Characterization and immunogenicity of the heat shock protein, hsp60 of Brucella abortus

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Characterization and immunogenicity of the heat shock protein, hsp60 of *Brucella abortus*

Gor, Dennis, Ph.D.

Iowa State University, 1994
Characterization and immunogenicity of the heat shock protein, hsp60 of Brucella abortus

by

Dennis Gor

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfilment of the
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DOCTOR OF PHILOSOPHY

Department: Zoology and Genetics
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Approved:

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For the Interdepartmental Major

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For the Major Department

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For the Graduate College

Iowa State University
Ames, Iowa

1994
This is dedicated to my people, the late Simeon Misiani Gor, Dora Jean Gor, Jennifer, Joyce, Bill, Doug, Nicky, Anyango, and Emilio.
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GENERAL INTRODUCTION

*Brucella abortus* is the causative agent of bovine brucellosis, a disease that causes spontaneous abortion in cows. The most effective and cost effective form of protection against this pathogen is immunization; however, *Brucella abortus* strain 19 (the standard vaccine against *Brucella abortus*) is not completely protective. The work described in this thesis details the vaccination of laboratory mice with the heat shock protein, hsp60 derived from *Brucella abortus*, using live attenuated recombinant *Salmonella typhimurium* expressing hsp60 as a delivery system, and the characterization of the immune response to *Brucella abortus* hsp60 over a 12 week time course following vaccination. The scientific studies described can be broadly divided into 4 main sections, as follows:

1. Identification, cloning, and characterization of the *Brucella abortus*-hsp60 (B-hsp60) gene.

2. Construction of plasmids expressing the *Brucella abortus*-hsp60 gene constitutively, and the characterization of that expression in *Salmonella typhimurium* χ4064 strains transformed with such B-hsp60 expressing plasmids.

3. Assessment of appropriate delivery of B-hsp60 to the immune system of BALB/c mice by various recombinant *Salmonella typhimurium* χ4064 strains expressing graded quantities of B-hsp60, by measuring humoral antibody titers against B-hsp60, as well as T-cell blastogenic responses to B-hsp60, in response to *in vitro* stimulation with B-hsp60.
4). Evaluation of T-cell effector functions late in the course of a primary recombinant *Salmonella typhimurium* χ4064 infection, by monitoring the production and secretion of the cytokines, interferon-gamma (IFN-γ), Interleukin-4 (IL-4), and Interleukin-10 (IL-10), in response to *in vitro* stimulation of splenocytes from recombinant χ4064 infected mice with purified B-hsp60 protein, and demonstration of cytolytic activity, specific for, and directed against B-hsp60 pulsed target cells.

Literature survey

*Brucella abortus* is the causative agent of bovine brucellosis, a disease which mainly affects cattle, causing abortion and infertility in these animals [1]. Humans that come in contact with infected animals, or contaminated products thereof may become infected, acquiring a debilitating disease which oftentimes becomes chronic, despite protracted antibiotic treatment. Other closely related *Brucella* species cause disease in goats, sheep, swine, dogs, and numerous other mammals [2]. *Brucella abortus* has worldwide distribution [3], and despite an active vaccination program, it is still economically important and prevalent in parts of the United States [4,5]. *Brucella* exists in the host animal primarily as an intracellular parasite [6,7] which, like other intra-cellular bacterial parasites such as *Mycobacterium, Salmonella, and Listeria* [8], is able to survive and replicate within phagocytic cells of the host's reticulo-endothelial system [9]. Late in the infection, the brucellae are sequestered in granulomas (focal aggregations of infected mononuclear phagocytes,
surrounded by sensitized T lymphocytes) wherein the bacteria are slowly killed [6,10]. In pregnant ruminants, brucellosis is characterized by infection of placental tissues, where the bacteria are preferentially localized within trophoblasts of the placenta [11,12]. In chronic bovine brucellosis, brucellae are found within macrophages of the mammary gland, where they may persist for the life of the cow, continually shedding organisms into the milk [1,14]. Vaccination remains the best form of protection against such pathogens, and such prophylactic measures have taken on new significance in the light of recent reports documenting the increasing occurrence of multiple drug resistant strains of bacterial pathogens [15], and a general resurgence of infectious diseases that have come about, mainly as opportunistic infections afflicting individuals whose immune system has been compromised, as seen in people infected with human immuno-deficiency virus (HIV) [16].

Over the years, there have been attempts made to develop vaccines with improved efficacy against Brucella abortus. To that end, live reduced dose vaccines [17,19,24], non viable whole cell vaccines [21-24], outer envelope fractions [25,26], cell free detergent extracts [22-24,27], phenol extracts [18,28], ribosomal preparations [29], lipopolysaccharides [30-34], and purified proteins [25,32,34,35] have been prepared. Of all these vaccine formulations tested, the most effective have been the attenuated live derivatives of the parent bacterium against which protective immunity is desired. Such attenuated strains are able to survive within the host for some time and to
express bacterial immunogens for presentation to the host's immune system [36-42], but do not cause serious disease [43]. There is no explanation as to why the generation of protective immunity against intracellular pathogens requires a live infection, but it has been hypothesized that such immunogens as are transported, or leak out from actively dividing bacteria within the phagosome are taken up by the phagocytes and processed for antigen presentation [44]. This obviates the need for protein antigens to be expressed on the bacterial surface, and explains the reactivity of sensitized T lymphocytes from infected animals to numerous cytosolic bacterial proteins [44]. The standard vaccine against bovine brucellosis is the live attenuated strain, *Brucella abortus* strain 19 [45]. To date, no suitable vaccine exists for humans.

There are two main problems associated with attenuated live vaccines, such as *B. abortus* strain 19: The first is the inability of health care/surveillance workers to distinguish between the immunological signatures of healthy vaccinated individuals and individuals infected with virulent strains of the same species [46,47]; and the second is that these vaccines do not adequately protect vaccinates [45,48].

Most of the information on acquired immunity to *Brucella abortus* has been obtained from experiments carried out in laboratory mice. When BALB/c strain of laboratory mice are infected intravenously with *Brucella abortus* strain 19, the organism replicates to large numbers in the spleen and reaches peak concentrations at 2 weeks post infection
(p.i.), followed by progressive clearance, such that the infection is cleared by about 8 weeks p.i. In contrast, infection of this same strain of mice with the virulent *Brucella abortus* strain 2308 produces a protracted infection without overt disease [25], which corresponds to brucellosis in cattle.

Despite a large body of literature available on the immunology of brucellosis, there is still no clear understanding of how the organism manages to evade/withstand the host's immune response. Brucellae are very resistant to direct killing by immune serum [49,50]. Macrophages from non-immune animals readily ingest brucellae, but the ingested organisms actively proliferate within these cells [51-54]. Neutrophils are capable of killing ingested brucellae, but do so slowly [55-57]. Experiments involving adoptive transfer of serum, or of various lymphocyte fractions from experimentally infected mice to naive mice have demonstrated that acquired immunity to *B. abortus* is mediated by cell-mediated immune responses [58,90], and to a lesser extent, humoral immune responses [90,91]. The protective effects of immune serum have been shown to be partly due to antibodies directed against the O-polysaccharide component of *B. abortus* LPS [61], and as such are thought to be effective only against secondary infections with *Brucella*. Studies carried out *in vitro* indicate that macrophages and immune T-cells can kill *Brucella* [53,54], and this bactericidal activity is enhanced in the presence of immune serum [51,53]. It is generally accepted that part of the cell-mediated immune response involves antigen specific T lymphocytes, which in response to bacterial antigens
secrete a variety of lymphokines, which in turn stimulate and enhance bactericidal mechanisms of phagocytes, thereby leading to killing of the ingested organisms [62-64]. In mice, CD 4+ T lymphocytes have been divided into two major subsets on the basis of non-overlapping profiles of lymphokines secreted after appropriate stimulation with antigens, or mitogens such as Concanavalin A [65]; thus TH1 lymphocytes typically secrete interleukin-2 (IL-2), and interferon gamma (IFN-γ), and are believed to be responsible for delayed type hypersensitivity (DTH) [66,67], while TH2 lymphocytes typically secrete IL-4, IL-5, and IL-10 [68], and are responsible for the helper function in antibody production by B-lymphocytes. IFN-γ is capable of activating three major pathways described for microbial killing, which are; the production of reactive oxygen intermediates [69], the production of reactive nitrogen intermediates [70], and the induction of 2,3 dioxygenase to catabolize tryptophan [71]. Current data suggests that a TH1 type T-cell response is required for bacterial killing and clearance of a Brucella infection [66,72], and consistent with this, IFN-γ, together with IL-2 are capable of mediating enhanced killing of brucellae when added to infected macrophages in vitro [73], and injection of recombinant IFN-γ into mice enhances resistance to Brucella [76], and other intracellular parasites [75], whereas injection of antibody to IFN-γ exacerbates infection with these intracellular parasites [74].

