GENERATION OF BACULOVIRUS-*BRUCELLA ABORTUS* HEAT SHOCK PROTEIN RECOMBINANTS; MICE IMMUNE RESPONSES AGAINST THE RECOMBINANTS, AND *B. ABORTUS* SUPEROXIDE DISMUTASE AND L7/L12 RECOMBINANT PROTEINS

by

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ABSTRACT

Brucella abortus is capable of resisting the microbicidal mechanisms of phagocytic cells and growing within phagocytic cells, usually macrophages. *B. abortus*, like several other intracellular bacteria responds to the hostile environment in macrophages by producing heat shock proteins (HSPs) which are induced by environmental stresses. Bacterial HSPs are very immunogenic, eliciting both cellular and humoral immune responses in the infected host. The significance of host cellular and protective immune responses directed against these proteins is currently unresolved. Baculovirus recombinants were generated in *Sf9* insect cells for *B. abortus* HSPs and the protein expression was optimized. Humoral (Western blot), cell mediated (CMI, IFN-γ- release by splenocytes, and $CD3^+CD4^+$, $CD3^+CD8^+$ T cell/ total splenocytes ratios) and protective immune responses of BALB/c mice (challenge with virulent *B. abortus* 2308) against

these recombinants, against *B. abortus* superoxide dismutase (SOD) and ribosomal L7/L12 proteins, inoculated alone or in various combinations with complete Freund's, Ribi and recombinant IL-12 as adjuvants, were analyzed. Vaccinia virus-GroEL recombinant as priming immunogen, followed by baculovirus-GroEL-Ribi booster, was explored. Androstenediol, an immune up-regulator, was tested for its ability to induce resistance against challenge.

None of the mice inoculated with individual, divalent or trivalent HSP-expressing *Sf9* cells combined with Freund's were protected against challenge and the *Sf9* cell-induced response masked the recombinant protein-specific CMI responses. Recombinant HSPs were purified and combined with Ribi. Although significant IFN-γ release was induced by immunization with the HtrA-Ribi combination, no mice were protected against challenge. Priming with vaccinia virus-GroEl recombinant and boosting with purified baculovirus-GroEL protein-Ribi combination did not induce protection. Androstenediol did not enhance *in vivo* resistance to challenge. IL-12 alone did not activate splenocytes but induced significant IFN-γ release in mice when combined with killed *B. abortus* RB51 vaccine, purified recombinant HtrA or purified SOD proteins, or L7/L12 expressing *Escherichia coli* cells. Significant protection was induced by SOD combined with IL-12. No correlation was seen between IFN-γ release by splenocytes and protection against challenge in the SOD/IL-12-immunized mice.

The results suggest that *B. abortus* HSPs are not highly immunogenic in mice and though various immune responses may be induced by one or another HSPs, protective immune response, unfortunately, is not among them. The results of this study reflect the difficulties in experimenting with immune responses against single or a limited number of recombinant *B.*

abortus proteins. This is particularly true when the task includes induction of a protective immune response and finding significant correlation between different types of immune response assays.

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DECLARATION OF WORK PERFORMED

I declare that, with the exception of the descriptions listed below, all works reported in this dissertation for Ph.D. degree have been performed by myself with the guidance of Dr. Thomas Toth and Dr. Gerhardt Schurig.

Running the flow cytometry instrument was carried out by Ms. Joan Kalnitsky.

B. abortus GroEL vaccinia virus recombinant was provided by Ms. Simge Baloglu, and *B. abortus* SOD- as well as L7/L12- recombinant protein expressing *E. coli* cells were provided by Mr. Ramesh Vemulapalli.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

DNA = deoxyribonucleic acid

kb = kilobase pair

kDa = kilodalton

ml = milliliter

µl = microliter

M = molar

mM = millimolar

g = gram

mg = milligram

µg = microgram

V = volts

RT = room temperature

EtOH = ethanol

SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

Amp = ampicillin

Kan = kanamycin

Tet = tetracycline

Gen = gentamycin

ELISA = enzyme linked immunosorbent assay

ACK = ammonium chloride, potassium carbonate lysis buffer

AED = androstenediol

APC = antigen presenting cell

BSA = bovine serum albumin

CD = cluster designation

CMI = cell mediated immunity

CFU = colony forming units

ConA = concanavalin A

CPM = counts per minute

DHEA = dehydroepiandrosterone

DMSO = dimethyl sulfoxide

EDTA = ethylenediaminetetraacetic acid

IFN = interferon

Ig = immunobglobulin

IL = interleukin

HSP = heat shock protein

LPS = lipopolysaccharide

MHC = major histocompatibility complex

MBP = maltose binding protein

PBS = phosphate buffered saline

RPMI = Roswell Park Memorial Institute, Buffalo, NY

SI = stimulation index

SOD = superoxide dismutase

TBS = tris buffered saline

TBST = tris buffered saline with Tween

TEMED = tetramethylethyl enediamine

TSA = trypticase soy agar

TSB = trypticase soy broth

CHAPTER 1.

LITERATURE REVIEW

I. Historical Aspects of Brucellosis.

According to Hughes (1897), Brucellosis was first described by Hippocrates (BC 460). An accurate description of the disease was made in 1861 by Marston, who was an assistant surgeon in the British Army stationed in Malta, and named it Malta fever. In 1897, Bernard Laurits Fredrick Bang, a Danish veterinarian from Copenhagen, identified an intracellular microorganism described as *'Bacillus abortus'* as the cause of abortion in cattle. The disease was named for him, 'Bang's Disease' (Bang, 1897). Bang's discovery was of interest only to veterinarians, dairy farmers and meat producers. It did not impact physicians at that time and no correlation was made between Bang's Disease and Malta Fever. As the organism in Malta Fever was described as 'Micrococcus' and in Bang's Disease it was described as 'Bacillus', the two diseases were not thought to be related. Twenty-one years later, an American microbiologist, Alice Evans, reported in 1918 the close relationship of *Bacillus abortus* to *Micrococcus melitensis*. *Micrococcus melitensis* was confirmed to be a bacillus and not, as originally described a micrococcus (Evans, A. C., 1918). She suggested a new name for Malta Fever and called it 'Brucellosis' for the discovery of the causative agent of the disease, David Bruce (Bruce, D., 1889). Two years later Meyer and Shaw (1920) suggested the genus *Brucella* rather than Bacteraceae.

In 1914, J. R. Mohler from the USA cultured an organism from the liver, kidneys and stomach of a premature pig. He identified the organism as similar to that of *B. abortus* with the exception that it grew readily on atmospheric air. The disease caused abortion in pigs. In 1927, Alice Evans identified one cause of brucellosis diagnosed initially as due to *B. abortus* but later confirmed to be *B. suis*. Other authors reported similar cases of *B. suis* (Blake and Oard, 1928, 1929). Other *Brucella* species were not discovered until 1953 when Buddle and Boyes reported *B. ovis*, an organism causing sterility in rams. Another, new *Brucella* organism was made by Stoenner and Lackman in 1957. They isolated *B. neotomae* from wood rats (*Neotoma lepida*). In 1966 Carmichael, an American veterinarian from Cornell University, was the first to report on abortions in beagles. The first report on *B. canis* in humans was in 1968 by the National Communicable Disease Center of the United States Public Health Service, in a laboratory technician who handled viable organisms (Madkour, 1989).

The urge to control and eradicate brucellosis in different parts of the world was not as intense as that experienced by the British Army in Malta. The motive behind the eradication programs was purely economical rather than prevention of human morbidity. In Sweden, Bjorkman and Bengtson (1962) noted: 'The bovine brucellosis in Sweden was serious in the early 1930s. Economic losses were heavy. At that time the annual losses due to the disease were estimated at 30-40 million Crowns'. It took Sweden 13 years (1944-1957) to achieve the eradication at a cost of 22 million Crowns, far less than the losses in one year in the 1930s. Eradication was first accomplished in 1952 by Norway (Madkour, 1989).

II. Pathogenesis of Brucellosis.

The term brucellosis is applied to a group of closely related infectious diseases, all caused by Gram-negative bacterial pathogens in the genus *Brucella*. Manifestation of the disease may range from abortion in the cow and to orchitis or epididymitis in the bull. Characteristically, all *Brucella* species establish persistent infection in the reticuloendothelial system of the natural host species. The most frequent symptoms of human brucellosis are fever, chills or shaking rigors, malaise, generalized aches and pains all over the body, joint and low back pain, headaches, anorexia, easy tiredness and general weakness (Marston, J. A., 1861; Bruce, D., 1889; Madkour, M. M., 1989). Man almost always receives the infection from infected animals, transmission from man to man rarely occurs (Thimm, 1982). Therefore, the research is focused at the control and eradication of the disease in the animal host. *Brucella abortus* causes bovine brucellosis, which is characterized principally by abortion and chronic infection within lymph nodes and in the mammary gland (Montaraz and Winter, 1977). In addition to cattle, *B. abortus* also causes infections in swine, goats, elk and bison populations (Nicoletti, P., 1990).

The *Brucellae* are facultative intracellular parasites. They usually enter the body through cuts and abrasions in the oral mucosa, nasopharynx, conjunctivae, or genitalia and even unbroken skin. After gaining entry to the body, the organisms have been found to survive within the cells of the reticuloendothelial system, particularly within macrophages/monocytes of the immune system. They can survive within cells derived from either ectodermal or mesodermal origin, but do not invade tissue of endodermal

origin. They can evade the bactericidal activity of phagocytic cells and replicate within them (Pomales-Leborn and Stinebring, 1957). They are transported to the lymph nodes, where macrophages and polymorphonuclear phagocytic cells (PMNs) die, releasing more bacteria. In animals where the acute infection is not controlled, the bacteria become disseminated and eventually localize in the spleen and liver (Cheers, C., 1984). The disease manifests itself differently for different hosts. For example, in ungulates, the organisms show a marked tropism for the placenta of pregnant animals probably due to the presence of the compound erythritol (Smith et al., 1961; Pearce et al., 1962; Keppie et al., 1965). Erythritol enhances the growth of the bacteria and in many cases, fetuses will abort because of endotoxic shock and/or fetal death caused by increased numbers of bacteria and increased concentrations of endotoxin. In humans, the disease is primarily one of the reticuloendothelial system, and very few cases of spontaneous abortions from infection with *Brucella* have been described. Reports of abortion after infection with *B. melitensis*, are not well documented (Sarram et al., 1974).

The acute disease, when properly diagnosed, can be cured by treatment with tetracycline and/or streptomycin. If untreated, it can cause serious sequelae such as arthritis and neurological disorders. Like many other intracellular organisms, the *Brucellae* are more sensitive to cell-mediated immunity rather than humoral immunity (Mackness, M. B., 1964; Splitter and Everlith, 1986), although Limet et al (1987) noted that some transient resistance to the disease can be transferred passively in serum to mice.

To date, little is known about how the organisms survive intracellularly. Even though the organisms have been found within phagocytic cells, they survive both in

phagocytic cells and nonphagocytic cells, such as epithelial cells. Survival within phagocytic, cells involves inhibition of phagosome fusion with primary and secondary granules (Kreutzer et al., 1979; Riley and Robertson, 1984) while survival within cells lacking phagocytic capability require a bacterial invasion mechanism.

Young et al. (1985) examined effects of phagocytosis by human PMN leukocytes on *B. abortus* and *B. melitensis.* They had earlier demonstrated that *B. abortus* induced the formation of granulomas and that *B. melitensis* induced formation of microabscesses (Young, E. J., 1979; Young et al., 1979). In studies using virulent and attenuated strains of *B. abortus* and *B. melitensis*, they demonstrated several things. First, both virulent and attenuated strains were rapidly phagocytosed only if they were opsonized with normal human serum. Second, they showed that normal human serum without anti-*Brucella* antibodies was bactericidal for *B. abortus* and not for *B. melitensis*, indicating that differences in membrane structure between the two types may be one reason why *B. melitensis* is a more virulent human pathogen. Apparently, this effect was not due to complement, since heating the serum inactivated this activity and addition of guinea pig complement did not restore it. Lastly, intracellular killing by human PMNs was demonstrated for virulent *B. abortus*, but not for *B. melitensis.* Attenuated strains of *B. abortus* and *B. melitensis* were killed to the same extent. This last fact may, in part, explain why *B. melitensis* is more virulent for humans than *B. abortus* (Spink, W. W., 1956).

III. Characteristics of *Brucella*.

The *Brucellae* are a family of small, nonmotile, Gram-negative coccobacilli, and facultatively intracellular bacteria that cause disease in a broad range of animal hosts. The genus *Brucella* consists of a small coherent group of very closely related bacteria. The organisms are coccobacilli 0.5-0.7 μm wide by 0.5-1.5 μm in length. They can occur as single cells, in pairs or in short chains. *In vivo* they often occur within the cytoplasm of cells in close-packed clusters. They do not form spores or true capsules and are invariably non-motile and aflagellate (Corbel and Brinley-Morgan, 1984). They are aerobic and do not grow under strictly anaerobic conditions but many strains, especially of the species *B. abortus* and *B. ovis*, are carboxyphilic and require supplementary CO_2 for growth. Metabolism is oxidative and energy is produced by utilization of various amino acids and carbohydrate substrates. For many strains *i*-erythritol is a preferred energy source. Most strains require complex media containing multiple amino acids, thiamine, biotin, nicotinamide and pantothenic acid for growth, especially on primary isolation (Corbel and Brinley-Morgan, 1984). The production of H_2S from sulfur-containing amino acids varies between species and biovars and is of value in differentiating these. Proteolytic activity is slight; urease activity is consistently high in *B. suis* and *B. canis* but variable in other species, being weak or absent in the case of *B. ovis*. Citrate cannot serve as the sole carbon source. Acid is not produced from glucose and o-nitrophenol-β-D-galactoside is not usually hydrolyzed. Litmus milk either remains unchanged or is rendered alkaline. Growth occurs in the range 20-40^{o} C, but the optimum

temperature is 37°C. The optimum pH for growth is between pH 6.6 and 7.4; growth usually results in alkalization of the medium (Swann et al., 1981). *Brucella* strains are fairly resistant to drying and can survive in biological material for long periods, especially at low temperature. They are sensitive to a wide variety of disinfectants including formaldehyde, hypochlorite, iodophors and phenols provided that excess organic matter is not present. The organisms are killed by heat under pasteurization conditions. Sensitivity to antibiotics is variable but most strains are sensitive to chloramphenicol, gentamycin, tetracyclines and ansamycins *in vitro*. Differences between the protein components of the species and even individual strains can be shown by high performance liquid chromatography of extracts of live cells. The antigenic composition of the various species and biovars is very similar (Shibata et al., 1962).

All strains show a very similar DNA composition, with ranges between 55 and 59 mole % guanine plus cytosine, very similar to that observed for *Escherichia coli* (50 %) (Hoyer and McCullough, 1968 (a)), and share >94% homology between species (Hoyer and McCullough, 1968 (a), (b)). The size of the *Brucella* genome is about 2.5 X 10^6 base pairs, which is less than that of *E. coli* (4 X 10^6), based on the results of pulsed field electrophoresis (Allardet-Servent, 1988). Although DNA-DNA hybridization has failed to differentiate between the species of *Brucella*, electrophoretic analysis of restriction enzyme digests has disclosed species differences (Verger et al., 1985).

Unlike certain organisms of these genera, for example, *Agrobacterium* spp., the pathogenicity of *Brucella* is not regulated by plasmids. Indeed repeated attempts in many laboratories have failed to detect extrachromosomal DNA in *Brucella*. *B. abortus*,

has however, been infected under experimental conditions with a plasmid derived from *E. coli* and resistance to antibiotics has been transferred by this means. Nevertheless, this process has not been observed under natural conditions (Corbel and Thomas, 1983).

Lytic *Brucella*-phages have been described in detail, and are used mostly for identification and taxonomy (Smith and Ficht, 1990). All of the *Brucella*-phages appear to belong to the same phage family. Presently six phage groups have been identified. It is unknown if lysogenic phages exist in the *Brucellae*. Currently, there is no evidence that all phages except Bk2 are lytic for the *Brucellae*.

Attempts to use transformation procedures effective for *E. coli* have not been promising. The *Brucellae* have an extremely waxy cell wall. This may be refractory to the effects of compounds typically used in transformation protocols, such as calcium chloride and rubidium chloride. The *Brucellae* are also inherently resistant to many of the antibiotics generally used for selection of plasmids in the laboratory. In addition, there are several antibiotics that cannot be used, such as tetracycline and streptomycin, since they are antibiotics of choice to treat the disease (Smith and Ficht, 1990).

IV. Classification of *Brucella*.

Largely on the basis of preferred natural host, which shows a fairly close correlation with phage sensitivity, colony morphology, CO_2 requirement and ability to oxidize certain substrates, the genus has been divided into six species, three of which have been subdivided into biovars. These six species are recognized and differentiated

according to antigenic variation and primary hosts: *B. abortus* (cattle), *B. melitensis* (goats), *B. suis* (hogs), *B. ovis* (sheep), *B. neotomae* (wood rat), and *B. canis* (dogs) (Corbel and Brinley-Morgan, 1984). Of these, *B. melitensis, B. suis,* and *B. abortus* are apparently the most virulent and cause the majority of human disease. *B. canis* has occasionally caused disease in humans, but is much more prevalent in the primary host, the beagle (Meyer, M., 1990). *B. canis* is a rough lipopolysaccharide (LPS) isolate that retained virulence. *B. ovis* has the distinction of being a rough isolate, incapable of using erythritol, but still retaining full virulence for its primary host.

The organisms of the genus *Brucella* maintain a close taxonomic relationship and can only be distinguished by rigorous metabolic, immunologic, and biochemical analyses. The similarities among the *Brucella* species extend to the genetic level at which all species share greater than 90 % DNA homology (Hoyer and McCullough, 1968 (a), (b)). This has led some researchers to describe the *Brucellae* as a monospecific genus (Verger et al., 1985). An alternative school of thought maintains that any differences among the organisms within this genus, no matter how small, can and should be used to obtain a distinct taxonomic relationship. This is supported by genomic DNA analysis, which can distinguish between species based on restriction enzyme analysis, and by analysis of restriction polymorphisms within specific genes via Southern blotting and hybridization (Allardet-Servent et al., 1988; Ficht et al., 1988).

The genus was formerly classified together with morphologically similar bacteria of the *Bordetella, Haemophilus and Pasteurella* genera. Subsequent studies have failed to establish any genetic relationship between these groups and this classification is now

untenable. Observations using ribosomal RNA-DNA hybridization have disclosed a genetic relationship between the genus *Brucella* and *Agrobacterium, Mycoplasma, Phyllobacterium, Rhizobium* and the unclassified Centers of Disease Control group Vd (De Ley et al., 1983). The ecology of the non-parasitic genera implies that the group has probably evolved from soil bacteria.

Most available mutants of the *Brucellae* arose spontaneously and involve membrane constituents, as described by smooth to rough changes or inability to be lysed by *Brucella*-phages, CO_2 utilization, erythritol catabolism, or H_2S production. The species can be differentiated on the basis of oxidative metabolism tests with selected carbohydrate and amino acid substrates. Thus, strains of *B. abortus* and *B. neotomae* oxidize a range of carbohydrates and amino acids, but few carbohydrates other than D-glucose and *i*-erythritol. *B. ovis* will oxidize only a few amino acids, whereas *B. canis* and *B. suis* will oxidize a complete range of substrates, including carbohydrates, amino acids and urea cycle intermediates. Oxidative metabolism patterns show a fairly close correlation with phage lysis patterns and both procedures are useful for identification of the species (Corbel and Brinley-Morgan, 1984).

Biovars within *B. abortus*, *B. melitensis* and *B. suis* can be differentiated by a range of properties including CO_2 requirement, H_2S production, serological properties and ability to grow in the presence of dyes. Within the biovars, strains used as vaccines occasionally can be differentiated by additional tests.

V. Virulence Factors of *Brucella*.

As is the case for other bacterial pathogens, several cellular components contribute to the survival and virulence of the *Brucella*. However, the identity of most of these components has not been elucidated. Research concerning the virulence factors expressed by the *Brucellae* has focused primarily on the structural components of the outer membrane. Components are included as virulence factors on the basis of the following classification (Lory and Tai, 1984): (1) components that when inactivated by mutation markedly decrease virulence, (2) components that mimic pathological effects when administered in purified form, and (3) components that elicit a partially protective immune response. In contrast to other Gram-negative pathogens, the outer surface of the *Brucellae* does not have complex structures, such as pili or fimbriae, nor does it have capsular material. The outer membrane contains only two components that have been identified as virulence factors: the lipopolysaccharide (LPS) and the outer membrane proteins (OMPs) (Lory and Tai, 1984).

1. Lipopolysaccharide (LPS).

The LPS has been classified as a virulence factor according to each of the categories described above. Of the potential virulence factors expressed by the *Brucellae*, the LPS is the best defined (Moreno et al., 1979). The availability of natural isolates lacking LPS and exhibiting reduced virulence, the toxic effect of purified LPS, and the protective immunity stimulated by this fraction suggest that LPS is a primary virulence

determinant of the *Brucellae*. Virulence is associated with the smooth colony morphotype, designated S, which we now know contains the full complement of *Brucella* LPS. The rough colony morphotype, designated R, arises by dissociation from the smooth form and its accumulation is typically observed *in vitro*. Dissociation is stimulated by changes in environmental conditions, including different nitrogen sources or exposure to D-alanine (Braun, W., 1947; Goodlow et al., 1952). The R morphotypes are characterized by their opaque appearance, and lusterless colony morphology (Henry, B. S., 1933). In general, for most *Brucella* species, smooth strains are virulent and rough and mucoid strains are not.

The LPS of smooth strains consists of lipid A, a core region containing flucose, mannose, quinovosamine and 2-keto-3-deoxyoctulosonic acid (KDO) and an O chain composed of a homopolymer of about 100 residues of N-formylated perosamine (Caroff et al., 1984). Two distinct epitopes occur on the O chain depending upon the species/biovar of the strain. In those typified by *B. abortus* biovar 1, the A epitope is present and probably represents terminal N-formylated perosamine residues which are linked by the 1 and 2 carbon atoms throughout the chain. In strains typified by *B. melitensis* biovar 1, the M epitope is present. In this case, the O chain consists of repeating units of four 1,2-linked N-formylated perosamine residues and one 1,3-linked residue (Perry et al., 1986). The A, and M epitopes can be detected by cross-absorbed polyclonal antisera. Other antigens present on or near the surface include the outer membrane proteins. These, like the smooth LPS complex, are involved in the protective immune response to *Brucella*. Intracellular antigens include a variety of proteins,

glycoproteins and polysaccharides. The latter may include the polysaccharide B and native hapten present in some strains, which have been employed in the radial diffusion test. The lower molecular weight polypeptide fraction of the intracellular antigens plays an important role in eliciting delayed hypersensitivity reactions in the intradermal test (Bundle et al., 1987).

The toxic effects of the *Brucella* LPS are attributable to the lipid A portion (Galanos et al., 1972). Increased survival of smooth organisms compared with rough avirulent organisms in macrophages suggests that the LPS plays a role in uptake and survival (Riley and Robertson, 1984; Braun et al., 1958; Kreutzer and Robertson, 1979). Differences in uptake of smooth and rough organisms have been demonstrated in experiments using guinea pig and bovine monocytes. LPS plays a role in enhancing intracellular survival. Experiments performed by Frost et al. (1972) indicate that *in vivo*-grown, smooth organisms may have an additional membrane component that increases survival and virulence. The mouse model system has been investigated extensively with respect to the protective immunity induced by antibodies directed against LPS. The protective immunity elicited by LPS in the cow has not been clearly established. Winter et al. (1988) have demonstrated effective protection in the mouse model using a combination of O-polysaccharide covalently linked to *Brucella* porin. Under identical conditions, porin linked to R-LPS did not stimulate a significant level of protection. Montaraz et al. (1986) were able to demonstrate effective immunization by passive transfer of monoclonal antibodies directed against the O-antigen, while monoclonal

antibodies directed against a number of outer membrane proteins induced no significant protection.

2. Outer Membrane Proteins (OMPs).

The OMPs have been classified as virulence factors according to category 3 of Lory and Tai (1984). Characterization of the OMPs as virulence factors has been hampered by the inability to identify variants lacking expression or exhibiting altered profiles on SDS-PAGE gels. Furthermore, careful examination of these profiles is hampered by the presence of the LPS, which interferes with the migration of the OMPs. Thus, the role of these proteins in the survival and virulence can only be hypothesized. Protective immunity stimulated by OMPs has been demonstrated following vaccination with rough or mucoid organisms or cell envelope fractions that lack LPS. Verstreate et al. (1982), Santos et al. (1984), and Douglas et al. (1984) have identified three major groups of proteins present in the outer membrane of the *Brucellae*. These were designated as group 1 (88 to 94 kDa), group 2 (35 to 40 kDa), and group 3 (25 to 30 kDa). Little variation among species has been observed in the OMP profiles (Santos et al., 1984). All three groups of proteins appear to be recognized by the bovine immune system during the course of infection. Protective immunity stimulated by OMPs has been documented for several Gram-negative organisms (Isibasi et al., 1988; Wang and Frash, 1984). Protection stimulated by *Brucella* OMPs is still a matter of debate, and despite considerable experimental effort, is far from resolved. The doses employed have either been insufficient to produce an immune response or presentation of the antigen in the killed

vaccines does not mimic the situation obtained with a living vaccine strain. Also, the reliability of the mouse as a model for bovine brucellosis, despite their experimental practicality, is questionable. Vaccines tested with good success in the mouse have been subsequently tested without success in cattle. The development of an improved laboratory animal model for bovine brucellosis would be a major step in the resolution of this problem. Recent vaccine trials performed in cattle suggest that under the appropriate conditions, protective immunity can be stimulated by rough cell envelopes, implying that an immune response directed against the OMPs is protective (Adams et al., 1987).

3. Toxins and Other Factors.

To date, no concrete evidence exists that toxins similar to those produced by other organisms (i.e., *Vibrio chloera, Corynebacterium diphtheriae, E. coli, Shigella dysenteriae, Clostridium botulinum, C. tetani*) are produced by the *Brucellae*. A toxin has been postulated to exist due to the severe effects of the disease on the central and autonomic nervous systems. Endotoxin, consisting of LPS, has been suggested as the causative factor.

The *Brucellae* also produce catalase. Huddleson and Stahl (1943) determined, that catalase activities decreases with changes from smooth to rough morphology. Although the biochemical advantage of expressing catalase in *Brucella* has not been identified definitively, it may not only protect the organisms from peroxide, but could also be

important in maintaining oxygen tension required for survival of the organism (McCullough, N. B., 1970).

VI. Immunology of and Protection against *Brucella.*

Brucellosis is an infectious disease caused by a bacterium that affects animals and man; it frequently progresses towards a chronic state involving different organs. Once the organism has transgressed the mucous membranes or the skin, it tends to localize in the local lymph nodes and then a systemic dissemination occurs affecting organs of the mononuclear phagocytic system such as spleen and liver (Ruiz-Castaneda M., 1954). Classic experiments by Mackaness (1964) and Mackaness and Blanden (1967) showed the role of cellular immunity and the fundamental part played by activated macrophages in resistance to, and in protection against, intracellular pathogens such as *Mycobacterium* sp., *Salmonella* sp., *Listeria monocytogenes* and *Brucella* sp.

1. Humoral Immune Responses.

A number of the constituents of *Brucella* cells are capable of inducing specific antibodies in their hosts (Freeman et al., 1970; Moreno et al., 1981; Tabatabai and Deyoe, 1984; Verstreate and Winter, 1984). The classic reports include studies of agglutinins, precipitins, complement fixing and blocking antibodies (Glenchur et al., 1963; Wilkinson, P. C., 1966; Diaz et al., 1968; Schurig et al., 1978). Previous studies in naturally acquired and experimental brucellosis have shown that there is an early marked elevation in the serum concentration of agglutinating antibodies. This IgM antibody may be detected one

week after entry of the bacteria and reaches a peak level four weeks later, at the same time that IgG antibody peaks. IgG agglutinating globulin has a delayed appearance and, although it is found mixed with IgM, within four weeks following the initial antigenic stimulus the IgM agglutinin level always exceeds the IgG agglutinin level. A third category of agglutinating globulin, IgA appears later in a significant concentration in the serum (White R. G., 1978). IgG agglutinating antibody usually arises a few days after IgM antibody and decreases more rapidly. IgM antibodies may persist several years after therapy and recovery, whereas IgG is likely to be present in much lower titers one year after the beginning of adequate treatment (Buchanan et al., 1974). IgE levels of antibody to *Brucella* have not been studied extensively, but the work of Escande and Serre (1982) indicates that IgE levels may be significantly high in brucellosis patients and vaccinated subjects. According to available evidence, the IgE anti-*Brucella* antibodies appear somewhat after the IgM antibodies and a little earlier than IgG.

Multiple precipitating antibodies have been reported to be usually detectable within 3-4 months of severe or prolonged active *Brucella* infection (Glenchur et al., 1962; Schurig et al., 1978). These antibodies belong mostly to the IgG globulin fraction and they are not as long lasting as the agglutinins (Glenchur et al., 1962). The anti-*Brucella* complement fixing activity present in sera has been mainly related to IgG, somewhat less to IgM and not at all to IgA (Heremans et al., 1963).

Blocking antibodies have been studied and reported in cases of human brucellosis (Griffiths, 1947). They appear later in the course of the disease than agglutinating antibodies (Zinneman et al., 1959). Blocking antibodies are thermostable, divalent and

occur in the IgG and IgA fractions (Heremans et al., 1963). Interestingly, both IgG and IgA have been reported to act as agglutinating or blocking antibodies. Moreover, both IgG and IgA blocking antibody responses are prolonged and sustained. However, a quantitative assessment of the relative amounts contributed by each globulin to the blocking effect cannot be made (Wilkinson, P. C., 1966). For all those characteristics, the blocking antibodies have always been related to a long-lasting or chronic brucellosis (Glenchur et al., 1961; Kerr et al., 1967; Coombs et al., 1978).

In spite of all the advances in the study of immunoglobulins, including structure, sequential production and persistence after antigenic stimulation, this knowledge has been applied only to the diagnosis of brucellosis. As a consequence, it has been the basis for the development of supplemental serological tests and for research on the identification of cell fractions and the antibody classes reactive with them. Nevertheless, very little has been done in this field or in understanding the biological role of this especially complex antibody response.

2. Cell-Mediated Immune Responses.

Antibodies play an important role in eliminating *Brucella* organisms but the development of a solid cell-mediated immune response is fundamental for the establishment of a protective state as observed in other infectious diseases caused by intracellular pathogens including *Brucella* sp., *Mycobacterium* sp. and *Salmonella* sp.

The discovery and definition of several T lymphocyte sub-populations (Cantor and Boyse, 1975; Moretta et al., 1979) has permitted the study of the individual response

towards the different *Brucella* antigens. Many attempts have been made to identify the antigen(s) responsible for the protective immunity as well as the one responsible for the chronic state. In that respect, two important sub-populations of T lymphocytes have been identified by cellular surface markers and by functional criteria: helper/effector cells and suppressor/cytotoxic cells. Studies using cell transfer assays have established that T helper cells are responsible for the response that confers protection (Kauffmann et al., 1979; Cheers C., 1984). The main function of these helper cells is the activation of macrophages which leads to an increase of the bactericidal capacity through the production of soluble factors, e. g. IFN-γ. The T helper cell population activity is controlled by several factors: suppression mediated by soluble factors produced by suppressor macrophages (Schultz R. M., 1980) and by T suppressor cells. Bacterial antigens may stimulate the level of activity by T suppressor cells. In fact, this has been observed in other intracellular infections such as leprosy in which the antigenic fractions that specifically activate T suppressor lymphocytes have been identified (Mehra et al., 1984).

All the available experimental evidence points towards the role that the cell-mediated immune response plays during the procedures of the *Brucella* infection. Success in elimination of *Brucella* depends basically on the adequate macrophage activation induced by the T lymphocyte response, stimulated by the bacterial antigens. Yet, the detailed nature of the mechanism involved in the interaction of all the cellular populations involved in this protective response is still largely unknown.

3. Phagocytosis.

3.1 Polymorphonuclear leucocytes (PMN)

Ingestion and killing by phagocytic cells is one of the early non-specific defense mechanisms against many microorganisms, *Brucella* included. This activity is carried out by polymorphonuclear leukocytes (PMN) which are attracted to the infection site by chemical stimuli originated by or derived from the microorganism (Wilkinson P. C., 1980; Birmingham et al., 1982). Once the phagocytic cells arrive at the infection site, the ingestion of the bacterium occurs and a series of events takes place. These events include an increased consumption of oxygen that leads to the appearance of hydrogen peroxide, superoxide radical and other oxygen-derived radicals, together with activation of myeloperoxidase. Lysosomes fuse with the *Brucella*-containing phagosomes and release their acid hydrolases, glycosidases, proteases and lipases.

B. abortus and *B. melitensis* are equally susceptible to phagocytic ingestion by PMN, even more so if the bacteria are opsonized with serum factors such as antibodies, complement components and a third group of substances defined as thermolabile natural antibodies (Young et al., 1985).

The ingested bacteria may survive the destructive mechanisms of the phagocyte, a phenomenon apparently related to virulence (Smith and Fritzgeorge, 1964). Events occurring after phagocytosis have taken place do not seem to be influenced by opsonins (Elsbach P., 1980), so that resistance or susceptibility of the bacterium seems to be an extrinsic phenomenon. Both attenuated and virulent strains of *B. abortus* and attenuated *B. melitensis* are eliminated by PMN, while virulent *B. melitensis* is more resistant to

intracellular death, a property which probably relates to its higher virulence to both man and animals (Young et al., 1985). However, some virulent strains of *B. abortus* survive within the PMN. Selective resistance of smooth versus rough strains of *B. abortus* suggests involvement of a surface component in the inhibition of the fusion of phagosome and lysosome (Riley and Robertson, 1984). Low molecular weight substances from *B. abortus* also specifically inhibit the bactericidal system of myeloperoxidase-H_2O_2- halide (Canning et al., 1985). Moreover, strains of *B. abortus* appear not to trigger the burst of oxidative metabolism so that no H_2O_2 is generated for the myeloperoxidase system (Kreutzer and Robertson, 1979). Although PMNs are the primary cells involved in the elimination of foreign pathogens, they are generally considered of low efficiency against *Brucella* so that these bacteria are able to survive and grow within the PMN. *Brucella* cells are also transported to organs of the mononuclear system and this is considered the reason for the persistence of the infection.

3.2 Macrophages

Polymorphonuclear leukocytes rarely serve as hosts for *Brucella*, but the bacteria thrive within macrophages. To carry out its bactericidal activity the macrophage produces most of the oxygen-derived radicals mentioned above. Substances derived from T lymphocytes stimulated by bacterial components promote macrophage activity in three main ways:

(1) By increasing the bactericidal capacity of the macrophage.

(2) By attracting circulating monocytes which mature in the tissues into macrophages.

(3) By inducing the local proliferation of macrophages.

Although the importance of antibodies in protection against *Brucella* has been clearly established (Plommet and Plommet, 1983), the exact mechanism has not been elucidated. Armstrong and Hart (1975) observed that antibody covering bacilli facilitate the fusion of phagosomes with lysosomes in infections by *M. tuberculosis*. This also seems to be the case of *Brucella* since antibodies help to reduce dissemination (Plommet and Plommet, 1983).

In experimental brucellosis the macrophage activation occurs early in the infection, therefore it is easy to demonstrate the development of a specific cellular response from the third day after infection. This early activation seems to be due to contact with bacterial components such as *B. abortus* lipopolysaccharide (LPS) (Birmingham and Jeska, 1981). This activation is notably increased as a result of the establishment of the cell-mediated immunity and reaches maximal bactericidal ability at the 14^{th} day. During this activation stage the macrophage undergoes a succession of important metabolic and functional changes that allow it to eliminate the ingested germ. Thus, an increase in production and liberation of lysosomal enzymes occurs, as well as in Fc and complement receptors that facilitate the adhesion and ingestion of the bacteria. Also an increased production of superoxide and peroxides, and an increase in the chemotactic response which produces a greater mobilization of the macrophage occurs to the infection site (Birmingham and Jeska, 1981).

Brucella plays an active role in securing its survival within the phagocytic cell. One of the fundamental processes used by the bacteria is to prevent the fusion of the phagosome with the lysosome. This effect seems to be mediated by surface antigens not

related to LPS. These surface components have been found in smooth virulent strains but not in smooth attenuated strains (Frenchick et al., 1985). The mechanisms how these *Brucella* components act has not been elucidated yet.

Other intracellular parasites such as *Toxoplasma gondii* (Jones and Hirsch, 1972), *M. tuberculosis* (Armstrong and Hart, 1971) and *M. microti* (Lowrie et al., 1975; Lowrie et al., 1979) also avoid phagosome-lysosome fusion. Apparently in these cases parasite products interact with lysosomal membranes and render them unable to fuse. These products include the mycobacterial sulphatides and polyanionic trehalose glycolipids (Goren et al., 1974).

There are two alternative or complementary mechanisms that help *B. abortus* to survive within the macrophage (Corbel and Brewer, 1980):

(1) Some *B. abortus* strains avoid the respiratory burst inside the phago-lysosome, avoiding the consequences of oxygen-derived radical formation.

(2) B. abortus is able to release cellular products, including RNA that might inhibit lysosomal enzymes.

4. Protection against *Brucella.*

Resistance to *B. abortus,* which is one of the facultative intracellular bacterial pathogens, predominantly depends on acquired cell-mediated immunity, characterized by the activation of T lymphocytes and subsequent activation of macrophages for increased killing of such organisms (Zhan et al., 1996). An important cell-mediated immune

mechanism responsible for protection against *Brucella* is the interferon gamma (IFN-γ) up-regulation of macrophages, the main host cellular reservoir for the bacterium (Baldwin et al., 1993). Thus, many investigators have considered that Th1 CD4+ T cells are responsible for macrophage activation and attraction of inflammatory effector cells and therefore they have been suggested to play a major role in acquired cellular resistance (Golding et al., 1994).

VIII. Rationale for This Dissertation.

The most commonly used vaccines against brucellosis are the live attenuated *B. abortus* strain 19 and *B. melitensis* strain Rev.1 vaccines; recently *B. abortus* strain RB51 has replaced strain 19 as the preferred bovine vaccine in the USA. Strain RB51 as a bovine brucellosis vaccine has overcome several limitations of strain 19. A distinct advantage of strain RB51-vaccinated animals is that they do not produce anti-O-side chain antibodies (Buhrman, D. L., 1989); thus they avoid the problem with strain 19, which induces anti-O-side chain antibodies, indistinguishable serologically from the naturally-infected animal. However, there still are controversies using these live attenuated vaccines in terms of safety (Kahler S. C., 1998; Palmer et al., 1996).

One potential alternative to live vaccines is that offered by subunit vaccines. Subunit vaccines represent further reduction of the inactivated products, such as viral particles or bacterial cells, since only a few components from the appropriate pathogen are formulated into the vaccine. Subunit vaccines are considered to be much safer than

their live attenuated counterparts because they contain no infectious agents. The safety of subunit vaccines over the inactivated whole cell vaccines is further enhanced because of their defined physical and chemical characteristics.

The identification of *Brucella* spp. antigens capable of eliciting protective immunity constitutes an aspect of great interest for the development of efficient subunit vaccines. The ability of a specific antigen (Ag) to induce preferentially a Th1 or Th2 subset response is important for the development of vaccines against pathogens (Mosmann et al., 1989). Therefore, identification and characterization of dominant T-cell stimulating Ag from *B. abortus* is an important strategy in the search for protective subunit vaccines. Few *B. abortus* genes have been cloned, and the proteins that they encode have not been characterized as T cell stimulating Ags (Ficht et al., 1988; Roop et al., 1994; Wergifosse et al., 1995). In addition, the potential for these proteins to elicit a protective cellular response against *B. abortus* infection has not been investigated (Oliveira et al., 1996). Therefore, the identity of the *B. abortus* Ag, which induces the formation of protective T cells, is unknown. However, recently, some levels of protection have been induced with the purified L7/L12 *Brucella* recombinant protein, indicating that, if the appropriate antigen is selected, subunit vaccine could be successfully developed (Oliveira et al., 1996).

We directed our attention toward heat shock proteins (HSPs) as candidates for protective antigens; we investigated the possibilities of inducing protective immune response against *B. abortus* infection by using HSPs in a mice model. HSPs are also stress proteins, and have important biological functions in protein biogenesis.

Circumstantial evidence suggests that the function of heat shock proteins in prokaryotic and eukaryotic cells is to protect cells from the ill effects of stress stimuli such as increased temperature, exposure to toxic oxygen radicals, and nutritional deficiencies, allowing the cells to recover and survive (Young and Elliott, 1989). HSPs from bacterial and fungal pathogens are among the major targets of the immune response to infection and are capable of inducing protective immunity in experimental animal models (Abu Kwaik et al., 1993). Bacterial stress proteins may also play important roles in allowing facultative intracellular pathogens to successfully adapt to the harsh environment of the host phagosome (Abu Kwaik et al., 1993; Buchmeier and Heffron, 1990).

At least three factors suggest that heat shock proteins are the major targets of the immune response in a broad spectrum of infections. First, the abundance of these proteins under conditions of stress could well play a role. Second, some heat shock proteins may be intrinsically antigenic. For example, they may be particularly amenable to processing and presentation to the immune system by antigen presenting cells. Finally, it is possible that immunological memory for cross-reactive determinants of this conserved protein is generated early in life and periodically restimulated by subsequent infections (Young and Elliott, 1989).

However, there is controversy about their significance as vaccines; HSP-specific immune responses may induce autoimmune diseases by generating immune responses against shared epitopes in the conserved HSP sequences and hence cross-reactions between foreign and self HSP (Georgopoulus and McFarland, 1993; Kaufmann et al., 1990). Such a risk may exist, but recent evidence suggests that the benefits of an anti-self,

stress protein immune response may be much greater than the risk of autoimmune disease (Young and Elliott, 1989). HSP65, (which belongs to the HSP60 family), is a major antigen, that induces humoral and cell-mediated immune responses against a variety of bacterial pathogens, including *Mycobacteria leprae* (leprosy), *M. tuberculosis* (tuberculosis), *Coxiella burneti* (Q-fever), *Treponema pallidum* (syphilis), *Legionella pneumophila* (Legionnaires' disease), *Borrelia burgdorferi* (Lyme disease) and *Chlamydia trachomatis* (ocular trachoma) (Kaufmann S.H.E., 1990). This antigen is a homologue of the ubiquitous common antigen of *Pseudomonas aeruginosa* and other Gram-negative bacteria and of the groEL HSP of *E. coli* (Young et al., 1987; Shinnick T. M., 1987; Thole et al., 1988). In this regard, we considered that the *B. abortus* HSPs could be good candidates as protective antigens for an understanding of the development of possible subunit vaccines in this current research.

A major difficulty encountered during development of some subunit vaccines is their weak immunogenicity. This problem has been overcome by the use of appropriate adjuvants (Rook and Stanford, 1995). In many studies, numerous potential advantages were gained by using novel vaccine adjuvants (Daynes et al., 1991; Manetti et al., 1993; Rook G. A. W., 1988). These include enhancement of the immunogenicity of weaker immunogens; reduction in the amount of antigen or in the frequency of booster immunizations needed to provide adequate protection; and improvement of the efficacy of vaccines used in newborns, the aged, and immunocompromised individuals. Adjuvants also can promote T cell proliferation and cell-mediated immunity.

We based the current study on the hypothesis that high levels of cell mediated immune responses can be induced by *B. abortus*-HSPs as protective antigens in combination with the appropriate adjuvant, resulting in protective immunity against *B. abortus* infection. In addition to the appropriate adjuvant(s) and protective antigen(s), the doses of HSP antigens and the delivery mechanism of the antigens were investigated as factors leading to proper antigen presentation and processing to induce a protective immune response.

REFERENCES

Abu Kwaik, Y., B. I. Eisenstein, and N. C. Engleberg. 1993. Phenotypic modulation by *Legionella pneumophila* upon infection of macrophages. Infect. Immun., 61:1320-1329.

Adams, L. G., R. P. Craford, T. A. Ficht, R. III Sowa, J. W. Templeton, J. D. Williams, and A. M. Wu. 1987. New approaches for vaccinating against bovine brucellosis, 40th Ann. Brucellosis Conf. Abstract. Chicago.

Allardet-Servent, A., G. Bourg, M. Ramuz, M. Pages, M. Bellis, and G. Roizes. 1988. DNA polymorphism in strains of the genus *Brucella*. J. Bacteriol. 170:4603.

Araya, L. N., and A. J. Winter. 1990. Comparative protection of mice against virulent and attenuated strains of *Brucella abortus* by passive transfer of immune T cells or serum. Infect. Immun. 58:254-256.

Armstrong, J. A. and P. D. A. Hart. 1971. Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes with phagosmes. J. Exp. Med. 134:713-740.

Bang, B. 1897. The etiology of epizootic abortion. J. Comp. Path. Therap. 10:125-161.

Birmingham, J. R. and E. L. Jeska. 1981. Characterization of macrophage functions in mice infected with *Brucella abortus*. Infect. Immun. 32:1079-1083.

Birminham, J. R., L. B. Tabatabai, B. L. Deyoe, E. L. Jeska, and M. Neussen. 1982. Generation of chemotactic factor for granulocytes and monocytes from serum by fractions of *Brucella abortus*. Immunology. 46:17-22.

Bjorkman, G. and H. Bengtson. 1962. Eradication of bovine brucellosis in Sweden. J. Am. Vet. Med. Assoc. 140:1192-1195.

Blake, F. G. and H. C. Oard. 1928-1929. Undulant Fever: a report of three cases occurring in Connecticut. Yale J. Biol. Med. 1: 128.

Blander, S. J. and M. A. Horwitz. 1992. Major cytoplasmic membrane protein of *Legionella pneumophila*, a genus common antigen and member of the hsp 60 family of heat shock proteins, induces protective immunity in a guinea pig model of Legionnaires' disease. J. Clin. Invest. 91:717-723.

Braun, W. 1947. Bacterial dissociation. Bacteriol. Rev. 11:75.

Braun, W., A. Pomles-Lebron, and W. R. Stinebring. 1958. Interactions between mononuclear phagocytes and *Brucella abortus* strains of different virulence. Proc. Soc. Exp. Biol. Med. 97:393.

Bruce, P. 1889. Observations on Malta Fever. Br. Med. J. 1:1101-1132.

Buchanan, T. M., C. R. Sulzer, M. K. Frix, and R. A. Feldman. 1974. Brucellosis in the United States, 1960-1972. An abattoir-associated disease. Part II. Diagnostic aspects. Medicine. 53:415-425.

Buddle, M. B. and B. W. Boyes. 1953. A *Brucella* mutant causing genital disease of sheep in New Zealand. Aust. Vet. J. 29:145.

Buhrman, D. L., 1989. The behavior and effects of *Brucella abortus* rough strain RB51 in mice and cattle. M. S. Thesis. Virginia Polytechnic Institute and State University, Blacksburg, VA.

Bundle, D. R., J. W. Cherwonogrodzky, M. Caroff, and M. B. Perry. 1987. The lipopolysaccharides of *Brucella abortus* and *B. melitensis.* Ann. Inst. Pasteur/Microbiol. 138:92-98.

Canning, P. C., J. A. Roth, L. B. Tabatabai, and B. L. Deyoe. 1985. Isolation of components of *Brucella abortus* responsible for inhibition of function in bovine neutrophils. J. Infect. Dis. 152:913-921.

Cantor, H. and E. A. Boyse. 1975. Functional subclasses of T-lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T-cell subclasses in a differentiative process independent of antigen. J. Exp. Med. 141:1376-1389.

Carmichael, L. E. 1966. Abortions in 200 beagles. J. Am. Vet. Med. Assoc. 149:1126.

Caroff, M., D. R Bundle, M. B. Perry, J. W. Cherwonogrodzky, and J. R. Duncan. 1984. Antigenic S-type lipopolysaccharide of *Brucella abortus.* Infect. Immun. 46:384-388.

Cheers, C. 1984. Pathogenesis and cellular immunity in experimental murine brucellosis. *Dev. Biol. Stand.* 56:237-246.

Coombs, R. A., L. Edebo, A. Feinstein and B. W. Gurner. 1978. The class of antibodies sensitizing bacteria measured by mixed reverse passive antiglobulin haemagglutination (MrPAH). Immunology. 34:1037-1044.

Corbel, M. J. and E. L. Thomas. 1983. The *Brucella*-phages: Their properties, Characterization and Applications, 2nd Edition. Ministry of Agriculture, Fisheries and Food, London.

Corbel, M. J. and W. J. Brinley-Morgan. 1984. *Brucella*. Pp.377-388. In Bergey's Manual of Systemic Bacteriology. Vol. I. N. R. Krieg (ed.). Williams and Wilkins. Baltimore.

Daynes, R. A., A. W. Meikle and B. A. Araneo. 1991. Locally active steroid hormones may facilitate compartmentalization of immunity by regulating the types of lympholines produced by helper T-cells. Res. Immun. 142:40-45.

Diaz, R., L. M. Jones, D. Leong, and J. B. Wilson. 1968. Surface antigens of smooth *Brucellae*. J. Bacteriol. 96:839-901.

Douglas, J. T., E. Y. Rosenberg, H. Nikaido, D. R. Verstreate, and A. J. Winter. 1984. Porins of *Brucella* species. Infect. Immn. 44: 37.

Elsbach, P. 1980. Degradation of microorganisms by phagocytic cells. Rev. Infect. Dis. 2:106-128.

Enright, F. M. 1990. The pathogenesis and pathobiology of *Brucella* infections in domestic animals, p. 301-320. In K.H. Nielsen and J. R. Duncan (ed.), Animal brucellosis. CRC Press, Inc., Boca Raton, Fla.

Escande, A. and A. Serre. 1982. IgE anti-*Brucella* antibodies in the course of human brucellosis and after specific vaccination. Int. Arch. Appl. Immun. 68:172-175.

Evans, A. C. 1918. Further studies on *Bacterium abortus* and related bacteria. II. A comparison of *Bacterium abortus* with *Bacterium bronchisepticus* and with the organism which causes Malta Fever. J. Infect. Dis. 22:580.

Evans, A. C. 1927. Human infection with organisms of contagious abortion of cattle and hogs. JAMA. 88:630.

Ferrero R. L., J. M. Thiberge, I. Kansau, N. Wuscher, M. Huerre and A. Labigne. 1995. The GroES homolog of *Helicobacter pylori* confers protective immunity against mucosal infection in mice. Proc. Natl. Acad. Sci. USA. 92:6499-6503.

Ficht, T. A., S. W. Bearden, B. A. Sowa, and L. G. Adams. 1988. A 36 kDa *Brucella abortus* cell envelope protein is encoded by repeated sequences closely linked in the genomic DNA. Infect. Immun. 56:2036.

Freeman, B. A., J. R. McGhee, and R. E. Baughn. 1970. Some physical, chemical, and taxonomic features of the soluble antigen of the *Brucellae*. J. Infect. Dis. 121:522-527.

Frenchick, P. J., R. J. F. Markham, and A. H. Cochrane. 1985. Inhibition of phagosome-lysosome fusion in macrophages by soluble extracts of virulent *B. abortus*. Am. J. Vet. Res. 46:332-335.

Frost, A. J., H. Smith, K. Witt, and J. Keppie. 1972. The chemical basis of the virulence of *Brucella abortus*. X. A surface virulence factor which facilitates intracellular growth of *Brucella abortus* in bovine phagocytes. Br. J. Exp. Pathol. 53:587.

Galanos, D., O. Luderitz, E. T. Rietschel, and O. Westphal. Biological activities of lipid A complexed with bovine serum albumin. Eur. J. Biochem. 31:230.

Glenchur, H., H. H. Zinneman, and W. H. Hall. 1961. Significance of the blocking antibody in experimental brucellosis. J. Immunol. 86:421-426.

Glenchur, H., U. S. Seal., H. Zinneman and W. H. Hall. 1962. Serum precipitins in human and experimental brucellosis. J. Lab. Clin. Med. 59:220-230.

Glenchur, H., U. S. Seal, H. H. Zinneman and W. H. Hall. 1963. Antigenicity of some *Brucella melitensis* cell fractions. J. Bacteriol. 85:363-368.

Goodlow, R. J., L. Tucker, W. Braun, and L. A. Mika. 1952. Effect of the isomeric configuration of the source of nitrogen on changes in population and metabolism in culture of *Brucella*. J. Bacteriol. 63:681.

Goren, M. B., O. Brockl, and W. B. Schaeffer. 1974. Lipids of putative relevance to virulence in *Mycobacterium tuberculosis*: Correlation of virulence with elaboration of sulfatides and strongly acid lipids. Infect. Immun. 9:142-149.

Griffiths, J. J. 1947. Agglutination and an agglutinin blocking property in serums from known cases of brucellosis. Publ. Hlth. Rep. 62:865-875.

Henry, B. S. 1933. Dissociation in the genus *Brucella*. J. Infect. Dis. 52:374.

Heremans, J. F., J. P. Vaerman, and C. Vaerman. 1963. Studies on the immune globulins of human serum. II. A study of the distribution of anti-*Brucella* and anti-diphtheria antibody activities among γSS. γIM and γIA, globuline fractions. J. Immunol. 91:11-17.

Hoyer, B. H. and N. B. McCullough. 1968 (a). Polynucleotide homologies of *Brucella* deoxyribonucleic acids. J. Bacteriol. 95:444.

Hoyer, B. H. and N. B. McCullough. 1968 (b). Homologies of deoxyribonucleic acids from *Brucella ovis* canine abortion organisms and other *Brucella* species. J. Bacteriol. 96:1783.

Huddleson, I. F. and W. H. Stahl. 1943. Catalase activity of the species of *Brucella* as a criterion of virulence, in studies of Brucellosis II, Mich. Agric. Exp. Stn. Tech. Bull., 182:57.

Hughes, M. L. 1897. Mediterranean, Malta or Undulant Fever. Macmillan, London, pp.1-10, 28, 80, 85, 148, 156, 166.

Isibasi, A., V. Ortiz, M. Vargas, J. Paniagua, C. Gonzalez, J. Moreno, and J. Kumate. Protection against *Salmonella typhi* infection in mice after immunization with outer membrane proteins isolated form *Salmonella typhi* 9, 12, d, Vi. Infect. Immun. 56:2953.

Jones, T. C. and J. G. Hirsch. 1972. The interaction between *Toxoplasma gondii* and mammalian cells. II. The absence of lysosomal fusion with phagocytic vacuoles containing living parasites. J. Exp. Med. 136:1173-1194.

Kauffmann, S. H. E., M. M. Simon, and H. Hahn. 1979. Specific Lyt 1, 2, 3 T cells are involved in protection against *Listeria monocytogenes* and in delayed hypersensitivity to listerial antigens. J. Exp. Med. 150:1033-1038.

Keppie, J., A. E. Williams, K. Witt, and H. Smith. 1965. The role of erythritol in the tissue localization of the *Brucellae*. Br. J. Exp. Pathol. 46:104.

Kerr, W. R., D. J. H. Payne, L. Robertson, and R. R. A. Coombs. 1967. Immunoglobulin class of *Brucella* antibodies in human sera. Immunology. 13: 223-225.

Kreutzer, D. L. and D. C. Robertson. 1979. Surface macromolecules and virulence in intracellular parasitism: comparison of cell envelop components of smooth and rough strains of *Brucella abortus*. Infect. Immun. 23:819-828.

Kreutzer, D. L., L. A. Dreyfus, and D. C. Robertson. 1979. Interaction of polymorphonuclear leukocytes with smooth and rough strains of *Brucella abortus*. Infect. Immun. 23:737.

Limet, J., A. M. Plommet, G. Dubray, and M. Plommet. 1987. Immunity conferred upon mice by anti-LPS monoclonal antibodies in murine brucellosis. Ann. Inst. Pasteur. Immunol 138:417.

Lory, S. and P. C. Tai. 1984. Biochemical and genetic aspects of *Pseudomonas aeruinosa* virulence. Curr. Top. Microbiol. Immunol. 118:53.

Lowrie, D. B., P. S. Jackett, and N. A. Ratcliffe. 1975. *Mycobacterium microti* may protect itself from intracellular destruction by releasing cyclic AMP into phagosomes. Nature . 254:600-602.

Lowrie, D. B., V. R. Aber, and P. S. Jackett. 1979. Phagosome-lysosome fusion and cyclic adenosine 3í:5î-monophosphate in macrophages infected with *Mycobacterium microti, Mycobacterium bovis* BCG or *Mycobacterium lepraemurium*. J. Gen. Microbiol. 110:431-441.

Mackaness, M. B. 1964. The immunological basis of acquired cellular resistance. J. Exp. Med. 120:105.

Mackaness, M. B. and R. V. Blanden. 1967. Cellular immunity. Prog. Allerg. 11:89-140.

Madkour, M. M. 1989. Brucellosis. Butterworths, London.

Manetti, R., P. Parronchi, M. G. Guidizi, M. P. Piccinni, E. Maggi, G. Trinchieri, and S. Romagnani. 1993. Natural killer cell stimulatory factor (interleukin 12) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4 producing cells. J. Exp. Med. 177:1199-1204.

Marston, J. A. 1861. Report on fever (Malta), Great Britain Army Med. Dept Rep. 3:486-521.

Mayhew M., Ana C. R. da Silva, J. Martin, H. Erdjument-Bromage, P. Tempst and F. Ulrich Hartl. 1996. Protein folding in the central cavity of the GroEL-GroES chaperonin complex. Nature. 379:420-426.

McCullough, N. B. 1970. Microbial and host factors in the pathogenesis of brucellosis, in Infectious Agents and Host Reactions, Mudd, S., Ed., W. B. Saunders, Philadelphia. 324.

Mehra, V., P. J. Brennan, and E. Rada. 1984. Lymphocyte suppression in leprosy induced by unique *M. Leprae* glycolipid. Nature. 308:194-196.

Meyer, K. F. and E. B. Shaw. 1920. A comparison of the morphologic, cultural and biochemical characteristics of *B. abortus* and *B. melitensis*: studies on the genus *Brucella*. J. Infect. Dis. 27:173.

Meyer, M. 1990. Current concepts on the taxonomy of the genus *Brucella* In: Animal brucellosis. Eds. Nielsen, K., Cuncan, J. R., pp.1-17. CRC Press, Boca Raton, Florida.

Mohler, J. R. 1914. Infectious abortion of cattle. Ann. Rep. US. Brv of Animal Industry. 1913-1914.

Montaraz J. A. and A. J. Winter. 1977. Brucellosis research: an evaluation. Report of the Subcommittee on Brucellosis Research, National Academy of Sciences. National Academy Press, Washington, D.C.

