in infecting hypochlorhydric individuals (92). Administration of sodium bicarbonate to neutralize stomach acid reduces the infective dose by 10-100 fold (174). The dose of sodium bicarbonate, or the timing of its administration in our study, may have allowed more or fewer live bacteria to pass through the stomach. This is unclear; the methodological details of the former study are not elaborated.

Boedeker et al. may have tested as few as 2 rabbits. In our work, one rabbit of seven fed $>10^{10}$ CFU remained well throughout the experiment. It is possible that, by chance, the rabbits Boedeker studied were more resistant than the average outbred population at large if the sample size was small. In mice, resistance to *S. typhimurium* infection is associated with expression of Nramp-1, a divalent cation transporter expressed solely in reticuloendothelial cells (formerly *Ity*) (87,175). Innately resistant (Ity^r) mice rapidly clear wildtype *S. typhimurium* C5 infection, probably by enhanced intracellular killing in phagocytes (87). In contrast, sensitive Ity^s mice (BALB/c, C57BL6) rapidly succumb to wildtype infection, unless specific humoral and cell mediated immunity has been primed by vaccination (152). Other host phenotypes that determine susceptibility to *Salmonella* and similar infections have also been identified. For example, a lipopolysaccharide binding protein plays a role in the host response to endotoxin (176). Humans express analogous protective factors (177). New Zealand White Rabbits are outbred, so a wider variability in susceptibility, possibly associated with differential expression of an Nramp-1 analogue, or any other genetic factor, is expected. This kind of inter-subject variability may better represent that expected in nature, or in the outbred human population.

Rabbits appear to be more sensitive to *S. typhimurium* SL3261 than mice are, and the pathogenesis of infection may differ between the two species. Doses of 10^{10} CFU SL3261 are commonly fed intragastrically to mice, sometimes for 3 consecutive days, without inducing illness: a difference that is more striking when the great difference in body mass between the species is considered. Species-related differences in

gastrointestinal anatomy and physiology might account for large differences in virulence of pathogens.

I did not know what enteric or systemic clinical signs to expect in feeding rabbits this attenuated strain. In susceptible mice, a disease analogous, but not identical, to typhoid in humans is elicited on infection with wildtype strains (82). In humans, typhoid fever has an incubation period of 5-20 days, depending on the health and immune status of the host and on the inoculum size (80). Symptoms of typhoid are constipation, abdominal pain and fever. Boedeker reported a typhoid-like illness on feeding H68 to rabbits, but *S. typhimurium* infection in animals other than mice is reported to cause gastroenteritis, and not to progress to systemic infection. In fact, Hanes et al. (178) recently developed a rabbit model of *S. typhimurium* gastroenteritis using an oral gavage procedure much like that tested in this study. The rapid onset of illness I observed in rabbits, and the observation of diarrhea as a sign of vaccine toxicity, suggest that *S. typhimurium* SL3261 infection in rabbits may be characterized by localized enteritis, rather than a systemic typhoid-like enteric fever. However, hectic fever was observed in some animals at high doses.

A large, direct inoculum might produce an unexpected disease effect. Certainly, massive quantities of LPS might induce a systemic inflammatory response (179). In our study, one rabbit died 5 days after inoculation with $1.7x10^{10}$ CFU of the *Salmonella* attenuate. Necropsy revealed disseminated intravascular coagulation and a distended gastrointestinal (GI) tract with a granular inflammatory infiltrate, suggestive of an overwhelming systemic inflammatory reaction, such as in bacterial sepsis. *Salmonella* flagella (180,181) and porins (182,183) also induce the production of proinflammatory

cytokines. However, in the rabbit model of gastroenteritis (178), 10^{11} CFU of heat killed *Salmonella* did not cause diarrhea; cell surface components alone may not cause the gastroenteritis I observed with attenuated vaccine bacteria. Alternatively, it is possible that live cells are required to gain sufficient proximity between LPS, porins or flagella and enterocytes for cytokine induction.