Experimental infection of mice with various intracellular microorganisms has revealed an additional cell mediated mechanism,
which involves the lysis of infected target macrophages by T-cells belonging to different subsets: Thus Major Histocompatibility Complex (MHC) class II restricted T-cells that mediate lysis of *Listeria* infected macrophages [114, 115], CD4+ T cells that mediate cytolysis of *Mycobacterium* infected macrophages [44], and CD8+ T cells that mediate lysis of *Salmonella* infected cells [116], have all been demonstrated in BALB/c mice. It has been hypothesized that such subsets of cytolytic T-cells play an important role in the eventual clearance of intracellular microorganisms by lysing chronically constipated infected macrophages that have been rendered anergic, to release their content of microorganisms, which can be then be effectively eliminated by fresh phagocytes, or by humoral bactericidal mechanisms [114]. At present, this hypothesis is backed only by circumstantial evidence, and the requirement for antigen specific cytolytic T-cells as part of a protective immune response has not been conclusively demonstrated.

*Brucella abortus* actively modulates selected phagocyte responses. Whereas macrophages containing brucellae appear activated by certain criteria, such as random locomotion, increased Fc receptors, and generalized phagocytic activity [77], there is an inhibition of phagosome-lysosome fusion, and a decreased oxidative burst associated with bacterial ingestion [78]. This inhibition has been partially accounted for in neutrophils, where the ingested brucellae apparently secrete adenine, and 5'-guanosine monophosphate, which inhibit degranulation of primary granules, resulting in decreased
myeloperoxidase dependent oxidative attack [79-81]. *Brucella* LPS has been shown to decrease the surface-bound expression of IL-1 on macrophages in the presence of IFN-γ, leading to a decreased ability to stimulate proliferation of T cells [85,86].

It is well established that macrophages infected with bacteria secrete increased amounts of prostaglandin E2 (PGE₂) [82,84]. PGE₂ has a direct down-regulatory effect on the activated state of macrophages, and in addition, selectively inhibits production of TH1 lymphokines [83] resulting in a further dampening of the protective infection clearing response, and promotes the establishment of conditions that favor chronic infection [84]. Thus, treatment of *Brucella* infected mice with indomethacin (a potent inhibitor of PGE₂ synthesis) results in enhanced clearance of the bacteria, compared to untreated mice [76].

The protective roles of various T-cell subtypes during *Brucella* infection are not well characterized, but it is known that double negative, CD4⁻ CD8⁻ T cells have no ability to confer immunity in cell transfer experiments [90], whereas both CD4⁺, and CD8⁺ T cells contribute to protective immunity in similar types of adoptive transfer experiments [90,91]. Purified CD4⁺ and CD8⁺ T cells from *Brucella* infected mice have recently been shown to secrete IFN-γ in response to stimulation with purified *Brucella* protein antigens *in vitro* [87]. However, it must be noted that the *Brucella* antigens used above were most likely contaminated with smooth *Brucella* LPS which may have confounded the reported results. LPS is the dominant humoral antigen during the course of *Brucella* infection and, due to its stickiness, also
contaminates all protein/antigen preparations derived from smooth Brucella strains [23, 33, 59]. Brucella LPS is a potent immunogen, and has been shown by itself to stimulate IFN-γ production from human CD4+ and CD8+ T cells [88,89]. This contrasts to LPS's of enteric bacteria, such as E. coli, and S. typhimurium, which do not stimulate IFN-γ production (this study). There is a fair amount of circumstantial evidence that supports the hypothesis that a switch in T cell type occurs during the course of a Brucella infection, from a TH1 type T-cell response associated with a bactericidal stage early, to a TH2 type T-cell response later on, characterized by increasing amounts of circulating antibody and immune T cells, and the persistence of large numbers of bacteria associated with suppression of macrophage bactericidal activity [84,90,92,93]. A similar pattern as has been demonstrated for experimental syphilis [84].

It can be seen from this brief overview that interactions between brucellae and their hosts are extremely complex; brucellae have evolved a multifaceted strategy for survival within the host, which includes resistance to killing by humoral factors, evasion of phagocyte killing mechanisms, and active downregulation of an aggressive immune response.

In order to design new vaccines, a better understanding is needed of the antigens capable of eliciting a protective response to infection with the intact organism. To date, there is no clear picture of what antigens evoke a protective immune response in brucellosis, because most of the work done to characterize the immunogenicity of Brucella
antigens utilized antigens prepared from smooth strains of the organism, and therefore suffered from the drawback of contamination of the proteins with LPS [60]. LPS is the major humoral antigen during a Brucella infection [59,60], and is capable of modifying, or masking immune responses to other antigens which may be present [32,35].

Research plan

In order design subunit vaccines, it is necessary to accurately assess the immunogenicity of individual proteins, separating the protein of interest from the rest of the pathogen from which it is derived.

The starting point for this work was to screen a genomic library for the gene encoding the protein of interest. The Brucella analog of hsp60 (B-hsp60), was chosen as a suitable test antigen in this study, because members of the heat shock protein family had been shown to stimulate strong cellular, and humoral immune responses during the course of mycobacterial, salmonella, and borrelia infection in mice [94]. Owing to the high degree of conservation of hsps from evolutionarily distant organisms, it was judged reasonable to expect B-hsp60, also to be a good cellular and humoral immunogen. Oral infection with live recombinant attenuated Salmonella typhimurium expressing the B-hsp60 gene was chosen as the delivery system for presentation of B-hsp60 to the immune system for several reasons; it (Salmonella typhimurium) provides long term exposure to the antigen, localizes the antigen to lymph nodes and spleen, and presents the antigen in a bacterial context, but decoupled from other possible modulatory
activities of Brucella. Also, vaccination with recombinant Salmonella has been shown to stimulate humoral, secretory, and cellular immunity directed against foreign antigens expressed within them [36-42,117]. One of the more practical objectives of this study was to critically evaluate the protective potential of immunization with a single antigen (in this case B-hsp60) against subsequent infection with the virulent parent organism (Brucella abortus). Using attenuated Salmonella as the delivery system had an additional advantage which was exploited in attempting to evaluate the protective potential of vaccination with B-hsp60, which is as follows: Previous infection of BALB/c mice with Salmonella typhimurium χ4064 (used in this study) protects such mice from a subsequent lethal challenge infection with virulent Salmonella typhimurium [117]. It was therefore hypothesized that the quality of the immune response generated against this Salmonella carrier would be indicative of a protective immune response, and because Salmonella, (like Brucella) is a bonafide intracellular pathogen, that the production of an immune response against B-hsp60 during the course of the recombinant Salmonella infection that was qualitatively similar to that elicited against Salmonella itself might allow a more objective assessment of the protective potential of such a single subunit vaccination scheme. Salmonella typhimurium χ4064 therefore served the function of a positive, and internal control, versus which to compare immune responses against B-hsp60.
MATERIALS AND METHODS

Identification, cloning, and sequencing of the *Brucella abortus* gro E operon

Clones containing *Brucella abortus* gro E gene sequences were positively selected by a low stringency hybridization, using the *Escherichia coli* gro EL gene (kindly provided by Costa Georgopoulos, University of Utah School of Medicine) [95] to probe a *Brucella abortus* strain 19 genomic DNA library, by plaque hybridization, as described [96]. Briefly, plaques from the *Brucella abortus* genomic library in bacteriophage lambda 1059 [97] were produced on a lawn of *E. coli* Q359 at a density of approximately 500 per 90 cm² plate, and transferred directly to nitrocellulose filter paper. The bound DNA was denatured in 0.1N NaOH, 1M NaCl and hybridization was conducted using 5x10⁶ cpm. of ³²P labeled plasmid PBS, containing *E. coli* gro EL DNA as a probe. The probe DNA was labeled by nick translation [98], in hybridization solution containing 6x saline sodium citrate (SSC), 0.25% nonfat dried milk, and 1% NP40 detergent, and either 0%, 25%, or 50% formamide, at 42°C for 24 hours. The filters were washed and exposed to X-ray film. Hybridization with 25% formamide appeared to discriminate positive from negative plaques. Positively hybridizing plaques obtained under optimal hybridization conditions (i.e. hybridization solution containing 25% formamide) were rescreened as described above, and DNA prepared from these plaques [99]. The Southern blot technique was used to identify appropriate DNA fragments that contained the *Brucella gro E* operon [100], which were
then sub cloned into pUC118 and pUC119 plasmid vectors [101].
Plasmids pBHS60-6-E-9 and pBHS-4-H-9 were used as the
experimental material for sequencing by the chain termination method
[102].