Montaraz, J. A., A. J. Winter, D. M. Hunter, B. A. Sowa, and L. G. Adams. 1986. Protection against *Brucella abortus* in mice with O-polysaccharide-specific monoclonal antibodies. Infect. Immun. 51:961.

Moreno, E., M. W. Pitt, L. M. Jones, G. G. Schurig, and D. T. Berman. 1979. Purification and characterization of smooth and rough lipopolysaccharides from *Brucella abortus*. J. Bacteriol. 138:361.

Moreno, E., S. L. Speth, L. M. Jones, and D. T. Berman. 1981. Immunochemical characterization of *Brucella* lipopolysaccharide and polysaccharides. Infect. Immun. 31:214-222.

Moretta, L., S. R. Webb., and C. F. Grossi. 1979. Functional analysis of two human T-cell subpopulations: help and suppression of B-cell responses by T cell bearing receptors for IgM or IgG. J. Exp. Med. 146:184-200.

Nicoletti, P. 1980. The epidemiology of bovine brucellosis. Adv. Vet. Sci. Comp. Med. 24:69-98.

Nicoletti, P. 1990. Vaccination. In: Animal brucellosis. Eds. Nielsen, K., Duncan, J. R., pp. 283-290. CRC Press, Boca Raton, Florida.

Nicoletti, P. and F. W. Milward. Protection by oral administration of *Brucella abortus* strain 19 against oral challenge exposure with a pathogenic strain of *Brucella*. Am. J. Vet. Res. 44:1641-1643.

Pearce, J. H., A. E. Williams, P. W. Harris-Smith, R. B. Fitzgeorge, and H. Smith. 1962. The chemical basis of the virulence of *Brucella* abortus. II. Erythritol, a constituent of

bovine fetal fluids which stimulates the growth of *B. abortus* in bovine macrophages. Br. J. Exp. Pathol. 43:631.

Perry, M. B., D. R. Bundle, and J. W. Cherwonogrodsky. 1986. The structure and serology of the A and M antigens of *Brucella abortus* and *Brucella melitensis* and the structure of the polysaccharide B of *Brucella* species. XIV International Congress of Microbiology (Manchester, England), Abstract p. G7-4.

Plommet, M. and A. M. Plommet. 1983. Immune serum-mediated effects on brucellosis in mice. Infect. Immun. 41:97-105.

Pomales-Lebron, A. and W. R. Stinebring. 1957. Intracellular multiplication of *Brucella abortus* in normal and immune mononuclear phagocytes. Proc. Soc. Exp. Biol. Med. 94:78.

Ralston, D. J. and S. S. Elberg. 1971. Serum-mediated immune cellular responses to *Brucella melitensis*. VII. The separation and assay of serum globulins responsible for macrophage stimulation and *Brucella* inhibition. J. Infect. Dis. 123:507-518.

Riley, L. K. and D. C. Robertson. 1984. Ingestion and intracellular survival of *Brucella abortus* in human and bovine polymorphonuclear leukocytes. Infect. Immun. 46:224-230.

Rook, G. A. W. 1988. The role of vitamin D in tuberculosis (Editorial). Am. Rev. Resp. Dis. 138:768-770.

Rook, G. A. W. and J. L. Stanford. 1995. Adjuvants, endocrines and conserved epitopes; factors to consider when designing 'therapeutic vaccines'. Int. J. Immunopharmac. 17:91-102.

Ruiz-Castaneda, M. 1954. Brucellosis. 2nd eds. Prensa Medica Mexicana, Mexico. pp.183-204.

Santos, J. M., D. R. Verstreate, V. Y. Perera, and A. J. Winter. 1984. Outer membrane proteins from rough strains of four *Brucella* species. Infect. Immun. 34:1000.

Sarram, M., J. Feiz, M. Foruzandeh, and P. Gazanfrpour. 1974. Intrauterine fetal infection with *Brucella melitensis* as a possible cause of second trimester abortion. Am. J. Obstet. Gynecol. 119:657.

Schultz, R. M. 1980. Macrophage activation by interferons. Lymphokine Rep. 1:63-97.

Schurig, G. G., L. M. Jones, S. L. Speth, and D. T. Berman. 1978. Antibody response to antigens distinct from smooth lipopolysaccharide complex in *Brucella* infection. Infect. Immun. 21:994-1002.

Schurig, G. G., R. M. Roop, T. Bagchi, S. Boyle, D. Buhrman, N. Sriranganathan. 1991. Biological properties of RB51; A stable rough strain of *Brucella abortus*. Vet. Microbiol. 28:171-188.

Shinnick, T. M., M. H. Vodkin, and J. C. Williams. 1988. The *Mycobacterium tuberculosis* 65 kilodalton antigen is a heat shock protein, which corresponds to common antigen and to the *Escherichia coli* GroEL protein. Infect. Immun. 56:446-451.

Silva, C. L., M. F. Silva, L. R. Pietro and D. B. Lowrie. 1994. Immunology. 83:341-346.

Smith, H., J. Keppie, H. Pearce, R. Fuller, and A. E. Williams. 1961. The chemical basis of the virulence of *Brucella abortus*. I. Isolation of *B. abortus* for bovine fetal tissue. Br. J. Exp. Pathol., 42:631.

Smith, H. and R. B. Fritzgeorge. 1964. The chemical basis of the virulence of *Brucella abortus*. V. The basis of intracellular survival and growth in bovine phagocytosis. Br. J. Exp. Pathol. 45:174-186.

Smith L. D. and T. A. Ficht. 1990. Pathogenesis of *Brucella*. Crit. Rev. Microbiol. 17:209-230.

Spink, W. W. 1956. The Nature of Brucellosis. University of Minnesota Press. Minneapolis. 112.

Splitter, G. A., and K. M. Everlith. 1986. Collaboration of bovine T lymphocytes and macrophages in T-lymphocyte response to *Brucella abortus*. Infect. Immun. 51:776.

Tabatabai, L. B. and B. L. Deyoe. 1984. Characterization of salt extractable protein antigens from *Brucella abortus* by crossed immunoelectrophoresis and isoelectric focusing. Vet. Microbiol. 9:549-560.

Thimm B. M. 1982. Brucellosis-distribution in Man, Domestic and Wild Animals. *Springer-Verlag Berlin, Heidelberg, New York.*

Verger, J., F. Grimont, and P. Grimont. 1985. *Brucella abortus* monospecific genes as shown by deoxyribonucleic acid hybridization. Int. J. Syst. Bacteriol. 35:292-295.

Verger, J., F. Grimont, P. Grimont, and M. Grayon. 1985. *Brucella*, a monospecific genus as shown by deoxyribonucleic acid hybridization. Int. J. Syst. Bacteriol. 35:292.

Verstreate, D. R., M. T. Creasy, N. T. Caveney, C. L. Baldwin, M. W. Blau, and A. J. Winter. 1982. Outer membrane proteins of *Brucella abortus*: isolation and characterization. Infect. Immun. 56:2953.

Verstreate, D. R., and A. J. Winter. 1984. Comparison of sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles and antigenic relatedness among outer membrane proteins of 49 *Brucella abortus* strains. Infect. Immun. 46:182-187.

Wang, L. I. and C. E. Frash. 1984. Development of a *Neisseria meningitidis* group B serotype 2b protein vaccine and evaluation in a mouse model. Infect. Immun. 46:408.

White, R. G. 1978. Immunoglobulin profile of the chronic antibody response: discussion in relation to brucellosis infections. Postgrad. Med. J. 54:595-602.

Wilkinson, P. C. 1966. Immunoglobulin pattern of antibodies against *Brucella* in man and animals. J. Immunol. 96:457-463.

Wilkinson, P. C. 1980. Leukocyte locomotion and chemotaxis: effects of bacteria and viruses. Rev. Infect. Dis. 2:293.

Winter, A. J., G. E. Howe, J. R. Duncan, M. J. Eis, J. Widom, B. Ganem, and B. Morein. 1988. Effectiveness of natural and synthetic complexes of porin and O polysaccharide as vaccines against *Brucella abortus* in mice. Infect. Immun. 56:3251.

Young, E. J., M. Borchert, F. L. Kreutzer, and D. M. Musher. 1985. Phagocytosis and killing of *Brucella* by human polymorphonuclear leukocytes. J. Infect. Dis. 151:682-690.

Zinneman, H. H., H. Glenchur, and W. H. Hall. 1959. The nature of blocking antibodies in human brucellosis. J. Immunol. 83:206-212.

CHAPTER 2.

CLONING AND EXPRESSION OF *BRUCELLA ABORTUS* HEAT SHOCK PROTEINS BY BACULOVIRUS RECOMBINANTS

INTRODUCTION

Bacterial heat shock proteins (HSP) are strong candidates for immunologic investigation because they are immunodominant targets of both the humoral and cellular immune responses (Hansen et al., 1988; Hindersson et al., 1987; Lin et al., 1992; Shinnick et al., 1988; Williams, J. C., 1990).

B. abortus GroEL and GroES are members of the family of hsp60 and hsp10 homologs, respectively. These proteins, of which *E. coli* GroEL and GroES are the archetypes, are involved in chaperone functions in the cell (Ellis and van der Vies, 1991). The chaperonin GroEL can mediate protein folding in its central cavity. GroEL-bound dihydrofolate reductase assumes its native conformation when the GroES cofactor caps one end of the GroEL cylinder, thereby discharging the unfolded polypeptide into an enclosed cage (Mayhew et al., 1996). GroES, the critical cofactor for GroEL in protein folding, is a heptameric ring of 10 kDa subunits. Under most conditions GroES forms an asymmetric complex with GroEL by capping one end of the GroEL cylinder.

Recently, this major bacterial heat shock protein, GroEL, has attracted considerable attention because of its unusual immunogenicity (Young and Elliott, 1989) and the potential for inducing immune protection against unrelated bacterial infections (Young et al., 1988; Shinnick et al., 1988). The immunodominance of *B. abortus* GroEL may be related to its high level of expression during infection of macrophages (Lin and Ficht, 1995). Vaccination with GroEL proteins induces protective immunity against intracellular pathogens, such as *Mycobacterium tuberculosis* (Silva et al., 1994) and *Legionella pneumophila* (Blander and Horwitz, 1992).

The ability of *Legionella pneumophila* Hsp60 to protect against Legionnaires' disease has been examined in a guinea pig model (Blander and Horwitz, 1992). Immunization of guinea pigs using purified *L. pneumophila* Hsp60 with Freund's adjuvant protects the animals from a lethal aerosol challenge with the organism. Cell-mediated immunity is critical to the host defense against intracellular *L. pneumophila* and animals immunized with purified *L. pneumophila* Hsp60 exhibited delayed-type hypersensitivity (DTH) to Hsp60, indicating that cellular responses had been elicited by the experimental vaccine (Horwitz, M. A., 1983).

Much less is known regarding the potential of the GroES protein to serve as a protective immunogen. However, Ferrero et al. (1995) did show GroES homolog confers immunity against mucosal infection in mice by *Helicobacter pylori*.

Members of the high-temperature requirement A (HtrA) class of stress response proteins are serine proteases which apparently function by degrading oxidatively or otherwise damaged proteins before they can accumulate to toxic levels in cells (Canning,

P., 1990; Enright, F. M., 1990; Johnson et al., 1991; Lipinska et al., 1989). Biochemical and genetic evidence indicates that these proteins represent important components of the cell's defenses against oxidative damage (Elzer et al., 1994). Oxidative killing pathways are generally thought to be the primary mechanism by which host phagocytes kill intracellular brucella (Baldwin and Winter, 1994; Enright F. M., 1990), thus the HtrA protein of brucella may contribute significantly to the successful survival in host phagosomes. This biochemical and genetic evidence is supported by the observation that *Salmonella typhimurium* HtrA mutants show significant increases in sensitivity to oxidative killing *in vitro* in comparison to wild-type strains (Cheers, C., 1983) and fail to replicate in cultured macrophages (Alton, G. G., 1990).

In this regard, we speculated that the three types of *Brucella abortus* HSPs, namely GroEL, GroES and HtrA may be capable of inducing cell-mediated and protective immunity and could be good candidates as protective antigens for the development of possible subunit vaccines.

The major obstacle to such studies of *Brucella* proteins is the difficulty in purification of large quantities of the protein due to the low concentrations of this protein in the microorganism and the inherent pathogenicity of this microorganism. Isolation of HSPs directly from the pathogen is a tedious as well as hazardous process yielding minuscule amounts of purified protein. A solution to these problems is the overexpression of the *B. abortus* HSPs in a heterologous system. In our laboratories, various approaches for expression of *B. abortus* HSPs are currently being employed. First, vaccinia virus is used as a cloning/expression vehicle to induce immune response to

B. abortus HSPs because this vector has numerous advantages including wide host range and stable recombinants. However, there are two major potential problems in the use of vaccinia virus recombinants as vaccines. First, since vaccinia virus is replication competent (live vaccine), the host must be immunologically competent. Second, since vaccinia virus proteins are highly immunogenic, vaccinia virus can most likely only be given one or two times since the induction of high titers of anti-vaccinia antibody responses which will neutralize subsequent doses of virus, thus reducing recombinant gene expression (Cooney et al., 1993; Etlinger and Altenburger, 1991; Kundig et al., 1993). Also, *B. abortus* HSPs have been successfully expressed in *E. coli* system (Roop R. M. et al., 1992; Roop R. M. et al., 1994) which can offer simplicity, short generation times, and large yields of product with low costs. But expression in prokaryotic cells has several possible drawbacks in obtaining purified protein: (1) they can have quality problems due to contamination by *E. coli* lipopolysaccharides; and (2) they often fail to fold properly and hence form insoluble inclusion bodies (Alberts et al., 1994). Protein extracted from these inclusion bodies is often biologically inactive. Therefore, we explored the utility of baculovirus as a potentially powerful eukaryotic expression system for the overexpression of the *B. abortus* HSPs. The rational was that baculovirus system (1) is capable of expressing very high levels of recombinant proteins because of very strong promoters for the polyhedrin gene, (2) can yield highly pure and biologically active recombinant proteins by avoiding lipopolysaccharide contamination and inclusion body production, respectively, and (3) is safe because baculovirus is non-pathogenic to mammals (O'Reilly et al., 1992; Luckow et al., 1993). This safe method should enable us

to obtain purified products of high quality and in high yields for functional analysis of *B. abortus* immunology.

Protein expression systems based on the *Autographa californica* baculovirus have wide applicability as an alternative to prokaryotic or other eukaryotic expression systems. The system is based on constructing recombinant baculovirus by replacing the polyhedrin gene with foreign DNA and then using this virus as a vector for the infection of insect cells. A wide range of foreign genes has been expressed using baculovirus vectors. The use of baculovirus as a gene expression vector has increased over the past few years (Makoff, A. J. et al., 1989; Miller, L. K., 1988; Young K. C., 1988).

In this study, we explored the use of the baculovirus cloning system for expression of *B. abortus* heat shock proteins to generate pure recombinant proteins as the first step in analyzing the potentials of developing bacterial subunit vaccines in eukaryotic cloning/expression systems.

MATERIALS AND METHODS

1. BAC-TO-BAC™ baculovirus expression system.

The BAC-TO-BAC™ Baculovirus Expression System (GibcoBRL, Grand Island, NY), a rapid and efficient method to generate recombinant baculoviruses (Luckow et al., 1993) was used. It is based on site-specific transposition of an expression cassette into a baculovirus shuttle vector (Bacmid) propagated in *E. coli.* The Bacmid contains the low-copy-number mini-F replicon, a kanamycin resistance marker, and a segment of DNA

encoding the lacZα peptide from a pUC-based cloning vector. The Bacmid propagates in *E. coli* DH10BAC cells as a large plasmid that confers resistance to kanamycin and can complement a lacZ deletion present on the chromosome to form colonies that are blue in the presence of a chromogenic substrate such as Bluo-gal or X-gal and the inducer IPTG. Recombinant Bacmids were constructed by transposing a mini-Tn7 element from a donor plasmid (pFASTBAC1) to the mini-attTn7 attachment site on the Bacmid when the Tn7 transposition functions are provided in trans by a helper plasmid in the cells. The mini-Tn7 in pFASTBAC1 contains an expression cassette consisting of a gentamycin resistance gene, the polyhedrin promoter from AcNPV (*Autographa californica* nuclear polyhedrosis virus), a multiple cloning site, and an SV40 poly(A) signal inserted between the left and right arms of Tn7. Genes to be expressed are inserted into the multiple cloning site of pFASTBAC1 downstream from the polyhedrin promoter. Insertions of the mini-Tn7 into the mini-attTn7 attachment site on the Bacmid disrupts expression of the lacZα peptide, so colonies containing the recombinant Bacmid are white in a background of blue colonies that harbor the unaltered Bacmid (Fig. 1). Using site-specific transposition to insert foreign genes into a Bacmid propagated in *E. coli* has a number of advantages over generation of recombinant baculovirus in insect cells by homologous recombination, which has been traditionally used. Recombinant virus DNA isolated from selected colonies is not mixed with parental, non-recombinant virus, eliminating the need for multiple rounds of plaque purification, which is the routine process in the traditional baculovirus system.

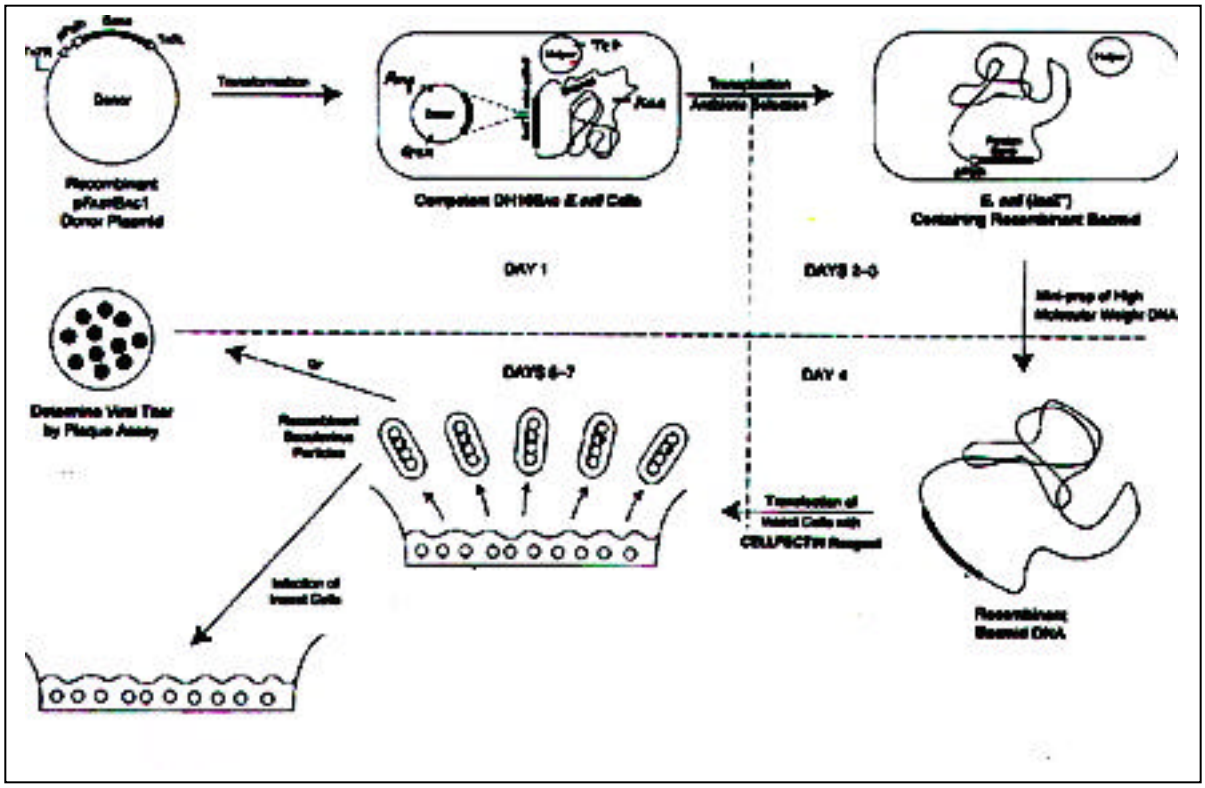

Figure 1. Generation of recombinant baculovirus and gene expression with the BAC-TO-BAC Expression System (Gibco-BRL).
B. abortus groEL, groES, or *htrA* gene was cloned into pFASTBAC1, and generated respective recombinant protein in insect cells.

2. Bacteria.

E. coli strains were grown in Luria-Bertani (LB) broth for routine cultivation (Sambrook et al., 1989). Competent *E. coli* DH5α cells were used to generate mini-preparations of plasmids. Competent *E. coli* DH10BAC cells were used for transformation of pFASTBAC recombinant donor plasmids to generate Bacmid (baculovirus shuttle vector) recombinant DNA. Antibiotics were used in growth media or plates for maintenance of Bacmid recombinant containing *E. coli* DH10BAC cells at the following concentrations: ampicillin (Amp), 100 μg/ml; gentamycin (Gen), 7 μg/ml; kanamycin (Kan), 50 μg/ml; tetracycline (Tet), 10 μg/ml.

3. Cells.

Sf9 cells, derived from *Spodoptera frugiperda* ovarian cells, were used for wild-type baculovirus infection and recombinant protein expressions. Serum free medium-adapted Sf9 cells were obtained and propagated in Sf900-SFM (serum-free media) (GibcoBRL, Grand Island, NY) and used in protein expression experiments. Cells were grown at 28^{o}C in adherent culture in the beginning stage or in suspension culture after adjustment, and maintained at more than 95 % cell viability and a maximum of 2 X 10^{6} cells/ml.

4. Development of recombinants.

4.1 Generation of *B. abortus* - baculovirus recombinants.

4.1.1 Preparation of competent *E. coli* cells.

With the following procedure, developed by Hanahan (1983), *E. coli* strain DH5α competent cultures can yield transformed colonies at frequencies of more than 5 X 10^8 per microgram of supercoiled plasmid DNA. *E. coli* strain DH5α was streaked directly from a frozen stock onto the surface of an SOB agar plate. The plate was incubated overnight at 37°C. Several colonies were transferred into 1 ml of SOB containing 20 mM $MgSO_4$. The bacteria were dispersed by vortexing at moderate speed, and diluted in 100 ml of SOB media (bacto-tryptone 20g, bacto-yeast extract 5g, NaCl 0.5g, 10 ml of a 250 mM solution of KCl, adjusted with dH_2O to 1 liter), and 5 ml of 2 M $MgCl_2$ containing 20 mM $MgSO_4$ in a 1-liter flask. The cells were grown for several hours at 37°C until the optical density of $(OD)_{600}$ was reached. The cultures were transferred to two sterile, cold 50 ml polypropylene tubes (Falcon 2070) and cooled by storing the tubes on ice for 10 minutes and centrifuged at 4000 rpm for 10 minutes at 4°C. The supernatant was discarded, the extra traces of media were evaporated under the hood, each pellet was resuspended in 5 ml of cold 50 mM $CaCl_2$, and the resuspended cells were stored on ice for 1 hr. $CaCl_2$-treated cells were centrifuged at 4000 rpm for 10 minutes at 4°C. The buffer was removed and the pellet was dried. The cells in each tube were gently resuspended in 5 ml of cold 50 mM $CaCl_2$ solution and kept on ice for 30 minutes. The cells were centrifuged as before and each pellet was resuspended in 2 ml of cold $CaCl_2$ solution. The cell suspensions were pooled into a tube. The contents were mixed very well and dispensed into prechilled sterile microfuge tubes into 200 µl aliquots. The tubes were kept on ice for 2 hrs and then moved into -70°C freezer.

4.1.2 Transformation.

Plasmid pMB*groELS* containing *B. abortus groEL* and *groES* genes and plasmid pBA32 containing *B. abortus htrA* gene were obtained from Dr. G. Schurig's (College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA). Baculovirus donor plasmid pFASTBAC was purchased from Gibco-BRL (Grand Island, NY). These plasmids were transformed into competent *E. coli* DH5α cells for mini-preparation of plasmid DNA (Sambrook et al., 1989). One hundred µl of $CaCl_2$-treated *E. coli* 5α competent cells was moved into a pre-chilled Falcon 2059 tube. Ten ng of each the above plasmids were added to the cells and stored on ice for 30 minutes. The contents were heat shocked for 45 seconds in a 42^{o}C waterbath. The tubes were rapidly transferred into ice and chilled for 2 minutes. One ml of SOC media (SOB media plus 20 mM glucose) was added to the tube and the culture was shaken at 37^{o}C for 45 minutes to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. One hundred µl of transformed competent cells were transferred and spread onto a SOB agar plate containing 20 mM $MgSO_4$ and the appropriate antibiotic. The plates were left under the hood until the liquid had been absorbed, inverted and incubated at 37^{o}C for overnight. The transformed colonies were observed the next morning and kept at 4^{o}C until used.

4.1.3 Mini-preparations of plasmid DNA.

Each single transformed bacterial colony (4.1.2) (pMB*groEL*S, pBA32, or pFASTBAC containing *E. coli* cells) was transferred into 20 ml of Luria-Bertani (LB)

medium containing appropriate antibiotic(s) in a loosely capped 200 ml flask. The culture was incubated overnight at 37^{o}C with vigorous shaking. The culture was dispensed into microfuge tubes with 1.5 ml aliquots and the tubes were centrifuged at 12,000 g for 30 seconds at 4^{o}C. The medium was removed and the bacterial pellet was dried under the hood. The bacterial pellet was resuspended in 100 µl of cold Solution I (50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA pH 8.0) in order to lyse bacterial cells by vigorous vortexing. Two hundred µl of freshly prepared Solution II (0.2 N NaOH, 1% SDS) was added to denature DNA and the contents were mixed by inverting the tube rapidly five times. The tubes were stored on ice for few minutes. One hundred fifty µl of cold Solution III (60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid, 28.5 ml of H_2O) was added in order to keep single stranded genomic DNA and the contents were vortexed shortly. The tubes were stored on ice for 3-5 minutes. The tubes were centrifuged at 12,000 g for 5 minutes at 4^{o}C and the supernatant was transferred to a fresh tube. Equal volume of phenol:chloroform was added and mixed by vortexing. After centrifuging at 12,000 g for 2 minutes at 4^{o}C, the supernatant was transferred to a fresh tube. The double-stranded DNA was precipitated with two volumes of ethanol at room temperature and mixed by vortexing. The mixture was left for 2 minutes at room temperature, and centrifuged at 12,000 g for 5 minutes at 4^{o}C. The supernatants were removed and the tubes were stood in an inverted position on a paper towel to allow all of the fluid to drain away. The pellet of double-stranded DNA was rinsed with 1 ml of 70% ethanol at 4^{o}C. The supernatants were removed and the pellets

of nucleic acids dried under the hood for 10 minutes. The nucleic acids were redissolved in 50 µl of TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) containing DNAase-free pancreatic RNAase (20 µg/ml). The contents were vortexed briefly and the DNA was stored at -20^{o}C (Sambrook et al., 1989).

4.1.4 Subcloning of *B. abortus* genes into baculovirus vector.

Brucella abortus-pFASTBAC1 recombinants were generated for the following *B. abortus* heat-shock proteins: the 60 kDa molecular chaperon GroEL (Roop et al., 1992), the 10 kDa GroES, and the 60 kDa protein HtrA (Roop et al., 1990; Roop et al., 1992; Roop et al., 1993), as follows. A 2.1 kb size *groEL* fragment was isolated from plasmid pMB*groELS* by restriction enzyme (RE) double digestions with *Eco*R I, *Kpn* I and ligated into the same RE sites of the 4.8 kb pFASTBAC1. The digestions using the REs as above identified the *groEL*-pFASTBAC recombinants (6.9 kb). A 0.4 kb size *groES* fragment was isolated from plasmid pMB*groELS* by double digestions with *Bam*H I, *Eco*R I and ligated into the same RE sites of the 4.8 kb pFASTBAC1. The digestions using the REs as above identified the *groES*-pFASTBAC recombinants (5.2 kb). A 1.9 kb size *htrA* fragment was isolated from plasmid pBA32 by single digestion with EcoR I and ligated into the same RE site of pFASTBAC1 in frame. The digestion using the RE as above identified the *htrA*-pFASTBAC recombinants (6.7 kb).

4.1.5 Southern blotting.

RE single or double-digested DNAs were transferred by capillary action from 0.7 % agarose gels to nylon membranes (Micron Separations Inc., Westboro, Mass) using

20X saline sodium citrate (SSC; 3 M NaCl, 300 mM sodium citrate, pH 7) as previously described (Sambrook et al., 1989; Southern, E. M., 1975). DNAs were covalently linked to nylon membranes by ultraviolet irradiation using a UV Stratalinker (Stratagene, La Joll, CA). The blot was incubated in prehybridization solution (5X SSC, 1 % (w/v) blocking reagent for nucleic acid hybridization, 0.1 % N-lauroylsarcosine, 0.2 % sodium dodecyl sulfate) for 1 hr at 68^{o}C for probe hybridization by blocking non-specific nucleic acid binding sites on the membrane. Digoxigenin-labeled probes were prepared for DNA hybridizations according to the manufacturer's directions (Boehringer-Maninheim Corp., Indianapolis, IN). The appropriate *groEL*, *groES*, or *htrA* fragments were obtained from NuSieve low-melting temperature agarose gels (FMC BioProduct, Rockland, ME). Double digestions of pMB*groELS* with *Eco*R I and *Kpn* I yielded the *groEL* fragment, double digestions with *Bam*H I and *Eco*R I yielded *groES* fragment, and a single digestion of pBA32 with *Eco*R I yielded *htrA* fragment. The desired fragments were isolated from the Nusieve gels after mixed with DNA isolation binding buffer (Bio-Rad, Hercules, CA) and spun down using a spin column (Promega, Madison, WI). The eluant was run on 1 % agarose gel to check the recovery. Hexanucleotide mixture, dNTP labeling mixture, and labeling-grade Klenow enzyme (Boehringer-Mannheim Corp., Indianapolis, IN) were mixed with the DNA. The reaction tube was incubated at 37^{o}C overnight. The digoxigenin labeled DNA probe was denatured before addition to the prehybridization solution, and the membrane was hybridized at 68^{o}C for overnight. The membranes were washed and developed with the colorimetric detection reagent NBT (nitroblue tetrazolium

salt in 70 % (v/v) dimethylformamide) solution and X-Phosphate according to the Genius System directions (Boehringer-Mannheim Corp., Indianapolis, IN).

4.2 Transposition and selection.

pFASTBAC recombinant donor plasmid was transformed into competent *E. coli* DH10BAC cells containing Bacmid and helper plasmid as described above (4.1.2). Recombinant Bacmids were constructed by transposing a mini-Tn7 element from a donor plasmid pFASTBAC1 to the mini-attTn7 attachment site on the Bacmid when the Tn7 transposition functions were provided in trans by a helper plasmid, which contains the tetracycline resistance gene. Due to the *lac*Z gene disruption during the transposition, colonies containing the recombinant Bacmid are white in a background of blue colonies that harbor the unaltered Bacmid. White colonies were selected on Luria Agar (GibcoBRL) plates containing 100 μg/ml ampicillin, 50 μg/ml kanamycin and 7 μg/ml gentamycin, 10 μg/ml tetracycline, 300 μg/ml bluo-gal, and 40 μg/ml IPTG.

4.3 Isolation of recombinant Bacmid DNA.

Before isolating DNA from colonies, white colonies were streaked on Luria-Bertani agar (GibcoBRL) plates once again to ensure the validity of the color. Then, recombinant Bacmid DNA were isolated by a special protocol which was developed for purifying large plasmids (>100 kb) and adapted for isolating Bacmid DNA (Gibco BRL, Instruction Manual of BAC-TO-BAC Baculovirus Expression System, 1995). The major steps are the same as in regular mini-prep, described above (4.1.3), with a few differences. RNAase A was included in Solution I to remove RNA because RNA interacts with

CELLFECTIN (Gibco BRL) causing a reduction of the efficiency for the subsequent transformation. Also, a low concentration of NaOH was used in Solution II to support a less harsh environment for DNA and maintain intact Bacmid DNA.

4.4 Identification of recombinant Bacmid DNA.

The presence of recombinant Bacmid DNA was indicated by the appearance of a band that migrated more slowly than the 23.1 kb fragment of the λ/Hind III markers when the undigested recombinant Bacmid DNA mini-prep was loaded on 0.5 % agarose gel according to the BAC-TO-BAC baculovirus expression system (Gibco BRL). Recombinant Bacmid DNAs were digested with the appropriate restriction enzymes as above (4.1.5) and used for Southern blotting to confirm the presence of inserts in Bacmid DNA.

4.5 Transfection of Sf9 Cells with recombinant Bacmid DNA.

S. frugiperda (Sf9) cells were transfected with the recombinant Bacmid DNA using CELLFECTIN (Gibco-BRL, Grand Island, NY) reagent as described (Inumaru and Yamada, 1991). CELLFECTIN- mediated transfection was carried out as follows: 9×10^5 cells were seeded per 35-mm well of a 6-well plate in a volume of 2 ml Sf-900 II SFM containing penicillin/streptomycin at 0.5X final concentration. Cells were allowed to attach at 27^{o}C for 3 hours. Then, two different solutions were prepared. For Solution A, 5 ml of mini-prep Bacmid DNA was diluted into 100 ml Sf-900 II SFM without antibiotics for each transfection. For Solution B, 6 ml of CELLFECTIN was diluted into 100 ml Sf-900 II serum-free medium without antibiotics. The two solutions were mixed

gently and incubated for 45 min at room temperature. For each transfection, 0.8 ml of Sf-900 II serum-free medium was added to each tube, containing the lipid-DNA complexes. After mixing, 1 ml of the diluted lipid-DNA complexes was overlaid onto the washed cells in the 6-well plate. For the negative control, no Solution A and B was added. For the positive control, cells were transfected with pFASTBAC-Gus-Bacmid, obtained from Gibco-BRL (Grand Island, NY). After incubation of the plate for 5 hours at 27^{o}C, the transfection mixture was removed from the well of experimental, and 2 ml of Sf-900 II serum-free media containing antibiotics was added. Cells were incubated at 27^{o}C for another 72 hours and morphological differences were observed between negative controls and experimental. Viruses were harvested 72 hours after transfection in the experimental plates, 2 ml of the supernatant was transferred to a sterile, capped tube. After a 5 min at 500 X g centrifugation, the virus-containing supernatant was transferred to a fresh tube. They were kept at -20^{o}C until needed.

4.6 Infection of insect cells with recombinant baculovirus.

To observe protein expression, 0.5 ml of viral stock was used to infect 50-ml cultures at 2 X 10^{6} cells/ml. The infected cells and resulting viruses were harvested at 48 hours post-infection.

5. Screening of recombinant baculoviruses by SDS-Polyacrylamide gel electrophoresis and Western blot analysis.

Polyacrylamide gel electrophoresis (PAGE) of infected cell lysates was performed on 10 % acrylamide-sodium dodecyl sulfate (SDS) gels. All cell lysates subjected to SDS-PAGE contained 5 x 10^5 cells per lane. The proteins on the gel were electrophoretically transferred using trans-blot (Bio-RAD, Hercules, CA) to nitrocellulose membrane (Micron Separations Inc., Westborough, MA) at 15 V for 30 min in a solution consisting of 25 mM Tris, 192 mM glycine, and 5 % (vol/vol) methanol. Polyacrylamide gels were either stained with Coomassie blue or transferred to nitrocellulose membranes for the Western blot analysis. For the Western blotting, the nitrocellulose membranes were incubated for overnight with 5 % (w/v) skim milk (Carnation, Glendale, CA) in TBS (0.15 M NaCl, 20 mM Tris), pH 7.4. The membranes were washed three times with TBS containing 0.05 % Tween 20 (TBS-tween 20), and each membrane was incubated with goat anti-*B. abortus* RB51, mouse monoclonal anti-*B. abortus* GroEL, goat monospecific anti-*B. abortus* GroES sera, and mouse monospecific anti-*B. abortus* HtrA, respectively, as a primary antibody. Mouse monoclonal anti-*B. abortus* GroEL antibody a gift from Dr. Mark Stevens in Iowa State University, and the remaining antibodies were supplied by Dr. G. Schurig at the Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University. After three washes with TBS-tween 20, the nitrocellulose membranes were incubated for 1 h with secondary antibody, which was either rabbit anti-goat or goat anti-rabbit immunoglobulins conjugated to horseradish peroxidase. The membranes were washed three times with TBS-tween 20. The bands were visualized by the addition of the color reagent made of 15 mg of 4-chloro-1-naphthol

(Sigma Chemical Co., St. Louis, MO) in 5 ml of methanol, combined with 25 ml of TBS containing 0.06 % hydrogen peroxide (Fisher Scientific, Fair Lawn, N.J).

RESULTS

1. Subcloning *B. abortus* genes into baculovirus vector.

1.1. *groEL* gene

The expected size of 6.9 kb *groEL*-pFASTBAC recombinant bands, composed of the 4.8 kb pFASTBAC1 vector and the 2.1 kb size *groEL* fragment, was identified by the restriction enzyme double digestions. The expected size of the bands was observed on agarose gel (Fig. 2 a).

1.2. *groES* gene

The expected size of 5.2 kb *groES*-pFASTBAC recombinant bands, consisting of the 4.8 kb pFASTBAC1 vector and the 0.4 kb size *groES* fragment, was identified by restriction enzyme double digestions. The expected size of the bands was observed on the agarose gel (Fig. 2 b).

1.3. *htrA* gene

The expected size of 6.7 kb *htrA*-pFASTBAC recombinant bands, consisting of the 4.8 kb pFASTBAC1 vector and the 1.9 kb size *htrA* fragment, was identified by restriction enzyme single digestion. The expected size of bands was observed on the agarose gel (Fig. 2 c).

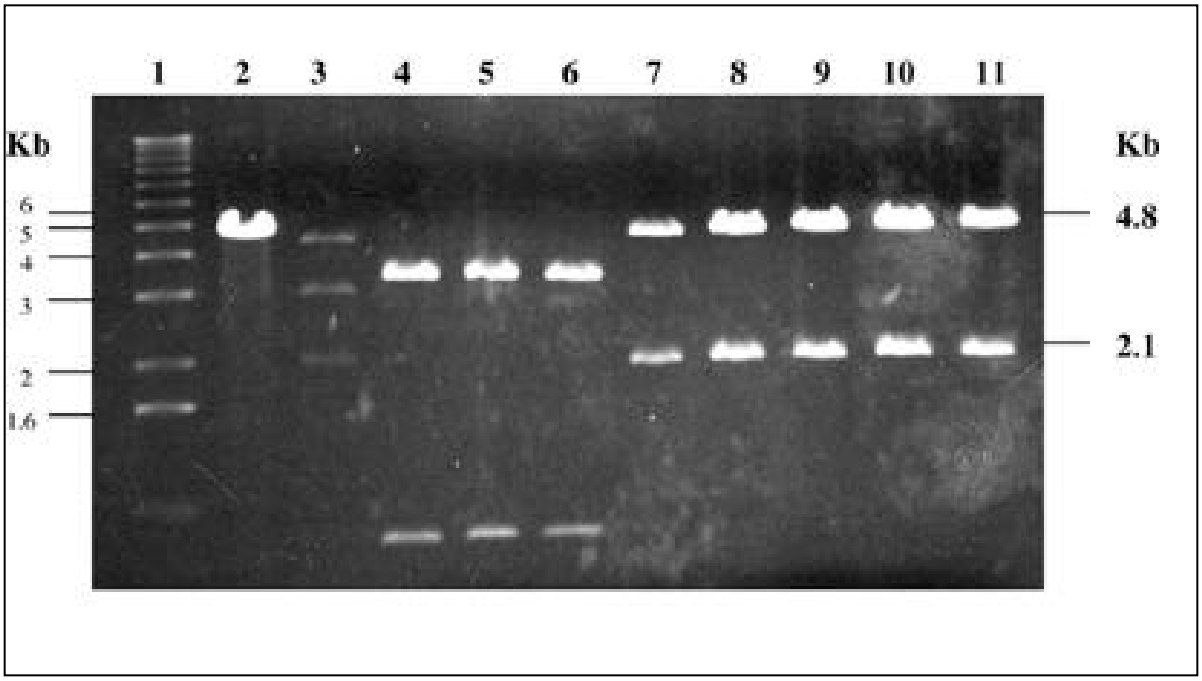

Fig. 2 (a). Gel electrophoresis for identification of *B. abortus groEL*-pFastBAC baculovirus recombinant plasmids.

Lane 1: One Kb molecular weight marker

Lane 2: 4.8 Kb pFastBAC vector, double-digested with *Eco*R I and *Kpn* I

Lane 3: pMB*groELS* parent plasmid for insertion, , double-digested with *Eco*R I and *Kpn* I

Lanes 4, 5 and 6: False positive recombinants

Lanes 7, 8, 9, 10 and 11: 4.8 Kb pFastBAC vector and 2.1 Kb *groEL* fragments of *groEL*-pFastBAC recombinants, double-digested with *Eco*R I and *Kpn* I

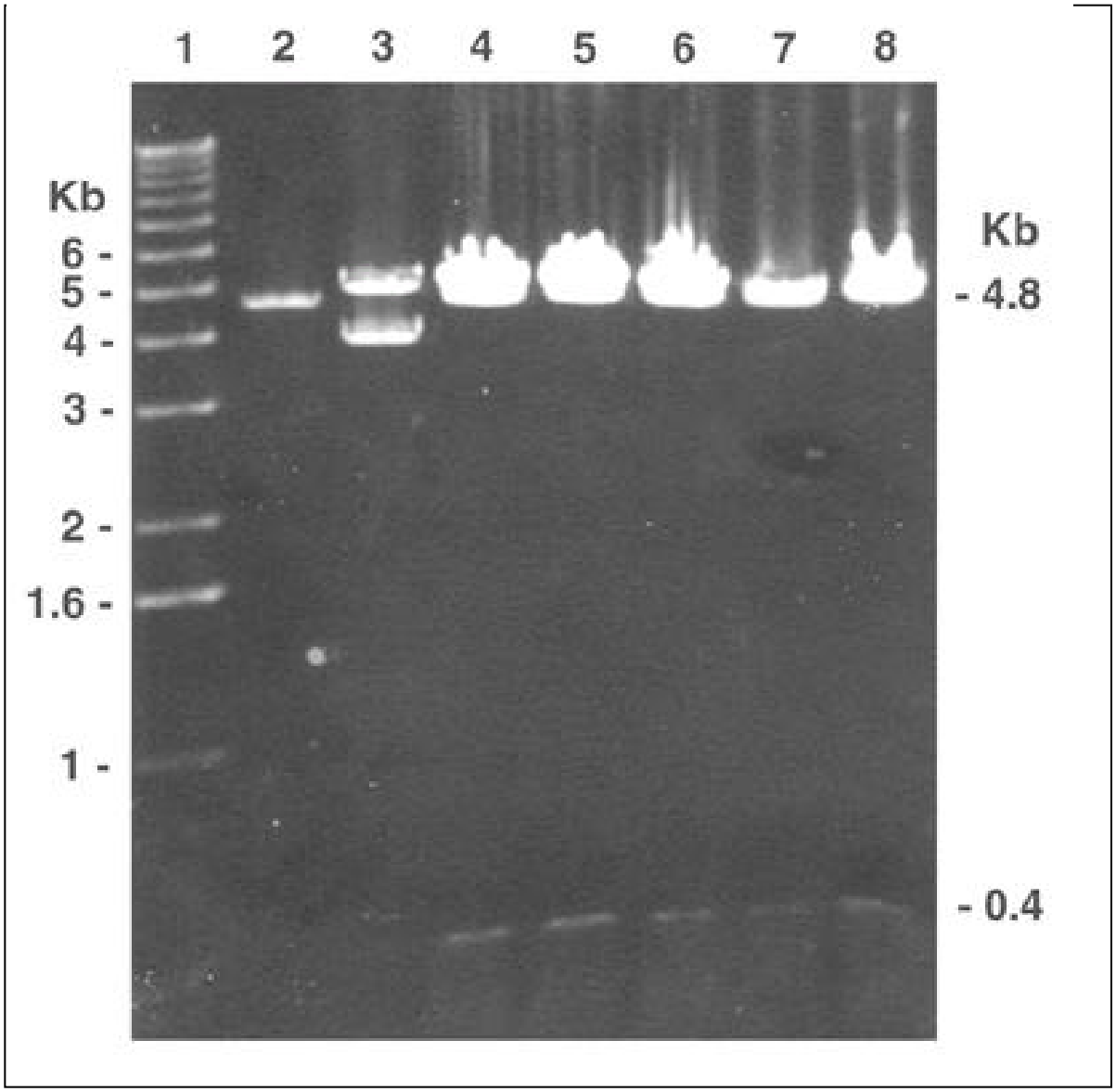

Fig. 2 b. Gel electrophoresis for identification of *B. abortus groES*-pFastBAC baculovirus recombinant plasmids.

Lane 1 : One Kb molecular weight marker

Lane 2 : 4.8 Kb pFastBAC vector, double-digested with *Bam*H I and *Eco*R I

Lane 3 : pMB*groELS* parent plasmid for insert, double-digested with *Bam*H I and *Eco*R I

Lanes 4, 5, 6, 7, and 8: 4.8 Kb pFastBAC vector and 0.4 Kb *groES* fragments of *groES*-pFastBAC recombinants, double-digested with *Bam*H I and *Eco*R I

2. Analysis of Bacmid DNA by colony hybridization, gel electrophoresis, and Southern blotting.

After each pFASTBAC recombinant was transformed into competent *E. coli* DH10BAC cells, white colonies were streaked from agar plates onto nitrocellulose membranes for colony hybridization, and the identity of the colonies containing recombinant Bacmid DNA was confirmed using digoxygenin-labeled specific DNA probes. White colonies containing the recombinant Bacmid DNA were selected for isolation of recombinant Bacmid DNA. The identity of recombinant Bacmid DNA was confirmed by the following two methods. The presence of recombinant Bacmids was detected on 0.5 % agarose gel through the appearance of a band that migrated more slowly than did the 23.1 kb fragment of the λ/Hind III markers (data not shown). The 2.1 Kb band *groEL*, 0.4 Kb band *groES*, or 1.9 Kb band *htrA* insert DNAs were identified by Southern blot to confirm the insert in each Bacmid recombinant (Figs. 3 a, b, c).

3. Transfection.

The morphology of Sf9 cells transfected with each recombinant Bacmid DNA was compared with both the negative control (untransfected cells) and the positive control (pFASTBAC-Gus Bacmid-transfected cells) under the microscope. The morphology of untransfected cells was circular without granules. Cells transfected with the recombinant Bacmid DNA and pFASTBAC-Gus Bacmid were elongated and had clear large granules.

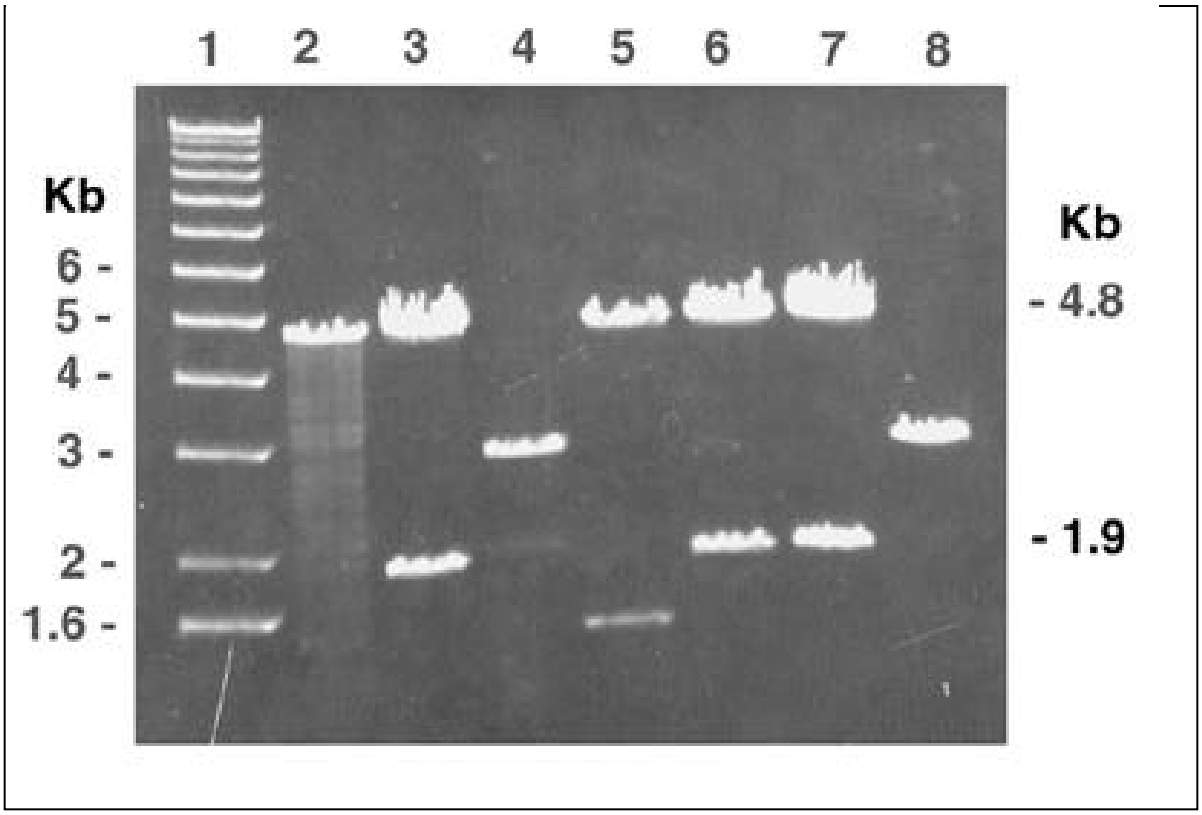

Fig. 2 c. Gel electrophoresis for identification of *B. abortus htrA*- pFastBAC baculovirus recombinant plasmids.
Lane 1: One Kb molecular weight marker
Lane 2: 4.8 Kb pFastBAC vector, single-digested with *Eco*R I
Lanes 3, 6 and 7: 4.8 Kb pFastBAC vector and 1.9 Kb *htrA* fragments of *htrA*-pFastBAC recombinants, single-digested with *Eco*R I
Lanes 4, 5 and 8: False positive recombinants

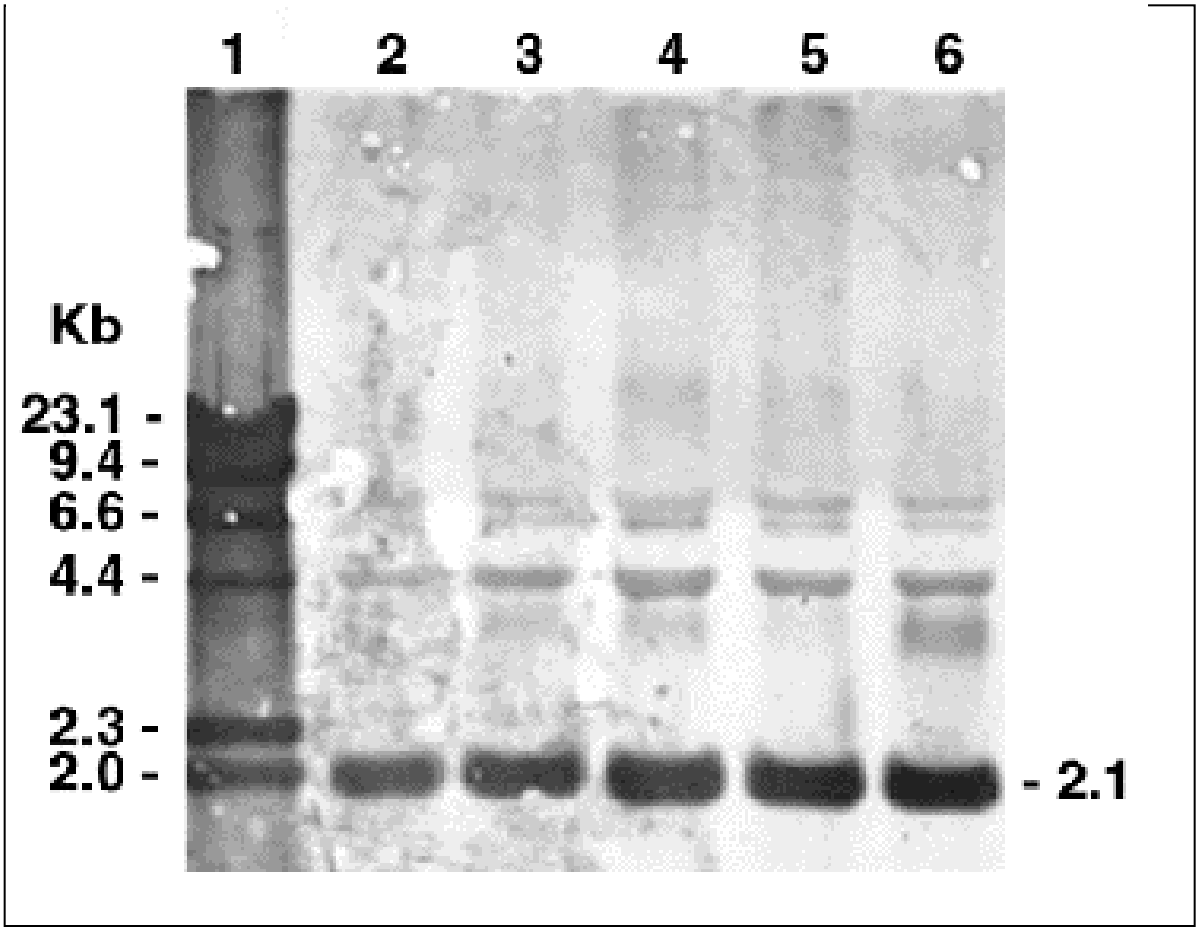

Fig. 3 a. Southern blotting for identification of *B. abortus groEL* insert from Bacmid (baculovirus shuttle vector) recombinants.

Lane 1: λ Hind III marker

Lanes 2, 3, 4, 5 and 6: 2.1 Kb *groEL* band from *B. abortus groEL*-Bacmid recombinant, double-digested with *Eco*R I and *Kpn* I

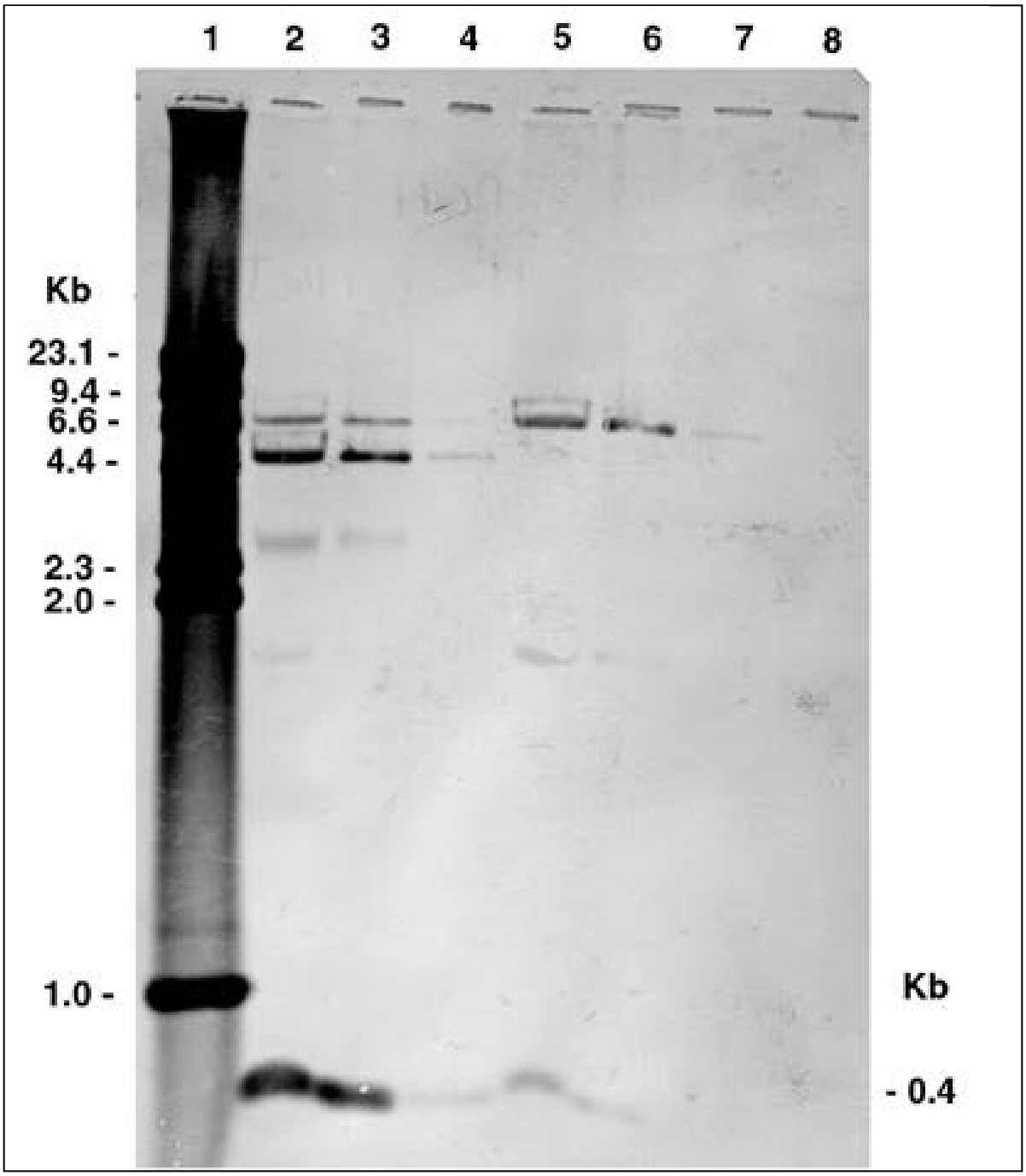

Fig. 3 b. Southern blotting for identification of *B. abortus groES* insert from Bacmid (baculovirus shuttle vector) recombinants.