I did not investigate the occurrence or persistence of the vaccine strain SL3261 in Peyer's patches, liver or spleen, nor its presence in blood cultures. However, Boedeker et. al. (172) sacrificed rabbits inoculated with the *aroA* strain H68 at 2 and 7 days post immunization. H68 was detected at 10^4 CFU/g in liver at day 2, but was not detectable at day 7. Peyer's patches were infected at 10^6 CFU/g at day 2 and 10^5 CFU/g at day 7. In adjacent segments of ileal tissue, H68 was detected at 2 logs lesser concentration, showing a preferential infection of Peyer's patches. White miliary nodules noted in appendices and Peyer's patches suggested bacterial replication within the gut-associated lymphoid tissue. Dunstan et al. (109) showed that colonization of Peyer's patches, but not spleen was an important correlate of immunity.

Dose-escalating study: the immune response to inoculation

I detected a strong SL3261-specific serum antibody response in rabbits fed SL3261. Titres rose to a maximum at 4 weeks, with consistent high titres in rabbits fed over 10^8 CFU. In rabbits to be challenged with *H. ducreyi*, the SL3261 antiserum titre was measured for 8 weeks following *Salmonella* inoculation, and in these animals, the week 4 maximum was maintained for the duration (not shown). Boedeker et. al. (172) followed serum IgG, and biliary IgA responses to H68 (*aroA*) at days 2 and 7 post

immunization. This timecourse is too short to compare with our findings of the anti-*Salmonella* immune response.

I did not investigate qualititative aspects of the immune response, as knowledge is limited for specialized immunologic study in rabbits. In mice, oral vaccination with strain SL3261 induces cell-mediated immunity characterised by CD4+ T lymphocytes that secrete proinflammatory cytokines such as IFN-γ, IL-2 (159), IL-12 and TNF-α. CD8+ cytotoxic T lymphocytes are also detected. A strong serum antibody response is induced as well, usually with IgG2a the predominant IgG subtype. Antibody secreting cells, with homing receptors for the intestinal, genital and oral mucosae, are also detected (160-162). I did not investigate secretory IgA in the rabbits. Experimental infection in the rabbit model of chancroid is cutaneous, and IgA responses are likely irrelevant in this context. As well, systemic immunity and mucosal immunity have been shown to move in concert in other immunizations. For example, the measles vaccine, delivered systemically, protects against a mucosal pathogen (184).

Differential growth characteristics of *H. ducreyi* 35000 from two laboratories in two broth media

For the temperature-dependent rabbit model of *H. ducreyi* infection to function as a quantitative virulence assay, it is critically important that input inocula are accurately quantified. Input inocula are a determining variable of the experimental outcome, and for valid comparisons, both within experiments and between laboratories, enumeration of input inocula should be standardized. I demonstrated superior growth of viable organisms

in GC versus MH broth and differential growth characteristics of the same reference laboratory strain (*Haemophilus ducreyi* 35000) from two labs and in two media, as measured by viable count plate titrations. In part this difference may be an artifact of the growth estimate procedure used; differences in growth were not observed when culture density was quantified by optical density. Gram stains show that, in GC broth, *H. ducreyi* grows in long chains that form loose networks at high culture densities. In contrast, in MH broth, cells grow in short chains that form dense clumps of cells at high culture densities. These different patterns of growth may differentially bias the estimates of cell density in the two methods, as I have demonstrated in this experiment. These differences were not observed between the two laboratory stocks. The standard medium used in the North Carolina laboratory is GC broth. Superior growth of the North Carolina stocks observed in GC broth may be due to adaptation of the organism with serial passage in the laboratory.

Construction of the recombinant strain *S. typhimurium* SL3261(pTETnir15)