Purification of *Brucella abortus* hsp60 protein (B-hsp60)

The entire *Brucella abortus* gro EL gene was cloned into a
regulatable high expression plasmid vector called pJE-7. This plasmid
features the pKK 223-3 derived tac (tryptophan/lactose hybrid)
promoter [103] to drive high expression of an appropriately cloned gene
construct. The 8 kilobase long EcoRI fragment from plasmid pBHS60-6-
E-9 was ligated into pJE-7 at the EcoRI site. The resultant plasmid was
named pDG-7. For purification of B-hsp60 protein, *E. coli* TG-1 (pDG-7),
(the *E. coli* clone expressing *Brucella* hsp60), was grown to mid-log
phase in Luria-Bertani (LB) broth [13] containing 75 micrograms per
mL ampicillin, and protein expression induced with Isopropyl β-D-
thiogalactoside (IPTG) [104] for 6 hours in a final concentration of 200
mg/mL ampicillin, to ensure plasmid retention. The bacteria were
washed and resuspended in buffer containing 50 mM Tris (pH 8.0), 5 mM
EDTA, 10 mM PMSF (TEP), and disrupted by lysozyme treatment and
sonication, as described [105]. The resulting lysate was subjected to
centrifugation at 100,000 x *g* for 1.5 hours, the supernatant loaded
onto a DEAE sepharose column equilibrated in TEP, and protein
fractions eluted with a continuous gradient of 0-0.5 M NaCl in TEP. An
elution profile was determined spectrophotometrically at 280
nanometers, and fractions enriched for B-hsp60 (identified by heavy
staining in coomassie blue stained Laemmli gels [107]) were pooled and used for preparative gel electrophoresis, followed by electroelution of the desired protein band [106]. This purified protein was emulsified in Freunds incomplete adjuvant and injected subcutaneously into rabbits to prepare anti B-hsp60 serum [108]. The antiserum obtained after one priming and one booster injection was subsequently used for monitoring the large scale purification of B-hsp60 by western blot [109]. Purification of milligram quantities of B-hsp60 was effected as described above, with the exception that B-hsp60 enriched fractions were subjected to an additional round of ion exchange chromatography on DEAE sepharose, in place of preparative gel electrophoresis as the final purification step. The purified protein was stored at -70°C until further use. In later preparations, substitution of Tris buffer with sodium phosphate buffer at 50 mM was found to greatly reduce the degradation of the protein during purification.

**Construction of plasmids expressing B-hsp60 constitutively**

A 2.5 kilobase long BamHI/EcoRI DNA fragment (derived from pBHS60-6-E-9) that contained the B-hsp60 gene was cloned into a series of plasmid expression vectors in *E. coli* TG1 host cells using standard molecular cloning techniques [115]. These plasmid vectors (named pJE-3 through pJE-6 [110]) all contain the ampicillin resistance [β lactamase] gene as well as constitutive promoters of differing strengths, that can direct the expression of appropriately cloned genes. The resulting plasmids were named pDG-3, pDG-4, pDG-5, and pDG-6.

**Construction of recombinant S. typhimurium χ4064 (B-hsp60) expression vectors**

In order to overcome restriction barriers that prevent the efficient transfer of DNA from one bacterial species to another, the pDG plasmids which were cloned in *E. coli* (described above) were first introduced into a restriction negative, modification positive, rough *Salmonella typhimurium* strain SL5283 by the method of Lederberg and Cohen [111]. Plasmid DNA was prepared from these intermediate recipients by the alkaline lysis method [113], and subsequently introduced into an avirulent nalidixic acid resistant *Salmonella typhimurium* SR 11 double deletion (Δcya/Δcrp) mutant *S. typhimurium* χ4064 (kindly provided by R. Curtiss III, Washington University, St. Louis, MO) by electroporation [112]. Accumulation of B-hsp60 protein in the various recombinant χ4064 strains was characterized by western blot [109] after electrophoresing total bacterial cell lysates into 11% SDS-polyacrylamide gels [107].

**Preparation of S. typhimurium χ4064 (pJE-3) soluble antigen**

Soluble antigen for use in *in vitro* blastogenesis, and antibody titration was prepared by the method of Shaible *et al.* [52]. Briefly, *S. typhimurium* χ4064 (pJE-3) was grown for 24 hours in LB broth containing 100 mg/mL ampicillin, with shaking (250 rpm at 37°C). The cells were washed three times by repeated pelleting and resuspension in fresh 0.01 M phosphate buffered saline, pH 7.2 (PBS) and disrupted by
sonication (model Braunsonic 1510, from B. Braun Instruments, S. San Francisco, CA.) eight times in 15 second bursts at 4°C. The sonicate was centrifuged at 10,000 x g for 10 minutes, the supernatant recovered and filter sterilized through 0.22 μm pore size filters. This preparation is called S. typhimurium χ4064 (pJE-3) or χ4064 S.A. throughout this thesis.

**Animals**

All vaccination experiments described below were performed with specific pathogen free laboratory mice aged 10 to 12 weeks at the start of each experiment. The mice were housed in the Laboratory Animal Resource Facility at the College of Veterinary Medicine, Iowa State University, and given sterile food and water *ad libitum.*

**Infection (vaccination) of mice with recombinant strains of Salmonella typhimurium χ4064**

BALB/cByJ strain of laboratory mice (Jackson Laboratories, Bar Harbor, ME) were vaccinated with different recombinant strains of *Salmonella typhimurium* χ4064 (i.e. χ4064 (pJE-3), χ4064 (pDG-3), χ4064 (DG-4), and χ4064 (DG-6) intra esophageally using an intubation needle (1-inch 21 gauge from Popper and Sons, Inc., New Hyde Park, NY), as described previously[40]. Log phase bacterial suspensions were prepared by first inoculating 5 mL of LB broth containing 75 μg per mL with each χ4064 strain and incubating the cultures overnight. These overnight cultures were diluted 20 fold in LB broth containing 75 μg per mL, and grown for 4 hours at 37°C with shaking (250 rpm) to achieve log phase growth. The log phase χ4064 cultures were pelleted and
resuspended in 0.01M phosphate buffered saline (PBS) at 1/50th the log phase culture volume and stored on ice. Prior to intubation, stomach acidity of the mice was neutralized with 30 μL of 10% sodium bicarbonate administered orally, and the mice were immunized with 4 x 10^8 recombinant *S. typhimurium* χ4064. The actual number of bacteria given was determined retrospectively, by standard plate counts of serial 10 fold dilution's on LB agar. At various time intervals after vaccination, groups of naive (control) and vaccinated mice were sacrificed. Following CO₂ asphyxiation, blood was collected by heart puncture using 1 mL syringes and 22 gauge needles, and used to prepare serum.

**Bacterial loads** The load of *S. typhimurium* in the small intestine, mesenteric lymph nodes, spleens, and livers, of vaccinated mice were determined as follows: The organs were aseptically excised from euthanized mice and suspended individually in sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI) containing 0.85% saline. The organs were homogenized using a Stomacher 80 Laboratory Blender (A.J. Stewart, London), and bacterial counts obtained by plating the homogenates on MacConkeys agar containing nalidixic acid [43]. *S. typhimurium* cells recovered from mice in this fashion were also tested for the retention of plasmids expressing B-hsp60, by sub-culturing the salmonellae that grew on MacConkeys agar containing nalidixic acid onto LB agar containing 75 μg/mL ampicillin. Only salmonellae that still retained the plasmids would be resistant to ampicillin.
Serum antibody responses  Serum antibody responses of naive and vaccinated mice to B-hsp60 were determined by enzyme linked immuno-sorbent assay (ELISA), as described [40]. Briefly, 96-well Immulon 4 microtiter plates (Dynatech, Chantilly, VA.) were incubated overnight at 4°C with 1 μg of either recombinant B-hsp60, χ4064 SA diluted in 0.01M PBS pH 7.2. All sera were diluted 1:10 in saline containing 0.05% Tween 20 (TS) prior to use. Total immunoglobulin was determined using alkaline phosphatase conjugated goat anti-mouse immunoglobulin G diluted 1:2000 in TS. Color was developed by addition of no. 104 phosphatase substrate (Sigma, St. Louis, MO), and absorbance read at 405 nanometers using an ELISA reader.