Lane 1: λ Hind III marker

Lanes 2, 3, 4, 5 and 6: 0.4 Kb *groES* band from *B. abortus groES*-Bacmid recombinant, double-digested with *Bam*H I and *Eco*R I

Lanes 7 and 8: False positive recombinants

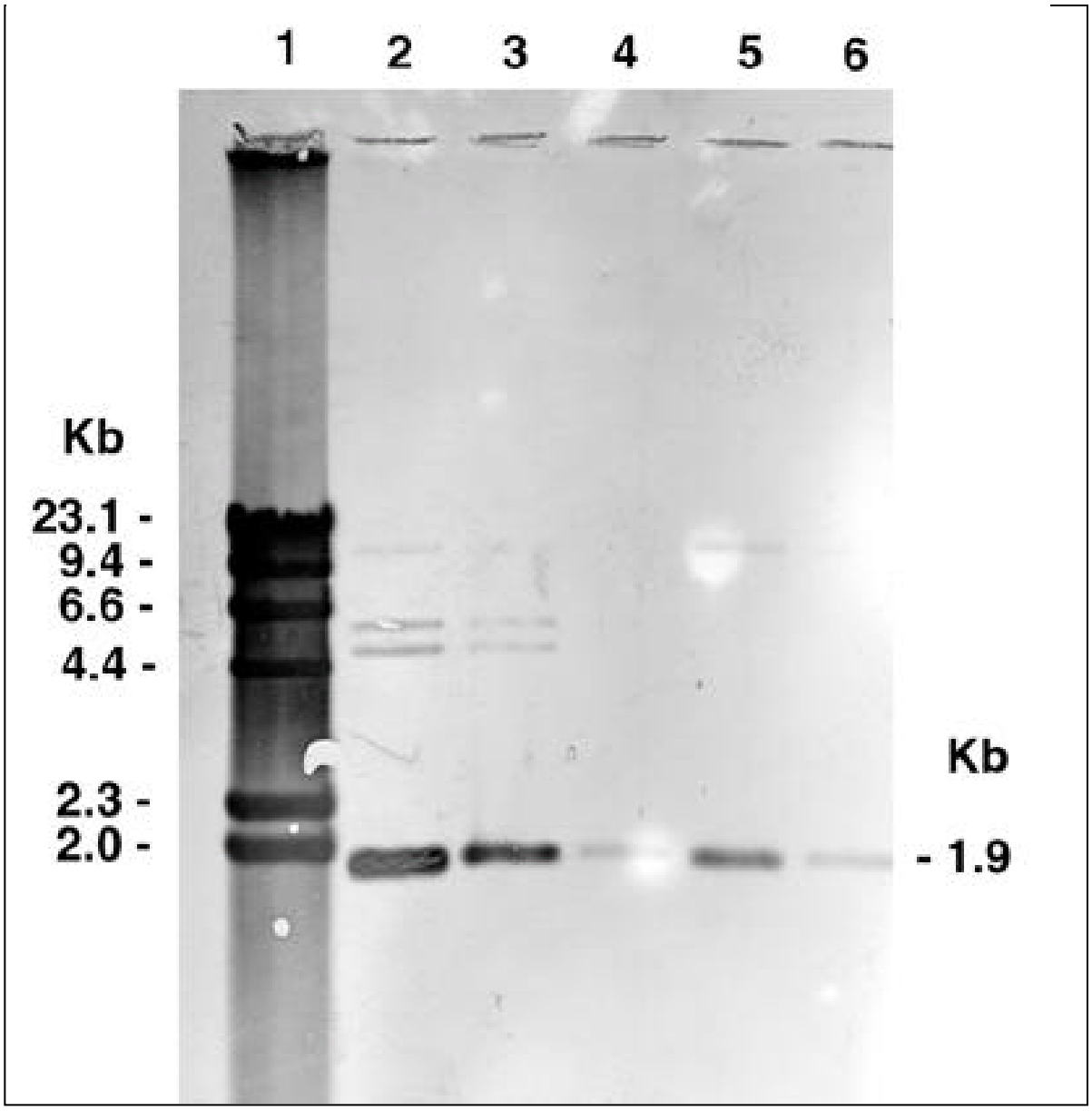

Fig. 3 c. Southern blotting for identification of *B. abortus htrA* insert from Bacmid (baculovirus shuttle vector) recombinants.

Lane 1: λ Hind III marker

Lanes 2, 3, 4, 5 and 6: 1.9 Kb *htrA* band from *B. abortus htrA*-Bacmid recombinant, single-digested with *Eco*R I

inside the cells 48 h after infection. The recombinant virus-containing supernatant was harvested and used for the infection of Sf9 cells.

4. Infection of Sf9 cells with recombinant virus and demonstration of recombinant protein expression.

Large numbers of Sf9 cells were infected with wild-type baculovirus (Fig. 4 a) or recombinant baculovirus (Fig. 4 b), and the morphological differences were compared with uninfected cells (Fig. 4 c). Wild-type baculovirus-infected cells showed very distinct morphology carrying polyhedra, which appears as dark granules, whereas the recombinant baculovirus-infected cells contained granules that were smaller and brighter than were the polyhedra. SDS-PAGE and Western blotting to determine the expression of each recombinant protein analyzed Lysates of recombinant virus-infected cells. The desired 60 kDa GroEL, 10 kDa GroES, and/or 60 kDa HtrA proteins were detected on nitrocellulose membranes using the following primary antibodies: Sf9 cells adsorbed goat anti-*B. abortus* RB51 antibody (Fig. 5), Sf9 cells adsorbed mouse anti-*B. abortus* GroEL monoclonal antibody (Fig. 6 a), Sf9 cells adsorbed goat anti-*B. abortus* GroES monospecific antibody (Fig. 6 b), and Sf9 cells adsorbed mouse anti-*B. abortus* HtrA monospecific antibody (Fig. 6 c). Peroxidase conjugated antibodies specific for the IgG of the species of the primary antibodies, were used as secondary antibodies. GroES recombinant protein was detected using Sf9 cells adsorbed goat anti-*B. abortus* RB51 antibody, but not detected using Sf9 cells adsorbed goat anti-*B. abortus* GroES monospecific antibody. However, GroES protein in RB51 was detected using Sf9 cells

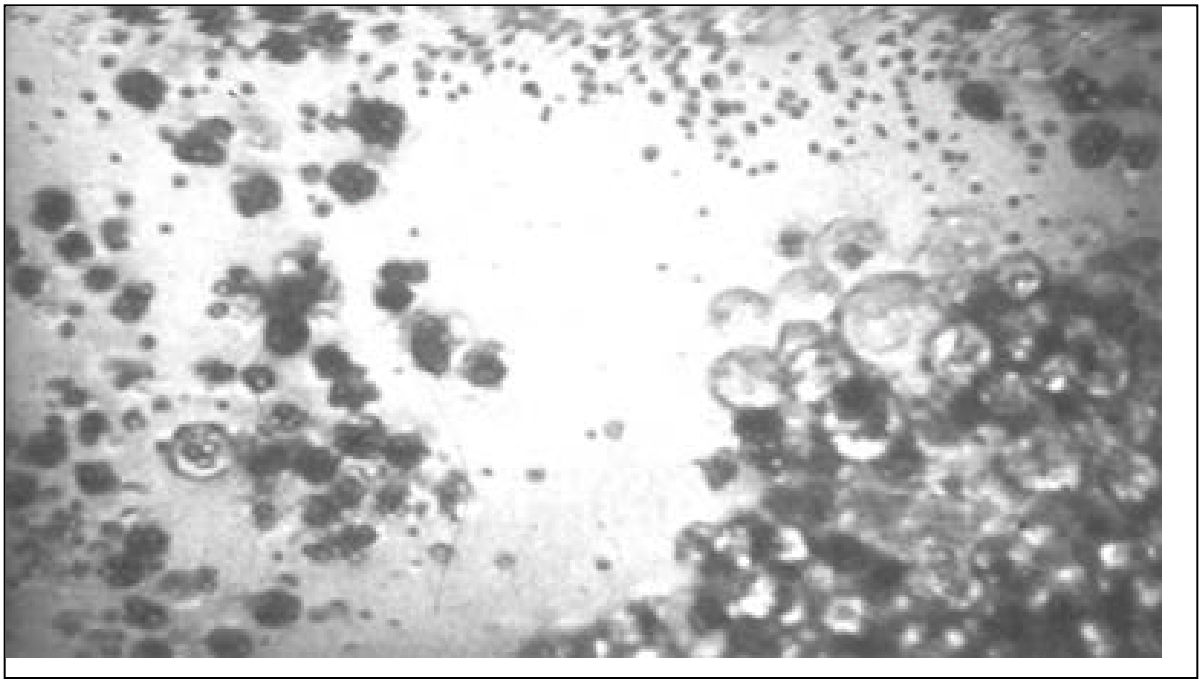

Fig. 4 a. Morphology of *Spodoptera frugiperda* ovarian (Sf9) cells
--- Wild-type baculovirus-infected cells

Wild-type baculovirus-infected cells show distinct polyhedra. The cells are disrupted in the late phases of the infection, 6 days after inoculation.

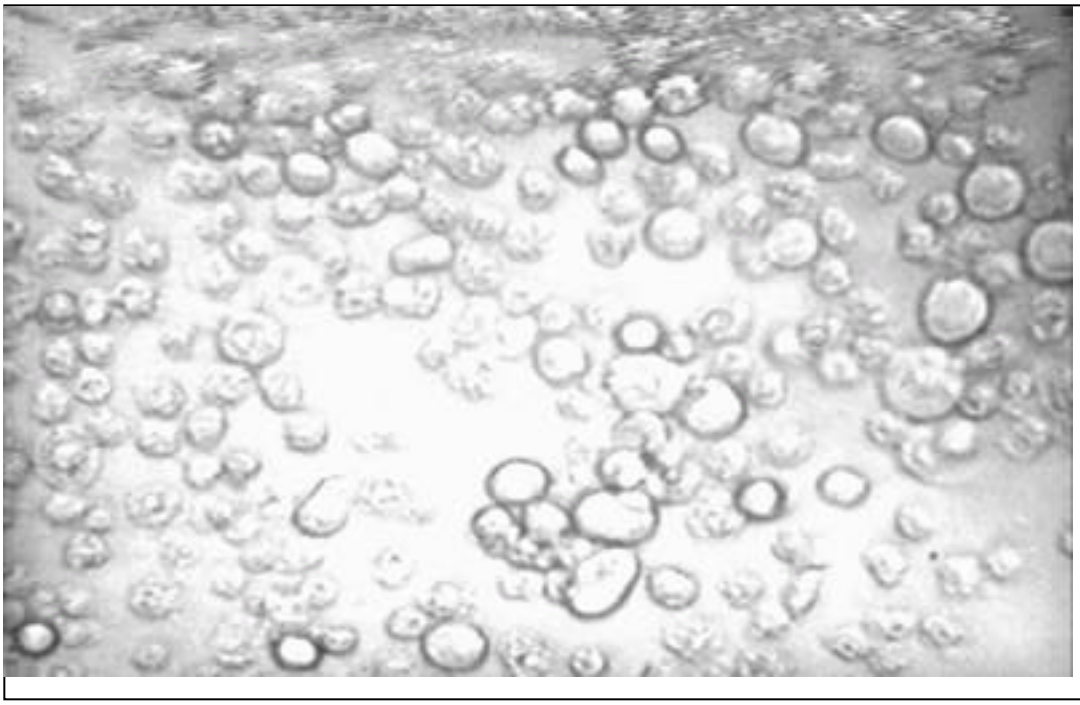

Fig. 4 b. Morphology of *Spodoptera frugiperda* ovarian (Sf9) cells --- recombinant baculovirus-infected cells

Recombinant GroEL virus-infected Sf9 cells show large number of granules in the late phases of infection. They are disrupted 8 days after inoculation. Size and darkness of granules are different from polyhedra of wild-type baculovirus-infected cells.

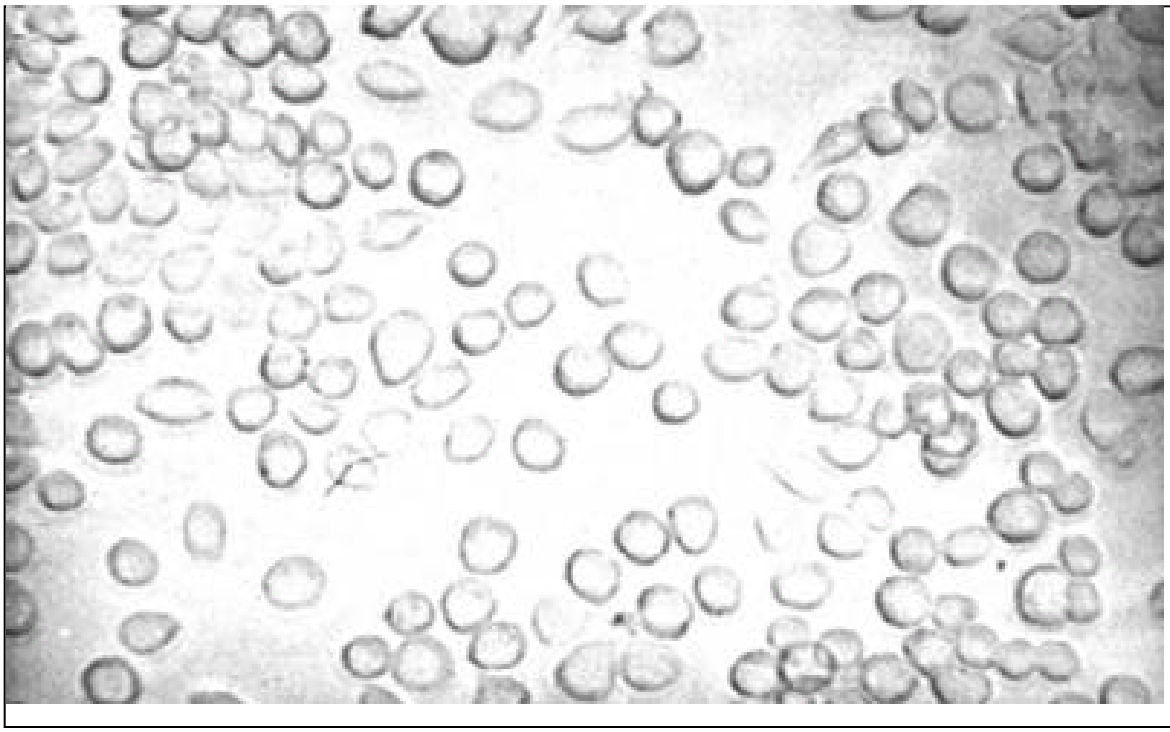

Fig. 4 c. Morphology of *Spodoptera frugiperda* ovarian (Sf9) cells --- Uninfected cells.

Uninfected cells are large and spherical.

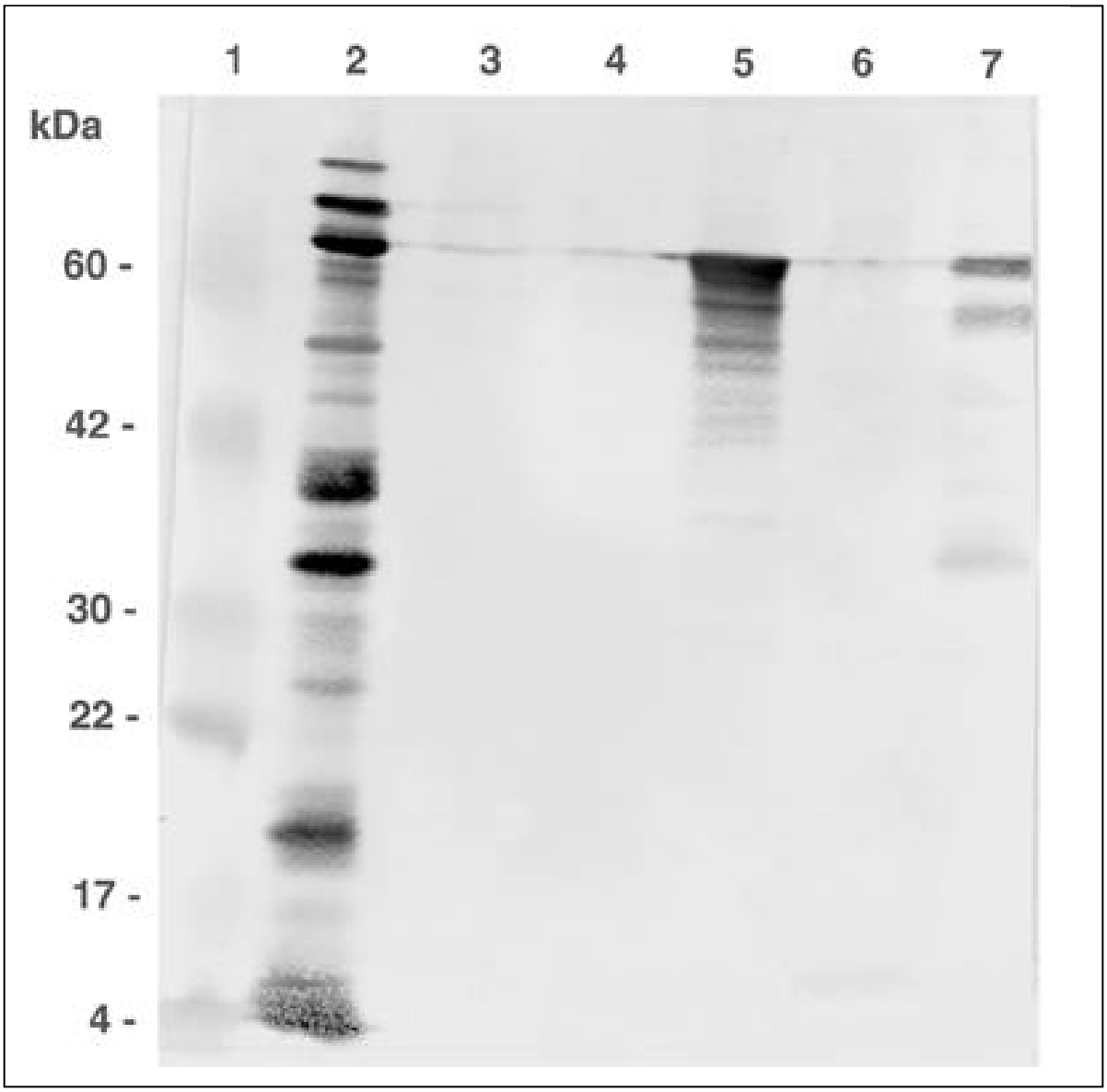

Fig. 5. Western blot of recombinant baculovirus-*B. abortus* heat shock protein expression in Sf9 cells, using Sf9 cells-adsorbed goat anti-*B. abortus* polyclonal antiserum as the primary antibody, and peroxidase conjugated rabbit anti-goat antiserum as the secondary antibody.

Lane 1: Molecular weight markers
Lane 2: *B. abortus* RB51
Lane 3: Lysate of uninfected Sf9 cells
Lane 4: Lysate of Bacmid only-infected Sf9 cells
Lane 5: Lysate of 60 kDa GroEL recombinant protein expressing Sf9 cells
Lane 6: Lysate of 10 kDa GroES recombinant protein expressing Sf9 cells
Lane 7: Lysate of 60 kDa HtrA recombinant protein expressing Sf9 cells

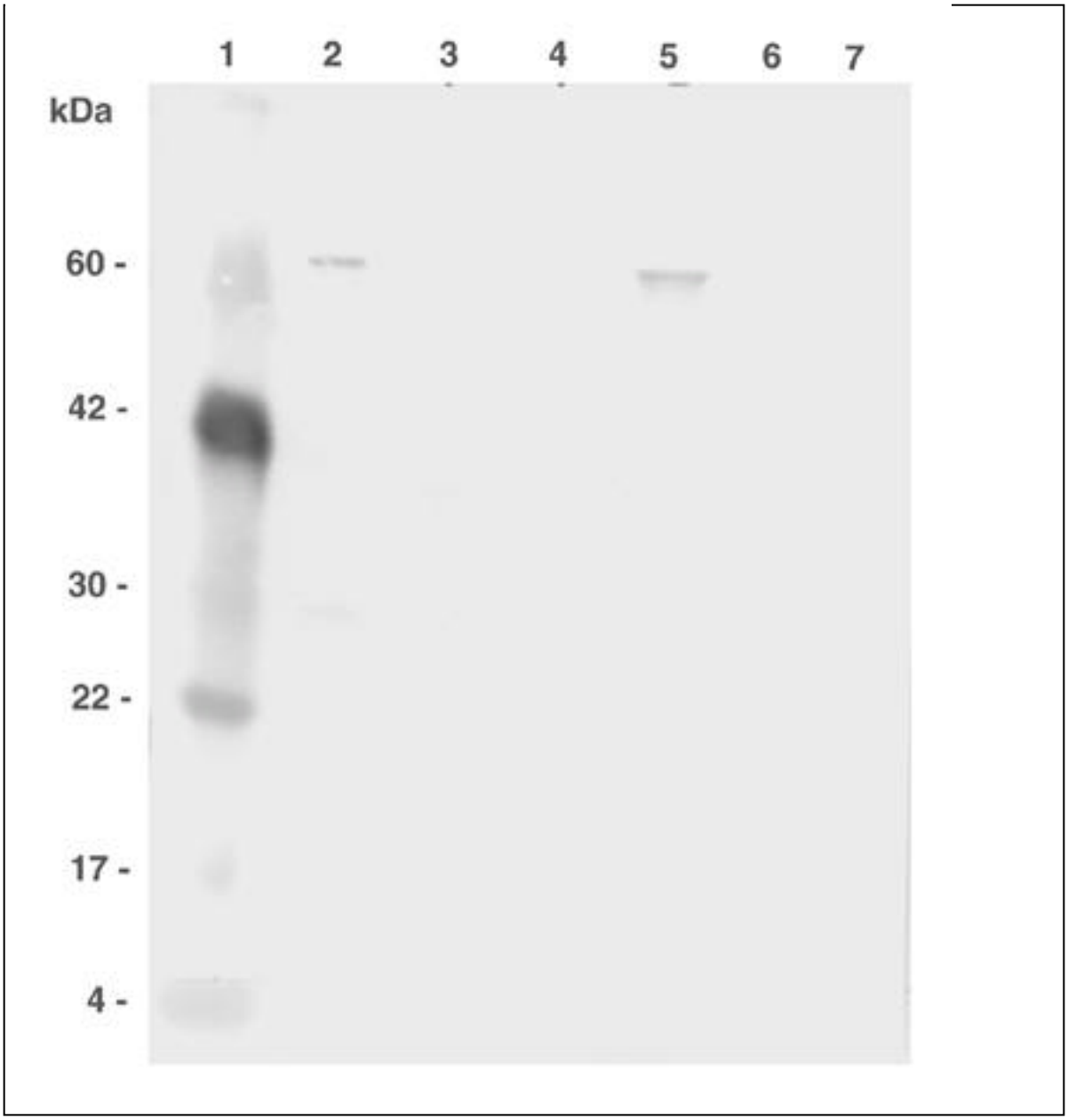

Fig. 6 a. Western blot of recombinant baculovirus-*B. abortus* GroEL heat shock protein expression in Sf9 cells using Sf9 cells-adsorbed mouse monoclonal anti-*B. abortus* GroEL antiserum as the primary antibody, and peroxidase conjugated goat anti-mouse antiserum as the secondary antibody.

Lane 1: Molecular weight markers
Lane 2: *B. abortus* RB51
Lane 3: Lysate of uninfected Sf9 cells
Lane 4: Lysate of Bacmid only-infected Sf9 cells
Lane 5: Lysate of 60 kDa GroEL recombinant protein expressing Sf9 cells
Lane 6: Lysate of 10 kDa GroES recombinant protein expressing Sf9 cells
Lane 7: Lysate of 60 kDa HtrA recombinant protein expressing Sf9 cells

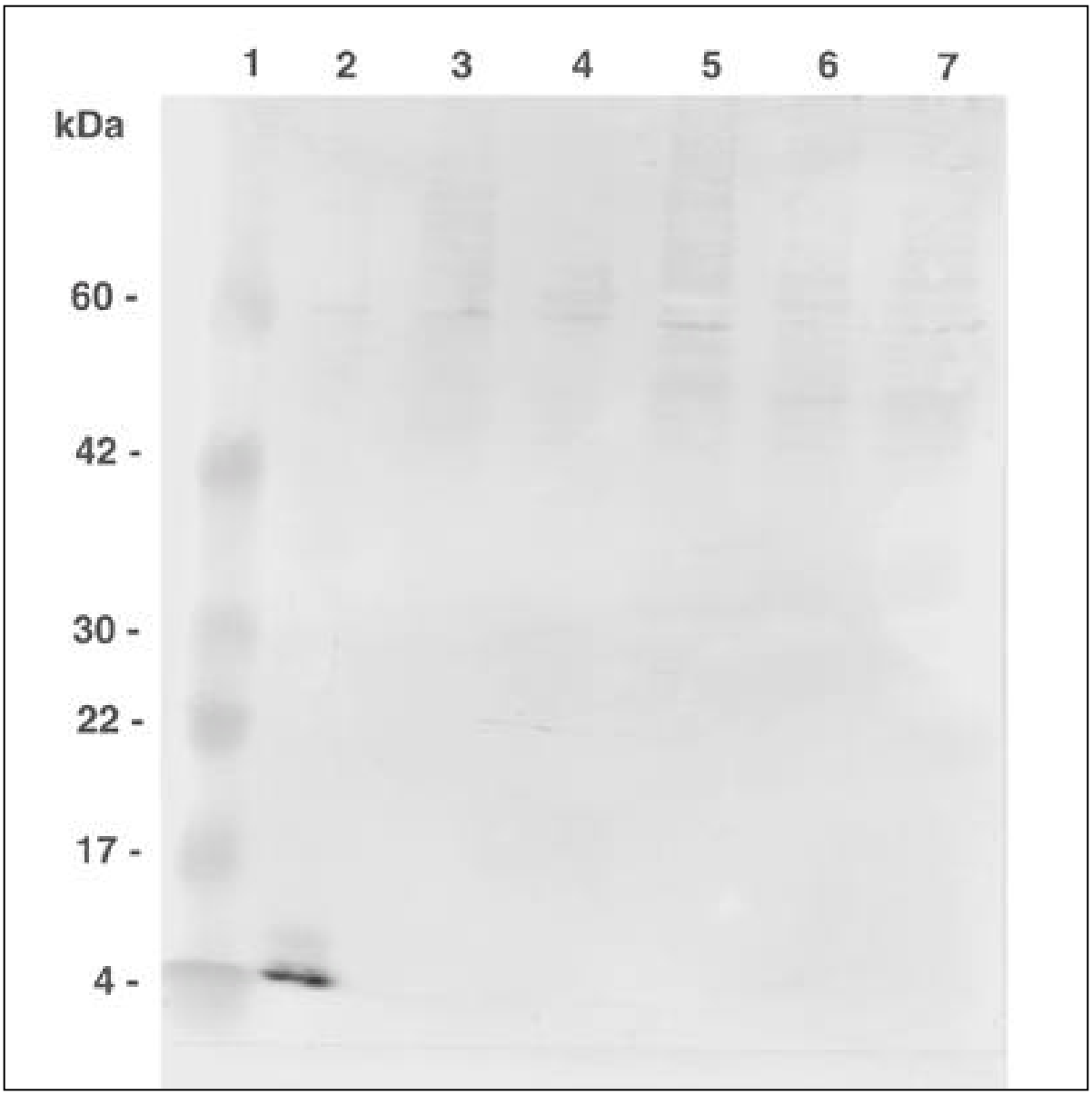

Fig. 6 b. Western blot of recombinant baculovirus-*B. abortus* GroES heat shock protein expression in Sf9 cells using Sf9 cells-adsorbed goat monospecific anti-*B. abortus* GroES sera antiserum as the primary antibody, and peroxidase conjugated rabbit anti-goat antiserum as the secondary antibody.

Lane 1: Molecular weight markers
Lane 2: *B. abortus* RB51
Lane 3: Lysate of uninfected Sf9 cells
Lane 4: Lysate of Bacmid only-infected Sf9 cells
Lane 5: Lysate of 60 kDa GroEL recombinant protein expressing Sf9 cells
Lane 6: Lysate of 10 kDa GroES recombinant protein expressing Sf9 cells
Lane 7: Lysate of 60 kDa HtrA recombinant protein expressing Sf9 cells

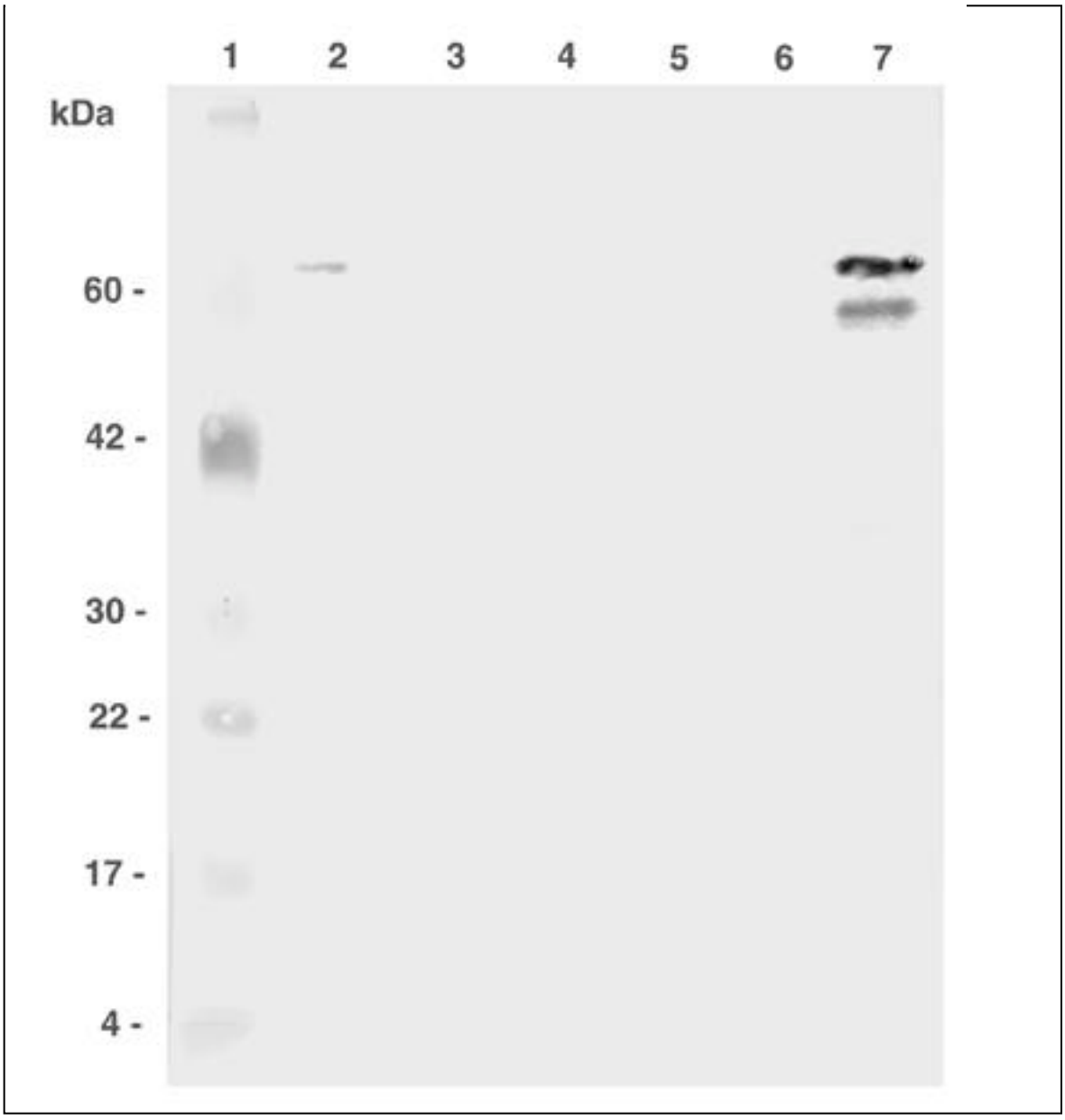

Fig. 6 c. Western blot of recombinant baculovirus-*B. abortus* HtrA heat shock protein expression in Sf9 cells using Sf9 cells-adsorbed mouse monospecific anti-*B. abortus* HtrA sera antiserum as the primary antibody, and peroxidase conjugated goat anti-mouse antiserum as the secondary antibody.

Lane 1: Molecular weight markers
Lane 2: *B. abortus* RB51
Lane 3: Lysate of uninfected Sf9 cells
Lane 4: Lysate of Bacmid only-infected Sf9 cells
Lane 5: Lysate of 60 kDa GroEL recombinant protein expressing Sf9 cells
Lane 6: Lysate of 10 kDa GroES recombinant protein expressing Sf9 cells
Lane 7: Lysate of 60 kDa HtrA recombinant protein expressing Sf9 cells

adsorbed goat anti-*B. abortus* GroES monospecific antibody. HtrA recombinant protein (60 kDa) was detected using polyclonal and monospecific antibodies.

DISCUSSION

Autographa californica nucleopolyhedrosis virus (AcNPV) is a baculovirus whose hosts include a number of *Lepidoptera* (Granados and Federici, 1986). This virus consists of a 128 Kb double-stranded, supercoiled, circular piece of DNA encased in an occlusion body (Smith and Summer, 1978). This occlusion body is primarily composed of polyhedrin, a 29 kDa structural protein (Rohrmann, 1986). The polyhedrin gene is dispensable for virus replication, and the expression of a foreign gene is based on the allelic replacement of the polyhedrin gene (Smith et al., 1983) under the control of the major late polyhedrin promoter (Luckow and Summer, 1988; Maeda, S., 1989; Miller, L. K., 1988). Recombinants are usually recognized by their polyhedrin-negative appearance.

Several bacterial proteins have been successfully expressed in the baculovirus systems including the protective antigen of *Bacillus anthracis* (Iacono-Connors et al., 1990; 1991), HD-73 delta endotoxin and crystal protein of *Bacillus thuringiensis* (Merryweather et al., 1990; Martens et al., 1990), tetanus toxin fragment-C of *Clostridium tetani* (Charles et al., 1991), and chloramphenicol acetyl transferase of *E. coli* (Carbonell et al., 1985). Also, few HSPs have been successfully expressed in the baculovirus system (Akins et al., 1994; Binart et al., 1995; Graham et al., 1996). The

recombinant baculovirus expressing chick HSP90 displayed the same physicochemical characteristics as the native protein, and scale-up of the recombinant baculovirus-infected cell culture has provided sufficient quantities of chick HSP90 for functional studies of the protein (Binart et al., 1995). The cDNA sequence encoding a novel desiccation stress protein (dsp28) from the beetle *Tenebrio molitor* was cloned into a baculovirus expression vector and the expressed protein was compared to native dsp28. Both the dsp28 expressed by the recombinant baculovirus and the native dsp28 are glycosylated and N-terminally processed (Graham et al., 1996). Only one report about cloning and expression of bacterial heat shock proteins in the baculovirus system has been made. This involved the overexpression of the *Mycobacterium tuberculosis* chaperonin 10 (Cpn10)-encoding gene (Akins et al., 1994). The product was immunoreactive with a Cpn10 monoclonal antibody (mAb) and had an electrophoretic mobility identical to authentic Cpn10. The baculovirus vector and purification methodology described in the Akins et al. (1994) study represents a very powerful system for the large-scale production of the *M. tuberculosis* Cpn10 that may allow structure-function analysis.

Our study is the first one expressing recombinant HSPs of *B. abortus* in the baculovirus system. *B. abortus groEL*, *groES*, and *htrA* genes were cloned into a baculovirus donor plasmid, and purified recombinant donor plasmids were confirmed by restriction enzyme analysis and Southern blots. Recombinant donor plasmids were transformed into DH10BAC competent *E. coli* cells, which contain the baculovirus shuttle vector (Bacmid) and helper plasmid. The gene of interest was placed from the donor plasmid into the Bacmid by the process of site-specific transposition. *E. coli* cells

containing recombinant Bacmids were selected resistant to four different antibiotics and the disruption of the *lac*Z gene. Purified recombinant Bacmids were identified by gel electrophoresis and Southern hybridization. Sf9 cells were transfected with recombinant Bacmid, and recombinant baculoviruses were harvested. Larger number of Sf 9 cells was infected with each recombinant virus, and the recombinant proteins were identified by Western blotting. The expression of each recombinant protein has been demonstrated by using polyclonal and compatible monoclonal or monospecific antibodies. GroEL recombinant protein was detected by polyclonal *B. abortus* RB51 and monoclonal antibodies. GroES recombinant protein was detected using polyclonal *B. abortus* RB51, but not detected using goat anti-*B. abortus* GroES monospecific antibody. Presumably, the low concentration of GroES recombinant protein might be the reason for the lack of detection or the goat anti-*B. abortus*. GroES mouse specific antibody is not against GroES. HtrA recombinant protein shows a distinct pattern of bands including 60 kDa and approximately 55 kDa proteins, using polyclonal and monospecific antibodies. The lower molecular mass size might be the degraded HtrA protein, and this smaller sized protein was also observed in the Western blotting of other *B. abortus* HtrA recombinant proteins (Toth et al., 1995). In conclusion, this study demonstrates the cloning and successful expression of three different *B. abortus* HSPs in the baculovirus system.

REFERENCES

Akins, D., H. Al-Ghusein, C. Prehaud, and A. R. M., Coates. 1994. Overproduction and purification of *Mycobacterium tuberculosis* chaperonin 10. Gene. 150:145-148.

Alberts M., C. Bruce, D. Bray, J. Lewis, M. Raff, K. Roberts, J. D. Watson. 1994. Molecular biology of the cell. Garland Publishing, Inc.

Alton, G. G. 1990. *Brucella melitensis*, p. 383-409. In K. H. Nielsen and J. R. Duncan (ed.), Animal brucellosis. CRC Press, Inc., Boca Raton, Fla.

Baldwin, C. L., and A. J. Winter. 1994. Macrophages and Brucella. Immunol. Ser. 60:363-380.

Binart, N., M. Lombes, and E. E. Baulieu, 1995. Distinct functions of the 90 kDa heat-shock protein (hsp90) in estrogen and mineralocorticosteroid receptor activity: effects of hsp90 deletion mutants. Biochem J. 311:797-804.

Blander, S. J. and M. A. Horwitz. 1992. Major cytoplasmic membrane protein of *Legionella pneumophila*, a genus common antigen and membrane of the hsp60 family of heat shock proteins, induces protective immunity in a guinea pig model of Legionnaires' disease. J. Clin. Invest. 91:717-723.

Buchmeier, N. A. and F. Heffron. 1990. Induction of *Salmonella* stress proteins upon infection of macrophages. Science. 248:730-732.

Canning, P. 1990. Phagocyte function in resistance to brucellosis, p.151-163. *In* L.G. Adams (ed.), Advances in brucellosis research, Texas A&M Press, College Station.

Carbonell, L. F., M. J. Klowden, and L. M., Miller. 1985. Baculovirus-mediated expression of bacterial genes in dipteran and mammalian cells. J. Virol. 56:153-160.

Charles, I. G., B. C., Rodgers, A. J. Makoff, S. N. Chatfield, D. E. Slater, and N. F. Fairweather. 1991. Synthesis of tetanus toxin fragment-C in insect cells by use of a baculovirus expression system. Infect. Immun. 59:1627-1632.

Cheers, C. 1983. Pathogenesis and cellular immunity in experimental murine brucellosis. Dev. Biol. Stand. 56:237-246.

Cooney, E. L., M. J. McElrath, L. Corey, S. L. Hu, A. C. Collier, D. Arditti, M. Hoffman, R. W. Coombs, G. E. Smith, and P. D. Greenberg. 1993. Enhanced immunity to human immunodeficiency virus (HIV) envelope elicited by a combined vaccine regimen consisting of priming with a vaccinia virus recombinant expressing HIV envelop and boosting with gp160 protein. Proc. Natl. Acad. Sci. USA. 90:1882-1886.

Ellis, R. J. and S. M. van der Vies. 1991. Molecular chaperones. Annu. Rev. Biochem. 60:321-347.

Elzer, P. H., R. W. Phillips, M. E. Kovach, K. M. Peterson, and R. M. Roop. 1994. Characterization and genetic complementation of a *Brucella abortus* high-temperature requirement A (*htrA*) deletion mutant. Infect. Immun. 62:4135-4139.

Enright, F. M. 1990. The pathogenesis and pathobiology of *Brucella* infections in domestic animals, p. 301-320. In K.H. Nielsen and J. R. Duncan (ed.), Animal brucellosis. CRC Press, Inc., Boca Raton, Fla.

Etlinger, H. M. and W. Altenburger. 1991. Overcoming inhibition of antibody responses to a malaria recombinant vaccinia virus caused by prior exposure to wild type virus. Vaccine. 9:470-472.

Ferrero, R. L., J. M. Thiberge, I. Kansau, N. Wuscher, M. Huerre, and A. Labigne. 1995. The GroES homolog of *Helicobacter pylori* confers protective immunity against mucosal infection in mice. Proc. Natl. Acad. Sci. USA. 92:6499-6503.

Georgopoulus, C. and H. McFarland. 1993. Heat shock proteins in multiple sclerosis and other autoimmune diseases. Immunol. Today. 14:373-375.

Graham, L. A., W. A. Bendena, and V. K. Walker. 1996. Cloning and baculovirus expression of a desiccation stress gene from the beetle, *Tenebrio molitor.* Insect Biochem. Molec. Biol. 26:127-133.

Granados, R. R. and B. A. Federici. 1986. The biology of Baculoviruses. CRC Press, Boca Raton, FL. 1986.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166:557-580.

Horwitz, M. A. 1983. Cell-mediated immunity in Legionnaires' disease. J. Clin. Invest. 71:1686-1697.

Iacono-Connors, L. C., C. S. Schmaljohn, and J. M. Dalrymple. 1990. Expression of the *Bacillus anthracis* protective antigen gene by baculovirus and vaccinia virus recombinants. Infect. Immun. 58:366-372.

Iacono-Connors, L. C., S. L. Welkos, B. E. Ivins, and J. M. Dalrymple. 1991. Protection against anthrax with recombinant virus-expressed protective antigen in experimental animals. Infect. Immun. 59:1961-1965.

Inumaru, S. and S. Yamada. 1991. Characterization of pseudorabies virus neutralization antigen glycoprotein gIII produced in insect cells by a baculovirus expression vector. Virus Res. 21:123-139.

Johnson, K., I. Charles, G. Dougan, D. Pickard, P. O'Gaora, G. Costa, T. Ali, I. Miller and C. Hormaeche. 1991. The role of a stress-response protein in *Salmonella typhimurium* virulence. Mol. Microbiol. 5:401-407.

Kindig, T. M., C. P. Kalberer, H. Hengartner and R. M. Zinkernagel. 1993. Vaccination with two different vaccinia recombinant virus: long-term inhibition of secondary vaccination. Vaccine. 11:1154-1158.

Lin, J. and T. A. Ficht. 1995. Protein synthesis in *Brucella abortus* induced during macrophage infection. Infect. Immun. 63:1409-1414.

Lipinska, B., O. Fayet, L. Baird, and C. Georgopoulos. 1989. Identification, characterization, and mapping of the *Escherichia htrA* gene, whose product is essential for bacterial growth only at elevated temperatures. J. Bacteriol. 171:1574-1584.

Luckow, V. A., S. C. Lee, G. F. Barry, and P. O. Olins, 1993. Efficient generation of infectious recombinant bauloviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli.* J. Virol. 67:4566-4579.

Luckow, V. L. and M. D. Summers, 1988. Trends in the development of baculovirus expression vectors. Bio/Technology. 6:47-55.

Maeda S. 1989. Expression of foreign genes in insects using baculovirus vectors. Annu. Rev. Entomol. 34:351-372.

Makoff, A. J., S. P. Ballantine, A. E. Smallwood, and N. F. Fairweather. 1989. Expression of tetanus toxin fragment C in *E. coli*: its purification and potential as a vaccine. Bio/Technology. 7:1043-1046.

Martens, J. W. M., G. Honee, D. Zuidema, J. W. M. van Lent, B. Visser, and J. M. Vlak. 1990. Insecticidal activity of a bacterial crystal protein expressed by a recombinant baculovirus in insect cells. Appl. Environ. Microbiol. 56:2764-2770.

Mayhew, M. A., C. da Silva, J. Martin, H. erdjument-Bromage, P. Tempst, F. U. Hartl. 1996. Protein folding in the central cavity of the GroEL-GroES chaperonin complex. Nature. 379:420-426.

Merryweather, A. T., U. Weyer, M. P. G. Harris, M. Hirst, T. Booth, and R. D. Possee. 1990. Construction of genetically engineered baculovirus insecticides containing the *Bacillus thuringiensis* subsp. kurstaki HD-73 delta endotoxin. J. Gen. Virol. 71:1535-1544.

Miller, L. K. 1988. Baculoviruses as gene expression vectors. Annu. Rev. Microbiol. 42:177-199.

O'Reilly, D. R., L. K. Miller, and V. A. Lucknow. 1992. Baculovirus Expression Vectors. A Laboratory Manual. W.H. Freeman and Company, N.Y.

Rohrmann, G. F. 1986. Polyhedrin structure. J. Gen. Virol. 67:1499-1453.

Roop, R. M., M. Price, B. E. Dunn, S. M. Boyle, N. Sriranganathan, and G. G. Schurig. 1992. Molecular cloning and nucleotide sequence analysis of the gene encoding the immunoreactive *Brucella abortus* Hsp60 protein, BA60K. Microbial Pathogenesis. 12:47-62.

Roop, R. M., S. M. Boyle, and G. G. Schurig. 1990. Expression of a 60K recombinant *Brucella* immunogenic protein in *E. coli*. 90th Annu. Meet ASM, Abstract #D-19. Anaheim, CA.

Roop, R. M., T. W. Fletcher, D. Dunn, S. M. Boyle, N. Sriranganathan, and G. G. Schurig. 1993. Identification of an immunoreactive *Brucella abortus* protein as an HrtA stress response protein homologue. 93rd Annu. Meet Am. Soc. Microbiol., Atlanta, GA, May 1993.

Roop, R. M., T. W. Fletcher, N. M. Sriranganathan, S. M. Boyle, and G. G. Schurig. 1994. Identification of an immunoreactive *Brucella abortus* protein as an HtrA stress response protein homologue. Infect. Immun. 62:1000-1007.

Sambrook, J., T. Maniatis, and E. F. Fritsch. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Shinnick, T. M., M. H. Vodkin, and J. C. Williams. 1988. The *Mycobacterium tuberculosis* 65 kilodalton antigen is a heat shock protein which corresponds to common antigen and to the *Escherichia coli* GroEL protein. Infect. Immun. 56:446-451.

Silva C. L., M. F. Silva, R. C. L. R. Pietro and D. B. Lowrie. 1994. Protection against tuberculosis by passive transfer with T-cell clones recognizing mycobacterial heat-shock protein 65. Immunology. 83:341-346.

Smith, G. E., M. J. Fraser, and M. D. Summers. 1983. Molecular engineering of the *Autographa californica* nuclear polyhedrosis virus genome: deletion mutants within the polyhedrin gene. J. Virol. 46:584-593.

Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Summer, M. D. and G. E. Smith. 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station Bulletin no. 1555.

Toth, T. E., J. A. Cobb, S. M. Boyle, G. G. Schurig. 1995. Selective humoral immune response of Balb/C mice to *Brucella abortus* proteins expressed by vaccinia virus recombinants. Vet. Microbiol. 45:171-183.

Young, K. C. 1988. Baculovirus vectors for expression of foreign genes. Adv. Virus Res. 35:177-192.

Young, D., R. Lathigra, R. Hendrix, D. Sweetser, and R.A. Young. 1988. Stress proteins are immune targets in leprosy and tuberculosis. Proc. Natl. Acad. Sci. USA. 85:4267-4270.

Young, R. A. and T. J. Elliott. 1989. Stress proteins, infection, and immune surveillance. Cell. 59:5-8.

CHAPTER 3

KINETICS OF THE OPTIMAL EXPRESSION OF *BRUCELLA ABORTUS* HEAT SHOCK PROTEINS BY BACULOVIRUS RECOMBINANTS

INTRODUCTION

Expression of proteins based on the baculovirus system has wide applicability as an alternative to prokaryotic or other eukaryotic expression systems. More than 100 laboratories are currently using this viral expression system, mainly because this system is capable of producing very high levels of recombinant proteins (Luckow and Summers, 1988). The other main reason is the benefit of generating less homologous heat shock proteins from eukaryotic host cells as compared to prokaryotic host cells. This is an important consideration because of the highly conserved nature of these proteins.

Following the development of *B. abortus* - baculovirus recombinants, described in Chapter 2, we turned our attention to defining the optimal conditions for maximal expression of *B. abortus* for each recombinant HSP are. The first step toward successful infection of insect cells with recombinant viruses was ensuring that the culture will not be rate limited by nutritional factors (i.e., amino acids or carbohydrate utilization) or environmental factors (i.e., pH, dissolved O_2, temperature, stir). These factors were incubating the insect cell cultures in a 28^{o}C incubator, appropriate media, suitable size of

CHAPTER 4.

IMMUNOLOGICAL CHARACTERIZATION OF RECOMBINANT BACULOVIRUS-*BRUCELLA ABORTUS* GroEL, GroES, HtrA HEAT SHOCK PROTEINS

INTRODUCTION

Vaccines against bacterial diseases have been introduced in the course of the past hundred years. Many have been in use without significant improvements. Some are well tolerated and provide a solid and long-lasting protection and can thus be considered optimal. Others cause adverse reactions and have only limited or temporary efficacy and therefore require fundamental improvements to become acceptable. The dramatic advances achieved in the fields of immunology and bacterial genetics have strongly stimulated and facilitated research directed at the development of more effective bacterial vaccines. Four different basic trends have been recognized (Lawrence and Pamela, 1999).

The first trend arises from the recognition that protection against some bacterial infections has only been achieved by vaccination with live attenuated bacteria that mimic the infection-induced immunity. The live attenuated vaccines induce immune responses similar to those elicited by natural infection, with little attempt on the part of the investigator to modify them. Examples of widely used live attenuated vaccines include the BCG vaccine against tuberculosis (Harboe et al., 1996; Lowrie et al., 1995), the Ty21A vaccine against typhoid fever (Germanier and Fuer, 1975), and strain 19 against

brucellosis (Nielsen and Duncan, 1988; Plommet and Plommet, 1988 a, b). Most of these types of vaccines were developed by *in vitro* passage of human pathogens before the era of molecular biology and biotechnology; therefore, the molecular mechanisms of their attenuation are unknown. Today such non-characterized vaccines would be difficult to introduce. The application of new recombinant DNA techniques allows for the development of genetically stable mutants, thus rendering obsolete the main concern in the use of live vaccines, namely reversion of attenuated strains to virulence. Well-characterized, live attenuated bacterial strains can be built by rational modification of the genome of the pathogen. Thus far, most of the work has been dedicated to obtaining attenuated strains of pathogens, such as *Salmonella*, by deleting or inactivating the genes coding for the synthesis of aromatic amino acids or components of regulatory pathways (Everest et al., 1995); in *Vibrio cholerae* by deleting the gene coding for the A subunit of cholera toxin (Kaper et al., 1995; Levin et al., 1988).

The second trend is the search for specific protective antigens, which can replace whole cells with purified components serving as subunit vaccines. Many adverse reactions caused by components irrelevant to a protective response can thus be eliminated. The first subunit vaccines to be developed were diphtheria and tetanus toxoids (Rappuoli, R., 1990). Both of these diseases are caused by a toxin produced by the bacterium, which suggested that serum antibodies able to neutralize the toxin could be sufficient to protect from disease. Therefore, the semi-purified toxins can be inactivated by chemical (formaldehyde) treatment and used as vaccines. A second example of subunit vaccines is polysaccharides and conjugated vaccines against encapsulated bacteria.

Jennings, H. J. (1983) and Gotschlich, E. C. (1984) observed that serum bactericidal antibodies against the capsular polysaccharide are enough to protect from invasive bacterial infection. Their observations suggested that purified capsular polysaccharides can be developed as vaccines. A third prototype subunit vaccine is the relatively recently developed acellular pertussis vaccine (Brennan et al., 1992). A considerable amount of work was initially required to identify the bacterial antigens that were able to induce protective immune responses. Pertussis toxin (PT) was identified as a major protective antigen; other antigens, such as adenylate cyclase, filamentous hemagglutinin, and fimbriae, were found to contribute to protective immunity. Therefore, these antigens were used in different combinations in candidate vaccines (Decker et al., 1995; Edwards et al., 1995; Moxon and Rappuoli, 1990).

The third trend, which is heat or chemical inactivation of bacteria, was the earliest approach to vaccines. Since all antigens present in the pathogen are included, it is not necessary to know which ones are protective. The disadvantage is that some of the vaccine components may responsible for toxic side effects. Today, this method of vaccine development is no longer common, although several vaccines of this type are still widely used: the whole-cell vaccines, for example, against pertussis (Manclark and Cowell, 1984).

The fourth trend is the use of naked DNA for the induction of immune responses. This new, but popular method in vaccinology has great potential (Ulmer et al., 1996). In this approach, the genes coding for the protective antigens are cloned into appropriate plasmid vectors under control of strong eukaryotic promoters and directly injected into

the host. A small fraction of the injected plasmid DNA is then taken up and expressed by antigen-presenting cells; the result is the elicitation of an immune response against the newly expressed foreign antigen. Although novel, this technology has been experimentally applied to many vaccines, often with very promising results. Tuberculosis and malaria are examples of infections that are now being applied by this fast-growing technology. Clinical trials over the next few years will confirm whether the promising results obtained in mice and primates lead to the development of a new class of vaccines providing protection against many of the diseases for which the classic technologies have failed or been inadequate (Lawrence and Pamela, 1999).

Recently, *B. abortus* live attenuated strain RB51 was approved by the United States Department of Agriculture as the official bovine brucellosis vaccine to replace strain 19. However, our research effort is also directed toward developing a *Brucella* vaccine for humans. It is unlikely that strain RB51 will be approved for human use since it is resistant to Rifampicin. In this study, we propose subunit vaccines as an alternatives to *B. abortus* live vaccines because of the advantages of modification. The research in this doctoral dissertation analyzed *B. abortus* heat shock proteins (HSPs) as protective antigens against *B. abortus* infection. We speculated that this bacterium requires the induction of HSPs such as GroEL, GroES, and HtrA for the intracellular survival and multiplication. GroEL (Hsp60) and GroES (Hsp10) are known as molecular chaperones involved in the correct synthesis of proteins. HtrA belongs to the family of high temperature requirement A stress response proteases and functions to degrade oxidatively

damaged proteins. The biological function of the *B. abortus* HtrA stress response protein homolog was studied by disrupting the *htrA* gene from the chromosome of *B. abortus* 2308 via gene replacement (Elzer et al., 1994). In contrast to the parental strain, the resulting *htrA* deletion mutant failed to grow on solid medium at 40^{o}C and demonstrated increased sensitivity to killing by H_2O_2 and O_2^-. In the study BALB/c mice were infected with strain 2308 and *htrA* deletion mutants to assess the effect of the *htrA* mutation on virulence. Significantly fewer *Brucella*e were recovered from the spleens of mice infected with *htrA* deletion mutant than from those of mice infected with strain 2308.

Using HSPs as antigens is controversial because of the potential for induction of autoimmune diseases by generating immune responses against shared epitopes in the conserved HSP sequences. Yet, bacterial HSPs are required for intracellular survival and are also very immunogenic during the course of infection, eliciting both cellular and humoral immune responses in infected hosts. Cell-mediated immunity is critical for the host defense against intracellular bacterial infections; several heat shock proteins of intracellular bacterial pathogens are known to induce cell-mediated immunity and to act as protective antigens. For example, animals immunized with purified *L. pneumophila* Hsp60 exhibit delayed-type hypersensitivity (DTH) to Hsp60, indicating that cellular responses are elicited by the experimental vaccine (Horwitz, M. A. 1983). Immunization of guinea pigs with purified *L. pneumophila* Hsp60 was found to protect the animals from a lethal aerosol challenge with this organism. *Mycobacterium tuberculosis* Hsp70 contributes to protective immunity in animal models of infection (Pal and Horwitz, 1992; Hubbard et al., 1992).

An immune response is initiated when foreign antigens introduced into the body are taken up by professional cells (antigen-presenting cells). These cells enzymatically process the antigens and present peptide fragments in the context of the class I or class II molecules in the major histocompatibility complex (MHC), a phenomenon known as antigen presentation. Migration of these cells to draining lymph nodes will trigger specific activation of T cells that recognize the MHC-peptide complex through their antigen-specific receptor. The pathway of antigen processing, either cytoplasmic or lysosomal, will dictate whether MHC class I or MHC class II molecules will be involved in antigen presentation, and in turn will dictate the type of T cells, $CD8^+$ or $CD4^+$, respectively, that will be activated and engaged in the proliferative phenomena (Germain, R. N., 1994). These T cells will then participate in effector functions against the microorganisms, either directly; for example, through cytolysis, production of cytokines such as IFN-γ by both $CD8^+$ and $CD4^+$ cells; or indirectly, for example, through the help provided by $CD4^+$ cells to B lymphocytes for the production of antigen-specific antibodies. Development of the appropriate $CD4^+$ Th subset toward Th1 during an immune response is critical for eradication of infectious organisms including *B. abortus* (Mosmann et al., 1986; Mosmann and Coffman, 1989; Sher and Coffman, 1992). Subsets of Th cells may be distinguished by the pattern of cytokines that they produce. Th1 cells produce IL-2 and IFN-γ, etc., mediate delayed-type hypersensitivity and play a critical role in directing cell-mediated immune responses, important for the clearance of

intracellular pathogens. Th2 cells producing IL-4, IL-5, IL-6, IL-10, IL-13, etc., have been associated with allergy, exhibiting a strong helper effect in the production of IgE, and are important for humoral responses (Romagnani, S., 1991; Parronchi et al., 1991; Yssel et al., 1992). Cytokines present during the initiation of a CD4+ T cell response can determine the development of a particular Th cells phenotype (Hsieh et al., 1992; Swain, S. L., 1993). Th2 cells develop when naive T cells are stimulated *in vitro* in the presence of IL-4 (Hsieh et al., 1992; Seder et al., 1992). Conversely, IL-12 is a critical factor driving the development of Th1 cells from antigen-specific naive $CD4^+$ T cells (Hsieh et al., 1993; Seder et al., 1993; Manetti et al., 1993); in addition, it enhances IFN-γ production by Th1 clones (Murphy et al., 1994) and human Th2/Th0-type clones and activated Th cells (Manetti et al., 1994; Kubin et al., 1994). Despite the induction of IFN-γ production by seemingly committed human Th2/Th0 clones (Manetti et al., 1994), such polarizing stimuli as IL-12 are the most effective when delivered at the initiation of the immune response, both *in vitro* (Hsieh et al., 1992; Seder et al., 1992; Manetti et al., 1993) and *in vivo* (Sypek et al., 1993; Heinzel et al., 1993; Afonso et al., 1994). A wide variety of factors can intervene in influencing the polarization of the $CD4^+$ T cell response toward one or the other functional phenotype. Genetic factors, the type of antigen-presenting cells, the amount of stimulating antigen, the type of co-stimulatory molecules, such as B7, and adjuvants have all been involved (Mosmann and Sad, 1996). Adjuvants can strongly influence the quality of the $CD4^+$ T-cell subpopulations that direct the immune response either toward Th2 or Th1 response, and, in turn, the quality of the antibody responses

elicited. This has been examined by comparing two very different adjuvant preparations, aluminum hydroxide and Freund's Complete Adjuvant (FCA), in immunizing mice against the same antigen. FCA activated Th1 cells and alum activated Th2 cells. The profile of activity of these cells has been studied in the mouse but it is likely that it is also relevant in humans (Morein et al., 1996; Del Giudice G., 1992). Besides the benefits of polarization of Th responses, adjuvants have generally been used with subunit vaccines to solve the problem of their weak immunogenicity. Adjuvants support the immune responses by increasing its magnitude. However, the appropriate adjuvant must not only enhance the immune response but should also drive this response to achieve the appropriate type of protective immunity in each situation. Bacterial components provide the major source of adjuvants. Even very low molecular weight synthetic components from Gram-negative bacteria are capable of altering the degree of immune response without being immunogenic themselves. They may have this property because they mimic the microbial structures that have provided the danger signal of infection from the beginning of the evolutionary history of host defenses. The conserved microbial components could also directly induce B cells or macrophages to express a potent co-stimulatory activity for $CD4^+$ T cell clonal expansion, as suggested by Janeway (1992). In our current investigation, several adjuvants were tested for their capacity to support the induction of cell-mediated and protective immune responses to this virulent pathogen, by polarizing responses to *B. abortus* HSPs toward Th1 subsets. Previous investigations and the justification for each adjuvant selected are described below.