Choice of plasmid pTETnir15

Plasmid pTETnir15 has several features that make it useful for future studies in this model. It is stable; in the absence of selective pressure *in vitro*, the plasmid is lost in <0.5% of cells per generation, and in mice, after 10 days, >90% of cells recovered from liver and spleen retained the plasmid (185). pTETnir15 carries the gene for fragment C of the tetanus toxin (TetC): an immunogenic antigen easily detected with commercially

available antibodies. Enhanced expression and immunogenicity of other pathogen-derived epitopes is achieved when they are incorporated in a fusion protein with TetC (146). The *nirB* promoter from the *E. coli* nitrate reductase gene, which is activated by Fnr under anaerobic conditions (135), controls TetC expression. p*nirB* was developed to promote plasmid stability (136), but also enhanced the efficacy of a vaccine construct already stabilized with an balanced lethal deletion of the *asd* gene (186) probably by appropriately directing the timing of antigen expression (187). Although two inducible promoters that are more efficient, p*pagC* (137) and p*htrA* (145), were recently identified, p*nirB* has been much studied and shown to be effective, and is still widely used (188,189). pTETnir15 also carries a β-lactamase (amp^r) gene for *in vitro* selection.

Introduction of pTETnir15 into *S. typhimurium* SL3261

The two-step transduction of strain SL3261 used in this study has several advantages. LB5010 (*galE*, r-, m+) has a partial deficiency in LPS (is a semi-rough mutant). The rate of transformation is higher in rough than in smooth cells for Ca+ shock methods (190), though this is less important for electro-transformation. On transformation, the restriction incompetent (r-) intermediate does not digest transforming DNA, labeled as foreign by its distinct methylation pattern (166). As the plasmid replicates inside the host cell, the methylation pattern is modified (m+) to match that of *S. typhimurium* (191).

Characterization of the recombinant strain

The LPS of recombinant strain SL3261 (pTETnir15) was present in native ("smooth") form, as demonstrated by SDS-PAGE/silver stain. Surprisingly, the high molecular weight LPS ladder was not precisely identical to that of the parent strain. P22 phage transduction selects for the smooth (LPS intact) phenotype because its cellular receptor specifically binds the LPS sidechain (166). Perhaps P22 LPS receptor favours a particular conformation of LPS, thus selecting a clone of the parent population with a different LPS conformation.

S. typhimurium SL3261(pTETnir15) expresses TetC, as established by SDS-PAGE/Western blot. Although there appeared to be more TetC protein in *E. coli*(pTETnir15) grown anaerobically, suggesting an anaerobic induction effect, and this was not apparent in SL3261(pTETnir15), as the 50 kDa band density was not quantified, so the presence or absence of an induction effect could not be determined.

Immunogenicity of TetC expressed by the recombinant strain and its influence on the temperature dependent rabbit model of chancroid

The immune response to vaccination

Because the mode of sub-pathogenic infection of attenuated vaccine strains may differ between mice and rabbits, it was important to test whether the attenuated *Salmonella* strain SL3261 could deliver a recombinant antigen to the rabbit immune system, and provide adjuvancy to stimulate its recognition. 2.0×10^9 CFU of the recombinant SL3261(pTETnir15) was administered to 4 rabbits. 4 more were given the

same dose of the parent strain SL3261, and 4 controls were fed PBS. This dose was above the target for tolerance, but no subsequent illness was noted.

Predictably, the immune response to the parent strain at this dose was markedly stronger than that seen in the previous trial run in which rabbits were underdosed with *Salmonella*, and was comparable to the immune response to similar doses in the dose-determination study. In mice, pTETnir15-recombinant strains persist in liver and spleen at one log lesser concentration than the parent strain (146). However, in this study, the recombinant and parent strains mounted an equally strong antibody response against SL3261 soluble antigen. This suggests either that carriage of the plasmid or expression of TetC did not compromise the recombinant strain, or that the dose administered was sufficient to achieve seroconversion in spite of a metabolic disadvantage.

A small, but statistically significant serum immune response to *H. ducreyi* crude soluble antigen was noted in rabbits fed the parent strain, SL3261, in this study. The EIA antigen was a crude preparation of the soluble portion of an *H. ducreyi* cell lysate. Some cross-reaction between *H. ducreyi* and *Salmonella* epitopes is predictable using this antigen, as it was chosen for sensitivity rather than specificity (192).

In contrast, for the relevant antigen, a strong TetC-specific antibody response was mounted by rabbits fed the recombinant strain SL3261(pTETnir15), but not in rabbits fed the parent strain. Thus rabbits, like mice, can recognize and mount an immune response to heterologous antigen delivered orally by an attenuated *Salmonella* vaccine strain.