Blastogenesis assays  Antigen specific T-cell proliferative responses to Brucella hsp60, and χ4064 SA from spleens of vaccinated mice were performed as described [40]. Briefly, spleens were collected from groups of naive or vaccinated mice (n=3 or 4), homogenized with the stomacher to produce single cell suspensions, washed using RPMI and inoculated into 96 well culture clusters (2.5 x 10^5 cells per well) containing one of the following: RPMI 1640 (Sigma), 0.5 mg per mL concanavalin A (Sigma, St. Louis, MO), 5 μg per mL E. coli LPS, B-hsp60 at 0.2, 1, or 5 μg per mL, or χ4064 SA at 0.2, 1, or 5 μg per mL. All cells were incubated for 5 days in RPMI 1640 containing 5% fetal bovine serum (Gibco-BRL), and 1% Serum Plus (Gibco-BRL). Cells were pulsed with [3H] thymidine for 12 hours prior to harvest, and counts were determined using a liquid scintillation counter (model 1500, Packard
Instrument Co., Inc., Downers Grove, IL). The results were calculated as stimulation indices for each mouse according to the general formula:

\[
\text{Stimulation index} = \frac{\text{cpm experimental splenocytes (i.e. plus antigen)}}{\text{cpm control splenocytes (i.e. medium only)}}
\]

**Preparation of culture supernatants for the determination of secreted lymphokines**

Groups of mice were vaccinated with approximately \(8 \times 10^8\) of the following *Salmonella typhimurium* strains: \(\chi 4064\) (pJE-3) (representing no B-hsp60 expressed); \(\chi 4064\) (pDG-6) (representing low levels of B-hsp60 expressed) \(\chi 4064\) (pDG-4) (representing moderate levels of B-hsp60 expressed). Four mice from each treatment group, including a group of non-vaccinated mice, were sacrificed at days 34, 64 and 84, post infection, and single cell suspensions prepared from their spleens. Individual splenocyte preparations were washed in Dulbecco's Modified Eagles Medium (DMEM) (Gibco-BRL, Bethesda, MD), and resuspended in fresh DMEM supplemented with 10% fetal bovine serum (Gibco BRL, Bethesda, MD), and 50 mg per mL gentamicin (Gibco-BRL, Bethesda, MD), and seeded into 12 well culture dishes (Costar Corp., Cambridge, MA) at 1 \(10^7\) cells per well, in a final volume of 2 mLs, together with one of the following; DMEM, purified B-hsp60 (10 \(\mu\)g per mL), or *S. typhimurium* \(\chi 4064\) (pJE-3) soluble antigen (10 \(\mu\)g per mL). Culture supernatants were harvested after 3 days (or 5 days, in the case of day 84 post-infection splenocytes), filtered through 0.22 \(\mu\)m Vanex filters (Vangard Int'l., Inc., Neptune, NJ), and used to determine cytokine profiles, or stored at -70°C until needed. The supernatants were
analyzed for the cytokines IFN-γ, IL-4, and IL-10, by sandwich enzyme linked immuno-sorbent assays (ELISAs), that utilized anti-mouse IFN-γ, anti-mouse IL-4, and anti-mouse IL-10 immunoglobulins (Pharmingen, San Diego, CA), according to a protocol supplied by the manufacturer. In all cases, known concentrations of purified IFN-γ, IL-4, and IL-10 (Genzyme Corp., Cambridge, MA) were included in the ELISAs to provide a basis for quantitative estimates of the respective cytokines.

**Cytotoxicity assays**

For the purpose of measuring the ability of splenocytes from recombinant χ4064 vaccinates to mediate lysis of antigen pulsed target cells, groups of mice were vaccinated with approximately $8 \times 10^8$ of the following *S. typhimurium* strains: χ4064 (pJE-3) (representing no B-hsp60 expressed); χ4064 (pDG-6) (representing low levels of B-hsp60 expressed) χ4064 (pDG-4) (representing moderate levels of B-hsp60 expressed). Four mice from each treatment group, including a group of non-vaccinated mice, were sacrificed at day 84, post infection, and single cell suspensions prepared from their spleens. Individual splenocyte preparations were washed in Dulbecco's Modified Eagles Medium (DMEM) (Gibco-BRL, Bethesda, MD), and resuspended in fresh DMEM supplemented with 10% fetal bovine serum (Gibco BRL, Bethesda, MD), and 50 mg per mL gentamicin (Gibco-BRL, Bethesda, MD), and seeded into 12 well culture dishes (Costar Corp., Cambridge, MA) at $1 \times 10^7$ cells per well, in a final volume of 2 mL together with one of the following: DMEM, purified B-hsp60 (10 μg per mL), or *S. typhimurium χ4064* (pJE-3) soluble antigen (10 μg per mL), to
stimulate generation of effector cytolytic lymphocyte populations. Splenocytes were harvested, and red blood corpuscles lysed by adding 6 mL of distilled water to non-adherent splenocytes in 2 mL of DMEM for 30 seconds, after which osmolarity was restored by adding 2 mL of 3.5% NaCl solution. The splenocytes were washed by repeated pelleting and resuspension in DMEM. For use as effector cells in cytotoxic assays, pooled splenocytes representing each treatment group (i.e. χ4064 (pJE-3) vaccinated, B-hsp60 stimulated, or χ4064 SA stimulated, etc.) were pooled, and resuspended in DMEM plus 0.5% fetal bovine serum, at 4 x 10⁶ cells per mL, and added to 96 well round bottom plates that contained the BALB/c mouse-derived macrophage-like cell line, J774 as target cells, in 0.5% fetal bovine serum, containing χ4064 (pJE-3) soluble antigen, or B-hsp60, at a final concentration of 20 μg per mL, in a total volume of 200 μL. Cytotoxicity was measured by the release into culture medium of the cytosolic enzyme, lactate dehydrogenase (LDH), using a non-radioactive cytotoxicity assay kit from Promega, according to a protocol supplied by the manufacturer (Promega, Madison, WI), in a fashion analogous to the more widely used ⁵¹Cr release assay. Percent cytotoxicity was quantitated for each splenocyte preparation as:

\[
100 \times \frac{((\text{experimental-effector spontaneous}) - \text{target spontaneous})}{\text{target maximum} - \text{target spontaneous}}.
\]

Flow cytometry

In order to confirm that the LDH release assay was accurately measuring cytotoxicity, the mixtures of effector and target cells used
in the LDH release cytotoxicity assay described above were incubated further at 37°C, for a total of 72 hours. Total cells were harvested from individual wells, fixed in 2% paraformaldehyde, and analyzed using an Epics Profile Analyzer (Coulter Corp., USA). Cytotoxicity was estimated by the accumulation of dead cells in each experimental culture (J774 cells that are dead at the time of fixation are clearly distinguishable from J774 cells that are alive at the time of fixation, on the basis of forward scatter, as well as side scatter. These parameters were therefore taken advantage of in order to obtain counts of dead cells from each experimental culture.

**Statistical analysis**

Statistical analyses were performed using Students T tests.

Differences were considered significant at p values of 0.05.
RESULTS

Identification and cloning, and sequencing of the *Brucella abortus* gro E operon

Identification of recombinant bacteriophage lambda plaques that contained the *Brucella* hsp60 gene was achieved using DNA hybridization conditions that allowed the radiolabelled *E. coli* hsp60 gene DNA probe to preferentially detect high copy numbers of the *Brucella* hsp60 sequence with lower sequence homology, versus lower copy numbers of *E. coli* hsp60 DNA with 100 percent sequence homology. Five identical lambda clones containing B-hsp60 were identified in the initial plaque hybridization screen, and one of these plaques was selected and subcloned into puc119 for further manipulation. Sequencing of the *Brucella* hsp60 gene was accomplished using appropriate sub-clones and oligonucleotide primers, by the chain termination method of Sanger *et al* [102]. An overview of the sequencing strategy is shown in Figure 1.

Sequencing demonstrated that in *Brucella*, as in many other bacterial species, that the hsp60 gene sequence is preceded by the smaller hsp10 gene sequence. Salient features of the *Brucella gro* E operon are 69% and 60% DNA sequence identity, and 68% and 50% predicted amino acid sequence identity, with the corresponding *E. coli* hsp60 (*gro* EL) and hsp10 (*gro* ES) genes, respectively (the sequence [20] is shown in appendix 1).
Figure 1. Sequencing overview of the *Brucella* gro E operon. Each arrow represents 2, and in most cases 3 independent sequencing runs. Open rectangles represent non-coding regions, and filled rectangles represent coding sequences.

**Construction of recombinant *S. typhimurium* χ4064 B-hsp60 expression vectors**

A 2.5 kilobase long Eco RI / Bam HI restriction digest DNA fragment containing the entire B-hsp60 gene, including its own Shine-Dalgarno sequence (ribosome binding site), but not the B-hsp10 sequence, was cloned into the plasmid expression vectors, pJE-3, pJE-4, pJE-5, and pJE-6 (these plasmids have been described in detail in Emily Chin (1993), Ph.D. Thesis, Iowa State University), resulting in the corresponding plasmids, pDG-3, pDG-4, pDG-5, and pDG-6. Plasmid pDG-3 was predicted to direct high expression, pDG-4 & 5 moderate expression, and pDG-6 low expression of the B-hsp60 gene.

The expression of the B-hsp60 gene by the various plasmids was characterized by western blot of total bacterial lysates of recombinant
Figure 2. Western immunoblot of recombinant strains of *E. coli* TG1 (lanes A-E) and *S. typhimurium* χ4064 (lanes F-J) showing relative accumulation of *Brucella* hsp60 protein. Bacterial strains containing either pJE-3 (E and J), pDG-3 (A and F), pDG-4 (B and G), pDG-5 (C and H), or pDG-6 (D and I) were grown to stationary phase in LB broth containing 100 mg/mL ampicillin. Total lysates were first electrophoresed through an SDS, 11% polyacrylamide gel, and then transferred to nitrocellulose. *Brucella* hsp60 expression was detected using rabbit antiserum raised against B-hsp60 (see Materials and Methods).