Previous investigators (Pugh and Tabatabai, 1994), who used various adjuvants for induction of cell-mediated immune response against *Brucella* proteins, found that subcutaneous immunization with the antigen in Ribi adjuvant (combined monophosphoryl lipid A and trehalose dimycolate) was the best protocol. This study shows the significant protection in mice when Ribi adjuvant and several *B. abortus* proteins were used. Monophosphoryl lipid A (MPL) is a highly refined nontoxic lipid A isolated from the mutants of *Salmonella typhimurium* and *S. minnesota.* Various cell-mediated immune responses are stimulated by MPL, including macrophage bactericidal activity and production of IL-1 and IFN-γ. Pugh and Tabatabai (1996) showed that MPL enhanced the suppression of splenic infection when given with the *B. abortus* salt-extractable protein (BCSP) vaccine. Trehalose dimycolate (TDM) is a glycolipid that is isolated from the cell wall of *Mycobacterium.* Antibody production, lymphocyte proliferation, and cell-mediated immunity to tumors are enhanced by TDM (Spargo et al., 1991). In a previous protection study (Tabatabai et al., 1990), TDM had no effect on mean colony forming unit (CFU) in the spleen of challenged mice when given with BCSP. However, incorporation of TDM resulted in a significant increase in mean CFU when given with proteinase-K-treated *B. abortus* lipopolysaccharide. Also, in other studies, investigators found that subcutaneous immunizations with the protective antigen in combination with Ribi adjuvant lead to significant protection in mice, guinea pigs and cow after the challenge with *Mycobacterium tuberculosis* (Singh et al., 1992), influenza virus

(Vanlandschoot P. et al., 1993), *Treponema pallidum* (Wicher K. et al., 1991), or *Fusobacterium necrophorum* (Saginala S., 1996).

Steroid hormone dehydroepiandrosterone (DHEA) functions to up-regulate the host immune response *in vivo* against infections and counteract stress-induced immunosuppression. DHEA is present in healthy adults at concentrations of 10^{-9} M in blood, but the declines during aging and correlates with a general decline of cell-mediated immunity and an increased incidence of malignancies. Therefore, this steroid hormone is considered as a physiologic regulator of the immune response. DHEA acts via two different mechanisms: (1) as an antiglucocorticoid, DHEA indirectly enhances Th1 because glucocorticoids suppress Th1 activity and synergize with Th2 cytokines; (2) DHEA also directly enhances Th1 T cell activity by influencing T cells to enhance IL-2 and IFN-γ secretion (Daynes et al., 1991; Suzuki et al., 1991). Several reports indicate that DHEA has antiviral, antibacterial, and anticarcinogenic effects in experimental murine models (Loria and Padgett, 1992; Daigle and Carr, 1998). Conceivably, the enhancing effect of DHEA on IL-2, IFN-γ secretion and induction of cytotoxic T cells contributes to its reported antiviral, antibacterial, and anticarcinogenic effects. This effect is detected at the mRNA level, suggesting that DHEA may act as a transcriptional enhancer of the IL-2 and IFN-γ genes in $CD4^+$ T cells (Rook and Stanford, 1994). After steroid hormones bind to their intracellular receptor, the resulting hormone receptor complexes gain DNA binding properties and interact with the cellular genome at specific sites to induce,

enhance, or sometimes repress transcription of relevant genes. Evidence indicates that the potential of both $CD4^+$ and $CD8^+$ T-cell subsets to produce IL-2 and IFN-γ can be enhanced by DHEA treatment. The cellular responsiveness to the effects of this steroid hormone are directly correlated to the presence of a high affinity intracellular receptor having specific binding affinity for DHEA (Meikle et al., 1992).

Cytokines that enhance immune responses to vaccines affect the balance between Th1 and Th2 responses. Cytokines may influence both the magnitude and the phenotype of the immune response to protein antigens. They can influence and amplify immune responses by affecting the proliferative capacity and differentiation of lymphoid and antigen-presenting cells. The presence or the absence of IL-4 or IL-12 and IFN-γ in primary *in vitro* cultures appears to play a dominant role in the differentiation of $CD4^+$ Th cells into Th2 and Th1 types, respectively. Other factors controlling the differentiation of $CD4^+$ Th cell subsets *in vivo* include the precise antigenic determinant and its affinity for MHC, antigenic dose, route of immunization, and pre-existing cytokine milieu. One of the most critical cytokines regulating the development of Th1 cells is the NK cell stimulatory factor, designated IL-12 (Scott P., 1993). This cytokine is produced by macrophages or dendritic cells and acts on NK cells to increase production of IFN-γ, thus favoring the development of Th1 cells. However, IL-12 may also prime IFN-γ production by directly acting upon Th cells. The biological role of IL-12 has been demonstrated in a number of intracellular models of infection. Several studies have

revealed the beneficial effects of recombinant IL-12 as a therapeutic treatment for mice infected with the intracellular parasites *Leishmania major* (Heinzel et al., 1993; Sypek et al., 1993), *Plasmodium chabaudi* (Stevenson et al., 1995), *Schistosoma mansoni* (Wynn et al., 1995), and *Toxoplasma gondii* (Gazzinelli et al., 1993) as well as with several viral systems including murine cytomegalovirus (Orange et al., 1994) and HIV (Clerici et al., 1993). In fact, depletion of IL-12 *in vivo* can exacerbate disease progression in infection models such as listeriosis (Tripp et al., 1994), malaria (Stevenson et al., 1995), and leishmaniasis (Sypek et al., 1993). The real value of IL-12 in vaccine development may be its ability to act as a form of immunologic adjuvant to potentiate host protective Th1 responses when delivered in conjunction with heterologous or recombinant antigen vaccines. The precedent for this work is a study by Afonso et al. (1994), who successfully protected a susceptible strain of mouse against infection with *Leishmania major* using soluble promastigote antigens co-administered with IL-12.

Besides immunization of mice with HSP in combination with various adjuvants described above, priming with vaccinia virus-HSP recombinants was tested as to their ability to enhance immune responses. Recombinant vaccinia viruses have been shown in numerous systems to induce both T-cell and antibody responses to the expressed recombinant protein (Bennink et al., 1984; Bennink and Yewdell, 1990; Fisher-Hoch et al., 1989; Lathe et al., 1987). The insertion of a gene for a weak or poor immunogen into vaccinia virus may render it a stronger immunogen due to the strong promoter of vaccinia virus and the resultant cascade of immunologic events such as local cytokine release and

T-cell infiltration. One of the potential drawbacks in the use of recombinant vaccinia virus, however, is that multiple administrations may not be feasible since preexisting and/or induced antibody and T-cell responses to vaccinia virus will prevent the spread of the inoculated recombinant vaccinia virus and thus diminish the expression of the inserted protein. Thus, immunization protocols would be limited to one or at most two administrations of recombinant vaccinia viruses, and would require other forms of the immunogen for booster inoculations. The Center for Molecular Medicine and Infectious Disease is in the unique position of having both vaccinia virus and baculovirus recombinants expressing *B. abortus* HSPs; in addition a previous study (Bei et al., 1994) shows enhancement of immune response by baculovirus recombinant antigen in mice primed with recombinant vaccinia virus.

Besides HSPs as experimental antigens, we included two *B. abortus* antigens (Cu/Zn superoxide dismutase, ribosomal protein L7/L2) as putative positive controls. Superoxide dismutase (SOD) activity is present in all oxygen metabolizing cells and protects cells from the toxic effects of reactive oxygen intermediates by converting superoxide radicals into hydrogen peroxide (H_2O_2) and oxygen (Fridovich, I., 1989; Hassan and Fridovich, 1980). Superoxide radicals (O_2^-) are generated as intermediates during reduction of molecular oxygen. In addition, these oxygen radicals undergo further reduction to form hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH*) (Ewang, D., 1983). These active oxygen species (O_2^-, H_2O_2, OH*) can damage DNA, RNA, protein and lipids (Mead, J., 1976; Bielski and Shiue, 1979) leading to disruption of cellular

architecture and activity (Farr et al., 1988). To protect themselves against these toxic species, cells that can grow in the presence of oxygen utilize antioxidant enzymes e.g. SOD, catalases and peroxidases. SODs also play an important role in bacteria by permitting them to survive phagocytic attack. Thus, SOD may be regarded as a virulence factor facilitating intracellular survival (Wollinsky, E., 1979; Beaman et al., 1983; Farr et al., 1988). Three forms of SOD have been characterized on the basis of their metal prosthetic groups. The evolutionary related manganese (Mn) and iron (Fe) SODs are commonly found in prokaryotes (Bannister and Rotillo, 1984). The copper-zinc (Cu-Zn) enzymes are mainly present in the cytosol of eukaryotes (Asada et al., 1973; Goscin and Fridovich, 1972; Lee et al., 1981), and in a few species of bacteria (Bannister and Parker, 1985; Steinman, H. M., 1987) and are considered the most recently evolved SOD (Britton et al., 1978). *B. abortus* possesses two forms of SOD. One has been preliminarily characterized as a Mn SOD (Sriranganathan et al., 1991), and the second has been identified as a Cu-Zn SOD (Beck et al., 1990). A periplasmic location for Cu-Zn SOD may be an important adaptation protecting *B. abortus* against the bactericidal oxygen intermediate produced within phagocytic cells (Tatum et al., 1992).

The role of SOD as a host defense mechanism against oxygen toxicity has been controversial. The SOD specific activity in crude extracts of *Brucella* strains is from 10 to 100 fold higher than that reported for other intracellular bacterial pathogens (Beaman et al., 1983; Mayer and Falkinham, 1986). However, the presence of large and similar amounts of SOD activities and lack of SOD activities in the culture supernatant among the *B. abortus* strains studied suggest that SOD is not a major virulence factor. Strain

RB51 is essentially avirulent in mice and none of the strains tested excrete SODs into the culture supernatant. It may well be that SOD contributes to the virulence of *B. abortus* but is only one of several factors (Canning et al., 1986). Others suggest that the presence of a novel Cu/Zn SOD in *Brucella* may be a critical virulence associated factor which allows the survival of the organism in the host (Beck et al., 1990). Tatum et al., (1992) suggest that Cu/Zn SOD contributes, but is not solely responsible, for increased survival of *B. abortus* 2308 within murine macrophages during early stages of infection. Stabel et al. (1994) supports the theory of Steinman and Ely (1990) that intracellular bacteria containing periplasmic Cu/Zn SOD, such as *B. abortus*, are able to better survive the initial extracellular or extracytoplasmic oxidative stress associated with neutrophil or macrophage phagocytosis. In the study of Stevens et al. (1994), lymph node cell mediated immune responses to Cu/Zn SOD and to three synthetic Cu/Zn SOD peptides were measured following vaccination of cattle with *B. abortus* S19 or RB51 to better understand the role of cellular immunity to SOD. Neither the strain 19 nor the strain RB51 vaccine induces antibody production to SOD and only the strain 19 vaccine induces lymph node cell-mediated immune responses to SOD. However, a recent study (Onate et al., 1999) showed the induction of a significant level of protection against *B. abortus* virulent strain challenge in BALB/c mice injected with 1×10^6 live *E. coli* cells expressing *B. abortus* SOD. Such a protective role and virulence for SOD has been demonstrated also for other facultative intracellular bacteria. The surface-associated and secreted SOD of *Nocardia asteroides* is protective against oxidative killing *in vivo* during all stages of infection (Beaman and Beaman, 1990). The Fe SOD of *Shigella flexneri* plays a role in

the pathogenesis of this organism, even though this protein is located in the cytoplasm (Franzon et al., 1990). Virulent forms of *Mycobacterium tuberculosis* secrete SOD into the medium, where it may act as a virulence factor (Anderson et al., 1991).

The immunological stimulation by ribosomal preparations from 28 different pathogens, including *B. abortus*, has been studied by several investigators (Gregory R. L., 1986; Youmans and Youmans, 1974; Cooper et al., 1980; Leon et al., 1980). These ribosomal antigens confer a high degree of protection when used as vaccines. Skeiky et al. (1995) isolated a gene from *Leishmania braziliensis* homologous to the eukaryotic ribosome associated eIF4A gene and shown that its recombinant protein is a potent antigen capable of stimulating strong Th1-type responses as well as IL-12 production in peripheral blood mononuclear cells of leishmaniasis patients. The ribosomal protein L7/L12 (12,200 Da) is located in the large ribosomal subunit (50S) of prokaryotes (Kaltschmidt and Wittman, 1970; Subramanian, 1975). This protein forms a well defined domain in the ribosome involved in interactions with translation factors during protein biosynthesis. The proteins L7 and L12 have identical sequences except for the presence of an acetylated amino-terminal serine residue in L7 (Terhorst et al., 1973). Both proteins are acidic but they differ slightly in their isoelectric points (Terhorst et al., 1972). Because of the close similarity they are often referred to in the literature as the L7/L12 protein. Protein L7/L12 has been studied in great detail because of the ease with which it can be removed selectively from the ribosomal subunits. The removal of L7/L12 reduces the accuracy and the rate of protein synthesis by an order of magnitude (Hamel et al.,

1972; Pettersson and Kurland, 1980). The L7/L12 ribosomal protein from *Mycobacterium bovis* was identified as a strong delayed-type hypersensitivity stimulus for sensitized guinea pigs (Tantimavanich et al., 1993); this observation heightened the interest in determining the importance of ribosomal antigen in protective immunity. *B. abortus* L7/L12 ribosomal protein has been identified as the most immunodominant antigen among 38 studied *B. abortus* proteins (Brooks-Worrell and Splitter, 1992; Oliveira et al., 1994). The nature of the T-cell response to L7/L12 protein has also been characterized. Recombinant *B. abortus* L7/L12 ribosomal protein induced a Th1 subset response from murine $CD4^+$ T cells featuring significant levels of IFN-γ production (Oliveira et al., 1994). Also, L7/L12 ribosomal protein from *B. melitensis* was identified as a major contributor to the antigenicity of Brucellergen for delayed-type hypersensitivity in *Brucella*-sensitized guinea pigs (Bachrach et al., 1994). Oliveira and Splitter (1996) tested the recombinant *B. abortus* L7/L12 to characterize the immunological importance as a T cell reactive ribosomal protein and showed that it conferred a significant degree of protection. Recently, the immune response to the *in vivo*-expressed *B. abortus* ribosomal L7/L12 gene in the muscle cells of mice was examined (Kurar and Splitter, 1997). This application established specific antibody and T-cell responses compared with negative controls and *B. abortus* strain 19 injected positive controls.

The objectives of this dissertation research were to analyze the potential of HSPs of *B. abortus* as protective antigens by assessing the optimal conditions for enhancement of immune responses using different adjuvants such as Freund, Ribi, or IL-12. Individual, divalent or trivalent recombinant HSP(s) expressing insect cells or individual/combined purified HSPs were injected into BALB/c mice in combination with different adjuvants. Priming by vaccinia virus GroEL recombinant and subsequent boosting by baculovirus GroEL recombinant with Ribi adjuvant was also tested. Androstenediol (AED) was tested to evaluate its function as an up-regulator. Adjuvanticity of IL-12 was tested by using putative positive control antigens *B. abortus* SOD and L7/L12. Humoral, cell-mediated immune response and protection studies were performed to evaluate the antigenicity of these *B. abortus* proteins. The results of this study contribute to the assessment of *B. abortus* HSPs as subunit vaccines to prevent brucellosis caused by *B. abortus*.

MATERIALS AND METHODS

1. Bacteria.

B. abortus strain RB51 was used as a live vaccine for positive control and strain 2308 was used as a virulent strain for challenge trials in protection studies. *B. abortus* RB51 and 2308 strains originated from stock cultures held in the infectious disease unit facility (biohazard level 3) at the Virginia-Maryland Regional College of Veterinary Medicine at Virginia Polytechnic Institute and State University. Trypticase soy agar

plates were streaked with Bacteria from a stock slant and incubated for 48 hours in 5% CO_2 air atmosphere at 37°C. A selected colony was inoculated into trypticase soy broth. The culture was incubated for 3 days at 37°C, harvested, and washed 3 times with PBS. Serial dilutions of the culture were performed, and 50 microliters of each dilution were plated on trypticase soy plates in 10 microliter drops and plates were incubated for 72 hours with 5 % CO_2 at 37°. Colony numbers were counted and colony forming units (CFU) were determined. The vaccine dose for strain RB51 was in the range of 1×10^8 to 4×10^8 CFU/mouse. Virulent *B. abortus* strain 2308 was used for all challenge inoculations at a dose of 2×10^4 CFU/mouse. Exact doses delivered were confirmed retrospectively by determining CFU of the inocula as given above.

2. Mice.

The BALB/c strain was chosen for this study due to its widespread use in brucellosis research (Phillips et al., 1989; Pugh et al., 1989; Araya et al., 1989; Enright et al., 1990). Clearance of *B. abortus* strains 2308 (Phillips et al., 1989; Enright et al., 1990), and RB51 (Schurig et al., 1991) in BALB/c mice corresponds closely to their respective virulence in cattle (Nicoletti and Milward, 1983; Nicoletti P., 1990; Schurig et al., 1991), suggesting that this inbred strain of mouse is an appropriate choice for initial vaccine trials. Female BALB/c inbred mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) were 5 to 6 weeks old upon arrival and were inoculated after a week of accommodation in our animal holding facilities. Mice were placed in mouse cages with no

more than five mice per cage and given feed and water *ad libitum*. The mice were killed by CO_2 inhalation at the times indicated in the protocols.

3. Adjuvants.

Complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO) (CFA) consists of heat killed and dried *Mycobacterium tuberculosis* (H3Ra, ATCC 25177), paraffin oil, and mannide monoleate. Incomplete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO) (IFA) consists of paraffin oil and mannide monoleate. Ribi adjuvant (Ribi Immunochemical Research, Inc., Hamilton, MO) consists of nontoxic monophosphoryl lipid A (MPL) produced from the endotoxin of *Salmonella minnesota* R595, trehalose dimycolate (TDM), squalene and Tween 80. The derivative of DHEA, AED was purchased from Sigma Chemical Co. (St. Louis, MO). Purified recombinant murine IL-12 was generously provided by Genetics Institute (Cambridge, MA).

4. Development of *Brucella abortus* -SOD, L7/L12 recombinants.

These recombinants were prepared by and obtained from R. Vemulapalli at the Center for Molecular Medicine and Infectious Disease in the Department of Biomedical Sciences and Pathobiology, VMRCVM, Virginia Polytechnic Institute and State University.

The *B. abortus* SOD recombinant protein was expressed in *E. coli* by cloning the *sod*C gene into pBluescript II phagemid vector (Stratagene, La Jolla, CA). The DNA

fragment containing the *sod*C gene and its promoter was initially obtained from a pUC9 genomic library of *B. abortus* strain 2308 (unpublished). The recombinant pBluescript plasmid was transformed into *E. coli* DH5α cells, and colonies containing recombinants were selected through ampicillin resistance and blue/white color screening. An *E. coli* colony expressing recombinant protein was grown in 1 liter culture. *B. abortus* Cu/Zn SOD protein was extracted from resuspended *E. coli* cell pellets mixed with lysis buffer (10 mM sodium phosphate buffer, pH 7.5, with 0.1 % Triton-X100). The identity of the SOD recombinant protein was confirmed by Western blot using goat anti-*B. abortus* RB51 sera and goat anti-Cu/Zn SOD sera.

The *B. abortus* L7/L12 ribosomal gene was amplified by PCR and subcloned into the expression vector pMAL-c2 (New England Biolabs, Beverly, MA.). This expression system was successfully used to express the L7/L12 protein in a previously published study (Oliveira and Splitter, 1994). Primers (Forward primer: 5'GGATCCATGGCTGATCTCGCAAAGATCGTT3', Reverse primer: 5'CTGCAGTTACTTGAGTTCAACCTTGG CGCCAGC3') were constructed based on the *B. abortus* L7/L12 gene sequence (Oliveira et al., 1994). PCR was performed in a 25 µl volume with 5 ng DNA template (genomic DNA of *B. abortus* 2308) and 1 µM of each primer. Ready-To-Go PCR beads (Amersham Pharmacia Biotech, Piscataway, New Jersey) were used for the amplification. PCR amplification was conducted in 35 cycles of a DNA thermal cycler using the following conditions: denaturation at 94^{o}C for 60 s, annealing at 62^{o}C for 90 S, and extension at 72^{o}C for 90 s. The PCR-amplified product

was purified by Geneclean (Bio 101, Inc., La Jolla, CA) and cloned into PCR2.1 plasmid of TA cloning system (Invitrogen, Carlsbad, CA). From the PCR2.1 plasmid, the L7/L12 gene was excised by *Bam*HI and *Pst*I digestion and ligated into the predigested pMAL-c2 vector, using DNA ligase (Gibco BRL, Gaithersburg, MD). The ligation reaction was used to transform *E. coli* DH5α, and single recombinant clones were selected. Positive *E. coli* clones possessing the pMAL-L7/L12 construct were selected, and expression of the fusion protein was induced by 0.6 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After 4 hrs of IPTG induction, bacterial cells were harvested by centrifugation at 4,000 X g for 20 min, and the pellet was washed with PBS three times. Cells were frozen at -80^{o}C and titration was done before use.

5. Protein Purification.

5.1. *B. abortus*-GroEL, GroES, HtrA, and SOD purification.

In addition to Sf9 cells expressing each recombinant protein, purified proteins were also used for immunization studies. Proteins were purified as follows. Lysates of *B. abortus*-GroEL, *B. abortus*-GroES, or *B. abortus*-HtrA recombinant protein expressing insect cells or *B. abortus*-SOD recombinant protein expressing *E. coli* cells were electrophoresed on 12.5 or 15 % polyacrylamide gels in a Protein II Vertical Electrophoresis cell (Bio-Rad, 15 cm X 15 cm X 0.1 cm) using the discontinuous buffer system of Laemmli (1970). Electrophoresis was performed at 100 V until the dye front reached the bottom. One slice of the gel was used for Western blot to verify the presence

and size of each recombinant protein. The required protein band was excised and electro-eluted using a Electroeluter Model 422 (Bio-Rad, Hercules, CA) at 10 mA per tube. Elution was done for 4 h for *B. abortus* 10 kDa GroES recombinant protein or *B. abortus* 17 kDa SOD recombinant protein, and for 6 h for *B. abortus* 60 kDa GroEL recombinant protein or *B. abortus* HtrA recombinant protein using elution buffer containing 25 mM Tris, 192 mM glycine, and 0.1 % SDS, pH 8.3. SDS was removed by adding Bio-Beads SM-2 Adsorbent (Bio-Rad, Hercules, CA) and rocking for 2 hrs followed by Sephadex G-10 column separation. Since *B. abortus*-SOD recombinant protein was expressed in the *E. coli* system, lipopolysaccharide was removed using polymixin B immobilized on agarose, Detoxi-Gel (Pierce, Rockford, IL). The polymixins are a family of antibiotics that contain a cationic cyclopeptide with a fatty acid chain. Polymixin B can neutralize the biological activity of endotoxins by binding to the lipid A portion of bacterial lipopolysaccharide. Purified proteins from which lipopolysaccharide was removed were dialyzed in cold distilled water for one day and lyophilized. The presence of purified protein was confirmed by further SDS-PAGE and immunoblotting. Protein concentration was determined by the method of Bradford using dye (Bio-rad, Hercules, CA) and bovine serum albumin (Fisher, Fair Lawn, N.J.) as the standard as described in Chapter3.

To obtain purified *B. abortus*-Cu/Zn SOD recombinant protein, an *E. coli* colony with the SOD gene was inoculated and cultured overnight in 1 liter of trypticase soy broth containing 100 μg/ml ampicillin. Cultures were harvested at 6,000 x g, for 20 minutes. The pellets were resuspended in lysis buffer (10 mM sodium phosphate buffer,

pH 7.5, with 0.1 % Triton-X100) due to the presence of SOD protein in the periplasmic space, and were incubated for 16-20 hours in a rotary shaker at 37^{o}C. The culture was lysed once again as before. Centrifugation was performed as above, and the extracts were separated on SDS-PAGE and Western blotted to detect the presence of *B. abortus*-SOD recombinant protein. *E. coli* cells identified as expressing *B. abortus*-SOD recombinant protein were purified by electro-elution as described before.

5.2. *B. abortus* L7/L12 ribosomal protein purification.

For the pMBP (maltose binding protein)-L7/L12 fusion protein purification, 10 ml of an overnight culture of *E. coli* containing fusion plasmid was inoculated in 1 liter of rich media (10 g tryptone, 5 g yeast extract, 5 g NaCl) containing glucose and ampicillin and cultured to 2 x 10^8 cells/ml (A_{600} = 0.5). IPTG, a transcription inducer, was added to a final concentration of 0.3 mM and the cultures were incubated at 37^{o}C for 2 hours. Cells were harvested (4000 x g, 20 minutes), and the pellets were resuspended in 50 ml column buffer (20 mM Tris-Cl, 200 mM NaCl, 1 mM EDTA, pH= 7.0). The culture was frozen in a dry ice-ethanol bath and thawed in cold water. A sample was placed in an ice-water bath and sonicated in short pulses of 15 seconds several times until release of the protein was observed using the Bradford assay. The sample was harvested (9,000 x g, 30 minutes), and this crude extract was diluted to 1:5 with column buffer. Diluted extract was poured in a column containing amylose resin (New England Biolabs, Beverly, MA) which was washed with 8 column volumes of column buffer. Diluted crude extract was passed through at a flow rate of about 1 ml/min for a 2.5 cm column. The column was washed with 10 column volumes of column buffer. The fusion protein was eluted with

column buffer containing 10 mM maltose. Fifteen fractions (3 ml each) were collected, and protein concentrations of each fraction were measured by Bradford assay, and purification of the protein was demonstrated by SDS-PAGE and Western blotting. Besides amylose resin affinity column purification, ammonium sulfate ($(NH_4)_2SO_4$) precipitation, and electro-elution were also performed. For ammonium sulfate precipitation, gradients of ammonium sulfate were used by mixing lysates of *E. coli* cells containing pMal-L7/L12 fusion protein with eleven different volumn of saturated ammonium sulfate solution. Samples were incubated at 37^{o}C for overnight. Samples were centrifuged (4000 x g, 20 minutes), and the pellets were redissolved with 1 ml dH_2O. Samples were dialyzed overnight to desalt. Electro-elution was also attempted as described above and eluted materials were concentrated by either ultrafiltration using an ultrafilter (membrane for molecular weight cut off 30 kDa or 60 kDa) (Micron Separations Inc., Westboro, MA) or lyophilization. The presence of purified protein was confirmed by SDS-PAGE and Western blotting.

6. Preparation of *E.coli* cells expressing pMBP only, and *E.coli* cells expressing pMBP-*B. abortus* L7/L12 fusion protein for mice immunization.

E. coli cells were cultured and lysed as described above (5.2), and the expression of MBP only or MBP-*B. abortus* L7/L12 protein were confirmed by SDS-PAGE and

Western blotting. Titration was done and bacterial cell numbers were adjusted into 1 x 10^7 cells/ml.

7. Immunization procedures.

Six to seven weeks old, female BALB/c mice were immunized for all following experiments. Mice were inoculated either intraperitoneally (i.p.) or subcutaneously (s.c.). For protective immune response analysis, mice were challenged with *B. abortus* virulent strain 2308 at a dose of 2 x 10^4 CFU/mouse, and mice were killed 14 days later for viable cell counts in the spleen.

Experiment I. : Humoral, cell-mediated immune (CMI) and protective immune responses of mice inoculated with recombinant protein expressing Sf9 cells combined with Freund's adjuvant

Nine groups of mice (3 mice/group, a total of 27 mice for CMI response, 5 mice/group, a total of 45 mice for protective immune response) were injected i.p. as indicated in Table 2.

INOCULA	**DOSAGE**
Sf9 cells + Freund's adjuvant	1st injection : 100 µl of 5 x 10^6 cells + 100 µl of 100 µg CFA 2nd injection : 100 µl of 1 x 10^7 cells + 100 µl of IFA
Bacmid/Sf9 cells + Freund's adjuvant	1st injection : 100 µl of 5 x 10^6 cells + 100 µl of 100 µg CFA 2nd injection : 100 µl of 1 x 10^7 cells + 100 µl of IFA
Freund's adjuvant only	1st injection : 100 µl of 100 µg CFA + 100 µl of saline 2nd injection : 100 µl of IFA + 100 µl of saline
B. abortus RB51	1st injection : 100 µl of 4 x 10^8 cells 2nd injection : none
GroEL/Sf9 cells + Freund's adjuvant	1st injection : 100 µl of 5 x 10^6 cells + 100 µl of 100 µg CFA 2nd injection : 100 µl of 1 x 10^7 cells + 100 µl of IFA
GroES/Sf9 cells + Freund's adjuvant	1st injection : 100 µl of 5 x 10^6 cells + 100 µl of 100 µg CFA 2nd injection : 100 µl of 1 x 10^7 cells + 100 µl of IFA
HtrA/Sf9 cells + Freund's adjuvant	1st injection : 100 µl of 5 x 10^6 cells + 100 µl of 100 µg CFA 2nd injection : 100 µl of 1 x 10^7 cells + 100 µl of IFA
GroEL-GroES-HtrA /Sf9 cells + Freund's adjuvant	1st injection : 100 µl of 5 x 10^6 cells each + 100 µl of 100 µg CFA 2nd injection : 100 µl of 1 x 10^7 cells each + 100 µl of IFA

Table 2. Inocula and dosage of each group for Experiment I.

DAY	WORK PERFORMED
0	1st injection for all groups of mice
14	2nd injection for all groups of mice except for RB51 group
35, 36, 37	Sample collections and preparations for the humoral and CMI response analysis (9 mice/day)
49	Challenge of all groups with *B. abortus* virulent strain 2308 for protective immune response analysis
63, 64	Protective immune response analysis : viable *B. abortus* virulent strain 2308 in spleen (25 mice on day 63, 20 mice on day 64)

Table 3. Experimental schedules for Experiment I.

Experiment II. : CMI response of mice inoculated with purified *B. abortus* recombinant HSPs combined with Ribi adjuvant.

Preliminary studies used eleven groups of mice (2 mice/group, a total of 22 mice) were injected s.c. as shown in Table 4. Then, six groups of mice (5 mice/group, a total of 30 mice) were injected as indicated in Table 5 s.c. Both experiments were performed by the schedule in Table 6.

INOCULA	DOSAGE
Saline	1st and 2nd injections : 200 µl
Ribi adjuvant	1st and 2nd injections : 100 µl of 100 µg adjuvant + 100 µl of saline
B. abortus live RB51	1st injection : 100 µl of 4 x 10^8 cells 2nd injection : none
GroEL + Ribi adjuvant	1st and 2nd injections : 100 µl of 1 µg antigen + 100 µl of 100 µg adjuvant
GroES + Ribi adjuvant	1st and 2nd injections : 100 µl of 1 µg antigen + 100 µl of 100 µg adjuvant
HtrA + Ribi adjuvant	1st and 2nd injections : 100 µl of 1 µg antigen + 100 µl of 100 µg adjuvant
GroEL + Ribi adjuvant	1st and 2nd injections : 100 µl of 10 µg antigen + 100 µl of 100 µg adjuvant
GroES + Ribi adjuvant	1st and 2nd injections : 100 µl of 10 µg antigen + 100 µl of 100 µg adjuvant
HtrA + Ribi adjuvant	1st and 2nd injections : 100 µl of 10 µg antigen + 100 µl of 100 µg adjuvant
GroEL + GroES + Ribi adjuvant	1st and 2nd injections : 100 µl of 10 µg each antigen + 100 µl of 100 µg adjuvant
GroEL + GroES + HtrA + Ribi adjuvant	1st and 2nd injections : 100 µl of 10 µg each antigen + 100 µl of 100 µg adjuvant

Table 4. Inocula and dosage of each group for Experiment II (preliminary studies).

INOCULA	DOSAGE
Saline	1st and 2nd injections : 200 µl
Ribi adjuvant	1st and 2nd injections : 100 µl of 100 µg adjuvant + 100 µl of saline
B. abortus live RB51	1st injection : 100 µl of 4×10^8 cells, 2nd injection : none
GroEL + Ribi adjuvant	1st and 2nd injections : 100 µl of 10 µg antigen + 100 µl of 100 µg adjuvant
GroES + Ribi adjuvant	1st and 2nd injections : 100 µl of 10 µg antigen + 100 µl of 100 µg adjuvant
HtrA + Ribi adjuvant	1st and 2nd injections : 100 µl of 10 µg antigen + 100 µl of 100 µg adjuvant

Table 5. Inocula and dosage of each group for Experiment II.

DAY	WORK PERFORMED
0	1st injection for all groups of mice
21	2nd injection for all groups of mice except for RB51 group
56, 57, 58, 59, 60	Sample collections and preparations for CMI response analysis (5 mice on days 56, 58 and 6 mice on days 57, 59 for preliminary studies, total of 22 mice) (6 mice/day for main studies, total of 30 mice)

Table 6. Experimental schedule for Experiment II.

<u>Experiment III</u>. : Protective immune response of mice inoculated with purified *B. abortus* recombinant HSP combined with Ribi adjuvant.

Eight groups of mice (5 mice/group, a total of 40 mice) were injected s.c. as indicated in Table 7, and experiments were performed by the schedule in Table 8.

INOCULA	**DOSAGE**
Saline	1st and 2nd injections : 200 µl
Ribi adjuvant	1st and 2nd injections : 100 µl of 100 µg adjuvant + 100 µl of saline
B. abortus live RB51	1st injection : 100 µl of 4 x 10^8 cells, 2nd injection : none
GroEL + Ribi adjuvant	1st and 2nd injections : 100 µl of 10 µg antigen + 100 µl of 100 µg adjuvant
GroES + Ribi adjuvant	1st and 2nd injections : 100 µl of 10 µg antigen + 100 µl of 100 µg adjuvant
HtrA + Ribi adjuvant	1st and 2nd injections : 100 µl of 10 µg antigen + 100 µl of 100 µg adjuvant
GroEL + GroES + Ribi adjuvant	1st and 2nd injections : 100 µl of 10 µg each antigen + 100 µl of 100 µg adjuvant
GroEL + GroES + HtrA + Ribi adjuvant	1st and 2nd injections : 100 µl of 10 µg each antigen + 100 µl of 100 µg adjuvant

Table 7. Inocula and dosage of each group for Experiment III.

DAY	WORK PERFORMED
0	1st injection for all groups of mice
21	2nd injection for all groups of mice except for RB51 group
56	Challenge of all groups with *B. abortus* virulent strain 2308 for protective immune response analysis
70	Protective immune response analysis : viable *B. abortus* virulent strain 2308 in spleen

Table 8. Experimental schedule for Experiment III.

<u>Experiment IV</u>. : Protective immune response of mice inoculated with androstenediol (AED) as an up-regulator.

Three groups of mice (5 mice/group, a total 15 mice) were injected s.c. only once as indicated in Table 9, and experiments were performed by the schedule in Table 10.

INOCULA	DOSAGE
Saline	200 µl
Vehicle only	200 µl of dimethyl sulfoxide (DMSO) and ethanol (EtOH) mixture
AED in vehicle	AED suspension in vehicle was injected into each mouse at a concentration of 320 mg/kg

Table 9. Inocula and dosage of each group for Experiment IV.

HOUR DAY	WORKS PERFORMED
0	injection for all groups of mice
4 hrs later	Challenge of all groups with *B. abortus* virulent strain 2308 for protective immune response analysis
Day 14	Protective immune response analysis : viable *B. abortus* virulent strain 2308 in spleen

Table 10. Experimental schedule for Experiment IV.

Experiment V. : Protective immune response of mice primed with vaccinia virus GroEL recombinant and boosted with *B. abortus* GroEL baculovirus purified recombinant protein.

Vaccinia virus recombinant GroEL was developed by and obtained from S. Baloglu at the CMMID in the DBSP, VMRCVM, Virginia Polytechnic Institute and State University (S. Baloglu, 1997). Four groups of mice (5 mice/group, a total of 20 mice) were injected i.p as indicated in Table 11,. and experiments were performed by the schedule in Table 12.

INOCULA	DOSAGE
Saline	1st, 2nd , and 3rd injections : 200 µl
B. abortus live RB51	1st injection : 100 µl of 4 x 10^8 cells, 2nd injection : none 3rd injection : none
Vaccinia virus GroEL Recombinant	1st injection : 1 x 10^7 Plaque Forming Unit (PFU) of Vaccinia virus GroEL recombinant 2nd injection : 100 µl of 100 µg Ribi adjuvant only 3rd injection : 100 µl of 100 µg Ribi adjuvant only
Vaccinia virus GroEL Recombinant + Baculovirus GroEL Recombinant	1st injection : 1 x 10^7 PFU of Vaccinia virus GroEL recombinant 2nd injection : 100 µl of 10 µg Baculovirus GroEL purified recombinant protein in 100 µl of 100 µg Ribi adjuvant 3rd injection : 100 µl of 10 µg Baculovirus GroEL purified recombinant protein in 100 µl of 100 µg Ribi adjuvant

Table11. Inocula and dosage of each group for Experiment V.

DAY	WORK PERFORMED
0	1st injection for all groups of mice except for RB51 group
14	2nd injection for all groups of mice except for RB51 group
21	Single injection for RB51 group
28	3rd injection for all groups of mice except for RB51 group
56	Challenge of all groups with *B. abortus* virulent strain 2308 for protective immune response analysis
70	Protective immune response analysis : viable *B. abortus* virulent strain 2308 in spleen

Table 12. Experimental schedule for Experiment V.

<u>Experiment VI</u>. : CMI response of mice inoculated with killed *B. abortus* RB51 combined with IL-12 as adjuvant.

Five groups of mice (5 mice/group, a total 25 mice) were injected i.p. as indicated in Table 13, and experiments were performed by the schedule in Table 14.

INOCULA	**DOSAGE**
Saline	1st and 2nd injections : 200 µl
B. abortus live RB51	1st injection : 100 µl of 4 x 10^8 cells, 2nd injection : none
B. abortus killed RB51	1st and 2nd injections : 100 µl of 4 x 10^8 cells
IL-12 only	1st and 2nd injections : 100 µl of 0.5 µg adjuvant
B. abortus killed RB51 + IL-12	1st and 2nd injections : 100 µl of 4 x 10^8 cells + 100 µl of 0.5 µg adjuvant

Table 13. Inocula and dosage of each group for Experiment VI.

DAY	**WORK PERFORMED**
0	Single injection for RB51 group
7	1st injection for all groups of mice except for RB51 group
21	2nd injection for all groups of mice except for RB51 group
35	Challenge of all groups with *B. abortus* virulent strain 2308 for protective immune response analysis
49	Protective immune response analysis : viable *B. abortus* virulent strain 2308 in spleen

Table 14. Experimental schedule for Experiment VI.

<u>Experiment VII</u>. : CMI and protective immune responses of mice inoculated with killed *B. abortus* RB51, purified *B. abortus* HtrA and Cu/Zn SOD proteins, and *B. abortus* L7/L12 protein expressing *E. coli* cells combined with IL-12 as adjuvant.

Eleven groups of mice (5 mice/group, a total of 55 mice) were injected i.p. as indicated in Table 15, and experiments were performed by the schedule in Table 16.

INOCULA	**DOSAGE**
Saline	1^{st} and 2^{nd} injections : 200 µl
IL-12 only	1^{st} and 2^{nd} injections : 200 µl of 0.5 µg adjuvant
B. abortus killed RB51	1^{st} and 2^{nd} injections : 100 µl of 4 x 10^8 cells
B. abortus Cu/Zn SOD	1^{st} and 2^{nd} injections : 200 µl of 10 µg antigen
B. abortus live RB51	1^{st} injection : 100 µl of 4 x 10^8 cells 2^{nd} injection : none
B. abortus killed RB51 + IL-12	1^{st} and 2^{nd} injections : 100 µl of 4 x 10^8 cells + 100 µl of 0.5 µg adjuvant
B. abortus Cu/Zn SOD + IL-12	1^{st} and 2^{nd} injections : 100 µl of 10 µg antigen + 100 µl of 0.5 µg adjuvant
B. abortus HtrA + IL-12	1^{st} and 2^{nd} injections : 100 µl of 10 µg antigen + 100 µl of 0.5 µg adjuvant
E. coli live cells expressing pMBP-*B. abortus* L7/L12	1^{st} injection : 100 µl of 1 x 10^6 cells 2^{nd} injection : none
E. coli live cells expressing pMBP-*B. abortus* L7/L12 + IL-12	1^{st} injection : 100 µl of 1 x 10^6 cells 2^{nd} injection : none
E. coli live cells expressing pMBP + IL-12	1^{st} injection : 100 µl of 1 x 10^6 cells 2^{nd} injection : none

Table 15. Inocula and dosage of each group for Experiment VII.

DAY	WORK PERFORMED
0	1st injection for all groups of mice except for *B. abortus* RB51, *E. coli* live cells expressing pMBP-*B. abortus* L7/L12, *E. coli* live cells expressing pMBP- *B. abortus* L7/L12 + IL-12 *E. coli* live cells expressing pMBP + IL-12,
14	2nd injection for all groups of mice
56	Challenge of all groups with *B. abortus* virulent strain 2308 for protective immune response analysis
70	Protective immune response analysis : viable *B. abortus* virulent strain 2308 in spleen

Table 16. Experimental schedule for Experiment VII.

8. Analysis of Humoral Immune Responses.

Mice were bled retroorbitally, sera prepared, and analyzed by Western blotting. Goat anti-*B. abortus* RB51 serum was used as a positive control; sera were diluted 1:50. Lysates of recombinant protein expressing cells were subjected to SDS-PAGE and electophoretically transferred to nitrocellulose paper (Micron Separations Inc., Westborough, MA) using trans-blot (Bio-RAD, Hercules, CA) at 15 V for 30 min in a solution consisting of 25 mM Tris, 192 mM glycine, and 5 % (vol/vol) methanol. The nitrocellulose papers were incubated overnight with 5 % (wt/vol) skim milk (Carnation, Glendale, CA) in TBS (0.15 M NaCl, 20 mM Tris-HCl), pH 7.4, washed three times with TBS containing 0.05 % Tween 20 (TBS-tween 20) and incubated with the appropriate sera diluted in TBS. After three washes with TBS-tween 20, the nitrocellulose papers were incubated for 1 h with anti-mouse IgG sera conjugated to horseradish peroxidase (Cappel, West Chester, PA). The membranes were washed three times with TBS-tween 20, and the bands were visualized by the addition of the color reagent made of 15 mg of 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, MO) in 5 ml of methanol combined with 25 ml of TBS containing 0.06 % hydrogen peroxide (FisherScientific, Fair Lawn, N.J).

9. Analysis of Cell-mediated Immune Responses.

Lymphocytes were obtained by preparing single-cell suspensions from mice spleens by dispersion of the tissue through a sterilized stainless steel mesh and lysing of

the red blood cells with Tris-HCl buffered ammonium chloride (pH 7.2). Splenocyte cultures were either prepared in 96 well cell culture plates for lymphocyte proliferation assay, IFN-γ ELISA or stained with several monoclonal antibodies for scanning through flow cytometry. For lymphocyte proliferation assay and IFN-γ ELISA or flow cytometry analysis, isolated spleen cells were cultured in either microtiter wells containing 5×10^5 cells or in tubes containing 3×10^5 cells, respectively, in a volume of 200 µl of RPMI 1640 supplemented with 5×10^{-5} M 2-mercaptoethanol, penicillin-streptomycin (Gibco-BRL, Grand Island, NY), 1 mM glutamine (Gibco-BRL, Grand Island, NY), and 5 % (vol/vol) fetal calf serum (Intergen, Purchase, NY). For lymphocyte proliferation assay and IFN-γ ELISA, for stimulating antigens, purified GroEL, GroES, HtrA, and SOD recombinant proteins were used at a concentration of 5 µg/well, killed *B. abortus* RB51 was used between 5×10^3 and 5×10^8 cells/well, and Concanavaline A (Con A) was used at a concentration of 1 µg/well as a positive control to demonstrate cell viability. For flow cytometry analysis, 1×10^5 cells of each sample were stained with different combinations of monoclonal antibodies as described below.

9.1 Lymphocyte proliferation assay.

Cellular proliferation was analyzed after 48 h of incubation of the cultures by pulsing with 1 µCi of [^{3}H] thymidine per well. After a further 18 h of incubation at 37^{o}C, plates were harvested and processed for liquid scintillation counting. The results are given in cpms and stimulation indices (SI = cpm of test sample/cpm of media only) were determined. All tests were carried out in triplicate.

9.2 IFN-γ ELISA.

Supernatants for the analysis of IFN-γ were harvested from the cultures after 4 days of incubation. Supernatants were placed in the wells of MaxiSorp Immuno plates (Nunc, Roskilde, Denmark) precoated with rat anti-mouse IFN-γ monoclonal antibody in 0.5 % bovine serum albumin in carbonate/bicarbonate buffer pH 9.6. For IFN-γ standards, 50, 25, 12.5, 6.3, 3.2, 1.6, 0.8, 0.4, 0.2, and 0.1 ng of recombinant mouse IFN-γ were added into each well of the plate described above. After incubation for 3 h, the plate was washed and incubated with biotinylated detecting antibody (PharMingen, San Diego, CA) for 1 h at room temperature. After washing the plates, horse radish peroxidase-streptavidin (Vector, Burlingame, CA) was added and incubated for 1 h. The plates were washed and then tetra methol benzadine (TMB) substrate (Dako Corporation, Carpinteria, CA) was added and incubated for 30 min. The reaction was terminated by adding 0.18 M sulfuric acid and absorbance was measured at 450 nm. Linear regression was performed on concentration versus (vs.) optical density reading of the standard curve. IFN-γ concentrations were converted from the optical density numbers based on the IFN-γ standard curve. All tests were carried out in triplicate.

9.3 Flow cytometry analysis.

For flow cytometry, 1 x 10^5 cells of splenocytes were stained with either fluorescein isothiocyanate (FITC)-conjugated hamster anti-mouse IgG_1 CD3e monoclonal antibody and R-phycoerythrin (R-PE)-conjugated rat anti-mouse IgG_{2a} CD4 (L3T4) or fluorescein isothiocyanate (FITC)-conjugated hamster anti-mouse IgG_1 CD3e monoclonal

antibody and fluorescein isothiocyanate (FITC)-conjugated hamster anti-mouse IgG_1 CD3e monoclonal antibody R-PE-conjugated rat anti-mouse IgG_{2a} CD8 (Ly-2) (PharMingen, San Diego, CA). An equal number of splenocytes were stained with FITC-conjugated hamster IgG_1 and R-PE-conjugated rat IgG_{2a}, κ monoclonal immunoglobulin isotype standard as an isotype-matched negative control to determine nonspecific bindings. Flow cytometric analysis was done on a Coulter Epics XL flow cytometer with air cooled 488 nm laser and fluorescent emission was detected using 525 nm and 575 nm filters, counting 5,000 cells per sample.

10. Analysis of Protective Immune Responses.

10.1 Mouse challenge.

Five mice from each group were challenged intraperitoneally with 2×10^4 cfu/mouse of *B. abortus* smooth strain 2308 thirty five days after the last immunization.

10.2 Counting *B. abortus* 2308 colony numbers in spleens.

The mice were euthanized 14 days after challenge, their spleens were removed and macerated by grinding them in 1 ml of Trypticase Soy Broth™ containing sterile sand using a Teflon coated pestle. The cell suspension was diluted serially in ten fold dilutions in TSB and five drops of 10 µl from each dilution were plated out on Trypticase Soy Agar (TSA) plates. The plates were incubated for four days at 37^o C in 5 % CO_2 incubator and colony numbers were counted. Based on the colony numbers, the value of

the average *B. abortus* 2308 log cfu per mouse spleen and log protection for each group were determined.

10.3 Statistical Analysis.

All mouse immune response experiments were performed under completely randomized design. All calculations were performed using the General linear model (GLM) procedure of Statistical Analytical System (SAS) (Version 6.12, SAS Institute, Cary, NC). Statistical analysis for all experiments, except for using IL-12 as adjuvant (Exp. VII), consisted of analysis of variance followed by single degree of freedom contrasts between positive control (RB51) and every other treatment group or between negative control and every other treatment group.

When undertaking multiple contrasts between two groups it has been suggested that it is appropriate to use a Bonferroni correction, which adjusts for multiple comparisons (Howell 1992; Perloff and Kinsey, 1992). The Bonferroni correction, which uses a p/n adjustment to modify the significance level, thus holds the experiment-wise error rate less than the preselected alpha (0.05). Thus, the Bonferroni correction was applied to all contrast P-values. For the statistical analysis of the experiment using IL-12 as adjuvant (Exp. VII), single degree of freedom contrasts were used to analyze between positive control (live RB51)- and antigen with IL-12- treated mouse groups or between antigen without IL-12- and antigen with IL-12- treated mouse groups. Statistical differences between groups with $p < 0.05$ were considered significant.

RESULTS

1. Purification of *B. abortus*-GroEL, GroES, HtrA, and SOD recombinant proteins.

B. abortus-GroEL, GroES, and HtrA recombinant HSPs, expressed in the baculovirus system, and *B. abortus*-SOD recombinant protein, expressed in the *E. coli* system, were purified by electro-elution. In addition to elimination of SDS and salts from each protein purification, lipopolysaccharide was removed for SOD protein purification. SDS-PAGE and Western blottings were performed to demonstrate the identity and correct size of each purified protein. Desired size of purified recombinant HSPs (60 kDa GroEL, 10 kDa GroES, 60 kDa HtrA) as well as purified recombinant SOD protein (17 kDa) were identified (Fig. 14, Fig. 15). Protein concentrations determined by Bradford protein assay showed that a total between 2 and 3 mg of each purified protein were obtained. These were sufficient amounts of antigens for the *in vivo* and *in vitro* immune response studies.

Purification for pMBP-*B. abortus* L7/L12 fusion protein was not successfully accomplished after several attempts of affinity chromatography, ammonium sulfate fractionation, or electro-elution. Failure of purification by affinity chromatography might have resulted from the loss of affinity of maltose binding protein to amylose resin, due to a structural change in the fusion protein. Mixing with different concentrations of ammonium sulfate did not result in corresponding differences in the precipitation of samples. In the attempts of protein purification by electro-elution, maltose was highly

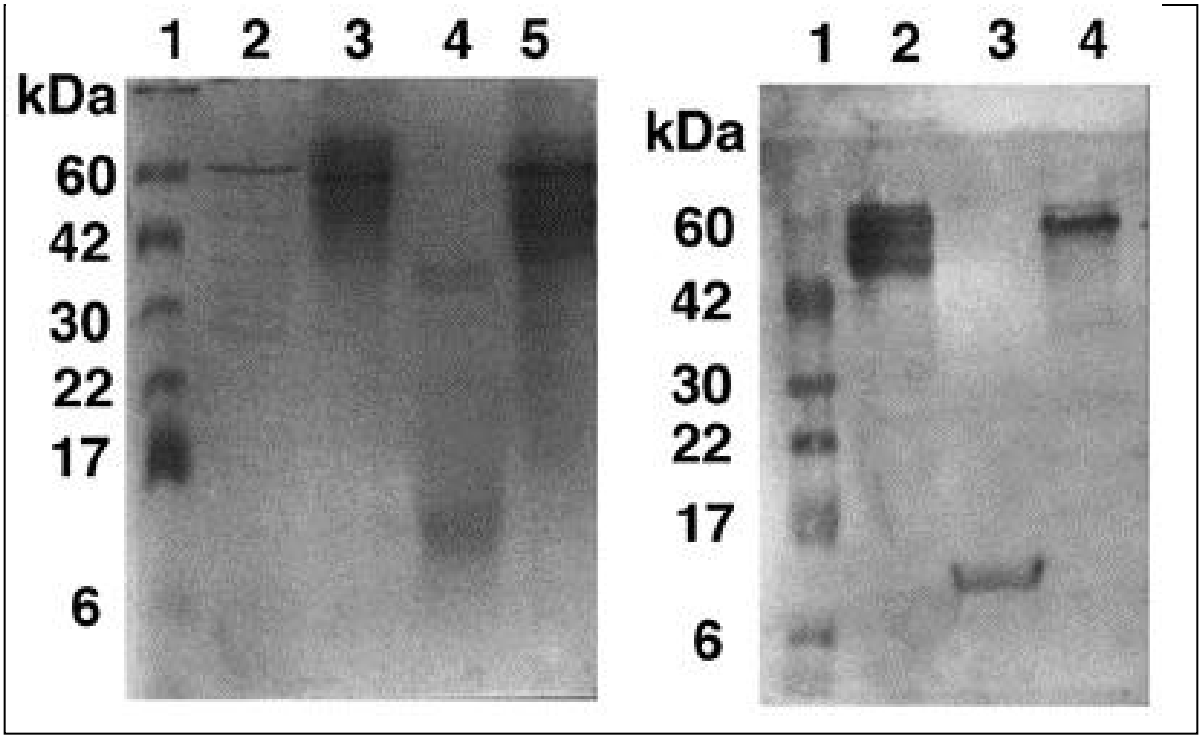

Figure 14. SDS-PAGE and Western blot analysis of electro-eluted baculovirus-*B. abortus* recombinant heat shock proteins.

Coomassie blue stained gel (Left),
Western blotting (Right); *B. abortus* anti-RB51 serum was used as the primary antiserum.

Left,
Lane 1: MW St
Lane 2: Bacmid only expressing Sf9 insect cells
Lane 3: 60 kDa purified GroEL recombinant protein
Lane 4: 10 kDa purified GroES recombinant protein
Lane 5: 60 kDa purified HtrA recombinant protein

Right,
Lane 1: MW St
Lane 2: 60 kDa purified GroEL recombinant protein
Lane 3: 10 kDa purified GroES recombinant protein
Lane 4: 60 kDa purified HtrA recombinant protein

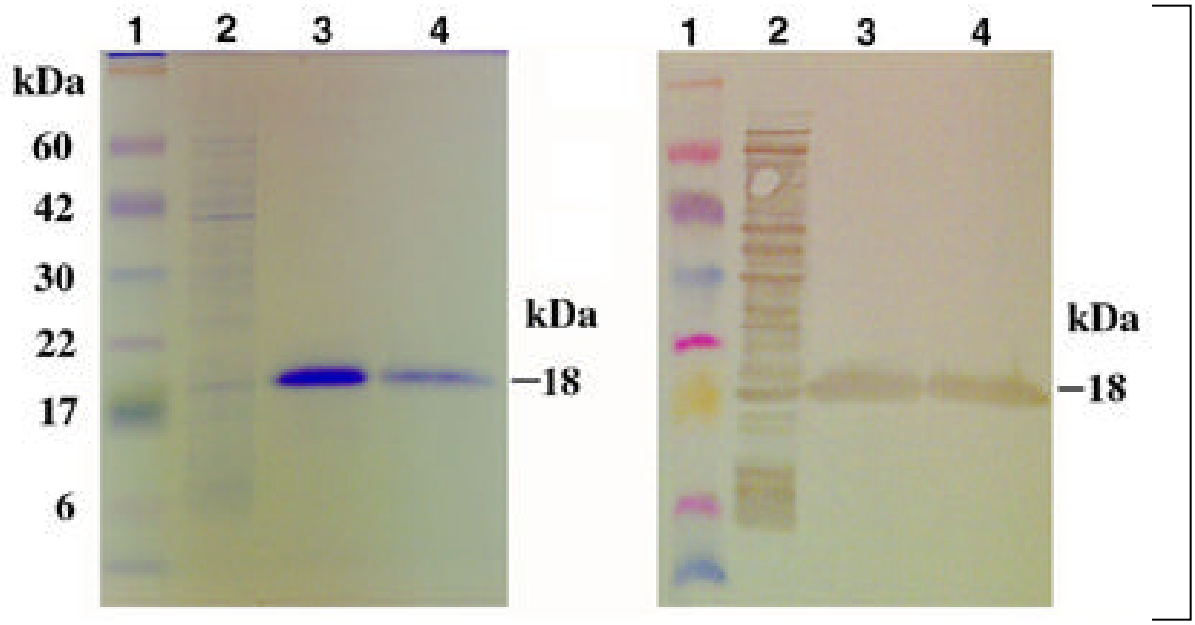

Figure 15. SDS-PAGE and Western blot analysis of electro-eluted baculovirus-*B. abortus* recombinant SOD protein.
Coomassie blue stained gel (Left),
Western blotting (Right); *B. abortus* anti-RB51 serum was used as the primary antiserum.

Both Figures,
Lane 1: MW St
Lane 2: SOD expressing *E. coli* cells
Lane 3: Purified SOD protein
Lane 4: Purified SOD protein

precipitated when eluted proteins were concentrated either by ultrafiltration or lyophilization. Therefore, instead of using purified L7/L12 purified protein, mice were injected with live *E. coli* cells expressing pMBP-L7/L12 fusion proteins as antigens in parallel with appropriate controls.

2. Identification of *B. abortus*-L7/L12 recombinant proteins.

Lysates of *E. coli* cells expressing only MBP or MBP-*B. abortus* L7/L12 fusion protein were analyzed by SDS-PAGE and Western blotting. Insertion of the L7/L12 gene inactivated the β-galactosidase α-fragment activity of the *malE* (gene encoding MBP)-lacZ fusion. Therefore, the MBP-L7/L12 fusion protein was identified as 59 kDa, composed of 47 kDa MBP and 12 kDa L7/L12. MBP expression without inserts showed a protein of similar size (57 kDa) because it is fused with β-galactosidase (10 kDa) instead (Fig. 16).

3. Immune responses.

3.1. Mice inoculated with *B. abortus* recombinant HSP(s) expressing Sf9 insect cells combined with complete and incomplete Freund's adjuvants (EXP.1).