TetC is soluble and expressed in the cytoplasm in this system (136), so it appears that in rabbits, surface expression of this antigen is not required to induce an anti-TetC antibody response.

Does the temperature-dependent rabbit model of experimental *H. ducreyi* infection function as a quantitative assay of comparative virulence after oral inoculation with attenuated *Salmonella*?

I did two trial runs of the temperature-dependent rabbit model of *H. ducreyi* infection to show that SL3261-immunized rabbits could serve as controls for comparison in future experiments with rabbits fed SL3261 bearing *H. ducreyi* antigen.

Preliminary trial: *S. typhimurium* SL3261- and PBS-fed controls

In a preliminary experiment, six rabbits were challenged with *H. ducreyi*. Of these, three controls had been sham-immunized orally with PBS, and 3 were fed *S. typhimurium* SL3261. Although the target dose for this group was between 10^8 and 10^9 CFU, the mean SL3261 inoculum fell significantly below this dose (1.2×10^7 CFU). Although a moderate but significant immune response was detected to SL3261 antigen, there was also a low-titre reaction to *H. ducreyi* antigen in other experiments (presumably cross reaction), and so interpretation of the results of this experimental run is limited. I have described the data without statistical comparisons as no differences had been predicted and the sample size was too small for adequate statistical power. In addition, the minimal ulcer-producing *H. ducreyi* inoculum in these animals was greater than 2×10^6 CFU; thus experimental infection in this run was not as predicted from past experience with the model, and this further limits analysis. The timing of culture harvest for inoculum preparation may have influenced the fitness of the bacteria for establishing

infection in the rabbits. The culture was harvested at 12 hours, or in late log phase as described in Figure 5. In future, the timing of culture harvest for inoculum preparation should be informed by growth curves. Nevertheless, culture positive ulcerative disease was produced, and the temperature-dependent rabbit model should function for the purposes of comparative virulence between rabbits sham immunized with *S. typhimurium* SL3261, and a recombinant derivative of SL3261 carrying *H. ducreyi* antigens.

The temperature-dependent rabbit model of experimental *H. ducreyi* infection functions as a quantitative assay of comparative virulence after oral inoculation with *S. typhimurium* SL3261, or SL3261(pTETnir15).

The 3 groups of rabbits, fed PBS, SL3261 or SL3261(pTETnir15) were experimentally challenged with *H. ducreyi*, to expand the preliminary *H. ducreyi* challenge experiment described above, and to establish the temperature-dependent rabbit model in the context of an irrelevant "control" antigen . Again, the sample size was too small to permit statistical comparisons. However, in all three experimental groups this time, at 10^5 CFU, ulcerative lesions were produced from which *H. ducreyi* cultures could be isolated. This is the minimum ulcer-producing inoculum predicted from previous work with this model in our laboratory. As expected, the duration and severity of disease was similar for all groups. Thus *S. typhimurium* SL3261 and SL3261(pTETnir15) can serve as appropriate controls in this context. Future assessment of inducible immunity conferred by oral inoculation with recombinant *S. typhimurium* SL3261 bearing an *H. ducreyi* antigen expressed from a derivative of pTETnir15 is feasible based on this experimental infrastructure.

Conclusion

In summary, a framework has been established for the development of a live oral chancroid vaccine in the rabbit model of *H. ducreyi* infection. I have identified oral dose ranges for tolerance and immunogenicity for an attenuated *Salmonella* carrier in rabbits, and have shown that a heterologous antigen expressed from a plasmid in this carrier induces measurable immune recognition of that antigen. An assay as simple as a "skin-test" to elicit a delayed-type hypersensitivity reaction to the purified antigen may also serve as a measure of inducible immune response. Finally, I have ensured that the *Salmonella* carrier and its plasmid permit measurable virulence, and appear not to influence the natural history of experimental rabbit *H. ducreyi* infection; thus the experimental model can be used to assay comparative virulence and protection in this setting. This framework may speed feasibility testing of live oral *Salmonella* recombinant constructs in pursuit of a human chancroid vaccine. Testing of *H. ducreyi* vaccine antigens can proceed using this system, based on this work.

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