χ4064 cells grown in LB medium for 24 hours, in the presence of 75 μg per mL ampicillin (Figure 2). This western blot confirmed that the accumulation of B-hsp60 protein by the various engineered bacterial strains was accurately reflected by the relative rates of constitutive expression predicted for each pDG plasmid. It can be seen that the relative amounts of B-hsp60 accumulation for a particular pDG plasmid are similar between the *E. coli* and *S. typhimurium* bacterial strains, confirming that the rules that dictate efficiency of initiation are similar between *E. coli* and *S. typhimurium*. 
Colonization of mice with recombinant *S. typhimurium* χ4064

Mice were orally infected (vaccinated) with strains χ4064 (pDG-3), χ4064 (pDG-4), and χ4064 (pDG-6), as described in the Materials and Methods. At predetermined time intervals, mice were sacrificed and analyzed for various parameters of infection (Figure 3) and immune response.

Following vaccination, the high B-hsp60 expressing *S. typhimurium* strain χ4064 (pDG-3) lost the plasmid (pDG-3) expressing

![Graph](image-url)  
**Figure 3.** Bacterial loads in *S.typhimurium* χ4064 (pDG-6) infected mice. Mice were orally infected with 2 x 10^8 to 4 x 10^8 *S. typhimurium* χ4064 (pDG-6). Mice were killed at intervals, and livers, spleens, small intestine, and mesenteric lymph nodes (MLN) were removed from each animal and homogenized. Viable counts of *S. typhimurium* χ4064 (pDG-6) were obtained by plating serial 10 fold dilution's of the homogenized organs on MacConkey agar plates containing 30 μg per mL of Nalidixic acid. Values reported are means of three mice per time point of one of two such experiments.
B-hsp60 within two days of their introduction into the mice (data not shown). These mice failed to produce detectable serum antibody responses to B-hsp60, a result that implies that strain χ4064 (pDG-3) failed to deliver sufficient amounts B-hsp60 (figure 4).

On the other hand, the B-hsp60 plasmids were stable in the moderate, and low B-hsp60 expressing strains of S. typhimurium, χ4064 (pDG-4), and χ4064 (pDG-6), respectively. All subsequent work was therefore performed using these two bacterial strains. The extent of colonization of the various tissues of vaccinated mice (a typical time course of bacterial loads in the mice, following infection is shown in Figure 3) are similar to the results of Stabel et al. [40]. In all cases, bacteria were virtually undetectable in spleens, livers, mesenteric lymph nodes, and small intestines by 6 weeks post-infection.

**Serum antibody responses**

Serum antibody titers against S. typhimurium χ4064 (pJE-3) soluble antigens (χ4064 S.A.) and B-hsp60 from mice infected with strains of recombinant S. typhimurium χ4064 did not begin to rise until approximately 4-5 weeks post-infection, and this observation was consistent for all the experiments performed (Figures 4-6). Strain DG-6 did not elicit significant immunoglobulin production against B-hsp60, while the titers against χ4064 SA, in the same mice were present, but only became significant 4 weeks post-infection (figure 6). This antibody response was specifically directed against B-hsp60 and not an S. typhimurium χ4064 derived antigen because vaccination of
Figure 4. Antibody responses in recombinant *Salmonella* infected mice (mean ± SEM, n=3). Mice infected with *S. typhimurium* χ4064 (pDG-3) were bled at intervals. The serum was assayed for antibodies against B-hsp60, or χ4064 S.A. as described in Materials and Methods.

mice with *S. typhimurium* χ4064 (pJE-3) (this strain carries plasmid without the B-hsp60 gene), failed to elicit antibodies that cross-reacted with B-hsp60 protein (see figure 7A). Mice infected with strain DG-4 showed high titers of antibody directed against B-hsp60 (Figure 5, and 7A).

The antibody responses against B-hsp60 in these experiments differ markedly from those reported for another *Brucella abortus* protein, BCSP31, when it was delivered to BALB/c mice in a similar type of experiment, using comparable numbers of recombinant *S. typhimurium* χ4064 [40]. In those experiments, antibody titers directed
against BCSP31 began to rise by 2 weeks post infection, and reached a maximum by 4 weeks post infection.

**Blastogenic responses following immunization**

Splenocyte blastogenesis in response to in vitro stimulation with B-hsp60 protein was observed in vaccinates from the earliest time point that it was measured (14 days post infection) (Figure 8). The stimulation indices for splenocytes derived from mice vaccinated with χ4064 (pDG-4) were consistently higher than the stimulation indices for splenocytes derived from χ4064 (pDG-6) vaccinated mice in
Figure 6. Antibody responses (mean ± SEM, n=3) in mice infected with recombinant S. typhimurium χ4064 (pDG-6). Sera were assayed for antibodies against B-hsp60, χ4064 S.A. as described in Materials and Methods. Results are representative of two separate experiments.

response to in vitro stimulation with B-hsp60. In all cases, splenocytes from vaccinated mice showed the highest stimulation indices when cultured in the presence of χ4064 soluble antigen in vitro. All stimulation indices reported are at least 2 times higher than those obtained from non-vaccinated mice treated in parallel at each time point (data not shown).

Cytokine secretion patterns

Splenocytes derived from mice vaccinated with the strains χ4064 (pJE-3) (representing no B-hsp60 expression), χ4064 (pDG-6) (representing low levels of B-hsp60 expression), and χ4064 (pDG-4)
Figure 7. Antibody responses in recombinant *Salmonella* infected mice (mean ± SEM, n=3) late during the course of infection. Non-infected control mice, and mice orally infected with $8 \times 10^8$ *S. typhimurium* $\chi_{4064}$ (pDG-4), $\chi_{4064}$ (pDG-6), or $\chi_{4064}$ (pJE-3), were bled at days 35, 64, or 84 post-infection, and serum from each mouse was analyzed for antibodies to B-hsp60 (A), or $\chi_{4064}$ S.A.(B), as described in the Materials and Methods.
Figure 8. Blastogenic responses of recombinant *S. typhimurium* χ4064-vaccinated mice. Mice infected with *S. typhimurium* χ4064 (pDG-4) (A), or *S. typhimurium* χ4064 (pDG-6) (B) were sacrificed on the indicated days, post infection. Single cell suspensions were prepared from spleens of these mice, and cultured in the presence or absence of B-hsp60, or χ4064 SA at 5 μg per mL. Blastogenic stimulation was calculated as described in Materials & Methods. Results are expressed as mean ± SEM for groups of 3 mice (A), or 4 mice (B), per time point.
(representing moderate levels of B-hsp60 expression, also see Figure 2) were tested for the secretion of the cytokines IFN-γ, IL-4, and IL-10, following in vitro stimulation with various antigens.

**IFN-γ production** At day 34 post-infection, splenocytes from all vaccinated mice showed vigorous stimulation of IFN-γ production in response to χ4064 S.A., but not to B-hsp60 when cultured for 3 days in the presence of these antigens. (Figure 9).

---

**Figure 9.** Interferon gamma secretion by recombinant χ4064-vaccinates at 34 days post-infection. Mice infected with χ4064 (pDG-4), χ4064 (DG-6), or χ4064 (pJE-3), and non-vaccinated mice were sacrificed at day 34 post infection. Single cell suspensions were prepared from spleens of these mice, and cultured in the presence of medium only, medium plus B-hsp60 (at 10 μg per mL), or medium plus χ4064 soluble antigens (at 10 μg per mL). After 3 days in culture supernatants were harvested and assayed for interferon-γ by ELISA, as described in Materials & Methods. Results expressed as mean (shaded rectangles) ± SEM (clear rectangles) for 4 mice per group.
At day 64 post-infection, splenocytes from all vaccinated mice showed stimulation of IFN-γ production in response to χ4064 S.A., and B-hsp60 when cultured for 3 days in the presence of these antigens. (Figure 10). The splenocytes collected from mice at day 84 post-infection showed very low responses to χ4064 S.A., and no stimulation in response to B-hsp60, when cultured for 3 days in the presence of these respective antigens (data not shown). However, detectable stimulation of IFN-γ was observed when the day 84 splenocytes were cultured for 5 days in the presence of χ4064 S.A. or B-hsp60 (figure 11),

![Figure 10. Interferon gamma secretion by recombinant χ4064 vaccinates at 64 days post-infection.](image)

Figure 10. Interferon gamma secretion by recombinant χ4064 vaccinates at 64 days post-infection. Mice infected with χ4064 (pDG-4), χ4064 (DG-6), χ4064 (pJE-3), and non-infected mice were sacrificed at day 64 post infection, and processed as described in Figure 9.
Figure 11. Interferon gamma secretion by recombinant Χ4064 vaccinates at 84 days post-infection. Mice infected with Χ4064 (pDG-4), Χ4064 (DG-6), Χ4064 (pJE-3), and non-infected mice were sacrificed at day 84 post infection, and processed as described in figure 9, except that the supernatants were harvested after 5 days in culture, as opposed to 3 days.