3.1.1. Humoral immune response.

Humoral immune response was demonstrated by Western blotting against GroEL (Fig. 17 e) and HtrA (Fig. 17 g), but not against GroES (Fig. 17 f). Three distinct bands

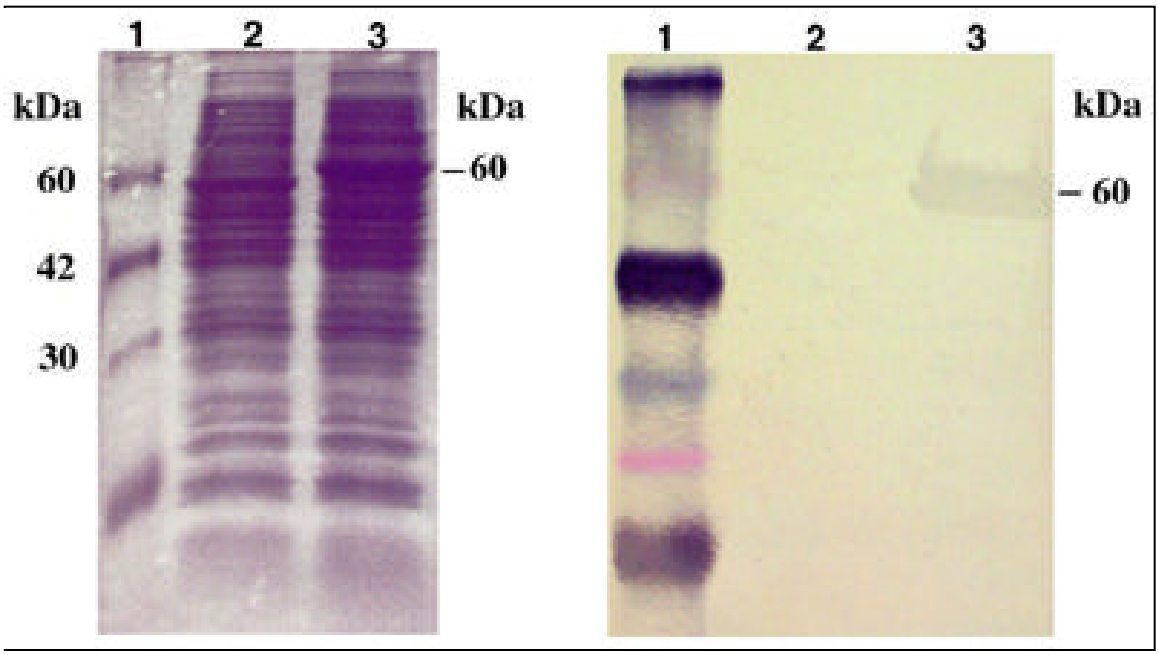

Figure 16. SDS-PAGE and Western blot analysis of baculovirus-*B. abortus* recombinant pMAL-L7/L12 protein.
Coomassie blue stained gel (Left),
Western blotting (Right); *B. abortus* anti-RB51 serum was used as the primary antiserum.

Lane 1: MW St
Lane 2: pMBP only expressing *E. coli* cells
Lane 3: pMBP-L7/L12 fusion protein expressing *E. coli* cells

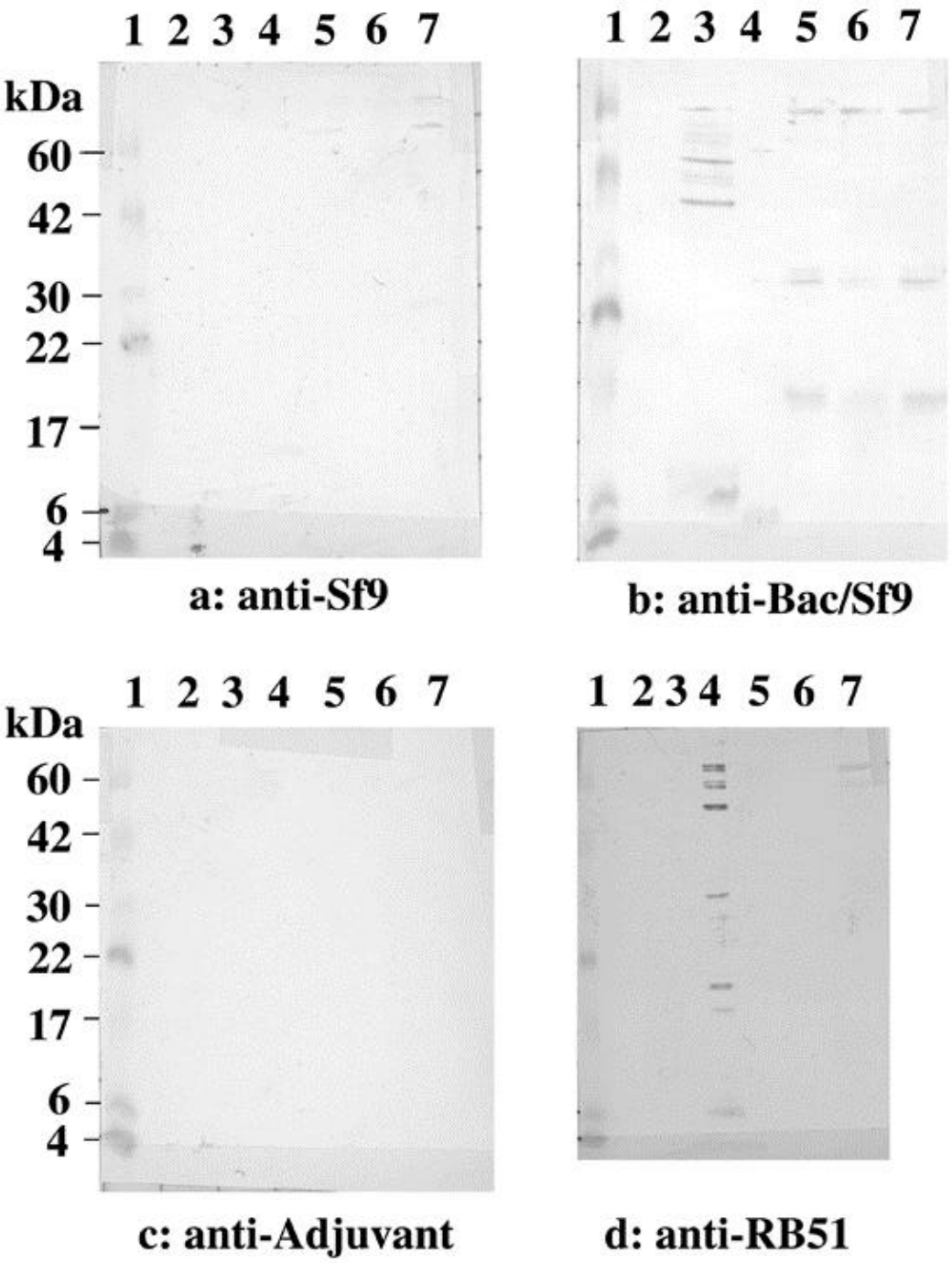

Figure 17 a, b, c, d. Humoral immune responses of BALB/c mice to baculovirus-*B. abortus* recombinant HSP(s) expressing Sf9 insect cells.

Sera from each group of mice inoculated with Sf9 insect cells (Fig. a), Bacmid/Sf9 (Fig. b), Freund's adjuvant only (Fig. c), or *B. abortus* RB51 (Fig. d), respectively, were applied as a primary antibody on each nitrocellulose membrane.

Lane 1: MW St. Lane 2: Sf9 Lane 3: Bacmid/Sf9 Lane 4: *B. abortus* RB51
Lane 5: GroEL/Sf9 Lane 6: GroES/Sf9 Lane 7: HtrA/Sf9

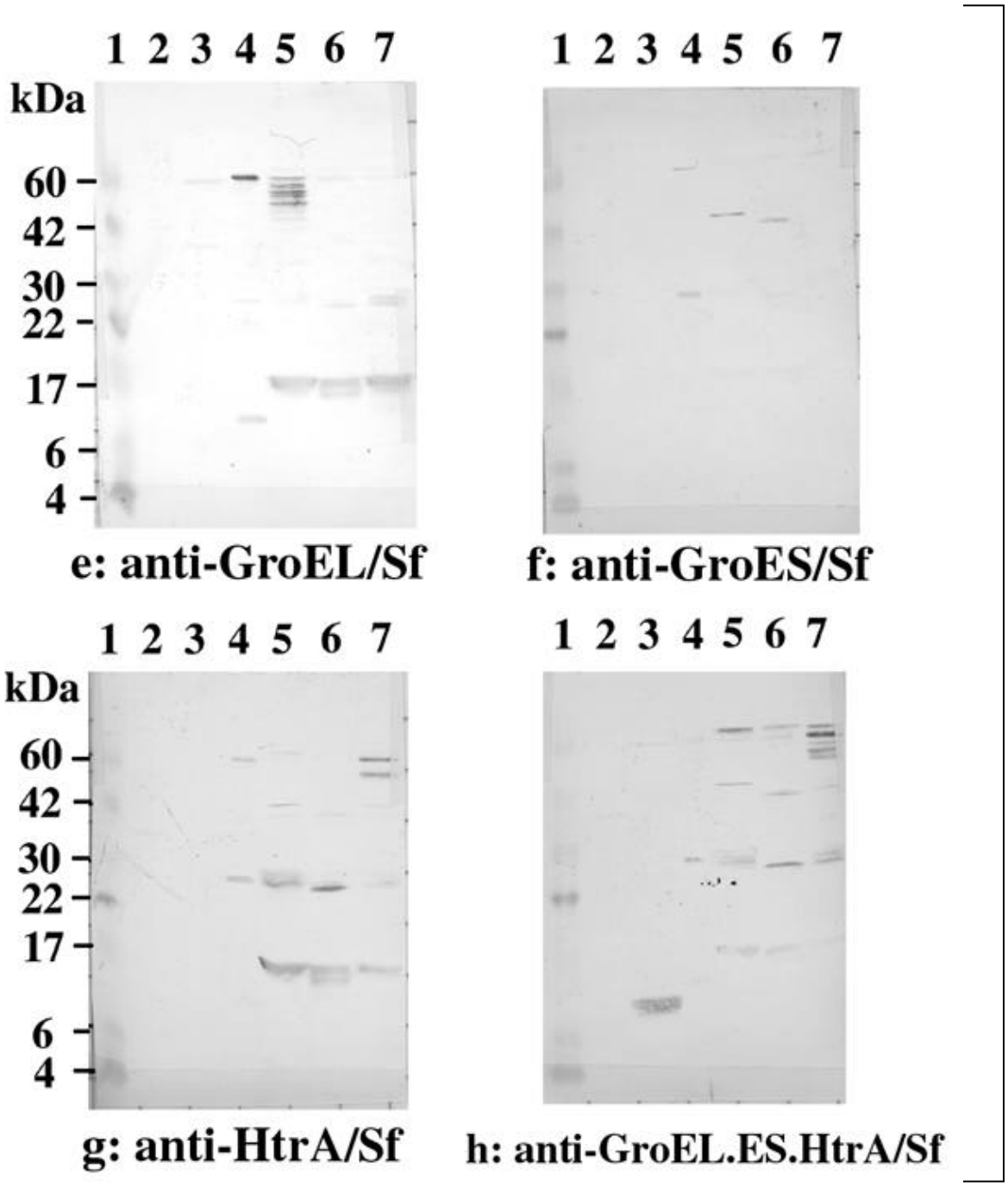

Figure 17 e, f, g, h. Humoral immune responses of BALB/c mice to baculovirus-*B. abortus* recombinant HSP(s) expressing Sf9 insect cells. Sera from each group of mice inoculated with GroEL/Sf9 (Fig. e), GroES/Sf9 (Fig. f), HtrA/Sf9 (Fig. g), or GroEL-GroES-HtrA/Sf9 (Fig. h), respectively, were applied as a primary antibody on each nitrocellulose membrane.

Lane 1: MW St. Lane 2: Sf9 Lane 3: Bacmid/Sf9 Lane 4: *B. abortus* RB51
Lane 5: GroEL/Sf9 Lane 6: GroES/Sf9 Lane 7: HtrA/Sf9

were observed in the results against Bacmid. This pattern of bands was consistent in the results with GroEL/Sf9, GroES/Sf9, and HtrA/Sf9 recombinant proteins. GroEL and HtrA antibodies were detected when the recombinant protein expressing Sf9 cells or *B. abortus* RB51 was used as an antigen (Fig. 17 e, g). However, the same antigens did not detect GroES antibodies (Fig. 17 f).

3.1.2. Cell-mediated immune responses (CMI).

Proliferation of lymphocytes was evaluated by incorporating ^{3}[H] thymidine into the lymphocytes for 48 hours. Several different antigens were used as stimulants to test antigen-specific proliferation of splenocytes. When Con A was used as a stimulant, splenocytes from each mice group showed high stimulation indices (Fig. 18 a); the splenocytes were normally reactive and had the capacity to react with specific antigen. When killed strain RB51 was used as a stimulant, only splenocytes from RB51-treated mice showed a high level of proliferation (Fig. 18 b), and splenocytes from other mice groups showed a significantly low level of stimulation. Splenocytes from mice treated with GroEL/Sf9, GroES/Sf9, HtrA/Sf9, or GroEL-GroES-HtrA/Sf9 combined with Freund's adjuvant showed a similar or even lower level of proliferation than did those from mice treated with negative control Bacmid/Sf9 with Freund's adjuvant by stimulation with killed RB51 (Fig. 18 b). Among experimentals, a relatively high level of, but not statistically significant, lymphocyte proliferation was consistently observed from mice treated with GroES/Sf in response to all antigens as well as to ConA. Thus, it does not reflect antigen-specific lymphocyte proliferation. Noteworthy, splenocytes from mice treated with any antigen containing a Sf9 cellular component (Sf9, Bacmid/Sf9,

GroEL/Sf9, GroES/Sf9, and HtrA/Sf9) reacted in the same pattern to the stimulating antigens Sf9, Bacmid/Sf9, GroEL/Sf9, GroES/Sf9, and HtrA/Sf9 (Figs. 18 c, d, e, f, g), indicating that the stimulation of splenocyte was due to Sf9 cells. These results indicate that there is strong splenocyte proliferation in mice against antigens of the Sf9 cells, masking potentially weak CMIs to GroEL, GroES and HtrA proteins.

3.1.3. Protective immune response.

Protection results are presented as mean log CFU/spleen and log protection (Table 17). The value for log protection is defined as the difference between mean log CFU/spleen in treated (inoculated with antigen or adjuvant) and negative control (inoculated with saline) mice. Achievement of protection was determined by statistical significance between saline and other experimental groups in the mean log CFU/spleen; if p value is less than 0.05, the experimental groups were considered as protected. Intraperitoneal challenge inoculation of virulent *B. abortus* strain 2308 showed that none of the recombinants induced protection. Only *B. abortus* RB51-inoculated (positive control) mice showed the level of protection ($p = 0.04$). The highest resistance in experimentals was shown by GroEL/Sf9-Freund's mice group, but no significant differences were observed between this group and saline-treated mice group (Table 17). The mean log CFU/spleen results were similar among the mice injected with GroES/Sf9, HtrA/Sf9, GroEL-GroES-HtrA/Sf9, and Freund's adjuvant only. The mean log CFU/spleen from mice injected with Sf9 cells expressing the various *B. abortus* recombinant heat shock proteins were somewhat higher, in other words, less resistant than those from mice injected with Sf9 cells (Table 17).

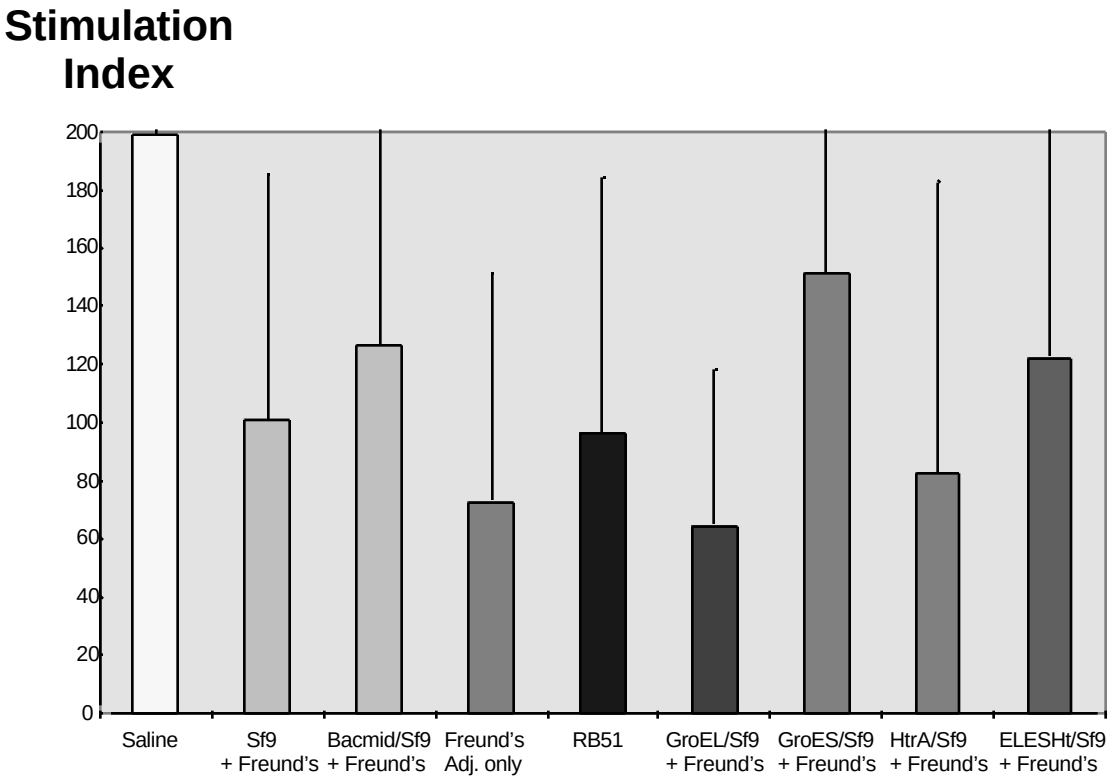

Fig. 18 a. Proliferation of splenocytes, stimulated by <u>ConA</u> *in vitro* (mean + standard deviation)

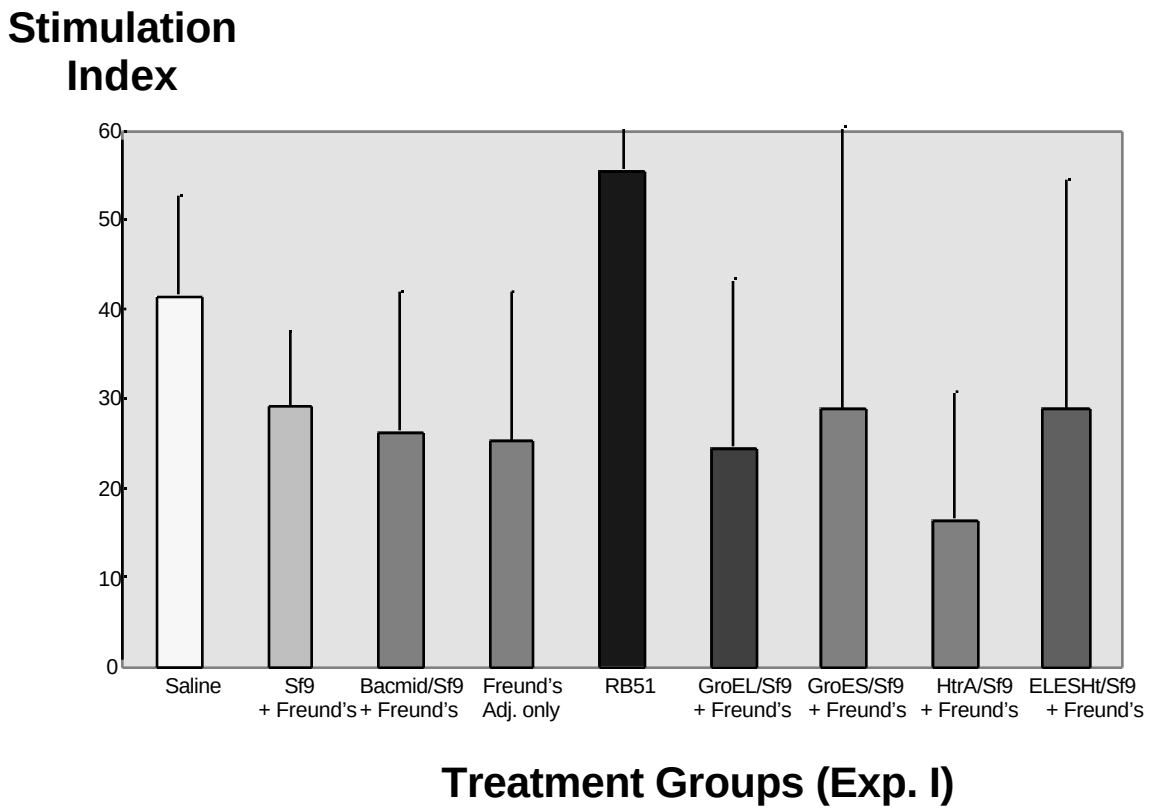

Fig. 18 b. Proliferation of splenocytes, stimulated by killed RB51 *in vitro* (mean ± standard deviation)

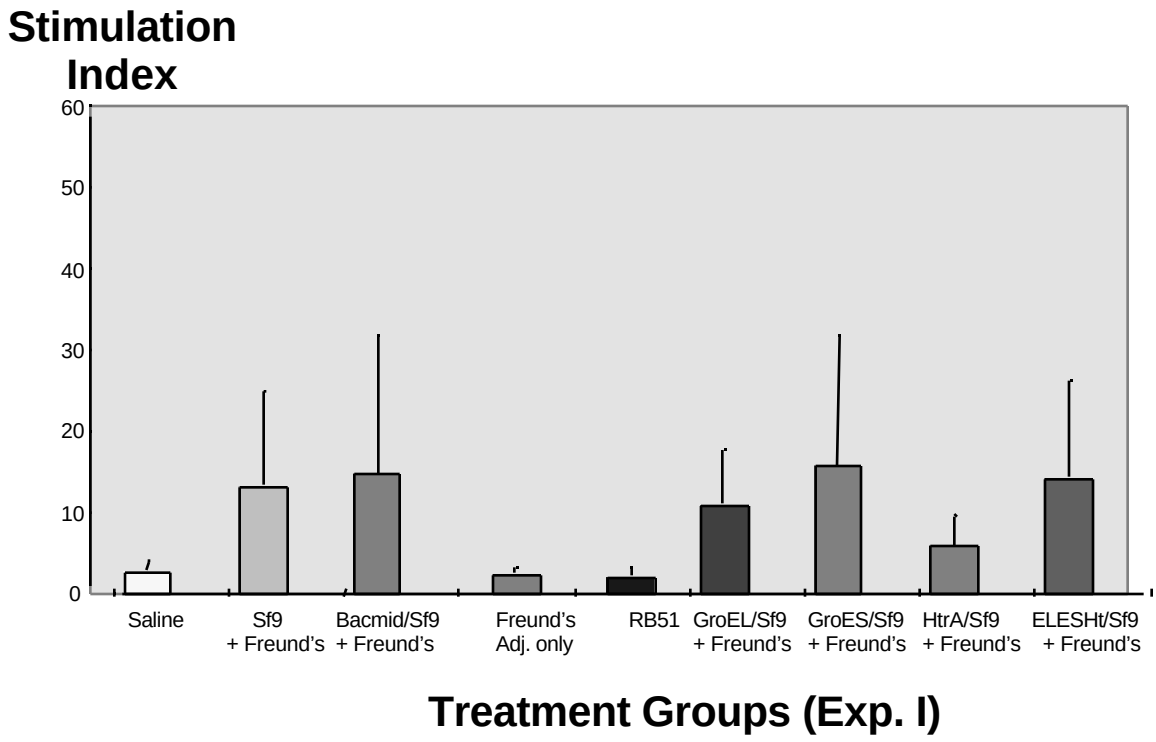

Fig. 18 c. Proliferation of splenocytes, stimulated by Sf9 cells *in vitro* (mean ± standard deviation)

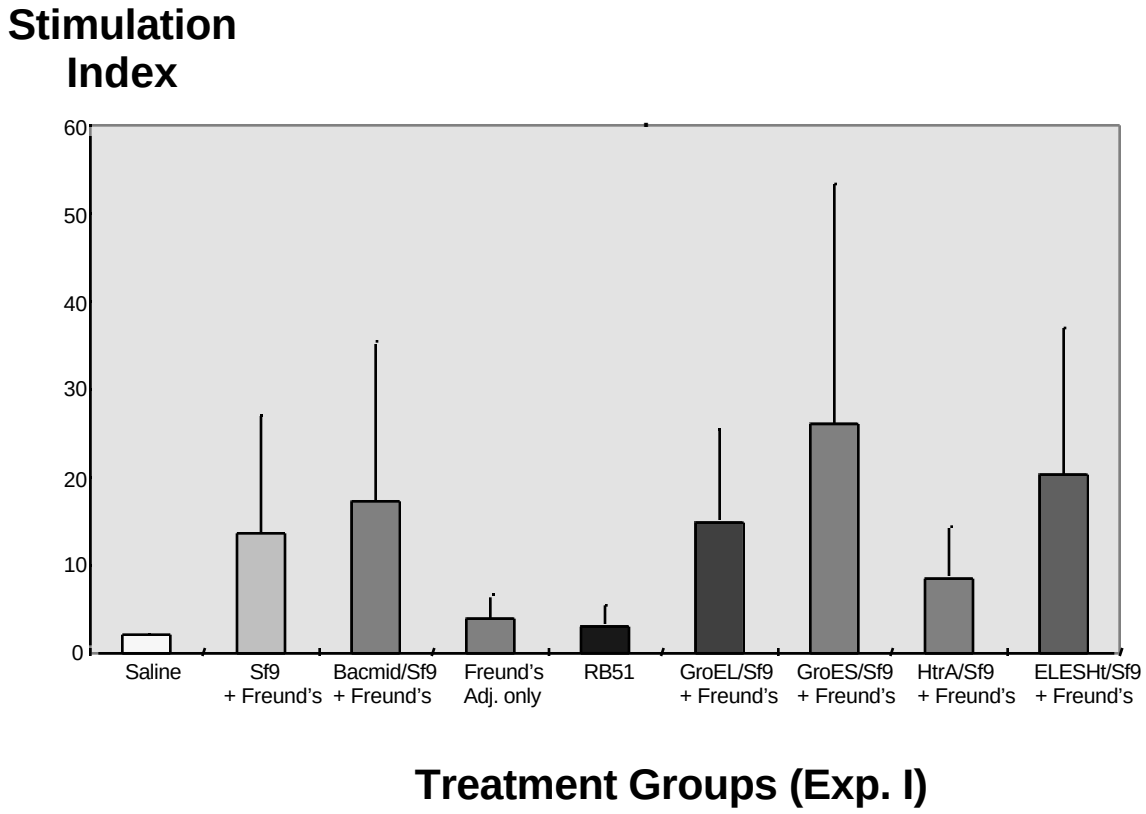

Fig. 18 d. Proliferation of splenocytes, stimulated by Bacmid/Sf cells *in vitro* (mean ± standard deviation)

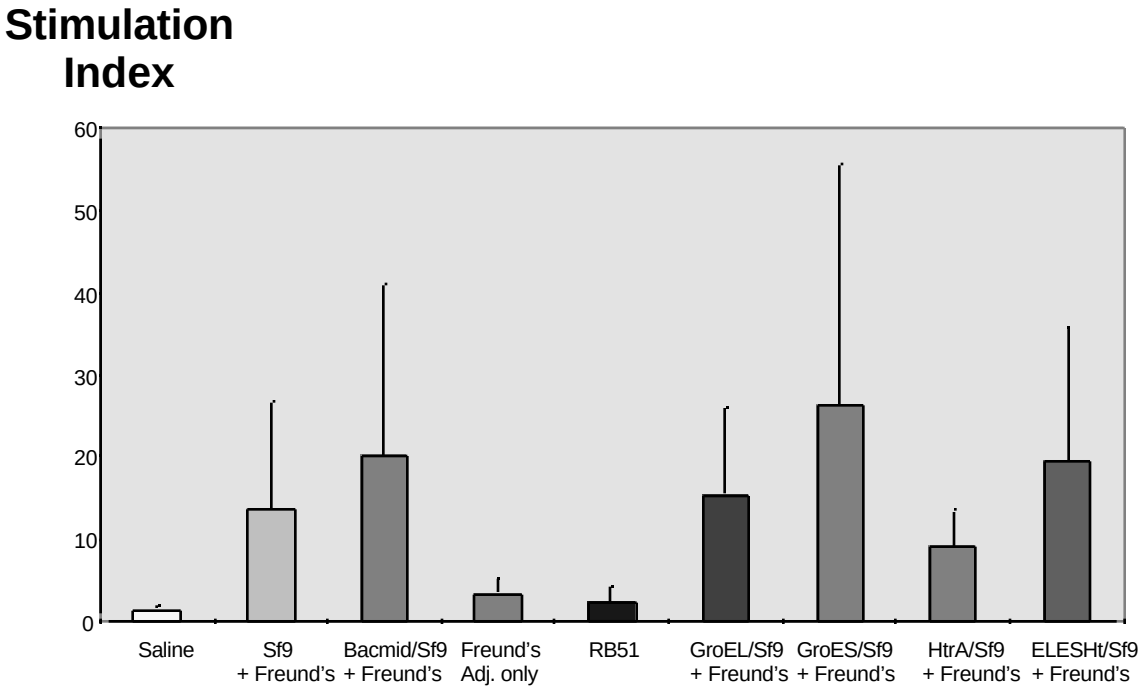

Fig. 18 e. Proliferation of splenocytes, stimulated by GroEL/Sf9 cells *in vitro* (mean ± standard deviation)

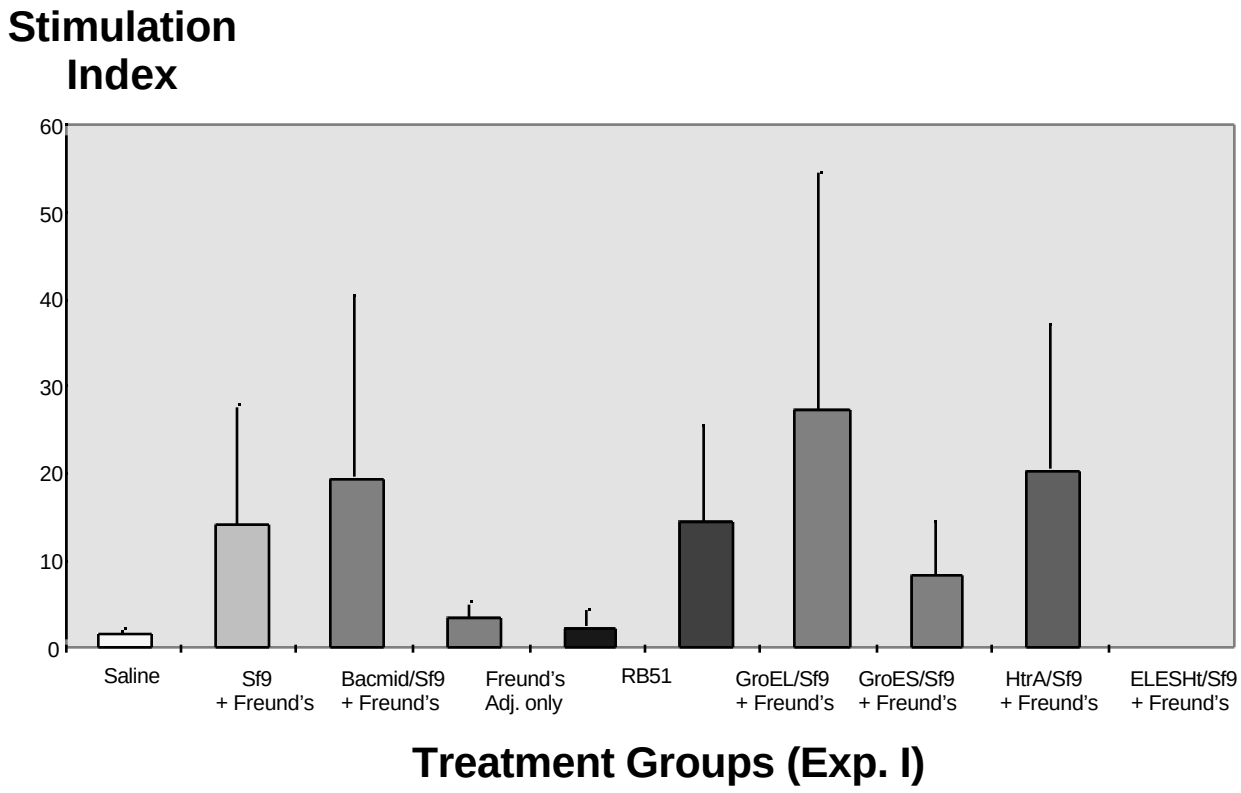

Fig. 18 f. Proliferation of splenocytes, stimulated by <u>GroES/Sf9</u> cells *in vitro* (mean <u>+</u> standard deviation)

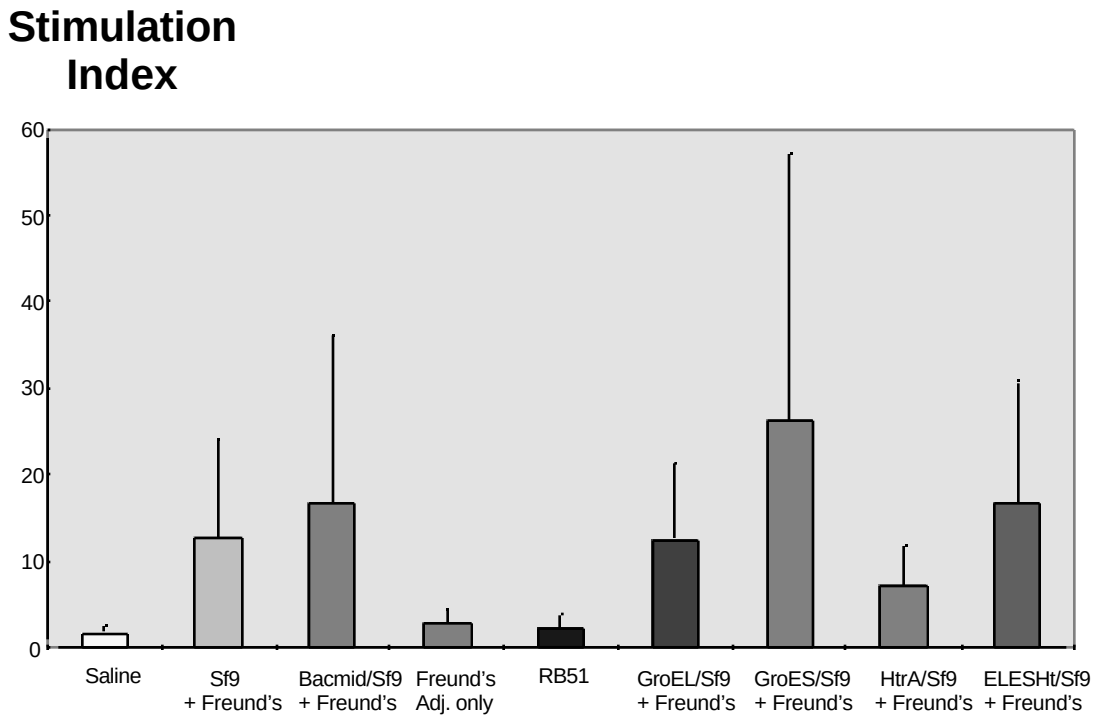

Fig. 18 g. Proliferation of splenocytes, stimulated by HtrA/Sf9 cells *in vitro* (mean ± standard deviation)

TREATMENT	LOG CFU/SPLEEN	LOG PROTECTION
Saline	5.26 ± 0.12	
Sf9 cells + Freund's adjuvant	4.57 ± 0.64 (p = 0.08)	0.69
Bacmid/Sf9 cells + Freund's adjuvant	4.54 ± 0.80 (p = 0.18)	0.72
Freund's adjuvant only	5.05 ± 0.14 (p = 0.20)	0.21
B. abortus live RB51	4.44 ± 0.48 (p = 0.04) *	0.82
GroEL/Sf9 cells + Freund's adjuvant	4.70 ± 0.84 (p = 0.17)	0.56
GroES/Sf9 cells + Freund's adjuvant	4.96 ± 0.33 (p = 0.19)	0.30
HtrA/Sf9 cells + Freund's adjuvant	5.16 ± 0.32 (p = 0.54)	0.10
GroEL-GroES-HtrA/Sf9 cells + Freund's adjuvant	5.08 ± 0.37(p = 0.49)	0.18

*** : Protection is achieved.**

Table 17.

***B. abortus* 2308 colony counts from lymphocytes of mice inoculated with *B. abortus* HSP(s) expressing Sf9 cells combined with Freund's adjuvant**

The high background in CMI responses and protection values of mice that were inoculated with Sf9 insect cell suggested that purification of recombinant proteins was required to generate the desired antigen-specific immune responses.

3.2. Mice inoculated with baculovirus *B. abortus* purified recombinant HSP(s) combined with Ribi adjuvant (Exp. II).

3.2.1. Cell-mediated immune response.

To explain the masking of recombinant protein-specific CMI and the lack of protection by Sf9 cells expressing recombinant protein, immune responses of mice to purified HSP(s) in combination with Ribi adjuvant were analyzed. Since low doses of HSPs as antigens are known to be more immunogenic in studies *in vivo* (DeNagel and Pierce, 1993), two different doses (1, 10 μg) of each HSP were evaluated in mice model. Relatively high IFN-γ release was observed in splenocytes from mice injected with 10 μg HtrA protein (Table 18). Receptors of CD3, CD4 or CD8 are found on the surface of mature T cells, helper T cells, and cytotoxic T cells, respectively. Highest proportions of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells/total number of splenocytes were also observed in splenocytes from mice injected with 10 μg HtrA protein (Table 19). These proportions of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells/total number of splenocytes in HtrA immunized mice were higher than those of the live RB51 immunized mice, which show the next highest value (Table 19).

Released IFN-γ in ng/ml

injected Ag / coated Ag	saline	Ribi	live RB51	GroEL 1 µg + Ribi	GroES 1 µg + Ribi	HtrA 1 µg + Ribi	GroEL 10 µg + Ribi	GroES 10 µg + Ribi	HtrA 10 µg +Ribi	GroEL-GroES 10 µg /each + Ribi	GroEL-GroES-HtrA 10 µg /each + Ribi
saline	0 ±0	.52 ±.11	0 ±0	.50 ±.09	0 ±0	1.40 ±.71	.52 ±.25	.49 ±.26	.80 ±.57	1.38 ±.79	0 ±0
killed RB51	0 ±0	.92 ±.45	>50	.92 ±.67	0 ±0	2.47 ±1.32	.90 ±.33	0 ±0	2.27 ±.98	.48 ±.23	1.43 ±.89
ConA	.87 ±.33	1.13 ±.78	5.87 ±1.95	4.80 ±2.45	2.33 ±.87	2.90 ±.89	1.50 ±.68	1.78 ±1.01	2.05 ±.92	3.43 ±1.12	1.72 ±.34
GroEL	0 ±0	1.22 ±.34	0 ±0	2.40 ±1.76	-----	-----	.44 ±.36	-----	-----	.73 ±.26	0 ±0
GroES	0 ±0	.42 ±.65	3.10 ±.79	-----	.50 ±.67	-----	-----	0 ±0	-----	.54 ±.33	0 ±0
HtrA	0 ±0	0 ±0	0 ±0	-----	-----	2.30 ±1.12	-----	-----	3.30 ±1.45	-----	0 ±0

-----: Not tested

Table 18. IFN-γ release by splenocytes of mice inoculated with purified recombinant *B. abortus* HSPs combined with Ribi adjuvant; preliminary study for determining optimal dosage of HSP.

$CD3^+CD4^+$, $CD3^+CD8^+$, and $CD3^+CD4^+/CD3^+CD8^+$ T cell ratio

injected with / stained with	saline	Ribi	live RB51	GroEL 1 µg + Ribi	GroES 1 µg + Ribi	HtrA 1 µg + Ribi	GroEL 10 µg + Ribi	GroES 10 µg + Ribi	HtrA 10 µg +Ribi	GroEL-GroES 10 µg /each + Ribi	GroEL-GroES-HtrA 10 µg /each + Ribi
γ/γ	0.0 ±0.0	0.4 ±0.0	0.0 ±0.0	0.2 ±0.0	0.3 ±0.0	0.2 ±0.0	0.4 ±0.0	0.3 ±0.0	0.1 ±0.0	0.3 ±0.0	0.4 ±0.0
CD3,4	22.8 ±2.2	25.0 ±3.7	27.6 ±2.9	27.1 ±4.7	23.7 ±3.3	28.0 ±2.3	20.0 ±4.1	20.1 ±1.5	31.1 ±5.5	18.5 ±2.3	21.8 ±3.3
CD3,8	7.3 ±0.5	5.7 ±1.1	7.8 ±2.2	7.4 ±2.0	6.7 ±0.6	6.9 ±2.8	6.3 ±1.9	5.8 ±3.3	8.5 ±2.2	5.4 ±0.9	5.7 ±1.9
Ratio of CD3,4 / CD3,8	3.1	4.4	3.5	3.7	3.5	4.1	3.2	3.5	3.7	3.4	3.8

Table 19. Percent of $CD3^+CD4^+$, $CD3^+CD8^+$, and the $CD3^+CD4^+/CD3^+CD8^+$ T cell ratio in splenocytes of mice inoculated with purified recombinant *B. abortus* recombinant HSPs combined with Ribi adjuvant; preliminary study.

It is not very well described in other studies whether there is a correlation between IFN-γ release and T helper ($CD3^+CD4^+$) or T cytotoxic ($CD3^+CD8^+$) cell proportions or between a specific cell-mediated immune response and T helper ($CD3^+CD4^+$) or T cytotoxic ($CD3^+CD8^+$) cell proportions. In this preliminary study, we did not observe any correlation between IFN-γ release and proportions of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in splenocytes. Since higher levels of IFN-γ release and $CD3^+CD4^+$, $CD3^+CD8^+$ T cell proportions were observed from mice injected with 10 μg HSP rather than 1 μg HSP, 10 μg of each HSP were tested in the main immunization studies. No significant level of IFN-γ release was observed by stimulation with saline (negative control)(Fig. 19 a). High level of IFN-γ release was observed by stimulation with ConA (no antigen-specific positive control)(Fig. 19 b). Mice injected with 10 μg of HtrA protein showed the highest IFN-γ release when their lymphocytes were stimulated by killed RB51 (Fig. 19 c). Thus, the preliminary (Table 18) and main (Fig. 19) studies are in agreement with respect to the highest level of antigen-specific IFN-γ release in the splenocytes from mice treated with 10 μg of HtrA, but the level of IFN-γ release was much higher in the main study. The level of IFN-γ release in the splenocytes from HtrA immunized mice was more than 50 ng/ml, exceeding the maximum reading scale by the spectrophotometer. Splenocytes of mice injected with GroEL or GroES protein released significantly less IFN-γ (= 25 ng/ml) in response to killed RB51 (Fig. 19 c). Results of IFN-γ release in splenocytes from mice injected with GroES or HtrA protein showed antigen-specific responses; higher responses

were observed to the specific coating antigens GroES and HtrA, respectively (Fig. 19 e, f). However, splenocytes from mice injected with GroEL protein did not show antigen-specific response (Fig. 19 d). Proportions of $CD3^+CD4^+$ (Fig. 20 a) and $CD3^+CD8^+$ T cells (Fig. 20 b) in the total number of splenocytes were not significantly different between mice injected with RB51 and any experimentals., Correlation was not observed between IFN-γ release and proportions of $CD3^+CD4^+$, $CD3^+CD8^+$ T cells in mice splenocytes in the main study, either.

3.2.2. Protective immune response (Exp. III).

In the results demonstrating mean log CFU/spleen, only *B. abortus* RB51 group mice showed protection to *B. abortus* 2308 challenge (log protection = 0.91). Mice injected with any of the experimental proteins, including HtrA, showed a similar level of mean log CFU/spleen and resistance to those injected with adjuvant only. No antigen-specific protection was observed among mice injected with any of the *B. abortus* purified HSP alone (GroEL, GroES, or HtrA), GroEL-GroES combined, or GroEL-GroES-HtrA combined in conjunction with Ribi adjuvant (Table 20). Therefore, no correlation was observed between the level of protection and IFN-γ release in mice splenocytes, nor between the level of protection and the proportions of $CD3^+CD4^+$, $CD3^+CD8^+$ T cells in mice splenocytes.

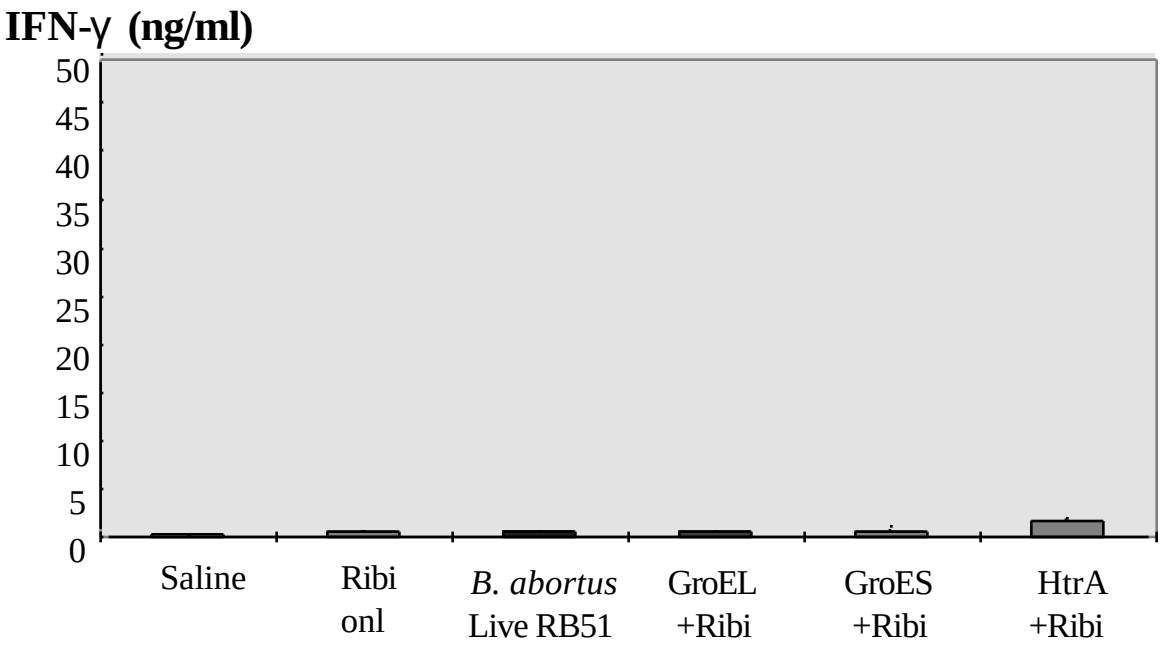

Fig. 19 a. IFN-γ release, stimulated by saline *in vitro*, (mean ± standard deviation)

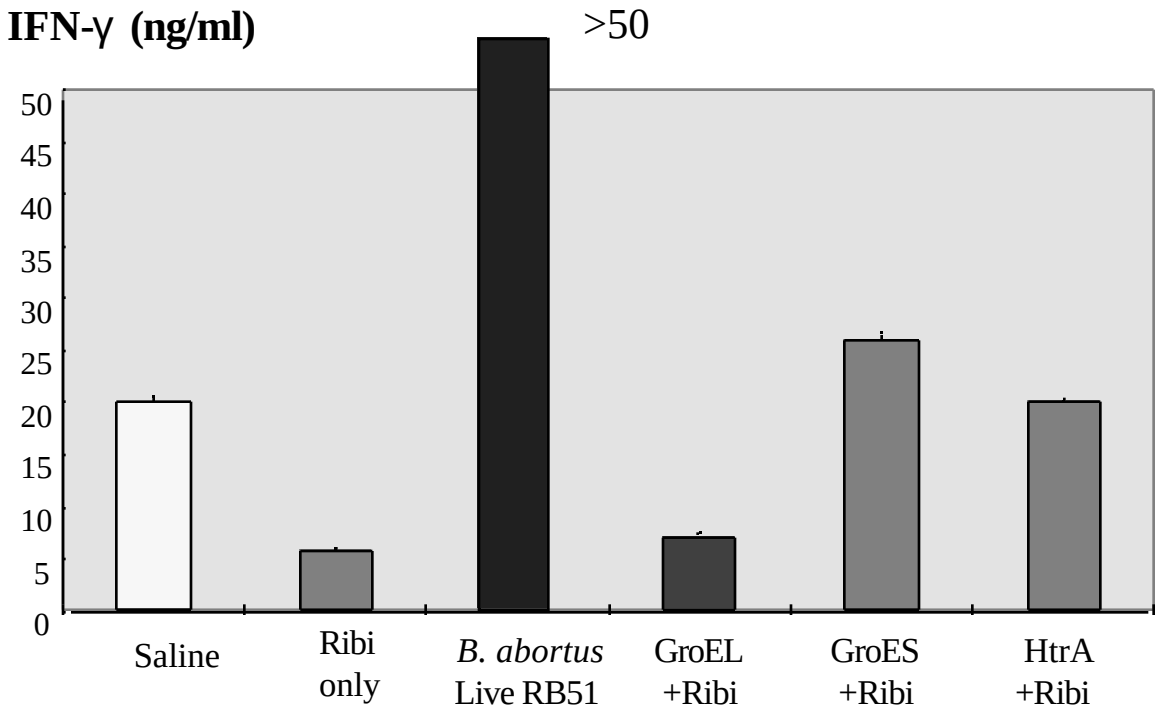

Fig. 19 b. IFN-γ release, stimulated by <u>ConA</u> *in vitro*, (mean <u>+</u> standard deviation)

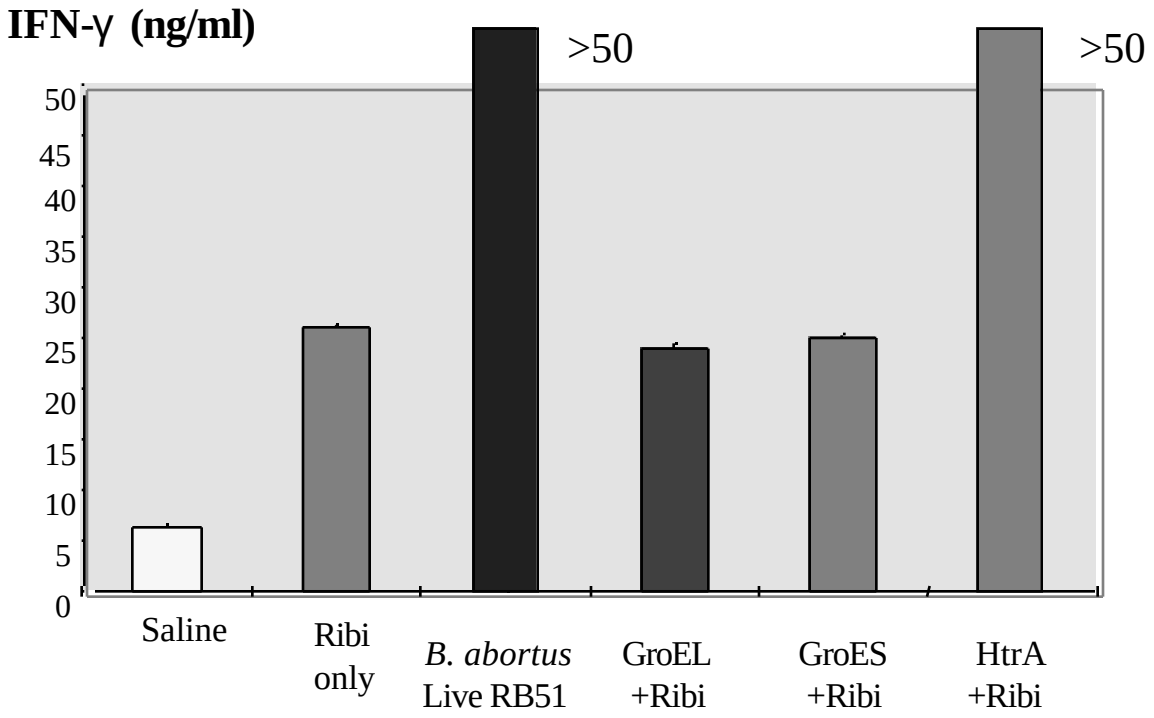

Fig. 19 c. IFN-γ release, stimulated by RB51 *in vitro*, (mean ± standard deviation)

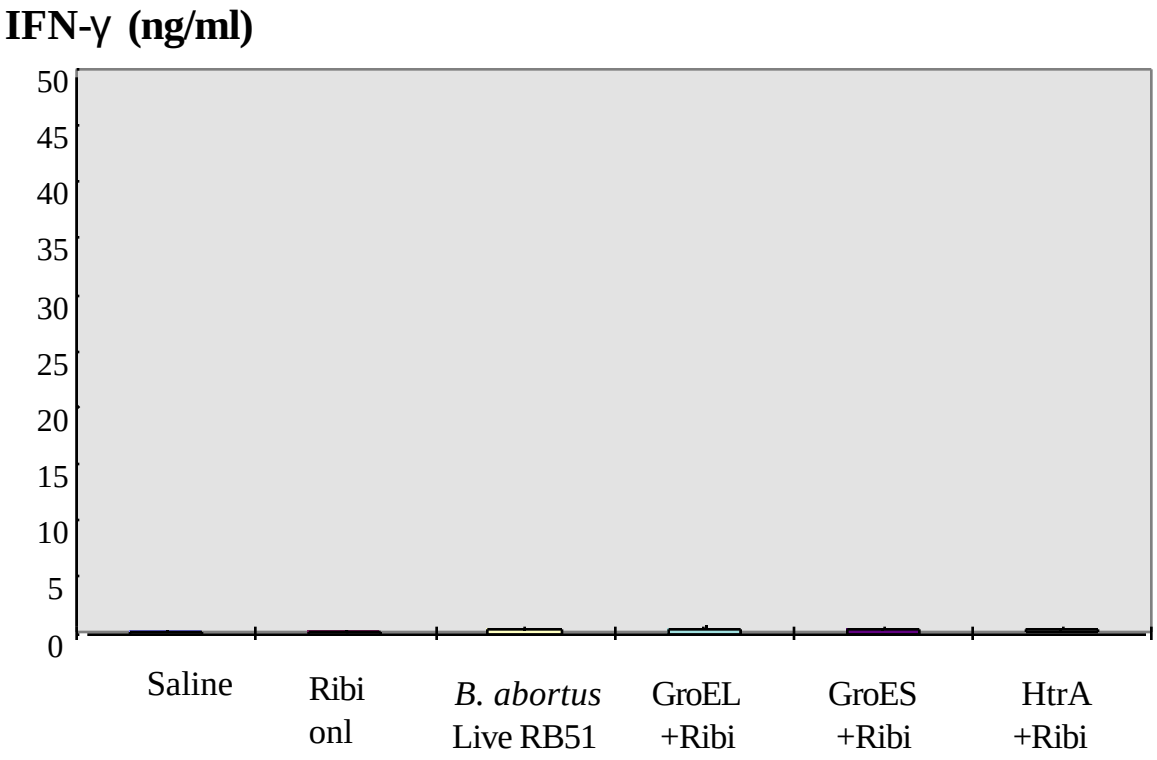

Fig. 19 d. IFN-γ release, stimulated by <u>GroEL</u> *in vitro*, (mean ± standard deviation)

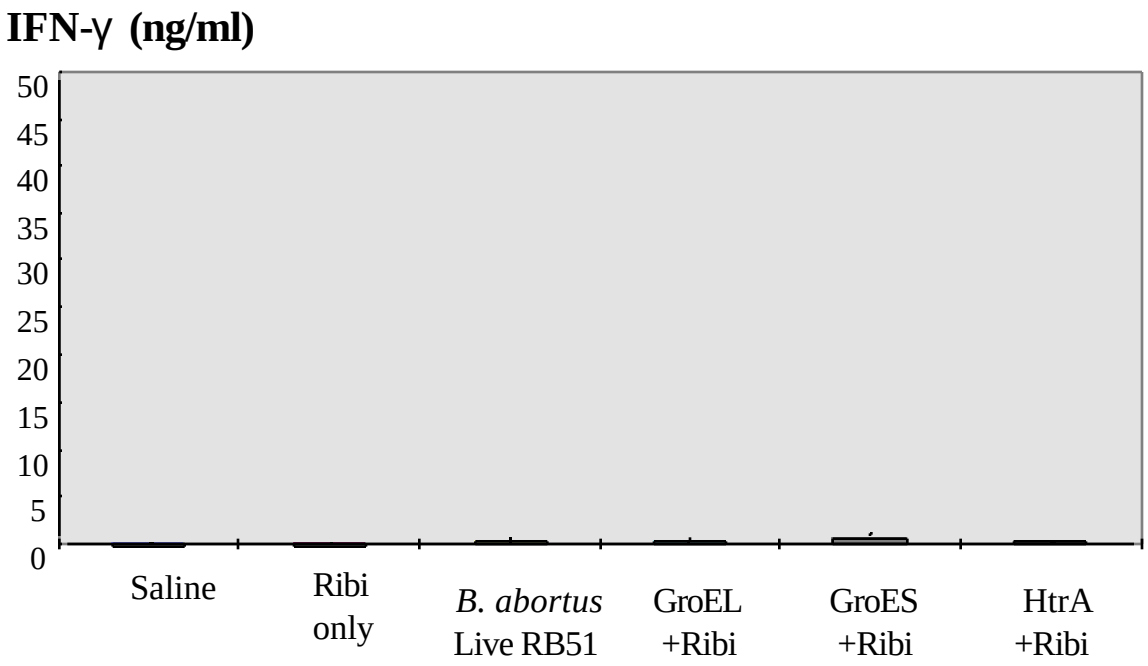

Fig. 19 e. IFN-γ release, stimulated by <u>GroES</u> *in vitro*, (mean <u>+</u> standard deviation)