A result consistent with the expected lag seen in the activation of a memory population of T cells [see ref. 119 for a recent review]. There were no statistically significant differences between the amounts of IFN-γ secreted in response to B-hsp60 by splenocytes from the 3 groups of recombinant Χ4064 infected mice at day 34, day 64, or day 84 post-infection. The fact that Χ4064 (pJE-3) vaccination elicited antigen specific IFN-γ production upon stimulation with B-hsp60 (which this Salmonella strain does not express) is not surprising, and is most likely due to cross-stimulation of splenocytes sensitized to S.
typhimurium χ4064's own endogenous hsp60, as a result of the high degree of homology that exists between heat shock proteins from diverse organisms. The stimulated secretion of IFN-γ by splenocytes of vaccinated mice in response to B-hsp60 or χ4064 soluble antigens was clearly an acquired response, since non-vaccinated control mice failed to produce IFN-γ when stimulated under similar conditions. In vitro stimulation of splenocytes from all groups of experimental and control mice with another Brucella abortus derived E. coli recombinant protein BCSP31 [35,40,60], and also purified S. typhimurium wild type LPS (purchased from Sigma, St. Louis, MO) failed to stimulate the secretion of IFN-γ (data not shown). A consistent observation was the secretion of higher concentrations of IFN-γ in response to stimulation of the splenocytes from all groups of recombinant χ4064 infected mice with χ4064 soluble antigen, as compared to B-hsp60. It should be noted however that the quantities of IFN-γ secreted by all the vaccinated mice in response to B-hsp60 at days 64 and 84 post-infection are in excess of the reported minimal concentrations of IFN-γ required to stimulate bactericidal activity of macrophages in vitro [73], and to result in a significant reduction of splenic bacterial loads of Brucella abortus in vivo [76].

IL-4 production At days 34, 64, and 84 post-infection, in vitro stimulation of splenocytes from vaccinated and non-vaccinated groups of mice with B-hsp60, χ4064 S.A. or S. typhimurium wild type LPS failed to elicit IL-4 production (less than 1 unit per 1 x 10^7 splenocytes) as illustrated in Figures 12 through 14. In all cases,
Figure 12. IL-4 secretion by recombinant \( \chi 4064 \) vaccinates at 34 days post-infection. Mice infected with \( \chi 4064 \) (pDG-4), \( \chi 4064 \) (DG-6), or \( \chi 4064 \) (pJE-3), as well as non-vaccinated mice were sacrificed at day 34 post infection. Single cell suspensions were prepared from spleens of these mice, and cultured in the presence of medium only, medium plus B-hsp60 (at 10 \( \mu \text{g} \) per mL), or medium plus \( \chi 4064 \) soluble antigens (at 10 \( \mu \text{g} \) per mL). After 3 days in culture supernatants were harvested and assayed for IL-4 by ELISA, as described in Materials & Methods. Results expressed as mean (shaded rectangles) ± SEM (clear rectangles) for 4 mice per group.

<table>
<thead>
<tr>
<th>(DG-4) S.A.</th>
<th>hsp60</th>
<th>NO ANTIGEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>(DG-6) S.A.</td>
<td>hsp60</td>
<td>NO ANTIGEN</td>
</tr>
<tr>
<td>(JE-3) S.A.</td>
<td>hsp60</td>
<td>NO ANTIGEN</td>
</tr>
</tbody>
</table>

| IL-4 (units per \( 10^7 \) splenocytes) |
|-----|-----|-----|-----|-----|-----|
| 0   | 2   | 4   | 6   | 8   | 10  |

The secreted amounts of IL-4 in response to B-hsp60, or \( \chi 4064 \) S.A. were similar in splenocytes from vaccinated, as well as non-vaccinated control mice treated in parallel (data not shown). This finding may explain the relatively long lag period seen in the rise of total immunoglobulin G responses against both B-hsp60 and \( \chi 4064 \) S.A. when BALB/c mice were vaccinated with recombinant \( S. \ typhimurium \ \chi 4064 \) (this experiment). However, it should be noted that IFN-\( \gamma \) is itself capable of promoting antibody production and immunoglobulin
Figure 13. IL-4 secretion by recombinant χ4064 vaccinates at 64 days post-infection. Mice infected with χ4064 (pDG-4), χ4064 (DG-6), or χ4064 (pJE-3), as well as non-infected mice were sacrificed at day 64 post infection, and processed as described in figure 12.

isotype switching [118], and could therefore be responsible for the humoral responses seen in these experiments against B-hsp60 and χ4064 S.A.

IL-10 production At days 34, and 64, and 84, post-infection, in vitro stimulation of splenocytes from vaccinated mice with χ4064 S.A. resulted in stimulation of IL-10 production (Figures 15 through 17). Stimulation of splenocytes recovered from vaccinated mice also elicited IL-10 production in response to B-hsp60. Whereas little difference was seen in IL-10 secreted at day 34 in response to B-hsp60, some differences in the amount of IL-10 secreted in response to B-hsp60 were observed between the groups of infected mice at both 64 and 84 days post-infection. Splenocytes from χ4064 (pDG-4) infected
mice secreted the most IL-10, followed by $\chi 4064$ (pDG-6) and $\chi 4064$ (pJE-3), in decreasing order (Figures 16, 17). The antigen specific stimulation of IL-10 secretion was found to be less stringently controlled than the antigen specific stimulated secretion of IFN-$\gamma$, in that a variety of antigens, including B-hsp60, $\chi 4064$ S.A, as well as S. typhimurium wild type LPS were all found to stimulate the secretion of low amounts of IL-10 from non-vaccinated mice (data not shown). This may be due to the fact that cell types other than T-lymphocytes are capable of IL-10 synthesis and secretion [56].

**Cytolytic activity**  
The ability of splenocytes from $\chi 4064$ vaccinates to mediate lysis of antigen pulsed cells was tested using

![Graph showing IL-4 secretion by recombinant $\chi 4064$ vaccinates at 84 days post-infection. Mice infected with $\chi 4064$ (pDG-4), $\chi 4064$ (DG-6), or $\chi 4064$ (pJE-3), as well as non-infected mice were sacrificed at day 84 post infection, and processed as described in figure 12, except that the supernatants were harvested after 5 days in culture, as opposed to 3 days.](image-url)
Figure 15. Interleukin-10 secretion by χ4064 vaccinates at 34 days post-infection. Mice infected with χ4064 (pDG-4), χ4064 (DG-6), or χ4064 (pJE-3), as well as non-vaccinated mice were sacrificed at day 34 post infection. Single cell suspensions were prepared from spleens of these mice, and cultured in the presence of medium only, medium plus B-hsp60 (at 10 μg per mL), or medium plus χ4064 soluble antigens (at 10 μg per mL). After 3 days in culture supernatants were harvested and assayed for IL-10 by ELISA, as described in Materials & Methods. Results expressed as mean (shaded rectangles) ± SEM (clear rectangles) for 4 mice per group.

vaccinated, as well as non-vaccinated mice, in response to B-hsp60. The levels of cytolytic activity correlated well with the relative amount of B-hsp60 originally delivered to the immune system; thus the highest level of cytolytic activity was seen in the case of χ4064 (pDG-4) vaccinates, and decreased for χ4064 (pDG-6) vaccinates. Mice infected with χ4064 (pJE-3) showed the lowest level of cytolytic activity, and there was no apparent difference between this cytolytic
Figure 16. Interleukin-10 secretion by recombinant χ4064 vaccinates at 64 days post-infection. Mice infected with χ4064 (pDG-4), χ4064 (DG-6), or χ4064 (pJE-3), as well as non-infected mice were sacrificed at day 84 post infection, and processed as described in figure 15.

Figure 17. IL-10 secretion by recombinant χ4064 vaccinates at 84 days post-infection. Mice infected with χ4064 (pDG-4), χ4064 (DG-6), or χ4064 (pJE-3), as well as non-infected mice were sacrificed at day 84 post infection, and processed as described in figure 15, except that the supernatants were harvested after 5 days in culture, as opposed to 3 days.
Figure 18. Cytotoxic activity of splenocytes from recombinant χ4064 vaccinates. Mice infected with χ4064 (pDG-4), χ4064 (DG-6), or χ4064 (pJE-3), as well as non-vaccinated mice were sacrificed at day 84 post infection. Single cell suspensions were prepared from spleens of these mice, and cultured in the presence of medium plus B-hsp60 at 10 μg per mL for 5 days. Cytolytic activity against J774 target cells was measured by release of LDH, as described in Materials and Methods. Results shown correspond to an effector to target ratio of 2:1 and are expressed as means of triplicate wells.

activity when compared to that of splenocytes collected from non-vaccinated mice. The results of cytolytic activity obtained using the release of lactate dehydrogenase (LDH) as an index of cytolysis of target cells (figure 18) was confirmed cytologically, where the
Figure 19. Cytolytic activity of recombinant χ4064 vaccinates. Splenocytes from non-infected (CTRL) or infected mice (χ4064), treated as described in figure 18 were cultured with identical numbers of J774 target cells in medium containing 20 μg per mL B-hsp60, at the indicated effector to target-cell ratios. J774 cells were harvested after 72 hours, and analyzed by flow cytometry for total number of dead cells in each experimental culture. Results shown are representative of two separate experiments.