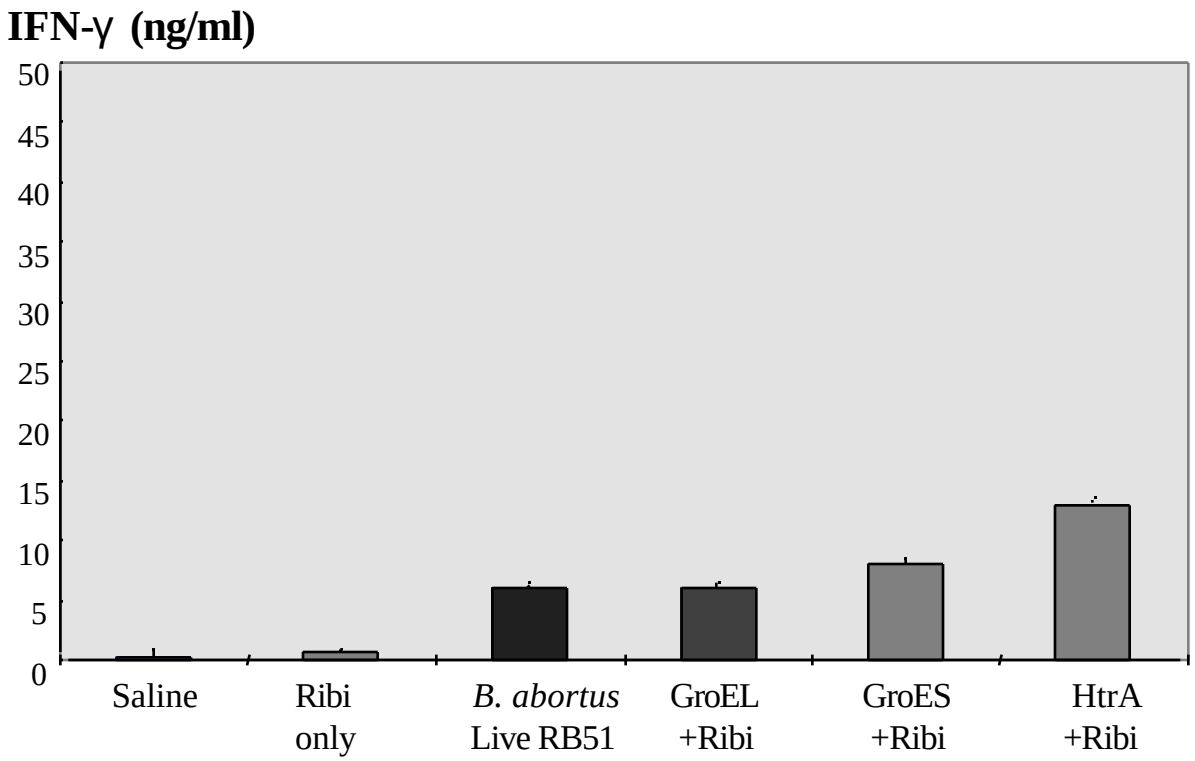

Fig. 19 f. IFN-γ release, stimulated by HtrA *in vitro*, (mean ± standard deviation)

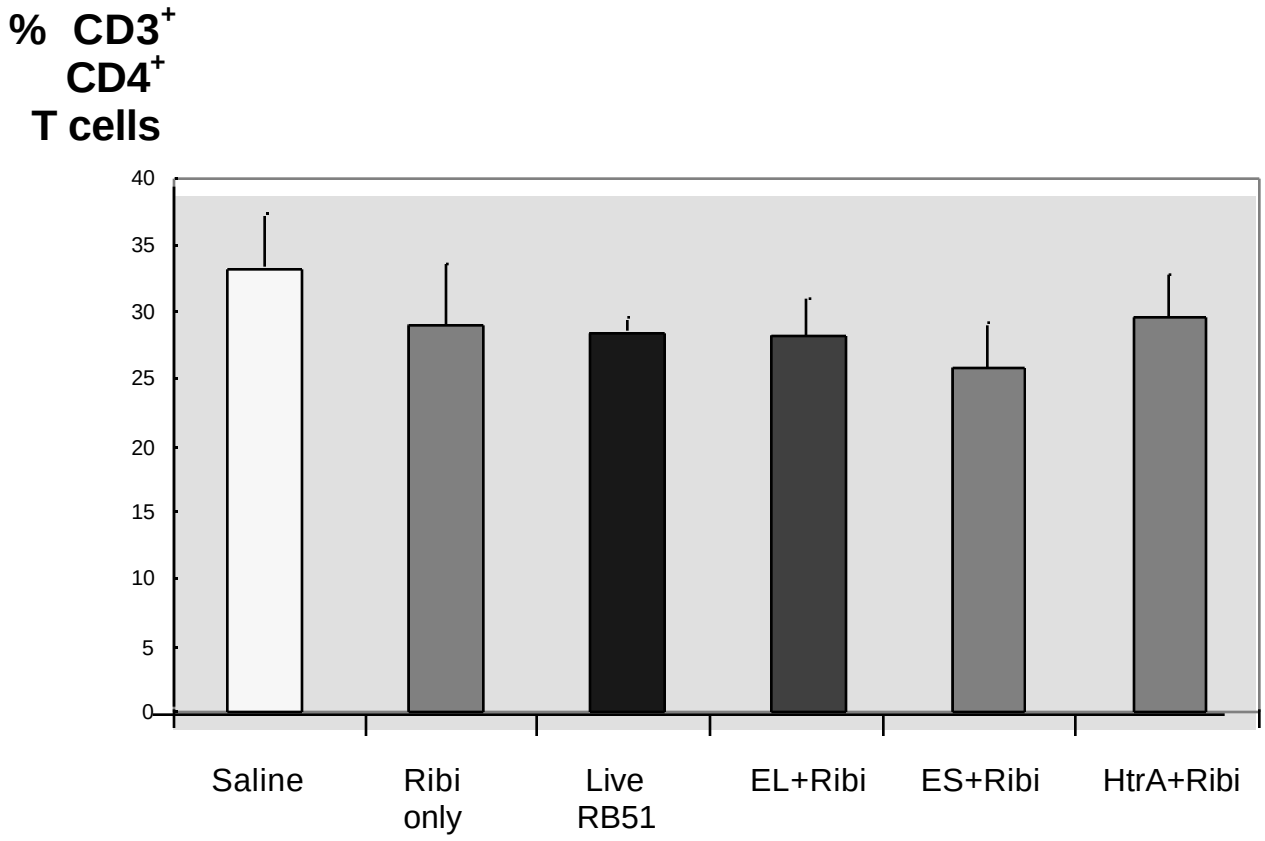

Fig. 20 a. Percent of $CD3^+$ $CD4^+$ T cells (mean ± standard deviation)

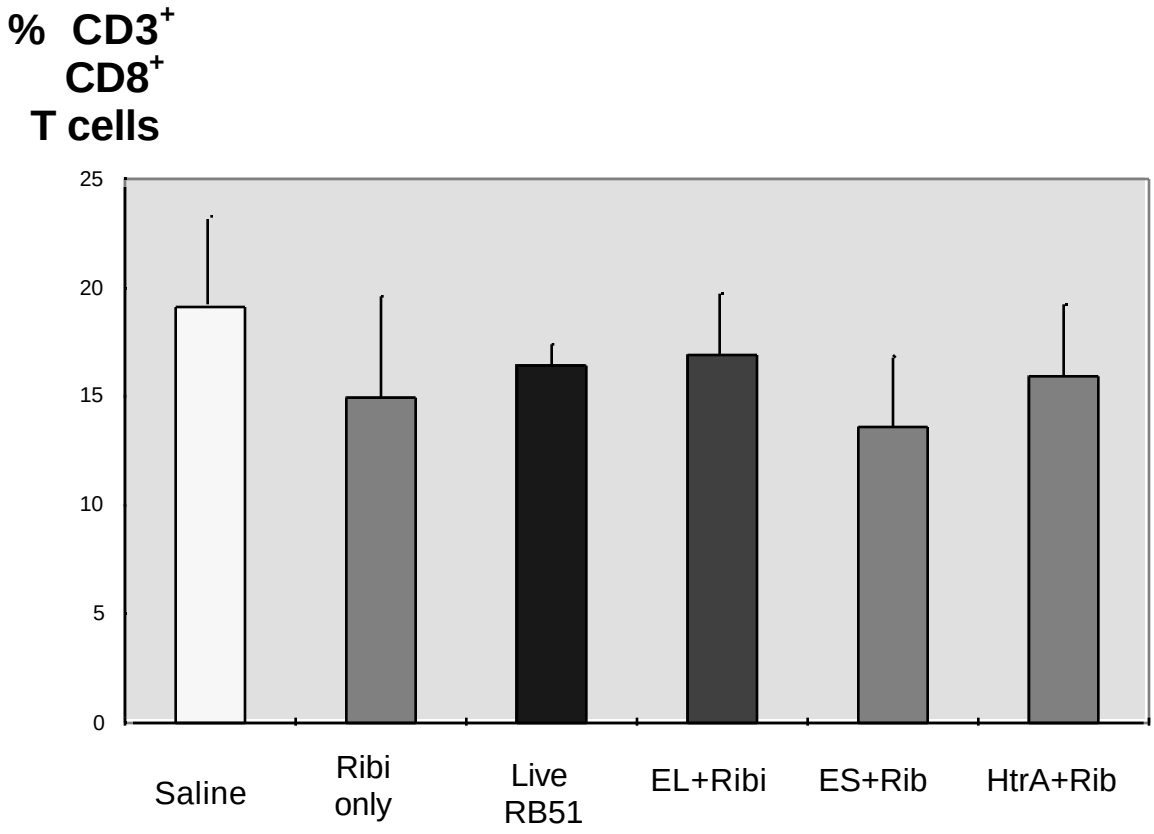

Fig. 20 b. Percent of $CD3^+$ $CD8^+$ T cells (mean ± standard deviation)

TREATMENT	**LOG CFU/SPLEEN**	**LOG PROTECTION**
Saline	4.96 ± 0.38	
Ribi adjuvant only	5.10 ± 0.36	0
B. abortus live RB51	4.04 ± 0.59 (p = 0.0005) *	0.91
GroEL + Ribi adjuvant	5.36 ± 0.05	0
GroES + Ribi adjuvant	5.38 ± 0.07	0
HtrA + Ribi adjuvant	5.62 ± 0.10	0
GroEL-GroES + Ribi adjuvant	5.25 ± 0.28	0
GroEL-GroES-HtrA + Ribi adjuvant	5.05 ± 0.27	0

*** : Protection is achieved.**

Table 20.

***B. abortus* 2308 colony counts from splenocytes of mice inoculated with purified recombinant *B. abortus* HSP(s) combined with Ribi adjuvant**

3.3. Mice inoculated with androstenediol (AED) as the immune up-regulator (Exp. IV).

3.3.1. Protective immune response.

The resistance of mice injected with AED was not statistically significant. No protection was observed in the mice injected with AED ($p = 0.26$) (Table 21).

3.4. Protective immune response of mice primed with vaccinia virus *B. abortus* GroEL recombinant and boosted with baculovirus GroEL purified recombinant protein combined with Ribi adjuvant (Exp V).

Mean log CFU/spleen from mice injected with saline or vaccinia virus recombinant GroEL, or primed with vaccinia virus recombinant GroEL and boosted with purified baculovirus GroEL recombinant combined with Ribi adjuvant did not demonstrate any significant differences in the level of protection. Only mice injected with *B. abortus* RB51 showed a significant level of protection (log protection = 1.25) (Table 22).

3.5. Cell-mediated immune response of mice inoculated with killed *B. abortus* RB51 combined with IL-12 as adjuvant (Exp. VI).

IFN-γ ELISA was performed 14 days after the second immunization. The highest level of IFN-γ release was observed by the splenocytes from killed *B. abortus* RB51 plus IL-12- group mice by stimulation with two different doses (5×10^3 cfu/ml, 5×10^8 cfu/ml) of killed RB51. The level of IFN-γ was higher than in the splenocytes from mice inoculated with *B. abortus* live RB51 alone, which was used as positive control (Table 23).

TREATMENT	LOG CFU/SPLEEN	LOG PROTECTION
Saline	5.46 ± 0.07	
Vehicle only	5.36 ± 0.13 (p = 0.60)	0.10
AED in vehicle	4.96 ± 0.54 (p = 0.26)	0.50

Table 21.

***B. abortus* 2308 colony counts from splenocytes of mice inoculated with AED as an up-regulator**

TREATMENT	LOG CFU/SPLEEN	LOG PROTECTION
Saline	5.46 ± 0.07	
B. abortus live RB51	4.21 ± 0.61 (p=0.03) *	1.25
Vaccinia virus GroEL recombinant + Ribi adjuvant	5.58 ± 0.14	0
Vaccinia virus GroEL recombinant + Baculovirus GroEL recombinant with Ribi adjuvant	5.62 ± 0.37	0

* : Protection is achieved.

Table 22.

***B. abortus* 2308 colony counts from splenocytes of mice primed with vaccinia virus GroEL and boosted with baculovirus GroEL recombinants combined with Ribi adjuvant**

Released IFN-γ in ng/ml

injected Ag / coated Ag	saline	live RB51	killed RB51	IL-12	killed RB51 + IL-12
saline	0 ± 0	0.17 ± 0.20	0.08 ± 0.15	2.17 ± 1.21	26.35 ± 2.20
ConA	2.7 ± 0.05	> 50, in 75 % mice used	> 50, in 75 % mice used	> 50, in 75 % mice used	> 50, in 75 % mice used
killed RB51 (5×10^3 CFU/well)	0 ± 0	1.08 ± 1.20	0.08 ± 0.15	2.29 ± 0.89	> 50, in 75 % mice used
killed RB51 (5×10^8 CFU/well)	0.08 ± 0	> 50, in 75 % mice used	> 50, in 75 % mice used (remaining: 1.83 ± 1.11)	1.87 ± 0.90	> 50, in 75 % mice used

Table 23. IFN-γ release by splenocytes of mice inoculated with killed *B. abortus* RB51 combined with IL-12; preliminary study.

3.6. Mice inoculated with killed *B. abortus* RB51, purified *B. abortus* HtrA and SOD proteins, and *E. coli* expressing *B. abortus* L7/L12 protein combined with IL-12 as adjuvant (Exp. VII).

3.6.1. Cell-mediated immune response.

IFN-γ ELISA was performed 42 days after the second immunization (Fig. 21 a, b, c, d, e, f). For statistical analysis, single degree of freedom contrasts were used to test of interest. First, contrasts between positive control live RB51 versus (vs.) antigen combined with IL-12 were analyzed. The level of released IFN-γ was compared between the live RB51- vs. killed RB51 combined with IL-12- groups, live RB51- vs. SOD with IL-12- groups, live RB51- vs. HtrA with IL-12- groups when stimulated with saline (Fig. 21 a), ConA (Fig. 21 b), *B. abortus* killed RB51 (5×10^3 cells/well) (Fig. 21 c), *B. abortus* killed RB51 (5×10^8 cells/well) (Fig. 21 d), *B. abortus* HtrA (Fig. 21 e) or *B. abortus* Cu/Zn SOD (Fig. 21 f). Second, contrast between antigen without IL-12- vs. the antigen with IL-12- group was analyzed. The level of released IFN-γ was compared between saline- vs. IL-12- groups, killed RB51- vs. killed RB51 with IL-12- groups, SOD- vs. SOD with IL-12- groups by stimulation with each antigen (Fig. 21 a, b, c, d, e, f). When saline or ConA were used as stimulants, no significant differences were detected in IFN-γ release between either positive control- vs. antigen with IL-12- groups or antigen without IL-12- vs. the antigen with IL-12- groups. Thus, it is demonstrated that IL-12 itself does not affect non-specific activation of lymphocytes.

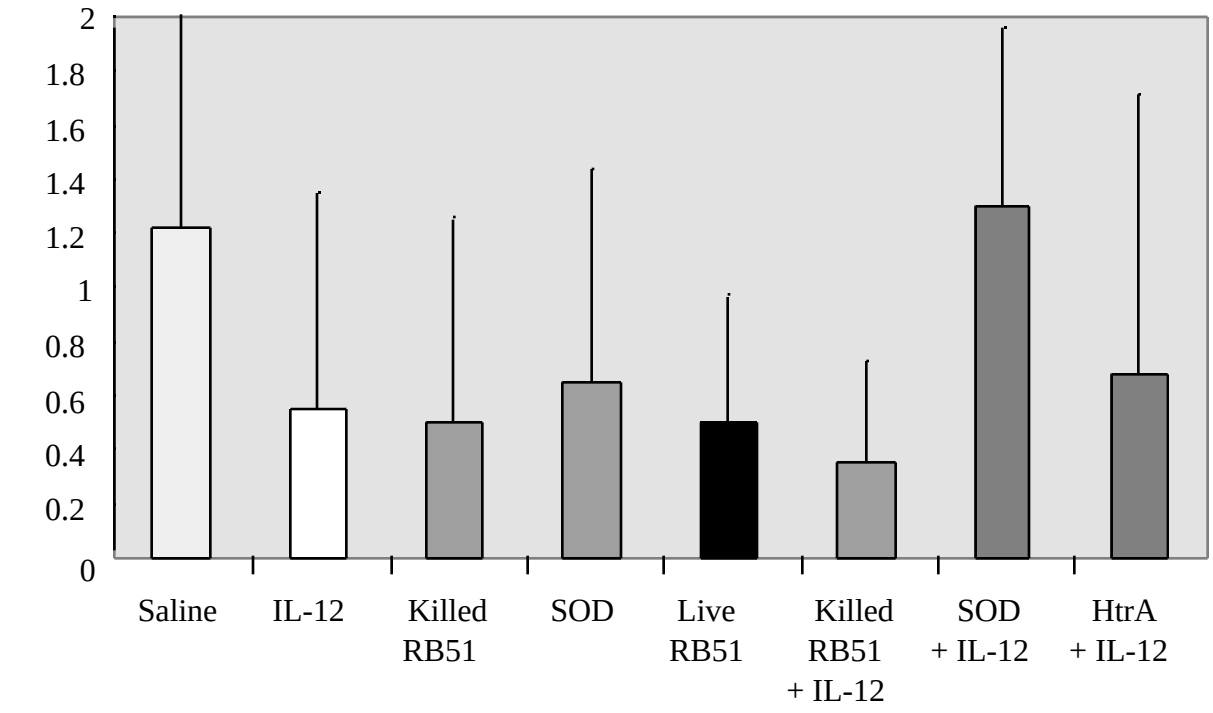

Fig. 21 a. IFN-γ release, stimulated by <u>saline</u> *in vitro*, (mean <u>+</u> standard deviation)

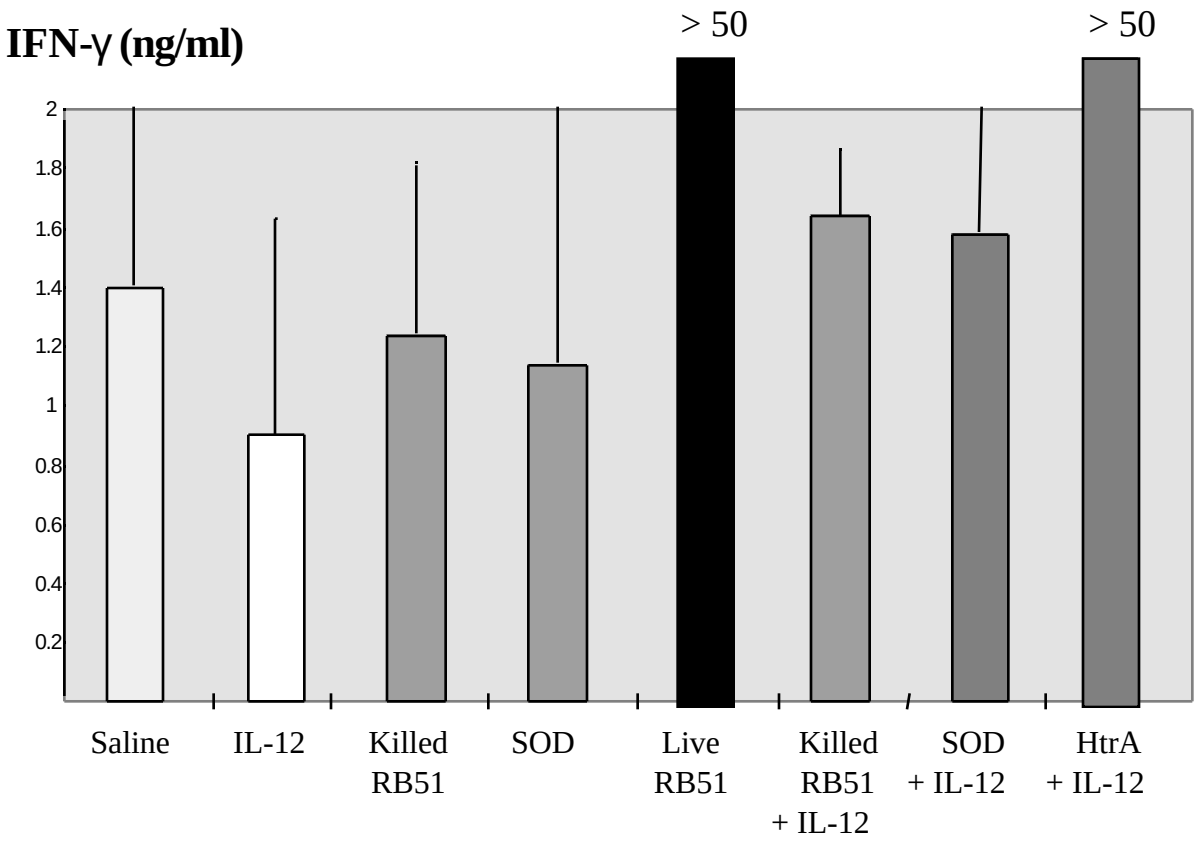

Fig. 21 b. IFN-γ release, stimulated by ConA *in vitro*, (mean ± standard deviation)

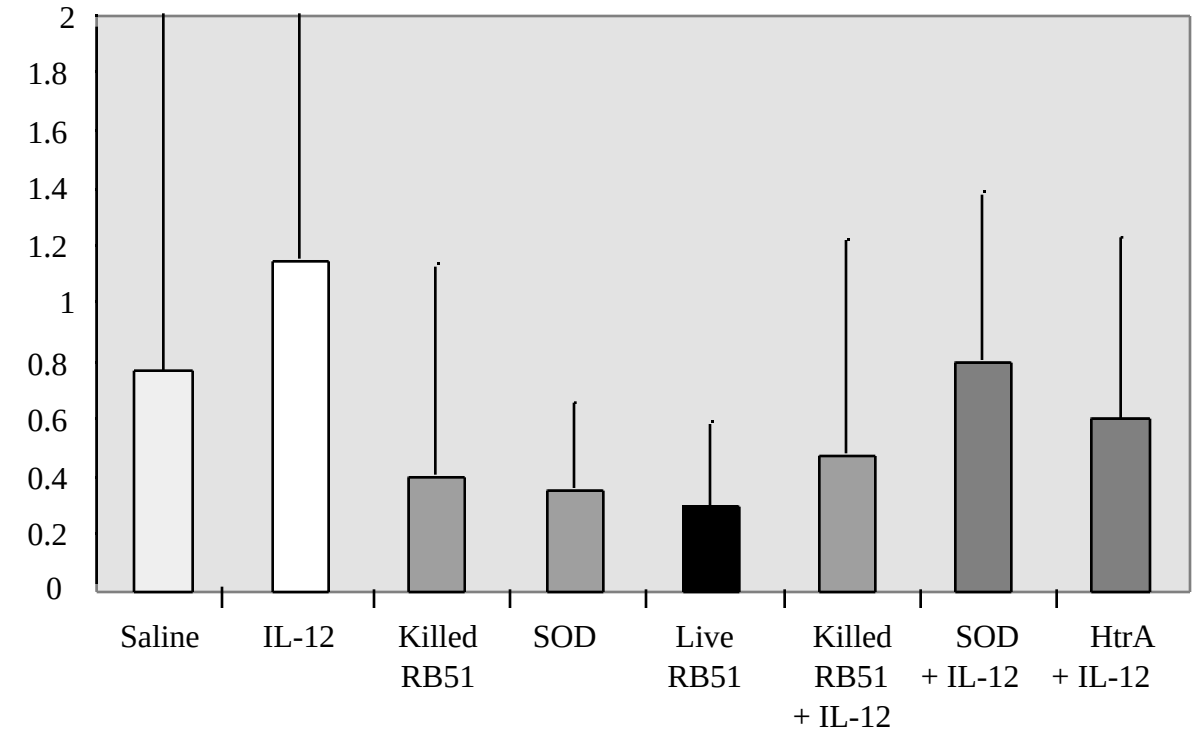

Fig. 21 c. IFN-γ release, stimulated by killed RB51 (5 X 10^3 cells/well) *in vitro,* (mean ± standard deviation)

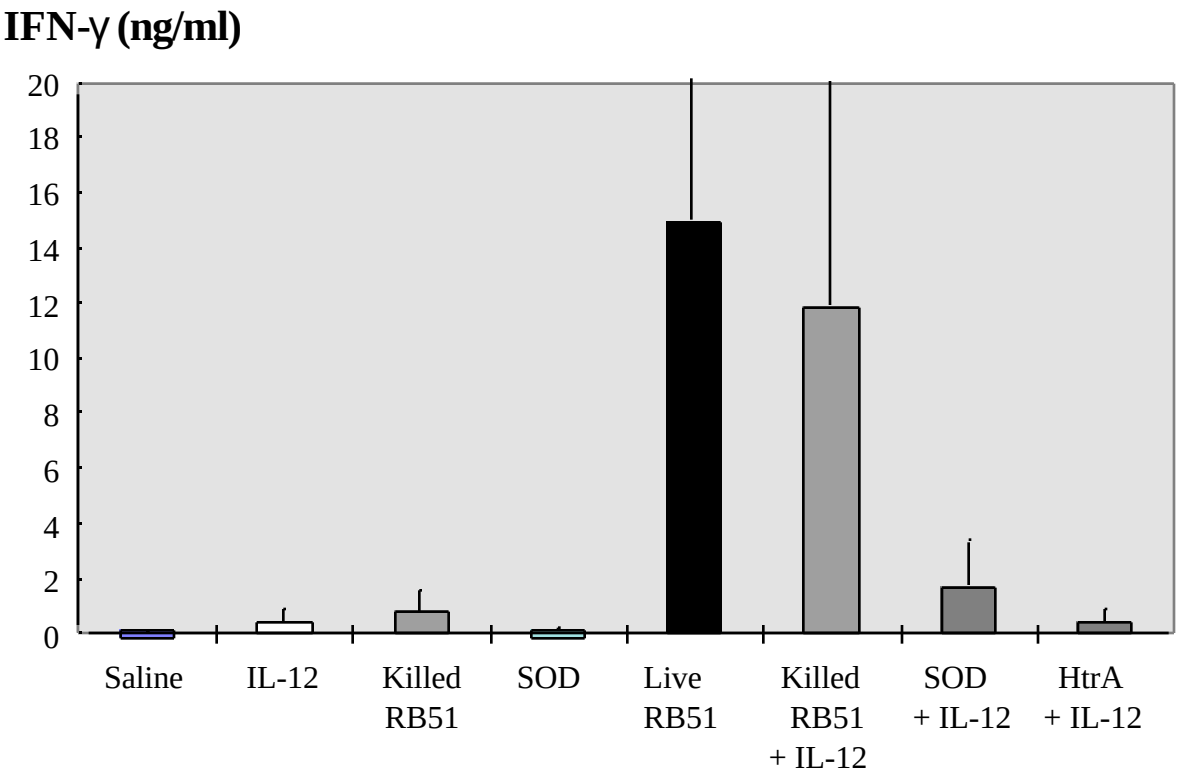

Fig. 21 d. IFN-γ release, stimulated by killed RB51 (5 X 10^8 cells/well) *in vitro*, (mean ± standard deviation)

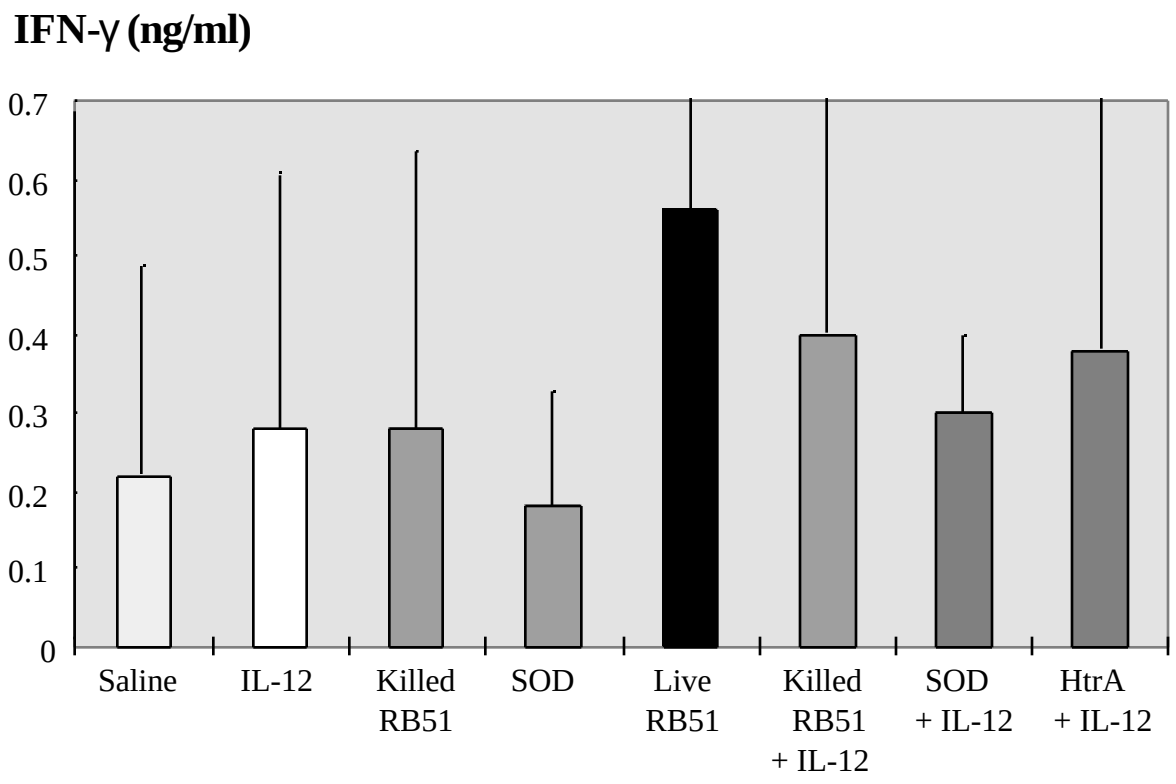

Fig. 21 e. IFN-γ release, stimulated by <u>HtrA</u> *in vitro*, (mean ± standard deviation)

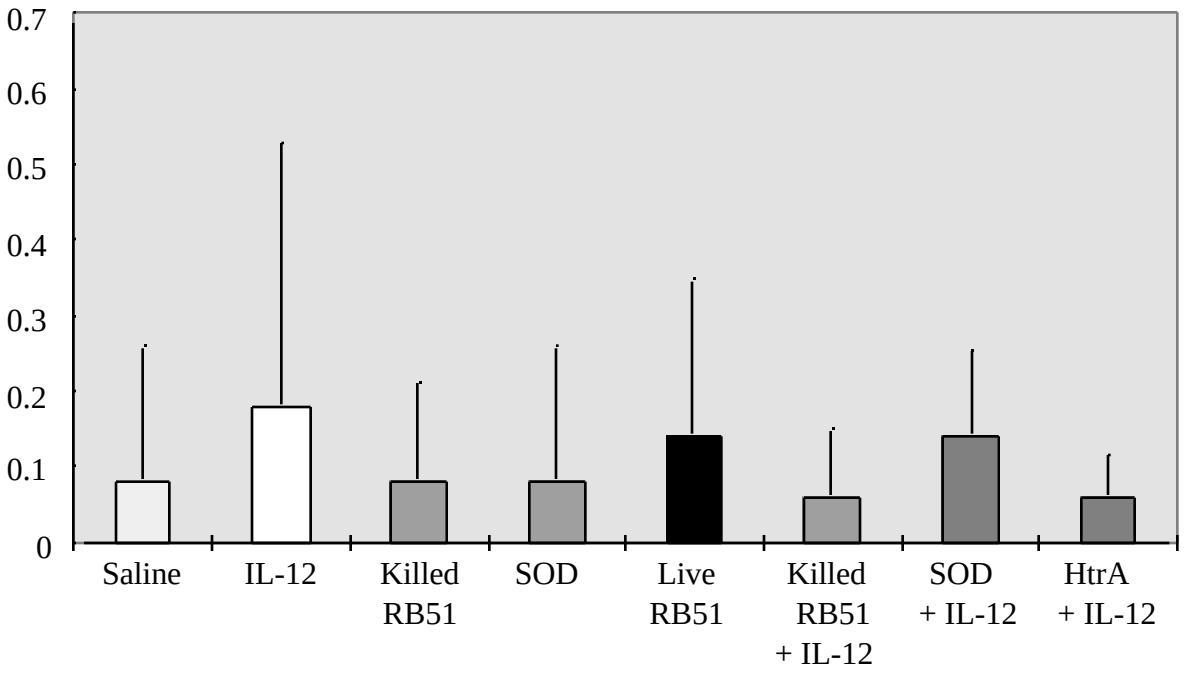

Fig. 21 f. IFN-γ release, stimulated by <u>SOD</u> *in vitro*, (mean <u>+</u> standard deviation)

When HtrA purified protein was used as stimulant, IFN-γ release in live RB51- and HtrA plus IL-12- treated groups was very low; no significant differences were observed in IFN-γ release between groups (Fig. 21 e). When purified SOD protein was used as stimulant, IFN-γ release in live strain RB51- and SOD plus IL-12- groups was very low, and no significant differences were observed in IFN-γ release between groups (Fig. 21 f). Much lower levels of IFN-γ release were observed in the splenocytes from all groups of mice by stimulation with HtrA as well as SOD compared to stimulation with killed strain RB51. No antigen-specific IFN-γ release was observed by stimulation with HtrA or with SOD protein.

When 5×10^8 cells/well of killed RB51 was used as a stimulant, no difference in the level of IFN-γ release was seen between saline- vs. IL-12- groups. There were statistically significant differences in the level of IFN-γ release between killed RB51- vs. killed RB51 with IL-12- groups ($p = 0.0442$), and SOD- vs. SOD with IL-12- groups ($p = 0.0256$) by stimulation with killed RB51 (Fig. 21 d). It appears that IL-12 acts to stimulate immune responses along with antigen, inducing significant IFN-γ release *in vivo*. Statistical contrast was analyzed to evaluate the proportions of $CD3^+CD4^+$ T cells (Fig. 22 a) and $CD3^+CD8^+$ T cells (Fig. 22 b). There were no differences in the specific T cell proportions between positive-control, live RB51 and any of the experimental- groups (live RB51 vs. killed RB51, $p = 0.58$; live RB51 vs. SOD, $p = 0.89$; live RB51 vs. HtrA,

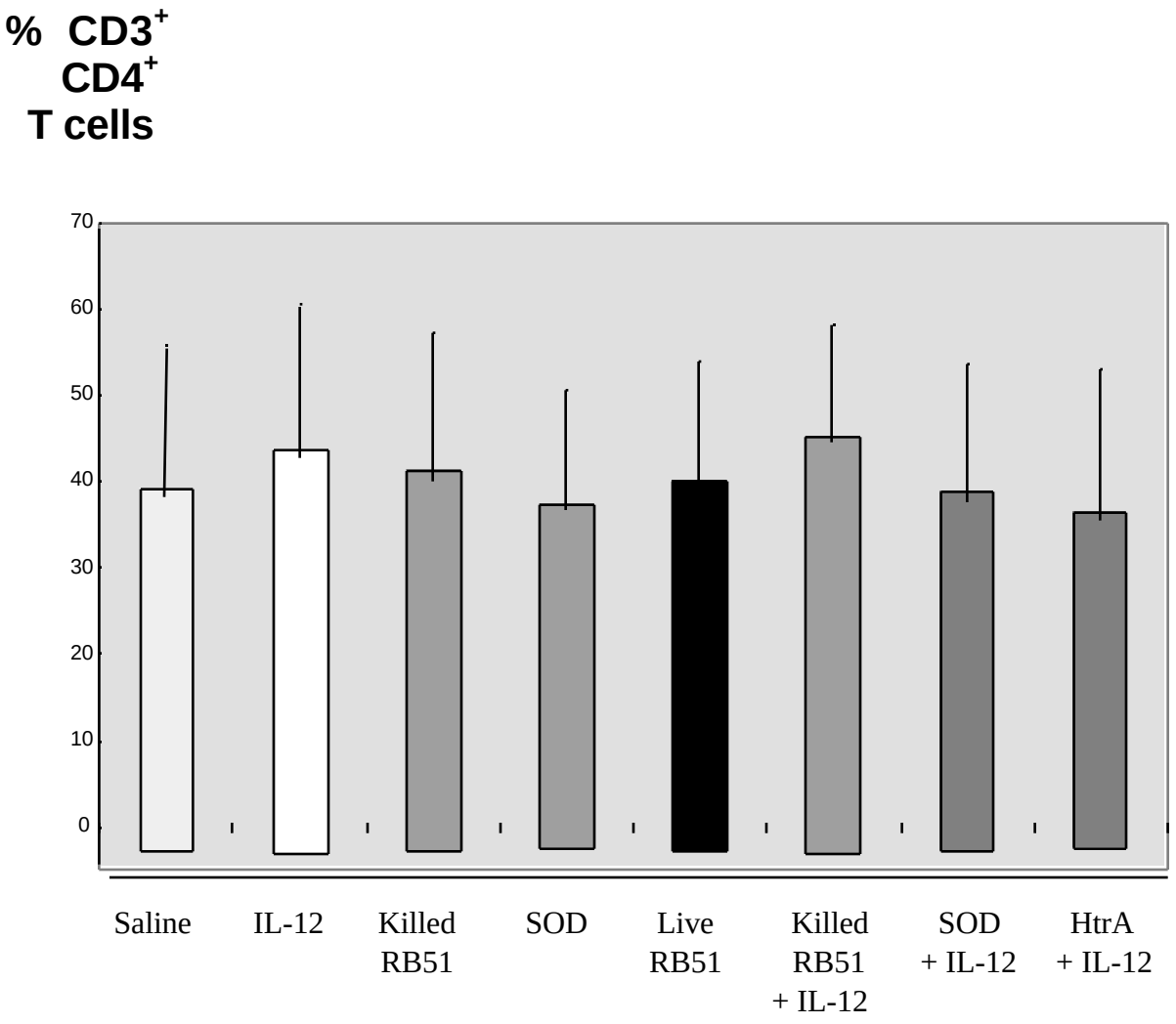

Fig. 22 a. Percent of $CD3^+$ $CD4^+$ T cells (mean ± standard deviation)

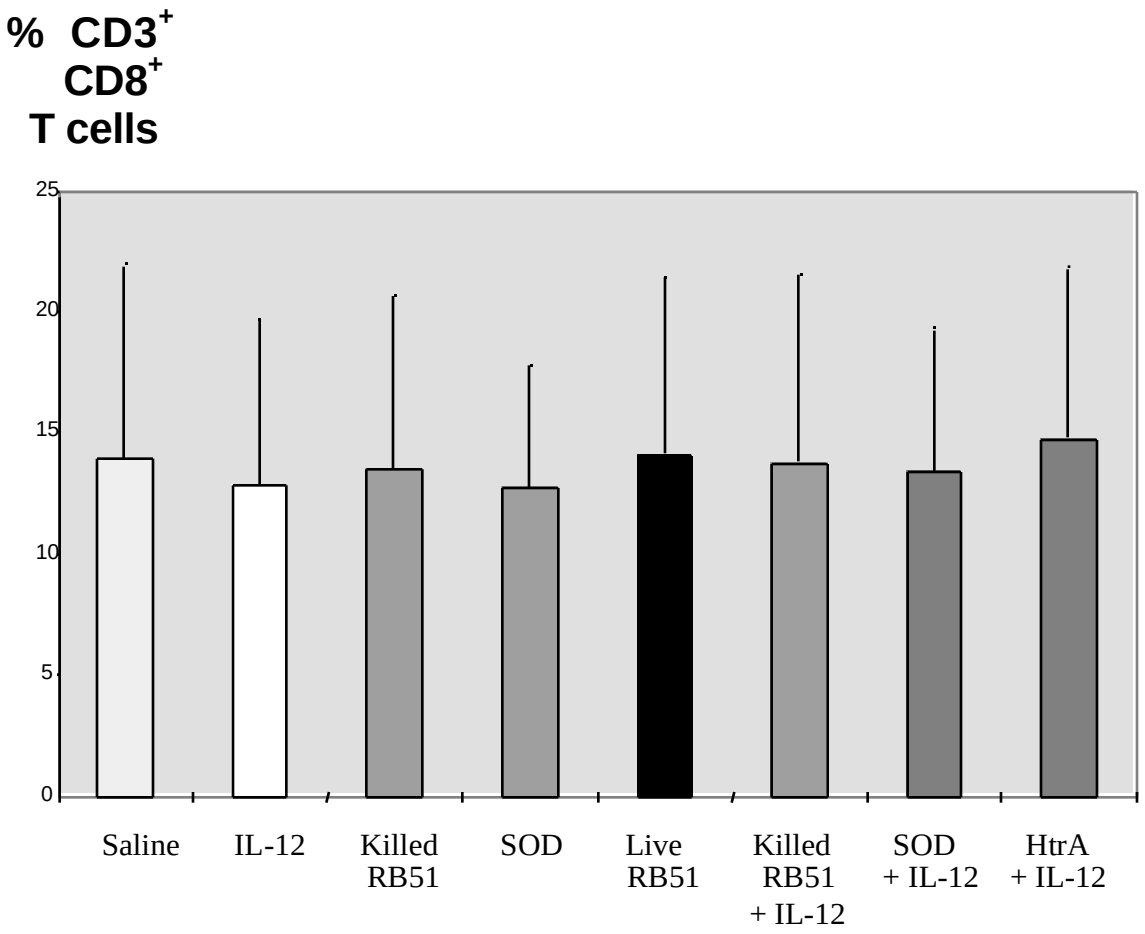

Fig. 22 b. Percent of $CD3^+$ $CD8^+$ T cells (mean ± standard deviation)

p = 0.73). Also, when the contrasts were analyzed between antigen alone vs. antigen with IL-12, no significant differences were shown between any of the groups (saline vs. IL-12, p = 0.63; killed RB51 vs. killed RB51 with IL-12, p = 0.66; SOD vs. SOD with IL-12, p = 0.89). Therefore, in these experiments, a distinct correlation does not exist between IFN-γ release in splenocytes and the proportions of $CD3^+CD4^+$ T cells or $CD3^+CD8^+$ T cells.

3.6.2. Protective immune response.

Significant differences were observed in mice groups injected with saline vs. other experimental groups. A significant level of protection was observed in mice injected with live *B. abortus* RB51 (p=0.003), SOD alone (p= 0.03), SOD plus IL-12 (p=0.0001) and live *E. coli* expressing pMAL-L7/L12 plus IL-12 (p=0.004) (Table 24). Higher resistance were observed in mice injected with IL-12 plus antigen (SOD vs. SOD plus IL-12, p=0.03 vs. p= 0.0001).

TREATMENT	LOG CFU/SPLEEN	LOG PROTECTION
Saline	5.37 ± 0.07	
IL-12	5.41 ± 0.29	0.00
B. abortus killed RB51	5.49 ± 0.13	0.00
B. abortus SOD	4.93 ± 0.35 (p = 0.03) *	0.44
B. abortus live RB51	4.75 ± 0.16 (p = 0.003) *	0.62
B. abortus killed RB51 + IL-12	5.42 ± 0.21	0.00
B. abortus SOD + IL-12	4.35 ± 0.45 (p = 0.0001) *	1.02
B. abortus HtrA + IL-12	5.19 ± 0.32	0.18
live *E. coli* expressing pMBP-*B. abortus* L7/L12	5.21 ± 0.24	0.16
live *E. coli* expressing pMBP-*B. abortus* L7/L12 + IL-12	4.77 ± 0.45 (p = 0.004) *	0.60
Live *E. coli* expressing pMBP + IL-12	5.10 ± 0.41	0.27

*** : Protection is achieved.**

Table 24.

***B. abortus* 2308 colony counts from lymphocytes of mice inoculated with *B. abortus* killed RB51, SOD, HtrA, and L7/L12 combined with IL-12**

DISCUSSION.

Most of the highly successful vaccines currently available were developed before 1970. They were not highly purified preparations but were shown to be capable of eliciting protective responses without unreasonable side effects. Subsequently, vaccine development slowed down as safety standards increased. Progress in peptide synthesis and recombinant DNA technology held out the promise of a new generation of vaccines and, in 1989, after reports were published by the USA National Academy of Sciences, it was predicted that 27 new vaccines would become available for general use within ten years (Jordan, W. S., 1989). Of these, 15 were to be subunit vaccines developed by chemical synthesis or biosynthesis. Subunit vaccines are a major goal of modern vaccine research because of a number of advantages (Blander and Horwitz, 1993). Subunit vaccines, consisting of one or a few epitopes, offer several potential advantages over traditional whole cell vaccines, which generally consist of avirulent or killed microorganisms. First, by focusing the immune system on a few key immunoprotective determinants, rather than on a complex mix of a few relevant and many irrelevant molecules, the subunit vaccine might achieve greater protective capability. Second, by excluding molecules capable of inducing suppression of an immune response, the subunit vaccine might allow the immune system to respond to immunoprotective moieties. Third, by being composed of a limited repertoire of molecular species, the subunit vaccine might be less likely to induce allergic or toxic reactions than whole cell vaccines. However, it is accepted that the prediction that 15 out of 27 new vaccines would be subunit vaccines

was too optimistic. Very promising immunogens have been designed, but, in most cases when tested in clinical trials, they have not induced sufficient levels of protective immunity under acceptable safety conditions to be considered for clinical use. A major difficulty encountered during development of modern vaccines was their weak immunogenicity (Audibert and Lise, 1993). This problem has been overcome by the use of suitable adjuvants. Aluminium-containing compounds such as hydroxides and phosphates (commonly referred to as alum) are the only adjuvants approved in the clinic, but they are not active with all immunogens and stimulate only humoral responses. For development of new subunit vaccines, the availability of improved adjuvants suitable for clinical use is crucial. Such adjuvants should display several different profiles of activity since an appropriate adjuvant must not only enhance the immune response but should also drive this response to achieve the appropriate type of protective immunity in each situation. Modern biotechnology methods now provide large numbers of adjuvant-active compounds to augment immune responses.

Adjuvants work by several mechanisms. First, adjuvants influence the Th1/Th2 ratio. Adjuvants operate through triggering cytokine release and prohormone conversion (Rook and Stanford, 1995). The released cytokines such as IL-12 or IL-10 from mast cells, macrophages, basophils, endothelial cells or NK cells influence selection of the immune response toward Th1 or Th2 subtype. IL-12 directs the immune response toward Th1, and IL-10 directs it towards Th2 (Hsieh et al., 1992, 1993; Seder et al., 1992, 1993; Manetti et al., 1993). Also prohormone conversion in macrophage influences the decision toward Th1 or Th2. When dehydroepiandrosterone sulphate (DHEAS) is

converted into dehydroepiandrosterone (DHEA), Th cells are differentiated into Th1 (Daynes et al., 1991; Suzuki et al., 1991), and when the prohormone 25-hydroxy cholecalciferol is converted into 1,25-hydroxy calcitrol, Th cells are differentiated into Th2 (Rook G. A. W., 1988; Rigby et al., 1987). The importance of IL-12 in the induction of cell-mediated immunity in general was confirmed when it was shown that co-injection of killed *Listeria*, which alone does not activate IFN-γ producing T cells, successfully induced cell-mediated immunity to this organism (Miller et al., 1996). DHEAS is the major adrenal steroid in the serum of both men and women. Specific cytosolic receptors for the free DHEA are found in T cells, and are known to have substantially higher concentrations in $CD4^+$ T-cells than in $CD8^+$ T-cells (Meikle et al., 1992). DHEA was found to up-regulate host immune response *in vivo* against infections and to counteract stress-induced immunosuppression (Araneo et al., 1993; Casson et al., 1993).

Second, adjuvants can influence antibody production (Morein et al., 1996; Del Giudice, G., 1992). Adjuvants that enhance cell-mediated responses, as evidenced by delayed-type hypersensitivity (DTH) reactions, elicit IgG2a antibody subclass responses in mice, possibly by acting on Th1-like cells. Adjuvants, which may primarily drive Th2 responses, enhance antibody production including IgG1, IgA, and IgE.

Third, adjuvants influence cytotoxic T lymphocytes (CTL) (Morein et al., 1996). The mechanisms of induction of CTL are not yet fully known, but several possibilities exist. Adjuvants increase the normal activity of antigen presenting cells (APC), particularly a subpopulation that is capable of presenting class I MHC antigen-restricted

CTL. The detergent property of the adjuvant could induce cell death and lysis at the site of injection, and the CTL response could be raised against the cellular debris. DHEA also enhances the generation of cytotoxic T cells because of the role of IL-2 in the induction of cytotoxic T cells. Adjuvants also affect CTL by nitric oxide production, tumor cell killing, and TNF-α release by very pure macrophage cultures in *in vitro* assays.

Fourth, adjuvants have a depot effect, which increases the biological half-life of antigens (Stites and Terr, 1991). The depot effect is highly effective with small antigens such as synthetic peptides that otherwise would be rapidly cleared from the body.

Fifth, adjuvants enhance antigen presentation (Stites and Terr, 1991). Adjuvants act as adhesive molecules, binding antigen and complement components to their hydrophilic component, and binding oil to their hydrophobic portion. This presentation helps to expose protective epitopes to cells.

Sixth, adjuvants can activate macrophages (Rook and Stanford, 1995). The maintenance of a consistent antigen concentration by the depot effect, particularly on the macrophage surface, ensures that the progeny of antigen-sensitive cells are highly likely to be further stimulated by antigen. Virtually all adjuvants stimulate macrophages, the majority probably through direct action, but complete Freund's adjuvant appears to act on the macrophage through the T-cell (Lawrence and Pamela, 1999).

The ultimate goal of our group in CMMID is the induction of higher protection in susceptible animals to *B. abortus* infection. Resistance to *B. abortus,* which is one of the

facultative intracellular bacterial pathogens, depends mostly on acquired cell-mediated immune response and activation of macrophages by IFN-γ producing T lymphocytes.

In the research for this doctoral dissertation, my attention has been focused on the examination of the capacity of heat shock proteins of *B. abortus* as protective antigen(s) against *Brucella* infection in a mouse model. Experiments were designed to determine whether it is possible to steer the immune responses to *B. abortus* HSPs toward IFN-γ producing Th1 lymphocyte proliferation and induction of protection by incorporating appropriate adjuvants, with or without the presence of insect cells.

In our studies, the lymphoproliferation assay was performed to determine the degree of proliferation of splenocytes in response to *B. abortus* HSP(s) and RB51. Results obtained in the lymphoproliferation assay showed a high variations of responses to a particular antigen from mouse to mouse, and the results did not give interpretable data. Thus, our attention was directed towards other types of CMI assays that could generate a meaningful information base. The IFN-γ ELISA was used to determine the amount of released IFN-γ in splenocytes from mice injected with each HSP as immunogen. The proportions of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells were also evaluated to correlate the relationship with the IFN-γ release. In Experiment I, Freund's adjuvant was selected since it influences the quality of the $CD4^+$ T cell subpopulation activated, and favors the induction of Th1 cells. In other investigators' studies using Freund's adjuvant (Blander and Horwitz, 1992), when a guinea pigs were injected with *L. pneumophila* HSP60 in conjunction with complete and incomplete Freund's adjuvant,

CMI and protective immunity were induced against *L. pneumophila* infection. In our first immune study, Sf9 insect cells expressing *B. abortus* HSP(s) were used as an antigen in combination with Freund's adjuvant. In humoral immune response analysis, antibodies were observed against *B. abortus* GroEL and HtrA recombinant proteins, but not against GroES recombinant protein (Fig. 17). In the lymphocyte proliferation (Fig. 18) and protection studies (Table 17), the background due to Sf9 cells was too high to demonstrate any specific responses to each HSP.

The possibility of using purified HSPs as protective antigens was evaluated using the adjuvant, Ribi (combined monophosphoryl lipid A and trehalose dimycolate). Either Ribi or one of the Ribi components has been found to be a powerful adjuvant in many other studies, by enhanced antibody production, lymphocyte proliferation, suppression of splenic infection, or induction of protection against the invasions of intracellular parasites including *B. abortus* (Spargo et al., 1990; Pugh and Tabatabai, 1996; Singh et al., 1992; Vanlandschoot et al., 1993; Wicher et al., 1991; Saginala et al, 1996). HSPs are known to be more immunogenic when they are injected at low dosage (DeNagel and Pierce, 1993). In order to evaluate the optimal low dosage of *B. abortus* HSPs to mice, CMI responses were examined by injection of two different dosages (1 μg or 10 μg/mouse/injection) of each HSP antigen. Based on this preliminary study showing that splenocytes of mice injected with 10 μg HSPs released a higher amount of IFN-γ than did splenocytes of mice injected with 1 μg (Table 18); this dose was determined to be used for the further immunological studies. No protection was observed in mice when any of

the purified each HSP alone, GroEL-GroES combined, or GroEL-GroES-HtrA combined in conjunction with Ribi adjuvant were injected (Table20).

Previously Loria et al., (1988, 1990) reported that a single subcutaneous injection of the steroid hormone dehydroepiandrosterone (DHEA) to inbred male mice resulted in more than 50 % protection against coxsackievirus B 4-induced mortality. Moreover, a 90 % reduction in herpesvirus type 2 encephalitis-mediated mortality in female inbred mice was achieved with s.c. injection of DHEA. Recently, DHEA was shown to protect mice from infection with West Nile virus, a neurovirulent Sindbis virus, and Semliki Forest virus (Ben-Nathan et al., 1991). This dissertation research was the first study to examine androstenediol under very limited circumstances as immune up-regulator against an intracellular bacterial infection. The failure of induction of increased resistance against *B. abortus* challenge could be from the lack of cellular responsiveness to the effects of this steroid hormone which could have been caused by the absence of intracellular receptors having specific binding affinity for this steroid hormone.

No protection was observed when a vaccinia virus GroEL recombinant was used to immunize mice against *Brucella* challenge (S. Baloglu, 1997). In this study, the possibility of inducing protection by injecting two different forms of recombinant was explored; priming with a live form (vaccinia virus GroEL recombinant) and boosting with a subunit form (baculovirus GroEL purified recombinant protein combined with Ribi adjuvant) was examined. The induction of protective immunity in immunized mice failed with either form of recombinant.

Cytokine IL-12 has a broad range of stimulatory effects that generally act to promote cell-mediated immunity. IL-12 was tested because of its ability to promote IFN-γ by αβ T cells and NK cells (Chan et al., 1991; Gazzinelli et al., 1993; Tripp et al., 1993) and by γδ T cells (Ziegler et al., 1994; Skeen and Ziegler, 1994) and Th1 cell generation (Trinchieri, G., 1993). The utility of IL-12 as a vaccine component was demonstrated in a murine model system in which a soluble leishmanial antigen preparation induced a protective Th1-type response when administered along with rIL-12 (Afonso et al., 1994). In other studies, IL-12 has been used as the adjuvant as an essential component of a subunit vaccines against *Listeria monocytogenes* (Tripp et al., 1944), *Toxoplasma gondii* (Gazzinelli et al., 1994), and *Mycobacterium tuberculosis* (Flynn et al., 1995), all of which are intracellular pathogens.

We began to study the adjuvant effects of IL-12 by using killed *B. abortus* RB51, which is known not to induce a significant level of cell-mediated immune responses when it is used as antigen alone. Using an ELISA, the level of IFN-γ release was measured in the splenocytes prepared 14 days after the second immunization in the preliminary study (Fig. 21). The highest level of IFN-γ was observed in mice immunized with IL-12 and was higher than that of live RB51 immunized mice. In the main experiment using IL-12 as adjuvant, cell mediated and protective immune responses were studied using additional immunogens. Since the level of IFN-γ release was somewhat high in IL-12-only-injected mice in our previous IL-12 experiment, CMI studies and challenge for protection were performed 6 weeks after the second immunization to reduce the background stimulation

due to the adjuvant itself. The level of IFN-γ release observed was considerably lower in IL-12- only injected mice than in the preliminary study. When splenocytes were stimulated with 5 X 10^8 cells/well of killed RB51, inoculation of mice with live RB51 induced a higher level of IFN-γ release from splenocytes than did inoculation with killed RB51 plus IL-12 (Fig. 21 d). This result is in conflict with those in the preliminary experiment and is probably due to the difference in the time lapse between the second inoculation and the CMI test: 12 days in the preliminary and 42 days in the main studies. Independent of whether splenocytes were stimulated with 5 x 10^3 or 5 x 10^8 cells/well of killed RB51, the level of IFN-γ release in the killed RB51 plus IL-12-group mice was considerably lower (Fig. 21 c, d) compared to the level in the preliminary studies. Therefore, it appears that the IFN-γ release induced by IL-12 in combination with the antigen does not last at high levels for 6 weeks. Although IL-12 may generate a Th1-type immune response, activate macrophages, and influence the release of IFN-γ in the early stage following injection, the influence by this cytokine appears diminished by 6 weeks after the injection. This fact may explain why CMI studies and/or challenge with the virulent strains were performed within two weeks after injection in most of the other studies that successfully used IL-12 as adjuvant (Noll and Autenrieth, 1996; Miller M. A. et al., 1996; Miller M. J. et al., 1996; Mountford et al., 1996; Xiong et al., 1997). In order to prolong the activity of adjuvant IL-12 and maintain its effects for a longer period of time, the delivery of IL-12 in liposomes or microspheres may provide a mechanism for releasing it at a slower rate.

In the flow cytometry analysis, no significant differences were observed in the proportion of $CD3^+CD4^+$ (Fig. 22 a) or $CD3^+CD8^+$ (Fig. 22 b) T cells/total number of splenocytes between positive control- and antigen plus IL-12- groups or between antigen only- and antigen plus IL-12-groups.

In Experiment 1, lymphoproliferation was not reproducible and showed much variation from mouse to mouse. Therefore, subsequent analysis of cell mediated immune responses used purified proteins as antigens and Ribi or IL-12 as adjuvant; furthermore instead of an lymphoproliferation assay, IFN-γ release and the proportions of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in splenocytes were evaluated.

Besides evaluation of IFN-γ release to measure cell-mediated immune responses, it was interesting to assess the proportions of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in splenocytes in order to determine whether a correlation exists between the amount of released IFN-γ and induction of protective immune responses. A direct correlation between the proportions of $CD3^+CD4^+$, $CD3^+CD8^+$ T cells and the release of IFN-γ by the splenocytes or the induction of protection including the positive control RB51-mice group was not observed. Perhaps testing splenocytes 5-6 weeks after the second injection in order to observe specific T cell proliferation is not the best choice of time. Since IFN-γ release is followed by the specific T cell proliferation, the time point might be only appropriate to measure IFN-γ release in spleen, but not the proportions of circulating $CD3^+CD4^+$ and/or $CD3^+CD8^+$ T cells. The proportions of $CD3^+CD4^+$ and/or

$CD3^+CD8^+$ T cells in lymph nodes or liver may be in closer correlation with IFN-γ release in splenocytes or induction of protective immune response.