accumulation of dead J774 target cells in the various experimental cultures in response to B-hsp60 stimulation (Figure 19), or χ4064 S.A. (Figure 20) were quantitated by flow cytometry. Reliable estimates of the numbers of dead cells were made by taking advantage of the decrease in overall size (forward light scatter), and granularity (side light scatter) of dead J774 cells. This protocol was standardized for J774 cells in earlier and dead cells was confirmed by the ability of live cells to exclude the cytoplasmic membrane-impermiant fluorescent
Figure 20. Cytolytic activity of χ4064 vaccinates. Splenocytes from non-infected (CTRL) and infected mice (χ4064), treated as described in figure 18 were incubated with identical numbers of J774 target in medium containing 20 μg per mL χ4064 (JE-3) soluble antigen, at the indicated effector to target-cell ratios, cells for 72 hours. J774 cells were harvested and analyzed by flow cytometry for total number of dead cells accumulated in each experimental culture. Results shown are representative of two such experiments.

nucleic acid dye, propidium iodide. Cytolytic activity of the splenocytes was tested late in the infection because it had been previously shown in the case of murine mycobacteriosis, that a low level of antigen specific cytolytic activity, mediated by CD4+ T cells was demonstrable relatively late in the course of the mycobacterial infection [44]. Cytotoxic T-lymphocytes of the CD8+ phenotype have previously been demonstrated, to arise during the course of murine salmonellosis [116].
DISCUSSION

As a group, intracellular bacterial pathogens present major conceptual and experimental challenges to the fields of immunology and microbial molecular genetics. For each pathogen, there exists a very poor understanding of how the microorganism is able to survive and multiply in the hostile environment of the animal body. For many of these same organisms, it has proven difficult to develop vaccines which provide lasting protection against re infection. Organisms included in this latter category include *Brucella abortus*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and *Salmonella typhi*. Each of these organisms can and does live and replicate within macrophages inside the living host, each can cause chronic disease, and for each, the best (though imperfect) existing vaccine is based on infection with a live attenuated bacterial strain. Many experiments suggest that for these organisms, only a prolonged infection can lead to protective immunity. In each case, a molecular understanding of how these organisms can survive and replicate in the macrophage, an understanding of how they are able to evade immune surveillance, and an immunological understanding of why prolonged infection is required to produce protective immunity and why this immunity is frequently imperfect, is lacking.

Live carrier vaccination would seem to be a powerful tool for studying all of these issues. With this system, the heterologous (test) antigen is separated from the rest of the biological activities normally
associated with the pathogen which should allow a clearer
terpretation of results, and a properly chosen host and carrier system
can provide both intracellular expression and prolonged infection of the
host. We chose, as the carrier, *S. typhimurium* χ4064 which is
attenuated by deletions in both the cya and crp genes and the BALB/c
mouse as the test animal model. As the test antigen, we chose the
*Brucella abortus* heat shock protein 60 (B-hsp60), in part, because
reports in the literature documented the strong immunogenicity of
other members of this protein family derived from other pathogens.
There are many potential questions which might be addressed by this
system. Some examples of these are: 1. Are the immune responses
generated by the mouse against a single antigen derived from *Brucella
abortus* sufficient to provide protection against *B. abortus* infection
when the antigen is delivered in the context of a prolonged (non-
*Brucella*) bacterial infection? 2. Does *B. abortus* modulate the nature
of the immune response differently than *S. typhimurium* during the
course of infection? 3. Do multiple antigens provide better protection
than a single antigen? 4. Is the immunity generated in this system
against different antigens qualitatively similar or different?

The first aim of this study was to determine whether or not B-
hsp60 was a suitable test antigen. The criteria for a suitable test
antigen was that when mice were infected with *S. typhimurium* χ4064
expressing B-hsp60, both humoral and cellular immunity would develop
to the protein during the course of the *Salmonella* infection. The
second aim was to characterize relevant aspects of the immune
response during the course of the infection; and the third aim was to
determine the effect of expressing different amounts of the test
antigen in the carrier salmonellae on measured aspects of immunity.
This later aim is of practical interest because expression of proteins
in heterologous organisms requires the selection of a promoter, and
high expression is frequently deleterious to the vaccine strain.

The results of this study have adequately demonstrated that B-
hsp60 was delivered to the immune system of mice by oral vaccination
with recombinant attenuated *S. typhimurium* χ4064 expressing the
protein, and that parameters of both humoral and cellular immunity
were generated under the appropriate conditions. Differences in the
quality of immune responses were seen between χ4064 (pDG-6), (the
strain with low constitutive expression of B-hsp60), and χ4064 (pDG-
4), (the strain with moderate constitutive expression of B-hsp60). This
was reflected in marked differences in humoral antibody production
seen at days 34 through 84 post-infection (high in the case χ4064
(pDG-4) infected mice, and none in the case of χ4064 (pDG-6) infected
mice). Although the humoral antibody responses were negligible in
χ4064 (pDG-6) infected mice, the blastogenic, as well as cytotoxic
responses due to stimulation with B-hsp60 in vitro support the notion
that the χ4064 (pDG-6) infected mice were exposed to an immunogenic
quantity of B-hsp60, even though this was insufficient to elicit a
significant humoral response. The ability of foreign (non-*Salmonella*)
antigens to trigger specific immune responses, such as antigen specific
cytotoxic T cells, in the absence of an apparent antibody response has
been reported in at least 2 different studies [120,121] and is reaffirmed in this study.

The results of the stimulated secretion of lymphokines are at first glance strange. The pattern of secreted lymphokines in response to in vitro stimulation of splenocytes from recombinant \( \chi 4064 \) (pDG-6) infected mice with either B-hsp60, or \( \chi 4064 \) soluble antigen revealed similar patterns of T cell stimulation, characterized by substantial quantities of interferon gamma and IL-10, but no secretion of IL-4. The presence of interferon gamma, in the absence of IL-4 is suggestive of a TH1 type T cell response. Although the presence of IL-10 is suggestive of a TH2 type T cell response, a recent report suggests that IL-10 levels are elevated in certain types of TH1 response [120]. Whereas the importance of interferon gamma in immunity against intracellular pathogens is well documented, the currently accepted models do not include IL-10 as a component of a protective immune response against intracellular bacteria. There are at least two explanations for the presence of IL-10 in the supernatants of antigen stimulated splenocytes from infected mice: one is that IL-10 constitutes part of a down-regulatory host reaction to a highly activated inflammatory state, following resolution of the \( \chi 4064 \) primary infection, and the other possibility is that IL-10 is a component of a protective response against \( S. \) typhimurium \( \chi 4064 \) in particular, and intracellular bacteria in general. IL-10 was originally discovered and characterized by its suppression of inflammatory responses, including inhibition of TH1 cytokine production [122] and inhibition of the production of reactive
nitrogen intermediates by macrophages[123]. While these reports favor IL-10 production as down-regulatory of the inflammatory state, a fairly strong case can be made for IL-10 production as a component of the protective response, although this is supported only by circumstantial evidence. IL-10 has been shown to be a cytotoxic T cell differentiation factor that markedly increases the clonal size, and lytic potential of cytotoxic T cell precursors [124]. This report is intriguing for a number of reasons. *Brucella abortus* has been shown to be capable of infecting a variety of phagocytic, as well as non-phagocytic cell types [27]. Because the vast majority of non-professional phagocytes lack a full repertory of bactericidal mechanisms, such infected cells may provide a safe haven for the bacteria. In such a situation, cytolytic cells capable of recognizing infected cells could come into play, lysing the infected targets to release the bacteria which could then be more effectively dealt with by circulating professional phagocytes. In support of this, the generation of antigen specific cytolytic T cells has been demonstrated during the course of infection for several intracellular bacterial species [44,114-116,120]. Furthermore, it has been recently demonstrated that bacterial antigens can access the MHC class I antigen processing pathway for effective in vivo priming of CD8+ cytotoxic T cells [125].

It is interesting that there is no evidence in the data presented here for a TH1 to TH2 switch during the course of the live carrier infection. Such a switch should be manifested by an increase in IL-4 and IL-10 and a decrease in IFN-γ during the course of the infection. It
is also interesting to speculate on whether or not the unusual cytokine profile of high IFN-γ, low IL-4, and high IL-10 observed in these experiments is a *Salmonella* specific response. In one recent report, immunization of BALB/c mice with killed *Brucella abortus* was consistently found to stimulate the elevated expression of genes coding for IL-10 and IFN-γ but not of the genes coding for IL-4 and IL-5 by CD4+ T cells [120]. Because comparable data does not exist for live infections with other intracellular bacterial parasites, we cannot say at this time whether or not this profile is the typical response to prolonged intracellular infections or if it is specific to certain subclasses of infections.