The correlation between IFN-γ release and protective immune response was not observed except for mice injected with the positive control RB51 injected mice. When injected with purified HtrA and Ribi adjuvant, the splenocytes exhibited the highest level of IFN-γ release (> 50 ng/ml), but no protective immune response was observed. When mice were injected with purified SOD and IL-12 as adjuvant, despite no significant release of IFN-γ release, a protective immune response was observed. Since the highest level of IFN-γ release did not correlate with the level of protection against *Brucella* infection, we could interpret these results as follows; (1) the maximum level of IFN-γ released (50 ng/ml) by splenocytes might not be high enough to reflect accurately the level of protection; and (2) the amount of released IFN-γ in the splenocytes is not the only factor needed for the induction of protective immune response. It appears that other mechanism(s) may be responsible for protection, which may include cytotoxicity.

The usefulness of *B. abortus* Cu/Zn SOD protein as a protective antigen has been controversial for years. Strain RB51 is devoid of the O-side chain and this deficiency may explain the low virulence regardless of the high content of SOD (Sriranganathan et al., 1991). To better understand the role of cellular immunity to SOD, lymph node cell-mediated immune responses to Cu/Zn SOD and to three synthetic Cu/Zn SOD peptides were measured in a study following vaccination of cattle with *B. abortus* S19 or RB51 (Stevens et al., 1994). The results suggest that neither strain 19 or strain RB51 vaccine

induce antibody to SOD; only the strain 19 vaccine induces cell-mediated immune responses to SOD. Recently, a significant level of protection was induced against the challenge of *B. abortus* 2308 when mice were injected with live *E. coli* expressing *Brucella* Cu/Zn SOD (Onate et al., 1999). In this study, vaccination with *E. coli* (pBSSOD) induced antibodies to Cu/Zn SOD and a strong proliferative response in splenocytes was observed when stimulated *in vitro* with a thioredoxin-Cu/Zn SOD fusion protein. Our finding supports this study (Onate et al., 1999) as we observed that a significant level of protection when mice were injected with purified recombinant *B. abortus* SOD protein combined with IL-12 ($p=0.0001$). These results suggest that when *B. abortus* SOD is used under optimal conditions, including dosage and the appropriate cytokine milieu, this protein can induce a protective immune response against *Brucella* infection. Also, when *B. abortus* SOD purified recombinant protein was used alone as an antigen without IL-12, protection at somewhat lower level ($p=0.03$) was observed. Therefore, our study supports the role of SOD as a protective antigen.

B. abortus L7/L12 ribosomal protein has been identified as the most effective immunogen among *B. abortus* proteins (Brooks-Worrell and Splitter, 1992; Oliveira et al., 1994). Oliveira and Splitter (1996) found that recombinant *B. abortus* L7/L12 ribosomal protein induced a Th1 subset response from murine $CD4^+$ T cells characterized by significant levels of IFN-γ production. Since any antigen that preferentially induces an IFN-γ producing Th1 subset response is a desirable subunit vaccine preparation against brucellosis, this protein has been identified as an immunodominant antigen. However, we

could not analyze IFN-γ release by stimulation with this antigen due to the failure of protein purification in our study. Other investigators (Bachrach et al., 1994) have identified the L7/L12 ribosomal protein from *B. melitensis* as a major antigen, which stimulates delayed-type hypersensitivity in *Brucella*-sensitized guinea pigs. In addition, Skeiky et al. (1995) have isolated a gene homologous to the eukaryotic ribosomal eIF4A from *Leishmania braziliensis*; they have characterized it as a potent antigen capable of stimulating strong Th1-type responses as well as IL-12 production in leishmaniasis patients. Having characterized the recombinant *B. abortus* L7/L12 as a T-cell, especially Th-1 type reactive ribosomal protein and knowing the immunological importance of ribosomal preparations, we analyzed the ability of this antigen in conferring protection to mice against brucellosis. In this study, we have demonstrated that recombinant *B. abortus* L7/L12 ribosomal protein conferred protective immunity to BALB/c mice against brucellosis when *E. coli* expressing pMBP- *B. abortus* L7/L12 fusion protein cells were used as antigen. Our data confirm the work of Oliveira and Splitter (1996) who reported that L7/L12 ribosomal protein is protective against *Brucella* and of others (Gregory R. L., 1986) who reported that ribosomal preparations from other pathogens are highly protective vaccines. Therefore, this work demonstrated the usefulness of *B. abortus* SOD and L7/L12 proteins, but not HSPs, for assessing the development of subunit vaccines against *Brucella* infection.

REFERENCES

Afonso, L. C. C., T. M. Scharton, L. Q. Vieira, M. Wysocka, G. Trinchieri, and P. Scott. 1994. The adjuvant effect of interleukin-12 in a vaccine against *Leishmania major*. Science 263:235-237.

Anderson, P., D. Askgaard, L. Ljungqvist, J. Bennedsen, and I. Heron. 1991. Proteins released from *Mycobacterium tuberculosis* during growth. Infect. Immun. 59:1905-1910.

Araneo, B. A., M. L. Woods, and R. A. Daynes. 1993. Reversal of the immunosenescent phenotype by dehydroepiandosterone: hormone treatment provides an adjuvant effect on the immunization of aged mice with recombinant hepatitis B surface antigen. J. Infect. Dis. 67:830-846.

Araya, L. N., P. H. Elzer, G. E. Rowe, F. M. Enright, and A. J. Winter. 1989. Temporal development of protective cell-mediated and humoral immunity in BALB/c mice infected with *Brucella abortus*. J. Immunol. 143:3330-3337.

Araya, L. N. and A. J. Winter. 1990. Comparative protection of mice against virulent and attenuated strains of *Brucella abortus* by passive transfer of immune T cells or serum. Infect. Immun. 58:254-256.

Asada, K., M. Urano, and M. Takhashi. 1973. Subcellular location of superoxide dismutase in spinach leaves and preparation and properties of crystalline spinach superoxide dismutase. Eur. J. Biochem. 36:257-266.

Audibert, F. M. and L. D. Lise. 1993. Adjuvants: current status, clinical perspectives and future prospects. Immunol. Today. 14:281-284.

Bachrach, G., M. Banai, S. Bardenstein, G. Hoida, A. Genizi, and H. Bercovier. 1994. *Brucella* ribosomal protein L7/L12 is a major component in the antigenicity of *Brucella* INRA for delayed-type hypersensitivity in *Brucella*-sensitized guinea pigs. Infect. Immun. 62:5361-5366.

Baldwin, C. L., X. Jiag, and D. M. Fernandes. 1993. Macrophage control of *Brucella abortus*: influence of cytokines and iron. Trends Microbiol. 1:99-104.

Bannister, J. V. and M. W. Parker. 1985. The presence of a copper/zinc superoxide dismutase in the bacterium *Photobacterium leiognathi*. Proc. Natl. Acad. Sci. USA 82:149-152.

Bannister, J. V. and G. Rotillo. 1984. A decade of superoxide dismutase activity. Dev. Biochem. 26:146-189.

Beaman, L. and B. L. Beaman. 1990. Monoclonal antibodies demonstrate that superoxide dismutase contributes to protection of *Nocardia asteroides* within the intact host. Infect. Immun. 58:3122-3128.

Beaman, B. L., S. M. Scate, S. E. Moring, R. Dean, and H. P. Misra. 1983. Purification and properties of a unique superoxide dismutase from *Nocardia asteroides.* J. Biol. Chem. 258:91-96.

Beck, B. L., L. B. Tabatabai, and J. E. Mayfield. 1990. A protein isolated from *Brucella abortus* is a Cu-Zn superoxide dismutase. Biochemistry 29:372-375.

Bei R., J. Kantor, S. V. S. Kashmiri, S. Abrams, and J. Schlom. 1994. Enhanced immune responses and anti-tumor activity by baculovirus recombinant carcinoembryonic antigen

(CEA) in mice primed with the recombinant vaccinia CEA. J. Immunotherapy. 16:275-282.

Ben-Nathan D., Feuerstein G. 1990. The influence of cold or isolation stress on resistance of mice to West Mile virus encephalitis. Experientia. 46:285-290.

Bennink J. R., J. W. Yewdell, G. L. Smith, C. Moller, and B. Moss. 1984. Recombinant vaccinia virus primes and stimulates influenza haemagglutinin-specific cytotoxic T cells. Nature. 311:578-579.

Bennink J. R. and J. W. Yewdell. 1990. Recombinant vaccinia virus as vectors for studying T lymphocyte specificity and function. Curr. Top. Microb. Immun. 163:153-184.

Bielski, B. H. J. and G. G. Shiue. 1979. Reaction rates of superoxide radicals with the essential amino acids. Ciba Found. Symp. 65:43-56.

Blander, S. J. and M. A. Horwitz. 1993. Major cytoplasmic membrane protein of *Legionella pneumophila,* a genus common antigen and membrane of the hsp60 family of heat shock proteins, induces protective immunity in a guinea pig model of Legionnaires' disease. J. Clin. Invest. 91:717-723.

Brennn, M. J., D. L. Burns, B. D. Meade, R. D. Shahin, and C. R. Manclark. 1992. Recent advances in the development of pertussis vaccines, In: Vaccines. New Approaches to Immunological problems. Ellis, R. W. (Ed.), Butterworth-Heinemann, Boston, London.

Britton, L., D. P. Malinowski, and I. Fridovich. 1978. Superoxide dismutase and oxygen metabolism in *Streptococcus faecalis* and comparison with other organisms. J. Bacteriol. 134:229-236.

Brooks-Worrell B. M. and G. A. Splitter. 1992. Antigens of *Brucella abortus* S19 immunodominant for bovine lymphocytes as identified by one- and two-dimensional cellular immunoblotting. Infect. Immun. 60:2459-2464.

Canning, P. C., J. A. Roth, and B. L. Deyoe. 1986. Opsonin-dependent stimulation of bovine neutrophil oxidative metabolism by *Brucella* abortus. J. Inf. Dis. 154:464-470.

Casson, P. R., R. N. Anderson, H. G. Herrod, F. B. Stentz, A. B. Straughn, and G. E. Buster. 1993. Oral dehydroepiandrosterone in physiologic doses modulates immune function in postmenopausal women. Am. J. Obstet. Gynecol. 169:1536-1539.

Chan, S. H., B. Perussia, J. W. Gupta, M. Kobayashi, M. Pospish, H. A. Young, S. F. Wolf, D. Young, S. C. Clark and G. Trinchieri. 1991. Induction of interferon gamma production by natural killer cell stimulatory factor: Characterization of the responder cells and synergy with other inducers. J. Exp. Med. 173:869-879.

Cheers, C. 1983. Pathogenesis and cellular immunity in experimental murine brucellosis. Dev. Biol. Stand. 56:237-246.

Clerici, M., D. R. Lucey, J. A. Berzofsky, L. A. Pinto, T. A. Wynn, S. P. Blatt, M. J. Dolan, C. W. Hendrix, S. F. Wolf and G. M. Shearer. 1993. Restoration of HIV-specific cell-mediated immune responses by interleukin-12 *in vitro*. Science 262:1721-1724.

Cooper M. D., R. P. Tewari, and D. V. Bowser. 1980. Immunogenicity of ribosomal preparations from *Neisseria gonorrhoeae.* Infect. Immun. 28:92-100.

Daigle J. and D. J. J. Carr. 1998. Androstenediol antagonizes herpes simplex virus type 1-induced encephalitis through the augmentation of type 1 IFN production. J. Immun. 160:3060-3066.

Daynes, R. A., A. W. Meikle and B. A. Araneo. 1991. Locally active steroid hormones may facilitate compartmentalization of immunity by regulating the types of lymphokines produced by helper T-cells. Res. Immun. 142:40-45.

Del Giudice, G. 1992. New carrier and adjuvants in the development of vaccines. Curr. Opin. Immunol. 4:454-459.

Decker, M. D., K. M. Edwards, M. C. Steinhoff, M. B. Rennels, M. E. Pichichero, J. A. Englund, E. L. Anderson, M. A. Deloria, and G. F. Reed. 1995. Comparison of 13 acellular pertussis vaccines: Overview and serologic response. Pediatrics 96:548-557.

DeNagel D. C. and S. K. Pierce. 1993. Heat shock proteins in immune responses. Crit. Rev. Immunol. 13:71-81.

Edwards, K. E., B. D. Meade, M. D. Decker, G. F. Reed, M. B. Rennels, M. C. Steinhoff, E. L. Anderson, J. A. Englund, M. E. Pichichero, M. A. Deloria, and A. Deforest. 1995. Comparison of thirteen acellular perussis vaccines: Overview and serological response. Pediatrics 96:548-557.

Enright, F. M., L. N. Araya, P. H. Elzer, G. E. Rowe, and A. J. Winter. 1990. Comparative histopathology in BALB/c mice infected with virulent and attenuated strains of *Brucella abortus*. Vet. Immunol. Immunopath. 26:171-182.

Everest, P., P. Griffiths, and G. Dougan. 1995. Live *Salmonella* vaccines as a route toward oral immunization. Biologicals 23:119-124.

Ewing, D. 1983. Synergistic damage from H_2O_2 and OH* radicals in irradiated cells. Radiat. Res. 94:171-189.

Farr, S. B., D. Touati, and T. Kogoma. 1988. Effects of oxygen stress on membrane functions in *Escherichia coli*: Role of HPI catalase. J. Bacteriol. 170:1837-1842.

Ficht, T. A., S. W. Bearden, B. A. Sowa, and L. G. Adams. 1989. DNA sequence and expression of the 36-kilodalton outer membrane protein gene of *Brucella abortus*. Infect. Immun. 57:3281-3291.

Fisher-Hoch S. P., J. B. McCormick, D. Auperin, et al. 1989. Protection of rhesus monkeys from fatal lassa fever by vaccination with recombinant vaccinia virus containing the Lassa fever virus glycoprotein gene. Proc. Nat. Acad. Sci. USA. 86:317-321.

Franzon, V. L., J. Arondel, and P. J. Sansonetti. 1990. Contribution of superoxide dismutase and catalase activities to *Shigella flexneri* pathogenesis. Infect. Immun. 58:529-535.

Fridovich, I. 1975. Superoxide dismutases. Annu. Rev. Biochem. 44:147-159.

Fridovich, I. 1989. Superoxide dismutase. An adaptation to a paramagnetic gas. J. Biol. Chem. 264:7761-7764.

Fridovich, I. 1975. Superoxide dismutases. Annu. Rev. Biochem. 44:147-159.

Gazzinelli, R. T., S. Hieny, T. A. Wynn, S. Wolf and A. Sher. 1993. Interleukin-12 is required for the T-lymphocyte-independent induction of interferon gamma by an intracellular parasite and induces resistance in T-cell-deficient host. Proc. Natl. Acad. Sci. USA 90:6115-6119.

Germain, R. N. 1994. MHC-dependent antigen processing and peptide presentation: providing ligands for lymphocyte activation. Cell 76:287-299.

Germanier, R. and E. Furer. 1975. Isolation and characterization of galE mutant and Ty21a of *Salmonella typhi*: A candidate strain for a live oral typhoid vaccine. J. Infect. Dis. 141:553-558.

Golding, B., M. Zaitseva, and H. Golding. 1994. The potential for recruiting immune responses toward type 1 or type 2 T cell help. Am. J. Trop. Med. Hyg. 50:33-40.

Gotschlich, E. C. 1984. *Meningococcal meningitis*, In: Bacterial Vaccines, pp. 237-255.

Germanier, R. and E. Fuer. 1975. Isolation and characterization of galE mutant and Ty21a of *Salmonella typhi* : A candidate strain for a live oral typhoid vaccine. J. Infect. Dis. 141:553.

Gregory R. L. 1986. Microbial ribosomal vaccines. Rev. Infec. Dis. 8:208-217.

Hamel, E., M. Koka and T. Nakamoto. 1972. Requirement of an *Escherichia coli* 50 S ribosomal protein component for effective interaction of the ribosome with T and G factors and with guanosine triphosphate. J. Biol. Chem. 10:805-814.

Hansen, K., J. M. Bangsborg, H. Fjordvang, N. S. Pedersen, and P. Hindersson. 1988. Immunochemical characterization of and isolation of the gene for a *Borrelia burgdorferi* immunodominant 60-kilodalton antigen common to a wide range of bacteria. Infect. Immun. 56:2047-2053.

Harboe, M., P. Anderson, M. J. Colston, B. Gicquel, P. W. M. Hermans, J. Ivanyi, and S. H. E. Kaufmann. 1996. Vaccines against tuberculosis. Vaccine. 14:701-716.

Hassan, H. M. and I. Fridovich. 1980. Superoxide dismutase: detoxification of a free radical. p. 311-322. In W. B. Jacoby (ed.), Enzymatic basis for detoxification, vol. 1. Academic Press, Inc., Orlando, FL.

Heinzel, F. P., D. S. Schoenhaut, R. M. Rerko, L. E. Rosser, and M. K. Gately. 1993. Recombinant interleukin 12 cures mice infected with *Leishmania major*. J. Exp. Med. 177:1505-1509.

Hindersson, P., J. D. Knudson, and N. H. Axelsen. 1987. Cloning and expression of *Treponema pallidum* common antigen (Tp-4) in *E. coli* k-12. J. Gen. Microbiol. 133:587-596.

Horwitz, M. A. 1983. Cell-mediated immunity in Legionnaires' disease. J. Clin. Invest. 71:1686-1697.

Howell, D. C. 1992. Statistical Methods for Psychology, 3rd edition, Belmont CA, Duxbury Press.

Hsieh, C. S., A. B. Heimberger, J. S. Gold, A. O'Garra, and K. Murphy. 1992. Differential regulation of T helper phenotype development of IL-4 and IL-10 in an ab-transgenic system. Proc. Natl. Acad. Sci. USA. 89:6065-6069.

Hsieh, C. S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy. 1993. Development of Th1 CD4+ T cells through IL-12 produced by *Listeria*-induced macrophages. Science. 260:547-549.

Hubbard, R. D., C. M. Flory, and Collins, F.M. 1992. Immunization of mice with mycobacterial culture filtrate proteins. Clin. Exp. Immunol. 87:94-98.

Janeway, C.A., Jr., 1992. The immune system evolved to discriminate infectious nonself from noninfectious self. Immunol. Today. 13:11-16.

Jennings, H. J. 1983. Capsular polysaccharides as human vaccines. Adv. Carbohydr. Chem. Biochem. 41:155-208.

Kalschmidt E. and H. G. Wittmann. Ribosomal proteins. XII. Number of proteins in small and large ribosomal subunits of *Escherichia coli* as determined by two-dimensional gel electrophoresis. Proc. Natl. Acad. Sci. U. S. A. 67:1276-1282.

Kaper, J. B., J. G. Morris, and M. M. Levine. 1995. Cholera. Clin. Microbiol. Rev. 8:48-86.

Kubin, M., M. Kamoun, and G. Trinchieri. 1994. Interleukin 12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T cells. J. Exp. Med. 180:211-222.

Kurar E. and G. A. Splitter. 1997. Nucleic acid vaccination of *Brucella abortus* ribosomal L7/L12 gene elicits immune response. Vaccine 15: 1851-1857.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lathe R., M. P. Kieny, and P. Gerlinger. 1987. Tumor prevention and rejection with recombinant vaccinia. Nature. 326:878-880.

Latimer, E., J. Simmers, N. Sriranganathan, R. M. Roop II, G. G. Schurig, and S. M. Boyle. 1992. *Brucella abortus* deficient in copper/zinc superoxide dismutase is virulent in BALB/c mice. Microbial. Pathogenesis. 12:105-113.

Lawrence C. Paoletti and Pamela M. Mclnnes. 1999. Vaccines ; from concept to clinic. CRC press, Boca Raton, FL.

Lee, Y. M., F. J. Ayala, and H. P. Misra. 1981. Purification and properties of superoxide dismutase from *Drosophila melanogaster*. J. Biol. Chem. 256:8506-8509.

Leon L. L., W. Leon, and L. Chaves. 1980. Immunization of mice with *Trypanoxoma cruzi* polyribosomes. Infect. Immun. 27:38-43.

Levin, M. M., D. Herrington, G. Losonsky, B. Tall, J. B. Kaper, J. Ketley, C. O. Tacket, and S. Cryz. 1988. Safety, immunogenicity and efficacy of recombinant live oral cholera vaccines, CVD 103 and CVD 103-HgR. Lancet I:467-470.

Lin, J., L. G. Adams, and T. A. Ficht. 1992. Characterization of the heat shock response in *Brucella abortus* and isolation of the genes encoding the GroE heat shock proteins. Infect. Immun. 60:2425-2431.

Lin, J. and T. A. Ficht. 1995. Protein synthesis in *Brucella abortus* induced during macrophage infection. Infect. Immun. 63:1409-1414.

Lin, J., L. G. Adams, and T.A. Ficht. 1996. Immunological response to the *Brucella abortus* GroEL homolog. Infect. Immun. 64:4396-4400.

Loria R. M. and D. A. Padgett. 1992. Androstenediol regulates systemic resistance against lethal infections in mice. Arch. Virol. 127:103-115.

Loria R. M., T. H. Inge, S. Cook, A. Szakai, and W. Regelson. 1988. Protection against acute lethal viral infections with the native steroid dehydroepiandrosterone (DHEA). J. Med. Virol. 26:301-314.

Loria R. M., W. Regelson, and D. A. Padgett. 1990. Immune response facilitation and resistance to virus and bacterial infections with dehydroepiandrosterone (DHEA). In:Kalimi M, Regelson W (ed). The biologic role of dehydroepiandrosterone (DHEA). W De Gruyter, Berlin, pp. 107-130.

Lowrie, D. B., R. E. Tascon, and C. L. Silva. 1995. Vaccination against tuberculosis. Int. Arch. Allergy. Immunol. 108:309-312.

Manclark, C. R. and J. L. Cowell. 1984. Pertussis, In: Bacterial Vaccines, pp. 69-106. Germanier, R. (Ed.), Academic Press, Inc., Orlando.

Manetti, R., F. Gerosa, M. G. Giudici, R. Biagotti, P. Parronchi, M. P. Piccinni, S. Sampognaro, E. Maggi, S. Romagnani, and G. Trinchieri. 1994. Interleukin 12 induces stable priming for interferon-γ (IFN-γ) production during differentiation of human T helper (Th) cells and transient IFN-γ production in established Th2 cell clones. J. Exp. Med. 179:1273-1283.

Manetti, R., P. Parronchi, M. G. Guidizi, M. P. Piccinni, E. Maggi, G. Trinchieri, and S. Romagnani. 1993. Natural killer cell stimulatory factor (interleukin 12) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4 producing cells. J. Exp. Med. 177:1199-1204.

Mayer, B. K. and J. O. Falkinham. 1986. Superoxide dismutase activity of *Mycobacterium avium, M. intracellulare, and M. scrofulaceum.* Infect. Immun. 53:631-635.

McCord, J. M., B. B. Keele, and I. Fridovich. 1971. An enzyme-based theory of obligate anaerobiosis: the physiological function of superoxide dismutase. Proc. Natl. Acad. Sci. U. S. A. 68:1024-1027.

Mead, J. 1976. Free radical mechanisms of lipid damage and consequences for cellular membranes. In: W. A. Pryor (Editor), Free Radicals in Biology, Vol.1. Academic Press, New York, pp.51-68.

Meikle, A. W., R. W. Dorchunk, R. W., and B. A. Araneo. 1992. The presence of a dehydroepiandrosterone-specific receptor-binding complex in murine T-cells. J. Steroid. Biochem. Molec. Biol. 42:293-304.

Miller, M. A., M. J. Skeen, and H. K. Ziegler. 1996. Protective immunity to *Listeria monocytogenes* elicited by immunization with heat-killed *Listeria* and IL-12. Annu. New. York. Acad. Sci. 797:207-227.

Miller, M. J., R. A. Wrightsman, and J. E. Manning. 1996. *Trypanosoma cruzi*: Protective immunity in mice immunized with paraflagellar rod proteins is associated with a T-helper type 1 response. Exp. Parasit. 84:156-167.

Misra, H. P. and B. B. Keele. 1975. The purification and properties of superoxide dismutase from a blue-green alga. Biochem. Biophys. Acta 379:418-425.

Misra, H. P. and I. Fridovich. 1977. Purification and properties of superoxide dismutase from a red algae, *Prophyridium cruentum.* J. Biol. Chem. 252:6421-6423.

Morein, B., K. Lovgren-Bengtsson, and J. Cox. 1996. Modern adjuvants. Functional aspects, In: Concepts in Vaccine Development, pp. 243-263, Kaufmann, S. H. E. (Ed.), Walter de Gruyter, Berlin, New York.

Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. 136:2348-2357.

Mosmann, T. R. and R. L. Coffman. 1989. Heterogeneity of cytokine secretion patterns and functions of helper T cell. Adv. Immunol. 46:111-147.

Mosmann, T. R. and R. L. Coffman. 1989. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. 7:145-173.

Mosmann, T. R. and S. Sad. 1996. The expanding universe of T-cell subsets: Th1, Th and more. Immunol. Today 17:138-146.

Mountford, A. P., S. Anderson, and R. A. Wilson. 1996. Induction of Th1 cell-mediated protective immunity to *Schistosoma mansoni* by co-administration of larval antigens and IL-12 as an adjuvant. J. Immunol. 156:4739-4745.

Moxon, E. R. and R. Rappuoli. 1990. *Haemophilus influenzae* infections and whooping cough. Lancet 335:1324-1329.

Murphy, E. E., G. Terres, S. E. Macatonia, C. S. Hsieh, J. Mattson, L. Lanier, M. Wysocka, G. Trinchieri, K. Murphy, and A. O'Garra. 1994. B7 and interleukin 12 cooperate for proliferation and interferon γ production by mouse T helper cell 1 clones that are unresponsive to B7 costimulation. J. Exp. Med. 180:223-231.

Nicoletti, P. 1990. Vaccination. In: Animal brucellosis. Eds. Nielsen, K., Duncan, J. R., pp.283-290. CRC Press, Boca Raton, Florida.

Nicoletti, P. and F. W. Milward. 1983. Protection by oral administration of *Brucella abortus* strain 19 against oral challenge exposure with a pathogenic strain of *Brucella*. Am. J. Vet. Res. 44:1641-1643.

Nielsen K. and J. R. Duncan. 1988. Antibody isotype response in adult cattle vaccinated with *Brucella* abortus S19. Vet. Immunol. Immunopathol. 19:205-214.

Noll A. and I. B. Autenrieth. 1996. Immunity against *Yersinia enterocolitica* by vaccination with *Yersinia* HSP60 immunostimulating complexes or *Yersinia* HSP60 plus interleukin-12. Infect. Immun. 64:2955-2961.

Oliveira, S. C., J. S. Harms, M. Banai, and G. A. Splitter. 1996. Recombinant *Brucella abortus* proteins that induce proliferation and gamma-interferon secretion by CD4+ T cells from *Brucella*-vaccinated mice and delayed-type hypersensitivity in sensitized guinea pigs. Cell. Immunol. 172:262-268.

Oliveira, S. C. and G. A. Splitter. 1996. Immunization of mice with recombinant L7/L12 ribosomal protein confers protection against *Brucella abortus* infection. Vaccine. 14:959-962.

Oliveira, S. C. and G. A. Splitter. 1994. Sequences of the rp1JL operon containing the L10 and L7/L12 genes from *Brucella abortus*. Gene 140:137-138.

Oliveira, S. C., Y. Zhu, and G. A. Splitter. 1994. Recombinant L7/L12 ribosomal protein and γ-irradiated *Brucella abortus* induce a T-helper 1 subset response from murine CD4+ T cells. Immunology 83:659-664.

Onate, A. A., R. Vemulapalli, E. Andrews, G. G. Schurig, S. Boyle, and H. Folch. 1998. Vaccination with live *E. coli* expressing *Brucella abortus* Cu/Zn superoxide dismutase protects mice against virulent *B. abortus*. Infect. Immun. 67:986-988.

Orange, J. S., S. F. Wolf and C. A. Biron. 1994. Effects of IL-12 on the response and susceptibility to experimental viral infections. J. Immunol. 152:1253-1264.

Pal P. G. and M. A. Horwitz. 1992. Immunization with extracellular proteins of *Mycobacterium tuberculosis* induces cell-mediated immune responses and substantial protective immunity in a guinea pig model of pulmonary tuberculosis. Infect. Immun. 60:4781-4792.

Parronchi, P., D. Macchi, M. P. Piccinni, P. Biswas, C. Simonelli, E. Maggi, M. Ricci, A. A. Ansari, and S. Romagnani. 1991. Allergen- and bacterial antigen-specific T cell clones established from atopic donors show a different profile of cytokine production. Proc. Natl. Acad. Sci. USA. 88:4538-4542.

Perloff, R. M. and D. Kinsey. 1992. Political advertising as seen by consultants and journalists. Journal of Advertising Research. 32:53-60.

Pettersson, I. and C. G. Kurland. 1980. Proc. Natl. Acad. Sci. USA 77:4007-4010.

Phillips, M., G. W. Pugh, B. L. Deyoe. 1989. Duration of strain 2308 infection and immunogenicity of *Brucella abortus* lipopolysaccharide in five strains of mice. Am. J. Vet. Res. 50:318-322.

Plommet M. and A. M. Plommet. 1988 a. Reactivation of a residual *Brucella abortus* 19 vaccine infection in mice by a virulent challenge or by injection of brucellin or of *Brucella* lipopolysaccharide. Ann. Rech. Vet. 19:245-251.

Plommet M. and A. M. Plommet. 1988 b. Virulence of *Brucella*: bacterial growth and decline in mice. Ann. Rech. Vet. 19:65-67.

Pugh, G. W., E. S. Zehr, V. P. Meador, M. Phillips, T. J. McDonald, and B. L. Deyoe. 1989. Immunologic, histopathologic, and bacteriologic responses of five strains of mice to *Brucella abortus* strain 2308. Am. J. Vet. Res. 50:323-328.

Pugh G. W. and L. B. Tabatabai. 1994. Alteration of protective and serologic responses in BALB/c mice vaccinated with chemically modified versus nonmodified proteins of *Brucella abortus* 19. Infect. Immun. 62:5327-5334.

Pugh G. W. and L. B. Tabatabai. 1996. Variation in *Brucella abortus* 2308 infection in BALB/c mice induced by prior vaccination with salt-extractable periplasmic proteins from *Brucella abortus* 19. Infect. Immun. 64:548-556.

Rappuoli, R. 1990. New and improved vaccines against diphtheria and tetanus, In: New Generation Vaccines, pp. 251-268, In Woodrow, G. C. and M. M. Levine (Eds.), Marcel Dekker, Inc., New York.

Ravindranath, S. D. and I. Fridovich. 1975. Isolation and characterization of a manganese-containing superoxide dismutase from yeast. J. Biol. Chem. 250:6107-6112.

Rigby, W. F., S. Denome, and M. W. Fanger. 1987. Regulation of lymphokine production and human T-lymphocytes activation by 1,25-dihydroxyvitamin D3. Specific inhibition at the level of messenger RNA. J. Clin. Invest. 79:1659-1664.

Romagnani, S. 1991. Human Th1 and Th2 subsets: doubt no more. Immunol. Today. 12:256-257.

Rook, G. A. W. 1988. The role of vitamin D in tuberculosis (Editorial). Am. Rev. resp. Dis. 138:768-770.

Rook, G. A. W. and J. L. Stanford. 1995. Adjuvants, endocrines and conserved epitopes; factors to consider when designing therapeutic vaccines. Int. J. Immunopharmac. 17:91-102.

Roop, R. M., T. W. Fletcher, N. M. Sriranganathan, S. M. Boyle, and G. G. Schurig. 1994. Identification of an immunoreactive *Brucella abortus* HtrA stress response protein homolog. Infect. Immun. 62:1000-1007.

Saginala S., T. G. Nagaraja, Z. L. Tan, K. F. Lechtenberg, M. M. Chengappa, and P. M. Hine. 1996. The serum neutralizing antibody response in cattle to *Fusobacterium Necrophorum* leukotoxoid and possible protection against experimentally induced hepatic abscesses.

Sambrook J., T. Maniatis, and E. F. Fritsch. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Schurig, G. G., R. M. Roop, T. Bagchi, S. Boyle, D. Buhrman, N. Sriranganathan. 1991. Biological properties of RB51: A stable rough strain of *Brucella abortus*. Vet. Microbiol. 28:171-188.

Scott P. 1993. IL-12 : Initiation cytokine for cell-mediated immunity. Science. 260:496-497.

Seder, R. A., R. Gazzinelli, A. Sher, and W. E. Paul. 1993. Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon γ production and diminishes interleukine 4 inhibition of such priming. Proc. Natl. Acad. Sci. USA. 90:10188-10192.

Seder, R. A., W. E. Paul, M. M. Davis, and B. Fazekas de St. Groth. 1992. The presence of interleukin 4 during in vitro priming determines the lymphokine-producing potential of CD4+ T cells from T cell receptor transgenic mice. J. Exp. Med. 176:1091-1098.

Sher, A. and R. L. Coffman. 1992. Regulation of immunity to parasites by T cells and T-cell derived cytokines. Annu. Rev. Immunol. 10:385-409.

Shinnick, T. M., M. H. Vodkin, and J. C. Williams. 1988. The *Mycobacterium tuberculosis* 65-kilodalton antigen is a heat shock protein which corresponds to common antigen and to the *Escherichia coli* GroEL protein. Infect. Immun. 56:446-451.

Silva, C. L., M. F. Silva, R. C. L. R. Pietro and D. B. Lowrie. 1994. Protection against tuberculosis by passive transfer with T-cell clones recognizing mycobacterial heat-shock protein 65. Immunology 83:341-346.

Simge Baloglu. 1997. Assesment of the expression of *Brucella abortus* heat shock protein, GroEL, in vaccinia virus to induce protection against a *Brucella* challenge in BALB/c mice. Master thesis. Virginia Polytechnic Institute and State University.

Singh I. G., R. Mukherjee, G. P. Talwar, and S. H. E. Kaufmann. 1992. *In vitro* characterization of T cells from *Mycobacterium* w-vaccinated mice. Infect. Immun. 60:257-263.

Skeen, M. J. and H. K. Ziegler. 1994. Activation of g/d T cells for production of IFN-γ is mediated by bacteria via macrophage-derived cytokines IL-1 and IL-12. J. Immunol. 154:5832-5841.

Skeiky, Y. A. W., J. A. Guderian, and D. R. Benson. 1995. A recombinant *Leishmania* antigen that stimulates human peripheral blood mononuclear cells to express a Th1-type cytokine profile and to produce interleukine 12. J. Exp. Med. 181:1527-1537.

Spargo B.J., L. M. Crowe, T. Ioneda, B.L. Beaman, and J. H. Crowe. 1990. Cord factor (a,a-trehalose 6,6-dimycolate) inhibits fusion between phospholipid vesicles. Proc. Natl. Acad. Sci. USA. 88:737-740.

Sriranganathan, N., S. M. Boyle, G. Schurig, and H. Misra. 1991. Superoxide dismutase of virulent and avirulent strains of *Brucella abortus*. Vet. Microbiol. 26:359-366.

Stabel, T. J., Z. Sha, and J. E. Mayfield. 1994. Periplasmic location of *Brucella* abortus Cu/Zn superoxide dismutase. Vet. Microbiol. 38:307-314.

Steinman, H. M. 1987. Bacteriocuprein superoxide dismutase of *Photobacterium leignathi*. J. Biol. Chem. 262:1882-1887.

Steinman, H. M. and B. Ely. 1990. Copper-zinc superoxide dismutase of Caulobacter crescentus: cloning, sequencing, and mapping of the gene and periplasmic location of the enzyme. J. Bacteriol. 172:2901-2910.

Stevens, M. G., L. B. Tabatabai, C. O. Steven, and N. F. Cheville. 1994. Immune responses to superoxide dismutase and synthetic peptides of superoxide dismutase in cattle vaccinated with *Brucella* abortus strain 19 or RB51. Vet. Microbiol. 41:383-389.

Stevenson, M. M., M. F. Tam, S. F. Wolf and A. Sher. 1995. IL-12 induced protection against blood-stage *Plasmodium chabaudi* requires IFN-γ and TNF-α and occurs via a nitric oxide-dependent mechanism. J. Immunol. 155:2545.

Subramanian A. R. 1975. Copies of proteins L7 and L12 and heterogeneity of the large subunit of *Escherichia coli* ribosome. J. Mol. Biol. 95:1-8.

Suzuki, T., N. Suzuki, R. A. Daynes, and E. G. Enleman. 1991. Dehydroepiandrosterone enhances IL2 production and cytotoxic effector function of human T-cell. Clin. Immun. Immunopath. 61:202-211.

Swain, S. L. 1993. IL-4 dictates T-cell differentiation. Re. Immunol. 144:567-651.

Sypek, J. P., C. L. Chung, S. E. H. Mayor, J. M. Subramanyam, S. J. Goldman, D. S. Sieburth, S. E. Wolf, and R. G. Schaub. 1993. Resolution of cutaneous leishmaniasis: interleukin 12 initiates a protective T helper type 1 immune response. J. Exp. Med. 177:1797-1802.

Tabatabai, L. B., G. W. Pugh, M. G. Stevens, M. Phillips, and T. J. McDonald. 1990. Monophosphoryl lipid A-induced immune enhancement of *Brucella abortus* salt-extractable protein and lipopolysaccharide vaccines in BALB/c mice. Am. J. Vet. Res. 53:1900-1907.

Tantimavanich S., S. Nagai, H. Nomaguchi, M. Kinomoto, and N. Ohara. 1993. Immunological properties of ribosomal proteins from *Mycobacterium bovis* BCG. Infect. Immun. 61:4005-4007.

Tatum, F. M., P. G., Detilleux, J. M. Sacks, and S. M. Halling. 1992. Construction of Cu-Zn superoxide dismutase deletion mutants of *Brucella abortus*: Analysis of survival in vitro in epithelial and phagocytic cells and in vivo in mice. Infect. Immun. 60:2863-2869.

Terhorst C., W. Moller, R. Laursen, and B. Wittmann-Liebold. 1973. The primary structure of an acidic protein from 50S ribosomes of *Escherichia coli* which is involved in GTP hydrolysis dependent on elongation factors G and T. Eur. J. Biochem. 34:138-152.

Terhorst C., B. Wittmann-Liebold, and W. Molle. 1972. 50S ribosomal proteins. Peptide studies on two acidic proteins, A 1 and A 2, isolated from 50S ribosomes of *Escherichia coli*. Eur. J. Biochem. 25:13-19.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. 76:4350-4354.

Trinchieri, G. 1993. Interleukin-12 and its role in the generation of Th1 cells. Immunol. Today. 14:335-338.

Tripp, C. S., M. K. Gately, J. Hakimi, P. Ling and E. R. Unanue. 1994. Neutralization of IL-12 decreases resistance to *Listeria* in SCID and C.B-17 mice. J. Immunol. 152:1883-1887.

Tripp, C. S., S. F. Wolf and E. R. Unanue. 1993. Interleukine-12 and tumor necrosis factor alpha are costimulators of interferon gamma production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. Proc. Natl. Acad. Sci. USA 90:3725-3729.

Ulmer, J. B., D. L. Montgomery, J. J. Donnelly, and M. A. Liu. 1996. DNA vaccines, In: Vaccine Protocols, pp. 289-300, Robinson, A., G. H. Farrar, and C. N. Wiblin (Eds.), Humana Press, Totowa, NJ.

Vanlandschoot P., G. Maertens, W. M. Jou, and W. Fiers. 1993. Recombinant secreted haemagglutinin protects mice against a lethal challenge of influenza virus. Vaccine. 11:1185-1187.

Weisiger, R. A. and I. Fridovich. 1973. Superoxide dismutase. Organelle specificity. J. Biol. Chem. 248:3582-3592.

Wergifosse, P., P. Lintermans, J. N. Limet, and A. Cloeckaert. 1995. Cloning and nucleotide sequence of the gene coding for the major 25-kilodalton outer membrane protein of *Brucella abortus*. J. Bacteriol. 177:1911-1914.

Wicher K., L. M. Schouls, V. Wicher, J. D. A. Van Embden, and S. S. Nakeeb. 1991. Immunization of guinea pigs with recombinant TmpB antigen induces protection against challenge infection with *Treponema pallidum* nichols. Infection and Immunity. 59:4343-4348.

Williams, J. C., T. A., Hoover, D. M. Waag, N. Banerjee-Bhatnagar, C. R. Bolt, and G. H. Scott. 1990. Antigenic structure of *Coxiella burnetii*: a comparison of lipopolysaccharide and protein antigens as vaccines against Q fever. Annu. New. York. Acad. Sci. 590:370-380.

Wolinsky, E. 1979. Nontuberculous mycobacteria and associated diseases. Am. Rev. Respir. Dis. 119:107-159.

Wynn, T. A., A. W. Cheever, D. Jankovic, R. W. Poindexter, P. Caspar, F. A. Lewis and A. Sher. 1995. An IL-12 based vaccination method for preventing fibrosis induced by schistosome infection. Nature 376:594-596.

Xiong, H., S. Ohya, Y. Tanabe, and M. Mitsuyama. 1997. Persistent production of interferon-gamma (IFN-γ) and IL-12 is essential for the generation of protective immunity against *Listeria monocytogenes*. Clin. Exp. Immunol. 108:456-462.

Youmans A. S. and G. P. Youmans. 1974. The effect of metabolic inhibitors and hydroxylamine on the immune response in mice to mycobacterial ribonucleic vaccines. J. Immunol. 112:271-284.

Yssel, H., K. E. Johnson, P. Schneider, J. Wideman, A. Terr, R. Kastelein, and J. E. de Vries. 1992. T cell activation epitopes of the house dust mite allergen Der p I. J. Immunol. 148:738-745.

Zhan, Y., L. Zhanqi, and C. Cheers. 1996. Tumor necrosis factor alpha and interleukine-12 contribute to resistance to the intracellular bacterium *Brucella abrotus* by different mechanisms. Infect. Immun. 64:2782-2786.

Ziegler, H. K., M. J. Skeen and K. M. Pearce. 1994. Role of α/β and γ/δT cells in innate and acquired immunity. In microbial pathogenesis and immune response. E. W. Ades, R. F. Rest and S. A. Morse, Eds. Annu. New. York. Acad. Sci. 730:53-70.

spinner flasks, and proper speed of stirring. Other factors and conditions were examined based on several parameters including multiplicity of infection (MOIs), times of replication of recombinant baculovirus, availability of oxygen, cell phases, cell densities, and cell lines (Ogonah et al., 1991; Scott et al., 1992; Licari and Bailey, 1990).

To determine the proper MOIs for optimal recombinant protein expression, the baculovirus life cycle must be considered. Late in the viral life cycle, nonoccluded, infectious virions of baculovirus are released from insect cells into the extracellular space. These secondary virions are then able to infect cells still in the exponential phase that were not originally infected (Granados and Federici, 1986; Luckow, V. A. and M. D. Summers, 1988). At extremely high MOI values, the cells may be synchronously infected (the primary infection), whereas at lower MOIs only a limited subpopulation may be infected. In the study of Licari and Bailey (1990), the effect of the MOI on product titer was determined by infecting cells with MOI values ranging from 0 to 100 and monitoring the production of β-galactosidase over time. For cells infected in the exponential phase, a logarithmic relationship existed between the final β-galactosidase product and the MOI used, with the highest MOI studied resulting in greatest protein synthesis in the early infection time. However, due to the very high infection at an early time, only a few uninfected cells had progeny cells which were to be infected by the secondary virions. Thus, the total number of cells infected with very high MOI will be less than the total number of cells infected with lower MOI, leading to a decrease in final product yields. Therefore, in baculovirus systems, viral titers between 1 and 10 are

highly recommended to yield optimal quantity and quality of recombinant proteins. The final product concentration as a function of the MOI was also highly dependent on the growth phase of the cells (Licari and Bailey, 1990). Dramatic decreases in product yields were observed if cells were infected during the stationary phase. Also, less variation in product titer occurred if the cells were infected in the exponential growth phase compared to infection in the stationary phase (Licari and Bailey, 1990). The maximum level of expression of the recombinant protein is also affected by the aeration rate of the culture (Scott et al., 1992). In their study, cultures at an identical cell density were infected with same MOI and stirred at either 30 revolution (rev)/min or 80 rev/min to test the effect of stirrer speed (and hence the availability of oxygen) on cell infection and recombinant protein production. At 80 rev/min, the percent viability of these infected cultures was 10-20% 96 h after infection, and at 30 rev/min, the percent viability was 75-85%, suggesting more infected cells were in the culture stirred at higher revolution because of higher oxygen availability. In both uninfected controls (stirrer speed: 30 rev/min., 80 rev/min.), the viability was greater than 95% at the same post-infection time. Thus, these results showed that available oxygen is one of the major parameters to affect the infectivity of recombinant baculovirus.

The optimal MOI varies by cell line and the relative infection kinetics of the recombinant virus. Thus, MOI should be established for each recombinant virus and cell line employed. Sf9 cells, derived from *S. frugiperda* ovarian cells, are commonly used to isolate and propagate recombinant AcNPV viral stocks and produce the recombinant protein of interest. High-Five™ cells, derived from *Trichoplusia ni* egg cell homogenates,

have been shown to be capable of expressing significantly higher levels of recombinant proteins compared to other insect cells, up to 25 fold higher expression of secreted proteins than Sf9 cells. These two different cell lines were used and compared for optimal recombinant protein expression in our studies.

The results of previous studies for factors and conditions of optimal recombinant protein expressions were not directly applicable to our studies because in most cases different vectors were used, or the experimental conditions were dissimilar. The evaluation of the conditions specifically optimal for the expression of *B. abortus* GroEL, GroES, and HtrA recombinant proteins is described in this chapter based on the results of our own set of parameters including cell lines, cell densities, post-infection times, and MOIs.

MATERIALS AND METHODS

1. Cells.

Sf9 cells, derived from *S. frugiperda* ovarian cells, were used for wild-type baculovirus infection, recombinant protein expression, and viral titration including plaque assay and end-point assay. These cells were purchased from GibcoBRL and propagated in Sf900-SFM medium (GibcoBRL). Sf21 cells, derived from *S. frugiperda* ovarian cells, were used for plaque assay. These cells were generously provided by Dr. Dwight E. Lynn at USDA/ARS and propagated in Grace's insect cell culture media (GibcoBRL). *Trichoplusia ni* cells from egg cell homogenates were used for recombinant protein

expression. These cells were purchased from Invitrogen (Carlsbad, CA) and propagated in Sf900-SFM medium (GibcoBRL). Cells were grown at 28°C, in either adherent or suspension culture, and were infected with either wild type or recombinant baculovirus while in the logarithmic phase of growth at greater than 95% cell viability and a density of 2 x 10^6 cells/ml. For high cell viability, stirrer speed of suspension culture was carefully adjusted depending on the status of the cell culture. Cells before exponential growth phase were stirred at 50 rev/minute because they are more shear-sensitive and less oxygen demanding. In the exponential phase, speed was adjusted to 95 RPM for supplying high level of oxygen.

2. Titration of recombinant baculovirus.

2.1 Plaque assay.

To standardize the amount of virus used to infect cells, log-phase cells without antibiotics were used in plaque assays to determine virus titers in plaque-forming units per ml (pfu/ml). Two ml of cell suspension was dispensed per well of 6-well plates under sterile conditions. The cells were allowed to settle to the bottom by incubating at room temperature for 1 h. The virus, diluted by 10-fold serial dilution steps in Sf900-SFM, was added to the cells and incubated for 1 h at room temperature. Following the 1-h incubation, the virus inoculum was removed from the wells and was replaced with 2 ml of the diluted agarose. The plates were incubated at 28°C in a humidified incubator for 4 to 10 days. Various conditions, such as cell lines (Sf 9, Sf 21, *T. ni*), cell numbers (1 X 10^7, 2 X 10^6, 1 X 10^6, 7.5 X 10^5 cells), viral dilutions (10^{-1} to 10^{-9}), replication times (4 to 9

days), types of agarose gel based on sulfate content and gel strength, percentages of agarose gels (0.75%, 1.5%), the presence or absence of serum, differences in supplied humidity and types of dyes (trypan blue to stain dead cells, neutral red to stain viable cells), were used as parameters. However, we failed to observe plaques in many trials. Thus, end-point assay has been performed as an alternative to titrate each recombinant baculovirus.

2.2. End-point median tissue culture infectious dose ($TCID_{50}$) assay.

Log- phase Sf9 cell culture was suspended into Sf900-SFM without antibiotics. Cell numbers were adjusted to 2 X 10^5 cells/ml. The GroEL, GroES, or HtrA recombinant viruses and wild-type baculovirus were diluted by serial 10 fold dilution steps from 10^{-1} to 10^{-9} using same media. Ten µl of each dilution of the viruses were added into 12 wells per dilution in 96 well plates, and then 100 µl volume of 2 X 10^4 cells were added into each well. In the last row of the plate, the same number of cells was added without virus inoculation to serve as a non-infected negative control. The plates were incubated at 28^{o}C for nine days in an air-tight container lined with moist tissue paper to prevent drying. The plates were monitored daily for signs of infection. After nine days of incubation, 10 µl trypan blue in 1:10 dilution was added to each well and incubation was continued for several hours at 28^{o}C. The total number of positive (infected, > 90% stained) and negative (non-infected, < 10% stained) wells were counted each of the next few days until no change was observed. When more than 90% of cells were stained and contained numerous granules, the well was regarded as positive. Based

on the results of infectivity in the plate, end-point dilution$_{50}$ or tissue culture infectious dose$_{50}$ ($TCID_{50}$) was determined. The infectious units expressed as $TCID_{50}$ units/ml were converted to an approximate titer in pfu/ml to standardize the inoculum dose of baculovirus. This conversion was done by a calculation applying the method of Reed and Muench, as described by Summers and Smith (1987), accepting the formula: pfu/ml = $TCID_{50}$ / ml x 0.69.

3. Preparation of high-titer viral stocks.

Adherent and suspension Sf9 cells in the exponential growth phase were infected with each recombinant virus and replicated for 48 hrs (Summer and Smith, 1987) to determine the condition yielding higher titer of virus. The titers were determined by end-point assay.

4. Kinetic studies of recombinant protein expression.

Studies for optimal expression of each recombinant protein have been performed with the high-titer stock of recombinant viruses.

4.1. Cell culture conditions.

In all experiments, cell suspensions were cultured in 50-ml spinner flasks at 28^{o}C and 95 rev/min. The screw caps of the spinner flasks were loosened to supply oxygen from the atmosphere.

4.2. Virus inoculation.

Cells in exponential phase of growth were centrifuged at 1,000 g for 10 minutes and then resuspended in fresh media. The final cell density was adjusted to 2×10^6 cells/ml and the viral inoculum was added.

4.3. Parameters analyzed.

Expression over time has been analyzed for each recombinant protein with different cell lines (*T. ni*, *S. frugiperda*), cell densities (1×10^6 cells/ml, 2×10^6 cells/ml), MOIs (1, 5, or 10), and replication times (24, 48, 72, 96, and 120 hrs). Each cell line has slightly different growth characteristics, and the kinetics of infection may vary. A 5-or 10-fold difference in the range of MOIs was tested in order to determine which will yield the maximal protein expression. An MOI of between 1 and 10 is generally recommended for baculovirus recombinant protein expression. Time points were set at 24 hour intervals to get a general idea of when each protein was being expressed at the highest level.

4.4. Analytical methods.

After collecting 1 ml of cells at different time points taken at 24 hour intervals, each sample was run on SDS-PAGE and Western blots. The optimal expression was determined on Western blot based on the differences of detection by using Sf9 cells adsorbed goat anti-*B. abortus* RB51 antisera as a primary antibody, rabbit anti-goat immunoglobulins conjugated to horseradish peroxidase as a secondary antibody and detected by using 4-chloro-1-naphthol.

5. Evaluation of recombinant protein concentrations.

The Bio-Rad protein assay, based on the Bradford dye-binding procedure, was used for measuring total protein concentration of recombinant protein expressing Sf9 cell lysates. Concentrations of bovine serum albumin (BSA) were used to generate standard curves. The total cellular protein concentration was measured at 595 nm using a spectrophotometer (Perseptive Biosystems, Inc., Framingham, MA). Protein concentration of each recombinant protein-expressing Sf9 cell lysates was determined, and appropriate concentrations were run on SDS-PAGE and stained with Coomassie blue staining solution. The stained gel was scanned through a densitometer (Model 300A, Molecular Dynamics, Sunnyvale, CA) to determine the proportion of each recombinant protein in the total cellular protein. The band corresponding to each recombinant protein was determined through Western blotting. Each baculovirus recombinant protein concentration was calculated by estimating the proportion of the specific recombinant protein relative to the total cellular protein concentration.

RESULTS

1. Growth condition of cell lines in suspension culture.

T. ni cells are up to 2.5 times larger than Sf9 cells. Unlike Sf9 and Sf21 cells, *T. ni* cells had high shear stress in the suspension culture so it was difficult maintaining them above 90% cell viability. Sf9 and Sf21 cell lines were used in plaque assay for viral titration, and Sf9 and *T. ni* cells were used in analyzing conditions of optimal protein

expression. All cells used in these studies were adapted to serum-free media to reduce foreign protein cross activity.

2. Titration of recombinant virus and preparation of high-titer viral stocks.

Plaque formation induced by recombinant virus was not seen under any of the various conditions tested. The pfu/ml titer was calculated by multiplying the $TCID_{50}$ units/ml with 0.69 (Summer and Smith, 1987). The cells infected with various dilutions of recombinant virus showed distinct morphology (Fig. 7 a, b, c). Cells infected with a higher dilution of recombinant virus showed a lower degree of lysis.

The titers of the recombinant viruses generated in adherent and suspension cultures were determined and compared (Table 1). Higher titer recombinant viruses were yielded in suspension cultures of each recombinant virus infected cells. The highest amount of infectious recombinant virus was 5×10^8 pfu/ml in suspension cultures, and 1.23×10^8 pfu/ml in adherent cultures.

	SUSPENSION CULTURE	ADHERENT CULTURE
GroEL recombinant virus	5×10^8 pfu/ml	1.23×10^8 pfu/ml
GroES recombinant virus	2.95×10^9 pfu/ml	0.84×10^8 pfu/ml
HtrA recombinant virus	3.23×10^8 pfu/ml	0.83×10^8 pfu/ml

Table 1. Titration of recombinant viruses.

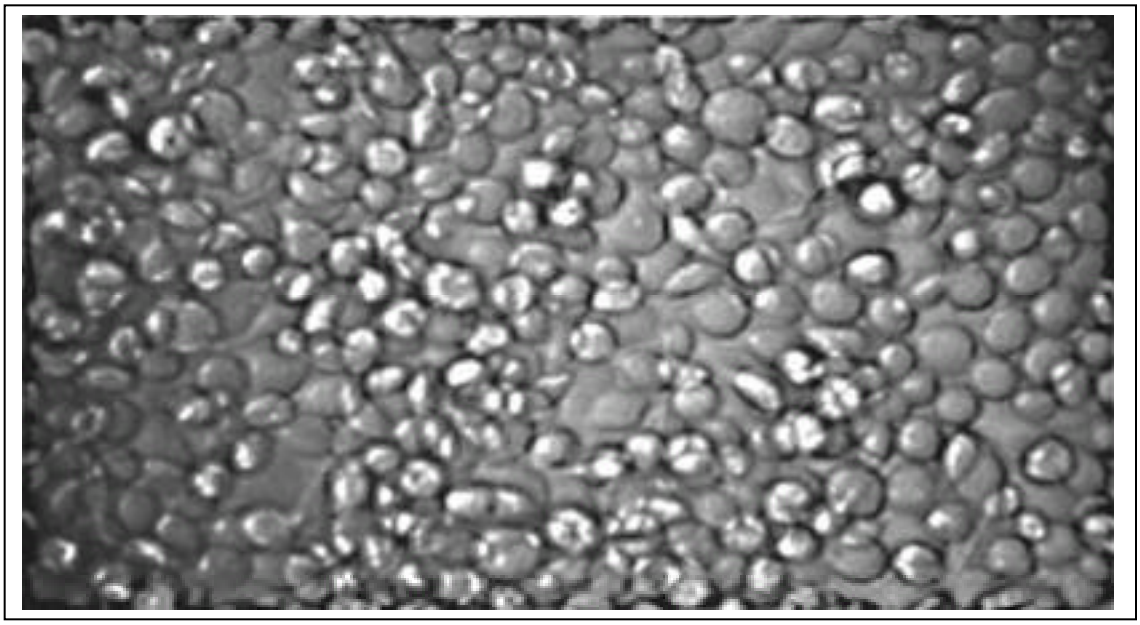

Fig. 7 a. Sf9 cells infected with GroEL recombinant virus at MOI of 2 x 10^{-7}.

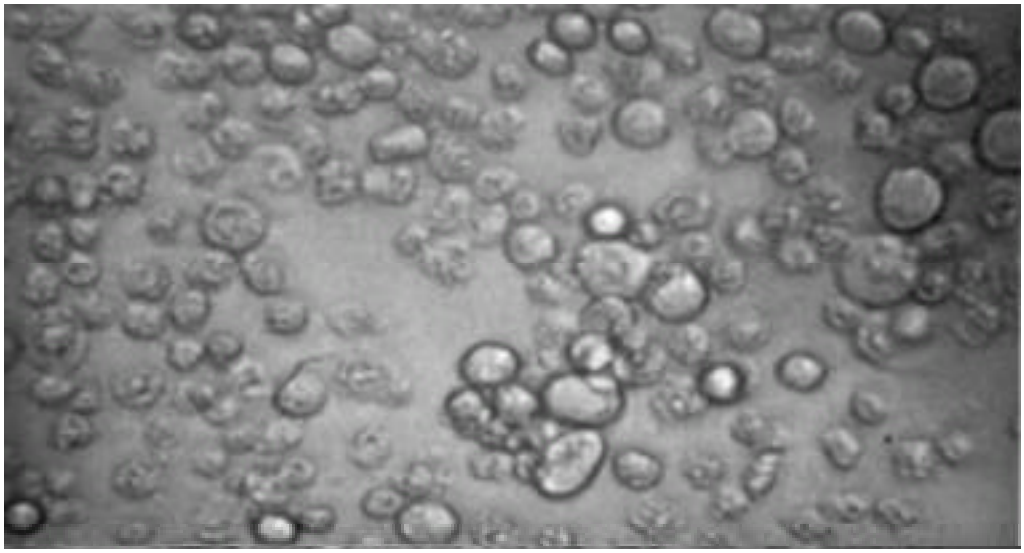

Fig. 7 b. Sf9 cells infected with GroEL recombinant virus at MOI of 2 x 10^{-2}.