In a number of instances, there were tantalizing, though not always statistically significant, differences in the responses to B-hsp60 when comparing infection with χ4064 (pDG4) with χ4064 (pDG6) [for example, see Figures 5, 6, 9, 10, 11, 16, 17, and 18]. These differences suggest that subtle differences in the immune response which are dictated by the concentration of the antigen within the pathogen itself. The significance of these differences is not presently understood. It remains possible that χ4064 (pDG6) is actually the better vaccine, and that over expression of an antigen may be counter productive by inducing tolerance, as has been reported for vaccination experiments using fractionated components of *Brucella abortus* [35].

From the data collected in this study it is not possible to predict whether or not vaccination of mice with χ4064 (pDG4) or χ4064 (pDG6) would be protective against brucellosis because it is not known
precisely what aspects of immunity are required for protection against *Brucella abortus* infection. It is encouraging that for all the parameters measured, the response to B-hsp60 is similar, though often lower, to the response to total *Salmonella* soluble antigen (S.A.). Others have shown that infection of BALB/c mice with χ4064 produces a high degree of protection against subsequent infection with the virulent parental strain of χ4064 [117]. Thus, it could be argued that the qualitatively similar immunity to B-hsp60 might be provide protection against *Brucella abortus* infection. It should be pointed out that not all antigens stimulate immune responses qualitatively similar to those elicited against χ4064 S.A. in this system. In an earlier paper, Stabel et al [40] demonstrated that another *Brucella* protein, BCSP31, is a strong humoral antigen but produces no detectable cellular response when delivered to the mouse by the same live carrier vaccination protocol used here.

In conclusion, this study has demonstrated that the immune response to B-hsp60 when presented to the immune system in the context of a live infection in BALB/c mice was qualitatively similar to the immune response generated against *Salmonella typhimurium* χ4064 itself. A study has been undertaken to assess the protective effects of this vaccination scheme against a subsequent infection with *Brucella abortus*. 
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110. A full description of the plasmid vectors belonging to the pJE series is given in Emily Chin (1993) Ph.D. Thesis, Iowa State University, Ames, IA.


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APPENDIX. Brucella abortus gro E DNA SEQUENCE

GCTGTTCCGCACAAAAACGCCAGCCTGAACGGAATCCCCTTGGACAAAACATTGGC 60

CTTCTATTTCAAGAACAGGTTAGCAGCTGGACGAATAGTGCTAACAGGCAGGCTTCC 120

CGTTCGTAAACAGGTTCAACGTCTATAACAcCAAGGGTTATACCATGCTGATATCA 180

AGTTCCGGCCATCTAGACCGGTAGTCGGTTGCCTCGGCTGATGACGGAAGCCAAGACTG 240

CGGCCCATCATCTCATCCAGACTGCCAACAGAAAGGCCAGGAAAGGGAGAAGTCGTTG 300

CAGCCGCTTCGCGCTCGAGCAAGCTGCAACTGCTCGGATGATGCAAGGGCTG 360

GCCACCCGGCTTCTTGCCCAAGTGGTCCCGGCAAGTCCGAGTCAAGTCGCGGAGACCC 420

TGCTGATCATGAAAGGAATCCGACATTCTGGGTATTGTCGGCTAAAAAATTCCTTTTGCT 480

TGCTTCACGCAACCAAGATTCCATTCAACGCTACACATTTACAAATCGACGGGATATT 540

CCCAGGAGAGTAAAATGGCTGCAAAAGACGTAAAATTCGGCGGATCTGCGCGAAAAGA 600

TGCTGCCTGCGTATTTCCGCTGACGCTGTTAAGGTCACGCTCGGCCCGAAGGGCC 660

GCAATGTGTTATCGAGAAGTTCTTCGCGCTCGGACATCCAAAGGAGCCTGCGCTGCGGAGG 720

TCGCAAGGAAGTCACTGAGAAGCTTTGGAAGGAGCCCCAAGAGAGAGGGATATT 780
AAGTGGCTTCCAAAGCAGGATCTGGCCTGACGGCACCACGGACCCGAGACGTTCTCG
luValAlaSerLysThrAsnAspThrAlaGlyAspGlyThrThrThrAlaThrValLeuG

GTCAGGCCCCCTGCTGAGAAGCGCCCAAGCCTGCTGACGGCATGAAACCAGTGGAC
lyGlnAlaIleValGlnGluGlyAlaLysAlaValAlaAlaGlyMetAsnProMetAspL

TGAAGCGCGCATGACCTGCTGCTGCTGACAGAAGTTGGCTGACTGCTCTAGAAGGCA
euLysArgGlyIleAspLeuAlaValAsnGluValValAlaGluLeuLeuLysAlaAl

AAAAGATCAACACTTCGGAAGGAAGCTGCCCAGGTTGGCACCATCGACGCTCGAC
ysLysIleAsnThrSerProValThrAlaGluThrGluThrGluGluLeuGluValGlyT

CCGAAATCCGCAAGATGTCGAGTGCAAGCCTGCTGCTGCTGCTGCGGTCAG
laGlulGluGlyLysMetIleAlaGluAlaMetGlnGluGlyValGlyValIleT

CGTGTAAAGCCAAGCCCGGCAAGGCACTGAGACTGAGCGCCATCGAGCT
hrValGluAlaLysThrAlaGluThrAlaGluLeuValValGluGluGlyMetGlnPheA

ACCGCGCTACCTGCTGCTGCTGCTGAGCTCAACCTGAGCTGAGCTGT
spArgGlyTyrLeuSerProTyrePhValThrAsnProGluValMetValAlaAspLueG

AAGACGCTACATCTTCGCAAGAAGCTGACCTCAGCTCTCGCTGCGC
luAspAlaTyrIleLeuLeuHisGluLysLysLeuSerAsnLeuGlnAlaLeuLeu

TTTTGAAAGCTGTCGTCCAGACCTCAGCACCCCTGCTTGGAGCTG
alLeuGluAlaValValGlnThrSerAlaAlaGluAspValGluValG

GCGAAGCTCTTGCACACCGCCGTGATCTGACGCGGCGGCGCCATGCTG
lyGluAlaLeuAlaThrLeuValValAlaAsnGluArgGlyGluGlyLeuGlyLeuAlaAlaV

TCAAGGCTCGGCTGGCTGCAACGCTGTCGCAAGCCTGCCGCTGCAAGC
alLysAlaProGlyPheGlyAspArgArgAlaMetLeuGluAspIleAlaIleLeuT

CTGGCCCTCGAGTCTCAGTCCAGAAGCTGAGCTTCAGTAAAAGGCTACGCT
hrGlyGlnValIleSerGluAspLeuGlyIleLysLeuGluSerValThrLeuAspM

TGCTGGCCGCGCCAGAAGGTTCGATCTCAGAAGACGATCCTGTCGAGG
etLeuGlyArgAlaLysValSerIleSerLysGluAsnThrThrIleLeuAspGlyA
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TTGCGGATCCGCGTTGCGCGGgTGCAAGGAAAGTTGAAGTGGAAGGAAAGAAGGACCG
alValIleArgValGlyGlyAlaThrValGluValGluValLysGluLysAspAsp

TTGACGCGCCCTGGAACCCCGCCTGCCGCTGCCGCGGCGGCGGCGG
alAspAspAlaLeuAsnAlaThrArgAlaAlaValGluGluGlylleValAlaGlyGly

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lyThrAlaLeuLeuArgAlaSerThrLysIleThrAlaLysGlyValAsnAlaAspGlnG

AAGCTGCGCATCAACATCGTTCCGGCAAGGAAAGGCTGCGCCGCAAGATCAGACCCAC
luAlaGlyIleAsnValIleValGlyLysIleLeuGluAsnThrSerGlyIleValAspP

ATGCGGTTGAAAGCTCGTTGGCAAGGAAAGGTTGAAGGAAAGAAGGACCG
snAlaGlyGluGluGluAlaSerValIleValGlyLysIleLeuGluAsnThrSerGlyThrP

TCGCTACAAACACGCGCAACTGCGCAATGCGACCTGCTGCCGCTGCCCGAATG
heGlyTyrAsnThrAlaAsnGlyGluTyrGlyAspLeuIleSerLeuGlyIleValAspP

CGGCTACAAACACGCGCAACTGCGCAATGCGACCTGCTGCCGCTGCCCGAATG
roValLysValArgThrAlaLeuGlnAsnAlaIleValAlaGlyLeuLeuIleT

CGACGGAAGCATGACGTCCGCAAGGCAAGGCACGCGCTCGGTGCGCGGCGCATG
hrThrGluAlaMetIleAlaGluLeuProLysLysAspAlaAlaAlaGlyMetProG

GCGGTATGGGCTGAGGGCCGACTGGGCACTGCCCTGTTGCGGCGCGCTG
lyGlyMetGlyGlyMetGlyGlyMetAspPheEnd

CTAAATGGCCTCCCGCGCAAAgcggcgccgggcccGGCAGTCGCTCCCAAGAAG
ACCAGGAAGGCAGGAAAGCCTGCCCAACTGGCCGACCAGAATTAAACACCTCCGGTT

69
70

CCCGGGAGGTTTTTT?ATTCGCTAAATAACTTCCATACCTGGTGACATTCAACGGGTGT 2400