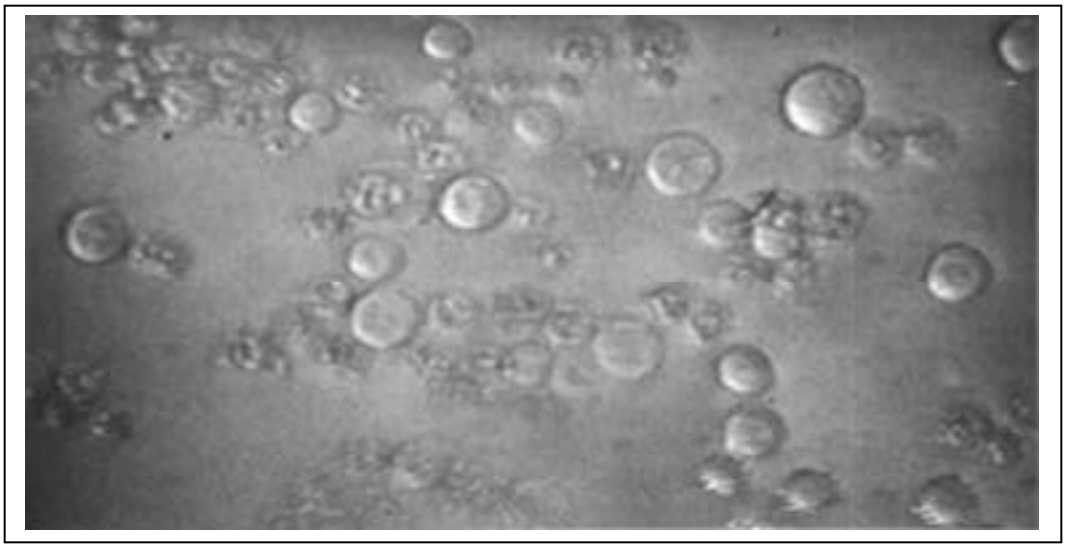

Fig. 7 c. Sf9 cells infected with GroEL recombinant virus at MOI of 2.

3. Analyzing conditions of optimal protein expression and concentration of the expressed recombinant proteins.

Based on the parameters described above, the optimal conditions for each recombinant protein expression were determined. The lysates of infected cells were tested by SDS-PAGE and Western blotting. The following conditions were determined as optimal for the maximal expression of recombinant proteins: 2 x 10^6 cells/ml of Sf9 cells infected with MOI of 10 for 96 hrs for GroEL (Fig. 8 a); 2 x 10^6 cells/ml of Sf9 cells infected with MOI of 1 for 120 hrs for GroES (Fig. 9 a) and for HtrA (Fig. 10 a). No GroEL and GroES recombinant proteins were expressed in *T. ni* cells (Fig. 8 b, Fig 9 b). HtrA recombinant protein was expressed in *T. ni* cells (Fig. 10 b), but the conditions for the maximal expression in *T. ni* cells were different from those for Sf9 cells. Since Sf9 cells are easier to maintain and produced good levels of all the recombinant proteins, they were selected for production of recombinants.

GroEL, GroES, and HtrA recombinant proteins were measured at 16%, 2%, and 8% in Sf9 cells, respectively, of the total cellular proteins. When GroEL was expressed under optimal conditions (2 x 10^6 cells/ml of Sf9 cells, MOI of 10, post-infection harvest time of 96 hrs), 105 μg/ml of GroEL recombinant protein was produced (Fig. 11). When GroES or HtrA were expressed under optimal conditions (2 x 10^6 cells/ml of Sf9 cells, MOI 1, post-infection time 120 hrs for both proteins), 15.25 μg/ml of GroES (Fig. 12) or 84.48 μg/ml of HtrA recombinant protein was produced (Fig. 13).

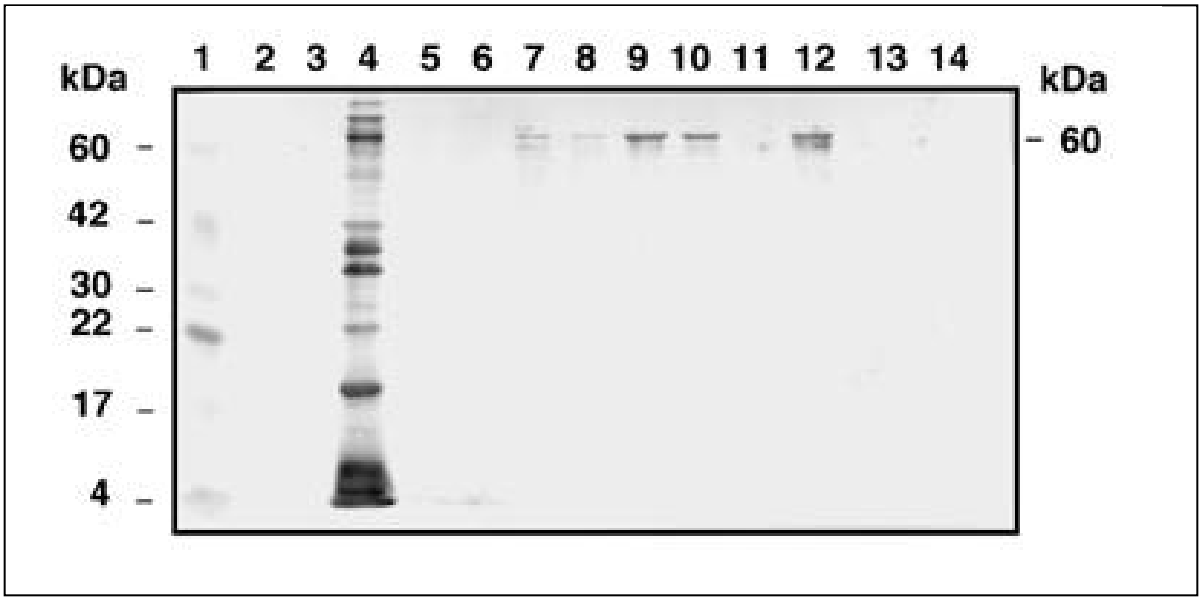

Fig. 8 a. Western blot analysis of the kinetics of the expression of GroEL recombinant proteins by *S. frugiperda* insect cells.

Five different post-infection times and two different MOIs were used as parameters. The conditions for the optimal expression of GroEL recombinant protein were when GroEL recombinant virus was infected to Sf9 cells with MOI 10 and replicated for 96 hours.

B. abortus anti-RB51 antiserum was used as the primary antiserum.

Lane 1: molecular weight markers
Lane 2: uninfected *Sf*9 cells
Lane 3: Bacmid only infected *Sf*9 cells
Lane 4: *B. abortus* RB51
Lane 5: MOI 5, 24 hrs post-infection (HPI)
Lane 6: MOI 10, 24 HPI
Lane 7: MOI 5, 48 HPI
Lane 8: MOI 10, 48 HPI
Lane 9: MOI 5, 72 HPI
Lane 10: MOI 10, 72 HPI
Lane 11: MOI 5, 96 HPI
Lane 12: MOI 10, 96 HPI
Lane 13: MOI 5, 120 HPI
Lane 14: MOI 10, 120 HPI

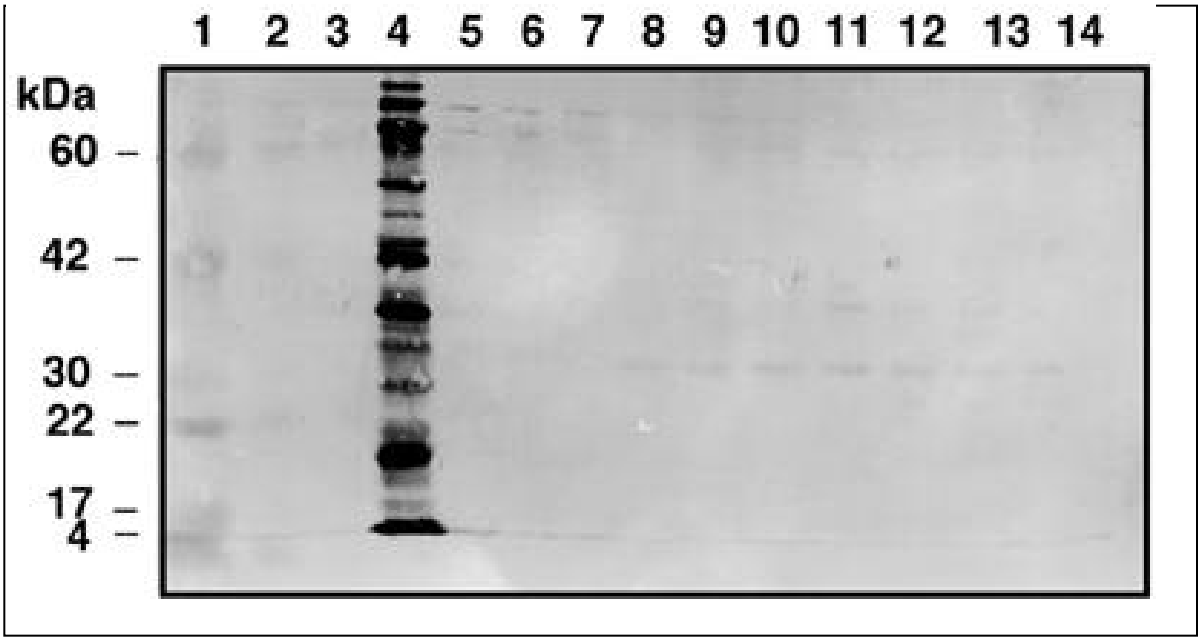

Fig. 8 b. Western blot analysis of the kinetics of the expression of GroEL recombinant proteins by *T. ni* insect cells.

Five different post-infection times and two different MOIs were used as parameters.

No GroEL recombinant expression was observed in this cell line. *B. abortus* anti-RB51 antiserum was used as the primary antiserum.

Lane 1: molecular weight markers
Lane 2: uninfected *T. ni* cells
Lane 3: Bacmid only infected *T. ni* cells
Lane 4: *B. abortus* RB51
Lane 5: MOI 5, 24 hrs post-infection (HPI)
Lane 6: MOI 10, 24 HPI
Lane 7: MOI 5, 48 HPI
Lane 8: MOI 10, 48 HPI
Lane 9: MOI 5, 72 HPI
Lane 10: MOI 10, 72 HPI
Lane 11: MOI 5, 96 HPI
Lane 12: MOI 10, 96 HPI
Lane 13: MOI 5, 120 HPI
Lane 14: MOI 10, 120 HPI

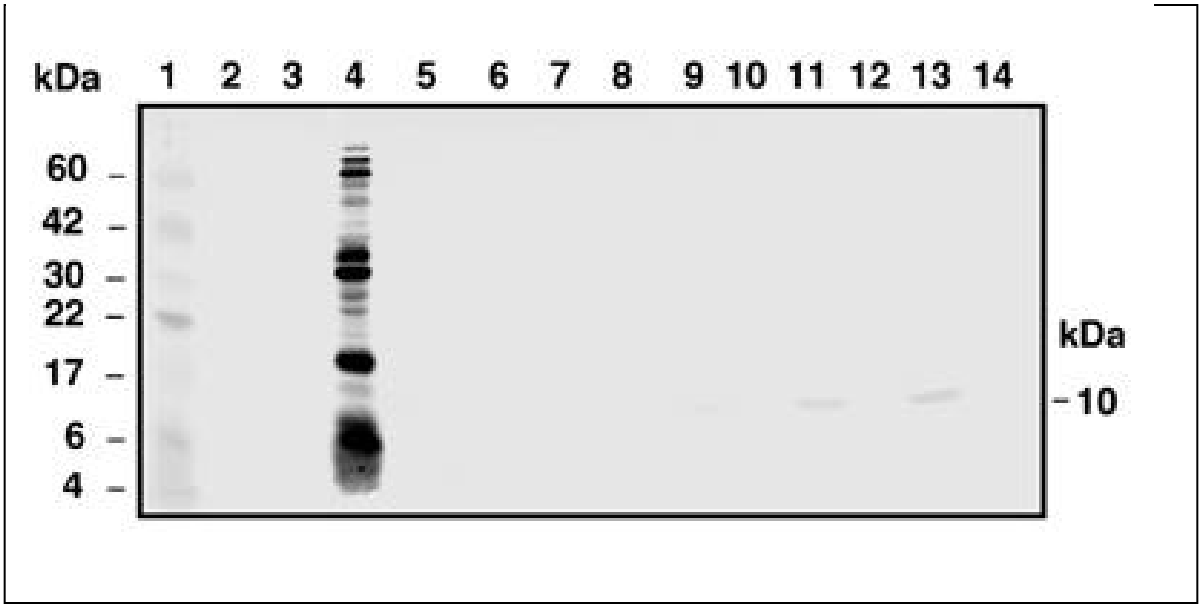

Fig. 9 a. Western blot analysis of the kinetics of the expression of GroES recombinant proteins by *S. frugiperda* insect cells.

Five different post-infection times and two different MOIs were used as parameters. The conditions for the optimal expression of GroES recombinant protein were when GroES recombinant virus was infected to Sf9 cells with MOI 1 and replicated for 120 hours.

B. abortus anti-RB51 antiserum was used as the primary antiserum.

Lane 1: molecular weight markers
Lane 2: uninfected *Sf*9 cells
Lane 3: Bacmid only infected *Sf*9 cells
Lane 4: *B. abortus* RB51;
Lane 5: MOI 1, 24 hrs post-infection (HPI)
Lane 6: MOI 10, 24 HPI
Lane 7: MOI 1, 48 HPI
Lane 8: MOI 10, 48 HPI
Lane 9: MOI 1, 72 HPI
Lane 10: MOI 10, 72 HPI
Lane 11: MOI 1, 96 HPI
Lane 12: MOI 10, 96 HPI
Lane 13: MOI 1, 120 HPI
Lane 14: MOI 10, 120 HPI

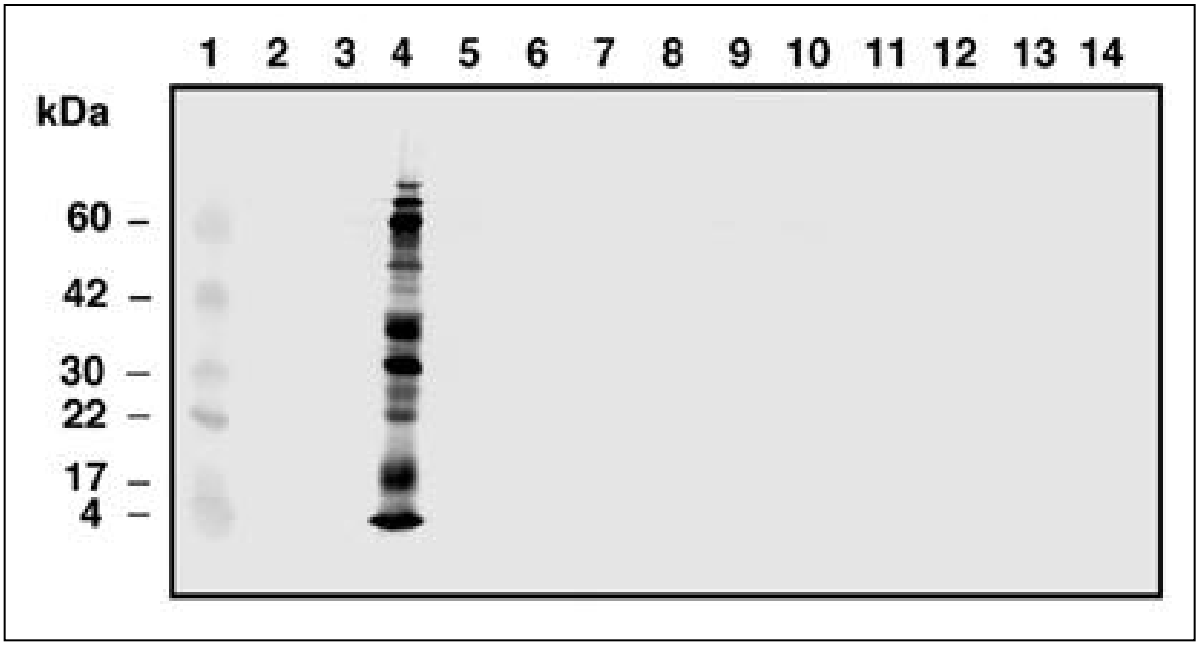

Fig. 9 b. Western blot analysis of the kinetics of the expression of GroES recombinant proteins by *T. ni* insect cells.

Five different post-infection times and two different MOIs were used as parameters.

No GroES recombinant expression was observed in this cell line. *B. abortus* anti-RB51 antiserum was used as the primary antiserum.

Lane 1: molecular weight markers
Lane 2: uninfected *T. ni* cells
Lane 3: Bacmid only infected *T. ni* cells
Lane 4: *B. abortus* RB51
Lane 5: MOI 1, 24 hrs post-infection (HPI)
Lane 6: MOI 10, 24 HPI
Lane 7: MOI 1, 48 HPI
Lane 8: MOI 10, 48 HPI
Lane 9: MOI 1, 72 HPI
Lane 10: MOI 10, 72 HPI
Lane 11: MOI 1, 96 HPI
Lane 12: MOI 10, 96 HPI
Lane 13: MOI 1, 120 HPI
Lane 14: MOI 10, 120 HPI

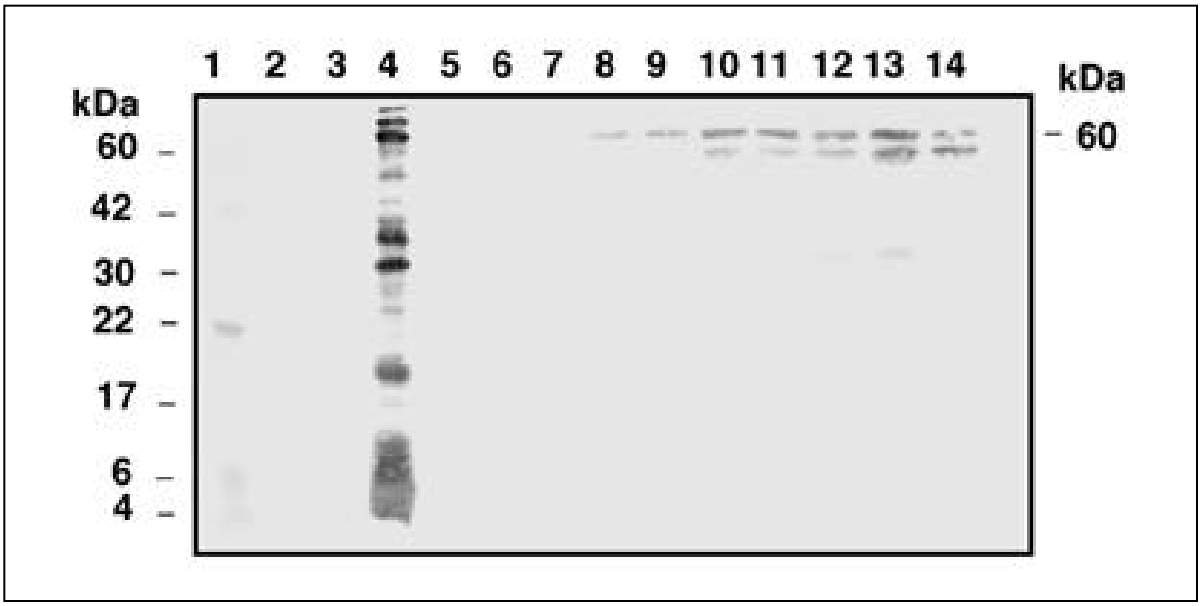

Fig. 10 a. Western blot analysis of the kinetics of the expression of HtrA recombinant proteins by *S. frugiperda* insect cells.

Five different post-infection times and two different MOIs were used as parameters. The conditions for the optimal expression of HtrA recombinant protein were when HtrA recombinant virus was infected to Sf9 cells with MOI 1 and replicated for 120 hours. *B. abortus* anti-RB51 antiserum was used as the primary antiserum.

Lane 1: molecular weight markers
Lane 2: uninfected *Sf*9 cells
Lane 3: Bacmid only infected *Sf*9 cells
Lane 4: *B. abortus* RB51
Lane 5: MOI 1, 24 hrs post-infection (HPI)
Lane 6: MOI 10, 24 HPI
Lane 7: MOI 1, 48 HPI
Lane 8: MOI 10, 48 HPI
Lane 9: MOI 1, 72 HPI
Lane 10: MOI 10, 72 HPI
Lane 11: MOI 1, 96 HPI
Lane 12: MOI 10, 96 HPI
Lane 13: MOI 1, 120 HPI
Lane 14: MOI 10, 120 HPI

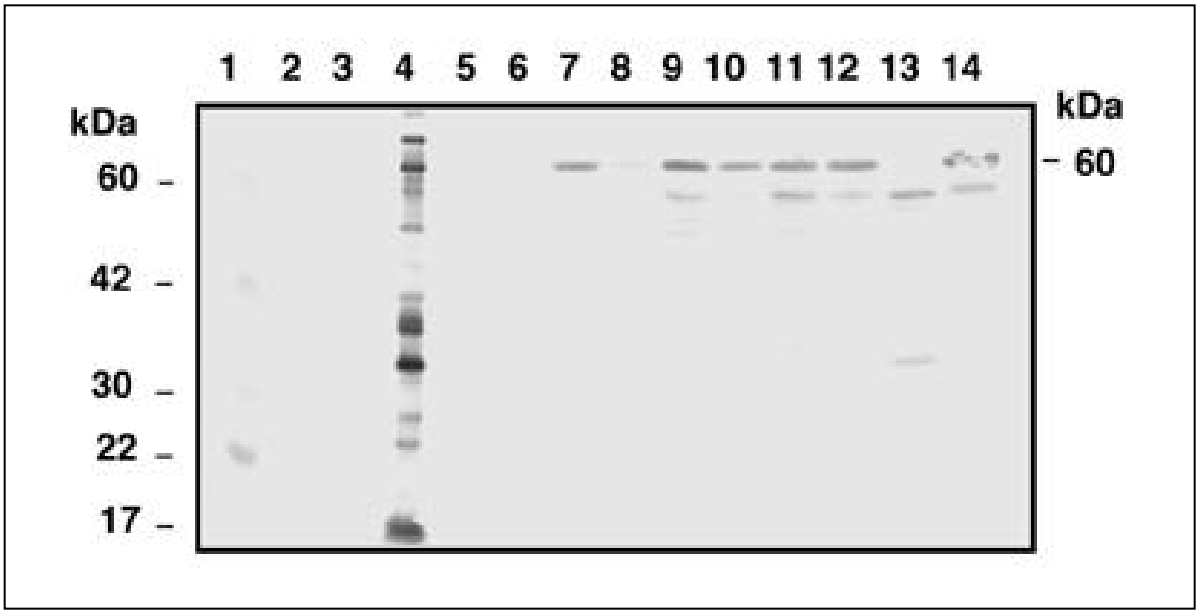

Fig. 10 b. Western blot analysis of the kinetics of the expression of HtrA recombinant proteins by *T. ni* insect cells.

Five different post-infection times and two different MOIs were used as parameters. The conditions for the optimal expression of HtrA recombinant protein were when HtrA recombinant virus was infected to *T. ni* cells with MOI 1 and replicated for 72 hours. *B. abortus* anti-RB51 antiserum was used as the primary antiserum.

Lane 1: molecular weight markers
Lane 2: uninfected *T. ni* cells
Lane 3: Bacmid only infected *T. ni* cells
Lane 4: *B. abortus* RB51
Lane 5: MOI 1, 24 hrs post-infection (HPI)
Lane 6: MOI 10, 24 HPI
Lane 7: MOI 1, 48 HPI
Lane 8: MOI 10, 48 HPI
Lane 9: MOI 1, 72 HPI
Lane 10: MOI 10, 72 HPI
Lane 11: MOI 1, 96 HPI
Lane 12: MOI 10, 96 HPI
Lane 13: MOI 1, 120 HPI
Lane 14: MOI 10, 120 HPI

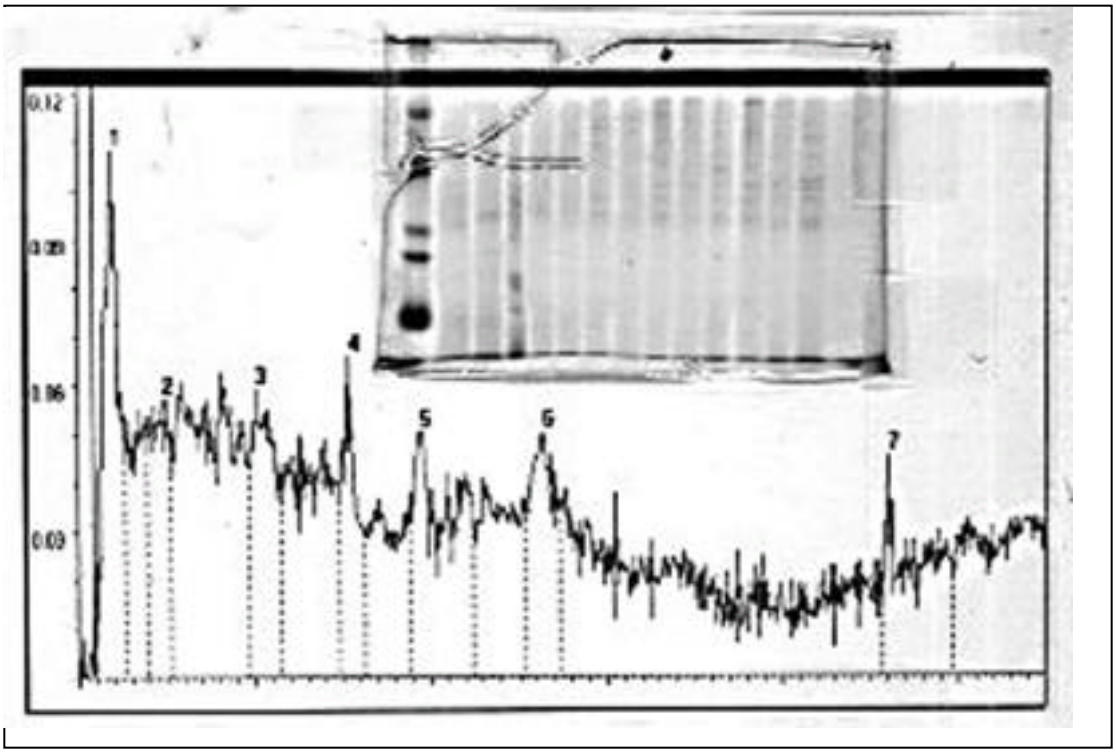

Fig. 11. Estimation of *B. abortus* GroEL-baculovirus recombinant protein concentration by scanning densitometry.
The figure shows the scanned Coomassie blue-stained gel and the densitometric profile of optimal GroEL recombinant protein expressing Sf9 cells. The 60 kDa GroEL recombinant protein was present in peak position #1 of scanning profile.

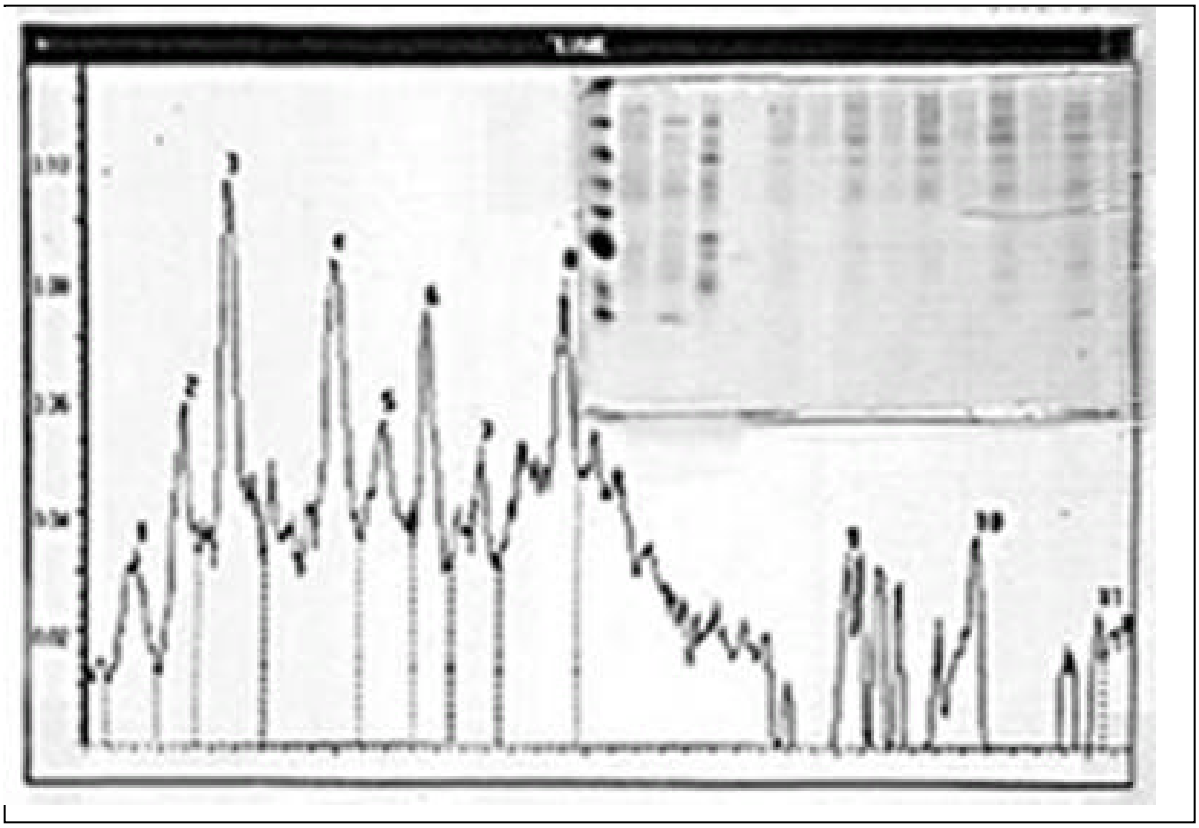

Fig. 12. Estimation of *B. abortus* GroES-baculovirus recombinant protein concentration by scanning densitometry.
The figure shows the scanned Coomassie blue-stained gel and the densitometric profile of optimal GroES recombinant protein expressing Sf9 cells. The 10 kDa GroES recombinant protein was present in peak position #10 of the scanning profile.

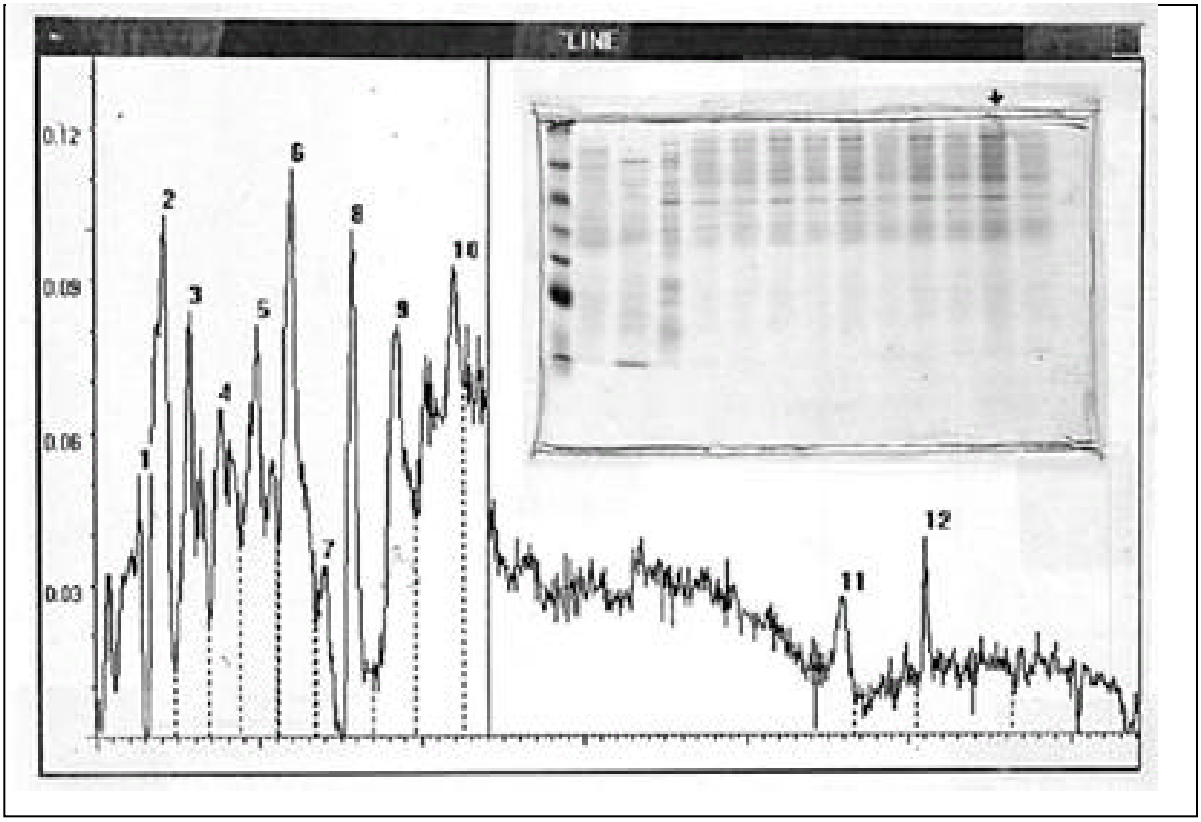

Fig. 13. Estimation of *B. abortus* HtrA-baculovirus recombinant protein concentration by scanning densitometry.

The figure shows the scanned Coomassie blue stained gel and the densitometric profile of optimal HtrA recombinant protein expressing Sf9 cells. The 60 kDa HtrA recombinant protein was present in peak position #4 of the scanning profile.

DISCUSSION

Two difficulties were revealed in the procedures using baculovirus expression system. One of the problems was with the method of determining titers of either wild-type or recombinant baculovirus. Historically, two methods have been used for baculovirus titration: plaque assays (Hink and Vail, 1973) and end-point tittering (Knudson and Tinsley, 1974). In our studies, determination of the titers of recombinant baculoviruses was attempted by both assays. According to some data (Hink and Vail, 1973), recombinant virus produced milky/gray plaques of slight contrast visible without staining or other detection methods. However, in our study, no plaque formation was seen under any of the various conditions examined including different cell lines, cell densities, viral dilutions, replication times, types of agarose gel, percentage of agarose gels, presence or absence of serum, supplied humidity to the cells, or types of dyes. Although this information seems to contradict published data, numerous communications with other researchers working with baculovirus recombinants indicated tittering recombinant baculoviruses by plaque assay is difficult. Thus, the titer pfu/ml of each recombinant virus was calculated after multiplying by 0.69 the $TCID_{50}$ units/ml based on the end-point assay results. In our case, the end-point assay proved to be a simpler procedure to perform and to yielded more reliable data than did the plaque assay. In a previous investigation (Lynn D. E., 1992), a wide variety of procedures - including the use of Leighton tubes, 60-well and 96-well microplates and three different cell lines – were described for reproducibly determining results of the endpoint assay to measure

baculovirus titers. In that study, three replicate assays were run with each cell line and each type of plate using the same original virus sample. The results of the study showed that the 96-well plates were significantly better ($p = 0.05$), in other words more consistent, than the 60-well microplates for each cell line, using analysis of variance and Duncan's multiple comparison of means. In our study, 96-well microplates were consistently used for titration by the end-point assay.

Another problem with using the baculovirus expression system was the difficulty of the scale-up of the insect cell culture with high viability, especially in the beginning stage, because insect cells are shear-sensitive and are reported to be damaged by gas production during adaptation to suspension (Tramper et al., 1986). Tramper et al. (1986) calculated the shear stress above which cell growth and division were suppressed. This value varies according to the cell line, and Sf9 and Sf21 cells have a higher tolerance for shear stress than do *T. ni* cells. The oxygen demand of insect cells is higher than that of mammalian cells (Maiorella et al., 1988; Weiss et al., 1982), and oxygen uptake increases after the viral infection (Streett and Hink, 1978). Thus, in our studies, stirring speeds were carefully adjusted for each stage of the cultures to maintain an optimal balance between less shear stress and more oxygen supply. When the optimal conditions were adjusted in the beginning stage of spinner culture, Sf9 insect cell cultures were well maintained with high viability and cell numbers without any further difficulties. It was much more difficult to reach 90% cell viability with *T. ni* cells than with Sf9 cells.

In the present work, we studied the kinetics of expression of three baculovirus - *B. abortus* HSP recombinants. In preliminary experiments, optimal expression of

recombinant proteins were evaluated based on two parameters: MOIs (between 1 and 10) and cell densities (1 x 10^6 cells/ml, 2 x 10^6 cells/ml). Cell density of 2 x 10^6 cells/ml had shown higher expression of each recombinant protein (data not shown); therefore, this cell density was used for further kinetic studies. In the subsequent studies, MOI, duration of recombinant virus replication, and cell lines were used as parameters for optimizing recombinant protein yields for each recombinant protein expression. In the baculovirus system, a viral titer of between 1 and 10 was highly recommended to yield optimal recombinant protein production in terms of quantity as well as quality. Thus, we used MOI between 1 and 10 for maximal expression of three recombinant proteins. Twenty four hours through one hundred twenty hours post-infection times were tested to allow replication of recombinant viruses in each insect cell line. Different MOIs and replication times were observed for optimal expression of respective recombinant proteins. *T. ni* cells are larger than Sf9 cells and the difference in average cell diameter - 13 vs. 18 mm - results in a cell volume for *T. ni* cells twice that of Sf9 cells (Tramper et al., 1986); *T. ni* cells were determined to be much more productive than Sf9 cells for expression of secreted proteins (Ogonah et al., 1991). However, there are no published data for non-secreted protein expression in this cell line. In our study, these two cell lines were used for optimal expression of *B. abortus* HSPs that are non-secreted proteins. Only *B. abortus* HtrA recombinant protein was expressed in this cell line. *T. ni* cells did not express GroEL and GroES recombinant proteins under our conditions. The reasons could be toxicity of protein to the cells, protein folding, or degradation, but these are only speculations. We also observed that *T. ni* cells grew well after a period of adaptation, but

the culture could not be maintained at 90% cell viability after about 30 days in suspension because of their high shear-sensitivity. We found that Sf9 cells were more suitable for large scale production of recombinant proteins because of their ease of cultivation, higher cell viability, and resistance to shear. In addition to these three parameters, intracellular protein degradation may be an important factor influencing heterologous protein yields in the insect cell-baculovirus system. Such protein degradation exists at all cell levels, encompassing both prokaryotes and eukaryotes (Goldberg and Dice, 1974; Rechcigl M., 1971). Given the cell-virus interactions, intracellular degradation of heterologous protein in the insect cell-baculovirus expression system is likely to happen. Shortly after infection, cells are synthesizing foreign proteins, and it is plausible that the cells activate some defense mechanism (Lacari and Bailey, 1990). Examples in both bacterial and mammalian cell systems have demonstrated the ability of a cell to identify a protein as foreign and target it for degradation (Ciechanover et al., 1985; Platt et al., 1970; Lin and Zabin, 1972). In addition to the cell targeting foreign proteins for proteolytic action, degradative action by the virus may play a role (Lacari and Bailey, 1990). In our study, we did not analyze the effect of intracellular protein degradation on optimal protein expression.

Previous studies reported maximum 50-70% of total cellular proteins as recombinant proteins in baculovirus systems (Luckow and Summers, 1988). We could not reach that high level of recombinant protein expression in this study. We speculate that it might be due to the characteristics of protein expressed in this system.

REFERENCES

Ciechanover, A., D. Finley, and A. Varshavsky. 1985. Mammalian cell cycle mutant defective in intracellular protein degradation and ubiquitin-protein conjugation. Prog. Clin. Biol. Res. 180:17-31.

Goldberg, A. L. and J. F. Dice. 1974. Intracellular protein degradation in mammalian and bacterial cells. Annu. Rev. Biochem. 43:835-869.

Granados, R. R. and B. A. Federici. 1986. The Biology of Baculoviruses. CRC Press, Boca Raton, FL 1986.

Hink, W. F. and P. V. Vail. 1973. A plaque assay for titration of alfalfa looper nuclear polyhedrosis virus in a cabbage looper nuclear polyhedrosis virus in a cabbage looper (TN-368) cell line. J. Invertebr. Pathol. 22:168-174.

Knudson, D. L. and T. W. Tinsley. 1974. Replication of a nuclear polyhedrosis virus in a continuous cell culture of *Spodoptera frugiperda*: Purification, assay of infectivity, and growth characteristics of the virus. J. Virol. 14:934-944.

Licari, P. and J. E. Bailey. 1990. Factors influencing recombinant protein yields in an insect cell-baculovirus expression system: multiplicity of infection and intracellular protein degradation. Biotech. Bioeng. 37:238-246.

Lin, S. and I. Zabin. 1972. Beta-galactosidase. Rates of synthesis and degradation of incomplete chains. J. Biol. Chem. 247:2205-2211.

Luckow, V. A. and M. D. Summers. 1988. Trends in the development of baculovirus expression vectors. Bio/Technology. 6:47-55.

Lynn, D. E. 1992. Improved efficiency in determining the titer of the *Autographa californica* baculovirus nonoccluded virus. Biotechniques 13:282-285.

Ogonah, O., M. L. Shuler, and R. R. Granados. 1991. Protein production (β-galactosidase) from a baculovirus vector in *Spodoptera frugiperda* and *Trichoplusia ni* cells in suspension culture. Biotech. Lett. 13:265-270.

Platt, T., J. H. Miller, and K. Weber. 1970. *In vivo* degradation of mutant lac repressor. Nature. 2281154-1156.

Rechcigl, M. 1971. Enzyme synthesis and degradation in mammalian systems. University Park Press, Baltimore.

Scott, R. I., J. H. Blanchard, and C. H. R. Ferguson. 1992. Effects of oxygen on recombinant protein production by suspension cultures of *Spodoptera frugiperda* (Sf-9) insect cells. Enzyme Microb. Technol. 14:798-804.

Smith, G. E. and M. D. Summers. 1978. Baculovirus structural polypeptides. Virology. 84:390-402.

Streett, D. A. and W. F. Hink. 1978. Oxygen consumption of *Tricgoplusia ni* (TN-368) insect cell line infected with *Autographa californica* nuclear polyhedrosis virus. J. Invert. Pathol. 32:112-113.

Summer, M. D. and G. E. Smith. 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station Bulletin no. 1555.

Tramper, J., J. B. Williams, D. Joustra, and J. M. Vlak. 1986. Shear sensitivity of insect cells in suspension. Enzyme Microb. Technol. 8:33-36.

Weiss, S. A., T. Orr, G. C. Smith, S. S. Kalter, J. L. Vaughn, and E. M. Dougherty. 1982. Quantitative measurement of oxygen consumption in insect cell culture infected with polyhedrosis virus. Biotechnol. Bioeng. 24:1145-1154.

SUMMARY AND CONCLUSIONS

In spite of the high degree of evolutionary conservation, heat shock proteins (HSPs) are such effective antigens that they are under consideration as potential components of subunit vaccines. Given the observation that immunity is associated with foreignness, it is paradoxical that HSPs are major immunogens in several parasitic infections, as examplified by *Plasmodium* (Dubois et al., 1984; Mattei et al., 1989: Kumar et al., 1990), *Trypanosoma* (Engmann et al., 1989) and mycobacteria (Britton et al., 1986; Adams et al., 1990). In addition, Hsp70 of *Mycobacterium tuberculosis* obviously possesses adjuvant properties when used as a carrier in immunization. The capability to induce protection in the case with *Brucella* HSPs, however, is unknown, although it is often hypothesized as capable of inducing specific cell-mediated immune responses (Lin et al., 1992; Phillips et al., 1995; Stevens et al., 1997).

The hypothesis of this study was that *B. abortus* SOD, L7/L12, or individual/combinations of HSP recombinants in combination with optimal adjuvant(s), or by primary immunization with vaccinia virus recombinant(s) could induce and direct cell-mediated immune responses, so that they conveyed protection against *Brucella* infection.

This investigation started by cloning and expression of *groEL*, *groES*, and *htrA* of *B. abortus* HSPs in the baculovirus system to yield large quantities of pure recombinant proteins. Following the identification of each recombinant HSP using specific antibodies, a titration for each recombinant virus was performed. The plaque assay did not work for viral titration and we obtained much more reliable results by end-point assay. The effects

of infection with different MOIs were reflected by the different degrees of cytopathic effects in infected cells. We could not understand exactly why recombinant baculovirus did not form plaques in insect cells in this study. However, communications with others working with baculovirus recombinants indicated that this was not an unusual situation.

Kinetic studies were carried out in order to establish optimal conditions for the maximum expression of each recombinant protein. Two different insect cells (Sf9, *T. ni*) were cultured first in adherent and later in suspension culture using serum free media. Even though it is established that *T. ni* cells yield higher levels of secreted proteins, no data are published in the case of expression for non-secreted proteins. For the expression of *B. abortus* HSPs, which are non-secreted proteins, *T. ni* cells were not a good choice. Only *B. abortus* HtrA baculovirus recombinant protein, but not GroEL and GroES recombinant proteins was expressed in this cell line. Sf9 cells expressed all three recombinant HSPs. Because Sf9 cells were much easier to grow and maintain in log phase due to less shear-sensitivity, this cell line was selected to obtain of recombinant proteins.

To exploit the potential of these proteins in vaccine design, humoral, cell mediated and protective immune responses were analyzed in BALB/c mice. Humoral immune response was demonstrated by Western blot using sera obtained from mice injected with each antigen combined with various adjuvants. Cell mediated immune (CMI) response was analyzed by either lymphoproliferation assay, or IFN-γ ELISA and/or flow cytometry for evaluating the proportion of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in splenocytes. Protective immune response was determined by measuring splenic clearance

of *Brucella* in mice immunized with various immunogens, and subsequently challenged with the virulent *B. abortus* 2308 strain. The level of protection was determined by the difference of log cfu/spleen between saline and specific antigen treated groups of mice.

In order to ascertain whether the Sf9 insect cells expressing recombinant *B. abortus* HSPs were capable of inducing specific cell-mediated immune responses, we started to analyze immune responses using whole insect cells combined with Freund's adjuvant as antigens. Cell mediated immune response of the mice to Sf9 insect cells antigens masked recombinant protein specific CMI. The lymphoproliferation assays were not reproducible and exhibited large mouse to mouse variations. For the subsequent analysis of CMI responses using purified proteins as antigens and Ribi or IL-12 as adjuvant, instead of lymphoproliferation, IFN-γ release and the proportions of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in splenocytes were evaluated.

To induce CMI and protective immune responses, individual, divalent or trivalent purified *B. abortus* HSP(s) were used with Ribi adjuvant. A preliminary study for the specific CMI response using two different doses (1, 10 μg) of each purified HSP combined with Ribi adjuvant showed that splenocytes of mice injected with 10 μg/mouse/injection released more IFN-γ than those inoculated with 1 μg/mouse. Thus, 10 μg/mouse/injection was used in the following immune studies. When Ribi was used as adjuvant in conjunction with 10 μg of each purified HSP, none of the recombinant proteins induced protection. However, significant level (> 50 ng/ml) of IFN-γ was released by the splenocytes from the mice injected with purified HtrA protein.

Relatively high proportions of $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells/total number of splenocytes were observed when purified GroEL or HtrA protein was injected combined with Ribi adjuvant. These proportions were similar to those of the positive control live RB51 inoculated mice group. However, the relatively high proportions of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells/total number of splenocytes did not correlate with the level of IFN-γ release and protection against *Brucella* infection.

Androstenediol did not increase resistance against *Brucella* infection. Injections of two different GroEL recombinants (live form and subunit form), by priming with live vaccinia virus recombinant and boosting with subunit baculovirus purified recombinant protein combined with Ribi adjuvant, did not induce protective immune response against *Brucella* infection.

The benefits of IL-12 as adjuvant, was demonstrated as various levels. Killed RB51 vaccine strain was injected combined with purified IL-12 and the level of IFN-γ release was measured two weeks after the second immunization. The highest IFN-γ release was observed in the splenocytes from mice injected with killed RB51 plus IL-12. This value was higher than that in the splenocytes from mice injected with positive control live RB51. The highest level of IFN-γ release, as well as the highest proportions of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells/total number of splenocytes, were observed in the mice injected with purified HtrA protein plus Ribi adjuvant. Therefore, this protein was selected from the heat shock proteins for further evaluation for adjuvanticity of IL-12. Besides killed *B. abortus* RB51 and purified HtrA, *B. abortus* SOD recombinant and live

E. coli cells expressing L7/L12 ribosomal protein were used as antigens in these studies. CMI response studies and challenging for protection were performed six weeks after the second immunization to reduce backgrounds of responses due to IL-12 itself. IL-12 itself did not induce non-specific activation of lymphocytes, but it appears that IL-12 acted to stimulate immune responses along with antigen, inducing significant IFN-γ release. Mice injected with live RB51 showed the highest level of IFN-γ release which was higher than in the mice injected with killed RB51 plus IL-12. Much lower levels of IFN-γ release were observed in the splenocytes from all groups of mice by stimulation with HtrA as well as SOD protein compared to stimulation with killed RB51. No antigen specific IFN-γ release was induced by stimulation with HtrA or SOD protein. No differences were observed in the proportion of $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells/total number of splenocytes either between positive control and antigen plus IL-12 treated group or between antigen without IL-12 and antigen plus IL-12 treated group. A direct correlation was not demonstrated between IFN-γ release in splenocytes and the proportions of $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells. A significant level of protection was induced when mice were inoculated with *B. abortus* SOD protein combined with IL-12.

The correlation between IFN-γ release in splenocytes and induction of protective immunity was not always straightforward. For example, no correlation was observed between IFN-γ release in splenocytes and protection in SOD immunized mice. Furthermore, no direct correlation between the proportions of $CD3^+CD4^+$ /$CD3^+CD8^+$ and protective immunity was observed.

In conclusion, protective immunity was not observed in any cases using *B. abortus* HSPs combined with various adjuvants or two different types of recombinants; vaccinia virus and baculovirus recombinant. Based on the results of these studies, we conclude that *B. abortus* HSPs do not have the capability to induce appropriate cell-mediated and protective immune responses against *Brucella* infection. It could be argued that the doses employed are either insufficient to produce an immune response or that presentation of the antigen in these killed vaccines does not mimic the situation obtained with a living vaccine strain. The prospect of observing the induction of a high level immune response still remains by using these antigens along with IL-12 by performing assays at the suitable time point. However, although HSPs alone did not induce protective immunity, it may be possible to use these proteins as carriers to deliver protective antigens *in vivo*.

A significant finding of this study was that *B. abortus* Cu/Zn SOD and L7/L12 recombinant proteins induced protection against the challenge with *Brucella* virulent strain. IL-12 does appear to direct immune responses to Th1 subtype, and protective immunity was induced when this adjuvant was used with the appropriate *B. abortus* antigens.

REFERENCES

Adams, E., R. J. Garcia, L. Helqvist, P. Holt, and A. Basten. 1990. T cell reactivity to the purified mycobacterial antigens P65 and P70 in leprosy patients and their household contacts. Clin. Exp. Immunol. 80:206-212.

Britton, W. I., L. Hellquist, A. Basten, and A. L. Inglis. 1986. Immunoreactivity of a 70 kDa protein purified from *M. bovis Bacillus Calmette-Guerin* by monoclonal antibody affinity chromatography. J. Exp. Med. 164:695-708.

Dubois, P., J. P. Dedet, T. Fandeur, C. Roussillon, M. Jendoubi, S. Pauillac, O. Mercerau-Pujalon, and L. Pereira da Silva. 1984. Protective immunization of the squirrel monkey against asexual blood stages of *Plasmodium falciparum* by use of parasite protein fractions. Proc. Natl. Acad. Sci. USA 81:229-232.

Engman, D. M., L. V. Kirchoff, and J. E. Donelson. 1989. Molecular cloning of mtp70, a mitochondrial member of the hsp70 family. Mol. Cel. Biol. 9:5163-5168.

Kumar, N., Y. Zhao, P. Graves, J. Perez, L. Maloy, and H. Zheng. 1990. Human immune response directed against *Plasmodium falciparum* heat shock-related proteins. Infect. Immun. 58:1408-1414.

Lin J., L. G. Adams, and T. A. Ficht. 1992. Characterization of the heat shock response in *Brucella abortus* and isolation of the genes encoding the GroE heat shock proteins. Infect. Immun. 60:2425-2431.

Phillips, R. W., P. H. Elzer, and R. M. Roop II. 1995. A *Brucella melitensis* high temperature requirement A (*htrA*) deletion mutant demonstrates a stress response

defective phenotype in vitro and transient attenuation in the BALB/c mouse model. Microb. Pathog. 19:227-284.

Mattei, D., A. Scherf, O. Bensaude, and L. Pereira da Silva. 1989. A heat shock-like protein from the human malaria parasite *Plasmodium falciparum* induces autoantibodies. Eur. J. Immunol. 19:1823-1828.

Stevens M. G., S. C. Olsen, G. W. Pugh, J. E. Mayfield. 1997. Role of immune responses to a GroEL heat shock protein in preventing brucellosis in mice vaccinated with *Brucella abortus* strain RB51. Comp. Immunol. Microbiol. Infect. Dis. 20:147-153.

APPENDIX

Procedure for Gel electrophoresis

I. **Samples to be prepared.**

1. Lyse recombinant protein expressing insect (Sf9, *T. ni*) or *E. coli* cells.
2. Centrifuge (3X) and discard pellet.
3. Aliquot, label, and freeze at -70^{o}C.
4. Use these as stock antigens.
5. When ready to use:

   --- Thaw 1 tube.

   --- Add 100 µl 2X Sample buffer and the antigens.

   --- Boil 10 minutes.

6. Run 30 µl/lane in small gels.

II. **Recipe for 2X Sample buffer.**

4% SDS

20% glycerol

10% 2-mercaptoethanol

0.004% bromophenol blue

0.125 M Tris HCl, pH 6.8

III. **Recipe for 15% resolving gel (2 gels).**

4.68 ml 40% acrylamide

1.9 ml resolving gel buffer

0.15 ml 10% SDS

7.57 ml HPLC-grade water

0.75 ml 1.5% ammonium persulfate

7.5 µl TEMED

IV. **Recipe for stacking gel (2 gels).**

0.94 ml 40% acrylamide

2.5 ml stacking gel buffer

0.1 ml 10 % SDS

5.96 ml HPLC-grade water

0.5 ml 1.5 % ammonium persulfate

10 µl TEMED

V. **Recipe for Resolving gel buffer;**

3M Tris-HCl (pH 8.8)

VI. **Recipe for Stacking gel buffer;**

5M Tris-HCl (pH 6.8)

VII. **Recipe for Reservoir buffer stock (10X);**

0.25M Tris, 1.92M glycine, 1% SDS (pH 8.3)

VIII. **Recipe for Coomassie Blue solution (1L)**

3g Coomassie Brilliant Blue R., 100 ml acetic acid (CH_3COOH), 450 ml ethanol (CH_3CH_2OH), 450 ml distilled water (dH_2O)

IX. **Recipe for Gel Destainer (1L)**

650 ml H_2O, 250ml 100% ethanol, 100 ml glacial acetic acid

X. **Procedure:**

1. Wash glass plates and spacers with 70% ethanol. Make sure that the plates are dried before putting together.
2. Assemble and check leaking with dH_2O.
3. Make the resolving gel solution, adding the TEMED only when ready to pour. Add the TEMED and pipette resolving gel solution between the glass plates --- make sure that does not leak. Leave enough room at top of plates for the stacking gel to be poured. Carefully add 70% ethanol to the top of the resolving gel before it polymerizes. This will help the gel polymerize more evenly and prevents any evaporation.
4. Make the stacking gel solution, again waiting until ready to pour to add the TEMED. Draw the distilled water off the resolving gel and place the comb between the top of the plates. Add the TEMED to the stacking gel solution and pipette around the comb --- do not allow air bubbles to form around the comb, because they will prevent even migration. Let stand until the stacking gel polymerizes well. Carefully pull the comb straight up and out from between the plates.
5. Wash the wells carefully with distilled water three times. Fill the wells and the back of the plates with reservoir running buffer after placing the apparatus in its tray.
6. Prepare and load the samples as described on above.

7. Add reservoir running buffer to the apparatus tray. Place lid on the apparatus, making sure that the reservoir buffer level is well above the samples. If the reservoir buffer level drops below the level of the top of the wells, the samples will not run.
8. Plug the apparatus into a power supply and run at 50 volts/gel until the leading dye reaches the bottom of the gel. The run on an average takes around 2 hours.
9. Place one gel to be stained into a dish containing Coomassie Blue dye. Gel that is being stained needs to remain in the dye 1 hr. Then, destain it with the destain solution until most of the background is gone. This takes as long as overnight.
10. The other gel is used for Western blot.

Procedures for Western Blot

I. **Recipe for transfer buffer**;

25 mM Tris,

192 mM glycine

20% methanol

II. **Recipe for TBS (Tris Buffered Saline);**

0.15 M NaCl

20 mM Tris

pH 7.5

III. **Recipe for TBST (TBS-tween 20) ;**

0.15 M NaCl

20 mM Tris

pH 7.5

0.05% Tween 20

IV. **Recipe for blocking buffer;**

5% non-fat milk in TBS

V. **Recipe for substrate;**

Mixture of 60 mg 4-chloro-1-naphthol in 10 ml MeOH and 60 µl 30% peroxide in 100 ml TBS

VI. **Procedure:**

1. Electrophoresis gel by previous procedure.
2. After gel electrophoresis, remove the gel from glass plates and cut off stacking gel.
3. Equilibrate the gel in transfer buffer for 30 minutes.
4. Prewet 4 pieces filter paper, and 1 piece of nitrocellulose membrane per gel in transfer buffer.
5. Transfer the proteins on the gel to the membrane by using semi-dry transfer cell (Bio-Rad, Hercules, CA) at 30V for 30 minutes.
6. Place the membrane into a blocking solution for 1 hour with agitation at room temperature.
7. Dilute primary antibody in TBS. Place the membrane in desired antibody with appropriate dilution and leave agitating for 1-2 hrs at room temperature.
8. Wash the membrane 3X in TBST. Place membrane in fresh TBST each time and agitate for 5 minutes.

9. Place the membrane in secondary antibody of appropriate dilution in TBS --- Use an anti-antibody IgG conjugated with horseradish peroxidase. Agitate for 1 hr at room temperature.
10. Wash the membrane 3X in TBST as in step 8.
11. Develop the membrane by using substrate.
12. Stop developing step with distilled water and dry at room temperature.

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